Discovery of novel cold-active antifungals from polar bacteria isolated from the Canadian high arctic that are active against major spoilage fungi in the cheese industry

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This thesis is dedicated to my family and friends.

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PREFACE

This thesis was prepared in accordance with the McGill University Thesis Preparation guidelines. This thesis is written and organized in the traditional monograph-style, containing six chapters; introduction, literature review, methods, results, discussion, and conclusion.

Contribution of Co-Authors

The six chapters of this thesis project were designed, performed, analyzed, and written by Adam Classen under the supervision of Dr. Jennifer Ronholm and Dr. Lyle Whyte. Adam Classen performed the *in silico* screening and *in vitro* assays on the arctic isolates. Dr. Roger Lévesque provided various arctic isolates for screening and preformed hybrid genome sequencing and assembly on isolates of interest. Dr. Stéphane Bayen and Dr. Lan Liu preformed LC-MS and data analysis of promising organic extracts. Evangelos Marcolefas preformed initial sample collection and helped design the initial extraction protocol.

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LIST OF ABREVIATIONS

AMPS	Antimicrobial peptides
AMR	Antimicrobial resistance
ANI	Average nucleotide identity
ATP	Adenosine triphosphate
A_W	Water availability
BGCS	Biosynthetic gene clusters
DAPG	2,4 diacetylphloroglucinol
GH	Glycosyl hydrolase
GRAS	Generally Recognized as Safe
GSLS	Glycosphingolipids
HPLC	High performance liquid chromatography
ISP2	International Streptomyces Project-2 Medium
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography–mass spectrometry
MCE	Mixed cellulose ester
MIGA	Microbial genomes atlas
MPP	MassHunter Profiler Professional
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetases
PDA	Potato dextrose agar

PKS	Polyketide synthases
TSB	Tryptic soy broth
TSA	Tryptic soy agar
YEME	Yeast extract-malt extract

ABSTRACT

The primary antifungal used in the cheese industry is natamycin – a polyene that inhibits fungal growth. Resistance to this antifungal is growing and consumer demand for *clean label* products is increasing. There are very few antifungal alternatives available to ensure cheese preservation. This project focuses on identifying novel antifungals from environmental bacteria isolated from the Canadian high arctic, which have the potential to be used in the cheese industry. We searched *in silico* for chitinases and glucanases as well as secondary metabolites in a collection of 146 arctic bacterial whole genome sequences and found a variety of biosynthetic gene clusters and hypothetical chitinases and glucanases. The entire collection was then screened *in vitro* for antifungal activity by co-culturing bacterial isolates with fungal strains common to the dairy industry. The most promising antifungal was produced by a novel species within the *Streptomyces* genus. Despite having a closed genome for this bacterium, we were not able to identify the presence of clear antifungal genes. Therefore, to identify the active antifungal component(s) produced by this species, organic extractions using ethyl acetate were conducted to produce crude extracts. LC-MS was performed on active crude extract but did not reveal any known antifungals, suggesting potential novelty. This project is the first step toward identifying novel cold active antifungals, specifically, an alternative to natamycin for the cheese industry.

RÉSUMÉ

La détérioration fongique du fromage entraîne une quantité importante de gaspillage alimentaire causant d'énormes pertes économiques à l'industrie fromagère. Une méthode utilisée pour lutter contre le gaspillage est l'addition de natamycine, un polyène inhibiteur de croissance fongique. La résistance des antimicrobiens envers les polyènes ainsi que la demande des consommateurs pour des produits dits c*lean label* sont en constante augmentation, laissant ainsi peu d'alternatives. Ce projet se concentre sur l'identification de nouveaux antifongiques provenant de bactéries arctiques utilisables pour l'industrie fromagère. Le projet a été divisé en trois objectifs; la recherche *in silico* d'antimicrobiens, l'investigation *in vitro* des bactéries et finalement la production d'extractions antifongiques et l'identification de leurs composants. Nos recherches *in silico* pour identifier des protéines antifongiques et des métabolites secondaires en utilisant 146 séquences de génomes entiers de bactéries arctiques nous ont permises d'identifier des groupes de gènes produisant des métabolites secondaires, des chitinases et des glucanases. L'activité antifongique de cette collection a alors été évalué *in vitro* avec des techniques de co-cultures entre les bactéries arctiques et les souches fongiques communes à l'industrie laitière. Une évaluation avec des données de séquence des isolats les plus prometteurs n'a abouti à aucun gène antifongique connu. Afin d'identifier le(s) composant(s) actif(s), des extractions organiques à l'aide d'acétate d'éthyle ont été menées pour produire des extraits bruts, qui après optimisation, ont permis l'inhibition de toutes les souches fongiques. Les extraits ont été analysés par LC-MS pour obtenir une liste d'agents bioactifs probables, mais aucun composé connu a été trouvé. Les étapes suivantes seront d'effectuer une analyse préparative par HPLC pour purification et de déterminer le composant actif. Ultérieurement, ce projet peut mener à d'importants avancements, tel que la réduction des pertes économiques et alimentaires d'origine fongique, tout en répondant

aux demandes des consommateurs pour des produits *clean label.*

CHAPTER 1. INTRODUCTION

1.1 General Introduction

The food industry sustains large losses due to food spoilage; and this accounts for significant food waste and economic losses (Leyva Salas et al. 2017; Petruzzi et al. 2017). Fungal food spoilage accounts for a 5-10% loss of all food products leading to economic losses (Pitt and Hocking 2009). Preventing food spoilage is in the best interest of the consumers and food companies. Less spoilage has positive impacts on brand names when consumers associate the two and allows prices to decrease as industry will not depend as heavily on the consumers to pay for lost profits due to spoilage (Leyva Salas et al. 2017). One of the causes of food spoilage is the growth of fungi which leads to off flavors, odors, and potentially adverse health effects (Leyva Salas et al. 2017). Traditionally, the food industry has used antifungals such as natamycin to prevent fungal food spoilage as well as other metabolites from lactic acid bacteria (LAB). However, studies have found fungal resistance to natamycin is growing, which causes concern for its continued use as an antifungal (Streekstra et al. 2016). The development of new preservatives has been aimed towards natural products on the basis of consumer demands and encouragement from public authorities to have *clean label* products (Streekstra et al. 2016). Natural antifungals could include the use of antagonistic microorganisms, microbial metabolites, and/or fermentates (Theis and Stahl 2004). Natural preservative organic acids have been extensively researched; however, research focusing on biopreservatives that are active at low temperatures for use in refrigerated products is lacking. This may be attributed by the unique challenges associated with working on microorganisms at cold temperatures and the slower discovery of new cold-adapted microorganisms. Therefore, further research is needed in these areas and for the development of novel and cold-active antifungals, specifically those that can

function in the cheese industry where most products are stored at refrigeration temperature.

1.2 Rationale

Few food grade antifungals exist for use in the cheese industry, and resistance is rapidly developing to those that do exist, such as natamycin. Arctic bacteria are a source of novel antimicrobials, and therefore are a potential source of novel antifungals for use in the food industry and beyond. This project will develop and use novel screening methods to identify novel antifungals from bacteria isolated from the Canadian high arctic which are effective against major spoilage fungi that are a problem in the cheese industry.

1.3 Research Hypothesis

Arctic bacterial isolates contain novel antifungal molecules active at low temperatures which can be used to inhibit the growth of common food spoilage fungi isolated by the cheese industry.

1.3 Research Objectives

To test this research hypothesis the following three objectives will be completed:

1) Conduct an *in silico* search of sequenced arctic bacterial genomes for homologues of known antifungals.
2) Using a high-throughput co-culture screening approach to identify arctic bacterial isolates with antifungal activity.
3) Produce and test soluble extracts from promising arctic bacterial isolates for the ability to inhibit the growth of a library of major food spoilage fungi.

CHAPTER 2. LITERATURE REVIEW

2.1 Fungal food spoilage

Spoilage is the process of food becoming undesirable for human consumption (Blackburn 2006). One of the many causes of food spoilage is fungi which spoil food by producing toxic chemicals leading to poor organoleptic qualities or adverse health effects (Hammond et al. 2015). The food industry attempts to prevent microbial spoilage by preventing microbes from accessing food or by inhibiting the growth of the microbes within the food (Hammond et al. 2015). These measures often involve multiple strategies or the application of the hurdle concept (Snyder and Worobo 2018). The hurdle concept is a food preservation method that increases food safety while maintaining food quality by combining various control techniques or so-called hurdles in synergy so that each control technique is applied at lesser intensity than if used individually (Leistner 2004). Some of the most used control techniques involve manipulating the temperature, water availability (A_w), acidity, or redox potential, or the addition of competitive microorganisms (Piližota 2014). Fungi are unique in that they are extremely resistant to a large array of conditions and can persist even after several prevention strategies have been implemented (Snyder and Worobo 2018).

2.1.1 Molds and Yeasts

Molds are a part of the kingdom fungi, which consist of heterotrophic organisms which obtain energy and carbon from organic compounds (Bullerman 2003; Li et al. 2021). Molds are multicellular eukaryotes that have hair like filaments and can be multinucleated. Their filaments are called hyphae and a bundle of hyphae forming a mold colony is called a mycelium (Bullerman 2003). The hyphae attached to food are called vegetative hyphae and function by absorbing nutrients and water, whereas the hyphae exposed to the surface are called fertile hyphae which are

the reproductive structures of the mold (Bullerman 2003). The reproductive structures include the conidiophores or sporangiophores which release millions of spores, conidiospores or sporangiospores (Bullerman 2003). Spores are produced at the tips of hyphae, and when given the correct conditions will develop into a germ tube and become a hypha (Bullerman 2003).

Yeasts are also a part of the kingdom fungi and are single-celled eukaryotic organisms, that are larger than bacteria (Bullerman 2003). Yeasts use asexual reproduction, more specifically a process called budding (Bullerman 2003). During budding, a bulge develops on the cell wall and fills with all the necessary material to allow for cell growth (Bullerman 2003). The growing bulge will eventually develop a cell wall between the parent cell and the new cell which allows them to separate (Bullerman 2003).

2.1.2 Cold adaptations for fungi

Fungi have several mechanisms that allow them to survive in cold temperature environments often at or below 0 °C and are thus commonly responsible for spoilage of refrigerated items (Wang et al. 2017). Cold temperatures will cause cell membranes to become stiff, inhibit enzymatic activity, denature enzymes, destabilize nucleic acids, and can lead to the formation of ice crystals which can kill the cell (Panikov 2013). Membrane modifications give fungi a selective advantage over other microorganisms at cold temperatures (Frisvad 2008; Panikov 2013). These modifications allow the cell membrane to maintain fluidity at low temperature (Gostinčar et al. 2009; Panikov 2013). Relative to bacteria, fungi have more unsaturated fatty acids in their cell membranes which allows the membrane to be less aligned and organized making it more fluid at lower temperatures (Panikov 2013). Additionally, fungi may have protein adaptations that have high catalytic efficiency at low temperatures and increase structural flexibility provided by a reduction in hydrophobic amino acids, smaller amino acids, and fewer covalent disulfide bonds (Panikov 2013). Cold-adapted fungi

can also have specialized proteins such as antifreeze proteins or cold shock proteins (Frisvad 2008; Panikov 2013). Cold shock proteins will bind to misfolded proteins or stabilize proteins maintaining their functionality, while antifreeze proteins will prevent the growth of ice crystals which can puncture the cell membrane leading to death (Frisvad 2008; Panikov 2013). One of the best examples of a cold-adapted fungi is the yeast *Rhodotorula glutinis* which is capable of growing at -18 °C and was discovered on spoiled stored peas (Collins and Buick 1989).

2.2 Cheese processing

Cheese is produced by the coagulation of casein in milk and separation of whey from the curd (Motarjemi et al. 2014). Cheese can be made in a variety of ways to produce different types of cheeses: hard, semi-hard, semi-soft, and soft – each with a different moisture content (Motarjemi et al. 2014). Generally speaking, softer cheeses have a shorter self-life. Cheese can also be made from either pasteurized or unpasteurized milk to achieve different taste profiles. To increase shelf life and develop characteristic flavor profiles many cheese producers will undergo a fermentation process, where they add LAB starter cultures to convert sugars in lactic acid (Kongo 2013). Other types of cheeses such as mold-ripened cheeses (blue cheese) will have additional cultures added, however, these mold cultures will deacidify the cheese (Gripon 1999).

2.2.1 Cheese spoilage by fungi

Yeasts and molds can grow in a wide variety of conditions and spoil several types of food. Yeast and molds can metabolize common substrates found in cheeses and tolerate low pH, low A_w, and low temperatures, which gives them several evolutionary advantages for growing on cheeses (Huis in't Veld 1996). Certain fungi are purposefully added to cheese to develop flavor profiles and increase shelf life. Some of those species are *Penicillium roqueforti* and *P. camemberti* which are

used in the cheese ripening process (Babel 1953). However, yeasts and molds are also common contaminants leading to the spoilage of cheeses.

2.2.2 Mold spoilage of cheese

The review article by Garnier et al. found that a total of 100 mold species have been discovered to spoil dairy products (Garnier et al. 2017). Those that were most commonly found on cheeses include: *Alternaria sp., Aspergillus sp., Cladosporium sp., Fusarium sp., Penicillium sp.,* and *Geotrichum sp.* (Bullerman 2003; Hymery et al. 2014) . *Alternaria sp.* can cause food spoilage in several types of foods, including vegetables, grains, and refrigerated meats and cheeses. Some species pose health risks as they can produce mycotoxins (Pitt and Hocking 2009). *Aspergillus sp.* is found ubiquitously and has species such as *A. niger* and *A. flavus* which are capable of mycotoxin production. *Aspergillus sp.* are considered xerotolerant and can grow in high salt foods with low A_w (Bullerman 2003; Hocking 2006). *Cladosporium sp.* have been isolated from the air and are well adapted to aerial dispersal. They have a wide temperature growth range, and can spoil refrigerated beef products, cheeses, and cause discoloration (black spots) on food (Bullerman 2003; Pitt and Hocking 2009). *Fusarium sp.* is commonly found in soil and infects grain plants. It is considered a plant pathogen and can cause health concerns for humans as it can also produce mycotoxins (Pitt and Hocking 2009). *Penicillium sp.* is an important mold in food spoilage and can grow at refrigeration temperatures, including in dairy products such as cheeses. Many *Penicillium* species can produce mycotoxins in certain foods and are therefore problematic, but other species are purposefully added to cheeses to improve flavor and texture (Bullerman 2003). Unfortunately, *Penicillium sp.* are also a common contaminant of some cheeses at refrigeration temperatures (Pitt 2006). *Geotrichum sp.* contains only one species of significance in food, *G. candidum*, which can grow in cheeses, meats, and frozen vegetables (Bullerman 2003). It causes issues in processing

facilities as it can grow on equipment, causing it to contaminate the food (Pitt and Hocking 2009).

Even with there being a large variety of molds that spoil cheeses, the genus with the largest prevalence is *Penicillium*. This genus is a well-known spoilage organism and can be xerophilic and psychrophilic (Kuehn and Gunderson 1963). *Penicillium* species are ubiquitous in the environment and can grow in inhospitable environments (Pitt and Hocking 2009). The second most common genus found in cheeses was *Aspergillus* sp. which are resistant to low A_w (Pitt and Hocking 2009). Without the addition of preservatives, cheese is predominantly spoiled by *P. commume* and *P. nalgiovense* and to a lesser extent *P. roqueforti* and *A. versicolor* (Filtenborg et al. 1996). However, Banjara et al. found *P. roqueforti* to be the most commonly isolated mold from a variety of cheeses (Banjara et al. 2015).

2.2.3 Yeast spoilage of cheese

Undesired yeast activity in cheese can lead to spoilage. Common signs of yeast spoilage in cheese include the development of gassiness as well as fruity, bitter, and yeasty off-flavors (Fleet 1990). There are several studies that have found spoilage yeasts in dairy products. Major yeast genera that contribute to cheese spoilage include *Debaryomyces* and *Candida* (Banjara et al. 2015; Fleet 1990; Garnier et al. 2017). A study by Nakase and Komagata found *Debaryomyces hansenii* and *Candida lipolytica* to be the most frequently isolated yeasts on cheese (Nakase and Komagata 1977). Their findings have been substantiated by other recent reports that found *D. hansenii* in 79% of all cheese products (Banjara et al. 2015). Additionally, a review article looking at the diversity of yeast spoilage concluded that *Candida* is the most frequently reported followed by *Debaryomyces* (Garnier et al. 2017). *Debaryomyces* have been found to grow on cheese and sausage products and are film yeasts that are on the surface of the food (Bullerman 2003). They can also tolerant high salt environments such as those found in olives, cheeses, and soy sauce koji (Pitt and Hocking 2009).

Candida have been found to grow on meats and spoil high acid, salty foods. One species *C. albicans* is known to cause infections in humans and animals (Bullerman 2003).

Saccharomyces sp. is the most important yeast used in the food industry because of widespread use in fermentation processes for products such as: beer and wine, kombucha, and bread. However, *Saccharomyces sp.* is considered a spoilage organism when it contaminates products that are not supposed to have active fermentation (Bullerman 2003).

