Signature page

An Assessment of Anti-Staphylococcal, Anti-Mycobacterial and Anti-Candidal Properties in Fungal Isolates of the Big Brown Bat (*Eptesicus fuscus*) Microbiome

By
Vanessa Noujaim

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfilment of the Requirements for
the Degree of Bachelor of Science with Honours in Biology

April 2020, Halifax, Nova Scotia

Approved: Dr. Clarissa Sit

Associate Professor
Supervisor

Approved:

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ABSTRACT

Antibiotic resistance is becoming an increasing problem in global health. The emergence of antibiotic-resistant bacterial and fungal infections is expected to increase over the next few years. In an effort to develop therapies to counteract this issue, previously unexplored environmental microbes are assessed in their anti-candidal, anti-staphylococcal and anti-mycobacterial properties. Five strains from the big brown bat microbiome are cross-cultured with three pathogenic strains: *Staphylococcus aureus, Mycobacterium smegmatis,* and *Candida albicans.* These pathogenic strains act as proxies for antibiotic-resistant pathogens. Working under the presumption of quorum-sensing occurring between microbes in close proximity, cross-cultures are conducted with the intent of activating dormant pathways. Bat strain growth and phenotypic changes are assessed in these cross-cultures and the possibilities of anti-pathogenic properties in bat strains are determined based on these findings. Two of the five bat strains studied have a strong likelihood of possessing anti-pathogenic properties. The remaining bat strains show promise in further investigations as well, but not to the same extent based on findings.

April 3, 2020

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List of Abbreviations

WNS: White-nose Syndrome
QS: Quorum-sensing
QSM: Quorum-sensing molecules
AR: Antibiotic-resistant
MC: Monoculture
CC: Cross-culture
SCV: Small-colony variant

INTRODUCTION

Modern medicine was initiated by the revolutionary discovery of antibiotics in the 1930's. Since then, antibiotic resistance has been mounting into a significant threat to the provision of healthcare. The World Health Organization maintains that antibiotic resistance is one of the largest threats to global health and food security (WHO 2018). This identified global epidemic has also placed a rather heavy economic strain on healthcare systems around the world, including that of Canada. The adaptation of microbes, bacteria in particular, to resist these anti-microbial compounds is a natural and rapid process. Bacteria have the ability to freely hybridize; genes can be transferred laterally between unrelated species via plasmids and conjugation. DNA in the extracellular environment can also be incorporated into the bacterial genome through the process of transformation. Even more, transduction facilitates mutation and adaptation where bacteriophages can carry and inject fragments of DNA from bacterium to bacterium. The increase in intensity of antibiotic resistance among pathogens is thus the inevitable result of antibiotic use.

However, the rate of acquired resistance in microbes is exacerbated by human activity: over-prescription of antibiotics, animal farming, poor sanitation and sewage treatment and increased international travel (Webster 2017; Aslam et al., 2018). This extensive use of antibiotics in such human interventions has created a strong selection pressure in environmental microbes (Thereutzbacher et al. 2019). The development of antibiotics indeed revolutionized medicine, and as such there is a real concern for a return to the pre-antibiotic age - in which the success rates of major procedures such as surgery and organ transplants will regress (Aslam et al. 2018). Given that these antibiotic-resistant (AR) microbes are slowly but surely pushing the state of healthcare back into a pre-modern age, there indeed is an indisputable need for the development of new antibiotics.

Traditionally, antibiotics have been isolated and developed from natural sources, such as environmental fungal strains, soil bacteria and aquatic organisms. Antibiotics are derived from secondary metabolites: compounds produced by an organism that do not have a direct effect on their own growth or reproduction (Bertrand et al., 2014). The natural products produced by actinomycetes and streptomycetes - classes of bacteria found in soil - are some of the primary sources for antibiotics (Malloy & Hertweck 2017; Bills, Bluer & An, 2013). Generally, these species are grown in monoculture that induces stress, such as lack of essential nutrients, temperature or pH alterations (Gould, Gunesekera & Khan 2019). These same products have been continuously tweaked to evade the acquired drug resistance of pathogenic microbes. This includes combining known antibiotics that possess different mechanisms of action or altering functional groups (Gould, Gunesekera & Khan 2019). Novel antibiotic production, however is at a stand-still; antibiotics developed in the last thirty years are merely variations of previously discovered products (Conly & Johnstone 2005; Gould, Gunasekera & Khan 2019). The discovery and development of new antibiotics has reached a lull for a variety of reasons. However, a fundamental issue in this is the lack of incentive for pharmaceutical companies to invest in the development of a drug that will prove to be ineffective after a short number of years (Theuretzbacher et al. 2019; Gould, Gunasekera & Khan 2019). New treatments for infections are virtually limited and the prevalence of AR microbes or 'superbugs' are on the rise. AR microbes are now developing a resistance to the 'last resort' drugs such as vancomycin (Aslan et al. 2018; Roemer, Schneider & Pinho 2014).

The harsh reality that acts as a deterrent in antibiotic development is the unfavourable cost and time invested into getting an antibiotic on the market, only for resistance to develop less than a year into its therapeutic use (Conly & Johnston 2005; Saha & Mukherjee 2019). Consequently, minimal resources are dedicated to antibiotic research, although some

organizations are recognizing the severity of the matter and have begun introducing some incentives (Conly & Johnston 2005; Aslam et al. 2018). Avenues that show promise for antimicrobial treatments have emerged nonetheless, such as nanotechnology and engineered bacteriophages (Kalhapure et al. 2014; Saha & Mukhurjee 2019). These developments also address the issue of the lack of specificity of some antibiotics. Broad-specificity antibiotics eliminate both detrimental and beneficial microbes in the human microbiome which provides an opportunity for the resistant strains to take root in this new, non-competitive environment.

Antibiotics have commonly been isolated from microbes that are in a laboratory monoculture, outside of their ecological context. In this process, innate secondary metabolites - products that are secreted by an organism in absence or response to an environmental stress - are extracted. That is, some secondary metabolites are secreted into the extracellular environment as chemical messengers and a stress such as competition does not need to be present for this to occur. However. the full 'biosynthetic potential' of these microbes is thus unrealized when they are grown in monoculture, since there are dormant pathways that are not expressed under laboratory conditions but rather are expressed in the natural environment in the presence of other microbes. Even well-studied microorganisms have vast quantities of undocumented biochemical pathways (Malloy & Hertweck 2017). The importance of emergent properties in the natural environment of microbes is exemplified by the human microbiome, where beneficial products are produced by the diverse population of bacteria in the gut and aid in immune defence (Braga, Dourado & Araujo 2016). Some diseases in humans arise due to an imbalance in the microbiota, because pathogens can maintain their colonization and infection of the host when it can successfully compete against commensal bacteria (Braga et al. 2016). In this lies the importance of emulating the natural environment as a means of unlocking novel secondary metabolite production.

