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CHARACTERIZATION OF BACTERIA AND YEASTS FROM THE ANTARCTIC PENINSULA AIMING AT THE BIOTECHNOLOGICAL APPLICATION OF ANTIFREEZE PROTEINS

Florianópolis, 2024

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RESUMO

A Antártica é um dos ambientes mais inóspitos do planeta, caracterizado por temperaturas extremamente baixas, limitação de água líquida e escassez de nutrientes. Para sobreviver nessas condições, microrganismos antárticos desenvolveram adaptações específicas, como a produção de proteínas anticongelantes (AFPs), que inibem a formação de cristais de gelo e protegem as células contra os danos do congelamento. Devido a essas propriedades, as AFPs despertam grande interesse biotecnológico, com aplicações potenciais em saúde, indústria e agricultura, especialmente em criopreservação e estabilização de biomoléculas. Este estudo teve como objetivo identificar e caracterizar microrganismos antárticos produtores de AFPs, avaliando seu potencial na criopreservação celular e na estabilidade de adenovírus recombinantes. Entre os objetivos específicos, destacam-se a criação de um banco de microrganismos produtores de AFPs, testes de resistência ao congelamento a -18 °C e -80 °C, análise da atividade de inibição de recristalização de gelo, caracterização molecular e estrutural dos microrganismos resistentes ao congelamento e avaliação da eficácia de extratos proteicos na preservação celular e estabilidade viral. Para alcançar esses objetivos, foram aplicadas várias técnicas analíticas: (1) uma revisão sobre microrganismos produtores de AFPs em regiões polares; (2) isolamento e identificação de microrganismos a partir de amostras antárticas utilizando sequenciamento molecular (ITS e RNAr 16S), seguido de testes de resistência ao congelamento a -18 °C e análise da atividade de inibição de recristalização em extratos proteicos; (3) 21 isolados foram submetidos ao teste de resistência a -80 °C e caracterização morfológica, sendo quatro selecionados para estudo aprofundado: *Rhodotorula sp. C01*, *Pedobacter sp. BGS4005*, *Psychrobacter sp. P61* e *Salinibacterium sp. P45*. Proteínas intracelulares e extracelulares desses microrganismos foram testadas para criopreservação de células *E. coli* e HEK293, e em ensaios de estabilidade de adenovírus recombinantes monitorados ao longo de 60 dias. Os resultados mostram que ambientes polares possuem uma diversidade de 172 microrganismos produtores de AFPs, destacando a importância desses ecossistemas como fontes de organismos adaptados ao frio. Ao todo, foram isolados 215 microrganismos, dos quais 118 foram identificados molecularmente. Destes, 24 demonstraram resistência ao congelamento a -18 °C e 14 exibiram atividade de inibição de recristalização, com quatro apresentando ambas as características. No teste a -80 °C, *Psychrobacter sp. P53* manteve viabilidade celular após o congelamento, sendo considerado altamente resistente. Nos ensaios de criopreservação, extratos extracelulares de *Pedobacter sp.* e *Psychrobacter sp.* conferiram viabilidades de 5% e 4%, respectivamente, em *E. coli*, enquanto a AFP tipo III proporcionou 10%. Em células *HEK293*, extratos intracelulares apresentaram eficácia semelhante ao DMSO (10-15%), enquanto a AFP tipo III alcançou 50%. Na preservação de adenovírus, o extrato de *Psychrobacter sp. P61* e a AFP tipo III mantiveram a estabilidade viral após 60 dias. Conclui-se que microrganismos antárticos representam fontes promissoras de AFPs, com significativo potencial biotecnológico para criopreservação celular e estabilidade viral, reforçando o papel da Antártica como um reservatório natural de inovação biotecnológica.

Palavras-chave: Adaptação ao frio, Antártica, Biotecnologia, Crioproteção, Microrganismos, Proteínas Anticongelantes.

ABSTRACT

Antarctica is one of the most inhospitable environments on the planet, characterized by extremely low temperatures, limited liquid water, and nutrient scarcity. To survive in such extreme conditions, Antarctic microorganisms have developed specific adaptations, such as producing antifreeze proteins (AFPs), which inhibit ice crystal formation and protect cells from freezing damage. Due to these properties, AFPs have attracted significant biotechnological interest, with potential applications in health, industry, and agriculture, particularly in cryopreservation and biomolecule stabilization. This study aimed to identify and characterize Antarctic microorganisms that produce AFPs, assessing their potential in cellular cryopreservation and the stability of recombinant adenoviruses. Among the specific objectives were the creation of a microbial database of AFP producers, conducting freeze-resistance tests at -18 °C and -80 °C, analyzing ice recrystallization inhibition activity, molecular and structural characterization of freeze-resistant microorganisms, and evaluating the efficacy of protein extracts in cellular preservation and viral stability. To achieve these objectives, several analytical techniques were applied: (1) a comprehensive review of AFP-producing microorganisms in polar regions; (2) isolation and identification of microorganisms from Antarctic samples using molecular sequencing (ITS and 16S rRNA), followed by freeze-resistance tests at -18 °C and analysis of ice recrystallization inhibition in protein extracts; (3) twenty-one isolates were subjected to resistance tests at -80 °C and morphological characterization, with four selected for further study: *Rhodotorula sp. C01*, *Pedobacter sp. BGS4005*, *Psychrobacter sp. P61*, and *Salinibacterium sp. P45*. The intracellular and extracellular proteins from these microorganisms were tested in cryopreservation assays on *E. coli* and HEK293 cells, as well as in recombinant adenovirus stability assays monitored over 60 days. The results show that polar environments contain a diversity of 172 AFP-producing microorganisms, highlighting the importance of these ecosystems as sources of cold-adapted organisms. A total of 215 microorganisms were isolated, of which 118 were molecularly identified. Of these, 24 demonstrated freeze resistance at -18 °C, and 14 exhibited ice recrystallization inhibition activity, with four displaying both characteristics. In the -80 °C test, *Psychrobacter sp. P53* maintained cell viability after freezing, considered a highly resistant strain. In cryopreservation assays, extracellular extracts from *Pedobacter sp.* and *Psychrobacter sp.* conferred viabilities of 5% and 4%, respectively, in *E. coli*, while Type III AFP provided 10%. In HEK293 cells, intracellular extracts showed similar efficacy to DMSO (10-15%), while Type III AFP reached 50%. In adenovirus preservation, the extract from *Psychrobacter sp. P61* and Type III AFP maintained viral stability after 60 days. In conclusion, Antarctic microorganisms represent promising sources of AFPs, with significant biotechnological potential for cellular cryopreservation and viral stability, reinforcing Antarctica's role as a natural reservoir for biotechnological innovation.

Keywords: Cold adaptation, Biotechnology, Cryoprotection, Microorganisms, Antarctica, Antifreeze Proteins.

RESUMO EXPANDIDO

Introdução

A Antártica, um dos ambientes mais extremos do planeta, é caracterizada por baixas temperaturas, escassez de nutrientes e limitação de água líquida. Nesse contexto, microrganismos desenvolveram adaptações específicas, como a produção de proteínas anticongelantes (AFPs), que inibem a formação de cristais de gelo e protegem as células contra danos causados pelo congelamento. Essas proteínas desempenham um papel crucial na sobrevivência desses organismos e apresentam aplicações promissoras em biotecnologia, com potenciais usos na saúde, na indústria e na agricultura.

Objetivos

O principal objetivo desta pesquisa foi identificar e caracterizar microrganismos antárticos com a capacidade de produzir AFPs, avaliando a aplicação dessas proteínas na criopreservação de células e na estabilidade de adenovírus recombinantes. Os objetivos específicos incluem: (1) criar um banco de dados de microrganismos produtores de AFPs; (2) realizar testes de resistência ao congelamento a -18 °C e -80 °C; (3) analisar a atividade de inibição de recristalização do gelo; (4) caracterizar molecularmente e estruturalmente os microrganismos resistentes ao congelamento; e (5) avaliar a eficácia de extratos proteicos intracelulares e extracelulares na preservação celular e na estabilidade viral.

Metodologia

No Capítulo 1, foi realizada uma revisão abrangente da literatura sobre microrganismos produtores de AFPs em ambientes polares, enfatizando a diversidade de organismos (bactérias, fungos, algas e arqueias), a caracterização anticongelante (testes de inibição de recristalização e térmica), a distribuição espacial dos microrganismos e as aplicações biotecnológicas. O Capítulo 2 descreve o isolamento de microrganismos a partir de amostras antárticas, sua identificação por sequenciamento molecular (ITS e RNAr16S) e a avaliação da resistência ao congelamento a -18 °C, acompanhada da análise da atividade de inibição de recristalização (IR) nos extratos proteicos. No Capítulo 3, 21 isolados foram submetidos a testes de resistência a -80 °C, caracterizados morfologicamente e quatro microrganismos (*Rhodotorula* sp. C01, *Pedobacter* sp. BGS4005, *Psychrobacter* sp. P61 e *Salinibacterium* sp. P45) foram selecionados para extração de proteínas intracelulares e extracelulares. Essas proteínas foram utilizadas em testes de criopreservação em células de *E. coli* e HEK293, bem como na avaliação da estabilidade de adenovírus recombinantes, monitorados por titulação viral ao longo de 60 dias. A AFP tipo III foi utilizada tanto nos testes de criopreservação quanto nos testes de estabilidade viral.

Resultados e Discussão

No Capítulo 1, a revisão bibliográfica identificou 172 microrganismos produtores de proteínas anticongelantes (AFPs) isolados tanto da Antártica quanto do Ártico, destacando a relevância dos ecossistemas polares como fontes de organismos adaptados ao frio. Essa diversidade ressalta a importância desses ambientes como repositórios valiosos de microrganismos que desenvolveram adaptações para sobreviver a condições

extremas de congelamento. As AFPs desempenham um papel crucial na proteção celular contra o congelamento, tornando esses microrganismos de interesse não apenas do ponto de vista ecológico, mas também biotecnológico. No Capítulo 2, foram isolados 215 microrganismos a partir de amostras da Antártica, dos quais 118 foram identificados molecularmente. Dentre esses isolados, 24 demonstraram resistência ao congelamento a -18 °C e 14 apresentaram atividade de inibição de recristalização, com quatro deles exibindo simultaneamente resistência ao congelamento e atividade de inibição de recristalização. Esses achados são significativos, pois indicam que os microrganismos antárticos não apenas sobrevivem, mas também prosperam em condições de congelamento, tornando-os candidatos ideais para a produção de AFPs. A identificação dessas propriedades representa um passo importante na seleção de cepas com potencial biotecnológico. No Capítulo 3, avaliou-se a resistência dos isolados a -80 °C, sendo que dos 21 isolados testados apenas *Psychrobacter sp.* P53 manteve a viabilidade celular sob essas condições extremas. Isso demonstra a robustez e a adaptabilidade de algumas cepas bacterianas em enfrentar desafios térmicos severos. A morfologia dos isolados, revelada por Microscopia Eletrônica de Varredura (MEV), mostrou formas de cocos, cocobacilos e bacilos. Nos ensaios de criopreservação, os extratos proteicos extracelulares de *Pedobacter sp.* BGS4005 e *Psychrobacter sp.* P61 garantiram viabilidade de 5% e 4% em *E. coli*, respectivamente, enquanto a AFP tipo III proporcionou uma viabilidade de 10%. Esses resultados são promissores, pois sugerem que as AFPs podem ser eficazes na proteção celular. Em células HEK293, a eficácia dos extratos proteicos intracelulares foi comparável à do DMSO (10-15%), um agente crioprotetor convencional, enquanto a AFP tipo III ofereceu proteção de até 50%, indicando que as AFPs podem ser uma alternativa viável. Além disso, na preservação de adenovírus, tanto o extrato de *Psychrobacter sp.* P61 quanto a AFP tipo III mostraram-se eficazes na manutenção da estabilidade viral após 60 dias, o que é crucial para aplicações em virologia e no desenvolvimento de vacinas.

Considerações Finais

Este estudo demonstra o potencial dos microrganismos de ambientes polares, com destaque para a Antártica, como fontes de AFPs com aplicações promissoras em biotecnologia. A diversidade e a capacidade anticongelante dos isolados reforçam a relevância da Antártica como um reservatório natural de inovação biotecnológica, destacando o papel das AFPs na criopreservação celular e na estabilidade viral.

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IBPs – Ice Binding Proteins

AFPs – Antifreeze Proteins

INPs- Ice Nucleating Proteins

IR – Inhibition Recrystallization

TH - Thermal Hysteresis

SUMÁRIO

1. INTRODUCTION

Antarctica is recognized as one of the most extreme environments on the planet, characterized by extremely low temperatures and harsh seasonal conditions (Scambos *et al.*, 2018; Turner *et al.*, 2021; Wei; Yan; Ding, 2019). In addition, this ecosystem exhibits low nutrient levels, frequent exposure to freezing, and limited availability of liquid water (Convey; Peck, 2019; Kim *et al.*, 2019; Pearce, 2017). Despite these challenging conditions, a diverse range of microorganisms demonstrates a remarkable ability to survive and thrive, adapting to abrupt temperature fluctuations, dehydration, and physical changes in the state of water (Beall, 1983). Among the adaptations developed by these microorganisms to survive extreme cold are metabolic efficiency at low temperatures, maintenance of plasma membrane integrity, and the preservation of essential biomolecules (Christner, 2010; Lopes *et al.*, 2024b; Vick-Majors; Singh; Singh, 2024). Additionally, many of them produce cryoprotective substances, such as antifreeze proteins (AFPs), which play a crucial role in protecting against cold-induced cellular damage (Chattopadhyay, 2006; Russell, 1997).

Antifreeze proteins possess two essential physicochemical properties: recrystallization inhibition (RI) activity and thermal hysteresis (TH) activity (Christner, 2010). During the recrystallization process, water molecules migrate from smaller ice crystals to larger ones, resulting in a more stable form of ice but with a greater capacity to damage the cells of organisms. Recrystallization inhibition activity, in turn, refers to the ability of AFPs to prevent the growth of large ice crystals, maintaining the formation of smaller crystals, thereby protecting cells (Knight; Duman, 1986).

Furthermore, AFPs reduce the freezing point of water in a non-colligative manner, that is, without affecting its melting point (Barrett, 2001). The difference between the freezing point and the melting point of a substance is known as thermal hysteresis. AFPs play an important role in modulating this thermal hysteresis, contributing to the survival of organisms in extreme cold conditions where constant freezing and thawing can be fatal. Evaluating the thermal hysteresis activity of AFPs is a fundamental approach to studying the efficacy of these proteins as antifreeze agents (Guo *et al*., 2012).

AFPs were initially discovered in the blood plasma of Antarctic fish inhabiting cold waters (Devries; Komatsu; Feeney, 1970), but they are also present in plants (Griffith *et al.*, 1992), insects (Wu *et al*., 1991), and a variety of microorganisms, including algae, fungi, bacteria, and archaea (Batista *et al.*, 2020; Desai *et al.*, 2020; Liao *et al.*, 2021; Raymond;

Remias, 2019). These proteins not only play a crucial role in the survival of these organisms in extremely cold environments but also have significant biotechnological potential, with antifreezing applications in health, agriculture, and industry (Naing; Kim, 2019; Sreter; Foxall; Varga, 2022).

In the health sector, AFPs can be used to increase the viability of organs for transplantation, preserving them for longer periods (Tas *et al*., 2021; Amir *et al*., 2004). Moreover, these proteins can be applied in medications and vaccines stored at 4 °C, preserving efficiency and viral stability (Arpagaus, 2023; Brandau *et al*., 2003; Rexroad *et al*., 2002). They are also useful in the cryopreservation of eukaryotic and prokaryotic cells, embryos, and sperm (Correia *et al*., 2024; Kawahara *et al*., 2009; Sreter; Foxall; Varga, 2022). In agriculture, genetically modified plants expressing AFP genes may acquire greater cold tolerance, which is valuable for regions prone to frost (Cho *et al.*, 2019). In the food industry, AFPs can enhance the quality and shelf life of frozen foods (Xiao *et al*., 2024), while in industrial applications, they can be applied to metal surfaces and pavements to prevent freezing (Gwak *et al*., 2015; Meng *et al*., 2024). Thus, the use of AFPs can help address issues associated with freezing, such as cell viability after thawing, the toxicity of cryoprotectants, toxic antifreeze substances for the environment, loss of efficacy in medications and vaccines, reduced quality of frozen foods, and crop damage due to frost (Gan *et al.*, 2022; Griffiths *et al.*, 1979; Verheijen *et al.*, 2019).

Given this context, our study tested three main hypotheses: first, that Antarctic microorganisms have the potential to produce cryoprotective substances, including AFPs, as a survival strategy during freeze-thaw cycles; second, that protein extracts from these microorganisms can exert a cryoprotective effect on eukaryotic and prokaryotic cells subjected to freezing at -18 °C; and third, that these protein extracts can contribute to the stability of recombinant adenovirus after 60 days of storage at 4 °C. The overall objective of the study was to identify and characterize Antarctic microorganisms with the potential to produce antifreeze proteins (AFPs) and evaluate their effects on cell cryopreservation and the stability of recombinant adenoviruses.

The thesis is structured in three chapters. Chapter 1 presents a literature review on AFP-producing microorganisms, including bacteria, archaea, algae, and fungi. Chapter 2 addresses the isolation and characterization of Antarctic microorganisms, assessing their survival under freezing at -18 °C and their ability to inhibit ice recrystallization. Chapter 3 explores the morphological characterization of isolates by electron microscopy, as well as evaluating their

resistance to freezing at -80 °C and the effect of their protein extracts on cell cryopreservation at -18 °C and viral stability at 4 °C after 60 days.

2. GOALS

2.1 GENERAL GOAL

To identify and characterize Antarctic microorganisms with the potential to produce antifreeze proteins (AFPs) and evaluate their effects on the cryopreservation of cells and recombinant adenoviruses.

2.2 SPECIFIC GOALS

a) To review and catalog microorganisms producing antifreeze proteins through a database and describe their potential for biotechnological application studies;

b) To evaluate the freeze resistance of Antarctic isolates at temperatures of -18 ºC and -80 ºC;

c) Evaluate the potential of microbial protein extracts in Ice Recrystallization Inhibition Activity;

d) To identify and characterize the freeze-resistant microorganisms using molecular biology methods and electron microscopy;

e) Evaluate the potential of proteins produced by Antarctic microorganisms in the cryopreservation of cells and the maintenance of viral stability.

3. CHAPTER I

FROST FIGHTERS: UNVEILING THE POTENTIALS OF MICROBIAL ANTIFREEZE PROTEINS IN BIOTECH INNOVATION

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ABSTRACT

Polar environments pose extreme challenges for life due to low temperatures, limited water, high radiation, and frozen landscapes. Despite these harsh conditions, numerous macro and microorganisms have developed adaptive strategies to reduce the detrimental effects of extreme cold. A primary survival tactic involves avoiding or tolerating intra and extracellular freezing. Many organisms achieve this by maintaining a supercooled state by producing small organic compounds like sugars, glycerol, and amino acids, or through increasing solute concentration. Another approach is the synthesis of ice-binding proteins (IBPs), specifically antifreeze proteins (AFPs), which hinder ice crystal growth below the melting point. This adaptation is crucial for preventing intracellular ice formation, which could be lethal, and ensuring the presence of liquid water around cells. AFPs have independently evolved in different species, exhibiting distinct thermal hysteresis and ice structuring properties. Beyond their ecological role, AFPs have garnered significant attention in biotechnology for potential applications in the food, agriculture, and pharmaceutical industries. This review aims to offer a thorough insight into the activity and impacts of AFPs on water, examining their significance in cold-adapted organisms and exploring the diversity of microbial AFPs. Using a meta-analysis from cultivation-based and cultivation-independent data, we evaluate the correlation between AFPs-producing microorganisms and cold environments. We also explore small and large-scale biotechnological applications of AFPs, providing a perspective for future research.