2.2.4 Sources of Fungal Contamination

Fungal contamination can occur at several stages along the production line to the consumers' table. Raw milk naturally contains high levels of fungi, but heat treatment of the milk will eliminate nearly all. Because of this, fungal contamination in pasteurized cheeses generally happens after pasteurization (Garnier et al. 2017). Fungal spores found in the air are a common source of contamination of dairy products and can be easily transmitted in factories (Pitt and Hocking 2009). Companies can use air filters which have been shown to reduce fungal spore contamination by 30 fold (Beletsiotis et al. 2011). Specifically, for cheese products, the slicing and cheese rind can be the cause of spoilage, as the machinery used can become contaminated from the air and be transferred to the cheese (Garnier et al. 2017). This is a particular issue for shredded cheeses as the cheese becomes more susceptible to contamination by air-borne fungal spores (Oyugi and Buys 2007). Additionally, contamination can arise from poor hygiene of workers in the ripening environment (Banjara et al. 2015). Yeast spoilage can be caused by exposure to contaminated brine, surfaces, equipment, or ingredients (Garnier et al. 2017). Once the cheese has been opened by the consumer, the air at the consumer's home can also contain molds which can cause spoilage (Garnier et al. 2017).

2.2.5 Control methods concerns with current antifungals used in cheese production

The dairy industry commonly uses pasteurization as its most effective control process to increase food safety, but for unpasteurized cheeses the dairy industry utilizes several alternatives to insure safe and more shelf stable food. Safety is maintained using the hurdle concept, rapid cooling and processing of milk, rapid acidification, and good hygiene practices (Motarjemi et al. 2014). Companies also use chemical preservatives to inhibit fungal growth and spoilage, but manufacturers want to develop alternative food preservations (Benedict et al. 2016). Although controversial, the safety comparison of raw milk vs. pasteurized milk cheeses is still debated (Yoon et al. 2016). Without pasteurization, pathogenic microorganisms can grow on ready to eat foods during the ripening process while pasteurized cheeses would not have the same ripening period requirements (Meunier-Goddik and Waite-Cusic 2019).

A current problem that the dairy/cheese industry faces is rising concern on the use of natamycin, a food preservative in ripened cheeses, because the development of antimicrobial resistance (AMR) and potential health effects of using non-natural alternatives (Streekstra et al. 2016). When it was first discovered, natamycin was approved for use and was thought to not contribute towards AMR (Additives and Food 2009). This is because natamycin only functions against fungi; however, after exposing fungi to natamycin for a prolonged period, some fungi can develop tolerance (Streekstra et al. 2016). This demonstrated that if fungi are exposed to continuous selection pressure from natamycin in food processing environments, there could be natamycin resistance, as well as cross-resistance to other medical polyene antifungals such as amphotericin B and nystatin (Streekstra et al. 2016). Polyene resistance can also be developed by the transfer of genetic material between microorganisms, known as horizontal gene transfer (Dalhoff and Levy 2015). Horizontal gene transfer has been shown between *Candida* spp. and *Aspergillus fumiagtus* which has led to the spread of polyene resistance (Dalhoff and Levy 2015). This is concerning

towards consumer health, as there is potential for the normal human microflora to acquire polyene resistance, which could then be spread to pathogenic *Candida* spp. potentially leading to polyene resistant infections (Dalhoff and Levy 2015). Therefore, several companies wish to remove natamycin from their products to further satisfy the consumer concerns (Asioli et al. 2017).

2.2.6 Consumer interest towards a Clean Label approach

Consumer interest in the processing, production, and contents of their foods has continually increased over the last few decades (Asioli et al. 2017). This has led people to become concerned with the potential adverse health effects and environmental impacts of certain preservatives, including chemical alternatives to natamycin, pushing consumers to pursue products with more natural methods of preservation (Leyva Salas et al. 2017). This push has caused companies to want to develop natural preservatives to respond to their customers' demands of having less processed foods without chemical preservatives (de Arauz et al. 2009). Companies can then market their products as being *clean label* when specific ingredients, including preservatives, are not used or when the product is *all natural* (Asioli et al. 2017).

2.2.7 Rise in AMR and lack of new antimicrobials

Since the mid-20th century antimicrobials have had a profound impact on humanity, easily treating once fatal infections and making them seem nonthreatening (Baker et al. 2018). The vast development and efficacy of antimicrobials made them become overused and misused and with time research has slowed in the field (Baker et al. 2018). Because 95-99% of all bacterial species are considered unculturable there was a lull in the development of novel antimicrobials produced by novel bacteria and leaves huge potential for new compounds to be discovered (Ling et al. 2015). With the lull in the research and the development of novel antimicrobials, AMR has become a huge

problem (Baker et al. 2018). It has been said that by 2050 infections with AMR organisms will cause more deaths annually than cancer and result in a loss of $100 trillion from the global gross domestic product (O'Neil 2014). The largest source of new antibiotics is from natural products, and exploring unique ecological niches is a promising area of research that must be done to identify novel compounds (Tedesco 2016).

2.2.8 Mycotoxins and controlling their production

Mycotoxins are fungal metabolites that are produced during the growth of fungi (Garcia et al. 2009). Food spoilage fungi including *Aspergillus, Fusarium,* and *Penicillium* are capable of mycotoxin production (Hymery et al. 2014). Mycotoxins can be classified by the area they effect, and can be considered teratogens, mutagens, carcinogens, and allergens (Bennett and Klich 2003). Humans can develop illnesses when the mycotoxin is absorbed, ingested, or inhaled (Garcia et al. 2009). The conditions that allow for mycotoxin production in cheeses are dependent on the A_w, temperature, substrate, gas composition, and presence of preservatives (Sengun et al. 2008). Some of the ways the industry controls mold growth in cheese is by using proper storage conditions such as refrigeration and modified atmospheric packaging (Sengun et al. 2008). These methods have shown to be effective at limiting mycotoxin production as the species that can produce the mycotoxins are not capable of growing at temperature ranges of 5-7 °C (Sengun et al. 2008). At these temperatures, *Penicillium* sp. are still capable of growing and other preservatives are needed (Dobson 2017). Other methods include adding NaCl as that will lower the A_w but may alter the desired organoleptic properties of the cheese (Dobson 2017). Modified gas compositions such as 80% CO_2 and 20% O_2 have been shown to reduce mold growth and thus mycotoxin production (Taniwaki et al. 2010). A point of potential risk during the cheese making process is the ripening stage. The temperature can be kept higher than normal refrigeration to mature the cheese

characteristics, but it can facilitate the growth of molds producing mycotoxins (van Egmond 1983). With these control methods, cheese is still susceptible to mold growth; however, the production of mycotoxins is currently considered much less problematic (Dobson 2017).

2.3 Bacterial antifungals

Bacterial metabolites have potential antifungal activity that can be used for either therapeutic or industrial applications (Matevosyan et al. 2019). Methods for testing and observing antagonistic activity in multi-species bacterial communities has increased in recent years (Matevosyan et al. 2019). These interactions between bacteria and other microorganisms leads to the production of metabolites to allow the bacteria to thrive by inhibiting or destroying other microbial competitors. Therefore, these metabolites are of great interest. The most dominant type of bacteria currently used in the food industry for food preservation are LAB which have been involved with food processing for centuries (Matevosyan et al. 2019). The major genera that are within the LAB group include *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus, Enterococcus, Oenococcus,* and *Weissella* (Matevosyan et al. 2019). The types of antimicrobial metabolites that are commonly produced by LAB are organic acids, hydrogen peroxide, lipids, and bacteriocins (Matevosyan et al. 2019). Other antifungal metabolites from bacterial origin include glucanases, chitinases, chitin synthesis inhibitors, and syringomycins which target components of the fungal membrane or parts in its biosynthesis (Figure 1). The primary fungal membrane targets include ergosterol, chitin synthase, and glucan synthases (Lima et al. 2019).

Chitin synthesis inhibitors such as nikkomycin and polyoxin were first isolated from *Streptomyces tendae* and *Streptomyces cacaoi* var. *asoensis* respectively (Vicente et al. 2003). Nikkomycins and polyoxins are analogues of UDP-Nacetylglucoasamine which is essential for chitin synthesis and both can block its biosynthesis (Vicente et al. 2003). Syringomycins are a group

of lipopeptides with antifungal activity that are synthesized by *Pseudomonas syringae* (Thery et al. 2019). Syringomycins have been shown to be effective in inhibiting *Candida* spp. (Thery et al. 2019). Chitin-binding proteins assert their inhibitory effect by binding to chitin during hyphal growth, limiting and changing their morphology (Theis and Stahl 2004). Chitinases degrade chitin by hydrolysis, and have been found in *Bacillus, Pseudomonas,* and *Streptomyces* (Theis and Stahl 2004). Glucanases have antifungal activity by catalyzing the hydrolysis of glucans which are a major part of the fungal cell wall (Theis and Stahl 2004). This leads to cell lysis when the fungal cell wall has become leaky (Theis and Stahl 2004).

2.3.1 Bacterial chitinases and glucanases

Bacterial chitinases are primarily a part of the glycosyl hydrolase (GH) 18 family (Stoykov et al. 2015). They catalyze the hydrolysis of 1,4 β-glycoside bond of N-acetyl d-glucosamine in chitin (Stoykov et al. 2015). There has been growing interest in chitinolytic enzymes, for their potential application in chitin waste clean-up, to allow enzymes trapped in chitin to be released, and medicine (Stoykov et al. 2015). Additionally, chitinase have shown promise as a biocontrol agent against various insects and pests (Rathore and Gupta 2015). Unfortunately, there are not many commercially used chitinases, due to their high costs (Stoykov et al. 2015). Glucanases are proteins a part of the GH 16 family and hydrolyze β 1,3 glucans and have been used in the brewing industry (Planas 2000). They breakdown barley gums and glucanases have been made to improve clarity and filterability of wines (Canal-Llaubères 2010).

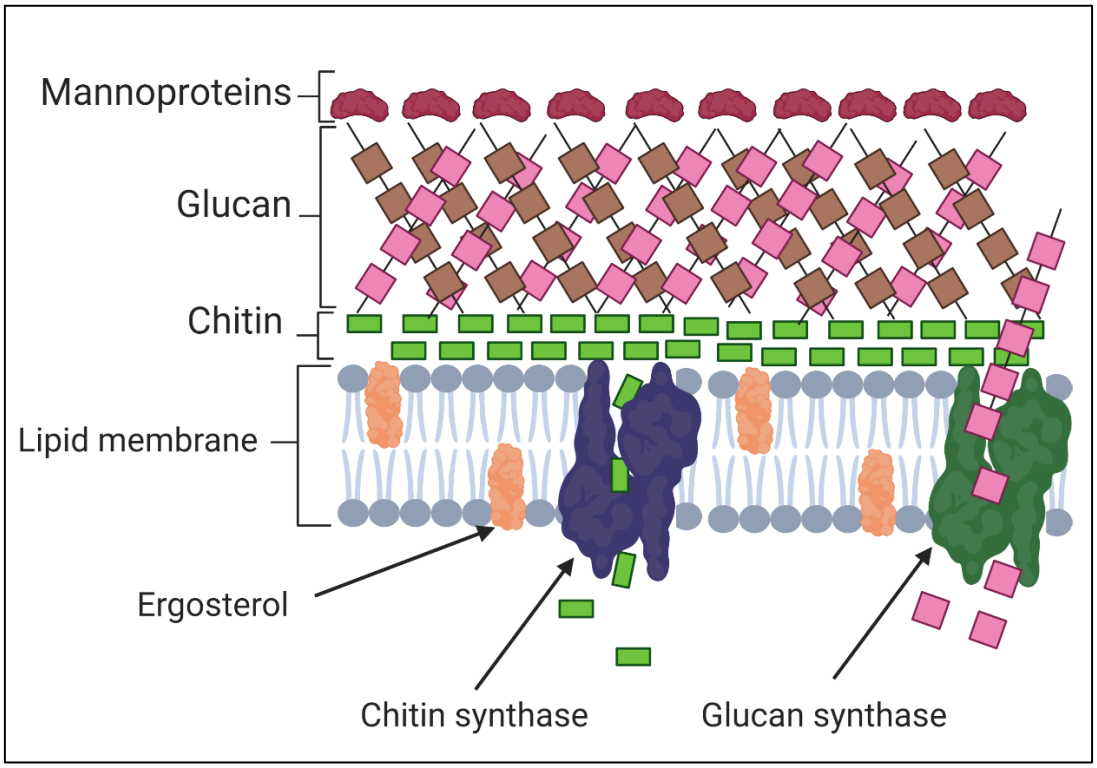


Figure 1. Fungal membrane targets. Bacterial glucanases and chitinases can degrade the chitin and glucan layer, while polyenes bind to ergosterol or nikkomycins inhibit chitin synthase. Created using BioRender.com.

2.3.2 Antifungals and mode of action

Historically, LAB have been a good source of antimicrobial compounds and have been tested extensively for their inhibitory affects against spoilage organisms (Siedler et al. 2019). Antifungals derived from bacteria can be very complex, involving bioconversions or peptide syntheses, or they can be quite simple such as organic acids and primary metabolites (Siedler et al. 2019). The mode of action that each antifungal can be different, but the mechanisms of currently described antifungals fall into 4 main processes: 1- cell wall permeabilization which can be done by modifying fatty acids, 2- peptides, 3- proton gradient interference blocked by organic acids and peptides, 4- enzyme inhibition and the production of reactive oxygen species (Siedler et al. 2019). Fungi possess a modified cell membrane which is a great target for antifungals, to allow them to be more specific towards fungi than to humans (Silva et al. 2014). The fungal membrane is composed

mainly of sterols, specifically ergosterol (Silva et al. 2014). Ergosterol allows the membrane to stay flexible and give the cell membrane stability (Silva et al. 2014). Ergosterol is a common antifungal target, as it is not present in humans and other higher eukaryotic organisms, which have cholesterol (Silva et al. 2014).

Another promising target for antifungal activity in the fungal cell walls are glycosphingolipids (GSLs) which are a part of the fungal cell membrane (Silva et al. 2014). GSLs contribute the structure of the membrane, but also serve important roles in cell to cell signaling, cell-cell interactions, and protein sorting (Silva et al. 2014). A study showed the effectiveness of GSLs as a target when antimicrobial peptides (AMPs) were successful in inhibiting fungi, as the AMP caused reactive oxygen species to be formed leading to apoptotic death (Thevissen et al. 2004). In general, the AMPs that induce membrane destruction must first bind to the negatively charged phospholipids on the cell surface (Thevissen et al. 2004). These AMPs are amphipathic, having both a positively charged and hydrophobic side, which allows them to bind both the outer layer phospholipid head groups and the inner hydrophobic tails (Silva et al. 2014).

Azoles and polyenes are considered to be the more traditional fungal inhibitors (Thery et al. 2019). The mode of action of azoles involves the inhibition of the synthesis of ergosterol while polyenes function by binding to membrane sterols (Ghannoum and Rice 1999). Azoles all function by inhibiting the synthesis of ergosterol, they accomplish this by inhibiting the P-450 dependent lanosterol 14α-demethylase in fungi, which blocks the lanosterol from becoming demethylated to ergosterol which is needed for fungal membrane growth (Vanden Bossche 1985). Therefore, azoles are mostly fungistatic and just inhibit growth rather than being fungicidal. Polyenes have an amphipathic macrocyclic ring, with one side being very hydrophobic because of the double bonds that are present, while the other side contains many OH groups making it hydrophilic (Welscher et al. 2008). These properties are what allows polyenes to bind to the fungal membrane (Welscher et

al. 2008). Resistance towards azoles has been documented where resistant strains modify the target of the antimicrobial or block the antimicrobial from reaching its target (Ghannoum and Rice 1999). Modifications include: overproduction of the target, limiting access of the target, efflux pumps, cell membrane/cell wall modifications and inactivation of key enzymes involved in the conversion of inactive to active state antimicrobials (Ghannoum and Rice 1999).

2.3.3 Nisin

There are 2 structural variants of the polypeptide nisin (A & Z) which is biosynthesized by *Lactococcus lactis* (de Vos et al. 1993). The nisin gene cluster is comprised of 11 genes, *nisABTCIPRKFEG* and is on a transposon (Trmčić et al. 2011). The gene cluster provides the necessary information to produce, regulate, and confer immunity to nisin (Trmčić et al. 2011). Nisin A and nisin Z differ only by a single amino acid at position 27, and contain a total of 34 amino acids (Additives et al. 2017). Nisin is considered a bacteriocin, meaning it can inhibit the growth of other bacteria (Additives et al. 2017). Nisin has been used in the food industry as a preservative against Gram-positive bacteria and has been said to have little to no activity on Gram-negative bacteria or fungi (Faustino Jozala et al. 2015). However, studies have demonstrated that nisin Z has some inhibitory activity against the growth and transition of the yeast *C. albicans* (Akerey et al. 2009; Le Lay et al. 2008). Therefore, more research is needed to better understand the effectiveness of nisin as an antifungal. The mechanism of action of nisin in Gram-positive bacteria involves pore formation in the bacterial membrane (de Abreu et al. 2016). The disruption of the membrane leads to cell death following the loss of proton motive force, imbalance in pH equilibrium, and hydrolysis of ATP (Adenosine triphosphate) (de Arauz et al. 2009). Additionally, nisin has the ability to bind lipid II, a peptidoglycan precursor, interfering with cell wall synthesis (de Arauz et al. 2009).

