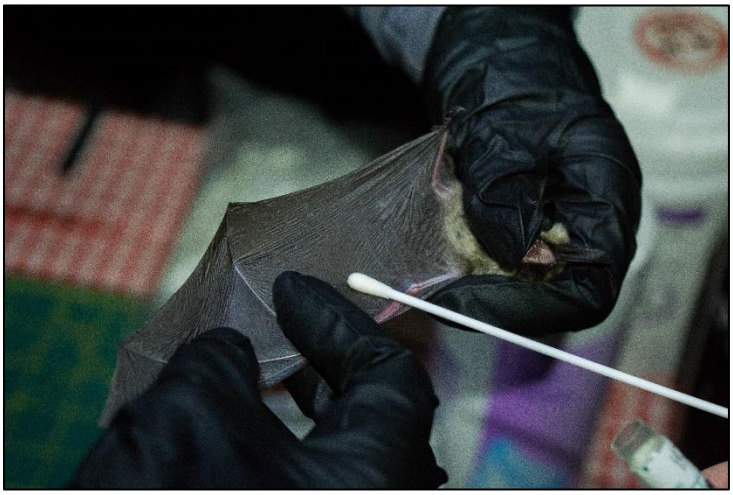
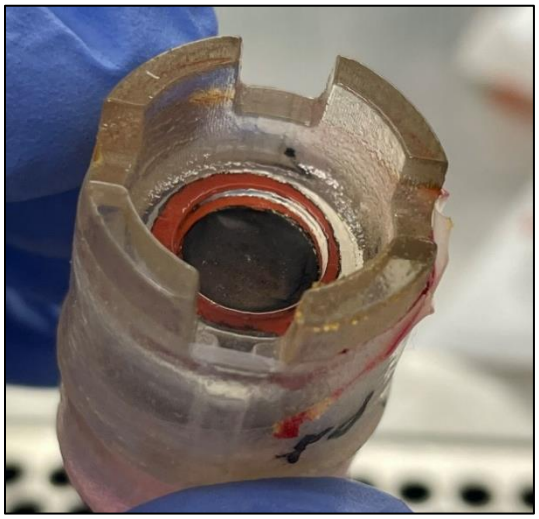
Developing and Evaluating Bat Mitigation Strategies





WCS Canada

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Fish & Wildlife Compensation Program
Coastal

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31 March 2021 (Updated July 2021)

Cover Photos: Left: Bat skin tissue within tissue explant device (Nick Fontaine); top-right: bat wing being swabbed (Aaron Wong); bottom-right: Colony Farms bat box array (John Saremba).

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EXECUTIVE SUMMARY

The goal of the Probiotic Project is to develop and test (in captive and wild trials) a cocktail of bacteria sourced from wings of B.C. bats which inhibits the fungus (*Pseudogymnoascus destructans*, *Pd*) that causes white-nose syndrome (WNS) in bats. Specific to the West, where few bat hibernacula are known, but many maternity roosts are known for building roosting species, our project aimed to take a prophylaxis approach; the strategy is that bats will be passively inoculated at their summer roosts, incorporate the probiotic bacteria into their wing microbiomes and then travel to hibernacula where the probiotic inhibit or slow growth of *Pd*, slowing or preventing the progression of WNS and reducing overwinter mortality.

Our study is multi-faceted and has been staged. In 2017 we swabbed bats from across B.C. and through culturing of bacteria in the lab, challenged with *Pd*, isolated anti-*Pd* bacteria. In 2018 we conducted a pilot captive trial to confirm that Yuma myotis (*Myotis yumanensis*) can be kept in captivity successfully. Using one anti-*Pd* bacteria we also experimentally determined we could alter the wing microflora of bats for an extended period of time. In 2019, using the finalized probiotic (4 anti-*Pd* bacteria), we tested the safety of this probiotic on bat health by conducting a comprehensive summer-long captive trial (May – August), and examined the persistence of probiotic cells in several bat boxes, including an experimental bat box in real field conditions, but closed off to bats. In October 2019, we prepared bats for a hibernation trial, acclimating them to cooling conditions and on 16 November they were placed into a modified fridge where they hibernated until 21 December. During the captive trials of 2019, we developed and tested an effective application method. In 2019, we piloted the probiotic in the field.

In 2018, 2019 and 2020, we conducted fieldwork in the Greater Vancouver area to select study sites, establish baseline ecological data, and locate alternate roosts used by the four selected maternity colonies: Colony Farm Regional Park, Alice Lake Provincial Park, Stave Lake BC Hydro Installation, and Deas Island Regional Park. All four colonies are mixed little brown (*M. lucifugus*) and Yuma myotis. The pandemic limited our 2020 field activities, but some capture, swabbing and probiotic application occurred. In 2020 at project field sites in the Greater Vancouver area, we conducted field application trials at 3 of our 4 study sites (Colony Farm Regional Park, Alice Lake Provincial Park, and Stave Lake BC Hydro Installation. Due to COVID-19 restrictions, no work took place at Deas Island Regional Park.

The field testing of this probiotic will continue in 2021, along with some final experiments. One of these experiments is to quantify efficacy of the probiotic cocktail on inhibiting *Pd* spore germination on wing skin cells kept alive ex vivo (tissue explants). We conducted a pilot of this experiment in fall 2020, refining methodology.

Since the commencement of this project, we have:

- Increased understanding of bat ecology including roost switching, dates of arrival and departure form summer roost sites, re-use of sites across years.

- Developed a probiotic cocktail made of four synergistic anti-*Pd* bacteria, for topical application to bats.
- Confirmed the absence of ill health effects attributed to the probiotic through captive trials.
- Confirmed positive response of the probiotic cocktail to hibernacula conditions (cool, humid), with bacteria increasing in concentration on the wings as hibernation progressed.
- Confirmed persistence of probiotic for at least 60 days on bat wings during summer in managed (*captive* trial) situation. The longest period from substrate inoculation to bat wing swabbing of *wild* bats that we could test was 3 weeks, at which point there was detectable probiotic on the wings of 30% of captured bats.
- Confirmed uptake of probiotic cells from inoculated roost structures to bats, in captive and free-living bats. Mean cell concentrations achieved on wings of captive bats ranged from 4 – 1.3 x 10^4 cells/cm^2 two months after a single probiotic application was made to their bat box application. This compares with mean concentrations ranging from 0.5 – 5.3 x 10^5 cells/cm^2 on wild bats found to have probiotic on their wings upon capture, nearly 3 weeks after a single probiotic application was made at the point of capture roost structure.
- Demonstrated that mean probiotic cell concentrations increase 12 fold or more over a one month period of hibernation.
- Demonstrated the summer persistence of probiotic bacteria in a bat box during hot summer temperatures, although further experimentation will be needed to confirm heat thresholds for each of the four strains of bacteria; to date, laboratory experiments have demonstrated growth of all strains at as warm as 37°C but mortality of all strains at 56°C.
- Collected evidence that probiotic bacteria survive winter in some bat boxes. Further sampling will take place in 2021-22, and we will also test persistence overwinter survival in building roosts.
- Determined that all four of the bacteria in our cocktail are of the *Pseudomonas fluorescens* family, a group of bacteria that contain common soil fungi and have been already proven effective at treating WNS in winter in hibernacula (reduced mortality from WNS; Hoyt et al. 2019).
- Through very preliminary searches of some soil databases in Canada, we have determined that at least 3 of our probiotic bacteria are common in soils outside of BC, suggesting this probiotic may be used in other areas of Canada without fear of introducing foreign microbes to the environment.

In 2021, we swabbed field roosting substrates in February and are testing for presence of probiotic (lab results pending). We will opportunistically sample wings of bats returning to roosts in spring to look for probiotic and *Pd*; collect guano for *Pd* surveillance; PIT-tag additional bats at all study sites (there is currently disparate percentage of PIT tagged bats among sites); apply probiotic (mid to late summer); sample bat wings and roosting substrates prior to hibernation; install acoustic detectors to determine if bats are found in study areas in winter; sample roosting substrates at end of the 2021-22 winter (including building and bat box substrates). Our laboratory goals in 2021 will be to improve amplification of the 4th probiotic bacteria (*P. antarctica*); improve our sampling procedures given that swab sampling may result in higher than desired variance in cell concentration measurements; and improve our methodology for assessing the viability of probiotic cells in samples from bat wings and from roost

substrates. We will continue to work closely with the Advisory Committee to make decisions regarding this cutting edge wildlife mitigation project.

Here we focus on summarizing 2020 field activities, and lab analyses. Because we have now analyzed stored samples from previous experiments, we are also presenting final results from the collective body of work (2019 – 2020), including the second captive trial, the hibernation simulation experiment, and the field pilot application.

This project addresses several high priority action items in the BC Bat Action Team's Bat Action Plan, last revised in December 2020 (available: https://bcbat.ca/wp-content/uploads/2021/02/BCBat-Action-Plan-Revised-Dec-2020_small.pdf) including winter monitoring of bats and mitigating WNS. As this project also takes place in the Stave Watershed and is funded in part by Fish and Wildlife Compensation Program, we highlight the fact that in the Stave Lake Watershed Action Plan, our project most closely aligns with the Ecosystem Chapter, Upland & Dryland habitat based action to "Determine presence, identify/protect bat maternity roosts & winter hibernacula." Environment Canada and Climate Change is also a significant funder of this project, and we note that our project addresses a high priority action in the Canadian Recovery Strategy for Little Brown Myotis to develop a treatment for WNS.

PROJECT TEAM AND ACKNOWLEDGEMENTS

PROJECT TEAM

We thank our team members since its inception in 2017:

GRADUATE STUDENTS – Thompson Rivers University (TRU) -- Nicolas Fontaine. McMaster University -- Adrian Forsythe. UBC Okanagan -- Leah Rensel.

LABORATORY and CAPTIVE TRIALS – TRU: Soumya Ghosh, Robyn McArthur, Rory Mckerchar, Gabrielle Kam, Aaron Wong, Brandon Hayashi, Julianna Bissonnette, Chadabhorn Insuk, Monique Nijjer, Paul Grand, Brian Burciaca, Ethan Herndier, Susan Dulc, Maria Collins, Taylor Kutzley. UBC: Dr. Julian Davies and lab staff. McMaster: Heather Yoell. Other: Leanne Mills. In University of Victoria we thank lab technicians Andrew Geppert and Alloysius Thomas in Dr. Caroline Cameron's lab for probiotic production and helping to establish a bulk production protocol.

FIELDWORK – Main field Contractors were Aimee Mitchell and Chris Currie. Patrick Burke led the sampling and monitoring at Burrvilla study site.

SUPERVISORY/ADVISORY - For serving on the project's Advisory Committee we thank: Dr. Glenna McGregor, Dr. Helen Schwantje, Dr. Dave Sedgman, Dr. Purnima Govindarajulu, Orville Dyer, Mandy Kellner, Dr. Leigh Anne Isaac, Dr. Craig K.R. Willis, Dr. Yvonne Dzal. Supervisors of students: Dr. Naowarat (Ann) Cheeptham (TRU), Dr. Jianping (JP) Xu (McMaster University), Dr. Cori Lausen (adjunct, TRU), Dr. Mark Patkeau (Committee Member, TRU), and Dr. Karen Hodges (UBC Okanagan).

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BACKGROUND

White-nose syndrome (WNS) has caused unprecedented mortality (>90% for some species) in hibernating bat populations in eastern North America, and the causative pathogen, *Pseudogymnoascus destructans* (*Pd*), continues to spread (USFWS 2021). In 2016, WNS made a giant leap into Washington (from Kentucky; Thapa et al. 2021), and has been found there in most subsequent years. It is now also in Montana for the first time in 2020. Without treatment/prevention, WNS is poised to possibly devastate hibernating bat populations in western Canada. British Columbia, with the highest bat species richness in Canada, could see up to 14 species impacted, two of which are already SARA-listed as Endangered (ECCC 2018).

In B.C. where nearly 20% of all small mammal species are bats, white-nose syndrome (WNS) puts biodiversity at risk. To date, most proposed chemical/biological 'treatments' involve spraying anti-fungal agents within WNS-affected hibernacula (Muller et al. 2013; Cornelison et al. 2014; Hoyt et al. 2015; O'Donoghue et al. 2015; Zhang et al. 2015; Cheng et al., 2016; Palmer et al. 2018) to target the causative agent, *Pd*. However, winter ecology, including hibernation sites, of most bat species in the Pacific Northwest remains poorly known and treatments that do not require knowledge of bat hibernacula are needed (e.g., Fletcher et al. 2020). Lack of knowledge of winter bat roosts challenges prospects for opportunities for treatments of infected bats at hibernacula (Tobin et al., 2017). An alternative strategy is to target maternity colonies for which an increasing number of locations are being identified, especially for building-roosting bats (e.g., Canadian national database of maternity roosts [www.batwatch.ca]; BC Community Bat provincial database [www.bcbats.ca]).

Bat species that roost in buildings and bat boxes in the Pacific Northwest are largely Yuma Myotis (*Myotis yumanensis*) and Little Brown Myotis (*M. lucifugus*), the two species accounting for the most WNS mortalities in western North America to date (Washington Department Fish and Wildlife 2019). Most if not all bat box or building roosts of these two widespread species contain hundreds or thousands of individuals, many of them reproductive adult females and their offspring (Kellner 2019 a,b). Citizen science efforts could be mobilized to deploy WNS prevention 'treatments' at many roosts, saving what is likely a biologically significant number of bats of these two species. Our work, and recent published findings, support probiotics as an effective approach to reducing WNS mortality (Hoyt et al. 2019a).

Our research group consisting of Dr. Naowarat Cheeptham (Thompson Rivers University), Dr. Jianping Xu (McMaster University), Dr. Karen Hodges (University of British Columbia Okanagan), Dr. Cori Lausen (Wildlife Conservation Society (WCS) Canada), graduate students Nicolas Fontaine, Adrian Forsythe and Leah Rensel, and field contractors Aimee Mitchell and Chris Currie (South Coast Bat Conservation Society) have worked closely with a project advisory committee (see Acknowledgements) to develop, test and now pilot a probiotic aimed at reducing the mortality caused by WNS.

We developed a probiotic cocktail of 4 anti-*Pd* bacteria which inhibit the germination and growth of *Pd*. Sourced from bats swabbed in B.C., this probiotic consists *Pseudomonas synxantha*, Strains A and B; *P. azotoformans*; and *P. antarctica*. These anti-*Pd* microbes were sourced from Big Brown, Townsend's

Big-eared and Long-eared Myotis bats in southern BC. Details can be found in Fontaine et al. (2019) and WCS Canada (2020), reports submitted to BC Ministry of Environment and Climate Change Strategy.

Since 2017, we have completed multiple stages of this project. Starting in 2017, we isolated anti-*Pd* microbes sourced from bat wings and identified one potential probiotic bacteria, which we applied to bats in our first captive trial. The first captive trial in 2018 was to confirm that we could successfully keep Yuma Myotis in captivity, and that a sustained change in wing microflora could be achieved. By 2019 we had a final probiotic cocktail that consisted of 4 bacteria that worked synergistically to inhibit *Pd*. We tested this probiotic in our second (2019) captive trial. Our objectives of this second trial were to determine that this probiotic was safe for bats, to develop an applicator, and to verify that the probiotic could be transferred to bats via their roost substrate. We developed an effective application method of spraying a mist of water followed by probiotic-laden clay powder onto the roosting substrates. We also conducted one winter hibernation simulation experiment in fall of 2019 using a subset of bats that had spent the summer in our captive trial. This simulation experiment examined the response of the probiotic bacteria to hibernation conditions (cold, humid).

In August 2019 we applied probiotic at roosts of two of the four maternity colonies that had been identified and previously studied to obtain baseline ecological information. Swab sampling of bats returning in spring 2020 was not possible due to COVID-19 field restrictions. We applied Probiotic again in late summer of 2020, although roost use was different at one of our treatment sites due to a temporary change made by BC Hydro in their infrastructure. This meant that fewer bats were likely to have been inoculated by the roosting substrates. We sampled bat wings in 2019 and 2020 and confirmed that wild bats are receiving the probiotic on their wings before departing maternity roosts at the end of summer. We have also swab-sampled roosting substrates to monitor presence of probiotic each year; in spring 2019, no probiotic could be found on the roosting substrates after winter, suggesting that the probiotic bacteria may not survive winter in bat boxes. We have swabbed substrates again (as of March 2021) but results are pending.

In fall 2020 we conducted one preliminary ex vivo explant test of anti-*Pd* effectiveness on bat wing tissue; this was a pilot experiment to establish methodology that would allow us to properly quantify spore inhibition by the probiotic cocktail. Pending final scanning electron microscopy results, the experimental design will be finalized and the experiment conducted. Due to funding limitations, all metagenomics swabs for this project had been frozen for processing at a later date. After securing funding in early 2021, we sent these swabs for processing to Integrated Microbiome Resource (IMR) at Dalhouse University. This lab determined that the samples were not of sufficient quality to amplify DNA. New wing metagenomics samples will be taken in 2021 in the field so that we can describe wing microflora of bats' wings prior to and following probiotic inoculation.

With the exception of the final ex vivo explant experiment, most of the work remaining to be conducted as part of this project is in the field. The four field study sites we selected are in the southwest region of BC where WNS may be imminent. As of March 2021, *Pd* had not been detected in BC. We continue to monitor bats at all four study sites (13+ roosts in total), and as of April 2021 will have a new PIT tag (Passive Integrated Transponders) reader system at the fourth study site. There will also be one more

system installed at Alice Lake Provincial Park. With all PIT tag readers in place, we are poised to collect data for individual bats including number of days roosting on probiotic inoculated roost substrates, and annual return. Our long term goal is to assess differences in overwintering survival between treated and untreated colonies should WNS/Pd be detected in coming years.

Here we report major results to date with emphasis on 2020 activities, and provide a list of 2021 activities.

METHODS

STUDY SITES

The WNS probiotic prophylaxis project conducted field research in 4 study areas around the Lower Mainland, British Columbia, from 2019 to 2021: Alice Lake Provincial Park, Deas Island Regional Park, Colony Farm Regional Park, and BC Hydro owned properties in the Stave River watershed (Figure 1).