Microbes have a complex mechanism of communicating and behaving with nearby organisms in its environment, referred to as quorum-sensing (QS). There is substantial literature on QS in colonies of bacteria, while QS in fungi represents a much more recent discovery (Barruso et al. 2018; Malloy & Hertweck 2017; Albuquerque & Casadevall 2012). Overall, QS involves the process of natural product secretion into the immediate extracellular environment (ECE) which are then detected by other organisms, and a course of action is taken depending on cell density (Bertrand et al, 2014). These microbes communicate through hormone-like molecules called quorum-sensing molecules (QSM) or 'autoinducers' (Albuquerque & Casadevall 2012). Microbes choose to ignore, cooperate or eliminate their neighbouring microbes in the ECE based on the number of signals received, which is proportional to the population density. Biofilm formation, competition and virulence factors in bacterial populations are facilitated by QS. In fungi, QSM have been observed to induce effects on its host and aid in its pathogenicity (Albuquerque & Casadevall 2012). Biofilm can also occur in a mixed form of both fungi and bacteria (Frey-Klett et al. 2011). In some cases, fungi provide 'biotic support' and facilitate biofilm establishment in bacteria (Frey-Klett et al. 2011). The interactions between bacteria, fungi and other microbes, for that matter, are comprehensive as well as innate in the natural environment.

Traditional antibiotics derived from natural products are a result of the isolation of innate QSM found in monocultures of various microbial strains. Interestingly, these innate QSM function as messengers in the ECE and are emitted at sub-lethal concentrations (Braga et al. 2016; Malloy & Hertweck 2017). Therefore, there are a plethora of natural products that could theoretically act as an effective, targeted antibiotic since these are secreted in response to a threat or competition in the environment. These induced secondary metabolites are 'defence antibiotics' while the innate metabolites are 'signaling antibiotics' (Malloy & Hertweck 2017). A certain

microbe's signalling antibiotic could also be a secretion of a lower concentration of its defence antibiotic (Malloy & Hertweck 2017; Bertrand et al., 2014).

With the knowledge that microbial interactions activate dormant pathways and incites the production of secondary metabolites that aid in the microbes' survival, one of the intentions of this experiment is to emulate the idea of the QS stimuli-response system in a controlled laboratory setting.

Three microbial strains will be used as a proxy or representative for pathogenic strains that are heightening in MDR; these pathogenic strains are Methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* and *Candida auris.* The MDR of MRSA and *M. tuberculosis* have been well-documented over the years. The first strain of MRSA was isolated in 1971, a couple decades after the realization was made that bacterial defences could evade the effects of antibiotics over time (Roemer, Schneider & Pinho 2013). MRSA infections are prevalent in hospitals, specifically in patients who are immunocompromised (Webster 2017). *M. tuberculosis* has also represented a rising issue in global health, though more recently there have been concerns over the lack of treatments for this particular infection. Conversely, *Candida auris* has been a more recent discovery, where it was first isolated in Japan and described in 2009 (Lone & Ahmad 2018). In 2017, the first case of MDR *C. auris* was reported in Canada (Lone & Ahmad 2018). Currently, there are no documented reliable therapeutic interventions for *C. auris* infections. This, in part, is due to the difficulty in differentiating it from related *Candida* species such as *Candida albicans* (Lone & Ahmad 2018). Thus, a need for effective anti-fungal treatments are highlighted.

Our experiments are conducted in a Containment Level 2 laboratory. As such, *Staphylococcus aureus, Mycobacterium smegmatis* and *Candida albicans* will act as the 'pathogenic strains' in place of MRSA, *M. tuberculosis* and *C. auris* respectively. The proxy

strains will then be inoculated on the same agar plate as the environmental strains. The environmental strains are retrieved from the microbial library of White-Nose Syndrome (WNS) resistant big brown bats (*Eptesicus fuscus)* compiled by Kolwich (2019). Dr. Paul Faure's group at the McMaster University Bat Lab sampled WNS-resistant big brown bats by swabbing the muzzle and the length of the forearms (Kolwich 2019). Pure microbial strains obtained from Dr. Myron Smith's lab at Carleton University will also be used in screening environmental microbes for potential antimicrobial properties. These strains were gathered from the soil and caves that serve as locations of hibernation for big brown bats.

Two strains from the Faure lab – F1, F3 – and three strains from the Smith lab – S2, S8, S9 – were selected for use in this experiment based on their anti-pathogenic performance against *Pseudogymnoascus destructans,* the fungus that causes WNS (Kolwich. 2019). These strains were isolated and have been identified by Kolwich to date as the following:

- F1 - *Trichoderma sp.*
- F3 – *Didymella sp.*
- S2 – *Unknown*
- S8 – *Unknown*
- S9 – *Penicillium canescens*

From here on, they will be referred to by using their original denotations: F1, F3, S2, S8, and S9.

Strains from the big brown bat epidermal microbiome and habitat are being screened against the pathogenic strains for anti-candidal, anti-staphylococcal and anti-mycobacterial properties for a few fundamental reasons. Firstly, the big brown bat poses as an unexplored area for potential antibiotics against human pathogens. It is evident that other sources of natural products should be examined since they represent an opportunity for the discovery of novel antimicrobial products. Secondly, the immunity of big brown bats against fungal infections is primarily dependent on their 'immuno-microbiome' rather than their biological immune system (Horrocks, Matson & Tieleman 2011). The microbes in and around their skin are the primary

defence for warding off fungal infections (Horrocks, Matson & Tieleman 2011). The strains obtained from the big brown bat essentially show promise in functioning against human pathogenic strains - bacterial and fungal - if their microbiota already shows a strong resistance to infection.

Bat strains that induce phenotypic variation and/or growth impediments in the pathogenic strains and/or demonstrate phenotypic variation in themselves will be identified as having potentially beneficial secondary metabolite production. These noted strains can be examined within future investigations in order to further isolate and identify the secondary metabolites in question. There also remains the question of if these secondary metabolites secreted by the environmental strains are induced or remain innate even in the presence of another microbe. Another question that should be addressed in further studies is the effectiveness of these environmental strains' defence mechanisms against the true pathogens in question - MRSA, *M. tuberculosis, C. auris* - since they possess unique characteristics that contribute to their pathogenicity.

In congruence with determining novel and effective antimicrobial products, this study aims to investigate a potential avenue for antibiotic discovery by inducing secondary metabolite production in co-cultures. The overall objective of this experiment is to conduct a baseline screening of anti-candidal, anti-mycobacterial and anti-staphylococcal properties in previously unexplored environmental strains from big brown bats and their habitats

MATERIALS AND METHODS

Materials:

Candida albicans (ATCC #10231), *Staphylococcus aureus* (ATCC #12600) and *Mycobacterium smegmatis* (ATCC #19420) were ordered and obtained from the American Type Culture

Collection. The F1 and F3 bat strains, which originated from the Faure Lab, were obtained from the preceding Kolwich project. The S2, S8 and S9 bat strains, which originated from the Smith Lab, were also obtained from the Kolwich project. Yeast malt agar was used as the growth medium in Petri dish cultures, the proportions being: 20g agar powder, 21g yeast malt powder for 1000L distilled H_2O.

Methods:

1-3-month-old stocks of pathogens and bat strains grown on YMA plates from freezer stocks were used to conduct CC trials.