2.3.4 Natamycin

Natamycin, also known as pimaricin, is an important preservative in the food industry to prevent mold growth and is used on the surface of cheeses and exhibits no effect on bacteria (Stark and Tan 2003). Natamycin is a part of the class of polyketide molecules, and more specifically a type of polyene macrolides whose chemical formula is $C_{33}H_{47}NO_{13}$ (Delves-Broughton et al. 2005). Natamycin was first discovered in 1955 and is made by *Streptomyces natalensis* (Delves-Broughton et al. 2005). The gene cluster required for *S. natalensis* to biosynthesize natamycin has been found and the reaction is catalyzed by a polyketide synthetase (Li et al. 2014). The cluster includes 16 open reading frames encoding 5 megasynthases (PIMS0-PIMS4) (Aparicio et al. 2000). Natamycin is tasteless and odorless, it is very stable, and does not have an effect on bacteria; therefore, this compound is well suited for use as an antifungal in the food industry. It has been used in the food industry for several years (Delves-Broughton et al. 2005). Since it does not have an effect on bacteria, it can be used without disrupting the fermentation or ripening processes (Delves-Broughton et al. 2005). The addition of natamycin on cheeses delays the growth of mycotoxins producing molds, which happens when cheeses are temperature abused, or during the ripening process (Delves-Broughton et al. 2005). The primary mechanism of action of polyene macrolides is that they bind ergosterol in the fungal cell membrane (Figure 2) (Aparicio et al. 2016). Natamycin is amphipathic, and the hydrophobic portion will irreversibly bind to the hydrophobic region of the sterol, disrupting the cell membrane, leading to cell death (Aparicio et al. 2004). Ergosterols are not found in bacteria, viruses, or protozoa; therefore, natamycin has no effect on them (Aparicio et al. 2016).

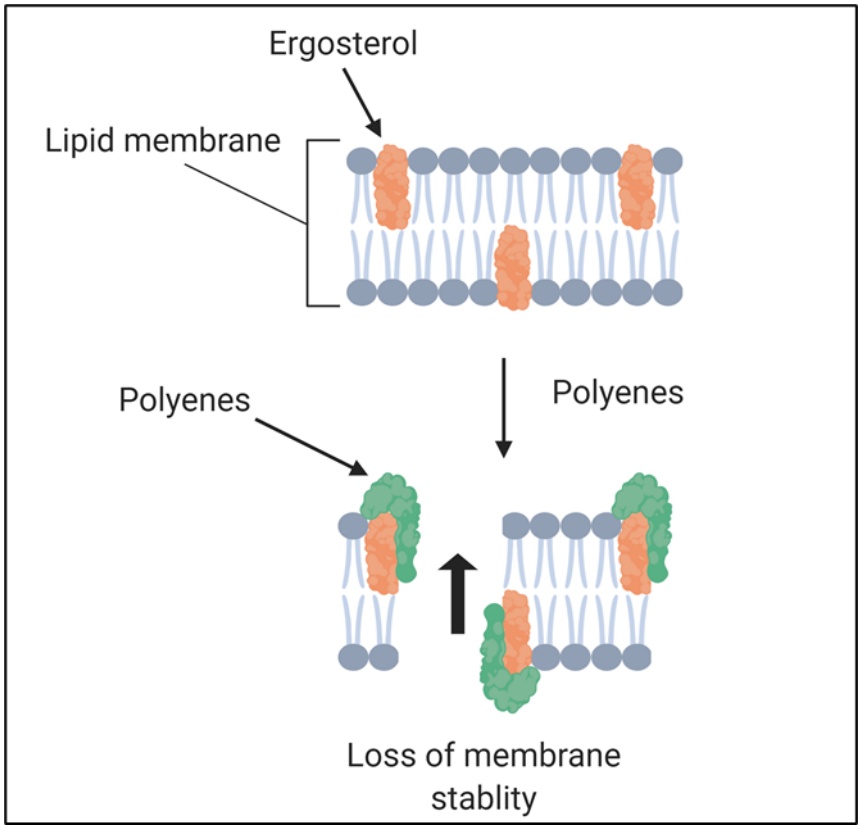


Figure 2. Mechanism of action of polyenes on fungal membranes. Created using BioRender.com.

2.4 Polar microbiology and applications to food industry

Cold temperatures (below 5 °C) cover most of the earth's biosphere, and these cold environments can cause many environmental stresses towards organisms living within it (Lo Giudice and Fani 2016). The most dominant type of organisms to colonize such cold environments are microorganisms (Lo Giudice and Fani 2016). Microorganisms that inhabit cold environments must produce cold-active enzymes to survive (Gerday 2014). Enzymatic activity is greatly reduced at lower temperatures and the cold-active enzymes are specially adapted to function adequately at very low temperatures (Gerday 2014). The cold environment stresses also increase competition for available resources between microorganisms, and as a result microorganisms may develop systems to antagonize other proximal microorganisms (Bell et al. 2013; Lo Giudice and Fani 2016). With limited nutrients microorganisms must compete or cooperate between one another to survive (Bell

et al. 2013). Bacterial and fungal interactions revolve around finding more nutrients (Deveau et al. 2018). Bacteria can produce antifungals to aid them in competing against the fungi in their ecological niche (Thery et al. 2019). Unfortunately, testing for the synthesis of antifungals in a laboratory is challenging, as many genes are silenced depending on the environmental condition (Ross et al. 2014). One way to induce the silent antimicrobial genes is to attempt at recreating the natural environment and competition, such as a co-culture experiment in a bioreactor at refrigeration temperatures (Ross et al. 2014). These relationships and adaptations between microorganisms have lead researchers to seek novel antimicrobial compounds for industrial and medical applications (Lo Giudice and Fani 2016).

The discovery of new cold-active enzymes has a large variety of applications (Cavicchioli et al. 2011). Being able to use enzymes at low temperatures can be useful when dealing with heat-sensitive substrates, or where there is a need to minimize other reactions that happen at higher temperatures (Cavicchioli et al. 2011). An example of cold active enzymes extracted from arctic bacteria are lipases (Kavitha 2016). Cold active lipases have a large variety of applications, for example they are used in laundry detergents, pharmaceuticals, environmental applications, and the leather industry (Kavitha 2016). The beneficial features of cold active enzymes also have the potential to be used in the food industry where spoilage can still occur at low temperatures and heat may alter the nutritional content or organoleptic properties of the food (Cavicchioli et al. 2011). An example of a cold active lipase used in the food industry is that from *Pseudomonas fluorescens* P38 which is used to synthesis a flavour compound (Kavitha 2016). These enzymes would also play a role in reducing energy consumption by requiring less heating to function (Cavicchioli et al. 2011).

2.4.1 Arctic bacteria with known antimicrobials

Actinobacteria from polar regions have been particularly well researched since they are well known sources of antibiotics (Lo Giudice and Fani 2016). Phylogenetically, they are a group of high G + C Gram-positive bacteria. A genus within this phylum includes *Streptomyces. Streptomyces* has been extremely successful in providing novel antimicrobials over the years, but has slowed significantly and testing other members of this genus in underexplored environments may be a source of new antimicrobial compounds (Lo Giudice and Fani 2016). Antarctic actinobacterial isolates have been found to possess non-ribosomal peptide synthetases (NRPSs) genes and have antimicrobial activity against bacteria and fungi (Gesheva 2010; Yi Pan et al. 2013). Other genera found in the arctic such as *Planococcus, Pseudomonas,* and *Arthrobacter* have been found to have antimicrobial properties (O'Brien et al. 2004). The study by O'Brian et al. found several isolates that could inhibit foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (O'Brien et al. 2004).

Pseudomonas is a genus of Gram-negative bacteria that can be found within the rhizosphere and are present throughout the environment (Garrido-Sanz et al. 2016). They can use multiple substrates for energy sources and have been known to produce several antibiotics (Gross and Loper 2009; Raaijmakers et al. 2002). Studies have found gene clusters for polyketide synthesis in *Pseudomonas* spp. (Bangera and Thomashow 1999). An antifungal polyketide, known as 2,4 diacetylphloroglucinol (DAPG) was found in multiple strains of *Pseudomonas* on a 6.5kb gene cluster (Bangera and Thomashow 1999). DAPG does protect plants from phytopathogens and bacteria; however, DAPG is also toxic towards various nematodes (Meyer et al. 2009).

Planococcus is a genus of Gram-positive, non-motile, halophilic bacteria (Engelhardt et al. 2001). They have also been found to contain NRPS and other biosynthetic pathways for

antimicrobials (Waghmode et al. 2019). Several *Planococcus* species have been shown to preform hydrocarbon degradation and have had several studies done on them for their potential use in bioremediation (Engelhardt et al. 2001; Waghmode et al. 2019).

Arthrobacter spp. has been isolated from arctic samples and is a known producer of antibacterial and antifungal compounds (Wietz et al. 2012). They are very adaptable and are able to utilize multiple substrates (Munaganti et al. 2016). A study with *Arthrobacter agilis* demonstrated the strain had the ability to produce antifungal organic compounds (Velázquez-Becerra et al. 2013). Other studies showed its effectiveness on wood-decaying fungi, and that it had potential for use in treated wood (Orozco-Mosqueda et al. 2015).

2.5 *In silico* search for antifungals

Advances in computer processing speeds and new bioinformatics software has allowed scientists to design and screen large number of compounds and genomes for antifungal activity before testing any of them *in vitro*. Genome mining can be used to identify genes involved in secondary metabolite synthesis (Belknap et al. 2020). This technique saves valuable time and money due to labor and material usage being significantly reduced (Lee et al. 2020). After *in silico* screening, *in vitro* assays can be performed to confirm initial predictions. This strategy has been used in the discovery of several antimicrobials and can be used in reverse if an unknown compound is bioactive and the goal is to identify potential unknown antimicrobials. For instance, a study used a secreted metabolite prediction software after isolating an antifungal producer to help identify genes involved in its production leading to the discovery of ibomycin (Robbins et al. 2016).

2.5.1 Gene Cluster Identification Technique

Secondary metabolites, are encoded by metabolic gene clusters or biosynthetic gene clusters

(BGCs) (Slot 2017). AntiSMASH is a bioinformatic tool that can be used to identify biosynthetic gene clusters (Wawrzyn et al. 2012) by using existing models and identifying conserved motifs for certain proteins, such as NRPS and polyketide synthases (PKS) (Medema et al. 2011). Both NRPS and PKS have highly conserved domains which antiSMASH will search for; the limitation with this technique is that the program may not have exact cut off points for the biosynthetic gene clusters and can miss highly novel antimicrobials. However, for preliminary screening purposes the program can be very useful for ranking isolates in terms of antifungal potential.

Non-ribosomal peptides are a diverse group of secondary metabolites that are made independently of the ribosome and are capable of producing antifungals (Martínez-Núñez and López 2016). They are generally made of macrocyclic structures with a peptide backbone and represent a new opportunity for the discovery of novel therapeutic agents (Singh et al. 2012). They are synthesized by NRPSs, which act as the machinery and the template (Singh et al. 2012). The NRPSs are made up of modules which contain 3 domains, adenylation domain, peptidyl carrier protein, and condensation domain (Figure 3) (Singh et al. 2012). The adenylation domain activates the substrate using ATP, the peptidyl carrier protein domain then binds the substrate and transfers it to the condensation domain which forms peptide bonds to other substrates on the NRPS (Bills et al. 2014). Each module catalyzes the reaction of the addition of a single amino acid to the growing polypeptide (Martínez-Núñez and López 2016). Due to the highly conserved nature of these domains, they can be used to find NRPSs in sequencing data (Etchegaray et al. 2004; Martínez-Núñez and López 2016).

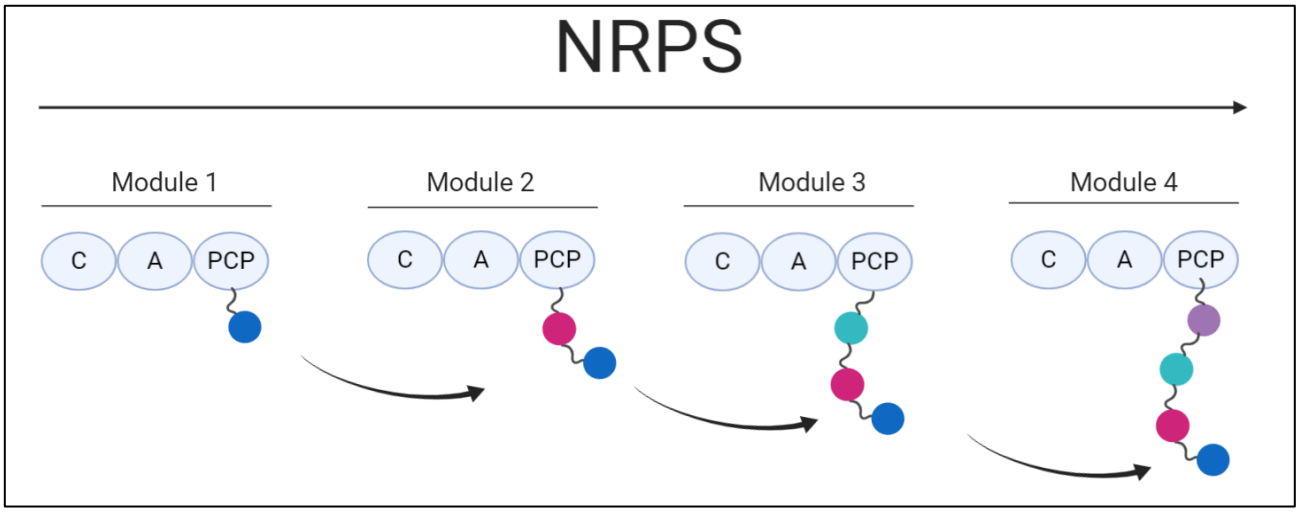


Figure 3. Non-ribosomal peptide synthesis. Each module of the NRPS is made of the 3 conserved domains, each adding a single amino acid to form the polypeptide. The C is the condensation domain, the A is the adenylation domain and PCP is the peptidyl carrier protein domain. Created using BioRender.com.

Polyketides are naturally produced by microorganisms and are formed by PKS when acetyl-CoA and malonyl-CoA are condensed (Daley et al. 2017; Kumar et al. 2004). An example of a polyketide is natamycin, which is biosynthesized by polyketide synthase genes in *S. natalensis* (Aparicio et al. 2000). They are secondary metabolites meaning they are not used for the growth or development of the microorganism, but are instead used for its defense (Daley et al. 2017). PKS operate using several domains, where groups of domains are organized into modules (Kumar et al. 2004). PKS gene clusters are numerous and several studies have shown the regions of conservation between different PKS (Kumar et al. 2004). The "core domains" include the acyl-carrier protein, β-ketoacyl-ACP synthase, and acyltransferase domains (Aparicio et al. 1996). Other domains: enoyl reductase, β-ketoacyl-ACP reductase, and dehydratase domains are not required in every module but are involved in the selection of extender units and chain modifications (Kumar et al. 2004). The acyltransferase domain causes the acetyl group from acetyl-CoA to be attached to the phosphopantetheine arm of the acyl-carrier protein domain (Kumar et al. 2004). The β-ketoacyl-ACP synthase domain accepts the polyketide from the upstream acyl-carrier protein and adds the

extender unit from the acyl-carrier protein domain in the same module (Kumar et al. 2004).

2.5.2 Genome annotation for antifungal proteins

Bacteria are capable of synthesizing a variety of antifungal proteins including chitinases and glucanases (Theis and Stahl 2004). Searching for GH domains based on conserved amino acid sequences could give insight into potential function of the organism. Bacterial chitinases are usually secreted or exposed to the environment which would require them to function in those conditions, meaning cold temperature expression should produce cold active proteins (LeCleir et al. 2004). Additionally, screening for specific genes such as chitinase has been done and could be a useful target when searching for bacteria with antifungal activity. Chitinases belong to family GH 18 (Rathore and Gupta 2015). The most well studied chitinolytic bacteria is *Serratia marcescens*, and it produces chitinases ChiA, ChiB, ChiC and Chitin binding protein 21 (Rathore and Gupta 2015). The ChiA gene is the most targeted chitinase sequence as it has highly conserved regions in a wide range of bacteria (Ramaiah et al. 2000). There are several bioinformatic tools available that can annotate bacterial genomes to help search for chitinases and glucanases, however, speed is a limiting factor for several of them when high throughput is required (Seemann 2014). Prokka is a tool that greatly increases the speed of genome annotation while simultaneously incorporating many genome feature prediction tools. These features include coding sequence, ribosomal RNA genes, transfer RNA genes, signal leader peptides, and non-coding RNA (Seemann 2014).

Chitinases and glucanases can be very challenging to annotate due to the large diversity in structure (Hamid et al. 2013). A more specialized annotation tool called dbcan2 was developed for annotating carbohydrate active enzymes which includes the GH families (Zhang et al. 2018). The software uses 3 methods to predict proteins, each method uses a search engine with a specific database. One of the databases is dbcan, which is similar to Pfam HMMs (Hidden Markov Models)

which is used in Prokka; however, dbcan uses HMMs for carbohydrate active enzymes families making the software better trained for annotating chitinases and glucanases (Huang et al. 2018).