ALICE LAKE PROVINCIAL PARK

Alice Lake Regional Park is located at the northern outskirts of Squamish BC, approximately 60 km north of Vancouver BC. Research in this area centered on 3 four chambered bat maternity boxes, as well as various buildings in the Park Headquarters complex which are used as maternity roosts by Yuma myotis (*Myotis yumanensis*) and little brown myotis (*Myotis lucifugus*). The park is a mixture of fairly mature Cedar/Hemlock forest, front country recreation areas, and several small lakes.

We have installed PIT tag readers and antennas on the three bat boxes. An additional PIT tag system was installed in spring 2021 at one of the main building (roof) bat entrances. We also installed an Anabat Roostlogger for acoustic monitoring in summer to verify presence of bats given that many bats are not yet PIT-tagged.

In recent lab analyses, substrate samples from this site revealed large quantities of probiotic cells at one of the bat boxes. It was deduced that this was a lab error in preparation of the sham clay application that inadvertently contained probiotic. As such, although this site has been considered a control site through to 2020, it will now be considered a treatment site and probiotic will be applied here. A new similar control site will be needed immediately and it is hopeful this can be established during summer of 2021.

DEAS ISLAND REGIONAL PARK

Deas Island Regional Park is an island at the upstream edge of the estuary of the south fork of the Fraser River, in Delta BC. Research in this park is focused on bats using the attic of the Burrvilla Heritage home. The park is largely mown fields with a perimeter of Cottonwood dominated mixed forest. Due to constraints posed by the COVID-19 pandemic, activities in this park were entirely suspended in 2020, aside from colony counts conducted by Burke Mountain Naturalists (BMN) and the BC Community Bat

Project. One of the two PIT tag readers installed at the roost was not functioning for most of the summer season in 2020 – this was not discovered in time to fix the problem for the 2020 reproductive season.

COLONY FARM REGIONAL PARK

Colony Farm Regional Park (Colony Farm) is a 260 ha park located along the lower reaches of the Coquitlam River, and comprised mainly of old-field and riverine habitats, with a few limited developed areas. Considerable industrial, commercial and residential development surrounds the park, though there is excellent connectivity to other natural habitats via a network of greenways.

Research at the park is centered on a cluster of four large, five-chambered maternity bat-boxes at the western edge of the park. These boxes have been monitored by members of the Colony Farm Park Association (CFPA) and BMN for several years via colony counts and guano collection.

A heat-induced mortality event in 2018 has led to the naturalist group modifying the bat boxes in an attempt to avoid a repeat occurrence. In 2019, the 4 existing bat boxes were painted white to reflect heat, as well as having ventilation pipes installed at the top of the rear chamber. After consulting with the WNS probiotic project team in winter 2020, the decision was made to repaint the boxes a more neutral colour so they would still absorb enough heat to be useful to pregnant females in the early part of the maternity season. As well, one aging box that had deteriorated was removed and another moved to the north side of the cluster, to provide cooler roosts that bats could move to in case of extreme heat events. Two new, unpainted four-chambered maternity boxes were added to the cluster, one facing north, and the other south.

There are now five bat boxes in the cluster. A PIT reader system was installed at this site in spring 2021 which records bats as they enter/exit the cluster. Note that due to budget constraints, the antenna system wraps the entire bank of boxes and does not record which box from which the bat is emerging.

STAVE LAKE WATERSHED

The Stave Lake watershed study area (Stave) is comprised of two BC Hydro owned properties – the Hayward Lake Recreation Area (Hayward) and the Stave Lake Lodge (Stave Lodge), located 2.25 km apart along the shores of Stave and Hayward Lakes.

At the Hayward, bats are roosting in a single four-chambered maternity box hung on the wall of a wooden maintenance building, as well as the walls and roof of a caretaker's residence 300 m away. Both roosts are in developed front country areas, which receive relatively heavy foot traffic by park users.

The Stave Lodge is an older building used for group rentals, weddings etc. It is gated and receives no use from the public except during bookings, which were eliminated entirely during the 2020 bat maternity season due to the Coronavirus pandemic.

There are two main roosts at the Stave Lodge site: The first is a basement closet housing three large hot water heaters, which bats access through small cracks in the exterior siding. The hot water heaters

generally keep the room at ~30-35° C. During the 2020 maternity season, the water pump supplying the water heaters failed, causing temperatures to return to ambient. BC Hydro staff have been unable to repair the pump, but this is planned for late spring 2021.

The other primary roosts are two four-chambered maternity boxes, hung on the exterior of the lodge roughly 20 -30 feet from the exit for the interior roost. Bats also occasionally use other areas of the building, which is riddled with gaps and holes in the siding.

We have installed PIT tag readers/antennas on the bat boxes as well as over the crack which provides the main entrance to the hot water closet.

Data from the PIT tag readers and banded bats at these sites have shown that bats frequently move between all these roosts at Hayward and the Stave Lodge, and therefore we consider it to be a single population.

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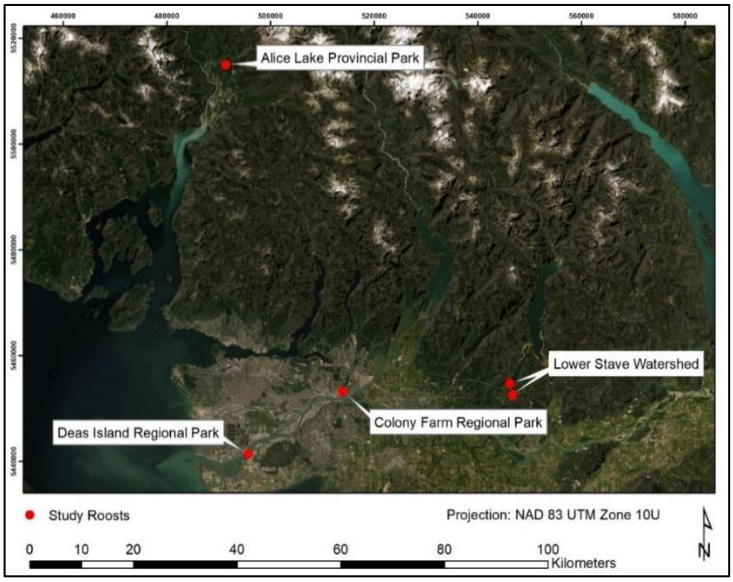

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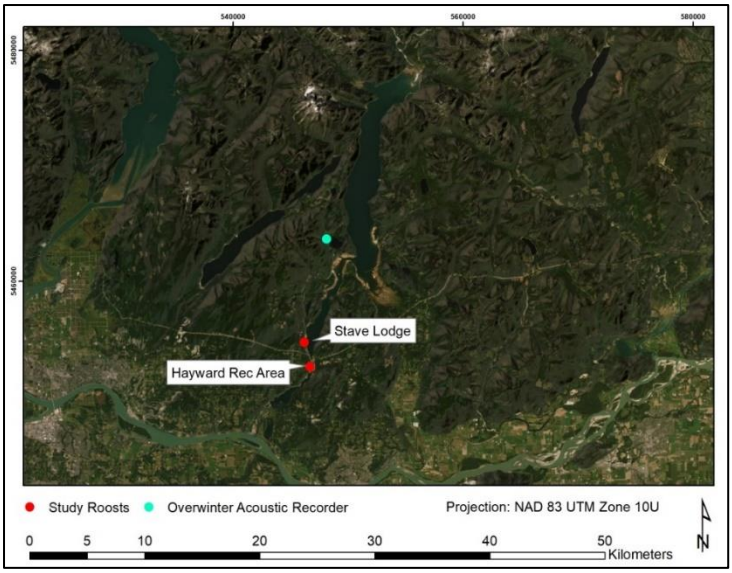

Figure 1. Study sites. A. Bat maternity colony monitoring sites in the Lower Mainland; B. Location of the Wildlife Acoustics SM2Bat deployed to monitor overwinter activity near potential hibernation habitat.

BAT CAPTURE

We monitored known maternity roosts at three sites across the Lower Mainland (Colony Farm Regional Park, Stave Lake BC Hydro, and Alice Lake Provincial Park) between March and November 2020 and captured bats during six sessions. Bats were not captured at Deas Island Regional Park because of COVID-19 restrictions (mist net capture was required at this site, but only harp traps had been approved by the province). The species and reproductive status were determined for each bat, and standard morphometrics recorded. We swabbed the wings of selected bats for analysis of the wing microbiome, including the presence of *Pd* on bats captured in early spring. Each female bat was implanted with a PIT tag, and we used tag readers to monitor bat presence within the roosts. Male bats were banded instead of PIT tagged.

COVID-19 AND WHITE-NOSE SYNDROME HYGIENE PROCEDURES

Due to concerns regarding Human-to-Bat and Human-to-Human transmission of the SARS-CoV-2 virus, we introduced several hygiene procedures in addition to standard WNS hygiene protocols. These procedures followed the recommendations developed by the Canadian Wildlife Health Cooperative, in conjunction with input from the BC Center for Disease Control (Erin Fraser), and 2 provincial veterinarians (Dr. Glenna McGregor and Dr. Helen Schwantje). Those recommendations in turn follow a guidance document released by the IUCN SSC Bat Specialist Group in June 2020 (CWHC 2020; IUCN 2020).

As well, we followed protocols set by the BC Center for Disease Control, local health authorities, and Worksafe BC to prevent transmission between researchers, park staff and park users (BC CDC 2020a; Worksafe BC 2020).

These procedures were revisited and adapted as new information became available, particularly in light of the state of the illness in the province. These protocols included:

- Following all current provincial and regional health authority guidelines (i.e. no international travel, maintaining a “bubble” of close contacts)
- Limiting research activities, particularly handling of bats, to essential activities
- Limiting the number of researchers to essential staff only, typically 2-4 researchers
- Researchers conducted a self assessment before traveling to site, and did not participate if any COVID symptoms, or any known exposure to COVID-19 had occurred
- Carpooling with anyone outside of researcher’s household bubble was avoided, and when necessary, followed safety procedures for carpooling set out by the BC CDC (BC CDC 2020b).
- Maintaining 2 m distance between researchers, and any Metro Vancouver Parks staff or park users that are in the vicinity
- Limiting bat handling time (less than 15 minutes per individual)
- Frequent hand washing or sanitizing
- Sanitizing all equipment between uses with 70% alcohol for 30 seconds

- Wearing PPE, including:
- A fresh set of long-sleeved clothes put on after arriving at the field site, and then removed and washed after handling bats
- Nitrile gloves
- Surgical masks
- Using compressed air, a squeeze bottle or handling techniques to get bats to release their bite, instead of blowing on the bat as was standard handling procedure prior to SARS-CoV-2
- In addition to the new COVID-19 procedures, we also continued to follow the standard provincial White-nose Syndrome hygiene protocols available at:

  (http://www2.gov.bc.ca/assets/gov/environment/plants-animals-and-ecosystems/wildlife-wildlife-habitat/wildlife-health/wildlife-health-documents/decon_protocol_for_bat_work_april_2016.pdf)

CAPTURE TECHNIQUES

Due to COVID-19 concerns, capture was limited to the use of harp traps and not mist netting, to circumvent the need to blow on bats to release mist-net fibres from their mouths. Standard trapping methods were used as described in the provincial RISC standards (1998) and Kunz & Kurta (1988). Custom-made harp traps were attached to the exits of occupied bat boxes, and captured bats were immediately removed from traps and placed in individual cotton bags prior to processing. Lactating females were offered water from a dropper. We processed and released bats directly into their roost structure within 3.5 hours of their initial capture.

MORPHOMETRICS, DEMOGRAPHICS, SPECIES IDENTICFICATION, REPRODUCTIVE ASSESSMENT

After capture, we visually inspected each bat for general body condition, and determined sex. Juveniles were differentiated from adults by examining the epiphyseal cartilage on the fifth finger, as well as secondary characteristics including tooth class, size and the lack of scarring on the wing membrane. We then measured forearm length, and weight for each bat. To ensure bats were released as soon as possible, other standard measurements (ear and pinnae length, nose width, thumb and tibia length etc.) were not taken except to differentiate any species that did not present as either Yuma Myotis *or* Little Brown Myotis. In individuals with intermediate characteristics between Little Brown Myotis and Yuma Myotis, we examined the anterior skull shape, fur length and sheen. We then conducted a “bag test” to record echolocation frequency of each bat before release, to further aid in species differentiation.

We determined reproductive condition of female bats by first gently squeezing the abdomen to determine if a bat was obviously pregnant. The standard method of examining nipples by blowing to part the fur was not possible due to COVID-19 precautions, and was instead accomplished using a wetted cotton swab. We attempted to express milk to determine if a bat was lactating, and noted the general characteristics of the nipple to assess whether a bat had previously nursed a pup. Male reproductive condition was determined by feeling for testes, and noting the condition of the epididymis.

PIT TAGGING

We implanted female bats with a Biomark MiniHTP8 8mm passive integrated transponder (PIT) tag. The process of implantation was as follows:

- Clean injection site with a swab soaked in 70% alcohol
- Pinch skin into a "tent" and insert only the tip of the transponder needle under the skin
- Depress plunger of applicator syringe to inject transponder under the skin; remove needle
- Gently massage the transponder away from the puncture
- Place a drop of VetBond on the puncture hole and allow to dry for up to one minute
- Monitor the individual 15-30 minutes post-injection

Females that were considered too small (< 4.3 g), in poor body condition, or those in which implantation failed, did not receive PIT tags, and were instead banded on their right forearm using Porzana split-ringed bands. Males were not PIT tagged, as they were considered unlikely to return to their natal roost, and were banded on their left forearm.

BAT CAPTURE

We monitored known maternity roosts at three sites across the Lower Mainland (Colony Farm Regional Park, Stave Lake BC Hydro, and Alice Lake Provincial Park) between March and November 2020 and captured bats during six sessions. Bats were not captured at Deas Island Regional Park because of COVID-19 restrictions (mist net capture was required at this site, but only harp traps had been approved by the province). The species and reproductive status were determined for each bat, and standard morphometrics recorded. We swabbed the wings of selected bats for analysis of the wing microbiome, including the presence of *Pd* on bats captured in early spring. Each female bat was implanted with a PIT tag, and we used tag readers to monitor bat presence within the roosts. Male bats were banded instead of PIT tagged.

COVID-19 AND WHITE-NOSE SYNDROME HYGIENE PROCEDURES

Due to concerns regarding Human-to-Bat and Human-to-Human transmission of the SARS-CoV-2 virus, we introduced several hygiene procedures in addition to standard WNS hygiene protocols. These procedures followed the recommendations developed by the Canadian Wildlife Health Cooperative, in conjunction with input from the BC Center for Disease Control (Erin Fraser), and 2 provincial veterinarians (Dr. Glenna McGregor and Dr. Helen Schwantje). Those recommendations in turn follow a guidance document released by the IUCN SSC Bat Specialist Group in June 2020 (CWHC 2020; IUCN 2020).

As well, we followed protocols set by the BC Center for Disease Control, local health authorities, and Worksafe BC to prevent transmission between researchers, park staff and park users (BC CDC 2020a; Worksafe BC 2020).

These procedures were revisited and adapted as new information became available, particularly in light of the state of the illness in the province. These protocols included:

- Following all current provincial and regional health authority guidelines (i.e. no international travel, maintaining a "bubble" of close contacts)
- Limiting research activities, particularly handling of bats, to essential activities
- Limiting the number of researchers to essential staff only, typically 2-4 researchers
- Researchers conducted a self assessment before traveling to site, and did not participate if any COVID symptoms, or any known exposure to COVID-19 had occurred
- Carpooling with anyone outside of researcher's household bubble was avoided, and when necessary, followed safety procedures for carpooling set out by the BC CDC (BC CDC 2020b).
- Maintaining 2 m distance between researchers, and any Metro Vancouver Parks staff or park users that are in the vicinity
- Limiting bat handling time (less than 15 minutes per individual)
- Frequent hand washing or sanitizing
- Sanitizing all equipment between uses with 70% alcohol for 30 seconds
- Wearing PPE, including:
- A fresh set of long-sleeved clothes put on after arriving at the field site, and then removed and washed after handling bats
- Nitrile gloves
- Surgical masks
- Using compressed air, a squeeze bottle or handling techniques to get bats to release their bite, instead of blowing on the bat as was standard handling procedure prior to SARS-CoV-2
- In addition to the new COVID-19 procedures, we also continued to follow the standard provincial White-nose Syndrome hygiene protocols available at:

  (http://www2.gov.bc.ca/assets/gov/environment/plants-animals-and-ecosystems/wildlife-wildlife-habitat/wildlife-health/wildlife-health-documents/decon_protocol_for_bat_work_april_2016.pdf)

CAPTURE TECHNIQUES

Due to COVID-19 concerns, capture was limited to the use of harp traps and not mist netting, to circumvent the need to blow on bats to release mist-net fibres from their mouths. Standard trapping methods were used as described in the provincial RISC standards (1998) and Kunz & Kurta (1988). Custom-made harp traps were attached to the exits of occupied bat boxes, and captured bats were immediately removed from traps and placed in individual cotton bags prior to processing. Lactating females were offered water from a dropper. We processed and released bats directly into their roost structure within 3.5 hours of their initial capture.

MORPHOMETRICS, DEMOGRAPHICS, SPECIES IDENTICFICATION, REPRODUCTIVE ASSESSMENT

After capture, we visually inspected each bat for general body condition, and determined sex. Juveniles were differentiated from adults by examining the epiphyseal cartilage on the fifth finger, as well as secondary characteristics including tooth class, size and the lack of scarring on the wing membrane. We then measured forearm length, and weight for each bat. To ensure bats were released as soon as possible, other standard measurements (ear and pinnae length, nose width, thumb and tibia length etc.) were not taken except to differentiate any species that did not present as either Yuma myotis *or* little brown myotis. In individuals with intermediate characteristics between little brown myotis and Yuma myotis, we examined the anterior skull shape, fur length and sheen. We then conducted a "bag test" to record echolocation frequency of each bat before release, to further aid in species differentiation.

We determined reproductive condition of female bats by first gently squeezing the abdomen to determine if a bat was obviously pregnant. The standard method of examining nipples by blowing to part the fur was not possible due to COVID-19 precautions, and was instead accomplished using a wetted cotton swab. We attempted to express milk to determine if a bat was lactating, and noted the general characteristics of the nipple to assess whether a bat had previously nursed a pup. Male reproductive condition was determined by feeling for testes, and noting the condition of the epididymis.