Two sets of CCs for each pathogen were conducted. One set remained at room temperature (about 21°C); the other set was incubated at 37°C.

Monocultures of the pathogens and bat strains acted as controls for this experiment. These monocultures were first grown at their ideal temperature for **7** days (Table 1), after which they remained at room temperature for the entirety of the experiment. The site of inoculation (SOI) for these monocultures was at the pole or end of the plate (Figure 1).

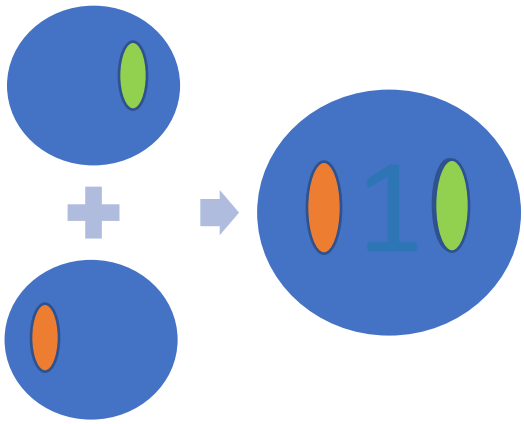

Figure 1: A diagram representing monocultures and cross-cultures of pathogens and bat strains, specifically the site of inoculation at either pole of the plate

In order to account for differences in growth rate among the pathogens and bat strains, some strains were given a 'head-start' to grow on the plate. *S. aureus, M. smegmatis* and bat strains (to be crossed with *C. albicans*) were inoculated and left to incubate at their ideal temperatures (Table 1). After **48** hours, CCs were completed for *S. aureus* and the bat strains, by inoculating the bat strains on the original *S. aureus* plates and *C. albicans* on the original bat strain plates. After **72-96** hours, *M. smegmatis* CCs were completed by inoculating the bat strains.

Growth and other phenotypic characteristics (color, structure) were observed at the **2**-day mark, **5**-day mark and **7**-day mark after completion of CCs.

An approximate area of each monoculture and each strain in CC was measured on the 8th day. Strains were measured in a rectangular area that loosely fit the actual area. For *S. aureus* and *M. smegmatis*, where growth is in distinct colonies, a rectangular area was measured based on how far the bacterial colonies reached (Figure 2). A percentage of growth in CC was calculated for each strain in a CC using these values (Equation 1).

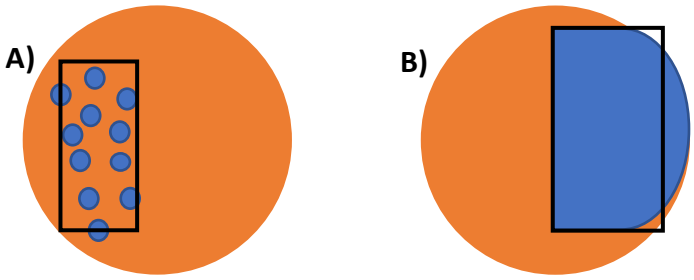

Figure 2: A diagram of the proposed method for approximate area measurement of A) bacterial/yeast growth and B) mold growth in monoculture and CCs.

Equation 1: Percentage growth = $\frac{\text{area of strain in cross-culture}}{\text{area of strain in monoculture}}$

Table 1: A qualitative growth profile of pathogens and bat strains gathered from experimental observation. The following data are approximations based on the environmental parameters that were set up for the experiment. Temperatures below 21°C were not tested, though it is believed that S2, S8, and S9 grow optimally in lower temperatures based on previous trials (Kolwich, 2018). Slow growth rate indicates any growth observed beyond 72 hours, mediocre growth rate indicates any growth observed between 24-72 hours, and fast growth rate is any growth observed up to 24 hours.

	Does growth extend past the area of inoculation? (Y/N)	**Qualitative growth rate (slow, mediocre, fast)**	**Optimal temperature**
Staphylococcus aureus	N	Mediocre	37°C
Mycobacterium smegmatis	N	Slow	37°C
Candida albicans	Y	Fast	37°C
F1	Y	Fast	37°C
F3	Y	Mediocre	21°C
S2	Y	Mediocre	21°C
S8	Y	Fast	21°C
S9	Y	Fast	21°C

RESULTS

Table 2: A general description of pathogen and bat strain phenotypes in both monocultures and cross-cultures. Bat strain phenotypes remained relatively consistent in all three pathogenic cross-

cultures, with a few exceptions which are noted in more detail in Table 3. The following summarizes the observed characteristics. 'Optimal conditions' and 'Early stages of growth' are indicative of phenotypic characteristics in monoculture, whereas 'Stressful conditions' is indicative of general observations of cross-cultures at the 7-day mark. Fungal strains in particular have distinct phenotypic changes at different stages of growth unlike bacterial strains.

	In optimal conditions	**In stressful conditions**	**In early stages of growth**
Staphylococcus aureus	Isolated, small, yellow-white circular colonies	No change observed	None
Mycobacterium smegmatis	Colonies are connected, sometimes circular; white-yellow in color with a smooth texture	No change observed	None
Candida albicans	Irregular appearance; creamy, white to beige and flat. Circular colonies scattered throughout in an irregular fashion Absence of mycelium	Mycelium visible	Absence of circular colonies; only flat, cream-white swashes of growth
F1	Brown-green, flat, moss-like appearance Color of YMA is brown	Clear yellow crystals present in mature cultures Fungus colored green with small buds, turns to white as it extends outwards	Green outgrowths of hyphae-like structures with white circular bud-like projections extending outwards
F3	Color of YMA is dark brown-orange-yellow; brown at SOI and fades into yellow as hyphae extend from SOI Clear orange crystals embedded in white puffy substances.	Absence of crystals	Absence of crystals
S2	White, fluffy, long hair-like appearance Color of YMA is pink-orange	Media is stained dark pink at site of interaction with pathogen; light pink to colorless elsewhere	None
S8	Orange-yellow mold like appearance with flecks of white fuzz Color of YMA is yellow-orange Presence of purple-pink hyphae at outer edges of growth	No changes observed	None

S9	SOI is pink with white, fluffy hair-like extensions growing upwards Color of YMA is pink at SOI; pink-orange elsewhere	Media is stained pink at point of inoculation and at site of interaction with pathogen	None