2.6 *In vitro* methods for screening and producing antifungals

Novel antimicrobial drug discovery is limited by the ability to identify natural antimicrobial compounds as well as avoiding the rediscovery of known compounds (Robbins et al. 2016). High-throughput co-culturing is a method that can be used to aid in screening large numbers of isolates for antagonistic activity against pathogenic or spoilage microorganisms (Marmann et al. 2014). Additionally, co-culture mimics the natural competitive environment experienced by microorganisms which can stimulate the expression of compounds that would not be seen in a monoculture (Ueda and Beppu 2017).

2.6.1 Factors effecting biosynthesis of antimicrobials in Streptomyces

Streptomyces spp. are known to produce antimicrobials substances; however, these substances are not produced under all growth conditions, and the conditions that stimulate optimal secondary metabolite must be found through a process of optimization (Pham et al. 2019). The life cycle and morphology of *Streptomyces* cells cause them to form clumps due to mycelial networks leading to poor culture heterogenicity (van Dissel et al. 2015). There are numerous variables that need to be correct for each specific *Streptomyces* strain to simply produce the natural product. In liquid cultures several parameters must be tested to optimize metabolite production, including: media composition and pH, incubation temperature, inoculation size, incubation period, culture volume, NaCl concentrations, carbon and nitrogen sources, and aeration (English et al. 2016; Ibrahim et al. 2019; Rajan and Kannabiran 2014). Additionally, scalability is important not only from an industrial viewpoint but also for downstream antimicrobial analysis (Núñez-Montero et al.

2019).

2.6.2 Organic extractions and compound isolation methods

After identifying antagonistic relationships between microorganisms, researchers need to be able to isolate the active component(s) causing the antimicrobial effect. The most common way to biosynthesize antimicrobials is through liquid fermentations; although, solid state fermentations can be used (Harms et al. 2017). After a desired fermentation period to obtain the antimicrobial of interest, liquid-liquid extraction can be performed to make a crude extract (Harms et al. 2017). Liquid extractions involve separating microbial cells to obtain a supernatant which can then be mixed with a solvent to then separate the solvent with dissolved antimicrobials from the cell-free solution (Núñez-Montero et al. 2019; Shetty et al. 2014). The solvent can then be removed via evaporation or desiccation. The selection of solvent greatly affects what type of compounds will be present in the final crude extracts. It is common to use ethyl acetate for *Streptomyces* with the goal of antimicrobial metabolite extractions (Núñez-Montero et al. 2019). Ethyl acetate is a good solvent when the active compound is unknown, as it has both polar and non-polar properties allowing for a large variety of compounds to be dissolved in it (Pintać et al. 2018).

With a crude extraction prepared it is possible to identify the active compound using a variety of chromatography techniques including, liquid chromatography mass spectrometry (LC-MS) (Pitt 2009). LC-MS allows for the separation of metabolites within crude extracts which can then be measured using a mass spectrometer. Using a similar technique called preparative liquid chromatography, it is possible to separate the different factions within the crude extract and then test fractions for bioactivity (Atlabachew et al. 2017; Latif and Sarker 2012). To determine the structure makeup of the purified active compound nuclear magnetic resonance (NMR) spectroscopy can be used (Hatzakis 2019).

CHAPTER 3. METHODS

3.0 *In silico* screening via genome annotation and antiSMASH

Draft genomes of 146 arctic bacterial isolates were previously constructed via short-read sequencing using Illumia Miseq (Marcolefas et al. 2019). The most promising isolates from the collection, based on broad spectrum antifungal activity from *in vitro* testing, were sent to Université Laval for Oxford Nanopore sequencing to produce a higher-quality genome using hybrid assembly by combining the short- and long-read sequencing data. Additionally, strains A165 and A28 were sent to Genome Quebec for whole genome sequencing using the Illumia MiSeq and was done using the reagent kit V3 in a 600-cycle format with read lengths at 300bp in each direction. The raw reads were then assembled using ProkaroteAssembly, and genome quality was assessed with Qualimap v.2.2.2-dev and potential contamination was checked by Confindr (García-Alcalde et al. 2012; Low et al. 2019). The hybrid genome assembly from Université Laval of A28 and normal assembly genome from Genome Quebec of A165 were uploaded to Microbial Genomics Atlas (MiGa) web tool to complete a TypeMat (Rodriguez-R et al. 2018). TypeMat was used to determine potential novelty of the isolate by preforming an average nucleotide identity (ANI) test.

Biosynthetic gene clusters were predicted from each genome using antiSMASH; each isolate of interest was submitted with all extra features enabled and detection strictness set to relaxed (Blin et al. 2017; Medema et al. 2011). Bacterial genome annotation was done with Prokka and dbcan (Seemann 2014; Zhang et al. 2018). Prokka was done with the default e-value threshold of 10^{-6} while using all feature prediction tools. Dbcan was done by uploading the nucleotide sequence of each draft genome and using HMMER, DIAMOND, and Hotpep with all default settings and thresholds settings.

3.1 Co-culture screening

Bacterial isolates were previously isolated from soil collected from the Canadian High Arctic on Axel Heiberg Island (Marcolefas et al. 2019). The isolates were stored in cryopreservation vials at -80 °C. Nine common cheese spoilage fungal isolates were supplied by Université Laval (Table 1). Fungal strains were stored in Microbank Cryoblock at -80 °C and cultured on potato dextrose agar (PDA) (Himedia sup. M096-500G) plates at 25 °C. The fungal strains were sub-cultured onto new PDA plates bimonthly, while being stored at 4 °C. Fresh cultures were prepared from the -80 °C stocks to confirm experiments with working cultures. Each arctic isolate was revived from long-term storage by streaking for isolation onto a tryptic soy agar (TSA) (BD Bioscience sup. 236950) plate then incubating it at room temperature (25 °C) for 72 hours. A single well isolated colony from each isolate was then streaked onto a fresh, square shaped TSA plate following co-culture diagram (Figure 4) and incubated at room temperature for roughly 48 hours or until colony establishment, this was done in replicates of 2. Fungal strains were then streaked by suspending spores in 1 mL of PBS and taking a loop-full of spores to streaking them perpendicularly to the bacterial isolates. The plates were then incubated at 25 °C for 72 hours to observe any growth inhibition.

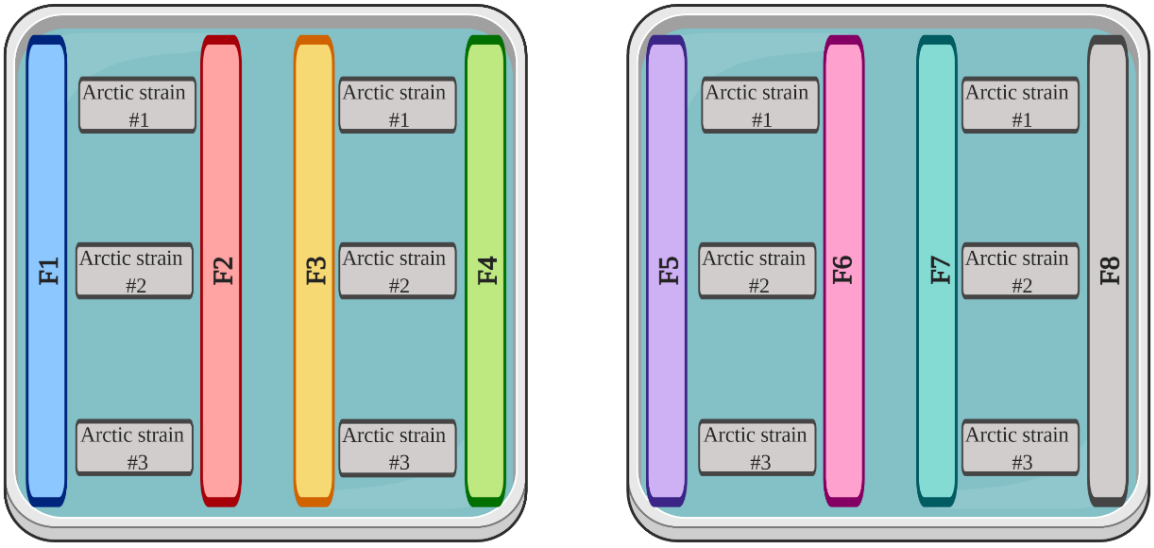


Figure 4. Co-culture diagram. Arctic isolates and fungal strains streaked perpendicular to one another to observe antagonistic relationships. Created using BioRender.com.

Table 1. Fungal strains obtained from Ismail Fliss at the Université Laval. Fungal strains originate from the dairy industry and were isolated from yogurt.

Penicillium crustosum	27159	F3
Penicillium roqueforti	27161	F4
Penicillium commune	27163	F5
Aureobasidium pullulans	27164	F6
Penicillium citrinum	27165	F7
Penicillium chrysogenum	ATCC 10106	F8
Aspergillus versicolor	LMA-370	F9

3.2 Crude extractions

Bacterial isolates of interest (A165 and A28) were cultured in 15 mL of tryptic soy broth (TSB) (BD Bioscience sup. 211768) in 125 mL Erlenmeyer flasks for 4 days at room temperature at 250 rpm. Cells were removed by 2 repeated centrifugation steps at 8000 rpm for 15 minutes. 100 µL of supernatant was then plated into 6 mm agar wells with fungal strains spread over to form a lawn. Plates were then incubated for 3 days at room temperature to observe any growth inhibition.

Organic extractions followed supernatant tests and started with a 15 mL TSB seed culture in

125 mL Erlenmeyer flasks for each isolate and were incubated at room temperature on a shaker set to 250 rpm for 24 hours. The seed culture was then sub-cultured into 3 fresh 15 mL TSB cultures with same incubation conditions as the seed culture with initial inoculum size being set to obtain an OD_{600nm} =0.05 in each subculture. The liquid culture was then transferred to a 50 mL falcon tube and centrifuged at 8000 rpm for 15 mins transferring the supernatant into a new falcon to repeat the process twice. After centrifugation, 7.5 mL of ethyl acetate (high performance liquid chromatography (HPLC) grade MilliporeSigma cat. EX0241-6) was added to the supernatant in the 50 mL centrifuge tube to give a final 2:1 (supernatant: solvent) volume ratio. The solution was then vortexed on high for 5 minutes and organic and aqueous layers were separated by centrifugation at 8000 rpm for 15 minutes. Organic layer was transferred using glass Pasteur pipette to a 40 mL amber borosilicate glass vial (Thermo Scientific B7999-6A). The extraction with ethyl acetate was then repeated a second time to increase potential yields. The organic layer was then left to evaporate by direct airflow filtered by 0.2 μm sterile syringe filter (Acrodisc cat.4652) until approximately 1 mL of extract remained in the 40 mL vial. The remaining extract was then transferred to a pre-weighed 2 mL amber borosilicate sample vial (Thermo Scientific cat. B7999-1A) with a glass pipette and left to completely dry by direct airflow. The dried extract could then be weighed and resuspended in methanol (HPLC grade MilliporeSigma cat. MX0475P-6) to conduct spot test assays. Spot assays were done by plating 30 μL of crude extract onto PDA plates and allowing the methanol to evaporate and then spreading the fungal strains from control zone to the dried extract and back to control zone.

To ensure consistent testing methods for the antifungal activity tests, the concentration of spores was calculated using a hemocytometer. Hemocytometer counting was done by placing 10 μL of suspension from the initial 1 mL PBS with 6 mm agar fungal plug into the hemocytometer and counting 5 squares, making sure to not recount the same spores by omitting any spores on outside

edges and by counting spores on lines only if they are on the top line or left side of the square.

3.3 Fermentation condition optimization

To optimize the organic extraction several parameters were tested. To optimize the incubation period, growth curves of each isolate were constructed by preforming viable plate counts and OD_{600nm} measurements in triplicate. Measurements were taken from a 50 mL TSB culture in a 500 mL Erlenmeyer flask incubated at room temperature on a shaker set to 250 rpm over the course of 24 hours. Additionally, organic extractions were performed on different incubation periods from 1-10 days and were tested for bioactivity. Media pH was tested from pH 5-8.5 in 0.5 increments and growth was measured using the plate reader with OD_{600nm}. Centrifugation method was adjusted for isolate A28 to best remove cells and debris from the supernatant by testing various rotors, speeds, duration, and deceleration settings. Various media types were tested including International Streptomyces Project-2 Medium (ISP2) (Appendix), yeast extract-malt extract (YEME) (Appendix) and TSB and organic extractions were performed to assess bioactivity. Organic solvent ratio to supernatant was tested, ratios included 1:3, 1:2, and 1:1.

The methods used to test bioactivity were also tested to obtain clear, repeatable results. After organic extractions and dried crude extracts were obtained a variety of parameters and methods were tested. Methanol was compared to ethyl acetate resuspensions when completing the bioactivity assays. The resuspension solvents were tested using agar wells and cellulose discs. Cellulose discs, agar wells and direct spot tests were compared for future experiments. Blank cellulose discs were loaded with 30 µL to 100 µL of crude extract and let dry before placing them on PDA plates with a fungal lawn to observe for growth inhibition, this was done in parallel with agar wells with the same volumes.

Crude extract stability and filter types were tested for future HPLC applications. After

organic extraction, 0.2 μm sterile MCE filters and 0.2 μm sterile polyvinylidene fluoride filters were used on the final methanol resuspensions. The extracts were then tested for bioactivity on PDA plates with a fungal tester strain (F3) over the course of 2 weeks while being stored at 4 °C until the crude extract ran out.

3.4 Larger scale extraction

To obtain larger and longer lasting extractions a scaled-up extraction was needed and changes to the optimized protocol were done to facilitate it. The same procedure for starting seed culture was done as the original protocol. The seed culture was the sub-cultured into 200 mL, pH 7.25 of TSB using a 1% v/v inoculum size and were incubated at 25 °C for 10 days with agitation at 250 rpm. Liquid cultures were centrifuged for 15 minutes at 5000 rpm using a swing bucket rotor to pellet all cells/debris and a serological pipet was used to collect supernatant. Due to the nature of the pellet being loose roughly 5 mL of supernatant was left above the pellet to ensure the pellet was not disturbed. Supernatant was then added to a 1L Erlenmeyer flask with a 1:1 ratio of ethyl acetate and shaken vigorously at 300 rpm at room temperature for 1 hour. The supernatant and ethyl acetate solution were then added to a separatory funnel and let to rest for 15 mins to allow for the separation of organic and aqueous layers. The organic layer was then collected into a round bottom flask and mounted to a rotary evaporator. The rotary evaporator settings were set to 80 rpm with a 40 °C water bath. Evaporation was let to continue in the rotary evaporator until approximately 10 mL of the crude extraction remained. Small amounts of aqueous layer were left behind in the round bottom flask while the organic layer was pipetted to a 40 mL amber borosilicate glass vial and evaporated by direct airflow filtered by a 0.2 μm sterile syringe filter. The dried crude extract was then resuspended in 5 mL of methanol and stored at 4 °C.

3.5 LC-MS

A subset of *Streptomyces* cultures (A28) turned dark after a few days of incubation, and the color change correlated to increased production of the antifungal substances. To compare dark and light cultures from the *Streptomyces*, and putatively identify the antifungal compound, LC-MS was performed. A total of 9 samples, 3 procedural blanks, 3 dark culture samples, and 3 light cultures samples were analyzed. The samples were separated into their components via a reverse phase C18 column. The mobile phases were water (A) and acetonitrile (B), both containing 5 mM NH4Ac and 0.1% formic acid. The liquid chromatography parameters used were as follows: 10 µL injection then an increasing linear gradient at 0-1 min 20% B, from 1-32 min up to 100% B, then down at 32.1-35 min 20% B at a flow rate of 0.25 mL/min.

The mass spectroscopy parameters are as follows: m/z range of 100-1700; capillary voltage set to 3500 v (-) or 4000 v (+); gas temperature of 250 °C; drying gas flow 10 L/min; nebulizer 35 psi; fragmentor 175 v; skimmer 65 v; all ion MS-MS: collision energy = 0, 10, 20, 40 v with the data acquisition rate set to 2 scans/s.

The data was then analyzed using the MassHunter Profiling software series. In each ion mode, data were first aligned using Agilent MassHunter Profinder (B.10.0) with the "Batch recursive feature extraction" with parameters set to mass window: +/- 20 ppm, retention time window: +/- 0.15 min and peak filter: >/= 300 counts. The statistical comparison of the chemical profiles among the dark extracts and light extracts was completed using MassHunter Profiler Professional (MPP, version B15.0). MPP screening parameters were as follows: normalization algorithm: 75% shift, baseline: median of all samples. Principal components analysis was applied to identify common or unique features among the two groups. A "fold change" analysis in MPP was applied to identify features whose intensity are significantly higher in dark extracts compared to the light extracts with a 2-fold change. The software could then compare the tentative molecular

formulas with the library database (Agilent Metlin_Metabolites_PCDL).