PIT TAGGING

We implanted female bats with a Biomark MiniHTP8 8mm passive integrated transponder (PIT) tag. The process of implantation was as follows:

- Clean injection site with a swab soaked in 70% alcohol
- Pinch skin into a “tent” and insert only the tip of the transponder needle under the skin
- Depress plunger of applicator syringe to inject transponder under the skin; remove needle
- Gently massage the transponder away from the puncture
- Place a drop of VetBond on the puncture hole and allow to dry for up to one minute
- Monitor the individual 15-30 minutes post-injection

Females that were considered too small (< 4.4 g), in poor body condition, or those in which implantation failed, did not receive PIT tags, and were instead banded on their right forearm using Porzana split-ringed bands. Males were not PIT tagged, as they were considered unlikely to return to their natal roost, and were banded on their left forearm.

PROBIOTIC

INOCULATION

Application of probiotic involved first spraying the roost surface with a fine mist of distilled water, and then spraying probiotic-clay using a bicycle pump attached to a tube in which the probiotic was placed. The clay was carefully sprayed onto each roost surface to ensure as even and thorough coverage as possible. We used 30 g of clay powder for each bat box chamber, and the same amount per area for the roost inside the building at the Stave Lodge. Each 30 g dose contained 375 million freeze-dried cells of each anti-*Pd* bacteria.

For field-testing the probiotic, roosts at Colony Farm (4 bat boxes) and Stave Lake (3 bat boxes, 1 building) were inoculated with probiotic laden clay powder in early August 2019 as a pilot initiative. In 2020 the following probiotic applications occurred:

Stave Lodge and 2 boxes – 18 July: Although probiotic was applied on all roosting surfaces. Note that the boiler room of the Stave Lodge was largely abandoned in 2020 due to hot water heaters being shut off. Probiotic was applied on the inside roosting surfaces and also on the outside at the entrance. Application was done during daylight hours as there were no bats roosting in the boxes.

Hayward bat box – 7 August: This site was treated later because in July there were always a few bats lingering in the boxes, even after emergence. Bats were not in the box at the time of application, but were seen roosting in this box again when it was checked 3 days after application. This was the only site where it can be confirmed bats were exposed to the probiotic prior to swabbing wings of captured bats later in August.

Colony Farm – 19 July, 26 July, 17 August and 12 Sept. Four boxes in the cluster, so applied to whichever ones they weren't using. They were using all boxes, then 3 out of 4, 2 of 4, then one. So

can't guarantee that they ever went back into inoculated boxes for very long (we released captures into there, but who knows how long they stayed.

Alice Lake (sham clay) – Although not a treatment site, we sprayed clay powder into the bat boxes at this site such that this control site was put through the same procedure as the treatment sites. It was our understanding that there was no probiotic in the clay. These sham applications occurred on 3 Aug, after emergence when there were no bats in the boxes. When these boxes were inspected one week later, there were ~5 bats using boxes. Additionally, there were PIT tag records showing that bats continued to use the boxes despite the clay application. This is positive as it indicated the disturbance to the roost was minor. As of June 2021, however, substrate swabs of this site revealed that probiotic was accidentally included in one of the clay powder vials used at one of the bat boxes. As such, this site will not be considered a control site moving forward and a new control site is being sought. This site will now be treated with probiotic and become a 3rd treatment site.

To confirm that any probiotic detected on bat wings following overwintering (i.e. those inoculated during 2019 pilot experiments), we sterilized the two treatment site roosts (Colony Farm and Stave Lake) with 70% alcohol in early spring 2020 before bats returned, prior to swabbing for microbiota – see below. Because some Yuma myotis returned to their Stave Lake roost in February 2020, we decided in 2021 to sterilize the roosts in early February, which coincided with our last swab sampling of the roost surfaces to look for persisting probiotic cells.

MICROBIOTA SWABS

The wings of adult female bats were swabbed with polyester-tipped swabs that were moistened in a 0.15M NaCl solution, which were placed in 2 mL centrifuge tubes with distilled water, and then frozen as the earliest opportunity. Bats were selected for swabbing based on a subset as time allowed during processing. Adult females were targeted at all study sites.

We swabbed along the forearm and down each finger on the dorsal and ventral sides of one wing to ensure the area swabbed on each wing was consistent and measurable for use in qPCR analysis. This process was completed on a subsample of bats before and after application of probiotic to roost substrates, to verify that probiotic was transferred to roost occupants. We are swabbing in spring to determine whether the probiotic component of the wing microflora is retained over the hibernation period.

Each treated roost substrate was swabbed along 30 cm of each bat box chamber or other roost surface. These samples were taken in spring 2020, and then again 1 month after inoculation, and once more in spring 2021.

SEARCHING SOIL DATABASES TO DETERMINE HOW COMMON THE BACTERIA ARE ACROSS CANADA

Using genetic sequence data, we did a preliminary search of several easily accessible soil sequence collections/databases: “Bog forest soil microbial communities from Calvert Island 51.62, -128.09; British Columbia; Canada - ECP12_OM2 metagenome”; “Farm metagenomes conventional and organic” for soils at 53.5 -

112.06, 50.34 -113.77, 50.35 -112.06; “Lichen metagenomic sequence reads from Peltigera sp.” At 49.5993 -125.496; “Mahoney Lake water and sediment samples” 49.2833 -119.583; “reference metagenomes” from 60.1987 -125.513 and 49.9543 -116.516; “Sandy soil microbial communities from University of British Columbia; Vancouver; Canada - MeOH1_35cm_T4_195 metagenome” from 50.4032 -113.261; and soil metagenomes at 53.93 -116.58.

PIT TAG READERS

To monitor year to year survival, we previously installed PIT tag readers on each available roost (see Study Sites). The antennae at Stave Lodge was upgraded in 2020. As of 2020, there were a total of eight roosts equipped with PIT tag readers: three boxes at Alice Lake, three boxes at Stave (two at the Lodge and one at Hayward Recreation Area), one building at Stave, and one building at Deas Island. At this latter site there are two readers, one for Biomark, the current PIT tag system being used at all study sites, and an older Trovan reader because some bats had received this type of PIT tag prior to the start of our study.

Each antenna was periodically tuned using Biomark’s Bioterm software, to reduce electromagnetic noise that could affect the ability of the reader to record tags passing the antenna.

Each site was fitted with a Biomark IS1001 reader which recorded each time a tagged bat entered or left the roost. Data were analysed using Biomark Log File Viewer and Tag Manager software.

Although bats were PIT tagged at the Colony Farm Regional Park roost, a reader had not yet been installed in 2020. Installation is currently planned for spring 2021.

ROOST MICROCLIMATE MONITORING

Temperature and relative humidity within roosts were monitored using Onset HOBO MX2302A or U23 Pro V.2 dataloggers. We installed two dataloggers on each bat box, with probes installed through holes in the front and rear chambers. Ambient temperatures were monitored with the same units installed in solar shields hung on the north side of nearby trees. We similarly monitored conditions inside the building roost at the Stave Lake Lodge.

OVERWINTER ACOUSTIC MONITORING

To monitor bat activity over winter, we installed at least one acoustic recorder at each of three sites (Stave Lodge, Hayward, and Alice Lake). At all three sites we deployed Anabat Roostloggers: there was a standard model roostlogger (records out to ~8-10 m) near the roost entrances at Alice Lake, Hayward box, and Stave Lodge. A high sensitivity model was used to detect bats out as far as an estimated 20-30 m and was deployed on up on the buildings at Stave Lodge and Hayward Rec Site. An SM4Bat (Wildlife Acoustics) detector was additionally deployed at Hayward on a nearby roof-top, which is likely to pick up bats flying along Stave Lake in winter or during spring migration. An SM2Bat running on solar power was deployed in the north part of Stave Lake at a site with potential hibernation habitat 9 km north of Stave Lodge, next to Sayres Lake (Figure 1B). Acoustic data were analysed using Anabat Insight and Analook Software from Titley Scientific, and Kaleidoscope Pro from Wildlife acoustics.

UPTAKE AND PERSISTENCE OF PROBIOTIC ON BATS

SUMMER - CAPTIVE BATS - SECOND CAPTIVE TRIAL – METHODS REVIEW

In May 2019 we began our second captive trial to test the effects of probiotic application on bat health and test for uptake and retention of probiotic cells from a bat box surface onto live roosting bats.

We captured 20 Yuma myotis, all of which were male except for one non-reproductive female. These bats were housed at the BC Kamloops Wildlife Park. Bats were split amongst three enclosures with one bat box in each enclosure, each representing a different experimental group. Two groups were 'test' groups and one group was a control. We applied 250 million cells of each probiotic bacteria (for a total of 1 billion bacteria applied) to bat boxes. All bats were tended to daily to ensure fresh water and food (mealworms) supplies. Once each week, bats were examined by a veterinarian and weighed, and wings were swabbed for examination of microflora and specifically to test for the presence and concentration of the four probiotic bacteria. See Processing of Swab Samples below.

The Control group (n = 6) did not have their bat box roost substrate coated with probiotic. Treatment 1 (n = 7) was exposed to multiple, within-year probiotic applications, with freshly applied probiotic on their bat box roost surfaces on 24 May, 24 and 29 June,. The Control and Treatment 1 bats were euthanized in July 2019 so that histology analysis could be completed in time for permitting of the field pilot in early August 2019.

Treatment 2 (n = 5) was exposed to a single probiotic application, with the probiotic applied only once through treatment of their bat box roosting substrate on 15 June 2019. Treatment 2 bats continued to be cared for and swabbed throughout summer of 2019. The bat box treated with probiotic was removed from the enclosure on 27 July 2019, and replaced with a box with no probiotic to simulate a limited exposure to probiotic that may occur in the field. The bats continued to be housed until mid-October 2019 when they were swabbed to determine concentration of probiotic cells and their viability (see Processing of Swab Samples below). This completed this portion of the experiment. These bats were then moved into a modified fridge for the hibernation simulation experiment (see below).

In this report we present all Treatment 2 group swab sample results because in previous reporting we were unable to report on the *P. antarctica* bacterial concentration because of difficulties with the primer annealing during qPCR procedures. These samples, taken June-October 2019, have now been analyzed and concentrations of all four probiotic bacteria are reported below (see Results). Concentration of each bacteria were averaged between all captive bats (n = 5) in Treatment 2, and presented relative to area of the skin in cm^2.

WINTER - CAPTIVE BATS - HIBERNATION SIMULATION EXPERIMENT – METHODS REVIEW

On 30 September, 2019, we treated the bat box of the Treatment 2 bats in preparation for the hibernation simulation experiment that was to begin in October. However, the start date of this experiment was delayed as an appropriate hibernation chamber was constructed. As such, we did an additional probiotic treatment of the bat box on 7 October. Bats were subsequently removed from their

enclosure at the Wildlife Park on 1 November 2019 and transferred to Kamloops where their acclimation into the fridge then began 3 November. The actual experiment began 16 November at which time bats were left for periods of ~10 days before removing briefly to swab sample, and offer water and mealworms. The hibernation simulation experiment ended 21 December 2019.

The hibernation chamber was a modified glass door wine fridge of approximate dimensions: width of 23.4", height of 33.7" and depth of 24.6". The temperature within the fridge was maintained between 4.5-8^{o}C and 90-100% humidity to replicate Yuma myotis hibernating environmental conditions. A reptile humidifier was used to achieve high humidity and an electronic on/off timer scheduled the device turning on. Temperature and humidity were monitored using an external HOBO thermostat and conditions were modified upon observation. A mesh screen was attached to the floor and right wall portions of the fridge using silicone. Mesh was placed at the entry to a cloth roosting pouch, a tin food dish, and a small water dish. Small sponges and marbles were placed inside the water dish to prevent accidental drowning or roosting within the water dish. Not disturbing the bats was a high priority, thus a wide-angle infrared camera was placed into the cooler. Researchers could monitor the bats activity by accessing video footage through a smartphone app. A temperature-sensitive transmitter was also affixed to each bat (Holohil) and VHF signal repetition rate was recorded by a Lotek SRX400 datalogger receiver to confirm torpid state of bats. The front of the cooler was covered with a thick blanket to prevent any light from disturbing the bats and the cooler's glass door prevented sound disturbances. Additionally, the room housing the fridge was kept dark. An oxygen tank was connected by an external hose and drilled into the cooler to prevent bat asphyxiation. Oxygen was monitored using an oxygen sensor and air levels were maintained between 19-24% oxygen using an external air tank. Once every ~10 days the door of the fridge was opened, bats were removed, weighed, warmed, swabbed (to test probiotic bacteria concentration) and then offered food (mealworms) and water. See Processing of Swabs section below.

PERSISTENCE OF PROBIOTIC IN ROOST STRUCTURES – BAT BOX EXPERIMENT – METHODS REVIEW

A four-chamber bat box was also treated with probiotic to determine how long probiotic clay would remain viable inside a bat box under typical hot summer conditions. No bats were involved, as the entrance was sealed off with mesh to prevent unwanted wildlife from roosting within it. It hung ~200m away from the control enclosure at the BC Kamloops Wildlife Park and was exposed to the same elements as our enclosures. The bat box was hung ~3 metres off the ground and faced the sunset (west) starting 17 May, 2019. Chambers were numbered starting with 1 as the back (immediately above the landing platform). The bat box was oriented on a pole such that the front chamber (1) faced west and part of the back chamber (4) was exposed to the east sun.

Four HOBO sensors were placed individually into each chamber of the bat house to measure temperature and relative humidity (no humidity sensor on chamber 4, just temperature). A HOBO datalogger was placed into each of the four chambers, 30 cm from the bottom opening of the box (see below) – all sensors were temperature and humidity, with the exception of chamber 4 which recorded temperature only.

On 20 July, we applied probiotic into each chamber (~30 cm up into the bat box). This experiment was terminated 25 August 2019.

Each clay treatment dose consisted of ~$2.5x10^6$ probiotic cells per chamber. We swabbed each chamber each week and treated the swabs as outlined in the Processing of Swab Samples section below.

PROCESSING OF SAMPLES

After swabbing a surface (bat wing or roost substrate), we placed the tips of the polyester swabs into Eppendorf tubes filled with 1ml of sterile water. These were subsequently refrigerated, and transported to Thompson Rivers University for further preparation before being shipped to McMaster University for analysis. These samples were examined for DNA through qPCR procedures.

As of 8 June 2019, we introduced an additional test to not only account for the DNA of the probiotic bacteria, but to also test if the bacteria were viable. To do this, we vortexed the Eppendorf vials containing the swabs and the swab tips were then removed. The 1 mL water sample was then divided in half: one half was placed into a separate Eppendorf containing 1mL of broth (Lysogeny Broth; LB broth), and the remaining half mL was frozen. The broth now containing half of the sampled cells was placed onto a shaker at 25°C on medium speed for 12 hours. Afterwards the broth was frozen and transported to McMaster University alongside our original frozen water samples for qPCR analysis. An increase in the DNA found in the broth versus water samples was indicative of viable cells. However, it was determined that this introduced error into the downstream qPCR's, possibly due to one or more of the following problems: 1. cell adherence onto swab tip; 2. unequal splitting of viable cells; 3. competition among wing microbes that were deposited into the rich nutrient broth; 4. inadequate incubation time depending on the starting cell concentration and state or rehydration (for cells from substrates). As such, we do not present results from broth here, but instead just water results. These water results may have experienced higher fluctuations in cell concentration (ie. higher variation in counts of cells) due to the procedural change to wash the swab tips prior to extraction. Alternative methods to testing viability of cells and to sampling wings and roost substrates, is forthcoming and will be employed in future sample collection and processing.

We used custom primers specific to each of the probiotic species to amplify DNA from each swab sample. We quantified the abundance of probiotic from each swab and as long as there was a known area that was swabbed, the results provided a per cm^2 quantification of concentration. All samples were run in triplicate and thus means of three qPCR results per swab sample are represented in most analyses, unless a replicate failed and was thus excluded from the mean.

EFFECTIVENESS OF PROBIOTIC AT INHIBITING *Pd*

PILOT EXPLANT EXPERIMENT

The purpose of the explant experiment is to test the *Pd* inhibition threshold of the anti-*Pd* cocktail bacteria on bat patagial tissue that is nourished to maintain cellular functions. This ex vivo experimental design allows the testing of the probiotic (challenged with *Pd* spores) on bat skin cells that are still

performing life reactions as a unit of skin tissue. The severed tissue explants are kept partially alive within specialized chambers that provide oxygen and nutrient uptake, thus preventing the associated interference of decomposition (see SOP in Appendix 2 for details of this experiment).

The goal of this pilot experiment was to establish the methodology for a full comprehensive explant experiment. This is a new technology from University of California Davis that has not been previously used for this purpose. We tested longevity of keeping tissue explants alive, and determined whether antibiotic would be needed, and whether this would interfere with the probiotic bacteria.

RESULTS

BAT CAPTURE

In 2020 we captured 299 individuals in two capture sessions at each of three sites (6 capture sessions total; Table 2 and Table 4). All bats were safely released back at their roost capture site within 3.5 hours of initial capture. One additional bat was captured at the Stave Lake Lodge on 15 March 2020 which was swabbed to test for the presence of *Pd*, and released. We implanted PIT tags in 205 bats (179 were adult females; Table 3), and banded 48 juvenile males. There were 43 bats captured that had been tagged in previous years. Three were released accidentally without marking.

SPECIES AND DEMOGRAPHIC COMPOSITION

Of the 299 bats captured (Table 1), the vast majority were Yuma myotis (271), with 5 individuals identified as little brown myotis. We were unable to conclusively identify to species 23 individuals as they had intermediate morphometric and/or acoustic characteristics between Yuma myotis and little brown myotis.

Unsurprisingly, the majority of bats captured were either adult females (62.2%) or juveniles born in 2020 (37.1%). Two adult males were captured at the Hayward Rec Site, two at Colony Farm Regional Park and one at Alice Lake.

In 2020, we swabbed the wings of 83 bats at Stave Lake, Alice Lake, and Colony Farm sites. The goal was to sample the wing microbiome for presence of the four probiotic bacteria. Swabs were taken from July through August 2020 (Table 2). One of these bats was a single capture in early spring that was wing swabbed for *Pd* surveillance. We also collected 68 swabs of the previously inoculated roosting substrates at Colony Farm and Hayward for detection of the 4 probiotic bacteria (using qPCR analysis; see Laboratory Methods). Of 35 bats sampled at Hayward (25 July, 14 August) 71% had significant concentrations of the probiotic bacteria present suggesting good coverage of probiotic at this site. Of 39 bats sampled at Colony Farm site 22 (56.5%) had significant probiotic on their wings. While we anticipated that Alice Lake bats would have no probiotic on their wings, as this is a control site, treated with sham clay only, we found that 3 of the 9 swabbed bats had significant amounts of probiotic. After further investigation and sampling of the boxes in spring 2021, we discovered that this site had in fact been inadvertently treated with probiotic, likely through a mislabelled vial at McMaster during probiotic

preparation. Probiotic application was delayed in 2020 due to COVID and less bats were sampled than was desirable; we anticipate higher sample numbers in 2021.