Keywords: *antifreeze proteins*, *Antarctica*, *Arctic*, *psychrophile*, *cryoprotection*

3.1 INTRODUCTION

Polar environments comprise a series of extreme physicochemical conditions for living organisms, including low temperatures, limited liquid water availability, long periods of high incident radiation, and permanently frozen areas (Duarte *et al.* 2012). Despite these stressors, many macro and microorganisms have evolved methods to cope with the harmful effects of extreme cold. One of the main strategies to survive subzero conditions is to avoid (or at least tolerate) intra and extracellular freezing. Many organisms prevent freezing by maintaining internal and surrounding water as a supercooled solution by producing small organic compounds (e.g., sugars, glycerol, and amino acids), by increasing solute concentration, or more efficiently by the production of special cryoprotective proteins (Baskaran *et al.* 2021). Proteins that interact with ice by adsorption to particular ice crystals are called ice-binding proteins (IBPs). Within this group, some proteins have been shown to inhibit ice crystals from growing (antifreeze proteins – AFPs), while others are capable of triggering the formation of new crystals at temperatures higher than the regular freezing point (ice-nucleating proteins – INPs) (Roeters *et al.* 2021; Soveizi *et al.* 2023).

Antifreeze proteins (AFPs), also known as thermal hysteresis proteins (THPs) and ice structuring proteins (ISPs), are a class of polypeptides found in cold-adapted organisms that bind into ice surface and inhibit the growth of ice crystals below the melting point (Ramløv and Friis 2020). Initially discovered in Antarctic notothenioid fishes (DeVries and Wohlschlag 1969), many cold-adapted organisms have been identified as AFP producers in the last decades, including bacteria, fungi, insects, and plants. The production of AFP has evolved as an efficient adaptation to survive in extremely cold environments since intracellular ice formation could lead to lethal damage to cells and tissues. Also, the production of AFP at the cell surface or close to its vicinity is an effective means to ensure liquid water around cells. Among other freezing adaptations, the production of AFP is considered one of the main strategies that cold-adapted organisms have developed to thrive in these ecosystems where freezing water can occur. Interestingly, the origin and evolution of AFP occurred convergently in different species, each one presenting distinct properties, such as their efficiency in inhibiting ice growth, the binding mechanism to the ice surface, and the effect of lowering the freezing temperature below the melting point (Davies 2022). From a biotechnological point of view, these proteins have attracted significant scientific interest over the years due to their potential applications in cryopreservation, such as in food, agriculture, and pharmaceutical industries.

This review aims to provide a comprehensive understanding of the AFP activity and effects on water, their role in cold-adapted organisms, and the diversity of microbial AFPs. We also used a meta-analysis approach of cultivation-based and cultivation-independent microorganisms to evaluate if AFPs are associated with different cold environments. Finally, we discuss their small- and large-scale biotechnological applications, offering a prospect for future research in this area.

3.2 AFP ACTIVITY AND EFFECTS ON ICE CRYSTAL GROWTH

Cooling water below the freezing point (0 °C) at atmospheric pressure does not freeze it spontaneously. Instead, water remains in a special liquid state called “supercooled”, i.e., a liquid below its equilibrium melting temperature. Thermodynamically, a supercooled liquid is an unstable system and will eventually freeze into its respective crystal by a two-stage mechanism known as *nucleation* and *growth* (Haji-Akbari 2020). Density fluctuations in the supercooled state cause water molecules to cluster and form an ice nucleus, starting the first stage of the freezing process known as *nucleation*. The nucleation process could occur either in

pure water (homogeneous) or in the vicinity of an interface (heterogeneous). Heterogeneous nucleation is the most prevalent form and involves fewer water molecules and little free energy compared to homogeneous nucleation (Margaritis and Bassi 1991). In either case, as the crystalline nucleus is formed, the thermodynamic free energy increases until it reaches a limit value, which acts as a free-energy barrier for nucleation (**Figure Chapter I 1**). At this point, the ice nucleus reaches a critical (maximum) size in the nucleation stage (Zhang and Liu 2018). Further increments in nucleus size (crystal surface area and volume) by surrounding water molecules will trigger the second stage of crystallization known as *growth* (Haji-Akbari 2020). In the growth stage, the free energy decreases as the ice nucleus expands into a macroscopic crystal (Eickhoff *et al.* 2019).

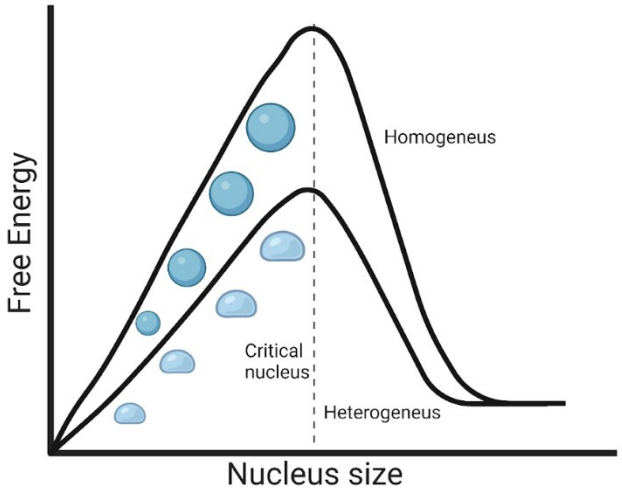

Figure Chapter I 1 - Schematic representation of the freezing nucleation dynamics: homogeneous nucleation and heterogeneous nucleation.

In general, organic and inorganic materials present in water may interact with water molecules and either increase or decrease the growth rate of ice nuclei. In this context, AFPs attach to ice surfaces and restrain their growth rate, lowering the water freezing temperature. On the other hand, INPs trigger ice growth by serving as a template for ice crystals.

Physicochemical and molecular studies on fish and insect AFPs have extensively characterized their antifreeze mechanism (Duman 2015; Duman and Newton 2020; DeVries 2020). The most well-documented observations are the AFP properties of inhibition of recrystallization, the thermal hysteresis, the ice-binding mechanism, and the protein concentration effect. AFPs obtained from various species have demonstrated varying outcomes in these characteristics, making them a common benchmark for assessing their effectiveness.

3.3 INHIBITION RECRYSTALLIZATION OF ICE (IR)

One of the most studied properties of AFP is the inhibition of ice recrystallization (IR) just below the melting temperature (Knight, Hallett, and DeVries 1988; Gruneberg *et al.* 2021). Recrystallization is a phenomenon by which water molecules migrate from small ice crystals to large crystals, resulting in a more stable (and dangerous) form of ice. This phenomenon has important effects on cell survival and is directly related to cooling and warming rates (Mazur 1984; Deller *et al.* 2014). Experimental observations of ice formation indicate that a high cooling rate leads to the creation of very small ice crystals. Conversely, applying high warming rates to these small crystals results in complete melting with minimal cell damage. However, subjecting small crystals to slow warming rates leads to their growth in size, contributing to a decrease in cell survival (Chang and Zhao 2021). This phenomenon, known as the recrystallization effect, involves the migration of water molecules from smaller to larger crystals, ultimately resulting in the formation of larger ice crystals over an extended period.

The recrystallization process is characterized by the melting of small crystals, the growth of large crystals, and the fusion of many crystals together (**Figure Chapter I 2**). This fusion reduces the overall number of crystals, producing fewer but larger crystals within a given ice phase volume. Additionally, a rounding process is observed, wherein crystals with initially rough surfaces become rounder through a thermodynamic ripening process. Notably, these recrystallization processes are particularly enhanced when temperature fluctuations occur, even though the primary observations take place at a constant temperature (Hartel 1998).

The IR activity is explained as the property of a molecule in preventing the formation of large ice crystals at the expense of small crystals, that is, an AFP with IR activity can inhibit the growth of large crystals, protecting the cell against ice damage (Knight and Duman 1986; Budke and Koop 2020). Experimental determination of IR is generally measured by looking at the behavior of ice crystals over time at temperatures below the melting point. This is usually

done in a phase contrast microscope adapted to work in cold chambers below 0 °C (Griffith and Yaish 2004) or attached to a cooling stage. The classic approach to observing ice crystals in the microscope was developed by Knight *et al.* (Knight and Duman 1986; Knight, Hallett, and DeVries 1988) and is known as the "splat cooling" method. Despite several modifications introduced since then (Tomczak *et al.* 2003; Graham *et al.* 2018; Biggs *et al.* 2019), the splat cooling method is still applied to investigate the IR activity of AFP and other ice inhibition molecules. The method consists of the preparation of a thin solution film between two glass slides (e.g., 300 μm thick film of 5–30 μL) and submitting this "sandwich" to subzero temperatures at rapid cooling rates (>10 °C.min^{-1}). At this cooling rate, ice nucleation will also occur at high rates, leading to the formation of small ice crystals. The temperature where the nucleation occurs depends on the molecules present in the water film but usually is within the range between -6 °C (Kawahara *et al.* 2007; Singh *et al.* 2014a; Xiao *et al.* 2010; Shah *et al.* 2012) and -8 °C (Mangiagalli *et al.* 2018). After the nucleation stage, the solution is heated to start the growth stage, where the ice crystals increase in size and the system's free energy decreases. Usually, this temperature is about -4 °C (Vance, Graham, and Davies 2018; Uhlig *et al.* 2011; Bayer-Giraldi *et al.* 2011) and the system is maintained under this condition for 24 h – a period called "annealing". During the annealing time, more AFP molecules attach to the crystal border, further restricting the overgrowth of ice fronts (Raymond and Devriesf 1977; Takamichi *et al.* 2007; Knight and DeVries 1994). Afterward, microphotographs are taken, and ice crystal parameters are analyzed, such as their abundance (number of crystals), size (diameter or length), and distribution (spatial organization) (Deller *et al.* 2014; Deller *et al.* 2018). The splat assay and its modifications are able to compare samples of putative AFP against a control solution, as well as the effect of different concentrations of the AFP on IR activity. It is important to note that the rate of ice recrystallization depends on solutes other than AFPs (Knight, Wen, and Laursen 1995; Smallwood *et al.* 1999), therefore, many IR experiments are usually carried out at high solute concentrations to diminish nonspecific effects (Griffith *et al.* 2005).

An alternative approach for IR analysis is the high-throughput AFP protocol (HTAP) designed by Gilbert *et al.* (2004), and further modified by Cid *et al.* (2016). Unlike the splat assay, this method employs a spectrophotometer to analyze the IR activity. The theory behind the HTAP is that small ice crystals (such as those formed when AFP is present) are visually more turbid, while larger crystals exhibit higher transparency (Cid *et al.* 2016). HTAP consists of preparing a 96-well microtiter plate containing a negative control/blank solution (e.g., 30%

sucrose), a positive control solution (e.g., AFP Type III 1 mg.mL^{-1} in 30% sucrose), and protein extracts (in 30% sucrose solution). The plate is frozen at ultra-low temperatures (-70 °C or -80 °C) for 10-15 min and then placed on a cooling stage at -6 °C. The plate is set to annealing at this temperature from hours to several days when crystals stabilize due to ice recrystallization. After this period, the microtiter plate is placed in a spectrophotometer and the absorbance of each solution is read at λ of 500 nm (Cid *et al.* 2016).

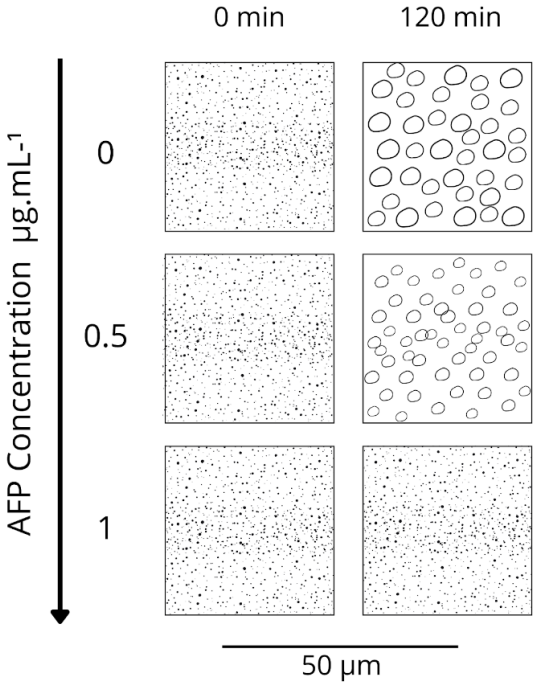

Figure Chapter I 2 - Representation of ice recrystallization as a spontaneous process in which water molecules migrate from small ice crystals to larger ice crystals, while temperature is held near the melting point. The scheme represents the IR activity of different concentrations of AFP (0.5 and 1 µg.mL^{-1}), before (0 min) and after (120 min) the annealing time at -8 °C. IR results in an enlargement of the average crystal size, accompanied by a decrease in the overall number of crystals over time. When AFP binds to the crystal border, ice growth is restricted, increasing the IR effect (Budke *et al.* , 2009; Nagel *et al.* , 2011).

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3.4 THERMAL HYSTERESIS (TH)

Along with the IR activity, thermal hysteresis (TH) is a well-studied property of AFPs. Thermal hysteresis is the phenomenon in which the freezing (T_f) and melting temperature (T_m) of a substance assume different values. In the case of pure water at atmospheric pressure, ice crystals form at 0 °C (nucleation stage) and grow at temperatures below this freezing point. Therefore, the T_f and T_m of pure water are both 0 °C. However, in the presence of molecules with TH activity such as AFPs, ice crystals stop growing even if the temperature is lowered several degrees below the freezing point, remaining in a supercooled state as previously

explained. The AFPs “hold” the crystal growth until a critical subzero temperature is reached ($T_{f\text{-}AFP}$), and a subsequent decrease in the temperature will cause the crystals to grow almost instantaneously. The difference between the T_m and $T_{f\text{-}AFP}$ is referred to as the hysteresis gap, or simply the TH value expressed as degrees Celsius. In simpler terms, AFPs lower the freezing point of water without changing the melting point of ice (Barrett 2001). For instance, AFP type III exhibits a thermal hysteresis of 1.6 ºC at a concentration of 1 mM when water is present (Can and Holland 2013).

AFPs with high TH activity play an important role in organisms inhabiting extremely cold environments. Assessing the TH activity of an AFP protein is a classic way of studying the antifreeze efficiency of these proteins (Guo *et al.* 2012). The standard method for measuring AFP's TH activity uses a cold stage coupled to a microscope with video output, where a single crystal is formed and the freezing point is determined with image analysis (Kubota 2011; Braslavsky and Drori 2013). The cooling stage is simply a sample holder or adapted microscope stage with temperature control, where the temperature is set with a Peltier element (thermoelectric component), liquid nitrogen, or a combination of the two. Just like the IR splat cooling method, conducting TH (thermal hysteresis) measurements involves swiftly cooling the sample, typically to around -40 °C. This rapid cooling process is necessary to produce a variety of ice crystals with diverse sizes, shapes, and orientations. Next, the temperature is slowly increased towards the T_m, until a single ice crystal is observed in the microscope. The sample is then kept at this temperature, which is considered the exact T_m value until the temperature is lowered at low rates and the behavior of the ice crystal is monitored by either visual inspection in the microscope or by a video recorder. The ice crystal remains stable within the hysteresis gap, but will instantaneously grow when the temperature reaches the $T_{f\text{-}AFP}$. The $T_{f\text{-}AFP}$ value is used along with T_m to calculate the TH. This method has been used for decades and many variations were introduced to enhance temperature control (Hashim *et al.* 2013, 2014; Singh *et al.* 2013; Lee *et al.* 2010; Xiao *et al.* 2010; Hoshino *et al.* 2003), sample loading, and image recording (Braslavsky and Drori 2013).

An alternative method for measuring TH activity is using different scanning calorimetry (DSC), originally developed by Hansen and Baust (1988) and later improved by many other authors (Ramløv, Devries, and Wilson 2005; Ding *et al.* 2014; Chen *et al.* 2021; Liu *et al.* 2023; Hassas-Roudsari and Goff 2012; Baier-Schenk, Handschin, and Conde-Petit 2005). The DSC is a technique that measures the thermodynamic properties (i.e., heat flow rate) of a material by comparing the energy change between the sample and a reference. It is one of the

most used methods for studying the enthalpy associated with the process of interest, such as the transition from liquid water to ice. In practice, the DSC promotes a temperature-controlled environment on two sample holders (one containing the sample, the other containing the control or reference) and measures the heat flow difference between both sample holders by performing a temperature scan. For the TH analysis, a sample containing the AFP (e.g., 1 $mg.mL^{-1}$ in 50 mM PBS buffer) is placed inside the sample holder, along with an empty sample holder as reference. After inserting the sample and reference in the DSC cells, two freeze-melt cycles are performed. First, the samples are cooled at a rate of 1 $°C.min^{-1}$ until completely frozen, such as -30 °C or -40 °C (T_f), and held for 3-5 minutes at this temperature for ice crystal stabilization. After that, the sample is heated at the rate of 1 $°C.min^{-1}$ until all crystals melt. The temperature where melting occurs is noted as T_m. Then, for the second cycle, samples are frozen again to -30 or -40 °C but using a higher cooling rate, such as 10 $°C.min^{-1}$. A rapid cooling rate creates very small ice crystals, which are stabilized again by holding at T_m for 3-5 minutes. Samples are then heated at a low rate of 0.1 $°C.min^{-1}$ until close to the T_m of the first cycle and held at this temperature for 5 minutes. If a microscope is attached to the DSC cold stage, a single ice crystal should be observed at this point. This hold temperature (T_{hold}) is recorded for the TH calculation. After that, samples are cooled up to complete freezing at a rate of 1 $°C.min^{-1}$. At this point, samples containing pure water will show an increase in crystal size as soon as the cooling starts. On the other hand, samples with AFP will be stable during the hysteresis gap, since the proteins will hold water at a supercooled state until the enthalpy of fusion is released as a "burst" exothermic event when the crystal grows instantaneously. The DSC thermogram will show a straight semi-horizontal line during the hysteresis gap and a heat flow peak during the exothermic event. With these data, the onset temperature (T_{onset}) is determined as the temperature that starts the exothermic event. Finally, the TH is calculated by the difference between T_{hold} and T_{onset} (Hansen and Baust 1988; Ding *et al.* 2014). Despite not using a microscope to visualize ice crystal growth, the DSC provides high-resolution data about the ice-liquid transition. This is an advantage compared to the cold stage/microscopy method because TH determination with DSC is not biased by visual interpretation.

AFPs are classified as moderate and hyperactive, based on the TH activity they present in equimolar concentrations (Mangiagalli *et al.* 2017). In quantitative terms, moderate AFPs (e.g., fish AFP Type III) present a TH activity of up to 1 °C in millimolar concentrations, while hyperactive AFPs (e.g., insect *Tm*AFP) produce higher TH in only micromolar concentrations (Scotter *et al.* 2006; Celik *et al.* 2010). Interestingly, the ice-binding mechanisms of moderate

and hyperactive AFPs are also different: moderate AFPs bind to prism and/or bipyramidal planes, while hyperactive AFPs bind to multiple crystal planes and eventually cover the ice surface (Drori *et al.* 2014; Pertaya *et al.* 2008; Knight, Cheng, and DeVries 1991; Park *et al.* 2012; Vance, Graham, and Davies 2018).

3.5 MICROBIAL AFP: DIVERSITY, EVOLUTION AND ANTIFREEZE ACTIVITIES

AFPs are widely distributed among microorganisms, most of them living in high latitudes or altitudes. The first report on AFP in microorganisms was published by Duman and Olsen (1993), and since then several other studies explored microbial AFPs in terms of their physicochemical properties, water binding effects, protein structure, genetic code, diversity, and applications. A summary of all AFP-producing microorganisms dated until 2023 can be found in the **Supplementary Table S1** of this review.