CHAPTER 4. RESULTS

4.0 *In silico* antimicrobial potential of Arctic bacterial isolates

To assess the overall antifungal potential of a culture collection of 146 unique bacterial isolates from the Canadian high arctic, various *in silico* search tools were used to screen them all. Identification of potential antifungal proteins such as GH 16 and GH 18 could only be done after genome annotation using Prokka or Dbcan, while secondary metabolite prediction could be done using antiSMASH (Medema et al. 2011; Seemann 2014; Zhang et al. 2018). After genome annotations were completed all GH 16 and GH 18 hypothetical proteins were tallied together for each isolate and labelled as a potential hit. Prokka gave an average of 1.85 hits per isolate with the highest number of hits from a single isolate being 12 hits (Figure 5). The more comprehensive list from Dbcan gave an average of 2.47 hits with the highest number of hits from a single isolate being 18 hits (Figure 6). Additionally, every isolate was scanned by antiSMASH and a variety of potentially antifungal gene clusters were found (Figure 7). Gene clusters with the most interest included the PKS (type I, II, III), as polyenes are known to be produced by them.

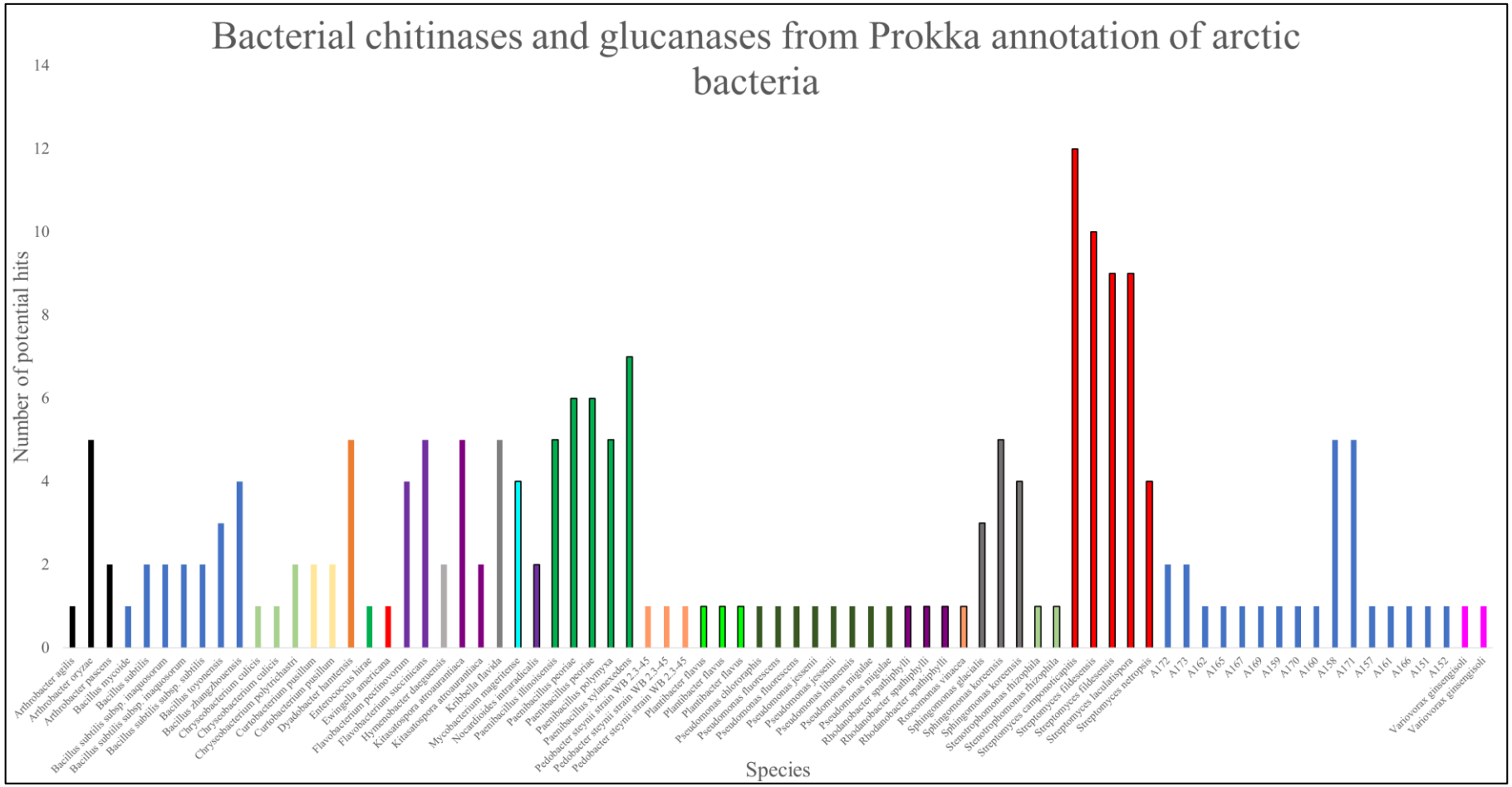


Figure 5. Protein annotation results from Prokka searching for glucanases or chitinases within the collection of arctic bacterial isolates. Potential hits are the number of proteins motifs predicted to be either glucanases or chitinase. The genera with the highest number of hits were *Streptomyces* and *Paenibacillus*. The average number of potential hits across all 146 isolates of was 1.85. Isolates are labelled using their closest relative based on 16s rRNA sequence, isolates without 16s RNA sequences were listed using their collection ID.

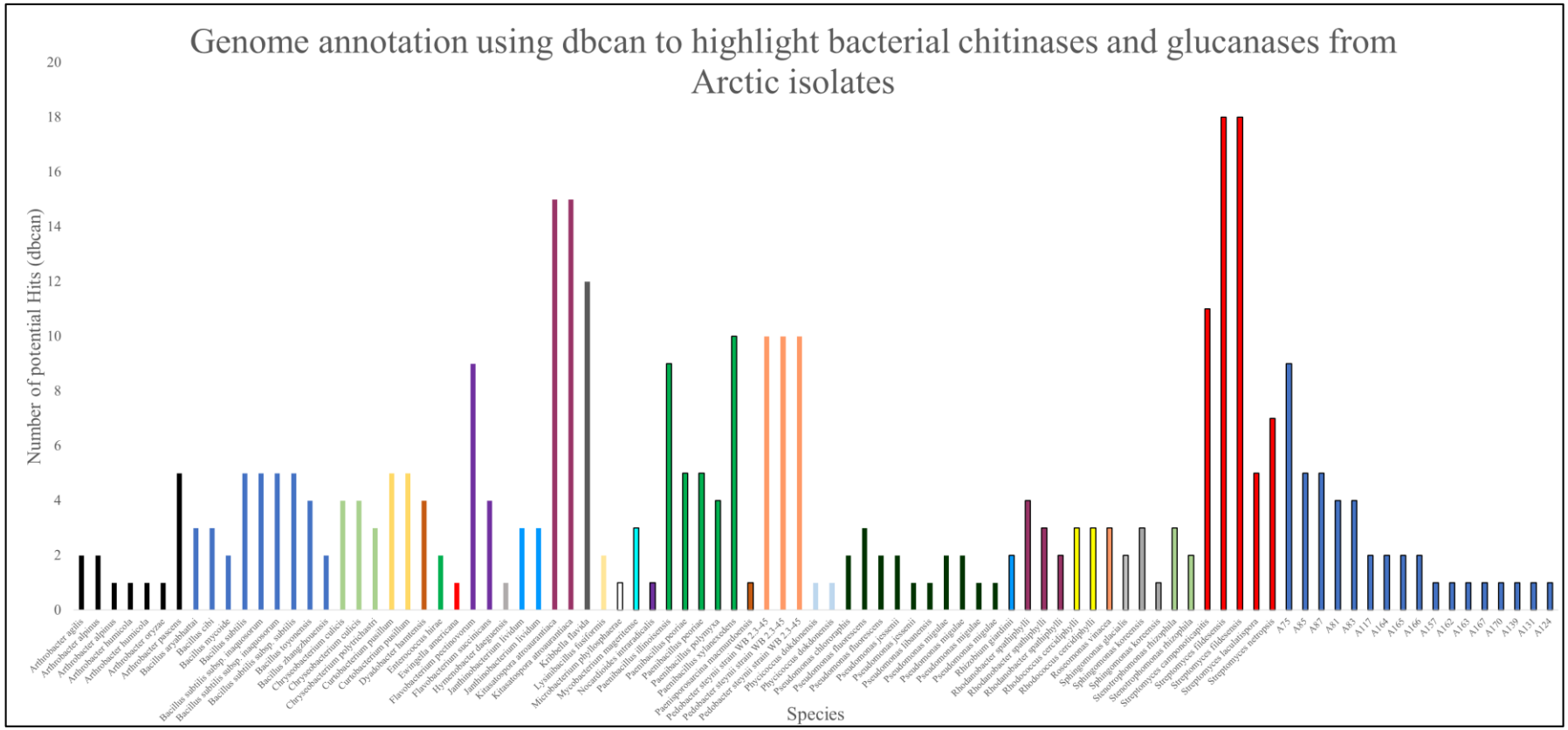


Figure 6. Protein annotation results from dbcan searching for glucanases or chitinases within the collection of arctic bacterial isolates. Potential hits are the number of proteins motifs predicted to be either glucanases or chitinase. The genera with the highest number of hits were *Streptomyces* and *Kitasatosporas* with the average number of potential hits across all 146 isolates being 2.47. Isolates are labelled using their closest relative based on 16s rRNA sequence, isolates without 16s RNA sequences were listed using their collection ID.

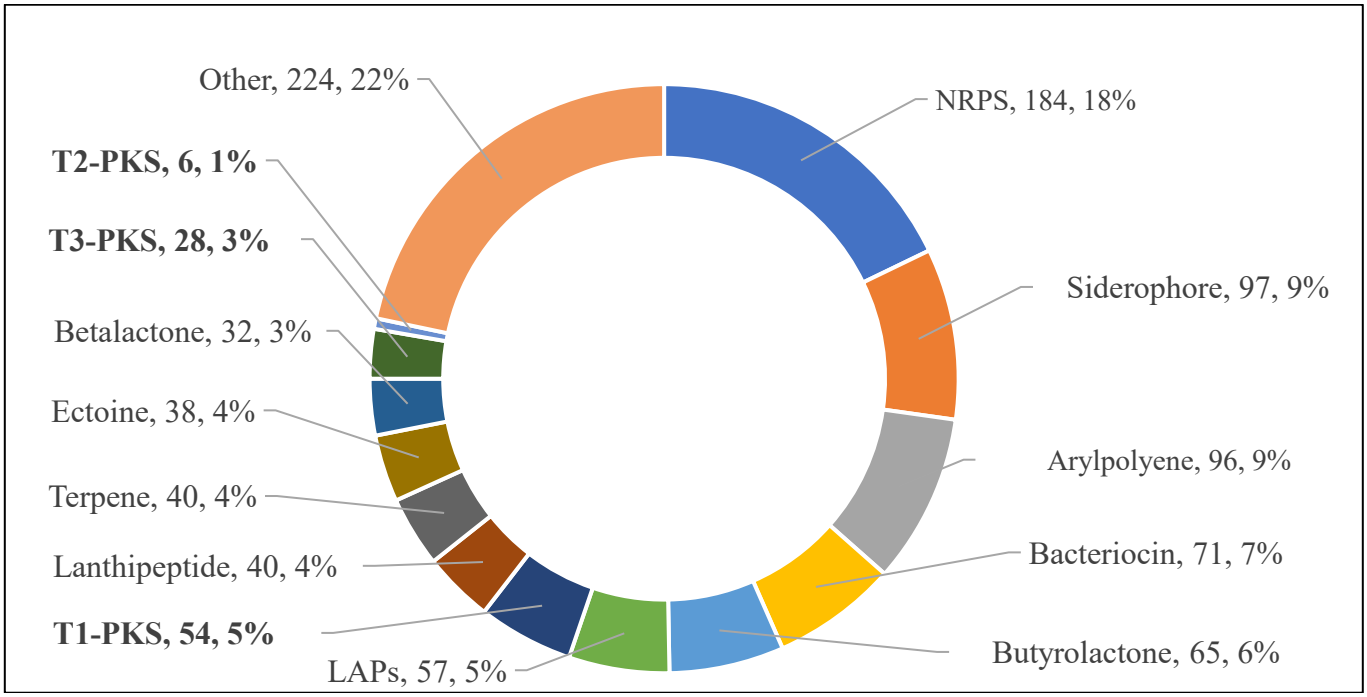


Figure 7. AntiSMASH summary of major BGCs in the arctic isolate database (n=146). Bolded BGCs are polyketide synthetase gene clusters that are known to produce numerous types of antifungals.

4.1 *In vitro* co-culture screening of arctic isolates and fungal strains

After initial *in-silico* screening and general outlook of the arctic isolate collection, culture-based methods could be used to narrow the collection to promising organisms with antifungal activity. A total of 80 arctic isolates were screened for antifungal activity (Table 2). Of the 80 arctic isolates, A165 and A28 produced very noticeable zones of inhibition across all fungal strains when co-cultured (Figure 8). Any antifungal activity that was observed on the co-culture screening plates were further confirmed using a single fungal strain against a single arctic isolate on additional agar plates (data not shown).

Table 2. Arctic isolate culture collection**.** 80 arctic isolates used in the co-culture screening assays with the closest relative based on 16S rRNA sequence data. Both identifiers are as follows; ID collection originated from Roger Levesque lab while ID original came from Lyle Whyte lab.

ID collection	ID original	Closest relative via 16S rRNA	% similarity
A1	EA101	*Pseudomonas libanensis*	99.47%
A10	B2B_RTpurple	*Janthinobacterium lividum*	99.79%
A106	EM_12	*Calidifontibacter indicus*	100.00%
A107	EA24	*Nocardioides intraradicalis*	97.05%
A11	EA3	*Stenotrophomonas rhizophila*	99.81%
A132	PV-56	*Arthrobacter tecti*	98.72%
A133	UVS1-I	*Rhodococcus cercidiphylli*	99.39%
A134	F2	*Kitasatospora atroaurantiaca*	98.09%
A136	AS1-U	*Arthrobacter agilis*	98.39%
A137	AV231	*Dyadobacter hamtensis*	96.65%
A138	PV-16	*Arthrobacter monumenti*	99.66%
A139	PV-55	*Paenisporosarcina macmurdoensis*	99.89%
A14	EA110	*Rhodococcus cercidiphylli*	97.96%
A140	B5	*Streptomyces fildesensis*	99.38%
A141	E6	*Streptomyces fildesensis*	99.31%
A142	PV-46	*Arthrobacter monumenti*	99.80%
A143	AV222	*Flavobacterium succinicans*	97.24%
A15	C6A_1_RT26	*Microbacterium phyllosphaerae*	99.87%
A151	2017_MAL_4_ABES_12_EC	N/A[1]	N/A[1]
A153	2017_CLMS_6_VMCS_4_BSPP	N/A[1]	N/A[1]
A154	2017_MAL_10_WYTK_25_ER	N/A[1]	N/A[1]
A155	2017_CLMS_7_VLYD_2_BSABEC	N/A[1]	N/A[1]
A156	2017_CLMS_7_VLYD_7_BSABEC	N/A[1]	N/A[1]
A157	2017_GS_1_SPSB_3_ER	N/A[1]	N/A[1]
A158	2017_GHHS_3_LBZX_4_BSSP	N/A[1]	N/A[1]
A159	2017_GHCE_5_JVZL_15_BSECSEAB	N/A[1]	N/A[1]
A16	C1B_RT18yellow	*Plantibacter flavus*	98.45%
A160	2017_GHHS_3_LBZX_18_BSSP	N/A[1]	N/A[1]
A161	2017_GS_7_EKIG_16_BS	N/A[1]	N/A[1]
A162	2017_AALPS_10_EMMH_23_BS	N/A[1]	N/A[1]
A163	2017_AALPS_2_EKRD_20_BS	N/A[1]	N/A[1]
A164	2017_AALPS_2_EKRD_7_BS	N/A[1]	N/A[1]
A165	2017_AALPS_10_JKNJ_7_PP	N/A[1]	N/A[1]
A166	2017_MAL_11_AHCHQX_12_BS	N/A[1]	N/A[1]
A167	2017_AALPS_10_MNAAK_13_BS	N/A[1]	N/A[1]
A168	2017_GHCE_5_JVZL_12_BSECSEAB	N/A[1]	N/A[1]
A169	2017_AALPS_4_MSMB_5_BS	N/A[1]	N/A[1]
A170	2017_GHHS_2_CGKF_16_BS	N/A[1]	N/A[1]
A171	2017_GHS_8_NWYW_5_BS	N/A[1]	N/A[1]
A18	C1B_RT18	*Pedobacter steynii strain WB 2.3-45*	99.93%
A19	B1_RT8	*Pedobacter steynii strain WB 2.3-45*	99.89%
A2	EA102	*Rhodococcus erythropolis*	100.00%
A20	B1_RT1	*Pedobacter steynii strain WB 2.3-45*	99.93%
A22	EA15diffused	*Pseudomonas frederiksbergensis*	99.88%
A23	EA14	*Arthrobacter agilis*	99.69%
A24	EA13entire	*Pseudomonas fluorescens*	99.28%
A26	EA13diffused	*Pseudomonas fluorescens*	99.28%
A27	EA11	*Streptomyces laculatispora*	98.28%
A28	EA10	*Streptomyces camponoticapitis*	98.02%
A29	EA6	*Stenotrophomonas rhizophila*	99.81%
A3	EA107	*Arthrobacter antarcticus*	98.88%
A30	EA5	*Microbacterium phyllosphaerae*	99.47%
A31	EA37beige	*Rhodococcus qingshengi*	100.00%