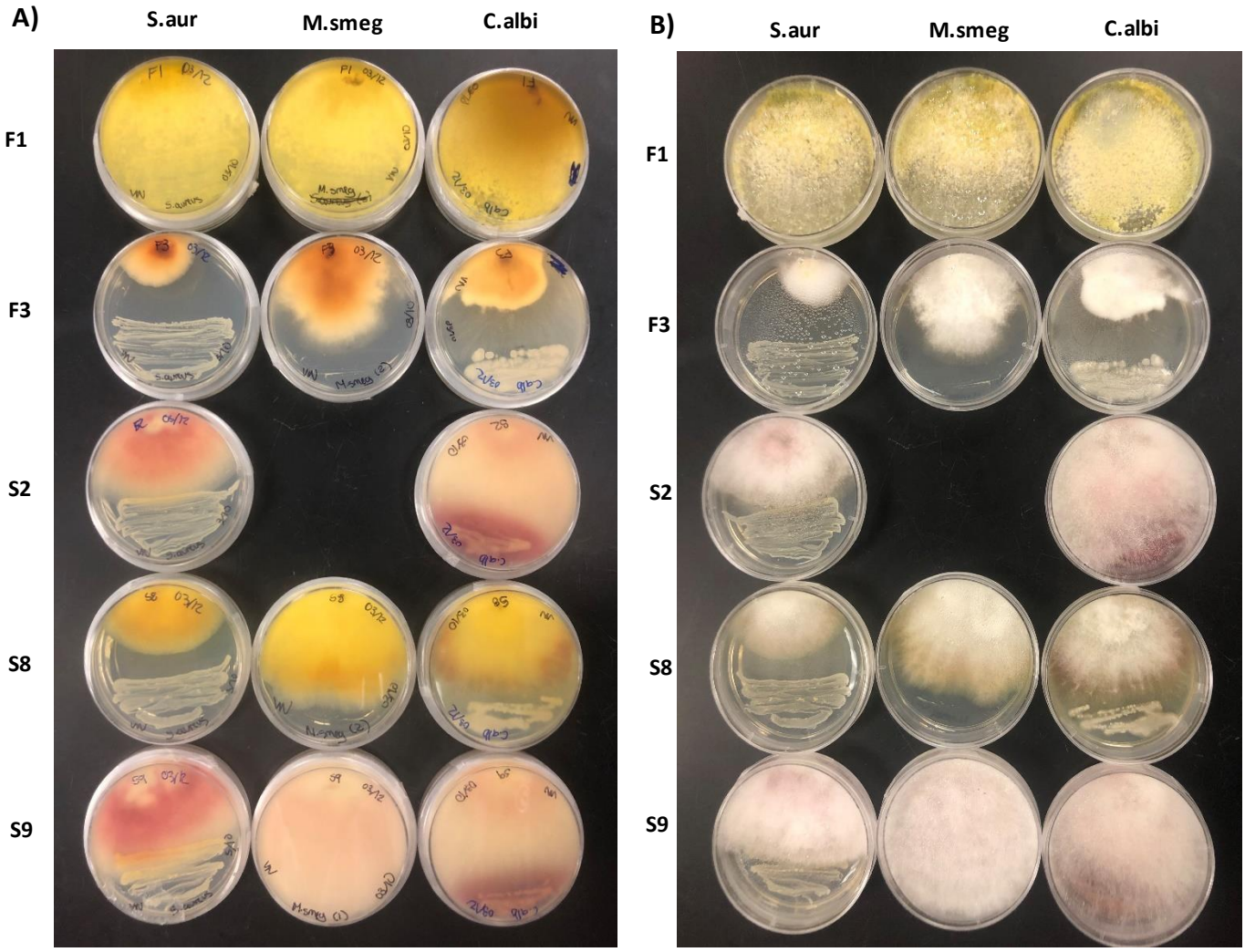

Figure 3: Cross-cultures at 21°C after 7 days of growth. Pathogens for each vertical row are indicated at columns, and bat strains are indicated at the rows. Photo **A)** shows cross-cultures from under the plate, with bat strains on the upper half of the plate and pathogenic strains on the lower half of the plate; Photo **B)** shows cross-cultures from the top of the plate, with pathogenic strains displayed on the upper half of the plate and bat strains displayed on the lower half of the plate.

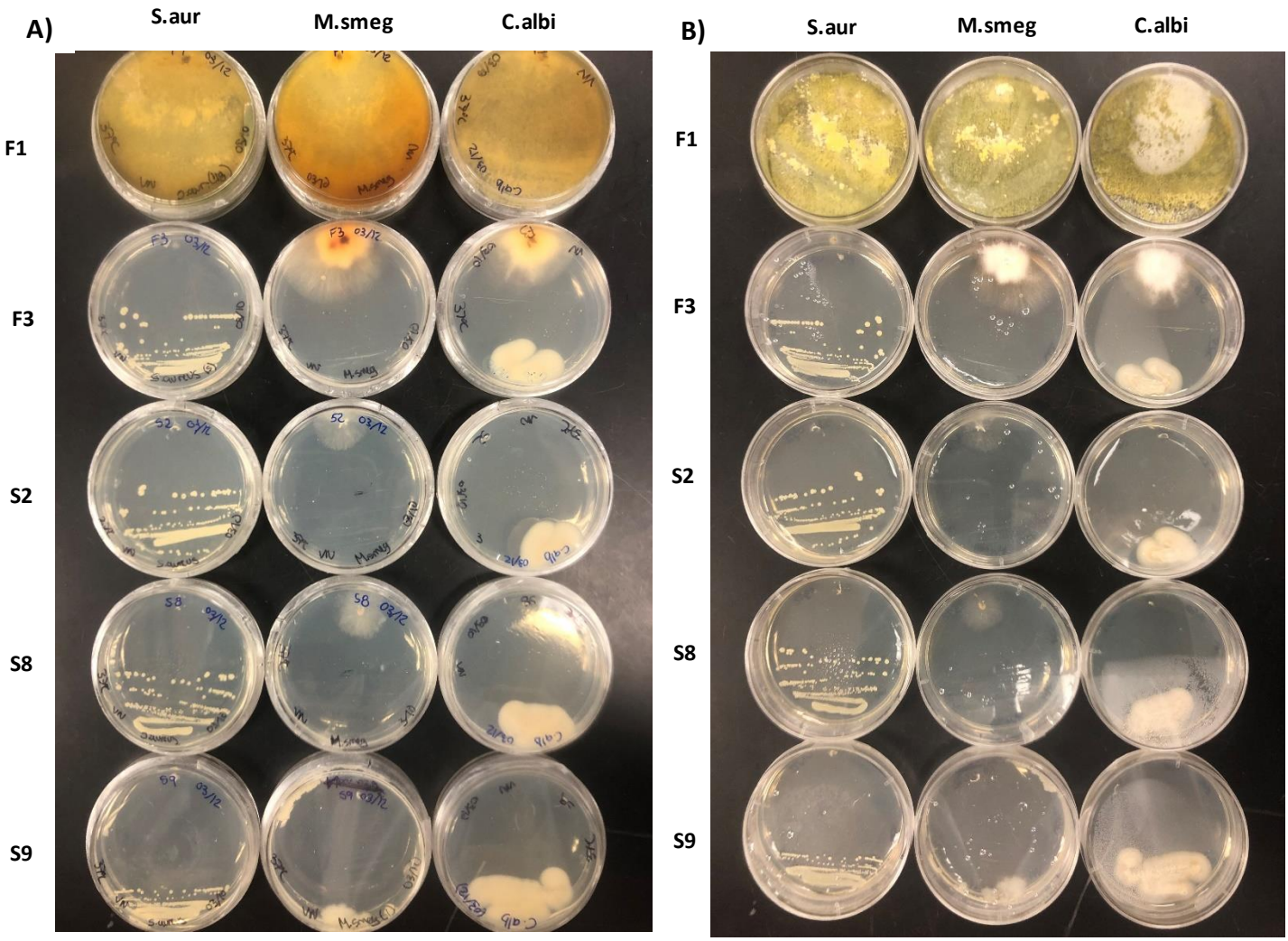

Figure 4: Cross-cultures at 37°C after 7 days of growth. Pathogens for each vertical row are indicated at columns, and bat strains are indicated at the rows. Photo **A)** shows cross-cultures from under the plate, with bat strains on the upper half of the plate and pathogenic strains on the lower half of the plate; Photo **B)** shows cross-cultures from the top of the plate, with pathogenic strains displayed on the upper half of the plate and bat strains displayed on the lower half of the plate.