3.5.1 AFP in Prokaryotes

Since their origin, about 4 billion years ago, both bacteria and archaea evolved on a cold planet (Gilichinsky, Wagener, and Vishnevetskaya 1995). Most of Earth's natural environments are under low temperatures, including two-thirds of the ocean at ~2 °C and semi-permanently frozen areas such as high-altitude mountains and polar regions. Considering temperature is one of the most important environmental parameters that regulate microbial activity and surveillance, it is no surprise that bacteria and archaea developed freeze-tolerance mechanisms during their evolution, including AFPs. Cold-adapted bacteria were first described in the 19th century by Forster (1887), who isolated bioluminescent bacteria from frozen fishes. These isolates were capable of growing at or near 0 °C, although their ability to produce antifreeze proteins (AFPs) was not initially understood. Nonetheless, this study shed light on microbial life at low temperatures and how they are adapted to permanent or semi-permanent frozen environments, such as the permafrost and glacial ice. For example, a bacterial strain isolated from Lake Vostok from samples of glacial ice at a depth of 3,519 m was identified for producing a 54 kDa AFP with recrystallization-inhibiting activity (Raymond, Christner, and Schuster 2008). The total RNA of the isolate was analyzed by reverse transcription-PCR, indicating constitutive expression of the IBP gene. This could explain how these microorganisms survive

in these frozen environments, since low but constant production of AFPs could control the ice crystals within the liquid layers of Antarctic ice for thousands of years (Achberger *et al.* 2011).

The first TH activity in bacteria was found in *Rhodococcus erythropolis*, isolated from the intestinal tract of beetle larvae, and *Micrococcus cryophilus*, isolated from frozen food (Duman and Olsen 1993). Later on, a cold-adapted strain of *Pseudomonas putida* was isolated from Canadian High Arctic soil and studied as a potential crop inoculant (Sun *et al.* 1995). While trying to evaluate their growth-promoting effects on plants, which was indeed shown for the strain, the authors also discovered that *P. putida* survived at -50 °C. This survival was associated with the production and secretion of a 164 kDa AFP into the medium (Sun *et al.* 1995; Xu *et al.* 1998). Further characterization of *P. putida*'s AFP showed that a 32-34 kDa breakdown product of the protein had ice-nucleating activity at -10 °C (Muryoi *et al.* 2004), while the entire protein showed TH activity. This curious combination of antifreeze and ice nucleation activity in the same protein suggests that *P. putida* could both induce and prevent ice crystals growth, enhancing its survival during temperature fluctuations. Later, other bacteria have been discovered to produce proteins with both thermal hysteresis and ice nucleating activities, such as *Pseudomonas fluorescens* KUAF-68 isolated from Antarctica (Kawahara *et al.* 2004).

Some bacterial AFP have been identified with both IR and TH activity simultaneously. For example, studies with *Flavobacterium xanthum* revealed the production of an intracellular AFP of 59 kDa with high TH activity (1.19 °C at 0.00086 $mg.mL^{-1}$) and IR activity (at 0.20 $mg.mL^{-1}$) (Kawahara *et al.* 2007). Do *et al.* (2014) investigated an antifreeze protein (FfIBP) encoded by another Flavobacterium, the PS1 Antarctic strain of *F. frigoris*. At the relatively low concentration of 0.12 $mg.mL^{-1}$, FfIBP demonstrated not only IR activity but also a 2.2 °C TH activity. The FfIBP is an example of an antifreeze protein with both IR and TH activities greater than those previously described for Flavobacterium and most other bacteria. For comparison, SfIBP_1 is a 25 kDa AFP from *Shewanella frigidimarina* that also inhibits ice recrystallization and has a 2 °C thermal hysteresis activity, but these effects were measured only at 2 $mg.mL^{-1}$ (Vance, Graham, and Davies 2018). A *Pseudomonas ficuserectae* isolated from Arctic cryoconite holes could efficiently inhibit ice recrystallization at low concentrations (0.05 $mg.mL^{-1}$), but the 2 °C TH activity was found only at higher concentrations of 2 $mg.mL^{-1}$ (Singh *et al.* 2014a).

The origin and evolution of AFPs is still unclear, particularly within the prokaryotes. While there is evidence for both convergent evolution (Bayer-Giraldi *et al.* 2010) and horizontal gene transfers (Sorhannus 2011) in eukaryotes, bacterial AFPs are just being explored in evolutionary terms. In 2020, the first report of a cyanobacterium producing AFP was also responsible for a brief insight on the evolution of bacterial AFPs. The genome of the cyanobacterium *Nostoc* sp. HG1 was sequenced and revealed 116 genes related to cold resistance, including an AFP called nIBP with the 3494 domain (Raymond, Janech, and Mangiagalli 2021). Several proteins containing this "domain of unknown function" (DUF) 3494 were classified as IBPs due to their capacity to bind into ice crystals (Vance *et al.* 2019). The DUF3494 was found in different organisms, from bacteria to yeasts and algae, being one of the most widespread IBP families known today, and considered an evidence of horizontal gene transfer among microorganisms. In agreement with this hypothesis, recent analysis of the nIBP amino acid sequence from *Nostoc* sp. HG1 suggested that this cyanobacterium acquired the AFP gene from the planctomycete *Singulisphaera* sp., which share 69% and 72% of similarity of amino acid and nucleotide sequences, respectively (Raymond, Janech, and Mangiagalli 2021).

Antarctica has been the focus of several studies on bacterial AFP. The extreme cold conditions of terrestrial and marine Antarctic ecosystems impose selection factors for freezing tolerant strains, making the continent an important source of AFP-producing bacteria. The first AFP studied from an Antarctic microorganism was a 52 kDa lipoprotein of *Moraxella* sp. (Yamashita *et al.* 2002). The AFP's N-terminal amino acid sequencing demonstrated high similarity with the membrane proteins of *Branhamella catarrhalis*, suggesting the *Moraxella* sp. protection against freezing occurs by inhibiting the ice formation on the cell surface. Two years after the first discovery, Gilbert *et al.* (2004) screened 866 bacterial isolates from Antarctic lakes, and found 11 isolates with IR activity at different protein concentrations. One of these isolates, identified as *Marinomonas primoryensis*, revealed a TH activity of 0.8 °C for the crude protein extract at a concentration of 11 $mg.mL^{-1}$ (Gilbert, Davies, and Laybourn-Parry 2005). In 2008, researchers purified and sequenced the 322-amino acid sequence of this *M. primoryensis* AFP, revealing it is a Ca^{2+} dependent AFP with about 2 °C depression on freezing point at concentrations of 0.5 $mg.mL^{-1}$, which classifies this protein as a hyperactive AFP (Garnham *et al.* 2008). Moreover, this AFP was characterized as an adhesin with a size of 1.5-MDa containing two important regions: region II, which has a role in ice binding; and region IV, which presents the antifreeze activity (Guo *et al.* 2012, 2013).

After the pivotal work by Gilbert *et al.* (2004), many other authors used Antarctica as a source for bioprospecting bacterial antifreeze proteins. In studies conducted by Cid *et al.* (2016), it was observed that 32 bacterial isolates obtained from the *Deschampsia antarctica* phyllosphere exhibited IR Activity (0.5 $mg.mL^{-1}$) when cultured under cold acclimation conditions, whereas only 5 of these isolates showed such activity among cultures that were not acclimated to the cold. Similarly, Muñoz *et al.* (2017) investigated microorganisms sourced from various locations in Antarctica to evaluate the thermal hysteresis of their crude extracts. Notably, isolates identified as *Sphingomonas* sp. GU1.7.1, *Plantibacter* sp. GU3.1.1, and *Pseudomonas* sp. AFP5.1 demonstrated significantly higher TH activity, measuring at 0.50, 0.46, and 0.41 ºC, respectively. Additionally, Moreira *et al.* (2022) explored the freeze resistance of non-virulent bacteria isolated from Antarctic waterlogged soils. Among the 65 isolates tested for antifreeze potential, 31 showed significant antifreeze activity, being one of the most successful screening for AFP-producing bacteria.

More recently, genomic and metagenomic approaches have been used to explore antifreeze proteins from bacteria. Searching for genes encoding AFPs has proven to be successful for several bacteria, as the example mentioned above on *Nostoc* sp. HG1 (Raymond, Janech, and Mangiagalli 2021). Genomes from psychrophilic bacteria are considered ideal targets for such methods, as genomic databases have grown exponentially in the last decades with data from novel species. As an example, the psychrophilic marine bacterium identified as *Paenisporosarcina antarctica* CGMCC 1.6503 has been isolated from King George Island in Antarctica and the genomic analysis revealed the presence of a gene (E2636_15475) related to the coding of an AFP (Rong *et al.* 2020). In addition to isolates, metagenomic studies have been carried out to explore the potential of antifreeze proteins from bacterial communities, especially those inhabiting Antarctic environments. (Raymond 2016) studied epiphytic bacteria from *Bryum argenteum* moss using a metagenomic approach. The results revealed the presence of bacterial genes (from Actinobacteria and Bacteroidetes populations) encoding the already mentioned DUF3494 domain, suggesting the role of epiphytic bacteria in increasing freeze resistance to the moss host. As an additional example, metagenomics was used to study the psychrophilic free-swimming ciliate *Euplotes focardii* and the symbiont bacteria, found in -1.8 °C marine water of the Terra Nova Bay, Antarctica. Although the symbionts were not grown as pure cultures, the metagenomic approach could unveil the coding sequence for an AFP (EfcIBP) within the symbiont genome (Pucciarelli *et al.* 2014). Not surprisingly, the EfcIBP gene codes for a protein that contains the DUF3494 domain (Mangiagalli *et al.* 2017). Further

characterization of EfcIBP revealed the expressed protein has a TH activity of 0.53 °C at 50 μM, and IR activity at a concentration of 2.5 nM, making it one of the most potent ice recrystallization inhibition agents described (Kaleda *et al.* 2019).

Despite several reports of AFP in bacterial isolates and genomes, limited attention was given to Archaea. To date, only one report of AFP-producing Archaea has been documented in the literature. Desai *et al.* (2020) explored the ability of haloarchaea (archaea adapted to high salt concentrations) to produce industrially important biomolecules, like proteases, amylases, and surfactants. Using a semiquantitative method to detect AFP on culture supernatant, the protein extract of several *Halobacterium* sp. strains showed antifreeze activity.

3.5.2 AFP in Eukaryotes

Eukaryotic organisms, from single-celled algae to complex multicellular life forms, also possess AFPs crucial for survival in cold environments. While molecular studies on fish and insect AFPs have provided insights into their mechanisms, the scope of AFP functionality widens considerably when considering other eukaryotic organisms. Despite AFPs being first reported in Antarctic fishes and later explored in many insects and plants, this review will limit the discussion on microbial eukaryotes that produce AFPs, such as filamentous fungi, yeasts, and microalgae.

The first report of filamentous fungi with antifreeze activity was in the early 1990's from soil samples collected during the winter season in South Bend, Indiana, United States (Duman and Olsen 1993). Isolates of *Flammulina velupites*, *Pleurotus ostreatus*, *Coriolus versicolor,* and *Stereum* sp., along with a couple of bacterial isolates, presented TH activity within the range of 0.3-0.35 °C. Interestingly, this pioneering work showed that fungi isolates were able to express TH activity only when field-sampled in winter, while the bacteria (*Rhodococcus* and *Micrococcus*) needed a 3-4 weeks acclimation at 3 °C for their thermal hysteresis.

Despite this initial study, investigations into fungal AFPs have progressed at a slower pace. Reports on the biochemical and physiological characterization of fungal AFPs emerged almost a decade later (Snider *et al.* 2000; Doucet *et al.* 2000; Hoshino *et al.* 2003). Doucet *et al.* (2000) investigated ice recrystallization (IR) activity in lichen species from maritime Antarctica, identifying five species with IR activity. While these authors did not work with pure

mycobiont cultures, the lichen extracts were able to inhibit ice recrystallization at concentrations between 0.00011 and 0.006 mg.mL^{-1}, which suggests the presence of highly active AFPs. Hoshino *et al.* (2003) investigated AFP production in fungi isolated from Arctic regions, identifying *Coprinus psychromorbidus* (LAFP-1 to LAFP-3, 25 kDa) and *Typhula ishikariensis* (TAFP-1 to TAFP-4, 22 kDa) as AFP producers.

Antarctic ecosystems are among the most important sources of fungi screened for AFP. During the last couple of decades, many authors have explored soils, snow, mosses, and algal mats for the isolation of fungi with AFP activity. (Kawahara, Takemura, and Obata 2009) explored AFP production in fungi (Ascomycota) isolated from Antarctic soils, singling out *Penicillium bilaiae* for its significant antifreeze activity, resistant even to protein and heat treatments. Later, Xiao *et al.* (2010a) isolated various strains from soils, mosses, and algal mats near the Chinese Station "Great Wall" on King George Island. While all fungal isolates grew at -1 °C, 13 of them produced some antifreeze activity. Nine of these AFP-producing isolates belonged to Basidiomycota, two to Ascomycota, one to Oomycota, and one to Blastocladiomycota (Xiao *et al.* 2010a). In the following work, these authors also compared the IR and TH properties of the AFPs produced by two Antarctic fungal isolates: *Antarctomyces psychrotrophicus* and *Typhula ishikariensis* (Xiao *et al.* 2010b). They discovered that *A. psychrotrophicus* produces a 28 kDa protein named AnpAFP. During experimentation, this protein demonstrated robust thermal hysteresis TH activity and effective IR Activity. Particularly noteworthy were the results indicating significant IR activity at a concentration of 0.05 mg.mL^{-1}, however a TH activity of 0.42 °C was observed at a higher concentration of 13.44 mg.mL^{-1}. Additionally, *T. ishikariensis* expressed seven AFP isoforms, with TisAFP6 and TisAFP8 exhibiting noteworthy activity. Particularly, TisAFP8 displayed a TH value of 1.9 °C and IR activity at 0.01 mg.mL^{-1}. Later, another Antarctomyces species isolated from Antarctica received attention for its AFP activity. *Antarctomyces pellizariae* was a new species isolated from snow samples on the Antarctic Peninsula and was considered endemic to Antarctica (Menezes *et al.* 2017). This psychrophilic, blue-colony forming Ascomycete was found to produce a 236 amino acid AFP encoded by the gene APELI_8493-RA. This AFP shares sequence similarities with two isoforms of *A. psychrotrophicus* and features the DUF3494 domain (Batista *et al.* 2020), which emphasizes horizontal gene transfer events also among the Antarctomyces.

Beyond filamentous fungi, AFPs have been discovered in yeasts as well. In the early 2000's, many psychrophilic and psychrotrophic yeasts were isolated from glacier ice, polar

soils, and other cold environments, but none were proven to produce AFPs until the isolation of *Leucosporidium* sp. AY30 (Lee *et al.* 2010). This yeast strain was isolated from ice cores collected in Svalbard (Norway) and produced an AFP with a molecular weight of 26.8 kDa. This AFP showed TH activity of 1.38 ºC at 7.25 mg.mL^{-1} and IR activity at 0.5 mg.mL^{-1}. The full-length cDNA sequencing of this transcript revealed a 261 amino acid sequence with high similarity to other AFPs found in fungi, diatoms, and bacteria.

After this first report of a yeast with antifreeze proteins, other yeasts were isolated from polar environments and had their AFP studied. *Glaciozyma antarctica*, isolated from Antarctic sea ice, was found to produce two AFPs - Afp1 and Afp4 (Hashim *et al.* 2013, 2014). The functional characterization of these AFPs revealed that Afp1 weights 15 kDa and exhibits a TH activity of 0.1 ºC, while Afp4 weights 25.3 kDa with a TH activity of 0.1 ºC at 5 mg.mL^{-1} and IR activity at 5 mg.mL^{-1}. *Rhodotorula svalbardensis* sp. nov., a new species originating from an Arctic cryoconite hole, was also discovered to produce AFPs. This yeast underwent growth in a culture medium maintained at -1 ºC for a duration of 2 months. Following this, 5 µL of the culture was subjected to a test to observe ice crystals, revealing their characteristic hexagonal shape—an indicative trait of antifreeze protein (Singh *et al.* 2013; Singh *et al.* 2014b). Villarreal *et al.* (2018) analyzed freeze tolerance and AFP production in yeasts from Antarctica, also identifying *Goffeauzyma gastrica* as an AFP producer.

Research on antifreeze proteins (AFPs) in marine algae, especially in polar diatoms, has revealed a chronological sequence of significant discoveries. In 2006, Janech *et al.* identified the diatom *Navicula glaciei*, isolated from a crevice in sea ice on Cape Evans, Antarctica, as a producer of a 25 kDa AFP (Janech *et al.* 2006). Subsequently, *Fragilariopsis cylindrus,* another marine diatom from the Weddell Sea region, demonstrated the production of an AFP of 25.939 kDa. This protein was named FcAFP and exhibited a TH activity of 0.9 ºC at 5.9 mg.mL^{-1} and IR activity at 0.03 mg.mL^{-1} (Bayer-Giraldi *et al.* 2011; Krell *et al.* 2008).

Raymond, Janech, and Fritsen (2009) investigated *Chlamydomonas* CCMP681, isolated from the Palmer Station in Antarctica, and characterized four isoforms of AFP with molecular masses ranging from 36.2 to 37.1 kDa, all displaying a hexagonal morphology in ice crystals (Raymond, Janech, and Fritsen 2009). Other findings include the diatom *Chaetoceros neogracile*, isolated from Marian Cove, King George Island, Antarctica, which produces a 29.2 kDa AFP named CnAFP. The heterologous expression of this AFP exhibited TH activity of 0.8 ºC at 1 mg.mL^{-1} and also induced hexagonal ice crystal morphology (Gwak *et al.* 2010).

Similarly, three AFPs from *Fragilariopsis cylindrus* were studied by heterologous expression, showing sizes of 30.9-52.8 kDa and TH activity ranging from 0.06 ºC at 0.06 $mg.mL^{-1}$ to 0.09 ºC at 0.04 $mg.mL^{-1}$ (Uhlig *et al.* 2011). Additionally, two AFP isoforms were discovered in a psychrophilic chlorophyte, *Pyramimonas gelidicola*, also isolated from Antarctica (Jung *et al.* 2012, 2014). The isoforms Pg-1-AFP and Pg-2-AFP ranged from 26.4-27.1 kDa and showed TH activity of 0.6 ºC and 0.25 ºC at 15 $mg.mL^{-1}$, respectively. Subsequent research by Raymond and Morgan-Kiss (2013) identified in *Chlamydomonas raudensis* UWO241 three AFP isoforms with molecular masses of 23, 4.5, and 36.3 kDa, all produced extracellularly, accompanied by ice crystal morphology in the form of dendrites (Raymond and Morgan-Kiss 2013). Lastly, *Kremastochrysopsis austriaca*, isolated from alpine snow in Austria, produces three AFP isoforms that form hexagonal ice crystals (Raymond and Remias 2019). Furthermore, *Chloromonas* sp. KNF0032 isolated from the Dasan station in the Arctic produces 6 genes encoding Type II IBPs and have been named CmIBPs (CmIBP1, CmIBP2, and CmIBP3) (Cho *et al.* 2019; Jung *et al.* 2016).

Some eukaryotes were found to produce a larger variety of AFP isoforms. Raymond *et al.* (2014) studied the microalgae *Chloromonas brevispina* isolated from snow samples, and 20 AFP isoforms were found (Raymond 2014). The species *Chloromonas*, isolated from the Palmer station in Antarctica, produces a 34 kDa AFP (ChloroIBP) with TH activity (0.4 °C at 5 $mg.mL^{-1}$), forming ice crystals in dendritic shapes (Jung *et al.* 2016). Another example is the microalgae *Chlamydomonas* sp. ICE-MDV isolated from Lake Bonney in Antarctica (Raymond and Morgan-Kiss 2017). The genomic analysis of this strain found the Chlamy-ICE gene, responsible for encoding 50 isoforms of DUF3494-type AFPs, which raises intriguing questions about evolutionary mechanisms and environmental selective pressures on this Antarctic microorganism. Certainly, the presence of the DUF3494 domain highlights the role of horizontal gene transfers between eukaryotes and prokaryotes, as explained earlier. Nevertheless, the existence of numerous isoforms prompts us to ponder the evolutionary forces that have led to such diversity and the potential functional significance underlying the need for multiple isoforms. According to the authors, the multiple isoforms may help the algae to survive in a greater variety of frozen environments than prokaryotes, which usually have a single IBP gene.