[1] Not available: incomplete, or missing 16S rRNA sequence

A32	EA35	*Bacillus aryabhattai*	99.84%
A33	EA27	*Leifsonia kafniensis*	98.15%
A34	EA26	*Leifsonia kafniensis*	97.68%
A35	EA23	*Arthrobacter humicola*	99.45%
A36	EA21	*Rhizobium giardinii*	99.11%
A37	EA20	*Microbacterium paraoxydans*	98.99%
A38	EA19	*Pseudomonas fluorescens*	99.21%
A40	EA15entire	*Pseudomonas jessenii*	99.14%
A41	EA93	*Arthrobacter antarcticus*	99.44%
A45	E6.1	*Sphingomonas glacialis*	99.71%
A46	EA44entire	*Arthrobacter antarcticus*	98.88%
A47	B9_A_RT14	*Plantibacter flavus*	98.45%
A48	B7B_B_RTpurple	*Janthinobacterium lividum*	99.77%
A49	E4	*Ewingella americana*	98.97%
A5	B2B_RT_3	*Pseudomonas fluorescens*	100.00%
A51	B2B_RTwhite	*Pseudomonas fluorescens*	100.00%
A52	E3	*Pseudomonas migulae*	99.41%
A53	EA52	*Plantibacter flavus*	98.83%
A7	EA99	*Arthrobacter alpinus*	99.08%
A90	EA31	*Deinococcus xibeiensis*	100.00%
A92	EA2orange	*Chryseobacterium polytrichastri*	97.63%
A96	EA104	*Arthrobacter antarcticus*	99.33%
A135	PV-21	*Arthrobacter monumenti*	99.66%
A152	2017_MAL_4_ABES_21_ECBS	N/A	N/A
A82	EA1	*Kribbella flavida*	98.54%
A89	EA28	*Kitasatospora atroaurantiaca*	98.09%

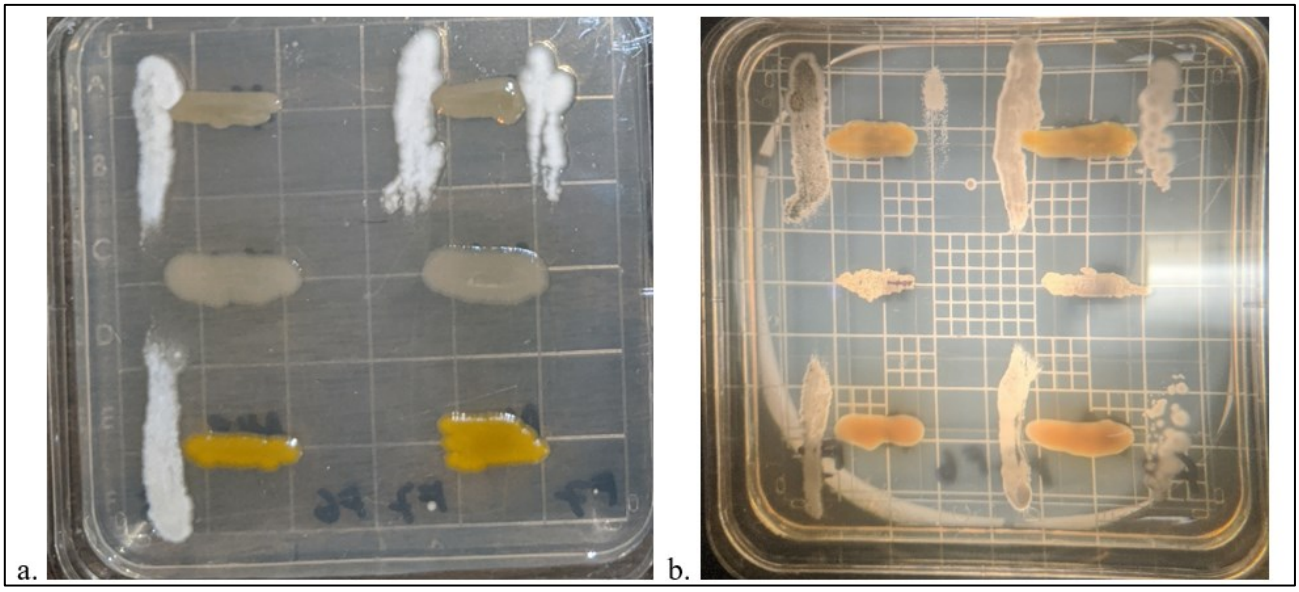


Figure 8. Promising arctic isolates showing antifungal activity against fungal strains. Zones of inhibition were produced by isolates A165 (a) and by isolate A28 (b).

4.2 Organic extraction and protocol optimization

Supernatant activity tests were conducted to determine if culture supernatants had antifungal activity. To increase the concentration of organic compounds crude organic extractions were made

using ethyl acetate as the organic solvent. Initial organic extractions had larger zones of inhibition, however, there was poor reproducibility of the extract's antifungal activity (Figure 9). Pre-extraction growth conditions, extraction procedures, and post-extraction storage were each optimized to produce organic extracts with the highest antifungal activity.

Three different media were used to examine the effect of media on antifungal activity (TSB, ISP2, and YEME media) but no antifungal activity was observed in ISP2 or YEME (data not shown). Changes to growth rate depending on media pH was observed in TSB with A28 where pH of 7.0-7.5 were optimal based on peak OD_{600nm} measurements and initial growth rate (Figure 10). It must be noted that the cell characteristics caused aggregates to form, even with vigorous shaking, which greatly increased the error on the curves. Lastly, the fermentation period was tested between 1, 2, 3, 4, and 10 days. Extracts became more consistent after 4 days, however the most reproducible crude extracts came from day 10 fermentations (data not shown). To test when the isolates achieve stationary phase -which is when most secondary metabolites are produced, growth curves were constructed for isolates of interest (Figure 11). The A165 strain reaches stationary phase at approximately 25 hours while A28 takes roughly 40 hours to reach stationary phase.

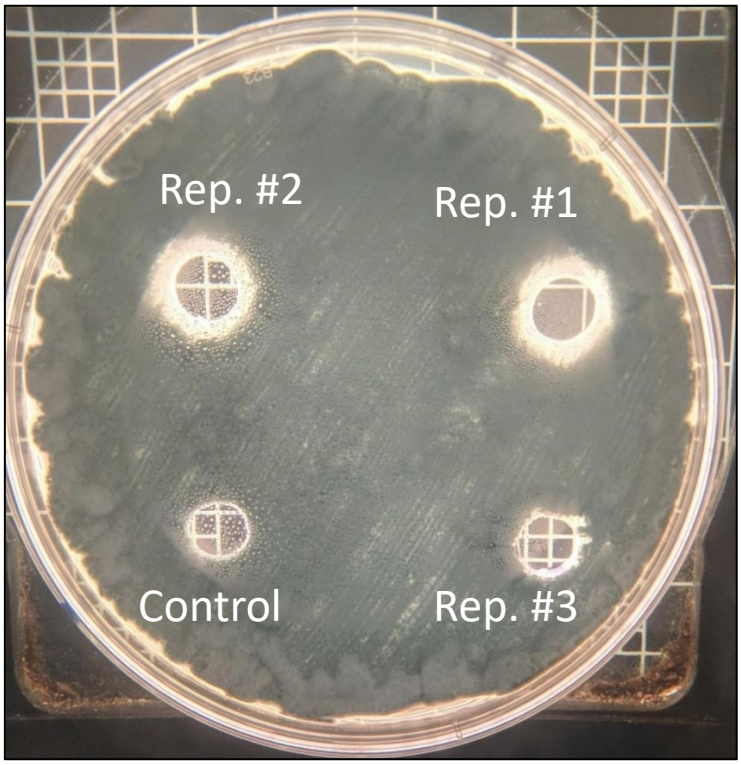


Figure 9. Organic extraction of A28 prior to completing optimization tested against F3. Replicates show low levels of antifungal activity and do not show the same levels of antifungal activity across the 3 replicates.

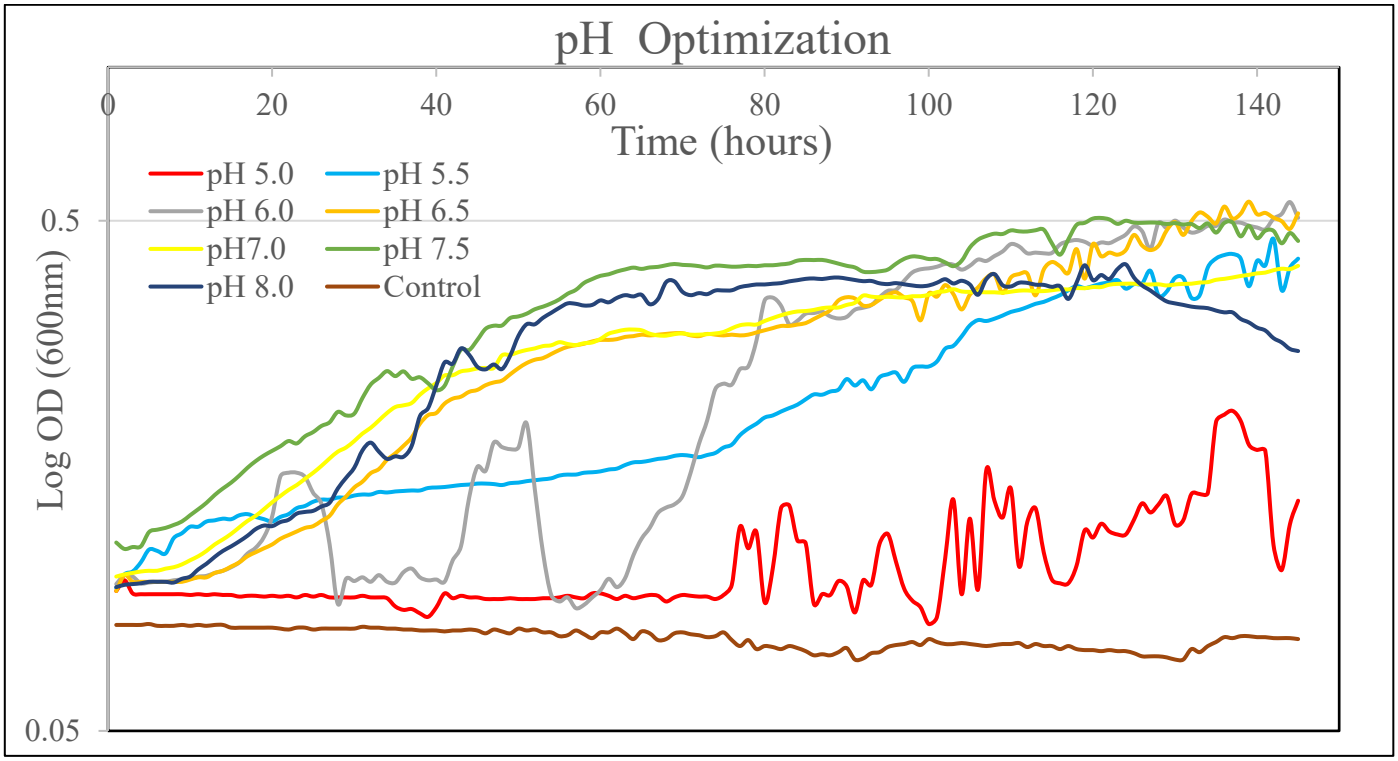


Figure 10. Growth curve measured by OD600nm of A28 cultures at various pH levels. Cultures aggregated into clumps which caused large variations in the OD600nm readings; however, growth was observed for pH levels above 5.0 and fastest growth came from pH 7.0-7.5.

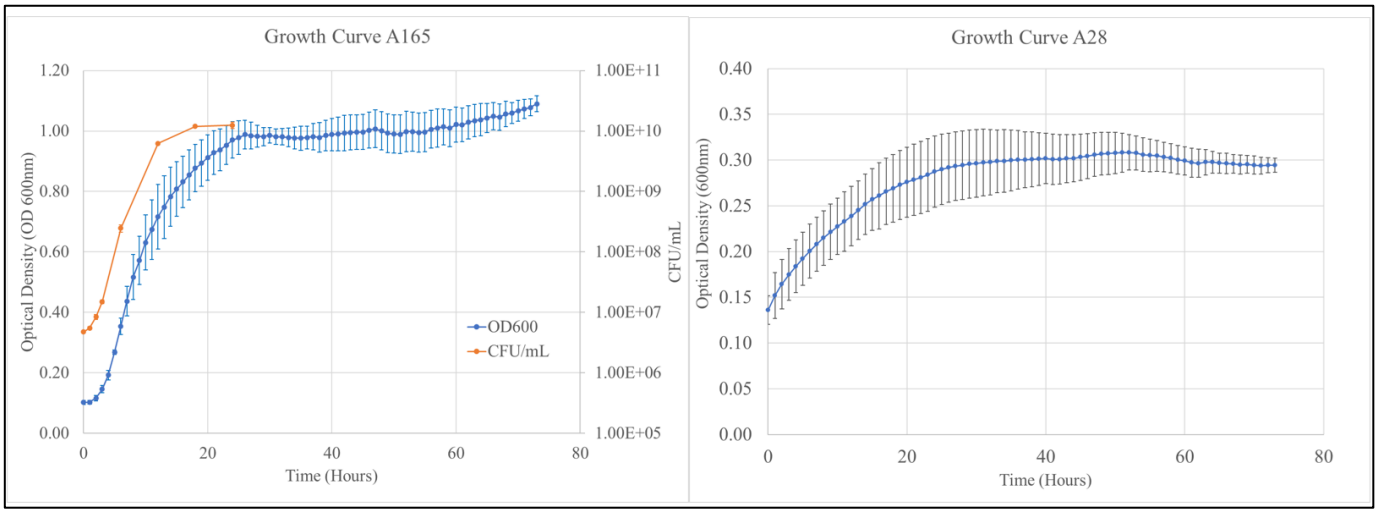


Figure 11. Growth curves of arctic isolates A165 (left) and A28 (right) in TSB at 25 °C. A165 reaches stationary phase at the 25-hour mark while A28 takes almost 40 hours to reach a stationary phase.

Optimization of the extraction process involved testing solvent ratios and centrifugation parameters. The solvent to aqueous layer ratios of 1:1, 1:2, and 1:3 were tested and no difference was visible in extract strength or reproductivity (data not shown). The centrifugation parameters were tested using an angled rotor at 10,000 rpm and a swing bucket centrifuge at 5,000 rpm (max speed) as well as testing the centrifugation time from 20 minutes, 30 minutes, and 1 hour. The clumping of cells was most affected by the rotor type, swing bucket rotors allowed the cells to pellet more evenly without having lots of debris left in the supernatant, while the time had minimal effects on recovery (data not shown).

Post-extraction optimization involved testing the best method for detecting antifungal activity and the stability of the extracts during storage. Crude extracts were compared using agar wells and blank antimicrobial susceptibility disc. The agar wells displayed zones of inhibition much more visible compared to the discs (Figure 12). The extract stability when stored in methanol and at 4 °C was measured over 2 weeks and after 35 days. Antifungal activity was observed in extract that had been stored at 4 °C was measured everyday over 2 weeks and after 35 days (data not shown).

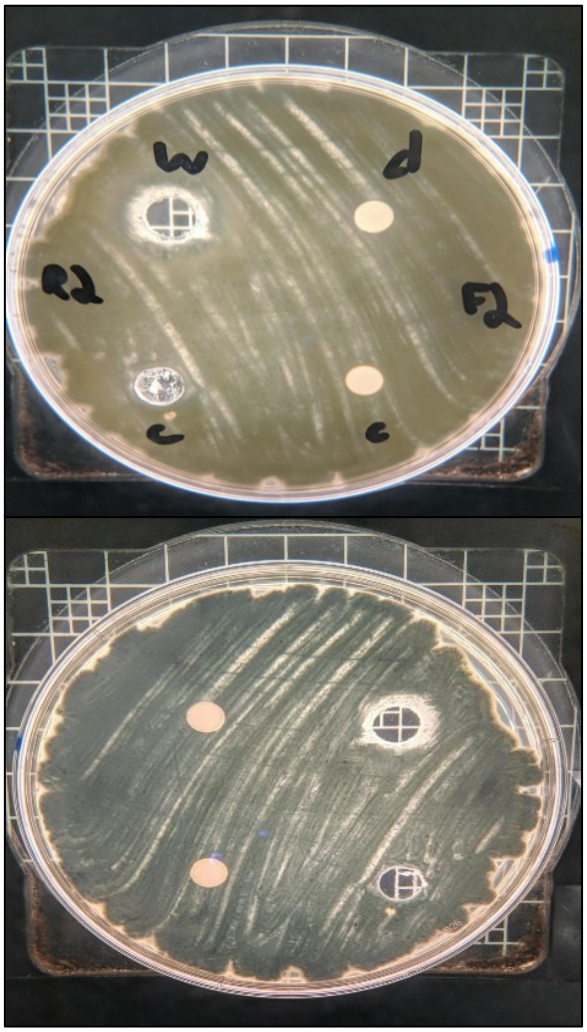


Figure 12. Comparing bioactivity testing method, using either agar wells or antimicrobial susceptibility discs. Agar well W performed well, showing clearly visible antifungal activity while discs D lacked any noticeable bioactivity while using the same extract and volume. Upper panel is the agar plate facing down, while the lower panel is the same agar plate facing up. Controls for both disc and agar well are labelled C.