Table 3: A summarization of phenotypic changes in cross-cultures at 21°C after 7 days.

	F1	F3	S2	S8	S9
S. aur	F1 covers plate. Small patch of light orange, fading to yellow (bottom). White-yellow moss-like texture with 'buds' (top)	F3 covers ¼ of plate. Dark brown-orange fading to yellow edges (bottom). White fuzz (top). Hyphae are thin and yellow in color.	S2 covers ½ plate. Dark pink-orange, fading to light pink-yellow (bottom). Pink fuzz fading to white fuzz (top). S. aur yellow in color at area of contact with S2; white in color elsewhere.	S8 covers 1/3 of plate. Orange yellow with white edges (bottom).	S9 covers 2/3 of plate. Dark pink fading to lighter edges (bottom). S.aur yellow in color in area of contact with S9; white in color elsewhere.
M. smeg	F1 covers plate. Small patch of light orange, fading to yellow (bottom). White-yellow moss-like texture with 'buds' (top)	F3 covers 2/3 of plate. Dark brown-orange fading to yellow edges (bottom). White fuzz (top). No hyphae.	Contaminated	S8 covers ¾ of plate. Yellow darkening to orange-yellow close to edge (bottom). White fuzz with orange yellow edges (top). Contact with M.smeg.	S9 covers plate. Uniform light pink color (bottom). White-pink fuzz (top).
C.alb	F1 covers plate. Dark brown turning to light yellow (bottom). White-yellow moss-like texture with 'buds' (top).	F3 body covering about 1/3 of the plate, with hyphae extending outwards. F3 dark orange in color to yellow edges (bottom). White fuzz (top). Hyphae are thin and yellow-orange in color No contact with C.alb	S2 covers entire plate. Light pink in color. Very dark pink at area of contact with C.alb (bottom). White-pink fuzz, not entirely covering C.alb (top).	S8 covers ¾ of plate Yellow, fading to light yellow, dark orange hyphae-like structures at the edge (bottom). No contact with C.alb	S9 covers plate. Light pink with dark pink at area of contact with C.alb (bottom). White fuzz covering C.alb (top)

Table 4: A summarization of phenotypic changes in cross-cultures at 37°C after 7 days.

	F1	F3	S2	S8	S9
S. aur	F1 covers plate. Green-brown with patches of yellow (bottom). Green-yellow moss-like texture with yellow-white patches (top).	Low growth of F3.	Low growth of S2	Low growth of S8	Low growth of S9
M. smeg	F1 covers plate. Brown-orange-green, fading out to light green and becoming darker again closer to M.smeg (bottom) Green moss-like texture with yellow-white patches (top)	F3 covers less than ¼ of plate. Dark-brown orange fading out to yellow (bottom). White fuzz (top). Hyphae extending out from body. M.smeg growth slightly visible	Low growth – hyphae visible. M.smeg growth not visible	Low –growth – hyphae visible. M.smeg growth slightly visible	Contaminated
C. alb	F1 covers plate. Green-brown with patches of light yellow where C.alb is (bottom). Dark green moss-like texture with yellow-white patches near C.alb (top).	F3 covers less than ¼ of plate. Dark-brown orange fading out to yellow (bottom). White fuzz (top). Hyphae extending out from body.	No growth S2	No growth S8	No growth of S9

Phenotype

The appearance of F1 differed between temperatures; at 21°C, the majority of F1's structure is composed of white buds (Table 3). At 37°C, F1 does not have white buds and is predominantly green (Table 4; Figure 4).

S2 varied in colour composition in CC with *S. aureus* as opposed to CC with *C. albicans*. S2 is a dark pink in the presence of *S. aureus* and light pink in the presence of *C. albicans* (Table 3; Figure 3). S2 within the *C. albicans* CC also appears to turn into a dark pink where it comes in contact with *C. albicans* (Table 3; Figure 3). When S2 comes into contact with *S. aureus*, this colour change is not observed. Rather, *S. aureus* changes colour from white to yellow when in contact with S2. These same observations between S2, *S. aureus* and *C. albicans* are seen with S9 (Table 3; Figure 3).

F3 also differs in phenotypic structure. F3 does have hyphae with the *M. smegmatis* CC, but does produce hyphae when CCd with *S. aureus* and *C. albicans* (Table 3; Figure 3). In contrast, F3 is observed to have hyphae with *M. smegmatis* and *C. albicans* CC at 37°C (Table 4; Figure 4). There were no hyphae observed with F3 x *S. aureus* due to insufficient growth of F3 (Table 4; Figure 7).

C. albicans and *S. aureus* varied in structure between temperatures. *C. albicans* appears to have many more visible circular colonies covering more surface area at 21°C (Figure 3). At 37°C, *C. albicans* is not as widespread, with little to no circular colonies (Figure 4). *S. aureus* appears as one fluid streak of bacterial growth at 21°C (Figure 3). At 37°C, *S. aureus* has distinct circular colonies (Figure 4).

M.smegmatis growth was inconsistent across both temperature trials, though colonies appeared to be slightly visible at 21°C compared to 37°C (Figure 3; Figure 4).

No data
No growth
<1 - 5% growth
6-24% growth
25-50% growth
51-74% growth
75-100% growth

	F1	F3	S2	S8	S9
S. aur	5	5	4	4	4
M.smeg	5	3	-	2	0
C.albi	1	4	3	3	2

Figure 5: A heat map displaying percent growth of pathogenic strains in cross-culture trials with bat strains at 21°C.

	F1	F3	S2	S8	S9
S.aur	0	5	5	4	3
M.smeg	0	4	5	5	-
C.albi	1	2	2	2	3

Figure 6: A heat map displaying percent growth of pathogenic strains in cross-culture trials with bat strains at 37°C.

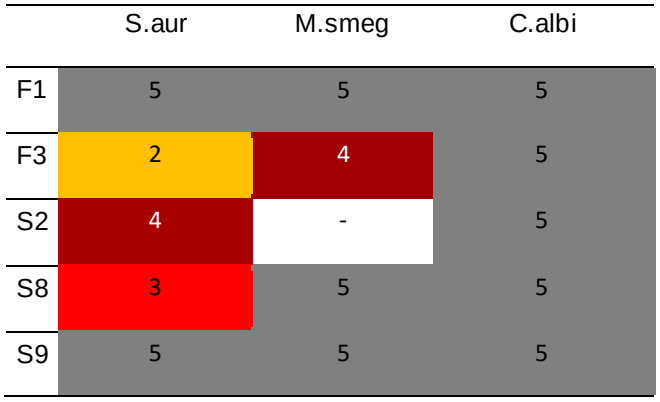

Figure 7: A heat map displaying percent growth of bat strains in cross-culture trials with pathogenic strains at 21°C.