Swab samples were taken from as many bats as time allowed (Table 2), with the preference being to swab adult females and bats that were captured at sites where probiotic was applied and bats were subsequently seen roosting.

Table 1. Demographic distribution of bats captured at 3 lower mainland sites, July-October, 2020.

Site	Date	Adult Female	Juvenile Female	Adult Male	Juvenile Male	Total Captures
Hayward Rec Site	25-Jul-20	18	2	1	3	24
Hayward Rec Site	14-Aug-20	60	11	1	4	76
Hayward Site Total		78	13	2	7	100
Colony Farm Regional Park	18-Jul-20	46	1	1	0	47
Colony Farm Regional Park	1-Aug-20	42	29	1	25	96
Colony Farm Site Total		88	30	2	25	143
Alice Lake Provincial Park	12-Aug-20	17	16	0	15	48
Alice Lake Provincial Park	14-Oct-20	3	4	0	1	8
Alice Lake Site Total		20	20	0	16	56
Total		**186**	**63**	**4**	**48**	**299**

Table 2. Bats that were swab-sampled.

Site	2020 Date	Adult Females	Adult Males	Juveniles	Total
Colony Farm	18 July	40	0	0	40
Colony Farm	1 August	5	0	5	10
Alice Lake	12 August	13	0	4	17
Hayward	25 July	14	1	4	19
Hayward	14 August	27	0	10	37
					123

REPRODUCTIVE CONDITION

Of 186 adult females captured, 77% reproduced in 2020, and 22% had reproduced in one or more previous years, but showed no signs of successful reproduction in 2020 (Figure 2). Three individuals showed no signs of ever having reproduced, and were likely one year old females born in 2019.

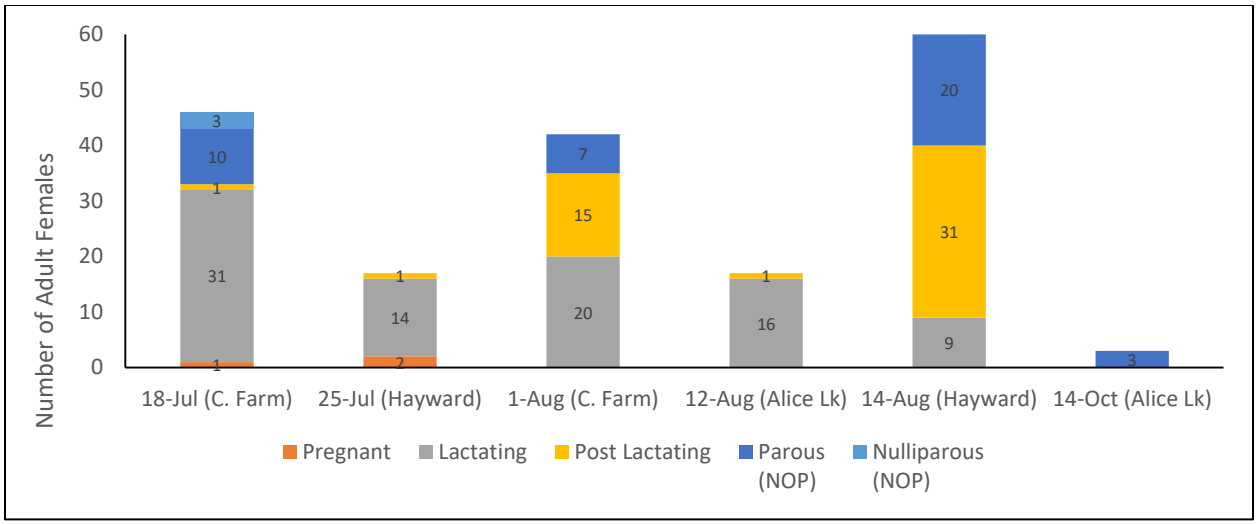

Figure 2. Reproductive condition of adult females captured at 3 lower mainland roost sites in 2020. NOP = not obviously pregnant. Parous = evidence of having reproduced in past. Nulliparous = no signs of current or past reproduction.

PIT TAG & BAND RECAPTURE

New individuals were PIT tagged at Stave Lake, Alice Lake and Colony Farm (Table 3). Burvvilla building roost at Deas Island Regional Park is not included in this summary as this site was not monitored in 2020 (entry to attic roost of residence did not occur due to COVID-19 restrictions); we hope this work resumes in 2021 and a summary of PIT tags will be provided in the next fiscal's report.

We implanted PIT tags in 179 bats in 2020 (Table 3). Recaptures are presented in Tables 4 and 5. Unfortunately, we had some difficulty keeping both PIT tag readers operational for the entire summer, which resulted in missing the presence of an unknown number of bats. All PIT-tagged bats re-sighted from previous years were females tagged as adults, except for 6 females tagged as juveniles in 2019 at the Stave site, and five males.

Two male recaptures were from Hayward bat box, and both had been originally identified as adults in 2019. One PIT-tagged individual was scanned by the reader on 13 March, then twice more in May and once in June. It was then physically recaptured 14 August. The other PIT-tagged male was scanned by the reader on 24 July and physically recaptured on 25 July.

Two male recaptures were from Colony Farm and both were physically recaptured 1 August. One had been banded in 2019 as an adult, the other was a juvenile at time of banding. We PIT-tagged them individuals to see if they show up in 2021.

One male was recaptured at Alice Lake – it was banded as a juvenile in 2018, was physically recaptured in 2019 and PIT-tagged at that time, and the reader picked it up on 16 April, after which time it was not recorded again.

All PIT-tag implantations at Alice, Stave and Colony Farm are listed in Table 4. PIT tag recaptures (re-sightings made by automated reader) for all adult females, are listed in Table 5A. And Table 5B provides a breakdown by adult females for each species and species-group. The dates of the first and last PIT tags scanned during the year are presented in Table 6, and coincided somewhat closely with acoustic recordings (see below), at the Stave sites, with bats occupying or abandoning the roosts within 7 days of the first or last recordings of *Myotis* spp. in 2019 and 2020.

Table 3. PIT tags implanted in female bats captured in 2020.

Female Age Class	Stave Lake	Alice Lake	Colony Farm
Adult	52	8	68
Juvenile	11	14	26
Total	63	22	94

Table 4. All bats captured and PIT-tagged or banded in 2019 and 2020.

	ALL BATS									
Study Area	**# Bats PIT Tagged in 2019**	**# Bats Only Banded in 2018-2019**	**Total # Bats Marked 2018-2019**	**Bats Captured (Physically) 2020**	**# 2018-2019 Marked Bats Physically Recaptured in 2020**	**Percent 2018-2019 Marked Bats Physically Recaptured in 2020**	**# 2019 PIT Tags Re-sighted (Scanned) in 2020**	**Percent 2019 PIT Tags Re-sighted**	**# 2020 New Bats Marked**	**Percent Bats Captured in 2020 That Were Previously Unmarked**
Alice	337	137	474	56	10	2.1	157	46.6**	35	82.14
Stave	494	103	597	101	26	4.4	312	63.2**	68	74.26
Colony Farm	0	223	223	142	7	3.1	NA	NA	102	95.07
Totals	831	463	1294	299	43		469		205	

See ** in Table 5.

Table 5. 2020 PIT tag and band recaptures from female bats tagged in 2018-2019: A. all females; B. females differentiated by species identification. Note that in the 2020 PIT tag data there were 18 tags that were recorded by the reader that were not in the capture database; these unknown tags have yet to be identified. MYLU = little brown myotis; MYYU = Yuma myotis.

A. RESIGHTING/RECAPTURE - ONLY ADULT FEMALES *ALL SPECIES*

Study Area	# Females PIT Tagged in 2019	# Females Banded Only in 2018-2019	Total # Females Marked 2018-2019	Adult Females Captured (Physically) 2020	# 2018-2019 Marked Females Physically Recaptured in 2020	% 2018-2019 Marked Females Physically Recaptured in 2020	# 2019 PIT Tags Resighted (Scanned) in 2020	% 2019 PIT tags Resighted**	# 2020 New Females PIT Tagged*	% 2020 Adult Females Captured that were Previously Unmarked
Alice	337	137	474	20	10	2.1	156	46.3	22	50
Stave	494	103	597	78	26	4.4	312	63.2	63	66.67
Colony Farm	0	156	156	88	7	4.5	NA	NA	94	92.05
	831	396	1227	186	43		468		179	

B. RESIGHTING/RECAPTURE - SPECIES BREAKDOWN (PIT Tagged Adult Females Only)

Study Area	# MYLU PIT Tagged in 2019	# MYLU Re--sightings 2020	% MYLU Re-sightings 2020	# MYLU/ MYYU PIT Tagged in 2019	# MYLU/ MYYU Re-sightings 2020	% MYLU/ MYYU Re-sightings 2020	# MYYU PIT Tagged in 2019	# MYYU Re--sightings 2020	% MYYU Re-sightings 2020
Alice	54	14	**25.9**	124	54	**43.5**	156	88	**56.4**
Stave	206	111	**53.9**	154	98	**63.6**	129	87	**67.4**

**We PIT-tagged Juv. Females as long as they were of sufficient mass (4.4 g or heavier).*

***It was determined after collecting recapture data from spring through to fall, that many bats captured and tagged in spring then roost elsewhere for the pup-raising portion of the summer and some return in late summer, possibly for social or breeding reasons. Thus, recapture rates may be low because a large portion of bats were PIT-tagged in spring when bats are not yet at their pup-raising roosts and it is unknown if these bats will return to the roost of capture at any point in coming years as site fidelity to these 'commuting roosts' is unknown (Rensel 2021).*

Table 6. Dates of the first and last PIT tags (Yuma myotis) of the year scanned at each study site.

	Last PIT Tag 2019	First PIT Tag 2020	Last PIT Tag 2020
Stave	05 Nov	22 Feb	02 Nov
Alice Lake	12 Oct	31 Mar	31 Oct

ROOST MICROCLIMATE MONITORING

Unfortunately, technical issues with numerous data loggers (i.e. batteries dying prematurely, inability to download data) resulted in incomplete data sets, and meaningful analyses were not able to be completed for the writing of this report. However, there are temperature and humidity data from 11 roosts and these will be analyzed in 2021 in conjunction with occupancy to assess patterns. More complete datasets are anticipated in 2021.

WINTER BAT ACTIVITY FROM ACOUSTIC MONITORING

Recordings of *Myotis* spp. were almost non-existent between late November 2019 and late February 2020, with 6 recordings of "50 kHz" bats (either Yuma myotis or California Myotis, *M. californicus*) at Alice Lake Provincial Park, and none in the Stave sites (Table 7). These recordings were sporadic, and consisted of single short sequences, often weeks apart. The 2020-21 winter recordings have yet to be downloaded and analysed -- recorders have been removed from the field but analysis is delayed until late summer 2021 due to analyst workload.

The first recording of a 50k Hz *Myotis* (presumably Yuma myotis) at Stave sites in 2020 was 17 February at the Hayward Lake bat box, near the time of the first PIT tag recorded on 22 February. Activity of 50 kHz bats increased substantially through March, which matched the rapid increase in detections of PIT tagged Yuma myotis.

The first spring 2020 recordings of a 40 kHz bat at Stave Lake Lodge and Alice Lake were 18 and 21 March, respectively; however, because there are two other species of *Myotis* bats known from SW BC that could produce this 40 kHz echolocation calls, it cannot be known what species this was, but may have been little brown myotis.

The last recording of a *Myotis* spp. in 2020 was 26 November at Alice Lake, though analysis of recordings from December 2020 to March 2021 were incomplete at the time of this report.

There were also sporadic recordings of Big Brown Bat (*Eptesicus fuscus*) and/or Silver-haired Bat (*Lasionycterus noctivagans*) (<10 sequences per month) over winter months on several detectors: Alice Lake roostlogger, Hayward roostlogger, Hayward SM4Bat, and Stave Lodge roostlogger. The SM4Bat at Hayward is the only site where a Silver-haired bat species identification could be confirmed (based on diagnostic call features). There is a SM2Bat recording in the upper Slave Lake area that has not been downloaded due to high snow levels yet.

Table 7. Dates of first and last acoustic detections of 40 and 50 kHz *Myotis* at lower mainland sites during winter 2019-2020 and fall/early winter 2020.

	Last Fall Recording 2019		First Spring Recording 2020		Last Fall Recording 2020*	
Site	50 kHz	40 kHz	50 kHz	40 kHz	50 kHz	40 kHz
Stave	12 Nov	21 Oct	17 Feb	18 Mar	02 Nov	30 Oct
Alice Lake	09 Dec	11 Oct	21 Jan	21 Mar	26 Nov	08 Oct

**Recordings only analysed until 30 November 2020; remaining data analysis is pending.*

UPTAKE AND PERSISTENCE OF PROBIOTIC ON BATS

SUMMER - CAPTIVE BATS – SECOND CAPTIVE TRIAL - TREATMENT 2

Bats were exposed to the freeze-dried probiotic via their clay-sprayed roosting substrate. Bats roosted on this treated substrate from 15 June to 14 August 2019. Probiotic bacteria were detected on captive bats roosting in an inoculated bat box, providing clear evidence of probiotic transfer from roost substrate to wings. Here we present the full Treatment 2 wing bacteria results of the second captive trial. Bat swabs taken from the Treatment 2 group had a detectable concentration of each bacterial species throughout the trial from 3 July until 14 August (Table 8).

Table 8. Concentration of cells adjusted for wing area (cm^2) of Treatment 2 captive trial bats, and field trial bats. Results are not available for *P. antarctica* due to poor PCR amplification. 2019 application dates of probiotic in bat box roosts were: 15 June, 7 August and 8 August for Treatment 2, Colony Farm and Stave Lake bats, respectively.

			Cell Concentrations per Probiotic Strain (per cm^2 of wing) (± standard error, (range), n = number of replicates per swab sample)		
Group of bats	Sample Size Description	Days since last roost substrate application	***P. azotoformans***	***P. synxantha* A**	***P. synxantha* B**
Captive Trial Treatment 2	n = 5 captive bats	14	112 ± 48 n=4	14 ± 9 n=6	58 ± 26 n=4
Captive Trial Treatment 2	n = 5 captive bats	60	77 ± 75 n=2	58 ± 24 n=2	1355 ± 1355 n=2
Field (wild bats) Colony Farm	n = 2 bats (of 2 captured)	8	1098 ± 868 n=6	20510 ± 16359 n=6	566 ± 447 n=6
Field (wild bats) Stave Lake	n = 10 bats found to have probiotic on wings (of 33 captured)	19	22 ± 7 n=9 from 8 bats	88,226 ± 87,663 n=8 from 6 bats	21 ± 7 n=3 from 3 bats

SUMMER – FREE-LIVING BATS

Bats captured at Colony Farm and Stave Lake at bat boxes previously inoculated with probiotic showed evidence of uptake of probiotic bacteria in the field (Table 8). Concentrations of probiotic species ranged greatly. In the field, bats had detectable probiotic which was highest in concentration soon after the roost was treated. At Stave Lake, where bats switch roosts frequently, not all bats captured had probiotic, but of those that did, concentrations were similar to what had been found on Treatment 2 captive bats. Mean cell concentrations achieved on wings of captive bats ranged from 4 – 1.3 x 10^4 cells/cm^2 two months after a single probiotic application was made to their bat box application. This compares with mean concentrations ranging from 0.5 – 5.3 x 10^5 cells/cm^2 on wild bats found to have probiotic on their wings upon capture, nearly 3 weeks after a single probiotic application was made at the point of capture roost structure. We compared only 3 of the 4 probiotic bacteria: the fourth bacteria *P. antarctica* did not amplify well and was excluded from our analysis. The custom PCR probe for this bacterial isolate will be improved in 2021 and may allow re-processing of some swab samples.

At Stave Lake, bats were caught with harp traps for swabbing on Bat Box 3 on 27 August, almost three weeks after inoculation of probiotic on 8 August. Bats were seen using each bat box as a night roost 5 days after inoculation of the probiotic. The boiler room's roosting substrate had not been inoculated in 2019, only the entrance point to the roost, so it was not clear how much transfer of probiotic was likely to occur at that roost. Bats captured for swabbing (at the Hayward Lake bat box) to test for the presence of probiotic could have come from anywhere in the Hayward area. Thirty-three bats were swabbed and 10 of them had detectable amount of probiotic on their wings. Swabbed bats were adult female Little Brown myotis with the exception of one that was a juvenile female and one that was a Yuma myotis (See Appendix 1 Table A1).

PROBIOTIC RESULTS FOR WINTER CAPTIVE BATS

All four probiotic strains (*P. synxantha* A and B, *P. azotoformans*, and *P. antarctica*) increased in concentration (at least 10.9 fold increase) on hibernating bats throughout the testing period from 16 November until 21 December (Table 9). All bacteria species increased in concentration from their original starting quantities from 16 November. This increase indicates that the probiotic bacteria were proliferating on the captive bat wings during hibernation.

Captive Myotis body weights slowly decreased throughout the trial and can be attributed due to their ~weekly disturbance during which time they were offered food and water. Bats were largely torpid throughout the experimental period.

Table 9. 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.

	Mean (± SE) Concentrations of Probiotic bacteria (per cm^2 of wing surface) n = 3 bats			
	P. azotoformans	***P. synxantha* A**	***P. synxantha* B**	***P. antarctica***
Start concentration (/cm^2)	1555 ± 187	27920 ± 3372	870 ± 103	1490 ± 95
End concentration (/cm^2)	22,397 ± 1482	410,853 ± 27,464	10,805 ± 810	$6.95 \times 10^9 \pm 3.90 \times 10^9$
Fold increase	***14.4***	***14.7***	***12.4***	***4.6×10^6***

PERSISTENCE OF PROBIOTIC IN ROOST STRUCTURES

SUMMER – EXPERIMENTAL BAT BOX

We monitored the temperatures of a four-chambered bat box that was deployed in the field in Kamloops Wildlife Park. Internal temperature was monitored in each chamber for 68 days (17 May to 15 August, 2019). The probiotic application occurred 23 May 2019, and no further applications were made. Starting 20 July, as the bat box was experiencing warm summer temperatures, we also monitored for concentration of probiotic cells.