Metagenomic approaches in eukaryotes have been conducted in the polar regions. In one study, the expression of antifreeze proteins in eukaryotic microbial communities within Arctic and Antarctic sea ice was analyzed *in situ* (Uhlig *et al.* 2015). Abundant transcripts of

antifreeze proteins (AFPs) were detected, with 89% of these transcripts being grouped with known AFP sequences. However, the majority represented new, uncharacterized sequences in cultured organisms. The significant expression of eukaryotic AFPs observed in sea ice communities underscores their essential role in the survival of many microorganisms residing in extreme conditions.

3.6 META-ANALYSIS OF PUBLISHED AFP DATA: ECOLOGICAL DISTRIBUTION, ANTIFREEZE EFFICIENCY, AND RESEARCH INSIGHTS

In this review, we obtained data from cultivation-based and cultivation-independent methods for a comprehensive analysis of microbial AFPs. After an extensive search, we found that 174 microorganisms have been described as potential producers of AFPs, and 127 of this total were taxonomically identified up to genus. The revised microorganisms are distributed in the groups of Algae, Fungi, Bacteria, and Archaea (**Figure Chapter I 3**). The taxonomic analysis of AFP-producing microorganisms revealed significant diversity across various groups. In the bacterial group, 24 isolates (19.05%) were identified as belonging to the Phylum Actinobacteria, 4 isolates (3.17%) from the Phylum Bacteroidetes, 1 isolate (0.79%) from the Phylum Cyanobacteria, 6 isolates (4.76%) from the Phylum Firmicutes, and 40 isolates (31.75%) from the Phylum Proteobacteria. Among fungi, 16 isolates (12.70%) were found belonging to the Phylum Ascomycota, 22 isolates (17.46%) from the Phylum Basidiomycota, and one isolate each from the Phyla Blastocladiomycota and Oomycota. In the algae group, diversity was also evident, with 3 isolates (2.38%) from the Class Bacillariophyceae, 6 isolates (6.87%) from the Class Chlorophyceae, as well as one isolate each from the Classes Pyramimonadophyceae and Chrysophyceae. A unique representative within the Archaea was identified in the Phylum Euryarchaeota. Overall, this taxonomic variety highlights the complexity and richness of the microbial community found in this study.

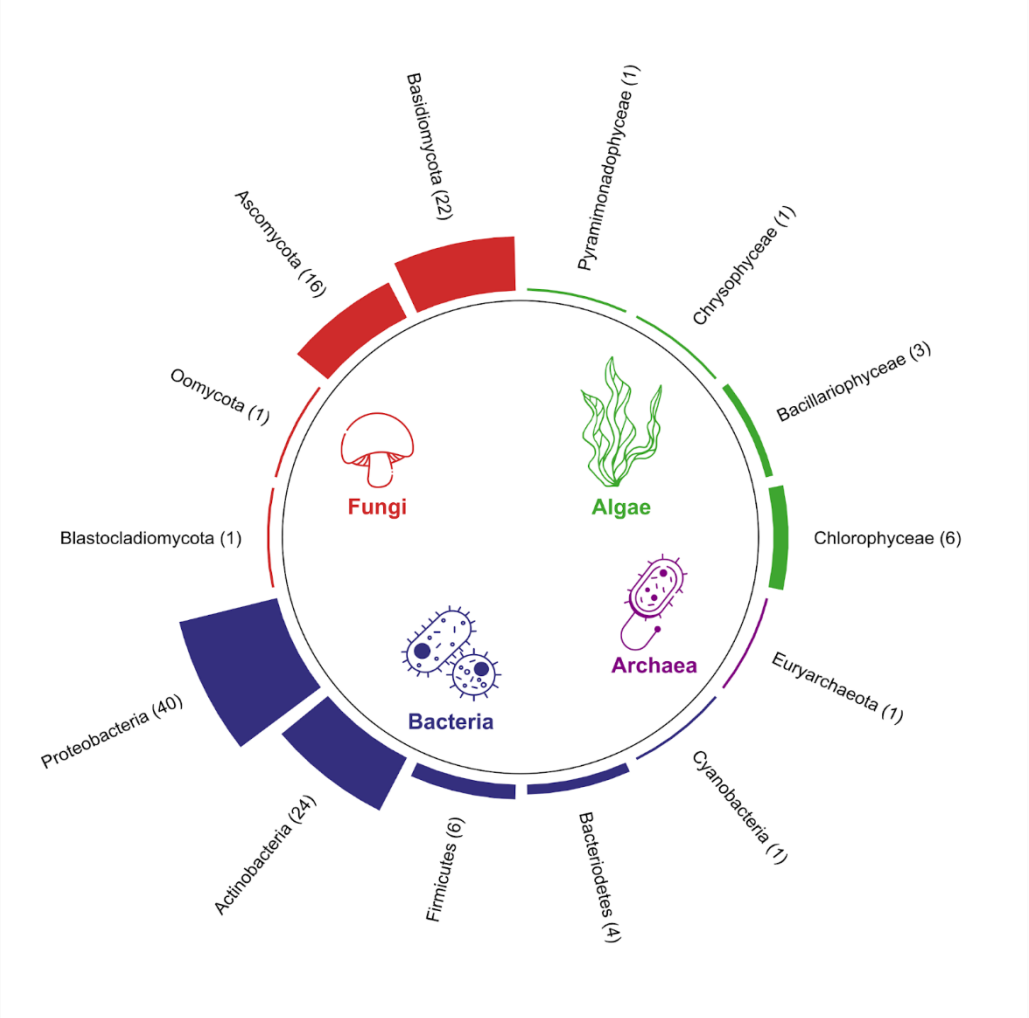

Figure Chapter I 3 - Groups of potential producers of antifreeze proteins (AFPs), in which taxonomic identification was investigated. The number of AFP-producing microorganisms is represented in parentheses. The microorganisms were distributed among Phyla and Classes of Fungi, Algae, Archaea, and Bacteria.

The taxonomic analysis of the algae group revealed a variety of genera and species across different taxonomic groups, but only one isolated strain was reported for each of them (**Figure Chapter I 4A**). In the Class Bacillariophyceae, *Navicula glaciei*, *Fragilariopsis cylindrus*, and *Chaetoceros neogracile* were found, each represented by a single isolate. In the Class Pyramimonadophyceae, a single isolate of *Pyramimonas gelidicola* was identified. In the Class Chlorophyceae, six species were found: *Chloromonas* sp. (2 isolates), and *Chloromonas brevispina, Chlamydomonas cf.* sp.*, Chlamydomonas raudensis* and *Chlamydomonas* sp. each represented by a single isolate. In the Class Chrysophyceae, *Kremastochrysopsis austriaca*, *Chlamydomonas* cf. sp., *Chlamydomonas raudensis*, and *Chlamydomonas* sp. were found, all represented by a single isolate each. The limited number of isolates in each taxon suggests that reported isolates may represent a small subset of the overall diversity within each algae species. Sampling efforts should be expanded to specific environments where AFP-producing algae are more prevalent or easily detectable, such as polar sea ice and marine water.

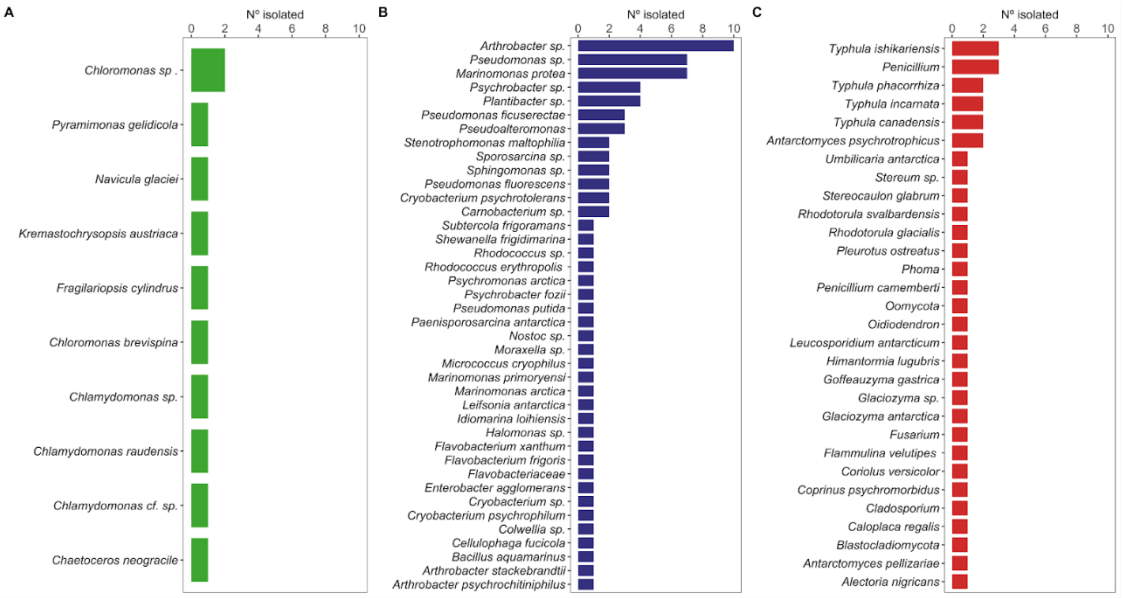

Figure Chapter I 4 - Distribution of AFP producers categorized by species of (A) Algae, (B) Bacteria and (C) Fungi isolates.

The bacterial group accounted for the majority of all AFP-producing microorganisms in our analysis, with a total of 75 identified bacteria, distributed into 5 Phyla (**Figure Chapter I 4B**). Within the Phylum Actinobacteria, various species were identified, including *Arthrobacter* sp. (10 isolates), *Leifsonia antarctica* (1 isolate), *Arthrobacter psychrochitiniphilus* (1 isolate), *Arthrobacter stackebrandtii* (1 isolate), *Rhodococcus* sp. (1 isolate), *Cryobacterium psychrotolerans* (2 isolates), *Cryobacterium psychrophilum* (1 isolate), *Cryobacterium* sp. (1 isolate), *Subtercola frigoramans* (1 isolate), *Plantibacter* sp. (4 isolates), *Micrococcus cryophilus* (1 isolate), and *Rhodococcus erythropolis* (1 isolate). In the Phylum Bacteriodetes, the isolated species included *Cellulophaga fucicola* (1 isolate), *Flavobacterium frigoris* (1 isolate), *Flavobacterium xanthum* (1 isolate) and Flavobacteriaceae (1 isolate). Within the Phylum Cyanobacteria, a single isolate of *Nostoc* sp. was identified. In the Phylum Firmicutes, *Sporosarcina* sp. (2 isolates) and *Carnobacterium* sp. (2 isolates) were found, along with *Bacillus aquamarinus* (1 isolate). Finally, the Phylum Proteobacteria exhibited a wide variety of isolated species, including *Sphingomonas* sp. (2 isolates), *Psychrobacter fozii* (1 isolate), *Marinomonas protea* (7 isolates), *Pseudomonas ficuserectae* (3 isolates), *Pseudomonas fluorescens* (2 isolates), *Pseudoalteromonas* (3 isolates), *Stenotrophomonas maltophilia* (2 isolates), *Pseudomonas putida* (1 isolate), *Marinomonas primoryensi* (1 isolate), *Colwellia* sp. (1 isolate), *Moraxella* sp. (1 isolate), *Shewanella frigidimarina* (1 isolate), *Pseudomonas* sp. (7 isolates), *Halomonas* sp. (1 isolate), *Enterobacter agglomerans* (1 isolate), *Psychrobacter* sp. (4 isolates), *Marinomonas arctica* (1 isolate), *Psychromonas arctica* (1 isolate), *Idiomarina loihiensis* (1 isolate), and *Paenisporosarcina antarctica* (1 isolate). The higher number of AFP-

producing isolates among the bacteria could be explained by some arguments. The taxonomic and functional diversity within bacteria is considered much higher than fungi and archaea, as they evolved in a wide range of environments for almost 4 billions of years. Also, bacteria are easier to cultivate in laboratory conditions compared to other microorganisms, which makes them the focus of many research efforts and sampling strategies.

Regarding the fungi group, these eukaryotes revealed a higher number of taxa and identified strains than the algae group, but fewer numbers when compared to the bacteria (**Figure Chapter I 4C**). In the Phylum Basidiomycota, the isolated species included *Coprinus psychromorbidus* (1 isolate), *Coriolus versicolor* (1 isolate), *Flammulina velutipes* (1 isolate), *Glaciozyma antarctica* (1 isolate), *Glaciozyma* sp. (1 isolate), *Goffeauzyma gastrica* (1 isolate), *Leucosporidium antarcticum* (1 isolate), *Pleurotus ostreatus* (1 isolate), *Rhodotorula glacialis* (1 isolate), *Rhodotorula svalbardensis* (1 isolate), *Stereum* sp. (1 isolate), *Typhula canadensis* (2 isolates), *Typhula incarnata* (2 isolates), *Typhula ishikariensis* (3 isolates), and *Typhula phacorrhiza* (2 isolates). Within the Phylum Ascomycota, various species were identified, including *Alectoria nigricans* (1 isolate), *Antarctomyces psychrotrophicus* (2 isolates), *Antarctomyces pellizariae* (1 isolate), *Caloplaca regalis* (1 isolate), *Cladosporium* (1 isolate), *Fusarium* (1 isolate), *Himantormia lugubris* (1 isolate), *Oidiodendron* (1 isolate), *Penicillium* (3 isolates), *Penicillium camemberti* (1 isolate), *Phoma* (1 isolate), *Stereocaulon glabrum* (1 isolate), and *Umbilicaria antarctica* (1 isolate). Additionally, Oomycota had four isolated species (4 isolates), while Blastocladiomycota was represented by a single isolate (1 isolate).

In addition to the Algae, Bacteria, and Fungi groups, there is only one report of AFP producers within the Archaea. A strain of *Halobacterium* sp. was discovered in 2020 from fermented fish sauce from different markets in India and was reported to produce AFP (Desai *et al.* 2020).

Various microorganisms have been isolated from diverse environments, reflecting a broad range of isolation sources within the reviewed microorganisms. In general, most AFP-producing microorganisms are found in cold or frozen habitats, mainly due to the selective stress of living in low temperatures (**Figure Chapter I 5**). Algae were found in diverse environments such as sea ice, snow, lake, ice, and transient pools, whereas fungi were found in environments such as sea ice, ice, snow, soil, cryoconite sediment, mosses, and algae mats (Hoham and Remias 2020; Santos, Meyer, and Sette 2020; Hirose *et al.* 2016). Finally, bacteria species were found in environments such as seawater, cryoconite sediment, soil, glacier ice, sea

ice, lakes, rock surfaces, associated with plants (*Deschampsia antartica*, *Bryum argenteum,* and others) and protists (*Euplotes focusdii*), as well as in sand, frozen food and the intestinal tract of beetle larvae. Overall, the main sources of AFP-producing microorganisms are different when Arctic and Antarctica are compared. While in Antarctica most reports come from microorganisms associated with *Deschampsia antartica*, most of the Arctic isolates come from soils. Interestingly, no reports of AFP-producing microorganisms were found on water and snow samples from the Arctic, which emphasizes the lack of studies in these environments.

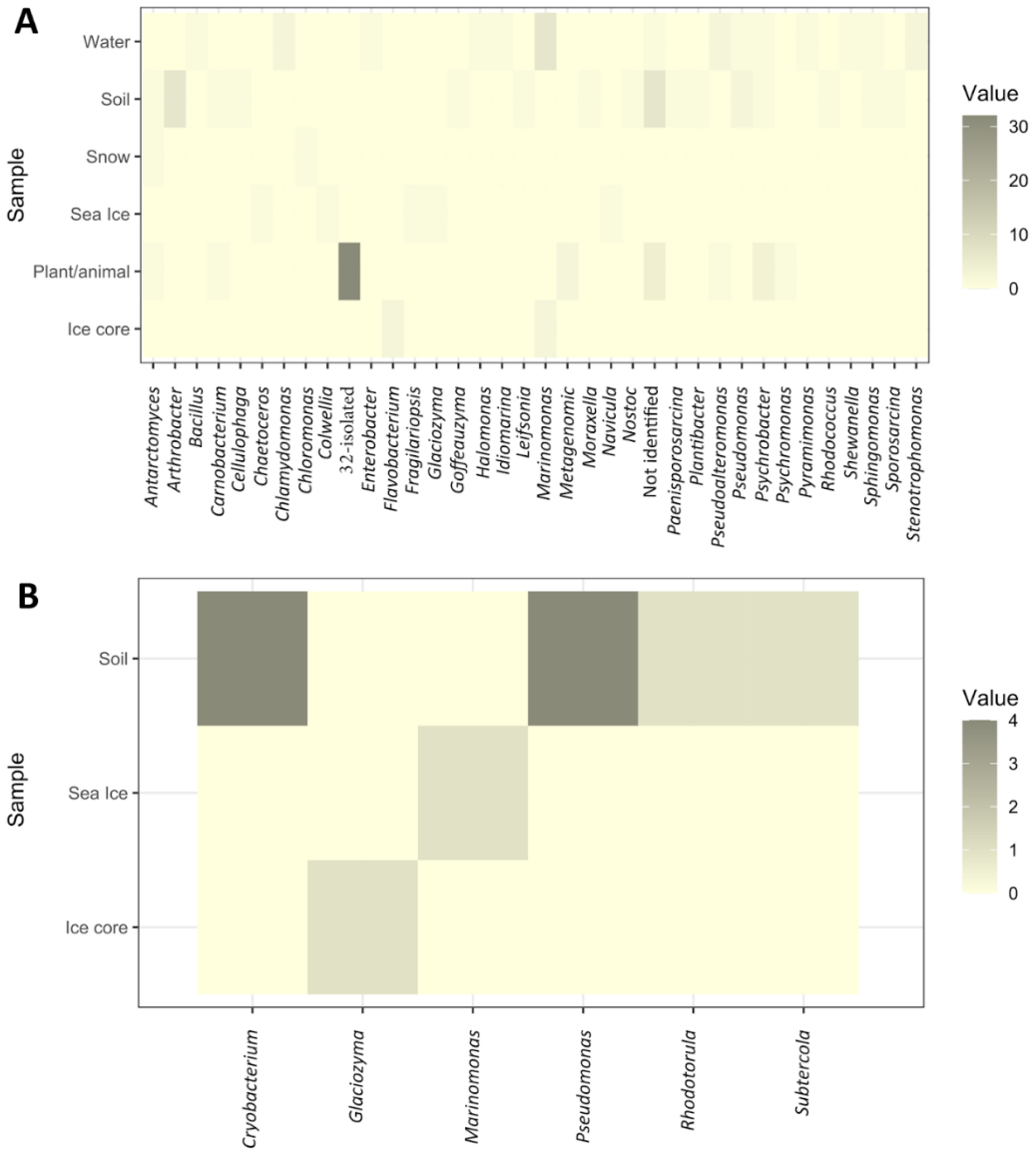

Figure Chapter I 5 - Heat map showing the number of AFP-producing microorganisms according to their taxonomic genus, in relation to the isolation source in Antarctica (A) and the Arctic (B).

3.7 CHARACTERIZATION OF ANTIFREEZE ACTIVITY

Among the studies gathered in this review, 66 microorganisms were screened and confirmed for IR activity. We used this data to plot the different concentrations used for IR activity assays, converting molarity data to $mg.mL^{-1}$ when possible (**Figure Chapter I 6**). Some studies did not show the protein weight, or did not quantify the protein extracts, therefore only 34 IR tests were valid for this comparison. While it is clear that no standard protein concentration was used for IR activity tests, most reports (58.82%; n = 20) tested protein extracts at a concentration between 1.5-3.3 $mg.mL^{-1}$, followed by tests in the range of 0.001-0.5 $mg.mL^{-1}$ (38.23%; n = 13).