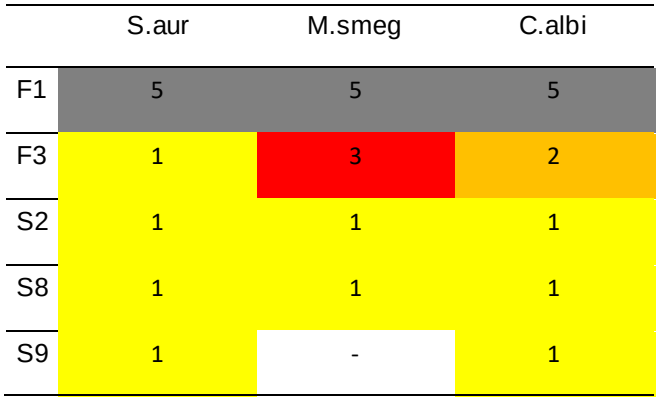

Figure 8: A heat map displaying percent growth of bat strains in cross-culture trials with pathogenic strains at 37°C.

There is no data for S2 x *M. smegmatis* (21°C) and S9 x *M. smegmatis* (37°C) due to contamination.

Growth

At 21°C, all bat strains showed 75-100% growth in *C. albicans* CCs (Figure 7). F1, S8 and S9 showed 75-100% growth in *M. smegmatis* CCs (Figure 7). Finally, only F1 and S9 showed 75-100% growth in *S. aureus* CCs (Figure 7). F1 and S9 were the only strains successful in 75-100% growth within all three pathogenic CCs (Figure 7),

At 37°C, S2, S8 and S9 showed little growth in all pathogenic CCs (Figure 8). F3 showed minimal growth in the *S. aureus* CC, compared to greater growth with *M. smegmatis* and *C. albicans* (Figure 8). F1 was the only bat strain in the 75-100% growth bracket for all three pathogens (Figure 8).

Pathogens generally grew above the 25% mark in most CCs with the exception of a few CCs. *M smegmatis* did not grow in the presence of S9 at 21°C (Figure 5). *C. albicans* growth was considerably less in the presence of F1 compared to the other bat strains at 21°C (Figure 5). *C. albicans* also showed the same extent of growth with F1 at 37°C (Figure 6). *S. aureus* and *M. smegmatis* did not show growth in F1 CCs at 37°C (Figure 6).

DISCUSSION

Overview of Pathogenic Strains

M. smegmatis

There appeared to be issues with significant *M. smegmatis* growth in both temperature trials of the experiment. *M.smegmatis* in monoculture had visible colonies (Appendix 2). In CCs, colonies were transparent, with very little growth of visible colonies seen (Figure 3; Figure 4). For the purpose of this experiment, *M. smegmatis* growth measurements were inclusive of these 'transparent' colonies. However, the success of bat strain growth against *M. smegmatis* cannot be attributed to possessing anti-mycobacterial properties. This is predominantly due to bat strain phenotypes in *M. smegmatis* CCs being quite similar to their phenotype in monoculture (Figure

3; Appendix 2). It is likely that what was observed in the *M. smegmatis* CCs were a dormant form of the bacteria, since a small area of bacterial growth was observed in certain CCs, such as that of F3 and S8 (Figure 3A; Figure 4A). Due to unfavourable conditions, *M. smegmatis* reverted to a dormant form upon inoculation from monoculture to CC.

As is the case with many soil bacteria, *M. smegmatis* requires H_2. Sequestering H_2 sustains respiratory cycles during the stationary phase of growth in many actinomycetes (Greening 2014). Aeration would have been beneficial for growth in *M. smegmatis* as to provide the essential atmospheric gasses it needs for cellular processes, along with an ideal nutritive medium such as MB7H10 (Prasad et al. 2019).

M.smegmatis is also a difficult microbe to assess in relation to anti-mycobacterial properties in CCs due to its rather unusual mechanism of asymmetrical division compared to other bacilli bacteria. Mycobacteria do not possess the molecular mechanism that ensures the placement of the division septum is at the centre of the cell, resulting in an elongated mother cell and a smaller, circular daughter cell (Aldridge et al. 2012; Ginda et al. 2017). Chromosomes are not evenly segregated during division, resulting in genetically distinct subpopulations of *Mycobacteria* colonies. Heterogeneity in the population increases the difficulty in treating mycobacterial infections like tuberculosis (Aldridge et al. 2012). A heterogeneous population also represents a source of inconsistency when inoculating CCs.

More CC trials involving bat strains must be conducted with *M. smegmatis* in order to properly assess the possibility of anti-mycobacterial properties within the bat strains.

C.albicans and *S. aureus*

C.albicans and *S. aureus* displayed similarities in the maintenance of growth and cell density in CC. Both pathogens generally displayed better growth in CCs at 21°C compared to 37°C (Figure

5; Figure 6). This observation does not lie in congruence with the optimal temperatures that were observed for both pathogens (Table 1). However, both *S. aureus* and *C. albicans* are considered to be successful pathogens due to their ability to survive in a wide range of environments including temperature fluctuations (Onyango et al. 2016; Sherwood et al. 2017; Du & Huang 2016).

Overall, the performance of *C. albicans* and *S. aureus* are relatively similar in the sense that these pathogens maintained their growth, though *S. aureus* better suppressed bat strain growth (Figure 4; Figure 5). The method in timing inoculation of CCs likely contributes to these findings. *C. albicans* was inoculated after providing the bat strains with a two-day head start because of its quick growth and robust properties as a microorganism.

The ability to switch between phenotypes – yeast or hyphal – makes *C. albicans* an effective pathogen in various environments, including the human microbiome (Fonzi 2002). The yeast state is beneficial for propagation of growth, whereas the hyphal state switches on in instances of infection or to increase virulence (Du & Huang, 2016). Hyphae generally facilitate penetration of host cells in cases of infection (Fonzi 2002; Jarosz et al. 2009; Brand 2011). pH greatly influences the transition between the yeast-phase and the hyphal-phase; *C. albicans* assumes a yeast-structure in an acidic environment while filamentation occurs in a neutral to basic environment (Fonzi 2002) (Du & Huang 2016).

Hyphae formation was not observed in *C. albicans* CCs, perhaps in part due to the pH of the media becoming slightly acidic in the presence of bat strains – since hyphal formation in *C. albicans* monocultures were observed in mature strains (Table 2; Appendix 2). It is also possible that the bat strains directly suppressed the ability of *C. albicans* to transverse into a hyphal form via QSM. There are other instances of microorganisms suppressing *C. albicans* hypha formation

through QSMs, such as the opportunistic bacterial pathogen *Pseudomonas aruginosa* (Jarosz et al., 2009).