We used qPCR to analyze the concentration of the probiotic bacteria in all four chambers (from 20 July to 24 August. As was found in swabs of bat boxes in the field sites, *P. synxantha* A had a significantly larger concentration than *P. syxantha* B, *P. antarctica*, and *P. azotorformans*, which all had similar quantities throughout the trial, regardless of chamber. Because there was no difference in humidity and mean temperature among the box chambers, and because probiotic cell concentrations were not different among chambers, we pooled results and present concentrations per probiotic isolate in relation to temperature (Figure 3). Temperatures inside the bat box fluctuated and in late summer exceeded 50°C (Figures 4, 5).

Although broth viability tests did not produce reliable results, in vitro experiments of temperature exposure from 2019 suggest that all strains of bacteria in the probiotic cocktail can withstand (and still grow) at least 37°C (see Appendix 3). Further in vitro and bat box tests will be conducted in 2021 to establish temperature thresholds for viability of each strain.

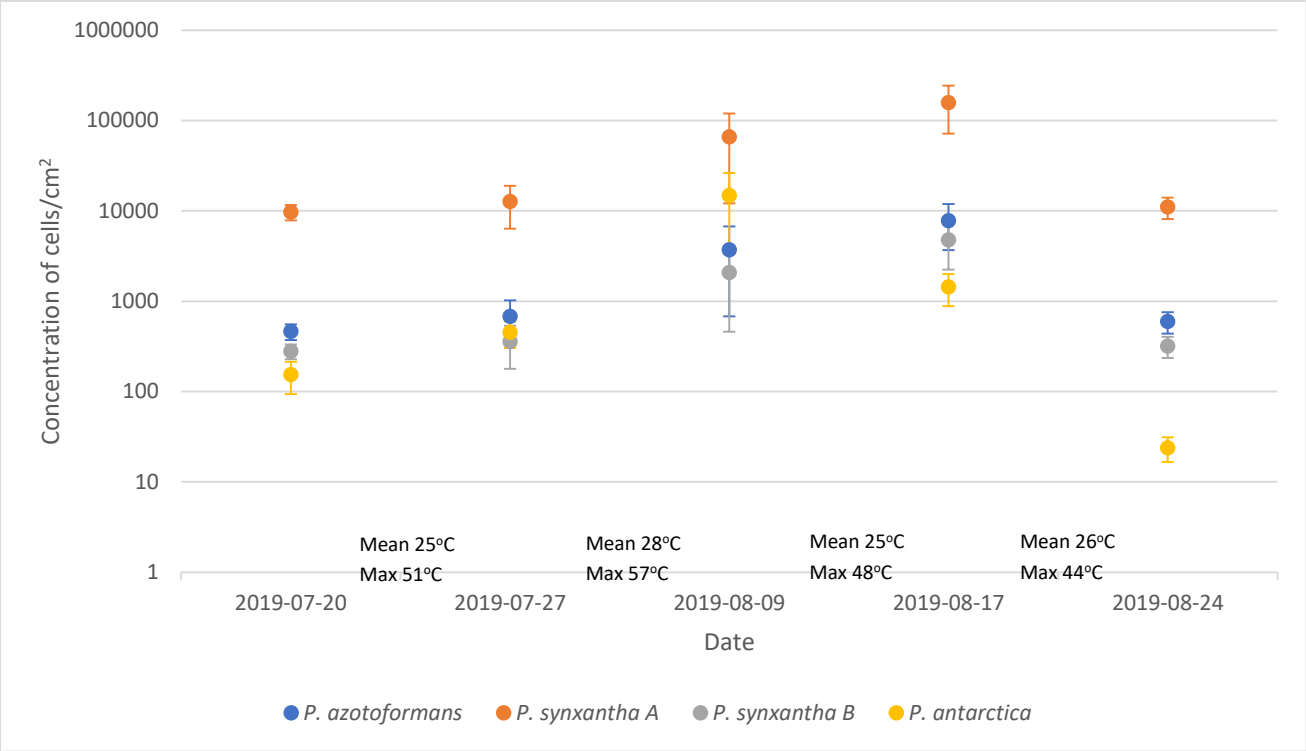

Figure 3. Probiotic in the temperature experiment four-chamber bat box. Concentration of each probiotic bacterial strain pooled across all four bat box chambers of the experimental bat box deployed in the field (no access to bats). Mean concentrations are presented with standard error bars. Mean temperature and maximum temperature reached inside bat box across chambers is presented for each time period (last set of temperatures are based on 4th chamber only).

Daily mean temperatures within each chamber ranged from 15.78°C-33.75°C across the entire monitoring period (Figure 4). 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 ($P=0.456$). Chamber 1 had a significantly higher maximum daily temperature when compared to chambers 2, 3 and 4 ($P<0.001$) (Figure 7) throughout the trial period. We saw no significant differences in average daily relative humidity or minimum temperatures among chambers ($P>0.5$) (results not presented).

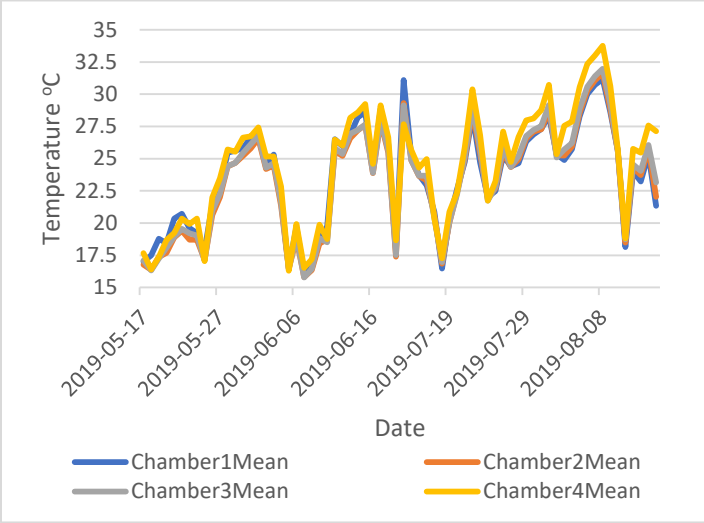

Figure 4. Average daily temperature for each chamber of the four-chamber bat box between 17 May 2019 and 15 August 2019.

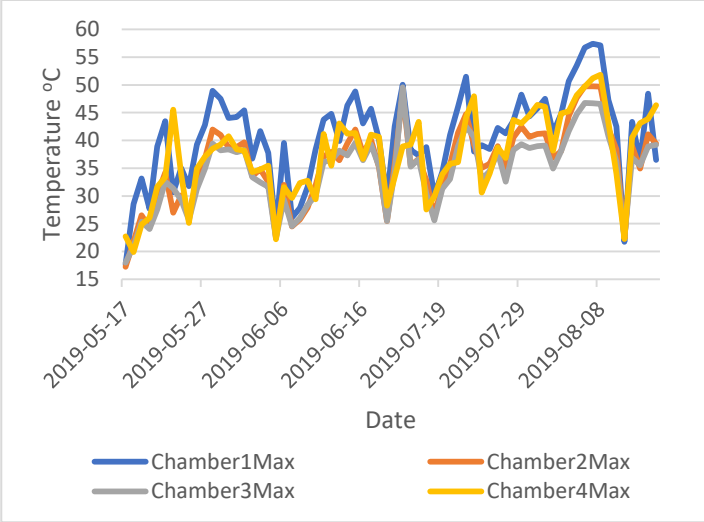

Figure 5. Daily maximum temperature for each chamber of the 4-chamber bat box between 17 May 2019 and 15 August 2019.

TREATMENT 2 CAPTIVE BATS – BAT BOX

Treatment 2 bat box swabs show a consistent concentration throughout the trial (Figure 6). Bacteria species appear to have stabilized since application on 15 June and began decreasing on the last swab, 9 August. The persistence of bacteria throughout the trial is indicative of survival of the probiotic on the bat box while being used by Treatment 2 bats. During the timeframe of 28 June – 9 August, the Treatment 2 bat box had a relatively consistent mean daily temperature ranging from 21 ± 6°C to 23 ± 7°C, and minimum daily temperatures ranged from 9 – 11°C, and maximum daily temperatures ranged from 31 to 37°C.

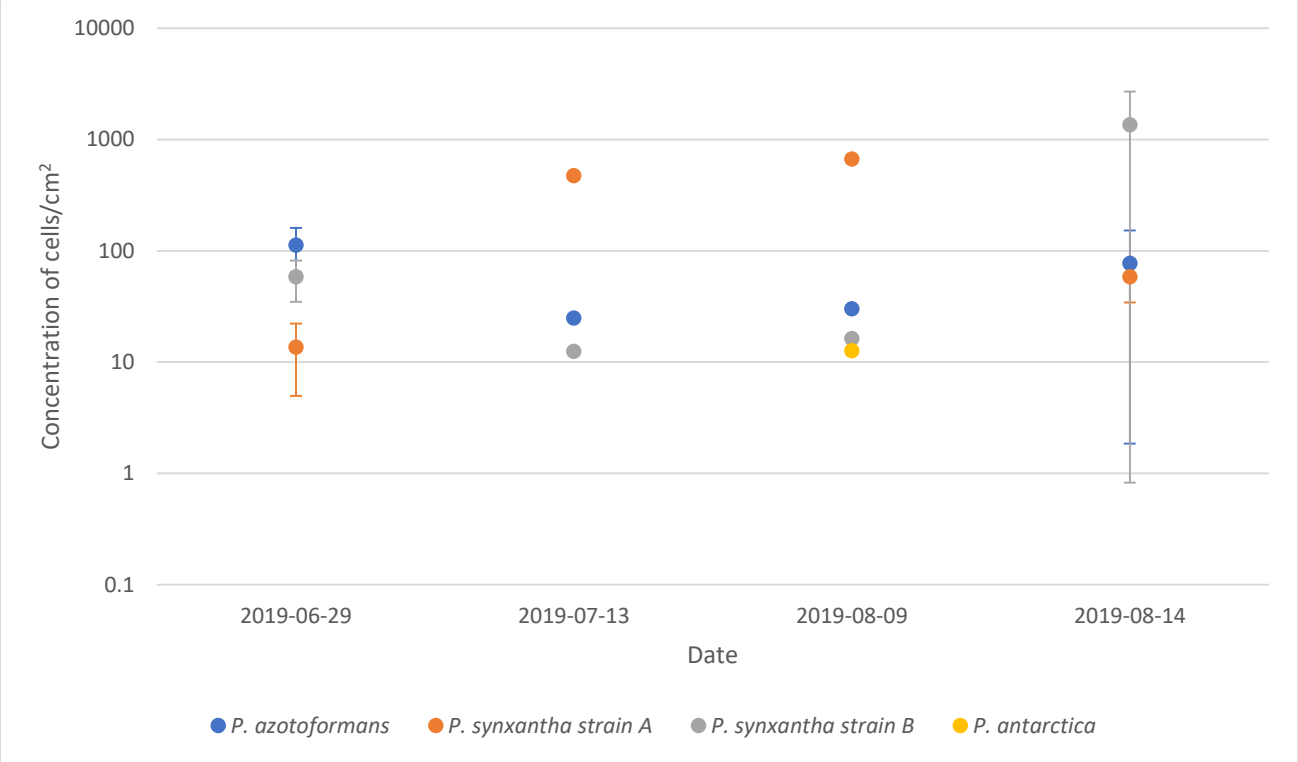

Figure 6. Concentrations of probiotic cells (from qPCR) detected on the bat box used by Treatment 2 captive bats. There was only one application of probiotic in the bat box and that occurred 15 Jun 2019. Bats remained roosting in this same bat box throughout the trial period.

SUMMER – FIELD SITES

In 2019, at Stave Lake, we swabbed three bat boxes (boxes 1 and 2 on Lodge building and Hayward bat box) and roost entrance of Stave Lodge on 22 August for detection of probiotic after being previously inoculated on 8 August. Two of the three bat boxes and the boiler room entrance had a detectable amount of the probiotic bacteria present. Bat box 1 inexplicably had no detectable amount of probiotic within the swabbed chambers. *P. synxantha* strain A was the most abundant bacteria within each chamber of the other bat boxes and the boiler room entrance. Overall bat box 3 had a greater number of probiotic bacteria present within each chamber compared to Hayward bat box, which may reflect

overall use of the box by bats and the adhering of cells to bat skin and fur that is expected to occur. Alternatively this could stem from application randomness when using the applicator and the amount of clay that stayed within each bat box. Support for the former hypothesis is the finding that the two most highly used chambers of Hayward box (chambers 1 and 2 nearest the landing platform entrance) were noticeably lower in quantity of all probiotic microbes compared to the least used chambers (3 and 4; Figure 7).

At all sampling locations, there was a 10-12.5 fold difference when comparing chambers containing detectable *P. synxantha* strain A to both *P. azotoformans* and *P. synxantha* strain B numbers combined (Figures 7 and 8). Presence of probiotic bacteria at the building roost was determined by taking two swabs at the entrance of the Lodge boiler room roost where the probiotic was initially applied (Figure 8). Both swabs had a detectable number of probiotic bacteria present. *P. synxantha* A had a marginally greater number of cells comparatively to both *P. synxantha* B and *P. azotoformans*.

Unfortunately due to problems with the *P. antarctica* probe, we had to exclude the fourth bacteria *P. antarctica* from our analyses. This means that we do not know exactly how well this bacteria has performed in the field; however, our goal in 2021 is to improve this probe and 4th bacteria results are thus still pending.

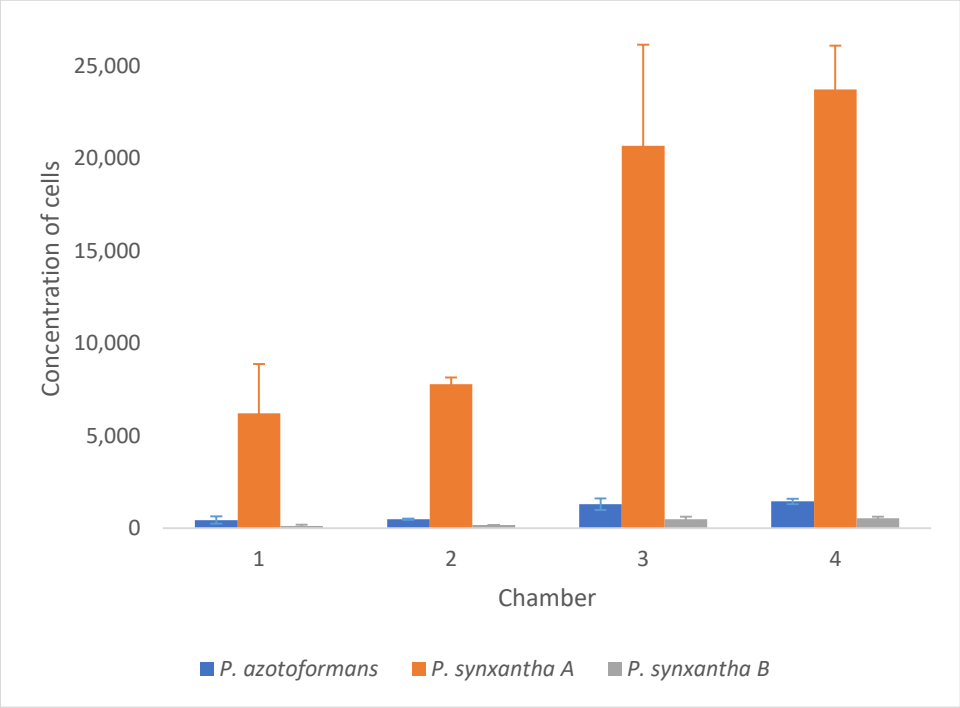

Figure 7. Hayward Bat Box at Stave Lake study site -- probiotic DNA concentrations (from qPCR) of *P. synxantha B* (left), *P. azotoformans* (centre), and *P. synxantha A* (right) from the four swabbed chambers on 22 August 2019. Noteworthy are: 1. the consistently lower quantity of DNA in chambers 1 and 2 (most likely to be occupied by bats as nearest the landing platform entrance) versus 3 and 4; 2. Greater quantity of *P. synxantha A* in all chambers (>10x greater quantities than the other 2 microbes).

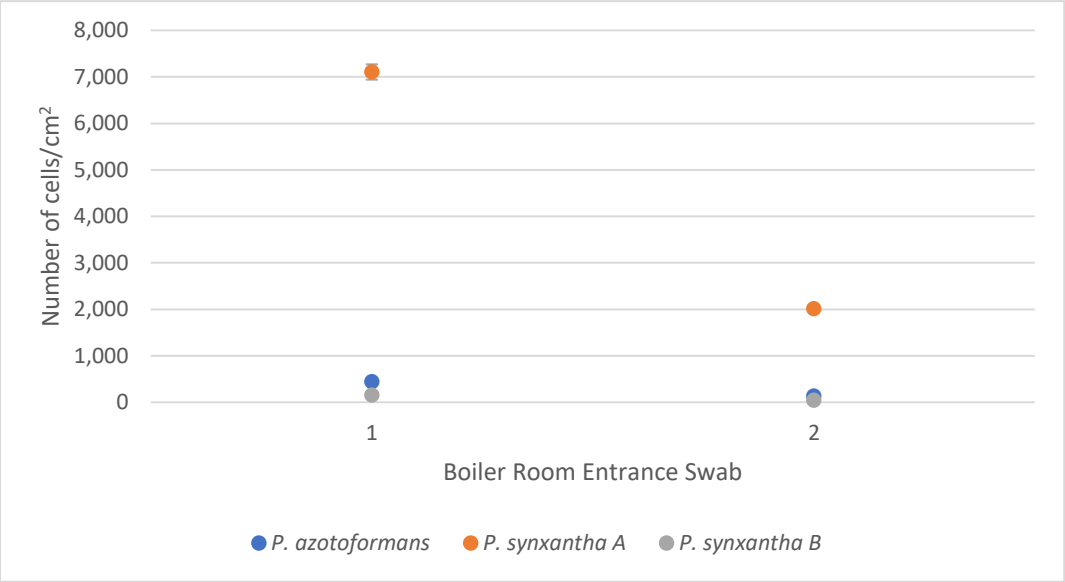

Figure 8. Boiler room roost entrance at Stave Lodge -- qPCR probiotic quantities of *P. azotoformans,* and strains A and B of *P. synxantha* from 2 swabs of the on 22 August, 2019. Noteworthy is the higher quantity of *P. synxantha A* in both locations suggesting this microbe may dominate the others despite equal concentrations in original probiotic application.