Based on the protein concentrations, ice recrystallization inhibition (IR) activity varies, with lower concentrations showing higher efficiency in IR activity. The most efficient AFPs on IR activity to date are found on *Alectoria nigricans*, *Caloplaca regalis*, *Himantormia lugubris*, *Stereocaulon glabrum*, and *Umbilicaria antarctica*, all belonging to the fungi category, exhibiting ice recrystallization inhibition activities of 0.001 $mg.mL^{-1}$, 0.006 $mg.mL^{-1}$, 0.001 $mg.mL^{-1}$, 0.001 $mg.mL^{-1}$, and 0.00011 $mg.mL^{-1}$, respectively. *Fragilariopsis cylindrus* (FcAfp), *Antarctomyces psychrotrophicus* (AnpAFP), *Pseudomonas ficuserectae* (Cry-n), and other strains all demonstrate ice recrystallization inhibition activities of 0.05 $mg.mL^{-1}$ or slightly higher. Also, *Flavobacterium frigoris* (FfIBP) exhibits an ice recrystallization inhibition activity of 0.12 $mg.mL^{-1}$, followed by *Flavobacterium xanthum* (FlAFP) and *Chloromonas* sp. (CmIBP) with 0.2 $mg.mL^{-1}$, and *Glaciozyma* sp. (LeIBP) and *Flammulina velutipes* with 0.5 $mg.mL^{-1}$ each. These organisms display moderate ice recrystallization inhibition activity, suggesting some capacity to inhibit ice recrystallization.

Most IR tests, however, were reported on a higher concentration. Likewise, the need for a higher AFP concentration to promote inhibition of ice recrystallization could be interpreted as a lower efficiency of these proteins. For example, *Idiomarina loihiensis*, *Psychrobacter* sp., and *Bacillus aquamarinus* demonstrate IR activities ranging from 1.32 to 1.5 $mg.mL^{-1}$, indicating a less efficient capacity to inhibit ice recrystallization. *Pseudoalteromonas* sp. and *Marinomonas protea* exhibit IR activities ranging from 1.8 to 3.33 $mg.mL^{-1}$, showing even less efficient abilities to inhibit ice recrystallization. Lastly, we find *Glaciozyma antarctica* (Afp4) with the lowest ice recrystallization inhibition activity of 5 $mg.mL^{-1}$, indicating a need for a concentration of about 100 to 1000 times higher compared to the most efficient microbial AFPs.

As explained earlier, the IR activity depends on the AFP concentration, but this relationship is not linear. Experimental observations showed that at least three effects influence the results: a threshold effect, a saturation point, and the optimal concentration for each AFP. In many cases, there is a threshold effect observed, where the IR activity of AFPs increases with concentration up to a certain point, beyond which further increases in concentration may not significantly enhance IR activity (C. A. Knight, DeVries, and Oolman 1984; Fletcher, Hew, and Davies 2001). Regarding the saturation behavior, at higher concentrations, AFPs ability to inhibit ice recrystallization reaches a plateau, and additional AFP molecules do not contribute significantly to further inhibition (Chao, Houston, and Hodges 1990). Finally, studies reported that there is often an optimal concentration range for AFPs where they demonstrate maximum IR activity (P. L. Davies and Hew 1990; Tachibana *et al.* 2004). It is important to note that these studies were made several decades ago, in experiments with non-microbial AFPs. Therefore, we highlight the importance of further exploring the relationship between the concentration of microbial AFPs and IR activity, especially setting a standard protein concentration and other experimental conditions for comparing different AFPs.

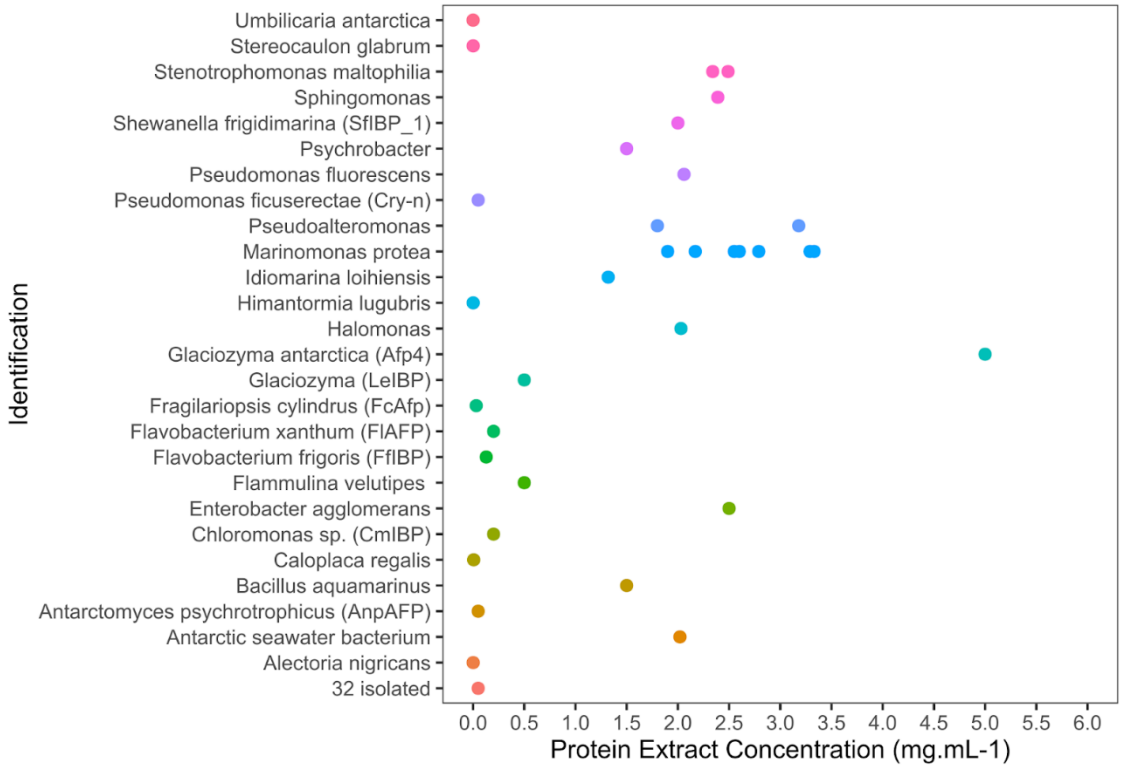

Figure Chapter I 6 - Identified microorganisms with Recrystallization Inhibition Activity, based on the concentrations of tested protein extracts.

In addition to the ice recrystallization, the TH activity was also evaluated (**Figure Chapter I 7**). Similarly, many studies did not report the protein concentration used for TH assays. From a total of 64 identified microorganisms tested for TH activity, only 31 could be used in this analysis. The result showed the TH data scattered on the plot graph, demonstrating that no standard concentration is used for TH tests. Overall, the highest TH values were reported when a lower protein concentration was used.

In the lower range of TH activity, we find *Flammulina velutipes*, demonstrating a TH activity of 0.01 ºC at a concentration of 5 $mg.mL^{-1}$. *Moraxella* sp. exhibits a TH activity of 0.1049 ºC at a concentration of 0.1 $mg.mL^{-1}$. As we progress along the scale, *Cryobacterium psychrotolerans* (Cry-c) and *Cryobacterium psychrotolerans* (Cry-21) show TH activities of 1 and 0.78 ºC, respectively, at concentrations of 4 $mg.mL^{-1}$ and 1.8 $mg.mL^{-1}$. *Pyramimonas gelidicola* (Pg-2-AFP) and *Glaciozyma antarctica* (Afp4) demonstrate TH activities of 0.25 and 0.08 ºC, respectively, at a concentration of 15 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$. In the higher TH activity range, *Cryobacterium* sp. (Cry-k) and *Pseudomonas ficuserectae* (Cry-g) exhibit TH activities of 0.78 and 2 ºC, respectively, at concentrations of 1.8 $mg.mL^{-1}$ and 2.4 $mg.mL^{-1}$. At the upper end of the TH activity scale, *Colwellia* sp. (ColAFP) and *Flavobacterium frigoris* (FflBP) show TH activities of 4 and 2.2 ºC, respectively, at concentrations of 3.3 $mg.mL^{-1}$ and 0.12 $mg.mL^{-1}$. If we consider a "TH index", calculated by dividing the TH value by the protein concentration, we find that FflBP of *Flavobacterium frigoris* scores 18.33, being the highest hysteresis per unit of concentration.

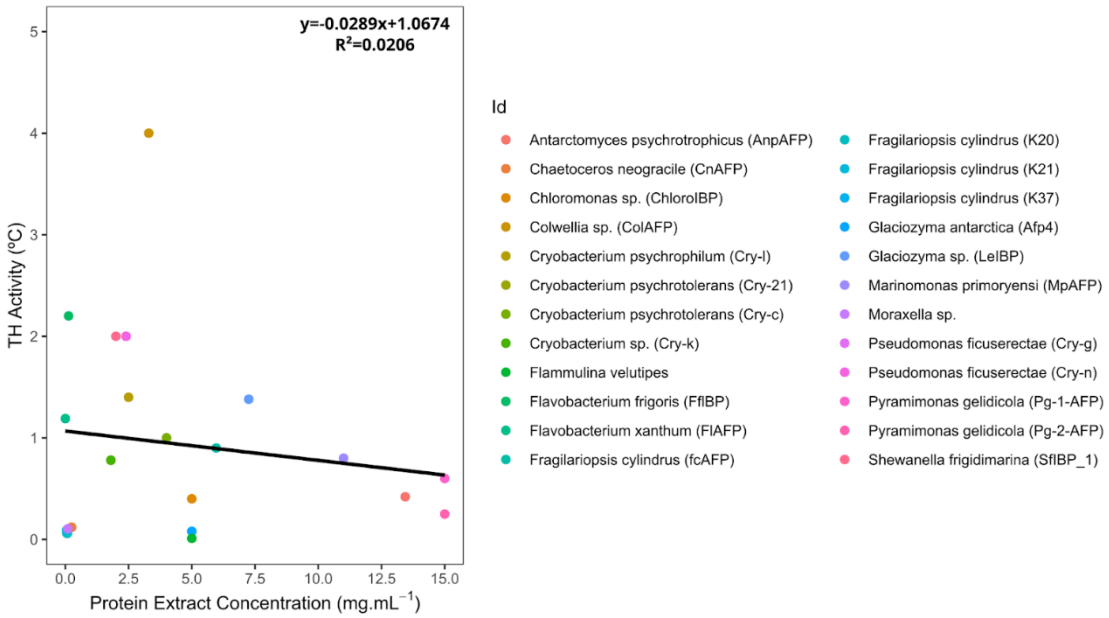

Figure Chapter I 7 - Relationship between the Thermal Hysteresis (TH) activity and the tested protein concentrations. The linear trend line was chosen for a better fit than the exponential model.

The weight of an AFP is typically among the initial properties determined upon the discovery of a new AFP, along with its IR and TH activity. In this context, we analyzed the relationship between the AFP molecular weight (kDa) and their TH values (**Figure Chapter I 8**). The peptide sequence of an AFP and its specific binding to ice planes share a significant role with the protein weight for determining the antifreeze efficiency. Considering the current models on ice-binding mechanisms and the protein size (or its weight), bigger AFPs could bind more effectively to ice than smaller proteins due to more ice-binding sites. Consistent with this idea, organisms producing several isoforms of AFPs that differ on both weight and activity showed higher antifreeze efficiency on bigger isoforms (DeVries 1988; Chao *et al.* 1996; Leinala *et al.* 2002). For example, the arctic ray-finned fish *Gadus ogac* (Greenland cod) produces both small (2.6-10 kDa) and larger (13.7-24.1 kDa) AFPs. Comparison of these isoforms at the same molecular concentration showed a higher activity on the large proteins (Wu *et al.* 2001). Also, experiments with combined proteins had shown a direct correlation between TH activity and the protein complex size, where TH activity increased by about 20% when the protein size was doubled (Chao *et al.* 1996; Leinala *et al.* 2002).

AFPs vary a lot in terms of weight, but in general, they are small monomeric proteins with 3-30 kDa (Ramløv and Friis 2020). Our evaluation revealed that 92% of the characterized microbial AFPs are distributed into two groups: smaller proteins of 22-34 kDa (72.4%), and bigger proteins of 41-59 kDa (20.7%). A few microbial AFPs were described out of this range, such as the 15 kDa AFP from *Glaciozyma antarctica* (Hashim *et al.* 2013) and the 80 kDa AFP from *Pseudomonas fluorescens* (Hidehisa Kawahara *et al.* 2004).

Comparisons of efficiency between the two size groups of microbial AFPs are yet to be done. Here we plotted the TH activity of 29 AFPs (8 algal, 15 bacterial, and 6 fungal AFPs) as a function of protein weight (**Figure Chapter I 8**). This analysis shows that the smaller group of microbial AFPs has an average TH of 1.16 °C (n = 21; range 0.033-4.0 °C), while the larger group has an average TH of 1.01 °C (n = 6; range 0.1-2.34 °C). These results suggest that the most efficient microbial AFPs are within the smaller protein group, and the TH activity has a negative correlation with protein weight, at least for microorganisms. It is important to note that these AFPs differ not only in weight, but also in structure, aminoacid sequence, and ice-binding planes, while also their TH was measured with different approaches and by different investigators. Therefore, the data should be interpreted with caution, but it serves as a starting line for future research. In this context of microbial AFPs, we suggest that future studies addressing the role of AFP weight on TH activity be evaluated by comparing protein isoforms

from the same microbial strain, or by changing the protein size through genetic engineering (mutational) studies.

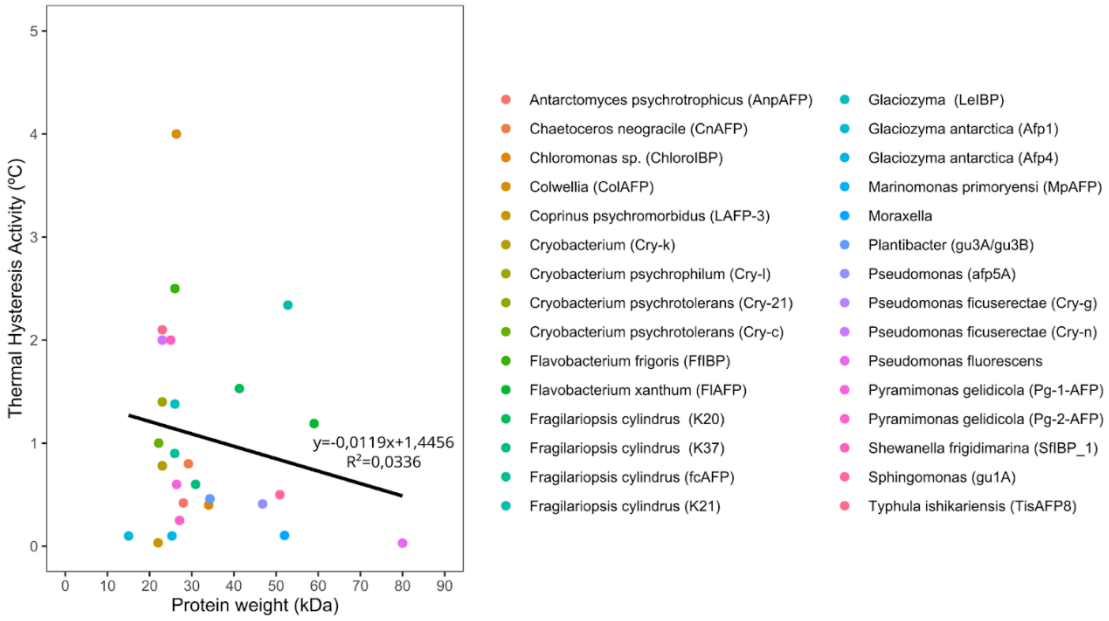

Figure Chapter I 8 - Relationship between the Thermal Hysteresis (TH) activity and the AFP weight in kilodaltons. The linear trend line was chosen for a better fit than the exponential model.

3.8 SPATIAL DISTRIBUTION OF AFP-PRODUCING MICROORGANISMS IN ARCTIC AND ANTARCTIC ENVIRONMENTS

Research on AFP-producing microorganisms occurs in cold ecosystems of both the Arctic and Antarctic. However, the number of AFP producers reported in Antarctica exceeds those in the Arctic by about an order of magnitude (**Figure Chapter I 9**). In our analysis, a total of 110 bacterial strains, 22 fungi and 8 algae from Antarctica have been reported to produce AFP, in contrast with only 10 bacteria, 3 fungi and 1 algae from the Arctic. While both polar regions have diverse microbial communities and a variety of cold ecosystems, these differences may reflect different efforts of the scientific community to prospect AFP-producing microorganisms. Also, considering the obvious difficulties in logistics for accessing Antarctic samples compared to the Arctic, special attention should be given to future prospection of AFP-producing microorganisms in the Arctic.

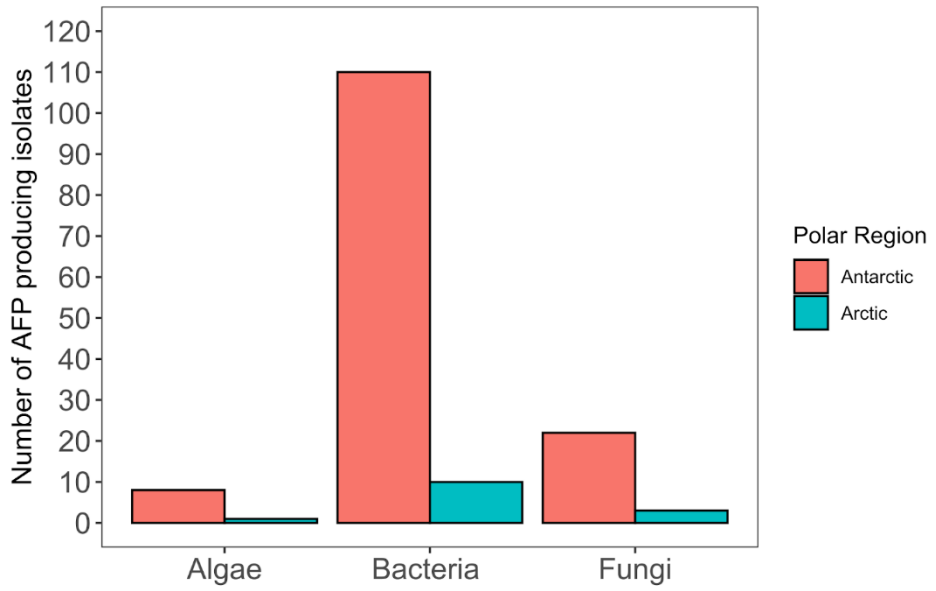

Figure Chapter I 9 - Distribution of AFP-producing isolates within groups of Algae, Bacteria, and Fungi in relation to the two polar regions.

The published data on microbial AFP was used for a spatial distribution analysis and comparison between the South and North Hemispheres (**Figures Chapter I 10 and 11, Supplementary Tables S2 and S3**). In Antarctica, there are 140 isolated AFP-producing microorganisms to date (8 types of algae, 22 types of fungi, 110 types of bacteria), in addition to 2 metagenomic studies that found AFP-producing genes of microorganisms associated with mosses and a free-living marine ciliate. The spatial distribution map of Antarctic isolates reveals a concentration along the coastline, mainly near research stations. These areas have easier logistic access for collecting samples, which is often a barrier for Antarctic research. Also, the proximity to research stations permits rapid processing of environmental samples, increasing the success rate in recovering microbial colonies. In addition, Antarctic research stations are usually surrounded by ice-free landscapes, where it is possible to access different ecosystems such as soils, rocks, snow, marine water, lake sediments, plants, and animals. In this context, the South Shetlands Islands and the McMurdo Valley concentrate about 36% and 25% of all microbial AFP studies in Antarctica, respectively (**Figure Chapter I 10**, upper-left and bottom-left squares). These areas concentrate most of the Antarctic research stations, since they are considered two of the most sensitive areas to climate change and are the focus of many research projects worldwide. The analysis of Antarctic studies also revealed that some environments were undersampled for AFP prospection. The Antarctic marine water and sea ice are accessible for most research stations but only a couple of studies identified AFP-producing microorganisms in these environments, more specifically on waters from Ross Sea and the

Bransfield Strait. The continental region also received a few studies. Despite being dominated by glacier ice, the interior of the Antarctic continent receives a significant input of air-borne microorganisms that precipitate with snow and become entrapped within the ice (Pearce *et al.* 2016). Since this is a permanently frozen ecosystem that preserves the microbial community for long periods of time (Hodson *et al.* 2008), future research on continental ice cores could reveal novel AFP-producing microorganisms.