After initial optimization steps on A165 cultures proved ineffective we continued with only using A28. Media for A28 variably turned dark after culturing, and the change in media color prior was highly correlated with the level of antifungal activity observed in the crude extracts (Figure 13). Darkened extracts consistently displayed antifungal activity while the lighter cultures had little or no antifungal activity (Figure 14). An attempt was made to identify the active component using LC-MS by comparing darkened and light culture extracts which found no known antifungal.

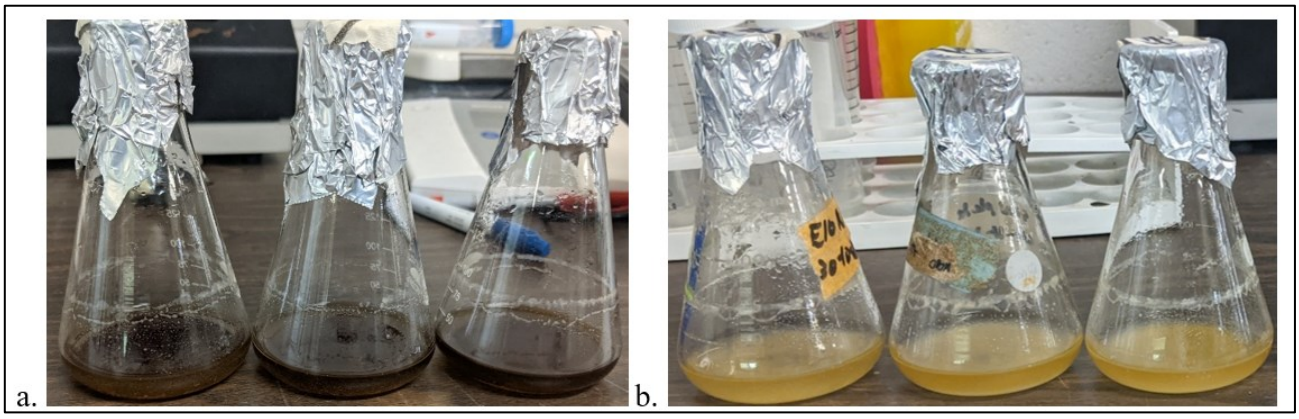


Figure 13. Media color change prior to extraction for isolate A28. (A) Dark cultures (B) Light cultures. Both dark and light cultures made with the same seed culture, inoculum size, and media composition would inconsistently change color after 10-day fermentation periods.

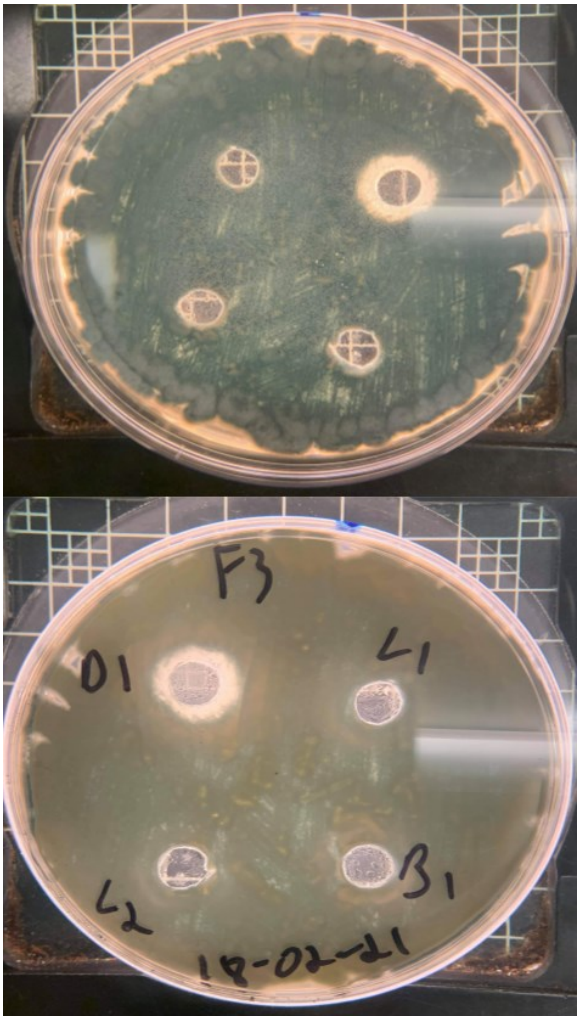


Figure 14. Antifungal activity of dark and light culture crude extracts. Top panel is agar plate facing up, while bottom panel is agar plate facing down. Agar well D1 is a dark extract showing antifungal activity, while wells L1 and L2 are light culture extracts showing no antifungal activity. Agar well B1 is the procedural control.

With the increase in extract reproducibility based on dark culture extraction it was time to move on to produce large scale extractions for downstream assays. The larger extraction was completed using optimized conditions including incubation in 1 L flasks with 250 mL of TSB at 25°C, with shaking at 250 rpm for 10 days. Extracts produced using the large-scale method yielded the best zones of inhibition on all 9 fungal strains (Figure 15).

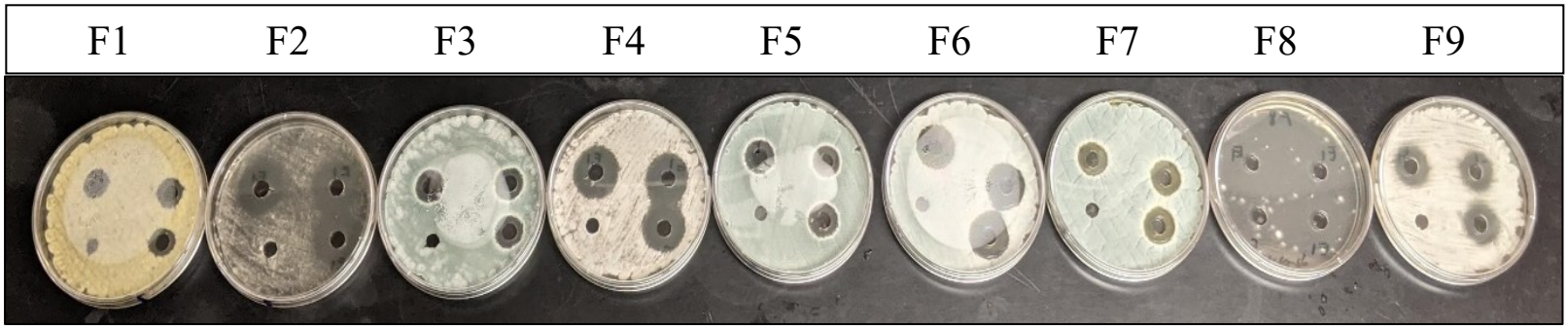
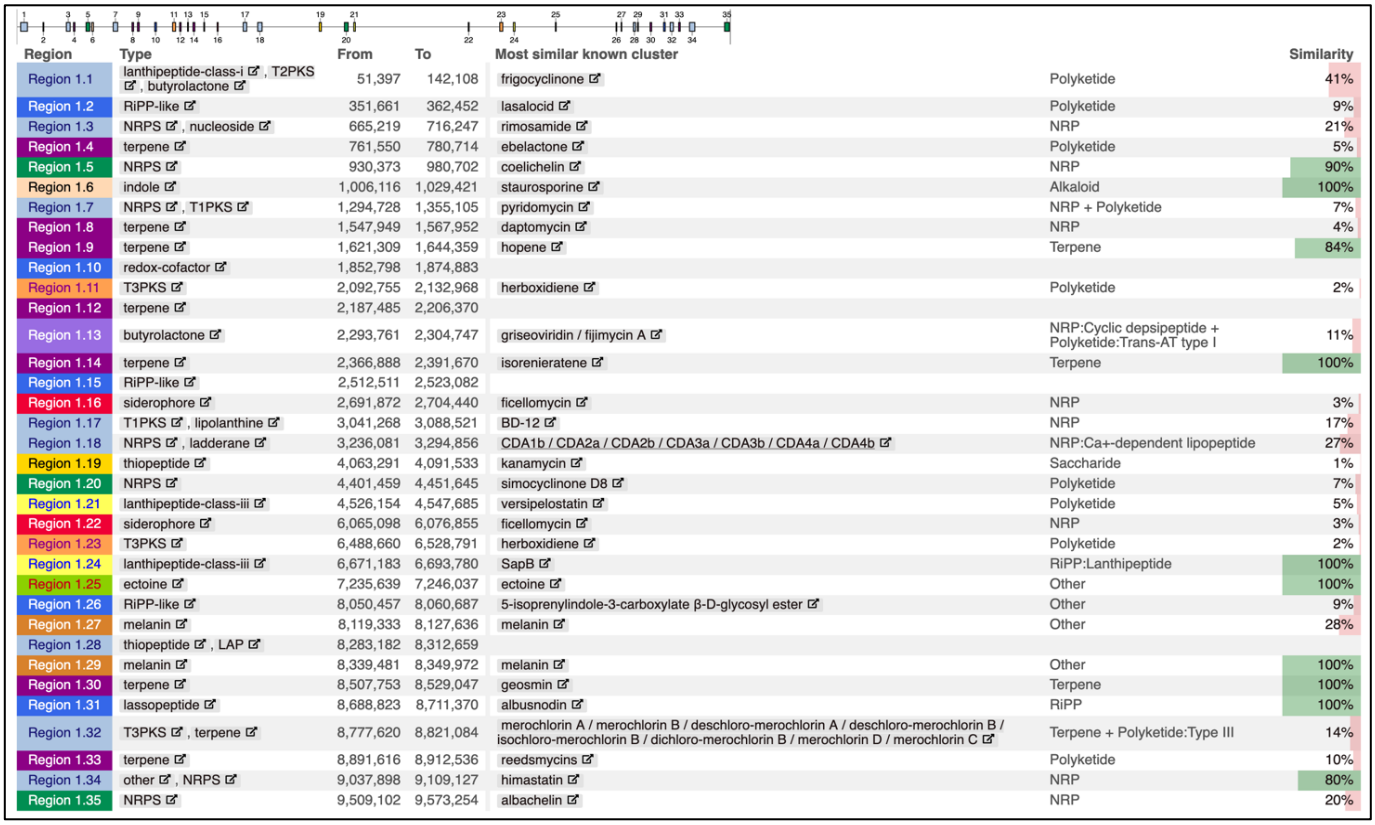


Figure 15. Larger scale organic extractions of A28. Testing antifungal activity on all 9 fungal strains (F1-F9). A single extract was tested in triplicate showing large zones of inhibition over a lawn of fungal growth (bottom left well is the control).

4.3 Whole genome sequencing and hybrid genome antimicrobial potential

Initial genome sequences showed potential quality issues for analysis of BGCs, specifically, a larger number of contigs (87) for A28. To remedy this and to obtain a more comprehensive overview, the 2 isolates A28 and A165 were sequenced for a second time at Genome Quebec using a larger number of cells for DNA extraction yielding 50 µL of DNA at a concentration of 956 ng/µL. Qualitmap analysis results for A165 showed a genome size of 6 Mb with 83 contigs and a mean coverage of 36.7x (García-Alcalde et al. 2012). Qaulitmap result for A28 showed a genome size of 9.7 Mb with 636 contigs and a mean coverage of 22.2x. Confindr results showed no contamination for either isolate (Low et al. 2019). Additionally, a hybrid genome (using both short and long-read sequencing technologies) of A28 was assembled by Université Laval giving a hybrid gnome of 9.9 Mb. with 7 contigs, thus a much higher quality genome. Using the hybrid genome for

A28 and the A165 genome from Genome Quebec, these strains of interest were analyzed for complete BGCs. AntiSMASH results for A28 showed no clear antifungal gene clusters but showed various potentially antibacterial clusters with low similarity (Figure 16) (Medema et al. 2011). AntiSMASH results for A165 showed very few BGCs compared to A28 although a potential aryl polyene with low similarity ranking was identified (Figure 17).



Region	Type	From	To	Most similar known cluster		Similarity
Region 1.1	lanthipeptide-class-i , T2PKS , butyrolactone	51,397	142,108	frigocyclinone	Polyketide	41%
Region 1.2	RiPP-like	351,661	362,452	lasalocid	Polyketide	9%
Region 1.3	NRPS , nucleoside	665,219	716,247	rimosamide	NRP	21%
Region 1.4	terpene	761,550	780,714	ebelactone	Polyketide	5%
Region 1.5	NRPS	930,373	980,702	coelichelin	NRP	90%
Region 1.6	indole	1,006,116	1,029,421	staurosporine	Alkaloid	100%
Region 1.7	NRPS , T1PKS	1,294,728	1,355,105	pyridomycin	NRP + Polyketide	7%
Region 1.8	terpene	1,547,949	1,567,952	daptomycin	NRP	4%
Region 1.9	terpene	1,621,309	1,644,359	hopene	Terpene	84%
Region 1.10	redox-cofactor	1,852,798	1,874,883			
Region 1.11	T3PKS	2,092,755	2,132,968	herboxidiene	Polyketide	2%
Region 1.12	terpene	2,187,485	2,206,370			
Region 1.13	butyrolactone	2,293,761	2,304,747	griseoviridin / fijimycin A	NRP:Cyclic depsipeptide + Polyketide:Trans-AT type I	11%
Region 1.14	terpene	2,366,888	2,391,670	isorenieratene	Terpene	100%
Region 1.15	RiPP-like	2,512,511	2,523,082			
Region 1.16	siderophore	2,691,872	2,704,440	ficellomycin	NRP	3%
Region 1.17	T1PKS , lipolanthine	3,041,268	3,088,521	BD-12	NRP	17%
Region 1.18	NRPS , ladderane	3,236,081	3,294,856	CDA1b / CDA2a / CDA2b / CDA3a / CDA3b / CDA4a / CDA4b	NRP:Ca+-dependent lipopeptide	27%
Region 1.19	thiopeptide	4,063,291	4,091,533	kanamycin	Saccharide	1%
Region 1.20	NRPS	4,401,459	4,451,645	simocyclinone D8	Polyketide	7%
Region 1.21	lanthipeptide-class-iii	4,526,154	4,547,685	versipelostatin	Polyketide	5%
Region 1.22	siderophore	6,065,098	6,076,855	ficellomycin	NRP	3%
Region 1.23	T3PKS	6,488,660	6,528,791	herboxidiene	Polyketide	2%
Region 1.24	lanthipeptide-class-iii	6,671,183	6,693,780	SapB	RiPP:Lanthipeptide	100%
Region 1.25	ectoine	7,235,639	7,246,037	ectoine	Other	100%
Region 1.26	RiPP-like	8,050,457	8,060,687	5-isoprenylindole-3-carboxylate β-D-glycosyl ester	Other	9%
Region 1.27	melanin	8,119,333	8,127,636	melanin	Other	28%
Region 1.28	thiopeptide , LAP	8,283,182	8,312,659			
Region 1.29	melanin	8,339,481	8,349,972	melanin	Other	100%
Region 1.30	terpene	8,507,753	8,529,047	geosmin	Terpene	100%
Region 1.31	lassopeptide	8,688,823	8,711,370	albusnodin	RiPP	100%
Region 1.32	T3PKS , terpene	8,777,620	8,821,084	merochlorin A / merochlorin B / deschloro-merochlorin A / deschloro-merochlorin B / isochloro-merochlorin B / dichloro-merochlorin B / merochlorin D / merochlorin C	Terpene + Polyketide:Type III	14%
Region 1.33	terpene	8,891,616	8,912,536	reedsmycins	Polyketide	10%
Region 1.34	other , NRPS	9,037,898	9,109,127	himastatin	NRP	80%
Region 1.35	NRPS	9,509,102	9,573,254	albachelin	NRP	20%

Figure 16. AntiSMASH result of isolate A28 hybrid genome. Several potential antimicrobials are present based on BGCs, however, there is no clear explanation for the observed antifungal activity.

Region	Type	From	To	Most similar known cluster		Similarity
Region 2.1	NRPS	594	137,915	pyoverdin	NRP	25%
Region 2.2	betalactone	328,319	351,921	fengycin	NRP	13%
Region 4.1	terpene	136,279	157,853			
Region 5.1	arylpolyene	205,860	249,465	APE Vf	Other	40%
Region 6.1	hserlactone	39,407	60,054	azomycin	Other	33%
Region 8.1	NAGGN	123,101	137,977			
Region 9.1	bacteriocin	246,990	257,829	bacillomycin D	Polyketide + NRP:Lipopeptide	20%
Region 15.1	NRPS	20,075	73,067	pyoverdin	NRP	20%

Figure 17. AntiSMASH result of isolate A165 genome. Predicted BGCs show low similarity to known antimicrobials, with no clear signs of antifungal potential.