WINTER – FIELD

Probiotic was not detected at Colony Farm bat boxes after one winter (spring 2020 swabbing). *P. azotoformans*, *P. synxantha* A, and *P. synxantha* B were not present in any of the swabs. This indicates that the probiotic cells had significant die off in the bat boxes by March in that year. However, probiotic was found at both the Colony Farm, and Stave Lake/Hayward sites when they were swab sampled in late January and February, respectively, in 2021. As such, it appears that probiotic can survive winters, but that varying conditions in bat boxes over winter may influence survivability. Further testing will be done.

EFFECTIVENESS OF PROBIOTIC AT INHIBITING *PD* - PRELIMINARY EXPLANT EXPERIMENT

Tissue explant chambers are a new technology from University of California Davis that we are adapting to answer our question of efficacy of probiotic on inhibiting *Pd* spore germination on live bat skin. In order to determine whether antibiotic was needed to keep cells in a semi-living state, we assessed qPCR starting quantities (SQ) among samples that were left incubating for 3.5 and 7.5 days, examining presence of probiotic in samples with and without antibiotic.

The gentamycin antibiotic was meant to reduce contamination within the nutritional media and prevent common molds and/or other bacteria from enveloping the explant in the study. Using coarse examination with the naked eye, we did not observe any mold or difference between explants that did

or did not receive the antibiotic. All samples with antibiotics had inconsistent results and/or showed no *Pd* in the qPCR analysis. Because the gentamycin antibiotic may have interfered with the qPCR process and prevented probes from attaching and qPCR from cycling, we conclude this should not be used in this procedure in the subsequent tissue explant experiment. (see Appendix 2 for details).

Starting quantities (SQ) of cells is indicative of how many cells were estimated to have been in a sample following PCR amplification using quantitative methods (qPCR). Most SQ numbers in this pilot experiment were low and are likely the result of antibiotic interference and swab retention of cells. Cells and spores may have stuck to swabs and prevented an accurate cell count. Swabbing for qPCR does not appear to be a reliable method for measuring probiotic and *Pd* quantity and thus we will not use this same methodology in future explant experiments.

Furthermore, when we inoculated each explant, the waxy surface of the skin prevented even distribution of inoculum onto the surface and splitting the explants into halves for swabbing may have been prevented accurate results. Inoculum would form a "bubble" upon inoculation instead of spreading over the entire explant.

The *Pd* control samples (*Pd* alone) tripled in quantity after 7.5 days compared to 3.5 days to 7.5 days and it was these latter explants that showed visible white fungal growth (Figure 2b). This provide assurance that *Pd* will grow under these conditions and during this time frame.

There was evidence of cross contamination within the data set. As this initial explant experiment was a pilot, this does not have direct impact on analyses, as this pilot experiment was aimed at determining methodology for a more comprehensive experiment (Explant Experiment #2 – spring 2021). In Explant Experiment #2, we will minimize the chance of cross contamination through use of parafilm and strategic timing of inoculation. Parafilmed explants without *Pd* will be inoculated first and placed into their own sterilized incubator and container. Afterwards, the remaining samples will be inoculated with *Pd* and/or probiotic and placed into a separate, sterilized incubator and container. Control explants will have their own container and be placed at a separate location within the respective incubator. In terms of processing samples, respective control samples will always be handled first (if need be), followed by non-*Pd* samples, then samples that have *Pd* will be handled last.

Conclusion: The methodology for the subsequent (spring 2021) comprehensive tissue explant experiment will eliminate swabbing and the use of antibiotics. Tissue explants will be qPCR'd directly rather than swab sampled (see Appendix 2 for details). Use of SEM is still uncertain (see Appendix 4). See Discussion section for implications and future directions regarding the second round of tissue explant experiment planned for spring 2021.

SEARCHING SOIL DATABASES TO DETERMINE HOW COMMON THE BACTERIA ARE ACROSS CANADA

All genetic blasts were done to locate these probiotic bacteria outside of BC. Three of the bacteria were found in at least one of the sources searched: *P. antarctica*, *P. azotoformans*, *P. synxantha* Strain A. We will search further sources of genetic information of soil micro-organisms to determine if we can locate *P. synxantha* Strain B.

DISCUSSION

FIELD WORK

Field work during 2020 was extremely challenging due to the logistical difficulties presented by COVID-19. Because of the uncertainty surrounding handling of bats and the possibility of disease transmission, we had to suspend field work March, and did not resume until late June. Additionally, COVID constraints on the lab at McMaster delayed the production of probiotic until the middle of July.

At the same time, spring weather was unusual and bats did not use the roosts as predictably as they had in previous seasons, and were often found using building roosts that we were unable to access, particularly given our reliance solely on harp traps and not mist netting. As such, we were unable to complete many of our planned field activities, while others had to be substantially modified.

BAT CAPTURE

As noted above, the behaviour of roosting bats diverged from our experience at these sites in the 2018 and 2019 maternity seasons. This was likely due to an unseasonably warm early spring, which was immediately followed by a prolonged period of unseasonably cold, wet weather. Bats appeared to be selecting building roosts during this period, presumably as they were warmer than bat boxes.

Additionally, the roost at the Stave Lake Lodge was almost entirely abandoned. This was certainly because the hot water heaters which had previously maintained a warm environment were unavoidably shut off when a water pump failed, and maintenance staff were unable to repair it. This caused a precipitous drop in temperature, after which bats used it only sporadically.

Species and Demographic Composition

As in previous years, the vast majority of bats captured were adult female or juvenile Yuma myotis. Only a small number of little brown myotis were observed, although there were a substantial number of bats with intermediate physical characteristics that could not be confidently identified to species. So called “bag tests” to assess the frequency of echolocation calls to resolve species identification were useful, but not successful in all cases, due to equipment failure on one capture night, and a reluctance from several bats to produce any measurable calls. In previous years, we observed bats identified as little brown myotis roosting more often in buildings than in bat boxes. Due to our inability to use nets in 2020 we could not investigate that observation further, as the harp traps were unsuitable for trapping bats exiting most building roosts.

Interestingly there were four adult males captured, all of which had also been captured in 2019 as adults. Sex ratios of juveniles were slightly skewed towards females, but not to a degree that would indicate it was not due to chance.

Reproductive Condition

As expected, most females (76.8%) successfully reproduced, though pup survival was not determined. However, there did seem to be a slightly unusual number of parous females that showed no signs of reproducing in 2020. This was most noticeable in the Stave (26%) and Colony Farm Regional Park (19.3%) colonies. This could perhaps be explained by the cold wet weather, which happened just as females were having their pups. The combination of low temperatures, reduced ability to forage in the rain, and the sudden loss of heat at the Stave Lodge roost may have caused females to be unable to carry pups to term, or produce enough milk to adequately raise them. Alternatively pups may have been lost for other unknown reasons. Unfortunately, as we were unable to capture bats in the spring and early summer, we have no data regarding females who were pregnant early in the season, and then showed no signs of pregnancy or nursing when they were captured later in the summer.

PROBIOTIC INOCULATION – ROOSTS AND BAT WING SAMPLES

Because the lab at McMaster was shut down due to COVID-19, we did not receive the probiotic clay until the end of July. By that time, pups had already been born, but many were not yet consistently flying, and roosts were almost continuously occupied. This largely prevented us from being able to apply the probiotic, which could not be done with bats inside the roosts. We were able to inoculate some roosts that were being used by small numbers of bats by waiting until after they had emerged to forage.

However, roosts that were used by large numbers of bats were never completely unoccupied, and therefore did not get inoculated until after the colonies had largely dispersed. Because of this, for many of the roosts, we were unable to confirm that bats had been roosting in inoculated structures before being swabbed, which should be considered when interpreting results from the lab (i.e. a swab that showed no presence of probiotic may not have been due to the failure of the probiotic species to grow on the bat, but simply because that bat had limited or no exposure to inoculated roost surfaces). We were still able however, to collect a significant number of swabs from bats that were confirmed to have been roosting for at least a week in an inoculated bat box.

The field trial of 2019 emphasized that probiotic can be successfully transitioned from inoculated human-made structures onto wild bat wings. Wild little brown myotis bats carried detectable amounts of the same probiotic species on their wings after application onto bat boxes and a roost entrance. Bats were still found at one inoculation site after applying the probiotic and caution is required to prevent bats from abandoning the roost. Future field trials will likely improve substantially given minor improvements and tweaking to the application process. Finally, the preliminary *Pd* explant experiment gives us guidance on how to tackle similar experiments in the future. The experiment is novel and required finessing before the primary experiment occurs in March and/or early April.

PIT TAG RECAPTURE

PIT tag records indicate that bats at the Stave Lake roosts are continuing to move back and forth between the two main roosts, with individuals being captured at both locations in the same year (Stave Lodge and Hayward Rec Area), as well as individuals that were tagged at one roost being subsequently

scanned in another. The number of bats roost switching was likely more pronounced in 2020 as bats abandoned the Stave Lake Lodge roost due to the loss of the heat source in the building roost, and many subsequently moved to roosts at Hayward bat box.

As noted above, bats spent a large portion of the early summer in building roosts which did not have PIT tag readers installed. This problem of not being able to scan PIT tags was exacerbated at Alice Lake, where one of the tag readers on a bat box was unplugged by park staff, and was not running for a little over three weeks during the warmer period in August, where bats were roosting inside the bat boxes.

At least one, and more likely two PIT tag readers will be installed at building roost locations at Alice Lake in 2021. In anticipation of installing a tag reader on the roosts at Colony Farm Regional park in 2021, we implanted PIT tags in females captured there in 2020, and will continue into the future.

The high proportion of adult female "return" bats compared to other cohorts is unsurprising, as very few males or juvenile females were implanted with tags in 2019 (11 juvenile females and 3 adult males) as we were unsure of whether low survival and return rates warranted the cost. That a similar proportion of PIT tagged juvenile and adult females survived and returned to the roosts demonstrates that there is value in tagging that age group. We PIT-tagged juvenile females in 2020 capture sessions.

Adult males were PIT tagged (or merely banded at Colony Farm where no PIT tag reader was in place), as they were considered an oddity, being found in a maternity roost during pupping season. As 4 out of 5 tagged adult males returned to the same roost site they were initially captured at, further investigation of that behaviour seems warranted.

The relatively close correlation at the Stave sites between first and last acoustic recordings of *Myotis* spp. each year, and the dates of the first and last PIT tag scanned, point towards bats generally not remaining in those areas around the roost sites during winter. Instead, bats seem to occupy the roost quickly after arriving in the area in early spring, and then leave the area completely soon after abandoning those particular roosts prior to hibernation.

The proportion of re-sighted individuals using passive PIT tag readers is substantially higher than the physical recapture of bats, and illustrates the value in PIT tagging as a recapture technique. Other studies in the province have also found a low recapture rate when using the traditional method of banding (Susan Dulc pers. comm.), which could be in part due to the learned behaviour of repeatedly captured bats to avoid nets/traps.

Our observations suggest that bat populations at any given roost site are not discrete, closed populations, and instead, bats appear to move fairly often between roosts, with only a small percentage of the true local population occupying a particular roost structure at any one time. This has been confirmed with emergence counts (Leah Rensel, pers. comm.) suggesting that a relatively consistent count does not necessarily mean that the same individuals are being counted each time. This has implications for long term monitoring of roosts that use emergence counts only.

The benefit of PIT tags being passively scanned also reduces the impacts of continued capture and handling of bats at these sites, as they do not have to be continually recaptured to re-sight bands to estimate survival. Although it should be noted that it is important for new cohorts of bats to be periodically tagged to effectively monitor populations.

More PIT tagging is planned for 2021, pending COVID-19 restrictions. Mist net capture will be important for sampling at Alice Lake, Deas Island and Stave Lake in order to capture bats in buildings.

ROOST MICROCLIMATE MONITORING

Unfortunately, technical issues with numerous data loggers (i.e. batteries dying prematurely, inability to download data) resulted in incomplete data sets and meaningful analysis was not able to be completed for the writing of this report.

OVERWINTER ACOUSTIC MONITORING

There were only a small handful of bat echolocation calls recorded at any of the monitored locations during winter, and very few of those were of *Myotis* species targeted in our study. The bulk of 2020-2021 data have yet to be downloaded from detectors that are still in the field at the time of this reporting.

No *Myotis* were recorded in the Stave Lake watershed between 12 November and 17 February in winter 2019-2020. The first and last recordings of the year were within 7 days of the first and last PIT tags recorded at a roost. As noted above, this suggests that bats do not linger around the roost sites once the decision to leave for hibernation has been made. Similarly, bats returning in the spring quickly find and enter roosts.

This tight correlation between PIT tag captures and acoustic captures did not entirely hold true at Alice Lake, where a handful of 50 kHz bats were recorded throughout the winter. However, those recordings may have been of California Myotis (*M. californicus*), which are consistently found flying in winter in our region, and not Yuma myotis that were occupying the monitored roosts. These two species are acoustically similar.

The only other bats recorded between late November and mid-February were either big brown bats (*Eptesicus fuscus*) and/or silver-haired bats (*Lasionycteris noctivagans*), which, like the California Myotis, are frequently observed flying in winter in coastal areas of the Pacific Northwest (Falxa 2007; Nagorsen et al. 1993). Big brown bats (*Eptesicus fuscus*) and silver-haired bats species are also acoustically similar.

LABORATORY DISCUSSION – ANALYSES FROM EXPERIMENTS AND TRIALS

To summarize results to date: We observed no detrimental effects of the probiotic on bats, as determined in two independent summer captive trials. The application method that we developed enables easy transport and delivery of freeze-dried cells onto roost substrates via clay powder. Probiotic bacteria were persistent on captive bats and bat boxes months after application, including when

exposed to high summer heat. However, we did note a potential reduction in probiotic in the range of 40-50°C bat box microclimates, and the thus upper lethal limit of these probiotic will be established through a final bat box and culturing experiment in summer 2021. We observed a substantial increase in viability and growth of all four probiotic strains (12 or greater fold increase) on bats in the hibernation trial and this was likely due to a combination of high humidity and little grooming behaviour. This was highly representative of the true hibernation conditions.

Researchers have explored many possible strategies to prevent WNS infection in hibernating bats. Chitosan, polyethelene glycol, *P. fluorescens*, *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. in 2019. Two field trials using *P. fluorescens* 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. They found that bats inoculated with anti-*Pd P. fluorescens* emerged from hibernation later with a five-fold increase in survivability. The study by Cheng et al. (2016) showed success when probiotic delivery occurred just prior to exposure to *Pd*. Probiotics have been considered the most promising method of preventing *Pd* infection, 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 & Willis 1998). Big brown bats 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). Published literature supports our bio-augmentation method to augment wing microbiomes of vulnerable bat species in British Columbia with anti-*Pd* bacteria prior to hibernation.

DISCUSSION OF PROBIOTIC CONCENTRATIONS ON WINGS AND SUBSTRATES

We are the first researchers to test an anti-*Pd* probiotic cocktail on captive bats in western North America and the first to test it as a prophylaxis approach. In this study we examined length of time and concentration in which probiotic could be sustained on the wing microbiome in summer and in hibernating conditions. We confirmed persistence of probiotic for at least 60 days on bat wings during summer in a managed (captive trial) situation. The longest period that we could test for presence of probiotic following roost substrate inoculation for wild bats at our summer field sites, was 3 weeks, at which time there was detectable probiotic on the wings of 30% of captured bats. We expect this percentage to improve in future years as we aim to inoculate bat boxes earlier in the summer, providing more time for individuals of each colony to visit the inoculated roosts. We also aim to discover more roosts used by each colony so that they can also be treated with probiotic. Although we cannot follow these bats into hibernation, our hibernation simulation experiment suggested that the probiotic cells persist and in some cases increase, in the cool humid conditions of hibernation.

We confirmed uptake of probiotic cells from inoculated roost structures to bats, in captive and free-living bats. Sustained cell concentrations achieved on wings of captive bats ranged from 8.5 – 197 cells/cm^2 two months after a single probiotic application was made to their bat box application. This

compares with cell counts ranging from 12.4 – 282 cells/cm^2 on wild bats found to have probiotic on their wings upon capture, nearly 3 weeks after a single probiotic application was made at the roost structure where they were captured. This suggests that we are seeing similar uptake of probiotic onto bat wings in the wild as we did in a captive situation. However, the wild bats at these sites are switching roosts frequently (Leah Rensel, pers. comm.) and therefore, not all bats captured had been sufficiently exposed to the probiotic and this may be due to our probiotic being applied late in the summer in 2020 (due to COVID-19 restrictions in the lab). We aim to apply probiotic earlier in the summer as long as the probiotic can be made available to allow more time for more individuals to circulate through the multiple roosts used by each maternity colony at each study site.

At Stave Lake, 3 of the 4 probiotic species were detected and quantified on 11 Little Brown bats from nearly 3 weeks after probiotic inoculation of their bat boxes. The fourth bacteria species, *P. antarctica* was unfortunately not recorded due to problems with our 4^{th} probe not annealing properly during qPCR analysis. Probiotic bacteria was not found on bats swabbed prior to inoculating the bat box substrates, suggesting that the presence of the *Pseudomonas* bacteria that we tested for (*P. synxantha*, and *P. azotoformans*) was due exclusively to inoculation of bats roosting on surfaces that had been treated with our probiotic cocktail.

At Colony Farms, 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 Farms 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.