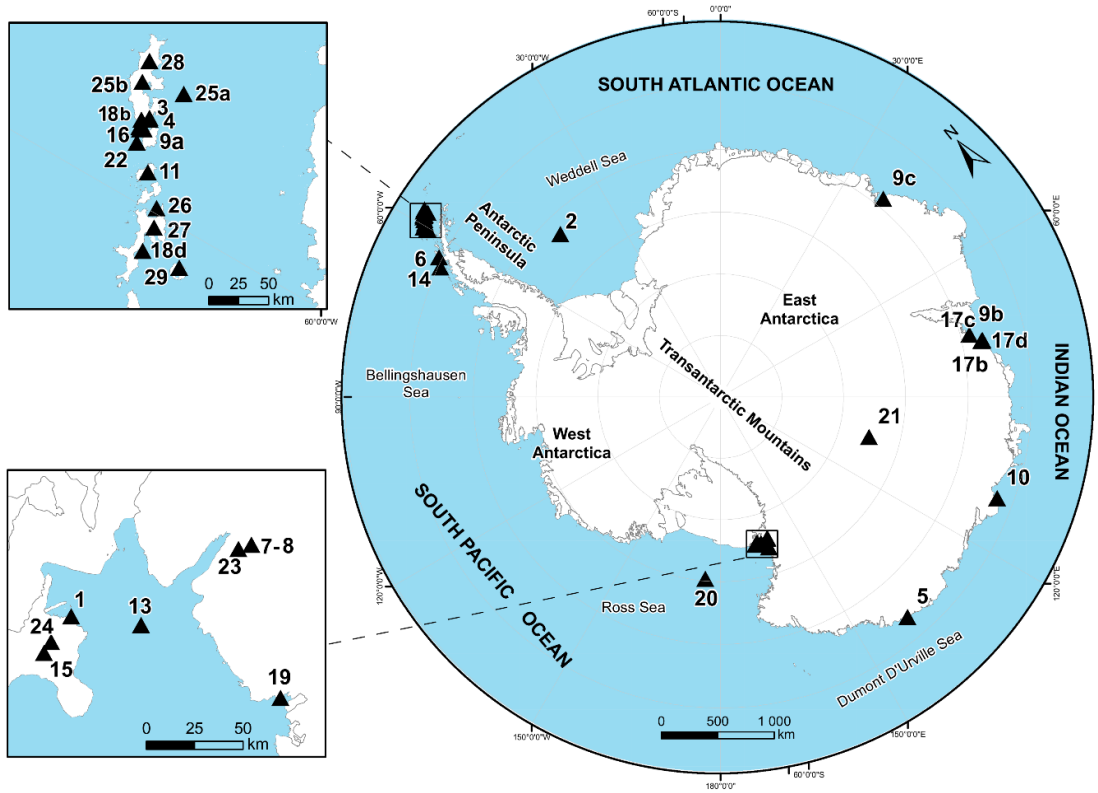

Figure Chapter I 10 - Map of the southern hemisphere (Latitude > 60° S) showing the locations where AFP-producing microorganisms (black triangles) were isolated in Antarctica. The upper-left and bottom-left squares show in detail the South Shetland Archipelago and the McMurdo Valley, respectively. The numbers above the triangles refer to different studies and a complete list with references is found in the Supplementary Material - Table S2. Database: Quantartica.

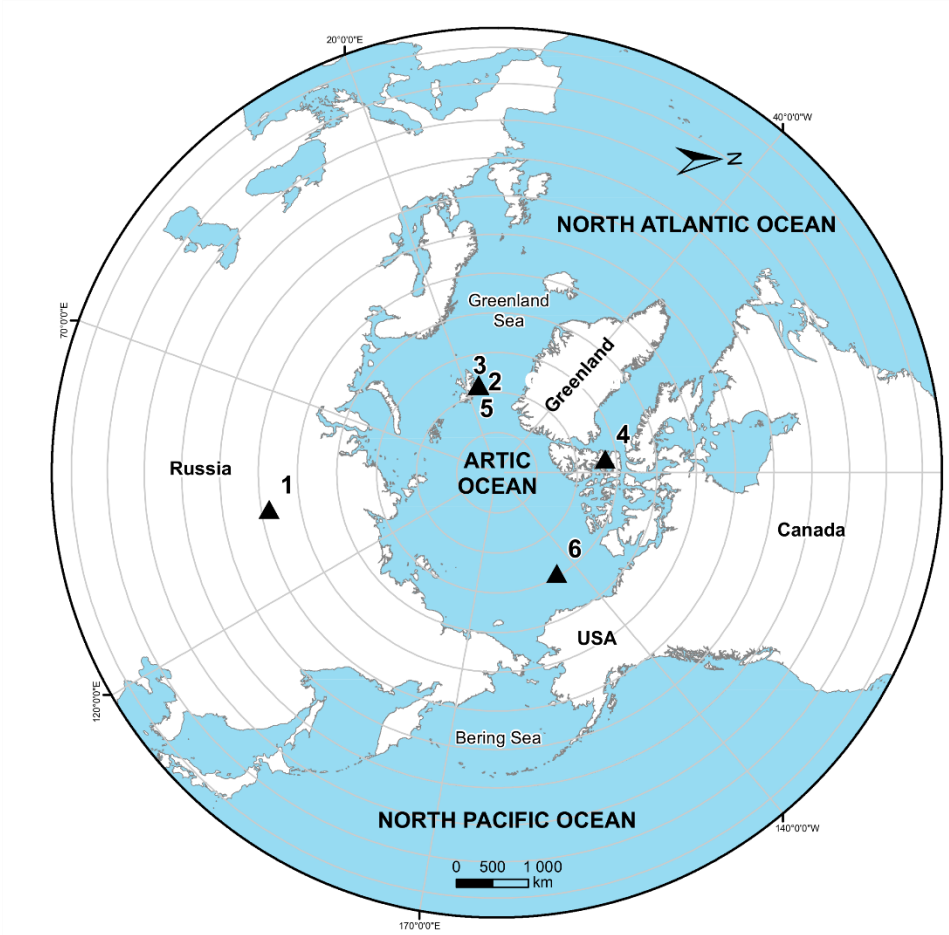

Figure Chapter I 11 - Map of the northern hemisphere (Latitude > 30° N) showing the locations where AFP-producing microorganisms (black triangles) were isolated in the Arctic. The numbers above the triangles refer to different studies and a complete list with references is found in the Supplementary Material - Table S3. Database: National Snow & Ice Data Center.

The Arctic presents fewer reports of AFP-producing microorganisms compared to Antarctica, with a total of 17 AFP-producing microorganisms (1 algae, 10 bacteria and 6 fungi). About 62% of these isolates were described in Svalbard Island, where the Norwegian research station Ny-Ålesund is located (Lee *et al.* 2010; Singh *et al.* 2013; Singh *et al.* 2014a). This is one of the main research stations in the Arctic, home to more than 20 international institutions engaged in long-term research and environmental monitoring. In addition, bacteria and fungi positive for AFP production were isolated from the Canadian High Arctic (Sun *et al.* 1995), Russian Siberia (Hoshino *et al.* 2003), and the Arctic sea ice (Liao *et al.* 2021). Despite the vast terrestrial area in the Arctic, many permanently and semi-permanently frozen environments are yet to be explored for microbial AFPs, including frozen lakes, glacier moraines, marine sediment, and permafrost. In the case of permafrost, for example, several studies have characterized the microbial communities frozen in the soil for thousands to millions of years (Yergeau *et al.* 2010; Vishnivetskaya *et al.* 2020; Sipes *et al.* 2021), but no reports of AFP-producing microorganisms were found. The closest example was a study of

Siberian permafrost isolates *Exiguobacterium* sp. 255-15 and *Psychrobacter* sp. 273-4 (Ponder *et al.* 2005). These bacteria were characterized for their physiology and capacity to grow at subzero temperatures, revealing cold adaptations in their membrane fatty acids and carbon source utilization, but no AFP production was found. The *Exiguobacterium* sp. 255-15 showed some soluble ice nucleation activity, but the presence of AFPs was not detected.

3.9 BIOTECHNOLOGICAL APPLICATIONS OF AFPs

The unique and useful properties of AFPs have generated significant interest for their potential applications in the medical and industrial fields, particularly for scenarios requiring low-temperature storage where ice crystallization must be avoided, such as cryopreservation processes. Cryopreservation is a method that preserves organelles, cells, and tissues by cooling them to sub-zero temperatures (Jaiswal and Vagga 2022; Whaley *et al.* 2021). Since the formation of intracellular ice leads to cell damage, some type of cryoprotectant is usually added to protect the biological material. These cryoprotectants can be permeable, such as dimethyl sulfoxide (DMSO), glycerol, and 1,2-propanediol, or impermeable, such as polyvinylpyrrolidone and hydroxyethyl starch (Jaiswal and Vagga 2022; Gao and Critser 2000). However, the majority of these cryoprotectants are highly toxic, and alternative technologies are needed to reduce its toxicity for human applications (Best 2015). In this context, AFPs are emerging as a promising alternative for cryopreservation in fields such as healthcare, agriculture and industry (Tas *et al.* 2021).

In the field of healthcare, AFPs enhance the long-term preservation of frozen cells and tissues, facilitating procedures like organ transplantation and vaccine production and storage (Chao, Davies, and Carpenter 1996; Amir *et al.* 2003; Tas *et al.* 2021; Ekpo *et al.* 2022). According to the Global Observatory on Donation and Transplantation (GODT-WHO, https://www.transplant-observatory.org/, accessed on 30 May 2024), a total of 157,494 organs are transplanted annually. Nevertheless, although organ transplantation is an effective treatment, conventional preservation methods are limited by short storage times (Chen *et al.* 2023). AFPs could extend the preservation time of organs and tissues during low-temperature storage, thereby increasing the viability window for more successful transplants. This could provide greater flexibility in logistics and potentially improve transplant outcomes by reducing the urgency and time constraints currently associated with organ transportation (Amir *et al.*

2004). AFPs can be also applied to improve the quality and viability of sperm and ovaries in the short and long term (Qadeer *et al.* 2016; Correia *et al.* 2021; Robles, Valcarce, and Riesco 2019; Correia *et al.* 2024). Additionally, cryopreserved sperm and ovaries can be used for bioconservation, as well as in artificial insemination and in vitro fertilization procedures (Ekpo *et al.* 2022; Mehdipour *et al.* 2021). In addition to transplants and cell preservation, vaccines have also played a crucial role in improving global health (Greenwood 2014). For example, the COVID-19 pandemic had led to over 660 million identified cases of respiratory syndrome-associated coronavirus 2 (SARS-CoV-2) (Chakraborty, Bhattacharya, and Dhama 2023), with an estimated 14 million deaths prevented from COVID-19 between 2020 and 2021 due to vaccinations (Watson *et al.* 2022). The storage temperature for COVID-19 vaccines can reach -80 °C , and this process can impact the vaccines effectiveness (Pambudi *et al.* 2022). In this context, AFPs have the potential to enhance the thermal stability of vaccines, particularly at cold temperatures, thereby significantly increasing their effectiveness. This is crucial, as current vaccines rely on a continuous cold chain to prevent loss of potency due to thermal denaturation or degradation. Such an approach would not only reduce the costs associated with refrigeration logistics but also minimize the loss of valuable vaccines due to exposure to inadequate temperatures (Kumar *et al.* 2022; Karp *et al.* 2015).

Antifreeze proteins hold significant importance in the pharmaceutical industry due to their ability to preserve the structural integrity and functionality of bio-active molecules during freezing and thawing processes. This includes effective medications for serious diseases, such as cancer and autoimmune diseases, and also on other molecules that rely on supply chains for global distribution, like antibiotics. (Fischer 2022; Tan, Mei, and Xie 2021). Antibiotics are a widely used therapeutic modality for the treatment of bacterial infections in various fields, including human health, agriculture, livestock, and aquaculture. By inhibiting ice crystal formation, AFPs help maintain the structural integrity of antibiotics during production, storage and transportation. In addition, AFPs have the potential to act as antimicrobial agents by binding to bacteria and altering biofilm formation. These proteins can interfere with bacterial defense mechanisms, offering a new approach to treating antibiotic-resistant infections (Heisig *et al.* 2014).

In agriculture, one of the most recurring issues is the loss of crops due to extreme low temperatures, such as frost damage. Climate changes have caused out-of-season frosts and snowstorms, exacerbating this problem. Among the numerous threats to food security, freezing has a significant impact on the productivity and yields of agricultural crops (Lee *et al.* 2024).

As a solution to this global challenge, genetic engineering is conducting research to develop genetically modified plants that produce AFP, creating more cold-tolerant plants (Fan *et al.* 2002; Hightower *et al.* 1991; Cho *et al.* 2019; Naing and Kim 2019).

The food industry has a particular interest in controlling ice crystal growth during freezing, storage, and transport of food products (Griffith and Ewart 1995; S. Xiao *et al.* 2024; Rosa *et al.* 2019; Zhou *et al.* 2024). For instance, the presence of AFPs can prevent the deterioration of food products by reducing undesirable changes in food quality, such as nutrient and flavor loss caused by freezing and thawing. For example, bakery products can have their shelf life extended by frozen storage, but at the cost of some issues such as reduced volume, weaker dough structure, poor texture, loss of flavor and decreased gas content. The use of AFPs have shown to improve dough quality during freezing storage, enhancing its softness and preserving loaf volume (Liu *et al.* 2018). Freezing storage is also a common long-term preservation method for products with high water content like vegetables and fruits. These frozen products could be used throughout the whole year, such as ingredients in jams, juices or fruit preparations. AFPs offer a promising solution for these long-term frozen products, preserving both nutrient quality and shelf life (Griffith and Ewart 1995; Xiao *et al.* 2024; Rosa *et al.* 2019; Zhou *et al.* 2024).

A recent and unusual branch of AFP application is in materials engineering and construction (Meng *et al.* 2024a; Meng *et al.* 2024b; Jung, Kim, and Jin 2020). The formation of ice in different surfaces causes several problems such as energy losses and structural damage to buildings and roads. In cold regions, for example, pavement freezing is a common issue for vehicle traffic. Traditional methods to remove surface ice, such as chemical and mechanical approaches, are expensive and can damage the pavement while contributing to environmental pollution. A promising alternative is the creation of antifreeze surfaces on pavements using APFs mixed with mineral powder, being an environmentally friendly solution while maintaining road performance capabilities (Meng *et al.* 2024a; Meng *et al.* 2024b; Jung, Kim, and Jin 2020). Other industrial applications include enhancing the cold resistance of equipments, especially on metallic surfaces like aviation and refrigeration storage sectors (Gwak *et al.* 2015; Jung, Kim, and Jin 2020).

Despite the wide variety of applications for AFPs, there is still limited knowledge about the effects of microbial proteins. Although many studies are intensifying in the large-scale production of AFPs through heterologous expression, the yield of produced protein is still

relatively low (**Supplementary Table S4**) (Kim *et al.* 2019; Do *et al.* 2014; Hanada *et al.* 2014; Kaleda *et al.* 2019; Mangiagalli *et al.* 2017; Raymond, Christner, and Schuster 2008; Raymond, Janech, and Mangiagalli 2021; Raymond, Fritsen, and Shen 2007; Vance, Graham, and Davies 2018; Kim *et al.* 2015a; Md Tab *et al.* 2017; Lee *et al.* 2013; Hashim *et al.* 2013, 2014; Park *et al.* 2012). Currently, the relatively high cost of AFPs is partly due to the need for isolation and purification of AFPs, which are time-consuming and expensive processes. The price remains high for commercial uses, with fish type III AFP costing about 10 dollars per milligram, depending on the product's purity (Ustun and Turhan 2020). However, compared to animal and plant AFPs, microbial proteins could be produced at lower costs due to their rapid growth coupled to simple and inexpensive nutrient sources for growth. Also, the production of microbial proteins could be enhanced by optimized conditions and easier genetic manipulation. Regarding the applications of antifreeze proteins from polar microorganisms, 12 studies covering the areas of health, industry, and agriculture have been identified (Lee *et al.* 2012; Muñoz *et al.* 2017; Koh *et al.* 2015; Gwak *et al.* 2015; Cho *et al.* 2019; Kim *et al.* 2015b, 2017; Khan *et al.* 2020; Sun *et al.* 1995; Lee *et al.* 2015; Liu *et al.* 2021; Nam *et al.* 2024) (**Supplementary Table S5**). Although a recent study has suggested that some insect AFPs may induce inflammatory responses on mammalian cells (Tran-Guzman *et al.* 2022), the potential side effects of microbial AFPs are yet to be studied.

Antifreeze proteins have garnered significant attention for their commercial applications across various fields, as evidenced by numerous patents revealed through the United States Patent and Trademark Office (USPTO), the European Patent Office (EPO) and the Japan Patent Office (JPO). While a patent, by itself, does not guarantee the commercial success of innovation efforts, it serves as an intermediate measure of innovation output (Kim and Lee 2015). Despite this broad interest, relatively few patents focus specifically on AFPs derived from polar microorganisms, and most existing patents focus on the processes for producing these proteins. For instance, the patent #US20140193854A1 outlines a method for mass-producing an antifreeze protein derived from the polar yeast *Glaciozyma* sp. This method involves synthesizing a recombinant polynucleotide, optimizing and altering a gene that codes for the antifreeze protein for a yeast expression system, and expressing it using this system.

According to Christopher Sidebottom and others (patent #US6774210B1), AFPs have not been widely applied in commercially available food products due to high costs and complex extraction processes. Additionally, suggested AFPs for frozen foods face challenges such as pasteurization, which can destabilize proteins due to denaturation. Despite these challenges,

patents already exist for the extraction of AFPs, their production, and their application in food products subjected to freezing. Furthermore, a patent by Takahisa Kusuura (patent #US8413462B2) describes a method for isolating nanoparticles in wastewater, which may accumulate in sludge and the food chain, posing health risks. This method involves preparing an aqueous mixture with nanoparticles and a thermal hysteresis molecule (AFP), regulating the temperature to form ice crystals that incorporate the nanoparticles, and then separating these ice crystals from the mixture. In addition to technological success, other crucial factors for bringing biotechnologies to market include regulations, policies, and social norms (Kim and Lee 2015).

The global market for antifreeze proteins is experiencing significant growth, with an estimated valuation of $10 million in 2023, projected to reach $50 million by 2028 (https://www.skyquestt.com/report/antifreeze-proteins-market, accessed on 30 May 2024). This increase is driven by a growing demand for solutions for storing and transporting cold-sensitive products. Among the leading companies in this sector are Japan's Nichirei Corporation and Kaneka Corporation, the US-based A/F Protein Inc., and the Netherlands' Unilever. These companies play a vital role in expanding and innovating the antifreeze protein market, providing high-quality products for a wide range of industrial and commercial applications on a global scale (Ishii and Inoue 2019).

3.10 CONCLUSIONS

Cold environments are important reservoirs for rare and undiscovered microorganisms, novel genes, and valuable sources of innovative biotechnological processes. The significance of cold environments becomes even more evident when considering the potential applications of microbial AFPs in the industrial, medical, pharmaceutical, and agricultural fields. Despite their relevance, a limited number of AFP-producing microorganisms have been thoroughly studied and characterized. In this review, we summarized all data about microorganisms reported to produce AFPs and evaluated their characteristics on IR and TH activities on a comparative basis. Furthermore, a meta-analysis of the reported studies was conducted to further investigate the relationship between microbial diversity and AFP properties. Our analysis suggests the search for novel AFPs in less representative microbial groups, such as members of Domain Archaea and non-traditionally-cultivated Phyla of Bacteria. Exploration of frozen and semi-frozen ecosystems in both the Arctic and Antarctica should be expanded,

especially on non-explored polar areas. We also propose setting standard methods for studying IR and TH activities, specially about the protein concentration used in experiments. Finally, studies on the origin and evolution of microbial AFPs should receive more attention, in order to better understand the role of horizontal gene transfer on AFP genes.