S. aureus has also been observed to undergo 'phenotypic switching' in periods of high stress such as low temperature (Onyango et al. 2012). *S. aureus* along with other staphylococcal species form 'small colony variants' (SCV) (Onyango et al. 2012; Mitchell et al. 2013). SCV contribute to virulence by increasing the rate of biofilm formation (Mitchell et al. 2013). SCV also produce distinct metabolites compared to their wild-type *S. aureus* counterparts (Wang & Zhu 2017). An increase in asymmetrical cell division is also seen in SCV (Onyngo et al. 2012). Asymmetrical division results in different composition of proteins and other cell wall components (Tropini, Rabbani & Huang 2012). So, the same effect in increased ability to respond to stress in environment as *M. smegmatis* is achieved by *S. aureus.* A great issue in treatment of *Staphylococcal* infections is the efficacy of drug delivery across biofilms (Mohammed et al. 2018).

The colour pigmentation and cell wall composition of SCVs are altered in comparison to the original 'wild-type' colonies (Onyango et al. 2012; Guerillot et al, 2019). SCV are also characterized by slow growth compared to wild-type *S. aureus* (Guerillot et al, 2019). Though slow growth is not particularly exhibited in the case of this experiment, the phenotypic difference observed in *S. aureus* colonies at 21°C and 37°C could still be explained by the presence of SCV (Figure 3; Figure 4). There is great genetic diversity between SCVs, dependent on the response that the environment elicits from *S. aureus* colonies (Guerillot et al, 2019). 21°C is not the ideal temperature for *S. aureus*, though growth at this temperature remains. This coupled with the stress that a CC induces on microorganisms could provide a trigger for SCV formation in *S. aureus.*

There is also a difference in *C. albicans* morphology between temperatures. Smaller, circular white colonies occur at 21°C (Figure 3). Larger, cream coloured circular colonies occur at 37°C (Figure 4). This is less likely due to bat strain influence and more likely to do with temperature. The cellular appearance and morphology of *C.albicans* has been observed to change (Du & Huang 2016). A significant amount of exposure to heat effectively inhibits hyphal formation in *C. albicans,* which increases the effectiveness of antifungal medications (Ikezaki et al. 2019).

Despite the slight difference in the success of *S.aureus* growth compared to *C.albicans,* this observed difference could be viewed as insignificant in terms of bat strain anti-pathogenic success. Both *C.albicans* and *S.aureus* posses properties such as phenotypic switching that contribute to their success as pathogens in colonizing an environment. In essence, an assertion of which pathogen is more resilient against bat strains cannot be made; but the anti-pathogenic potential of the bat strains themselves is slightly more conclusive. The extent of growth observed in bat strains in the presence of aggressive pathogens such as *C. albicans* and *S. aureus* show promise.

Overview of Bat Strains

Cell density as a function of Quorum-sensing

This investigation is working under the presumption that QSM are secreted into the extracellular medium by a microorganism when it is in the presence of another microorganism. There are a few issues to consider within this framework of microbial interaction, specifically cell density of the microorganism and quantity of QSM.

The rate of growth of any microorganism certainly has major implications in their ability to establish QS networks, at both the chemical and physical levels. First, more cells mean more QSM produced, which would propagate the intended effects of these molecules, whether it be to

increase virulence factors in a host or act as messengers to nearby organisms (Moreno-Gamez et al. 2017). In the initiation of a quorum-sensing network, autoinducers – a type of QSM – are released without the trigger of an environmental stress (Barrussio et al. 2018). The quantity of auto-inducers produced is directly proportional to the cell density of the microorganism (Moreno-Gomez et al. 2017). Once autoinducers reach a certain quantity in the ECE, quorum sensing with other microorganisms is commenced by triggering biofilm formation and/or the production of virulent QSM (Bettenworth et al. 2019).

QSM, including autoinducers, produced by bat strains could potentially be more lethal against pathogens in higher quantities. The difference lies in the quantity at which the QSM are secreted; autoinducers are secreted in smaller quantities. When a threat is detected, the microorganism then increases the quantity of that autoinducer or other QSM, diffusing into the ECE with the purpose of initiating competition with a nearby microorganism (Bettenworth et al. 2019).

A high growth rate is beneficial in the natural environment where competition for resources is inherent due to the usual microorganism-rich habitats such as soil and water (Frey-Klett et al. 2011). The relatively rapid growth rate of bat strains in this experiment could have provided these fungi with an advantage in colonizing an environment and secreting secondary-metabolites.

F1

Identified as *Trichoderma spp.*, there is no data to date on the presence of possible anti-pathogenic properties. F1 was the most successful bat strain in growth at both temperatures and in the presence of all three pathogens. The differences in phenotype between 21°C and 37°C could be attributed to spore formation. F1 at 21°C was not as mature since it grew optimally at 37°C (Table 1; Table 2). Even in a less mature state, F1 was still effective in colonizing the entire plate in the presence of pathogens (Figure 3). This strain in particular poses a possibly

fruitful avenue of further investigation, given its ability to thrive in varying temperatures as well as effectively limiting the growth of *C. albicans* and *S. aureus*, which possess effective virulent factors themselves.

F1's success in CCs could be a result of its relatively high growth rate (Table 1), the production of anti-pathogenic secondary metabolites, or a combination of these two possibilities. F1 exhibits a rapid growth rate and with a high cell-density fungus, a larger quantity of QSM could theoretically be produced. It is possibility that some of these F1-produced QSM are anti-pathogenic, yet this cannot be said for certain until these QSM are isolated and inserted into a growth medium with the pathogens in question.

F3

Identified as *Didymella* spp., there is no data to date on the presence of possible anti-pathogenic properties. Based on F3's growth at both temperature trials in the presence of pathogens with the exception of S. *aureus* at 37°C, investigating this fungus is yet another avenue worth pursuing in future investigations. The hyphal extensions seen in both trials (Figure 3; Figure 4; Table 3; Table 4) are indicative of fungal growth. It is likely that F3 would eventually cover the entire plate, in similar fashion to F1, if the CCs were observed at a point where more time has elapsed. The formation of hyphae extending outwards is strong evidence for more significant growth to occur (Abubakar 2013). F3 can then be considered as yet another successful bat strain that can grow in the presence of pathogens, marking it as a possible source of anti-pathogenic metabolite production.

S2

To date, there has been no identification of S2. Based on its similar appearance to S9 in monoculture and CCs, it is likely that the two strains are closely related (Figure 3; Appendix 2).

There is a slight difference between S2 and S9 seen in the rate of growth in CCs. *C. albicans* exhibited a higher surface area of growth in S2 CC as opposed to S9 CC (Figure 5). Conversely, growth of *S. aureus* in S2 and S9 CCs are similarly ranked (Figure 5). Due to the lack of definitive data in differentiating the performance of S2 and S9 in CCs, more trials are needed to discern the difference between the two strains, if any. For the purpose of this investigation, any discussion pertaining to observations of S9 in CC are applicable to S2.