We quantified probiotic survival inside a four-chamber bat box during summer conditions, and although probiotic cells were still detected with qPCR at the end of the summer, despite bat box temperatures exceeding 50°C, we do not know if these bacteria were still viable. Although the LB broth inoculation and incubation method was used for the bat box swabs, this ended up being unreliable (see Processing of Swabs section in Methods). In most swab samples of all bat boxes in the field and in our captive trials, *P. synxantha* A increased in concentration 24 – 35 times that of *P. synxantha* A, *P. azotoformans* and *P. synxantha* B, despite all of these starting from the same concentration. This suggests that *P. synxantha* A is viable in all bat box conditions tested to date, although in the 4 chamber bat box experiment, all bacteria were decreasing in number by the end of the summer in relatively the same proportion suggesting some of the extreme temperatures (>55°C may cause all of these microbes to die). To determine upper lethal temperatures for each of these bacterial strains, this bat box treatment experiment will be repeated in 2021 to assess viability of probiotic in varying field conditions, and the thermal culturing experiment will be expanded to include incremental temperatures ranging from 38°C to 55°C (to date we know that all strains grow in conditions between 4 – 37°C, but die at 56°C. The results of our post-winter swabbing of at boxes in 2019 suggested that the probiotic did not survive winter conditions in bat boxes; however, our 2020 results at four sites (Stave Lake, Hayward, Colony Farm and Alice Lake) verified that probiotic did survive winter in bat boxes (and inside and outside a

building roost). Additional sampling post-winter (2021 and 2022) will assess whether probiotic has differential survival in building roosts (heated and unheated). If the probiotic does not survive some winter conditions in some roosts, and especially in bat boxes, then annual re-inoculations will be needed in some areas under some winter conditions. We hope to include freezing conditions in our thermal culturing experiments to determine the lowest temperature that each bacterial strain can withstand.

These four probiotic bacteria belong to the *P. fluorescens* group, a group of bacteria that have been used in many efforts to inhibit white-nose syndrome because of their natural abundance in bat microbiota, including little brown myotis WNS survivors and big brown bats (Lemieux-Labonté et al., 2017; Lemieux-Labonté et al., 2020). *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; Nikolaidis et al. 2020). Sequencing of 16s rRNA in the late 20th century resulted in many bacteria being re-classified from the *Pseudomonas* genus (Anzai et al. 2000). All four species in our probiotic belong in 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 female *E. fuscus* caught from a mine near Salmo, 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 an adult *Corynorhinus townsendii* caught from a maternity roost in Deroche, British Columbia. Strain B was isolated from a *M. yumanensis* at a mine hibernaculum near Salmo, British Columbia. *P. antarctica* was first isolated in Antarctica and 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.

There are many factors that can influence the growth and proliferation of bacterial strains and this may explain a lot of the variation we measured in cell concentrations. Although the swabbing protocol remained consistent, weather, nutrients, humidity, where bats were sourced, grooming behaviours, and other wing microbes, are all variables that should be considered when interpreting our results. The wing microbiomes likely varied significantly between sites and individuals (Avena et al. 2016; Lemieux-Labonté et al. 2016; Winter et al. 2017). In 2021 we will make sample and process a larger range of individuals for metagenomic characterization of microbiomes. The swabs themselves may also introduce some error if the bacteria are not fully released from the fibres during mixing with water. Future processing of samples will try to minimize use of swabs when possible and use direct extraction from tissue biopsy punches. We will also use the entire swab tip in the DNA extraction process.

Anti-*Pd* antifungal compounds are produced by bacterial biofilms. We did not see biofilm production to date, however, an increased dosage with more bacteria can promote biofilm when used with water and clay, and is possible to achieve in our field inoculations with an increased dosage. *Pseudomonas* bacteria have been shown to use clay as a mineral source when producing biofilm (Alimova, et al. 2009). Clay has been shown to promote biofilm in *Pseudomonas* bacteria (Alimova, et al. 2009) and assists with

adhesion within bat box chambers during application. As such, clay alone may provide suitable enough conditions for the probiotic bacteria, especially when the roost substrates are slightly moistened with water prior to spraying with cell-laden clay powder. A final lab experiment should be conducted to determine extent of anti-*Pd* activity from freeze-dried cells in clay versus rehydrated cells, to determine if further nutrient supplementation may be beneficial to promote production of secondary metabolites (Duffy & Défago 1999).

One of the main mechanisms of WNS spread is the transfer of spores when huddling during hibernation (Lorch et al. 2011). Similarly, bats who hibernate together may rub probiotic species on each other in hibernation. It is possible that a biofilm anti-*Pd* bacteria could be promoted on bats in hibernacula because of its above freezing, humid and stable refrigerator-like temperatures (Fenton and Robert 1980, Gennari and Dragotto 1992).

EXPLANT EXPERIMENT

Our results suggested that antibiotics to prevent unwanted bacteria infection and mold growth were not necessary. No mold or visible morphological change of the explants occurred and explants that were not exposed to antibiotics were indistinguishable from ones that did. We determined that we will need to have the explants incubating for at least 7 days to see *Pd* germination – longer would be better, but the risk would increase that other microbes would grow (due to no antibiotics present), and/or that the skin cells will die completely.

The goal of this pilot explant experiment was to establish a methodology for a comprehensive explant experiment in which we test the reduction our probiotic has on *Pd* spore germination on bat wing skin. This will provide the desired quantification of efficacy for our prophylaxis approach. Scanning electron microscopy results are being interpreted to evaluate SEM as a tool to analyze spore germination. SEM may supplement qPCR results to provide a better estimate of probiotic influence on *Pd*. We determined that it is not ideal to conduct qPCR from a swabbing process, and thus a direct submission of explant tissue for qPCR is likely to be needed. A meeting of the Probiotic Project Advisory Committee will take place in April 2021 to finalize methodology for the experiment based on these pilot results.

The results from the pilot experiment were promising in that a negative interaction between *Pd* spores and our probiotic anti-*Pd* bacteria was observed; however, as the sample sizes were very low, it is impossible to draw any conclusions from these results. We will require much larger sample sizes in order to make conclusions.

Summary of Next Steps for Explant Experiment

Our finalized explant experiment will use different methodology for sampling and testing. We are proposing more defined methodology that does not use antibiotics, has less categories, and has a greater sample size. We would test explants in the following groups: (1) without probiotic and *Pd* as a control, (2) only inoculated with *Pd*, (3) only inoculated with the probiotic, and (4) with probiotic and *Pd*

inoculation together. Explants that involve *Pd* will have a greater number of explants compared to controls. Skin viability will be tested at the end of the trial on additional explants within each category to confirm the tissue is living. Although skin had the same texture and no signs of decay were present in the preliminary experiment, we did not conclude this with a cross section and/or histological examination. Finally, sampling will be vastly different in the next experiment. Instead of swabbing each half of the explant, we will use the entire explant for qPCR analysis. Inconsistencies seen in the preliminary experiment are largely due to difficulty swabbing and evenly distributing inoculum (spores and/or probiotic) across the explant surface area. Here we propose using the entire explant submerged in buffer solution and then quantify it for probiotic and *Pd* numbers using qPCR. This is a robust technical solution for the swabbing difficulties seen in the preliminary experiment. Strategies will be employed to avoid cross-contamination (see Contamination section above). And the use of SEM to examine *Pd* spore germination is still being evaluated (see Appendix 4).

NEXT STEPS (PLAN FOR 2021-22)

In 2020, COVID-19 interrupted and substantially delayed lab analyses, production of probiotic for field applications and swabbing of bats and roost surfaces.

Table 10. Activities in spring, summer 2021 and proposed for winter 21-22.

Timeframe	Activity	
Spring 2021	1	Refine the probe for our 4th probiotic bacteria (McMaster University). NOW COMPLETED.
Spring 2021	2	Install more PIT tag readers at Alice Lake and Colony Farm sites. NOW COMPLETED.
Spring 2021	3	Sample roosting substrates in spring for presence of probiotic (completed, but lab results are pending). COMPLETED. ALICE LAKE FOUND TO BE CONTAMINATED WITH PROBIOTIC FROM A LIKELY LAB ERROR. Conclusion: Convert Alice Lake to a 3rd Treatment Site and bring on a new Control Site in summer 2021. This will require additional funding and more contractor time to obtain baseline data, and mark bats with PIT tags; and install PIT tag readers.
Spring 2021	4	Swab sample bats returning to roosts in spring to look for probiotic and *Pd*. COMPLETED. Conclusion: it is difficult to capture bats in spring and the history of most captures were unknown as they were unmarked bats. This suggests that these bats may not be part of the colony and quite likely did not get exposed to the probiotic on the treated roosts. Moving forward, unless it can be confirmed that a bat has been in a probiotic treated roost the previous summer, it will not be spring swabbed.
Spring 2021	5	Conduct WNS surveillance at our study sites - collect guano and observe bat wings for scarring or signs of *Pd*. COMPLETED. No Pd was detected that we are aware of (BC Min of Agriculture conducted testing; however, some guano samples have yet to be processed).
Spring 2021	6	Complete the Tissue Explant Experiment. COMPLETED. Experiment was successful. Results pending.
Spring/ summer 2021	7	Replicate the bat box experiment to test viability of bacteria under varying bat box microclimates. Culture to confirm viability.
Spring/ summer 2021	8	Perform thermal experiments in vitro to establish upper (and lower if possible) lethal thresholds for each probiotic strain.
Spring/ summer 2021	9	Perform experiments to examine freeze-dried vs fresh/rehydrated probiotic bacterial anti-*Pd activity.* And experiment with a variation on the application method that may be more effective in applying probiotic to roost surfaces.
Early summer 2021	10	Use results from Explant experiment to determine if observed efficacy warrants continued field trials.
Mid summer 2021	11	If results of explant experiment are definitive and positive, continue with field applications in 2021, but inoculating roosts earlier in the summer compared to previous years, ensuring that a large portion of each test colony receives probiotic. UPDATE AS OF JUNE 30, 2021: The University of Victoria Lab has been unable to mass produce the probiotic, thus delaying the roost treatments. Production is anticipated for mid July and thus roosts will be treated as soon as pups are volant and roosts can thus be accessed at night while bats are out foraging.
Summer 2021	12	Based on 30 June 2021 results from McMaster lab verifying probiotic was accidentally applied at the Control sites, select a new control site, secure funding for this site, establish baseline data, PIT tag bats, and install readers. Sham inoculations of Control roosts will either be ceased or the sham clay will be sourced from a lab not making the probiotic. An Advisory Committee will be convened to make these methodology decisions.
Summer 2021	13	PIT tag a substantial number of bats at each of our 4 study colonies (and the new 5th colony as described in the above activity). This will require mist net capturing. Special BC

		MOE permission has been received to conduct this work (in accordance with COVID restrictions).
Summer 2021	14	Collect and genetically analyze wing biopsies for species identification of captures that are ambiguously Yuma or Little Brown Myotis.
Summer 2021	15	Sample wings of bats at all study sites for metagenomics analyses of wing microbiomes. At the treatment sites, this will include swabbing pre- and post- application of probiotic at roosts.
Late summer 2021	16	Sample bat wings and roosting substrates prior to hibernation to quantify probiotic cells.
Late winter 2021-22	17	Sample roosting substrates at end of the 2021-22 winter (including building and bat box substrates) to determine persistence of probiotic in winter. Also test viability of these bacteria.
Fall 2021	18	Install acoustic detectors to determine if bats are found in study areas in winter.
Ongoing	19	Begin discussion with BC MOECSS to determine if upscaling of this probiotic treatment is warranted to other areas of southern BC to protect other maternity roosts.
Ongoing	20	Continue to encourage media uptake for this project to inform the public of our progress. To date, this project has received substantial media attention (e.g. CBC 2019a,b; Telus 2019).

SUMMARY

Our goal is to reduce the mortality of bats in the Pacific Northwest using a probiotic (sourced from regional bats) which inhibits or slows the growth of the fungus that causes WNS. We know of few places where bats hibernate in BC and these places are often not accessible in winter and have few bats; it is not feasible nor biologically significant to apply any mitigation during winter at hibernacula.

We have developed a 4-strain probiotic that has demonstrated its ability to inhibit the growth of *Pd* in a lab situation. We do not yet have a measure of how well it reduces *Pd* on bat skin, and our explant experiments aim to quantify its efficacy. We have some data to suggest that these anti-*Pd* microbes can withstand high temperatures of bat boxes, although we have more work to do to identify upper critical temperatures for each strain. Bats successfully pick up these microbes off their inoculated roosting substrate and we have been able to show successful transfer has occurred to bat wings even weeks after application at roost – we've shown this in captive trials and the field trials on wild bats. To date our data suggest the probiotic may not be able to survive winter conditions in bat boxes; building-roost survival data are pending.

Our method of application using powered clay works well and is reasonably cost effective for upscaling. It is simple and could be deployed at building and bat box roosts around BC by interested and engaged citizens. We have demonstrated that these microbes do not pose any health concerns to bats, and thus this probiotic may be able to safely reduce the growth of *Pd* on wings of bats during hibernation, thereby reducing the overwinter mortality of bats.

Although our project's progress was slowed by COVID-19 in 2020, we hope our upcoming field trails and the last of our laboratory and field experiments will fill in the remaining knowledge gaps, including

quantifying efficacy against *Pd* on bat wings, thermal resilience in bat boxes, and overwinter survival on bat wings and in building and bat box roosts. The actual question of how well the probiotic reduces WNS-caused bat mortality is yet to be answered and could be several years off until *Pd* arrives in our BC study area. However, we are looking to sites in Washington to expedite answering this question, given that *Pd* is spreading within this state.

In 2021 we are beginning a field collaboration with Washington Department of Fish and Wildlife to identify study sites with and without *Pd*, and establish baseline data at these sites, with the goal of applying probiotic at these WA sites once permits have been approved. As *Pd* is present in this state, there is an opportunity to compare post-hibernation return rates of bats at 'control' and 'treatment' maternity sites, to quantify any realized reduction in mortality rates associated with the application of the probiotic.

RECOMMENDATIONS

Because probiotic can be easily deployed at building and bat box roosts by interested and engaged citizens, and because we have demonstrated that these microbes do not pose any health concerns to bats, it may be time to for plans to upscale in the Pacific Northwest. Although *Pd* has not yet been detected in BC, a suspicious bat that appeared to be infected with WNS was reported in the San Juan Islands ~10 km from Victoria (Washington Department of Fish and Wildlife, A. Tobin, pers. comm., March 2021). It seems inevitable that WNS will arrive in southern BC, and so it is recommended that we plan for the probiotic upscaling soon, so that bats are protected *prior* to the arrival of WNS at their hibernacula.

Upscaling could be beneficial at the *population* level for bats in BC, especially building roosting species for which we can target large colonies of adult females. We recommend that the BC Government and WCS Canada plan to upscale this probiotic to other major maternity roosts in southern BC. For example, there are other major maternity roosts known in the Greater Vancouver area and in Victoria. Because *Pd* is now found in the US Columbia Basin, it also seems prudent to consider use of the probiotic at the large and well-monitored roosts of Yuma and little brown myotis in the Columbia Basin, in particular in the Creston area. As the implementation of the probiotic could take several years of planning, we suggest it might be useful to start this planning in 2021. We plan to discuss this recommendation with the Probiotic Project Advisory Board.

In order to upscale outside of B.C., we recommend verifying that the presence of the bacterial strains in our probiotic are already found in environments outside the province. To date, searching very few data sources, we have found a good match for 3 of our 4 bacteria. The fourth one that has not been found to date is *P. synxantha* Strain B. While the 4 bacteria have been found to work synergistically and thus it would be good to keep all 4 in the cocktail, this Strain B was not one of the particularly active bacteria. This is in contrast to Strain A that performed exceptionally well in hot conditions of summer roosts, and *P. antarctica* that thrived in winter hibernation conditions. Thus if Strain B were to be removed from the cocktail, if it cannot be found in other 'soil databases', then the cocktail's efficacy may not suffer. McMaster University will be examining further sources of genetic sequences of microbes in 2021 and will provide conclusions about the 'commonness' of the probiotic bacteria outside of B.C.

Recommendations specific to the Stave Watershed in particular include the following: 1. Continue to PIT tag bats and apply probiotic annually (once there are a sufficient number of tagged individuals, the upkeep of this project should become minimal); 2. Continue to sample in spring for WNS surveillance; 3. Continue to monitor return rates and swab for persistence of probiotic (spring and fall); 4. Continue to acoustically monitor in the region (e.g., North American Bat Monitoring Program) as WNS surveillance (increase in winter activity and decrease in summer activity) and of species-specific disease impacts; 4. Maintain the hot water heaters in the boiler room of Stave Lake Lodge to facilitate reproduction and provide a probiotic-inoculated roost for this mixed species colony; 5. Pursue increasing the number of roosts that can be monitored for this large 'meta-colony' of little brown and Yuma myotis – additional PIT tag readers and inoculation locations would be beneficial in this Stave Lake area, although this would

require cooperation of residents in identified house roosts (including BC Hydro staff housing, and private landowners).

As this project is cutting edge use of soil microbes to prevent disease in bats, we recommend continuing to work closely with the Advisory Committee and as we get closer to potentially upscaling (e.g., to Washington state in 2022 as tentatively planned with the WA Department of Fish and Wildlife; and to other parts of B.C.) that this committee be expanded to include more stakeholder groups including the WNS Coordinator for Washington state, and the USFWS WNS Coordinator for Region 1. We also would recommend including Jordi Segers, Canadian WNS Coordinator and possibly a CWS staff member from the Pacific region.

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APPENDIX 1: CHARACTERISTICS OF BATS CAUGHT FROM STAVE LAKE

Table A1. Characteristics and qPCR starting quantity of bats caught at Stave Lake on 27 August, 2019. MYLU = *M. lucifugus*, Little Brown; YULU = either *M. lucifugus* or *M. yumanensis* (Yuma myotis).