SUPPORTING INFORMATION

The data used in this review are available in its online supporting information Table S1, Table S2, Table S3, Table S4, and Table S5.

SUPPORTING INFORMATION LEGENDS

Table S1. Summary of known AFP-producing microorganisms, including the isolation source, taxonomic identification, sequence database Ids, and their AFP properties. Numbers in column ID Map correspond to keymaps on Figure 10 (for Antarctic) and Figure 11 (for Arctic).

Table S2. List of Antarctic microorganisms with AFP activity. Numbers on the Code column correspond to their respective location shown in Figure 10. References for these isolates could be found in Table S1.

Table S3. List of Arctic microorganisms with AFP activity. Numbers on the Code column correspond to their respective location shown in Figure 11. References for these isolates could be found in Table S1.

Table S4. Summary of microbial AFPs explored for biotechnological applications, including the tested conditions for AFP production and/or characterization.

Table S5. Summary of microbial AFPs explored for biotechnological applications.

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4. CHAPTER II

FREEZING AND THAWING IN ANTARCTICA: CHARACTERIZATION OF ANTIFREEZE PROTEIN (AFP) PRODUCING MICROORGANISMS ISOLATED FROM KING GEORGE ISLAND, ANTARCTICA

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ABSTRACT

Antarctic temperature variations and long periods of freezing shaped the evolution of microorganisms with unique survival mechanisms. These resilient organisms exhibit several adaptations for life in extreme cold. In such ecosystems, microorganisms endure the absence of liquid water and exhibit resistance to freezing by producing water-binding molecules such as antifreeze proteins (AFP). AFPs modify the ice structure, lower the freezing point, and inhibit recrystallization. The objective of this study was to select and identify microorganisms isolated from different Antarctic ecosystems based on their resistance to temperatures below 0 °C. Furthermore, the study sought to characterize these microorganisms regarding their potential antifreeze adaptive mechanisms. Samples of soil, moss, permafrost, and marine sediment were collected on King George Island, located in the South Shetland archipelago, Antarctica. Bacteria and yeasts were isolated and subjected to freezing-resistance and ice recrystallization inhibition (IR) tests. A total of 215 microorganisms were isolated, out of which 118 were molecularly identified through molecular analysis using the 16S rRNA and the ITS regions. Furthermore, our study identified 24 freezing-resistant isolates (down to -18 °C), including two yeast and 22 bacteria. A total of 131 protein extracts were subjected to the IR test, revealing 14 isolates with positive AFP production. Finally, four isolates showed both freeze-resistance and IR activity (*Arthrobacter* sp. BGS04, *Pseudomonas* sp. BGS05, *Cryobacterium* sp. P64, and *Acinetobacter* sp. M1_25C). This study emphasizes the diversity of Antarctic microorganisms with the ability to tolerate freezing conditions. These microorganisms warrant further investigation to conduct a comprehensive analysis of their antifreeze capabilities, with the goal of exploring their potential for future biotechnological applications.

Keywords: *Antarctica; antifreeze proteins; extremophiles; freezing-resistance*

4.1 INTRODUCTION

Although freeze-thaw cycles limit the survival of most organisms, [1] several microorganisms can survive in the Antarctic extreme environments due to their resistance to freezing. These microorganisms present a series of adaptations to cold, including increased membrane fluidity and more efficient subzero metabolism, as well as the production of cryoprotective substances, such as exopolysaccharides [2,3] and antifreeze proteins (AFP) [4,5]. Previous studies have reported the freezing resistance of microorganisms [6–10], with some producing AFPs that effectively inhibit ice formation. AFPs present two distinct properties: Thermal Hysteresis (TH) and Ice Recrystallization Inhibition (IR). TH is a phenomenon where the freezing and melting points of a solution are different. AFPs in aqueous solution depress the freezing point of water, stopping ice crystals from growing even at subzero

degrees. In contrast, the IR activity refers to the prevention of the formation of large ice crystals at the expense of small crystals, thus protecting the cell against freezing damage. Therefore, AFPs play an important role as a survival mechanism for microorganisms living at sub-zero temperatures [4,10,11].

AFPs were first identified in the blood plasma of Antarctic fish inhabiting frigid environments [11] and subsequently found in a diverse array of organisms including plants, insects, algae, fungi, archaea, and bacteria [12–17]. Among bacteria, the description of AFP-producing species has increased substantially over the last years, including *Rhodococcus erythropolis [18]*, *Micrococcus cryophilus* [18], *Marinomonas protea* [19], *Pseudomonas putida* [20], *Moraxella* sp. [21], *Flavobacterium xanthum* [22], *Marinomonas primoryense* [23], *Colwellia* [24], Flavobacteriaceae [25], *Pseudomonas ficuserectae [26]*, *Flavobacterium frigoris* [27][28], *Sphingomonas*, *Pseudomonas*, *Plantibacter* [29], *Shewanella frigidimarina* [30], *Paenisporosarcina antarctica [31], Nostoc* sp.[32] *Marinomonas arctica* [17], and others [28]. Among yeast species, *Goffeauzyma gastrica* [10], *Rhodotorula svalbardensis [33]*, *Glaciozyma* sp. AY30 [34] and *Glaciozyma antarctica* [35,36] were also described as AFP producers. In addition to its role in cell survival, AFPs can be used in the cryopreservation of vaccines [37], foods [29], gametes and embryos [38]. Despite this potential, few studies have focused on biotechnological application of AFPs. In this context, our study investigates freezing-resistant microorganisms isolated from diverse Antarctic environments, with the aim of identifying efficient strains capable of producing AFPs for prospective biotechnological applications.

4.2 MATERIALS AND METHODS

4.2.1 Antarctic Environmental Samples

A variety of environmental samples were collected in the King George Island (Antarctica) during the summers of 2014/2015 and 2015/2016 as part of the MICROSFERA project (CNPq#407816/2013-5). Soil was collected using sterile laboratory spatulas at depths of 0-10 cm on the Baranowski and Collins Glacier forefields. Soils at distances of 0, 50, 100, 200, 300, and 400 m from the Baranowski and Collins southwest-face glacier front were sampled, and at distances of 0, 25 and 50 m from Collins southeast-face. Mosses from Collins and Baranowski soils were sampled for the isolation of endophytic microorganisms. Five

permafrost samples were collected near the Russian Station "Bellingshausen" during the 54th Russian Antarctic Expedition in the summer of 2008/2009 at depths of 4.6 to 9.2 m on-site A11/09, which corresponds to a frozen period of approximately 2250 to 7500 years before present [39]. A surface marine sediment sample (B2) from Maxwell Bay was collected at a depth of 20 m using a Van Veen grab. All samples were kept frozen during fieldwork, transportation, and storage (**Figure Chapter II 1, Table S1**).

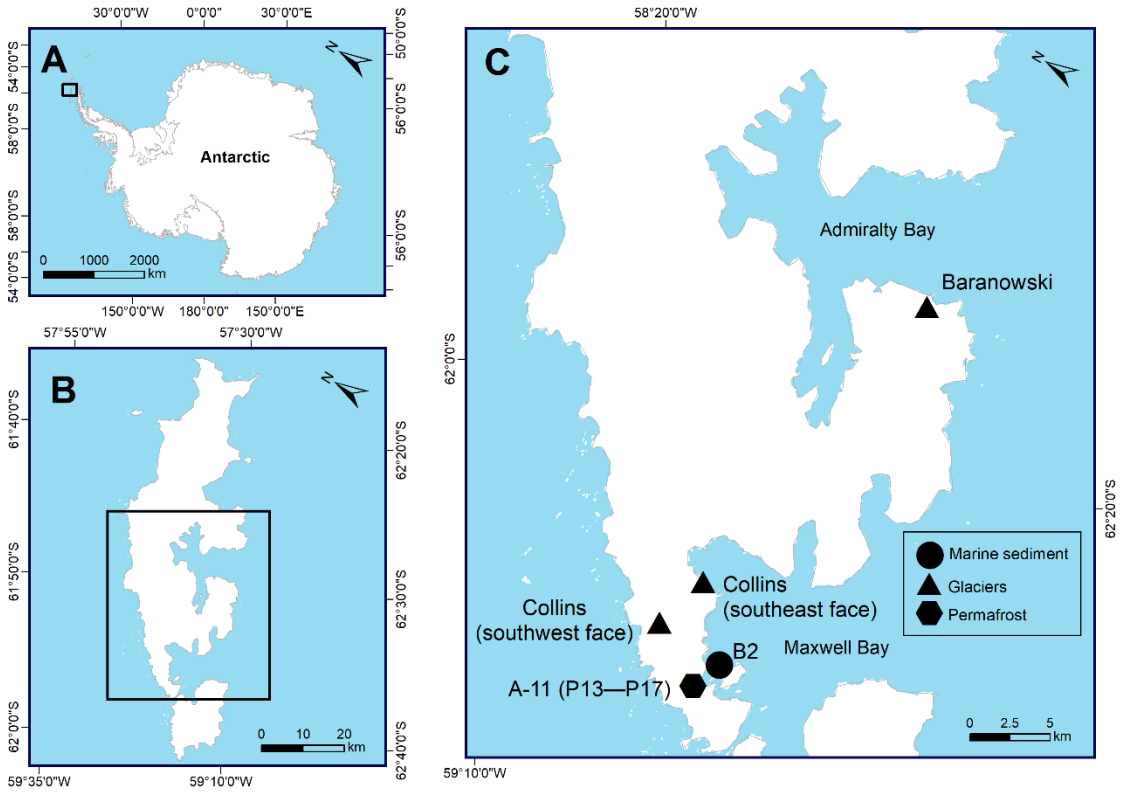

Figure Chapter II 1 - Map illustrating the sampling locations. A. Antarctic continent with the location of King George Island in the square; B. King George Island with the rectangle indicating the sampling areas; C. Detailed map of the King George Island showing the sampling sites of the Baranowski Glacier, Collins Glacier both southeast and southwest faces, the marine sediment site B2, and the permafrost borehole A-11 (samples P13-P17).

4.2.2 Isolation and Characterization of Antarctic Bacteria and Yeasts

Isolation techniques varied based on the characteristics of each environmental sample. For soil, isolation involved diluting a portion of the sample (3.0 g of moist soil) in a 50 mL Falcon tube containing 27 mL of 1X PBS buffer (comprising 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1000 mL of distilled water). The soil suspension was vortexed for 30 seconds, and 100 µL were inoculated onto plates containing R2A medium (Difco) composed of 18 g Agar, 0.5 g Yeast Extract, 0.5 g Hydrolyzed casein, 0.5 g Glucose, 0.5 g Soluble starch, 0.3 g K_2HPO_4 , 0.3 g Sodium pyruvate, 0.25 g Peptone, 0.05 g $MgSO_4$ heptahydrate, 1000 mL

of distilled water at concentrations of 100% and 10% (w/v). Additionally, plates were prepared with Soil Extract Medium, consisting of 20% sterile soil extract (w/v) from Collins or Baranowski glaciers diluted in sterilized distilled water and 15 $g.L^{-1}$ agar. For the preparation of the medium, 192.5 g of Antarctic soil and 0.5 g of calcium carbonate were weighed and diluted in 500 mL of distilled water. This solution was autoclaved, filtered (lã de vidro), and transferred to bottles containing agar. For the isolation of endophytic microorganisms, moss samples were used following the methodology described in Rosa *et al.* [40]. The methodology consisted of fragmenting the moss into 1x1cm pieces, then soaking the moss in 70% ethanol for one minute, followed by immersion in 2% hypochlorite for three minutes, and rinsing twice in autoclaved water for two minutes each. After sterilization, the moss was placed on plates containing R2A medium. The methodology described by Vishnivetskaya *et al.* [41], designed for recovering and isolating bacteria from Siberian permafrost served as the basis for isolating microorganisms from our permafrost samples. For the isolation of microorganisms from permafrost and marine sediment, TSA medium was used (15 g Tryptic Casein Digest, 5 g Soy Peptone, 5 g Sodium Chloride, 15 g Agar, 1000 mL of distilled water). The incubation temperatures of 25, 15, and 6 °C were used in all isolation experiments. The colonies derived from soil, moss, and permafrost were counted and characterized according to their morphology, encompassing features such as shape, size, and pigment. Gram staining was employed to determine the shape, arrangement, and bacterial cell wall type.

4.2.3 Selection and Identification of Isolates

Isolates were genetically differentiated by comparing the repetitive DNA band profile generated by the BOX-PCR [42]. Total bacterial DNA was extracted using the boiling method, where one colony was diluted in 20 µL of sterilized MilliQ H_2O, followed by boiling at 99 ºC for three min. The BOX-PCR was carried out as described by Versalovic *et al.* [42] for a volume of 25 µL, consisting of 2.5 µL of 10X Buffer, 1.9 µL of 50 mM $MgCl_2$, 1.25 µL of 100% DMSO, 0.6 µL of 25 µM Box-A1R primer, 0.2 µL of 100 mM dNTPs, 0.1 µL of 5 $U.\mu L^{-1}$ Taq Polymerase, and 1 µL of the extracted DNA. The amplification started with a hot start at 94 ºC for 7 min, followed by 35 cycles of 94 ºC for 7 min, 53 ºC for 1 min, 65 ºC for 8 min, and a final extension at 65 ºC for 15 min. The PCR products were verified by 1% of agarose gel electrophoresis in 1X TAE at 40V for 3 hours, and stained with GelRed (Invitrogen). Gels were exposed in a UV Chemidoc MP transilluminator (Bio-Rad, Inc.), and photographed using

ImageLab software (Bio-Rad, Inc.). The bands generated were grouped by similarity, and a dendrogram was constructed with the UPGMA method and Dice coefficient in BioNumerics v.5.10 software (Applied Maths, Belgium). Distinct phylotypes (isolates) were selected for identification based on 16S rRNA gene sequencing.

Selected isolates were subjected to PCR with degenerate primers amplifying the 16S rRNA gene of bacteria, specifically 27F [43] and 1492R [44]. The PCR reaction for a final volume of 25 µL consisted of 0.75 µL of 50 mM $MgCl_2$, 0.20 µL of 100 mM dNTPs, 0.25 µL of 20 µM 27F primer, 0.25 µL of 20 µM 1401R primer, 0.10 µL of 5 U Taq Polymerase, and 1 µL of extracted DNA. The amplification began with a hot start phase at 95 ºC for 10 minutes, followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, and extension at 72 ºC for 30 seconds. Lastly, a final extension step was carried out at 72 ºC for 10 minutes. A positive control containing DNA extracted from *Escherichia coli* ATCC 25922 (25 ng) and a negative control (MilliQ water) were prepared. The PCR products were verified as described above. For fungal isolates, yeast DNA extraction followed the protocol of Green and Sambrook [45]. Extracted DNA underwent PCR using primers ITS-1 and ITS-4 [46]. PCR reactions comprised 2.5 µL of 10X buffer, 1 µL of 50 mM $MgCl_2$, 0.2 µL of 100 mM dNTPs (25 mM each), 0.5 µL of each primer (20 µM each), 0.2 µL of 5 U Taq polymerase, 1 µL of extracted DNA, and sterile ultrapure water to a final volume of 25 µL. The PCR started with an initial step at 94 °C for 3 minutes, followed by 35 cycles consisting of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 2 minutes. A final extension was performed at 72 °C for 10 minutes. Both bacterial and fungal PCR products were purified [47], and quantified on NanoDrop. Samples containing a minimum of 30 $ng.\mu L^{-1}$ of amplified DNA were sent for Sanger sequencing at Macrogen Company (South Korea).

Bacterial 16S rRNA sequences were identified using the SILVA database [48]. Initially, sequences files were converted from AB1 to .fasta and .qual using a custom Python script, and these files were assessed using Mothur v.1.48.0 for sequencing quality analysis [49]. Sequences were checked for nucleotide quality using a window size of 20 bases and a minimum average Phred score equal 20. Nucleotide regions below this criteria were trimmed out towards the 3' end of the sequence. Sequences were aligned and taxonomically classified on Mothur against the SILVA 138 database. Fungal ITS sequences were identified similarly, but using the GenBank database.

4.2.4 Evaluation of Freezing Resistance in Antarctic Isolates

For the freezing test, the isolates were cultured on R2A and TSA medium at 25 °C to obtain isolated colonies. Subsequently, one colony from each plate was collected using a platinum loop and inoculated into 25 mL of R2B and TSB medium (without agar) in a 50 mL Falcon tube. The inoculum was incubated at 6 °C for two weeks. After growth, 1 mL of each culture was transferred to a 1.5 mL tube, and serial dilutions were prepared up to 10^{-6} on a 96-well plate to enable the initial counting ("N0") of colony-forming units (CFU). For this, each well of the plate was filled with 90 µL of PBS 1x buffer solution and 10 µL of the culture, in triplicate. Subsequently, the initial cell suspension (N0) was subjected to freezing at -18 ºC for 24 hours. The cell suspension was thawed at room temperature for 30 minutes, and a serial dilution was prepared on a 96-well plate as described above ("N1"). Aliquots of 10 µL from the dilutions of plates N0 and N1 were inoculated on R2A and TSA medium, according to the medium used in isolation, for CFU counting, in triplicate.

The survival rate was calculated using an equation presented by Kwon *et al.* (2018) [50]:

$$S\ (\%)\ = \frac{N1}{N0} \times 100$$

where S (%) represents the survival rate; "N1" the viable cell density after thawing, and "N0" the initial cell density. *Escherichia coli* ATCC 25922 was used as a negative control.

4.2.5 Assessment of Ice Recrystallization Inhibition Activity in Microbial Protein Extracts

For the recrystallization inhibition test, 131 isolates were selected and evaluated for AFP production using the method of Gilbert *et al.* [4] modified by Cid *et al.* [28], where AFP presence is indirectly detected by recrystallization inhibitory activity (IR). Initially, a cellular extract was prepared from bacterial cultures in flasks containing 100 mL of the R2B, incubated at temperatures of 4 ºC or 15 °C for 1 week with constant agitation. Cell lysis was achieved by adding a protease inhibitor buffer (25 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM PMSF in ethanol, 2 mg Pepstain A.mL^{-1} in methanol) with 0.3 g glass beads (Sigma) on vortex for 1

minute and cooled on ice for 1 minute (repeated 5 times). The supernatant was transferred to new tubes and frozen at -20 °C. Protein concentration was determined using a Multireader Infinite M200 TECAN spectrophotometer at 500 nm with the Bradford method and a BSA standard curve [51]. The cellular extract (50 µL) of each isolate was mixed with an equal volume of a 60% sucrose solution (w/v) and distributed in a 96-well plate. The positive control was prepared with the AFP III (A/F Protein Inc., USA) (0.5 $mg.mL^{-1}$ in 30% sucrose), and the negative control was made with 30% sucrose and protein extract from *E. coli* JM109. An aqueous solution of 30% sucrose (w/v) was used as blank. AFP screening in protein extracts was performed using a concentration of 0.5 $mg.mL^{-1}$ in 30% sucrose, as standardized for the positive control of AFP III. A 96-well plate containing protein extracts and controls in triplicate was frozen at -80 °C for 15 minutes, followed by incubation at -6 °C in the Thermo Scientific HM525 NX Cryostat for 2 days for the formation and stabilization of ice crystals. Subsequently, the plates were read on the Multireader Infinite M200 TECAN spectrophotometer at a wavelength of 500 nm, following the methodology of Cid *et al.* 2016 [28].