S8

To date, there has been no identification of S8. Along with its S2 and S9 counterparts, S8 showed minimal growth at 37°C (Figure 8). At 21°C, S8 exhibited more growth than F3 with *S. aureus* (Figure 3; Figure 7). Despite the larger growth seen in S8 compared to F3, this does not necessarily classify S8 as a possibly better anti-pathogenic microbe than F3. When considering growth coupled with phenotypic changes, F3 represents a better opponent of *S. aureus* than S8. Though surface area coverage is smaller in F3, hyphae are also observed (Table 3), indicating the continuation of growth.

In contrast, S8 may have covered more area, but no hyphae are observed (Table 3). Growth of S8, then, could possibly have been halted since hyphae are the mechanism in which immobile fungi grow. Hyphae are seen in S8 x *C. albicans* as growth is continued (Figure 3). Despite the larger surface area covered by S8 x *S. aureus*, F3 remains a more promising avenue of study due to the high possibility of continued growth indicated by hyphae presence.

S9

Identified as *Penicillium canescens*, S9 is a soil fungus and forms mycorrhizal associations with plants (Sinitsyna et al. 2003). Documentation of its anti-pathogenic properties and possible explanations of the distinct coloration seen with *C. albicans* are also minimal (Figure 3). A

possible theory to explain the colour change from light pink to dark pink at the site of interaction with *C. albicans* could be the up-regulation of certain QSM in response to this pathogen's presence. It could also be a reaction between S8 QSM and *C. albicans* QSM. Another explanation could be the production of spores. Sporulation, in addition to extension of hyphae, is another indicator of fungal growth (Abubakar 2013).

It is difficult to discern sporulation or QSM production, due to the 'reverse' colour change seen in S9 x *S. aureus* compared to S9 x *C. albicans* (Figure 3A). S9 has patches of light pink at the pole of the plate, changing to a dark pink and then to a light pink along the outer edges where it comes into contact with *S. aureus* colonies (Figure 3A). S9 x *C. albicans* is light pink throughout, only turning dark pink at the site of interaction with *C. albicans*. The same difference in colour is observed in S2 x *S. aureus* and S2 x *C. albicans* (Figure 3A). There are a multitude of possible explanations that could be put forth, such as the possibility that S9 appears different in CC with *S. aureus* as a response to an increase of competition and stress. S9 grew to the full extent of the plate area in CC with *C. albicans,* which strongly supports the idea that a significant amount of stress was not inflicted on S9 by *C. albicans.*

Considerations for Future Investigations

Competition or Mutualism

Another question these findings raise is whether the interaction between pathogen and bat strain is one of competition or one of mutualism/symbiosis. F1 and S9 were the only bat strains to encompass the plate, but pathogenic colonies remained visible (Figure 3A). There is a likelihood that this interaction between bat strain and pathogen is one of mutualism and not competition, decreasing the secretion of anti-pathogenic QSM molecule by the bat strains. To confirm competition, the isolated secondary metabolites or QSM produced in a bat strain x pathogen CC

could then be added in larger quantities to the growth medium for a pathogen monoculture. The presence of anti-pathogenic properties in the bat strains could then be accurately be observed.

pH

pH was a parameter that remained unaltered in this investigation. It is known that pH affects phenotypic switching in *C. albicans* and *S. aureus* (Sherrington et al. 2017). pH is considered a crucial signal for opportunistic pathogens in cases of infection, where changes in physical and metabolic changes can be seen (Sherrington et al. 2017).

pH also affects growth and sporulation in most fungi, the majority of which prefer acidic environments (Abubakar et al. 2013). Spore production generally decreases in high pH, with the exception of a few fungal species, and mycelium growth is inhibited (Abubakar et al. 2013). Altering pH to potentially create a slightly less favourable environment for bat strains, in addition to competing for resources with pathogenic strains, could trigger stress-induced metabolic pathways in which secondary metabolites are secreted.

Nutrition

It is possible that YMA media, though it facilitated growth for the diverse selection of microorganisms studied in this experiment, did not favour the production of QSM in some strains. For example, *Penicillium chrysogenum* produces penicillin when glucose is used as a carbon source as opposed to lactose (Cepeda-Garcia et al. 2014). Altering carbon sources for the bat strains could possibly induce the production of QSM by triggering otherwise dormant pathways in the organism that responds to stress.

CONCLUSION

There are endless combinations of parameters – temperature, nutrition, pH, aeration – that could elicit a stress response in microorganisms and, subsequently, the production of desired anti-pathogenic secondary metabolites. Within the temperature, nutritional and time parameters set by this experiment, it was found that bat strains F1 and S9 show the most promise in anti-pathogenic potential due to their ability to grow over the total surface of the plate after one week. F1 in particular greatly suppressed the growth of pathogens at both temperatures. F3 did not exhibit great growth but shows potential since it was continuing to grow past the one-week mark. The results of S2 CCs were nearly the same as those of S9. Identification of S2 is needed to confirm these strains are genetically distinct. S8 demonstrated potential anti-pathogenic properties against *C. albicans*, but growth of S8 was inhibited by *S. aureus.*

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APPENDIX 1

Area of growth calculations for 37°C trials (cm^2)

F1: 20.8 S.aureus: < 1	F1: 20.8 cm^2 M.smegmatis: 0 cm^2	F1: 20.8 C.albicans: < 1
F3: < 1 S.aureus: 10.14 cm^2	F3: 6.96 cm^2 M.smegmatis: 8.74 cm^2	F3: 4.94 C.albicans: 3.6
S2: < 1 cm^2 S.aureus: 8.63 cm^2	S2: 1.08 cm^2 M.smegmatis: 7.98 cm^2	S2: < 1 C.albicans: 3.38
S8: < 1 cm^2 S.aureus: 7.35 cm^2	S8: 0.87 cm^2 M.smegmatis: 9.12 cm^2	S8: < 1 C.albicans: 3.05
S9: < 1 cm^2 S.aureus: 5.89 cm^2	S9: contaminated M.smegmatis: contaminated	S9: < 1 C.albicans: 9.52

Area of growth calculations for 21°C trials (cm^2)

F1: 20.8 S.aureus: 9.02	F1: 20.8 M.smegmatis: 9.31	F1: 20.8 C.albicans: <1
F3: 3.81 S.aureus: 8.82	F3: 14.04 M.smegmatis: 3.51	F3: 18.4 C.albicans: 6.67
S2: 12.3 S.aureus: 7.78	S2: contaminated M.smegmatis: contaminated	S2: 20.8 C.albicans: 3.64
S8: 8.97 S.aureus: 6.48	S8: 19.8 M.smegmatis: 1.47	S8: 20.2 C.albicans: 4.18
S9: 16.8 S.aureus: 6.46	S9: 20.8 M.smegmatis: 0	S9: 19.76 C.albicans: 1.76

Approximate area of monocultures:
Bat strains: 20.8 cm^2 (full plate area)
S. aureus: 9.03 cm^2
M.smegmatis: 8.87 cm^2
C. albicans: 13.03 cm^2

APPENDIX 2

Monocultures at ideal temperature after *two weeks* of growth