Bat Swab #	Probiotic Species	Cell Quantity	Cells/cm	Age	Sex	Species
1	*P. azotoformans*	445.64	18.96	A	F	MYLU
1	*P. synxantha* A	16216.68	690.07	A	F	MYLU
1	*P. synxantha* B	420.10	17.88	A	F	MYLU
2	*P. azotoformans*	395.57	16.83	J	F	MYLU
2	*P. synxantha* A	0.00	0.00	J	F	MYLU
2	*P. synxantha* B	0.00	0.00	J	F	MYLU
3	*P. azotoformans*	79.07	3.36	A	F	MYLU
3	*P. synxantha* A	0.00	0.00	A	F	MYLU
3	*P. synxantha* B	0.00	0.00	A	F	MYLU
4	*P. azotoformans*	1374.40	58.49	A	F	MYLU
4	*P. synxantha* A	21844.19	929.54	A	F	MYLU
4	*P. synxantha* B	654.75	27.86	A	F	MYLU
5	*P. azotoformans*	448.01	19.06	A	F	YULU
5	*P. synxantha* A	0.00	0.00	A	F	YULU
5	*P. synxantha* B	0.00	0.00	A	F	YULU
6	*P. azotoformans*	0.00	0.00	A	F	MYLU
6	*P. synxantha* A	5639.29	239.97	A	F	MYLU
6	*P. synxantha* B	0.00	0.00	A	F	MYLU
7	*P. azotoformans*	395.78	16.84	A	F	MYLU
7	*P. synxantha* A	0.00	0.00	A	F	MYLU
7	*P. synxantha* B	0.00	0.00	A	F	MYLU
8	*P. azotoformans*	0.00	0.00	A	F	MYLU
8	*P. synxantha* A	5127.30	218.18	A	F	MYLU
8	*P. synxantha* B	0.00	0.00	A	F	MYLU
9	*P. azotoformans*	335.64	14.28	A	F	MYLU
9	*P. synxantha* A	6616.92	281.57	A	F	MYLU
9	*P. synxantha* B	176.60	7.51	A	F	MYLU
10	*P. azotoformans*	10.52	0.45	A	F	MYLU
10	*P. synxantha* A	0.00	0.00	A	F	MYLU
10	*P. synxantha* B	0.00	0.00	A	F	MYLU
11	*P. azotoformans*	0.00	0.00	J	M	MYLU
11	*P. synxantha* A	10370765.26	441,309.16	J	M	MYLU
11	*P. synxantha* B	0.00	0.00	J	M	MYLU

APPENDIX 2: MYOTIS EXPLANT SOP

Thompson Rivers University

The purpose of the explant experiment 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

<u>Overview of Procedure</u>

Yuma myotis bats (n = 3) were euthanized by overdosing via isoflurane (Thompson Rivers University). Each bat's patagium was separated into 1 cm diameter samples using a biopsy punch to collect full-thickness samples of skin (n = 29). Biopsy punches were placed into the individual explant chambers quickly to prevent decomposition/dehydration; one side of the explant was exposed to Eagle's modified minimal essential medium supplemented with or without antibiotics (gentamycin, 10ug/ml). The other side was inoculated with the following cell concentrations of the four bacteria present in the probiotic cocktail: 500 cells of *P. azotoformans*, 8500 cells of *P. synxantha* strain A, 200 cells of *P. synxantha* strain

B, and 200 cells of *P. antarctica*. We applied 1800 *Pd* spores onto the side of the explants that was inoculated with the probiotic.

Explants were separated into two groups with differing endpoints (3.5 days and 7.5 days duration), and two subgroups (with and without gentamycin antibiotic) (see Table A2.1). Samples that were exposed to antibiotic media (n = 16) were compared to samples that did not have any exposure to antibiotic media (n = 13) (total n= 29). Half of the surface area of the explants were swabbed with a sterile cotton tipped swab before being placed into the incubator. This swab was placed into sterile water to be used as a baseline in qPCR analysis. After 3.5 days, explants in the 3.5 day group were swabbed to quantify probiotic and *Pd* spore numbers. Similarly, explants in the 7.5 day group were swabbed after 7.5 days to quantify probiotic and *Pd* spore numbers. Five explants were visualized using Scanning Electron Microscope (SEM; McMaster University) to quantify *Pd* germination and visualize microscopic interactions between the probiotic bacteria, *Pd* and gentamycin, at the end of the 3.5 and 7.5 day trials. We received SEM results for analyses only a few days before this report and have thus included them only as an Appendix (see Appendix 4). Interpretation and recommendations stemming from these results will be included in subsequent communications.

Details of 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 4000 rpm 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 -80C 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.

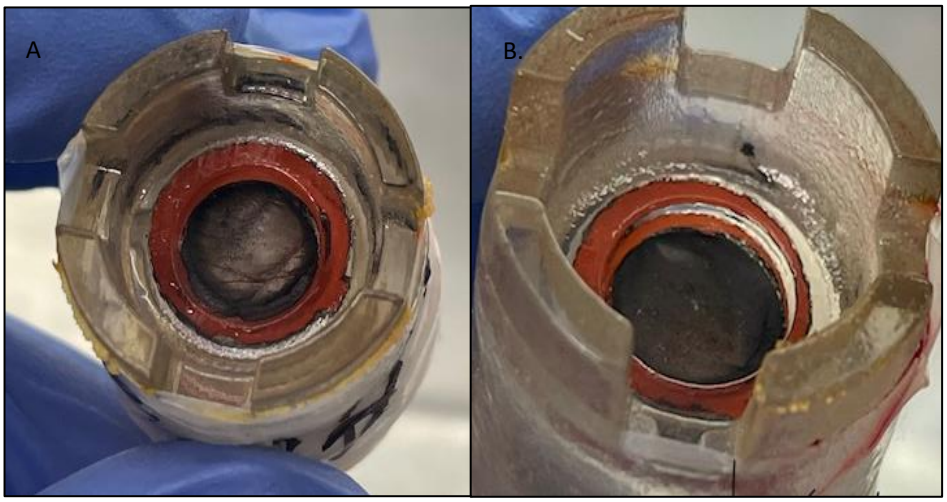

Figure Appendix 2. Explant chambers which contain separated Myotis patagium A) Myotis patagium with antibiotic, *Pd* and probiotic; and B) Myotis patagium with only *Pd* and no probiotic or antibiotics, with white fungal growth evident.

Experimental Design of this Pilot Experiment

Table A2.1. Number of wing tissue explants in each category that were exposed to media with antibiotics, media without antibiotics, or sent for scanning electron microscopy (SEM) analysis.

Endpoint / duration	Treatment	Media with antibiotic (analysis: qPCR)	Media without antibiotic (analysis: qPCR)	Media with antibiotic (analysis: SEM)	Media without antibiotic (analysis: SEM)
3.5	Control: No Probiotic or *Pd*	2	2	0	0
3.5 days	Probiotic	3	2	0	0
3.5	Probiotic + *Pd*	2	2	0	0
3.5	*Pd*	0	0	0	1

7.5	Control: No Probiotic or *Pd*	2	1	1	0
7.5	Probiotic	2	1	1	0
7.5	Probiotic + *Pd*	2	2	1	0
7.5	*Pd*	0	1	0	1
Total samples		13	11	3	2

Results of this Pilot Experiment

Table A2.2. Baseline and endpoint swabs -- starting quantity results of *P. azotoformans* (Cy5), *P. synxantha* strain A (FAM), *P. synxantha* strain B (HEX), and *P. antarctica* (HEX(2)), and *Pd* from the 3.5 day group 1 explants. Inconsistent or unexpected results are highlighted in yellow.

	BASELINE					***ENDPOINT***				
Explant	**Cy5**	**FAM**	**HEX**	**HEX(2)**	***Pd***	**Cy5**	**FAM**	**HEX**	**HEX(2)**	***Pd***
Treatment (Sample)	**qPCR 1.5 days with antibiotic**					**qPCR 3.5 days with antibiotic**				
With probiotic (A)	0	0	0	0	0	0	0	0	4.93E+10	0
With probiotic (B)	0.895	0.948	1.002	486.552	0	0.131	0.079	0.226	0	0
With probiotic (C)	0.084	0.118	0.166	0	0	0.078	0.092	0.112	0	0
Control (A)	0.105	0.005	0	0	0	0	0	0	3.21E+10	9.959
Control (B)	0	0	0	0	0	0	0	0	0	0
Probiotic + *Pd* (A)	0	0	0	263.039	2.961	39.776	42.313	41.808	74447.890	0
Probiotic + *Pd* (B)	0.218	0.236	0.303	154.715	1.935	0.408	0.428	0.500	763.842	3.069
Probiotic + *Pd* (C)	0	0	0	0	7.715					
	qPCR 1.5 days without antibiotic					**qPCR 3.5 days without antibiotic**				
With probiotic (A)	0.365	0.333	0.582	0	0	0	0	0	0	0
With probiotic (B)	2.255	2.137	2.575	204.683	0	0.486	0.413	0.880	374.456	0
Control (A)	0	0	0	0	0	0	0	0	0	0.983
Control (B)	0	0	0	0	0	0.197	0.017	0	0	0
Probiotic + *Pd* (A)	0.994	0.924	1.221	0	0.978	0	0	0	0	0.693
Probiotic + *Pd* (B)	1.361	1.367	1.683	340.744	0.782	1.051	0.978	1.338	89.739	0.836

Table A2.3. Baseline and endpoint swab starting quantity results of *P. azotoformans* (Cy5), *P. synxantha* strain A (FAM), *P. synxantha* strain B (HEX), and *P. antarctica* (HEX(2)), and *Pd* from the 7.5 day group 2 explants. Inconsistent or unexpected results are highlighted in yellow.

Explant	Cy5	FAM	HEX	HEX(2)	*Pd*	Cy5	FAM	HEX	HEX(2)	*Pd*
	qPCR 1.5 days with antibiotic (BASELINE)					qPCR 7.5 days with antibiotic (ENDPOINT)				
With probiotic (A)	0.015	0.019	0.023	0	0	0	0	0	0	0
With probiotic (B)	0.099	0.130	0.179	0	0	0.028	0.025	0	104.840	0
With probiotic (C)	0.024	0.035	0.028	0	0					
Control (A)	0	0	0	0	0	0.024	0.032	0.050	277.477	0
Control (B)	0	0	0	0	0					
Control (C)	0	0	0	0	0	0.078	0.096	0.129	0	0
Probiotic + *Pd* (A)	0	0	0	0	0	1.240	1.281	1.142	0	0
Probiotic + *Pd* (B)	0.154	0.173	0.234	411.981	1.309	0	0	0	0	0
Probiotic + *Pd* (C)	0	0	0	0	0					
	qPCR 1.5 days without antibiotic (BASELINE)					qPCR 7.5 days without antibiotic (ENDPOINT)				
With probiotic (A)	0.480	0.416	0.593	28.448	0	740.498	838.659	819.002	10476.202	0.490
With no probiotic (A)	4.254	16.847	0	0	0	0	0	0	0	0
Probiotic + *Pd* (A)	0.733	0.673	1.154	12.597	2.795	0.490	0.526	0.784	0	1.480
Probiotic + *Pd* (B)	1.265	1.073	1.802	0	0.383	0.118	0.133	0.161	0	1.802

Table A2.4. Starting quantity results of an explant only inoculated with *Pd* and sampled after 3.5 and 7.5 day periods.

Explant	Cy5	FAM	HEX	HEX(2)	*Pd*
Pd Only (3.5 days)	0	0	0	0	0.4069
Pd Only (7.5 days)	0	0	0	0	1.2770

Conclusion: These pilot results have informed a new methodology for this explant experiment. In the comprehensive experiment in spring 2021, new techniques will be employed to reduce chance of contamination. More controls (e.g. *Pd* alone, probiotic alone, no treatment) will be employed, and the entire tissue explant will be qPCR'd instead of using swab-sampling. Antibiotic will not be used and the experimental time period will need to be at least 7 days, ideally longer. Use of SEM is still uncertain, pending further analyses (see Appendix 4).

APPENDIX 3: LAB RESULTS OF TEMPERATURE STRESS EXPERIMENT (2019)

In vitro results of each isolate used in the probiotic cocktail (Tables A3.1 – 4). Growth in petri dish is quantified as follows:

(-) No growth
(+) Slight growth
(++) Moderate growth
(+++) Fully grown

Table A3.1. Growth of 1 *P. azotoformans* on an LB agar plate at various temperatures.

Date	4℃	8℃	15℃	25℃	37℃	56℃
05-16-2019	(-)	(-)	(-)	(++)	(-)	(-)
05-17-2019	(-)	(-)	(++)	(+++)	(-)	(-)
05-21-2019	(+)	(++)	(+++)	(+++)	(++)	(-)
05-22-2019	(+)	(++)	(+++)	(+++)	(+++)	(-)
05-23-2019	(+)	(++)	(+++)	(+++)	(+++)	(-)
05-27-2019	(+++)	(+++)	(+++)	(+++)	(+++)	(-)

Table A3.2. Growth of *P. synxantha B* on an LB agar plate at various temperatures.

Date	4℃	8℃	15℃	25℃	37℃	56℃
05-16-2019	(-)	(-)	(+)	(++)	(+)	(-)
05-17-2019	(-)	(+)	(++)	(+++)	(++)	(-)
05-21-2019	(++)	(+++)	(+++)	(+++)	(++)	(-)
05-22-2019	(++)	(+++)	(+++)	(+++)	(+++)	(-)
05-23-2019	(++)	(+++)	(+++)	(+++)	(+++)	(-)
05-27-2019	(+++)	(+++)	(+++)	(+++)	(+++)	(-)

Table A3.3. Growth of *P. Antarctica* on an LB agar plate at various temperatures.

Date	4℃	8℃	15℃	25℃	37℃	56℃
05-16-2019	(-)	(-)	(+)	(++)	(-)	(-)
05-17-2019	(-)	(+)	(++)	(+++)	(++)	(-)
05-21-2019	(++)	(+++)	(+++)	(+++)	(++)	(-)
05-22-2019	(++)	(++)	(+++)	(+++)	(+++)	(-)
05-23-2019	(++)	(+++)	(+++)	(+++)	(+++)	(-)
05-27-2019	(+++)	(+++)	(+++)	(+++)	(+++)	(-)

Table A3.4. Growth of *P. synxantha* A on an LB agar plate at various temperatures.

Date	4℃	8℃	15℃	25℃	37℃	56℃
05-16-2019	(-)	(-)	(+)	(++)	(+)	(-)
05-17-2019	(-)	(+)	(++)	(+++)	(++)	(-)
05-21-2019	(++)	(+++)	(+++)	(+++)	(++)	(-)
05-22-2019	(++)	(+++)	(+++)	(+++)	(+++)	(-)
05-23-2019	(++)	(+++)	(+++)	(+++)	(+++)	(-)
05-27-2019	(+++)	(+++)	(+++)	(+++)	(+++)	(-)

APPENDIX 4: PRELIMINARY SCANNING ELECTRON MICROSCOPY RESULTS FOR TISSUE EXPLANT EXPERIMENT

Samples from the pilot explant experiment were prepared by fixing the tissue in 2.5% glutaraldehyde for 1h and then washing 3 times with PBS. There was some concern this may not fix all microbes to the surface, especially light weight *Pd* spores, but this potential problem has yet to be assessed and we are in communication with McMaster Scanning Electron microscopy lab.

Five samples were submitted for SEM analysis:

1. Inoculated with *Pd* spores and a anti-*Pd* probiotic bacteria and exposed to gentamicin antibiotic. It sat for 7 days in the explant chamber.
2. Inoculated with *Pd* spores and sat for 3 days. No antibiotic.
3. Inoculated with Pd spores only and sat for 7 days. This had visible fungal growth that I could see. No antibiotic.
4. Exposed to antibiotic media, but had no inoculations. This is the closest sample that we had to a control. It sat for 7 days.
5. Inoculated with probiotic only and exposed to antibiotic media. It sat for 7 days.

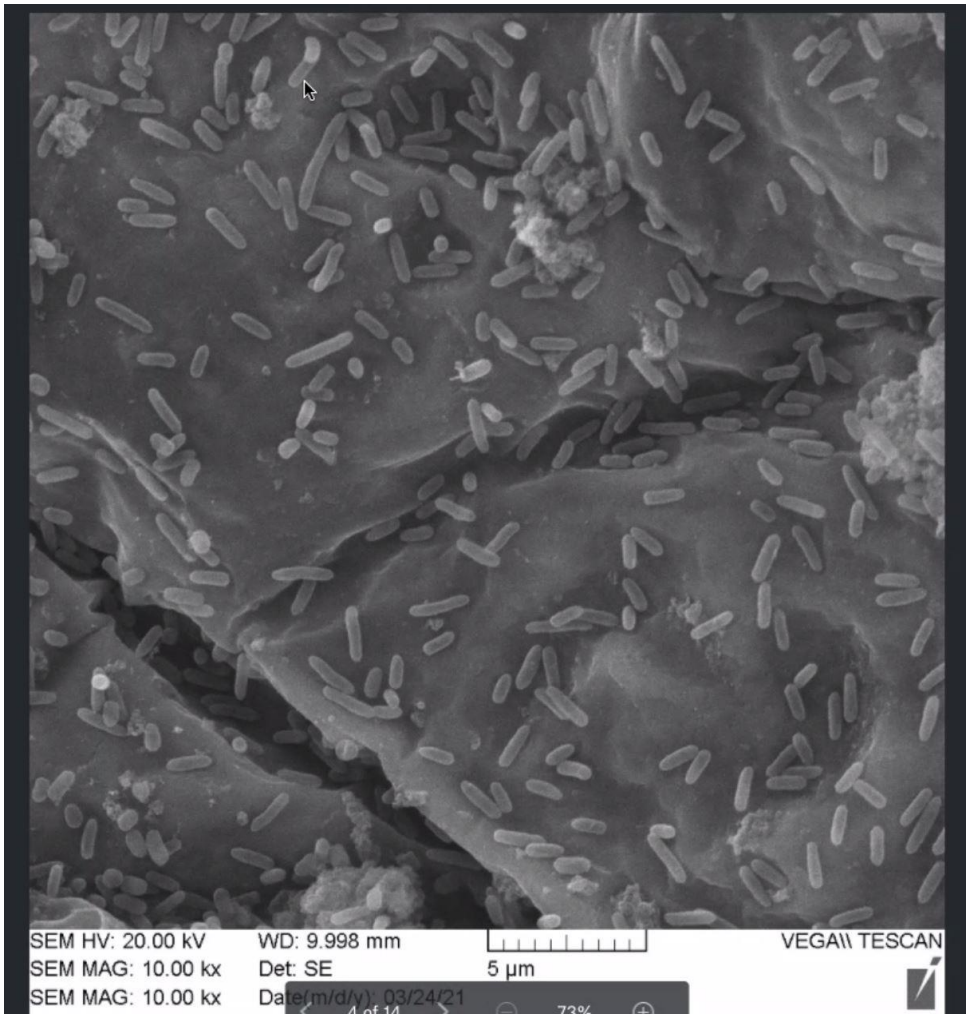

Figure A4.1 Scanning electron microscopy photo. Cursor at top left points to the potential hyphae growing from what is possibly a *Pd* spore. The imagery needs to be further assessed for presence of *Pd* spores and the ability to differentiate these from probiotic cells and determine germination rate of hyphae. Preliminary analysis suggests that few spores seen and even less are germinating; this may be in part due to the fixation process, and due to the short experimental period of 7 days in which few spores are likely to germinate.