Both genomes of A165 and A28 were uploaded to MiGa using the TypeMat tool to assess the ANI of the isolates (Rodriguez-R et al. 2018). Based on the TypeMat analysis results the A28 isolate belongs to a novel species within *Streptomyces* genus, with a P-value 0.0427 for the isolate being in the *Streptomyces* genus but only a P-value of 0.502 for the isolate belonging to the highest matched known species of *S. chryseus* (Figure 18). Only 71.36% of the genome from A28 is shared with *S. chryseus*, highlighting the novelty of the isolate A28. The TypeMat results for A165 showed that the isolate is a new subspecies of *Pseudomonas prosekii*, sharing 92.31% of its genome with it (Figure 19).

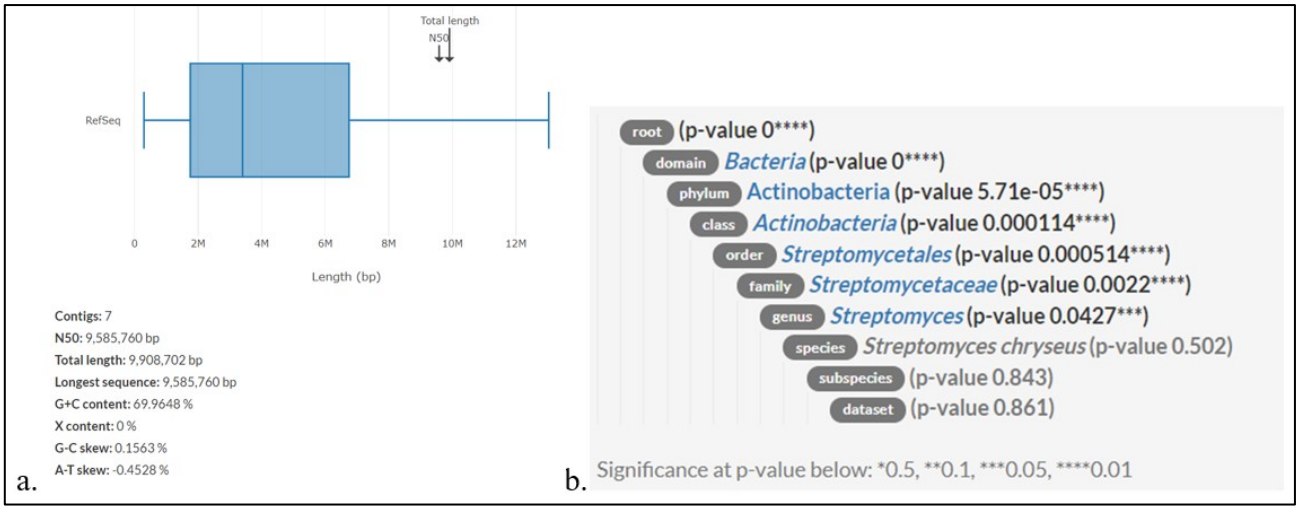


Figure 18. MiGa TypeMat results for isolate A28. A) Genome assembly information. B) ANI test for isolate A28 compared to MiGa database showing A28 is a novel species of *Streptomyces*.

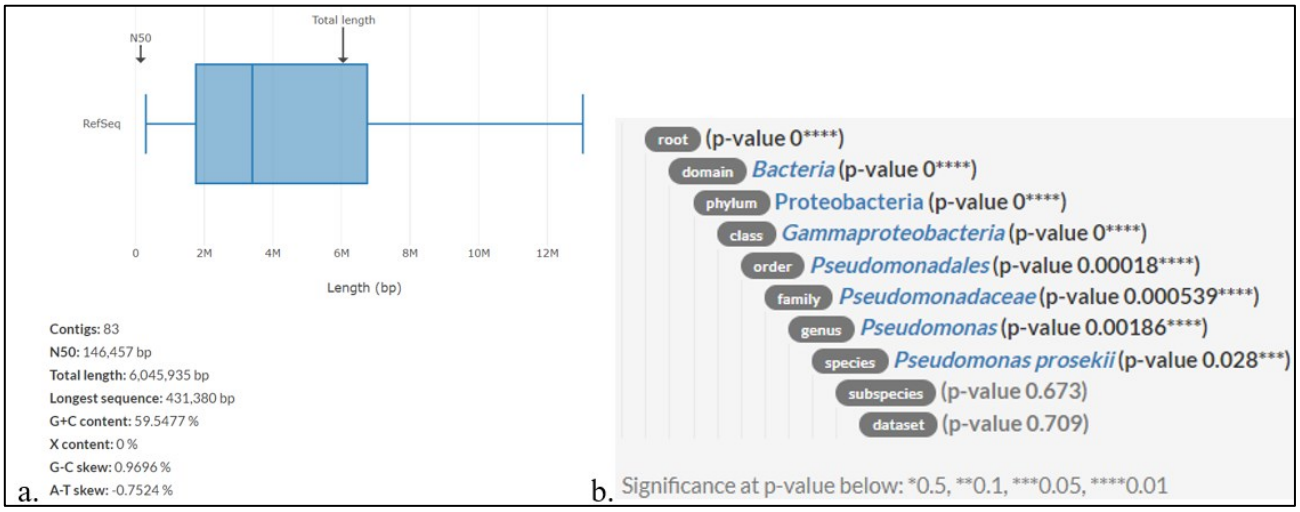


Figure 19. MiGa TypeMat results for isolate A165. A) Genome assembly information. B) ANI test for isolate A165 compared to MiGa database showing A165 is a novel subspecies of *Pseudomonas prosekii*.

4.4 LC-MS

After concluding that darkened cultures of A28 produce crude extracts with antifungal activity, we then started to search for the active compound(s) in the extract. To search for the active compound(s) we used the knowledge that darkened culture media produces extracts with antifungal activity while light culture media produces crude extracts with no antifungal activity, we sought to compare them using LC-MS. The compounds present at 2-fold higher concentrations in the dark extracts compared to the light extracts were analyzed to generate a list of potential candidates. Using the LC-MS negative ion mode resulted in no molecular features of significance while the positive ion mode resulted in a total of 30 candidates, of which 16 had no known name in the reference database (Table 3).

Table 3. List of suspect candidates from positive ion mode using LC-MS. Candidate compounds with 2-fold or more increase in concentration in darkened extracts compared to the light were selected. Candidates are presented with their putative name, molecular formula, and retention times in minutes.

Name	**Formula**	**Retention time (minutes)**
Adenine	C5 H5 N5	2.384
Adenine	C5 H5 N5	1.776
Anhydroglycinol	C15 H10 O4	3.55
Aloe-emodin	C15 H10 O5	5.04
Harmanine	C12 H10 N2 O	5.17
1-Nitrosonaphthalene	C10 H7 N O	2.839
Cypripedin	C16 H12 O5	3.65
Betavulgaroside X	C46 H70 O18	7.069
1,6-Dihydroxy-3,7-dimethoxy-2-(3-methyl-2-butenyl)-8-(3-hydroxy-3-methyl-1E-butenyl)-xanthone	C25 H28 O7	6.594
PIP(16:0/22:2(13Z,16Z))	C47 H88 O16 P2	27.611
Cubebinone	C23 H26 O8	8.18
Salviaflaside	C24 H26 O13	1.761
(S)-Rutaretin	C14 H14 O5	1.828
Compound 19	C12 H19 N3 O7	5.850
Compound 5	C10 H19 Cl N3 O P S3	2.384
Compound 8	C34 H72 F3 N7 P	34.14
Compound 14	C13 H Cl6 F N3 O7 P2 S5	34.192
Compound 16	C3 H4 Cl N3 O2 S	2.635
Compound 17	Unknown	34.104
Compound 18	C8 H11 F N5	5.677
Compound 20	Unknown	28.985
Compound 24	C41 H21 N5 O2	25.895
Compound 28	C7 H2 Cl5 F3 N4 S2	34.412
Compound 30	C10 H14 N3 O S3	1.762
Compound 31	C15 H5 F2 P S10	34.339
Compound 32	Unknown	28.936
Compound 35	Unknown	34.462
Compound 37	C28 H58 F4 N18 P	9.632
Compound 46	C18 H8 Cl F S	2.371

CHAPTER 5. DISCUSSION

This study was conducted to search for novel antifungals, produced by bacteria isolated from the environment in the Canadian high arctic, that could be used in the cheese industry to inhibit fungal growth. A three-step method was used to identify potentially novel antifungals: (1) an *in silico* analysis of the whole genome sequences of the bacteria, (2) an *in vitro* co-culture experiment, and (3) testing crude extract from isolates of interest for antifungal activity. This study has identified two potentially novel antifungals from two different isolates and has demonstrated that antifungal compounds produced by arctic bacteria have the potential to be used as novel antifungals in the cheese industry to prevent fungal food spoilage.

Fungal resistance to antifungals is increasing in both the food industry as well as within medicine. There are fewer antifungals available compared to antibiotics, and this is causing fungal infections, as well as fungal spoilage, to pose serious problems (Berman and Krysan 2020). Fungal resistance to antifungals such as azoles and polyenes typically occurs via mutations that alter the drug target, or the increased expression of drug efflux pumps, or the presence of drug transporters (Cowen et al. 2014). To help combat the evolution of antifungal resistance, researchers are testing natural compounds such as seaweed, thyme, lavender, tea tree, clove, and peppermint oils for the presence of antifungal activity which some have showed promise but there has yet to be implementation in the food industry (Puškárová et al. 2017; Silva et al. 2018). Additionally, researchers are performing tests on huge libraries of prospective compounds and using high throughput screening methods to filter small molecules with potential for antifungal activity (Brauer et al. 2019).

One aspect that is important to consider when searching for antifungals to be used in a food spoilage setting is the origin of the antifungal (Leyva Salas et al. 2017). Consumers and public

authorities have been advocating for *clean label* products that do not contain synthetic preservatives, and are thus shifting toward identifying natural preservatives and preservation techniques (Leyva Salas et al. 2017). In this project, we were able to produce an antifungal crude extract from environmental bacteria and, therefore, possibly meet consumer demands for *clean label* products if it would be implemented in food.

Searching for novel antimicrobials is challenging because of the high likelihood of rediscovering already known compounds. The use of high throughput screening systems in combination with *in silico* analysis to filter possible candidates has helped many researchers find novel antimicrobials (Lee et al. 2020). Researchers have found potential drug targets using either genomic or protein structure data to aid in the discovery of novel antimicrobials. Using structure based methods phytochemicals including; Melianoninol, Nimbinene, Vilasinin, and Fraxinellone were found to have potential against *Fusarium graminearum* (Joshi et al. 2020). This can be used for predicting antimicrobials using molecular docking analysis which, can be done on compounds to test for binding affinity/off target effects (Ndaba et al. 2020). Researchers can compare known compounds such as amphotericin B to other compounds by comparing the binding affinity to drug targets like cholesterol and ergosterol (Ferdosiyan and Sardari 2010). This method can be used to quickly narrow the number of compounds from a large database; however, *in vitro* testing still needs to be done to confirm and further filter compounds from the most promising candidates. Genome mining is the term used for identifying genes involved in natural product biosynthesis (Belknap et al. 2020). Popular genome mining tools such as antiSMASH were first released in 2011; however, there are now a variety of tools and strategies used for this purpose (Blin et al. 2017). In this project, the genome mining from the most promising isolate (A28), based on *in vitro* tests, found a total of 35 BGCs (which is slightly below the average number of BGCs present in *Streptomyces* at 39.65), but it contained a low amount of NRPS gene clusters at just 7 in total (4 of which are hybrid BGCs

containing 2 or more classes of BGCs) compared to the average of 17.66 (Belknap et al. 2020). Ten of the BGCs from A28 had high similarity to known antimicrobials gene clusters such as NRPS, terpenes, and ribosomally synthesized and post-translationally modified peptides. Although, these BGCs have likely already been discovered, they could still prove novel and useful as homologs of the known compounds produced by mesophilic bacteria.

Antimicrobial discovery from Antarctic *Streptomyces* has previously been done and has shown a large diversity and low similarity of BGCs within the genome, similar to the clusters found within this study's most promising isolate A28 (Núñez-Montero et al. 2019). Interestingly, Núñez-Montero et al. found melanin BGCs, in the *Streptomyces fildesensis* So13.3 genome, which are known to produce a brown pigment, like the color change found in the active antifungal crude extracts of A28. Melanins are negatively charged macromolecules that can be antimicrobial (El-Naggar and El-Ewasy 2017). The genome of A28 possesses 2 melanin BGCs, one with high similarity to known genes and the other with low similarity. The LC-MS data did not show any differential production of melanin between the dark and light extracts on the negative ion scan which suggests that melanin is not causing the antifungal activity in this instance; however, more tests are needed to confirm the source of the antifungal activity and potential antifungal novelty within the A28 genome. The cause of the antifungal activity correlating with the darkening of the culture is still unknown but it could potentially be explained by a type of phase variation acting as an on/off mechanism for the antifungal expression (Sumby and Smith 2003). Further *in vitro* characterization of the extracts produced by A28 are needed to elucidate the active component(s) but based on the isolate's genome and low similarity scores to several potential antimicrobials suggests, A28 could be a valuable reservoir for new drugs.

In vitro antifungal drug discovery can be done in multiple ways. Initial co-culture experiments between fungi and bacteria are a common method to induce antifungal expression.

Many microorganisms silence genes involved in antimicrobial synthesis depending on environmental conditions, and the close proximity of fungi and bacteria in a co-culture will likely induce the silent antimicrobial by recreating the natural competition (Ross et al. 2014) which can be done using the co-culture methods. After co-culturing, crude extracts are made to test against the fungi; unfortunately, depending on how the extracts are made, the expression of antifungals may not be present without the co-culturing method. This matches our results, where we saw 2 isolates (A165 and A28) with antifungal activity during co-culture, but only A28 was capable of producing antifungal extracts when fermented in a monoculture. This suggests that the antifungal activity of A165 is inducible but is not constitutively expressed under normal fermentation conditions. A165 is a part of the genus *Pseudomonas,* where gene expression of antimicrobials is often tightly regulated to stop the bacteria from needlessly synthesizing them (Duffy and Défago 1999; Gionco et al. 2017).

To identify the compounds in crude extracts, a variety of chromatography methods including LC-MS and thin layer chromatography can be used as effective tools to elucidate the antimicrobial agents (Jesionek et al. 2015). In this project, we used LC-MS due to the high resolution and separation power of the crude extracts to compare the light and dark culture extracts (Pitt 2009). The LC-MS results showed several unknown compounds indicating the need for further characterization of the extract. Of the known compounds found to be upregulated in the dark culture extracts, the compounds with the greatest potential for antifungal activity included 2 different terpenes and 2 different polyketides. Terpenes are a diverse group of compounds found in nature and have a wide variety of uses, including antimicrobial therapeutics (Cox-Georgian et al. 2019). Betavulgaroside X and N-(1-Deoxy-1-fructosyl)histidine are both terpenes with extremely limited information on their antimicrobial activity and therefore their extract fractions should be tested for antifungal activity. Both polyketides that were found, Anhydroglycinol and Aloe-emodin, were originally discovered in

plants. Aloe-emodin is an anthraquinone molecule that is a derivative of emodin (Pecere et al. 2000). Emodin contains antifungal properties; however, aloe-emodin has not shown any antifungal activity but has shown anticancer properties (Chen et al. 2010). There is limited data available on Anhydroglycinol but it is a pterocarpans which can have antimicrobial properties (Jiménez-González et al. 2008). In summary, the LC-MS analysis showed many potential compounds with antifungal activity, but with no clear antifungal molecule, that explains the observed antifungal activity, as well as the darkening of antifungal producing cultures, was identified.

The next short-term step for this project would be to elucidate the active component within the A28 crude extract. Another common method used for the identification of an active molecule from natural organic extractions is the use of a preparative-HPLC where purified fractions of the crude extract can be tested for antifungal activity (Schneider et al. 2018). By combining the current genomic and LC-MS data, future studies can be better equipped and guided towards identifying the active fraction(s) in the extract. Additionally, functional genomic analysis would be useful in identifying the gene cluster(s) of interest. This could involve transcriptomics searching for BGCs that are upregulated in the dark cultures compared to the light cultures or knocking out BGCs to determine which is causing the antifungal activity. The long-term steps needed to apply the antimicrobials found in this study would require structure identification via NMR spectroscopy, extensive toxicology testing as well as accessing the stability of the purified antifungals within the food matrix.

CHAPTER 6. CONCLUSION

This project's goal was to assess the antifungal potential of arctic bacterial isolates to be used in the cheese industry. Based on the data presented, there is strong evidence to suggest there are many undiscovered antifungals produced by arctic bacteria that can inhibit fungal pathogens common to the cheese industry. Additionally, based on genomic and LC-MS results, the most promising antifungal crude extracts are presumed to be novel, which could lead to a variety of applications. The number of unknown compounds found within the arctic *Streptomyces sp. nov* A28 genome highlights that bacteria from understudied polar environments could provide a valuable reservoir for novel antimicrobials with various applications.

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APPENDICES

Media recipes

1.0 YEME:

Per litre: 3g Yeast Extract, 3g Malt Extract, 5g Peptone, 10g Glucose, 340 g sucrose and fill to 1 L dH_2O.

1.1 ISP-2

Per liter: 4 g Yeast extract powder, 10 g Malt extract powder, 4 g Dextrose, 20 g Agar, and fill to 1 L dH_2O.

Recipes from (ActinoBase 2021).