4.2.6 Statistical Analyses

For the freezing test, the S (%) of each isolate was determined and compared with the negative control (*E. coli*). The data were assessed for normality and homogeneity using the Kolmogorov-Smirnov and Cochran tests, respectively. Analysis of variance (ANOVA) was used to identify significant differences in population density before and after freezing cycles. Subsequently, the Student-Newman-Keuls (SNK) post hoc test was used to identify differences between means ($p > 0.05$). For the ice recrystallization inhibition (IR) test, the results were interpreted according to the principle of ice recrystallization, in other words, the presence of AFP in a solution will limit the growth of large ice crystals, favoring the formation of small ice crystals [52]. Absorbances with values higher than the negative control were considered positive for AFP presence by the Student's t-test ($p < 0.05$). All analyses were performed using RStudio software (http://www.rstudio.org/).

4.3 RESULTS

4.3.1 Isolation and Identification of Antarctic Microorganisms

A total of 215 microorganisms were isolated after the enrichment medium procedure (**Table S2**). Of these, 91 were isolated from Collins soils, 55 from Baranowski soils, 52 from the marine sediment, 13 from the permafrost and 4 from mosses. Our study isolated 121 bacteria and two yeast from R2A medium (100%), 62 bacteria and three yeast from TSA, 18 bacteria from R2A (10%), and 9 from soil extract (9 bacteria). Regarding different temperature exposures, 102 bacteria and two yeast were obtained at 25 ºC, 15 bacteria at 15 ºC, and 96 bacteria and three yeast at 6 ºC. About 58% of the isolates were Gram positive and 42% stained as Gram negative. Analysis of the BOX-PCR dendrogram (Figure S1) showed a redundancy of isolates found in different environmental samples, culture media and isolation temperatures.

A total of 118 isolates were identified through the sequencing of the 16S rRNA gene and the ITS region (**Table S2).** The most frequent bacterial phyla were Actinobacteriota (54.24%), Proteobacteria (20.34%), Bacteroidota (6.78%), Deinococcota (3.39%), and Firmicutes (11.02%). The fungal phyla found among the identified isolates were Ascomycota (2.54%) and Basidiomycota (1.69%). The identification of isolates revealed a total of 28 bacterial genera, with the most representative ones being *Arthrobacter* sp., *Planococcus* sp., *Pseudarthrobacter* sp., and *Psychrobacter* sp. Additionally, two yeasts were identified as *Rhodotorula* sp. through the ITS region **(Figure Chapter II 2A, Table S2)**.

A higher number of isolates was recovered from 25 °C compared with 6 °C (**Figure Chapter II 2B, D**). *Pseudarthrobacter* sp. was the genus with the highest number of identified isolates isolated under 25 °C. The isolates obtained at 15 ºC were represented by *Arthrobacter* sp., followed by *Pedobacter* sp., *Rhodococcus* sp., *Leifsonia* sp., *Paeniglutamicibacter* sp. and *Salinibacterium* sp. (**Figure Chapter II 2C**). In contrast, *Planococcus* sp. and *Psychrobacter* sp. represented the highest number of identified isolates at 6 ºC. We noticed the highest number of identified isolates in R2A 100% (n = 77), followed by TSA (n = 31), R2A 10% (n = 7), and soil extract (n = 3), whereas Collins Glacier soil samples provided the highest number of identified isolates (n = 54), followed by Baranowski Glacier soil (n = 31), marine sediment (n = 17), permafrost (n = 13) and moss (n = 3).

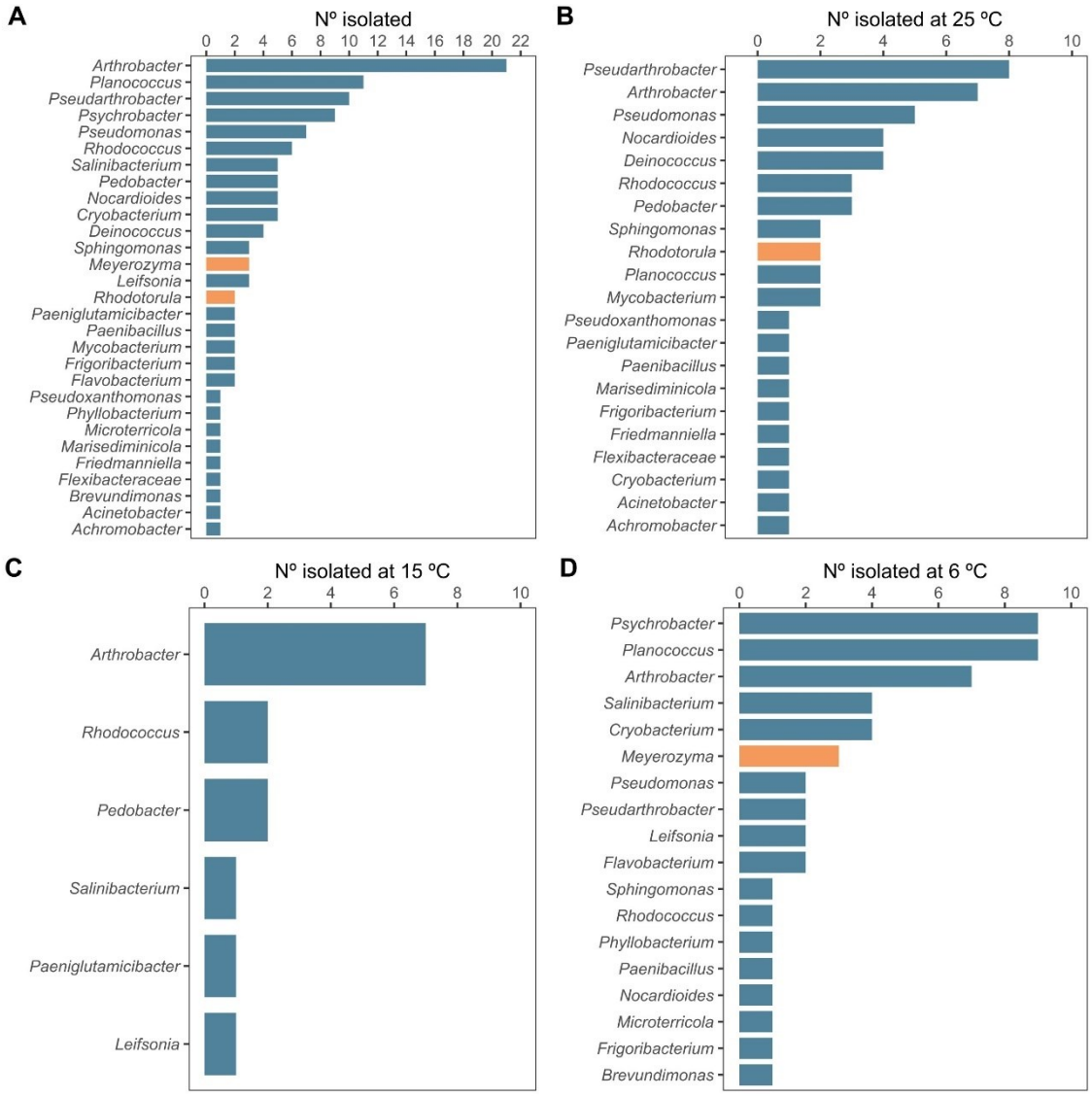

Figure Chapter II 2 - Total number of isolates related to bacterial (blue bars) and fungal (orange bars) genera identified by 16S rRNA and ITS region sequencing (A), and the number of isolates distinguished by the incubation temperature at 25 °C (B), 15 °C (C), and 6 °C (D).

4.3.2 Evaluation of Freezing Resistance in Antarctic Isolates

Antarctic isolates were tested for freezing resistance at -18 °C and a total of 24 isolates exhibited a survival rate greater than 50% when compared to their respective cell concentration before freezing ($p > 0.05$), indicating the presence of some mechanism of resistance to the lethal effects of ice (**Figure Chapter II 3A**).

Among the freeze resistant microorganisms, 22 bacterial isolates from different Antarctic samples and two yeasts (*Rhodotorula* sp. C01 and *Rhodotorula* sp. C1001) isolated from the Collins Glacier retreat soil showed a freezing survival rate equal to or greater than

50%. We identified a predominance of freezing-resistant isolates obtained from Antarctic permafrost (n = 14) and glacier retreat soils (n = 9), compared to a single freezing-resistant isolate obtained from moss (**Table S2)**.

Isolates obtained from permafrost were evaluated for freezing resistance in two different culture media (**Figure Chapter II 3B-C**). Among the 26 permafrost isolates tested in the R2A culture medium, nine (34.61%) achieved a survival rate close to 100%. Concerning the TSA culture medium, 15 permafrost isolates were tested and three were freeze-resistant, with an average survival rate of 96,65% (*Salinibacterium* sp. P49), 86,49% (*Planococcus* sp. P25) and 76,93% (*Planococcus* sp. P6).

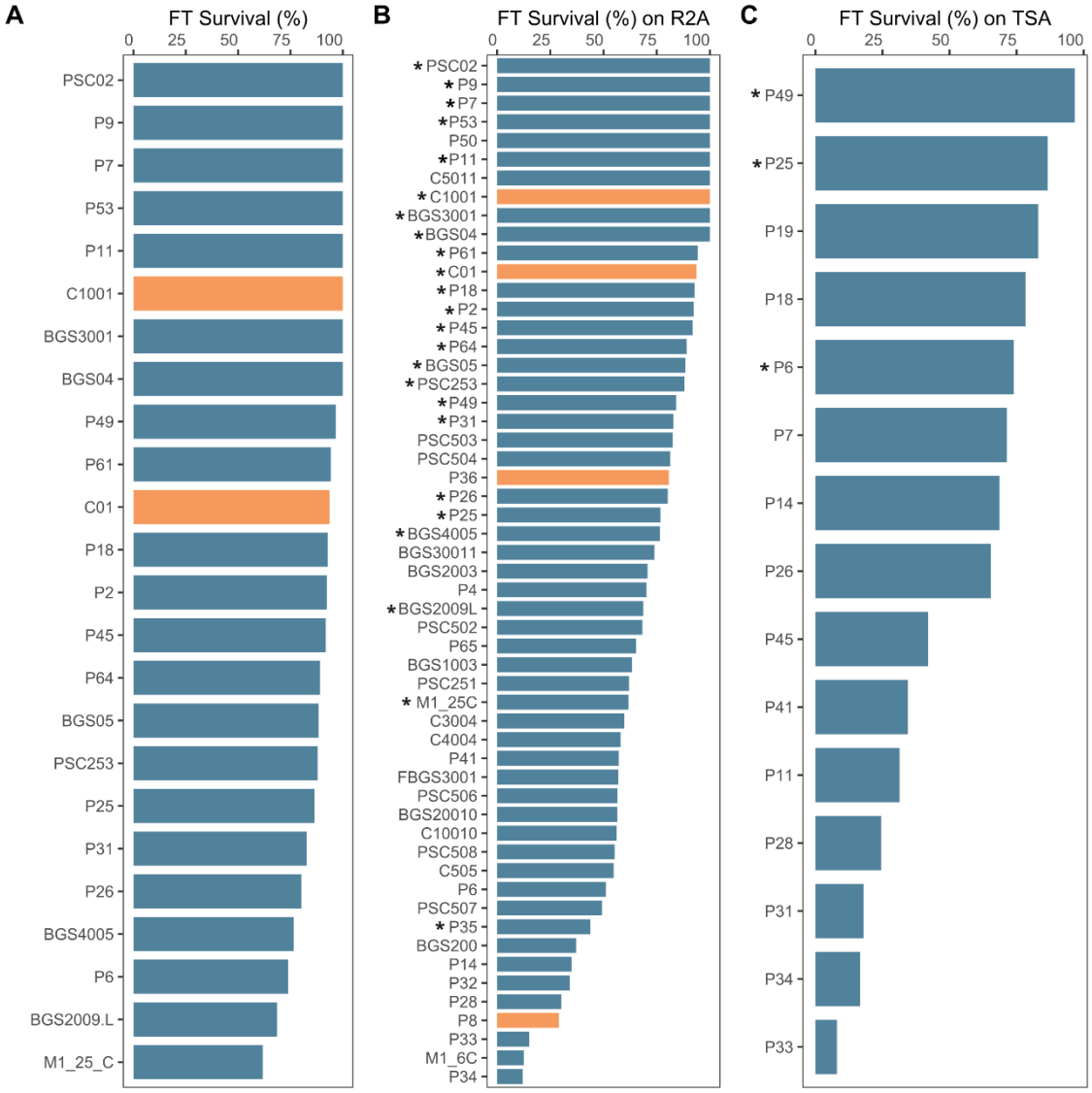

Figure Chapter II 3 - Bacterial (blue bars) and fungal (orange bars) isolates resistant to freezing at -18 °C (A); Freezing resistance profile at -18 °C concerning R2A/R2B culture media (B) and TSA/TSB (C). Isolates marked with asterisks (*) represent those with significative ($p<0.05$) freeze resistance. Some isolates showed statistical significance on both tested culture media. The experiment was conducted in triplicate.

Out of a total of 18 identified isolates obtained from glacier retreat soils, 9 (50%) were freeze-resistant: two (22.22%) from Collins Glacier-southwest face (*Rhodotorula* sp. C01 and *Rhodotorula* sp. C1001), two (22.22%) from Collins Glacier-southeast face (*Microterricola* sp. PSC02 and *Psychrobacter* sp. PSC253), and the remaining five (55.56%) from Baranowski Glacier (*Arthrobacter* sp. BGS04, *Pseudomonas* sp. BGS05, *Rhodococcus* sp. BGS2009L, *Rhodococcus* sp. BGS3001, and *Pedobacter* sp. BGS4005).

Out of the two microorganisms isolated from moss samples obtained at Collins Glacier (*Paenibacillus* sp. M1_6C and *Acinetobacter* sp. M1_25C) tested for freezing resistance, *Acinetobacter* sp. M1_25C showed freezing resistance with a survival rate of 61.82%, while *Paenibacillus* sp. M1_6C showed a lower survival rate of 12.54%.

4.3.3 Assessment of Ice Recrystallization Inhibition Activity in Microbial Protein Extracts.

The IR activity, which indicates the presence of AFP in aqueous solution, was detected using the methodology developed by Cid *et al.* [28]. First, in order to select the most sensitive absorbance that reveals IR under our conditions, a spectrophotometer scan was performed for the negative controls BSA (at concentrations of 0.25, 0.5, and 1 $mg.mL^{-1}$) and *E. coli* (protein extract at 0.5 $mg.mL^{-1}$), the positive control fish AFP III (concentrations ranging from 0.01 to 1 $mg.mL^{-1}$), and the blank (water + 30% sucrose).

The standardization of the IR detection protocol under our conditions showed that increasing concentrations of BSA (**Figure Chapter II 4A**) and AFP III (**Figure Chapter II 4B**) also enhances light absorbance, reaching a plateau after about 400 nm the spectra. When comparing the positive and negative controls, the scan showed different absorbances of light at the same wavelengths (**Figure Chapter II 4C**). Based on these results, and in agreement with the report of Cid *et al.* (2017), we selected the wavelength of 500 nm for IR activity detection. This wavelength was able to distinguish the positive (AFP III) and negative (BSA) controls at different concentrations (**Figure Chapter II 4D**).

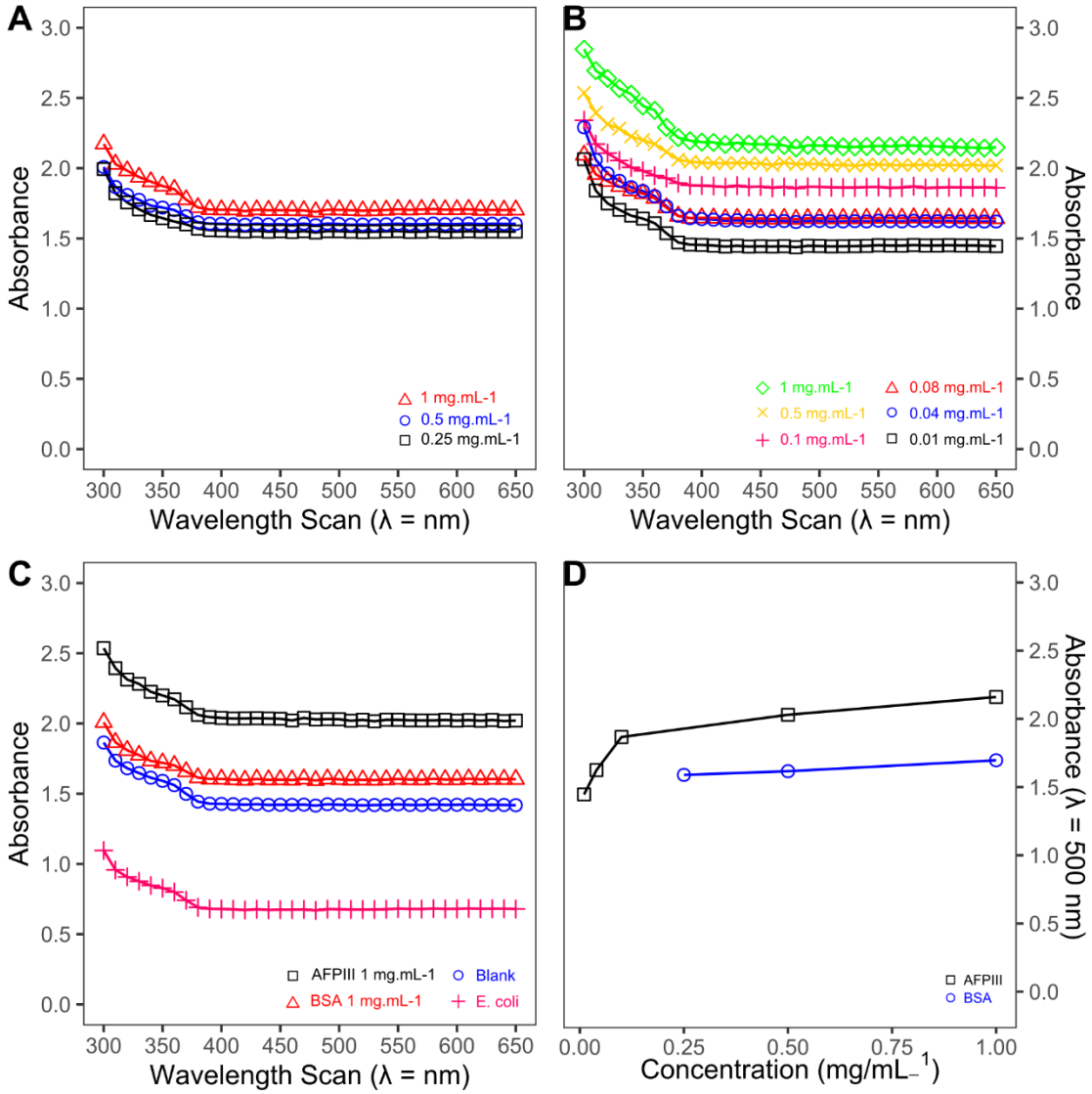

Figure Chapter II 4 - Scanning of concentrations of bovine serum albumin (BSA; 0.25 to 1 mg.mL^{-1}) (A), AFP type III (0.01 to 1 mg.mL^{-1}) (B), negative (Blank, 1 mg.mL^{-1} BSA, and *E. coli*) and positive (1 mg.mL^{-1} AFP III) controls (C), and different concentrations of AFP III and BSA at 500 nm (D). The experiment was conducted in triplicate.

Out of the 131 bacterial protein extracts tested, 81 bacterial protein extracts were positive for IR activity, showing absorbance values above the negative control (*E. coli* protein extract at 0.5 mg.mL^{-1}). After the statistical analysis, 14 of these protein extracts resulted in significant ($p < 0.05$) absorbances compared to the *E. coli* control, indicating the presence of IR activity in these extracts (**Figure Chapter II 5**). Except for P64, all other protein extracts showed absorbance values similar ($p > 0.05$) to the positive control AFP III. This result suggests that 13 isolates could inhibit ice recrystallization as well as the purified AFP III, while isolate P64 protein extract has IR activity but is weaker than AFP III.

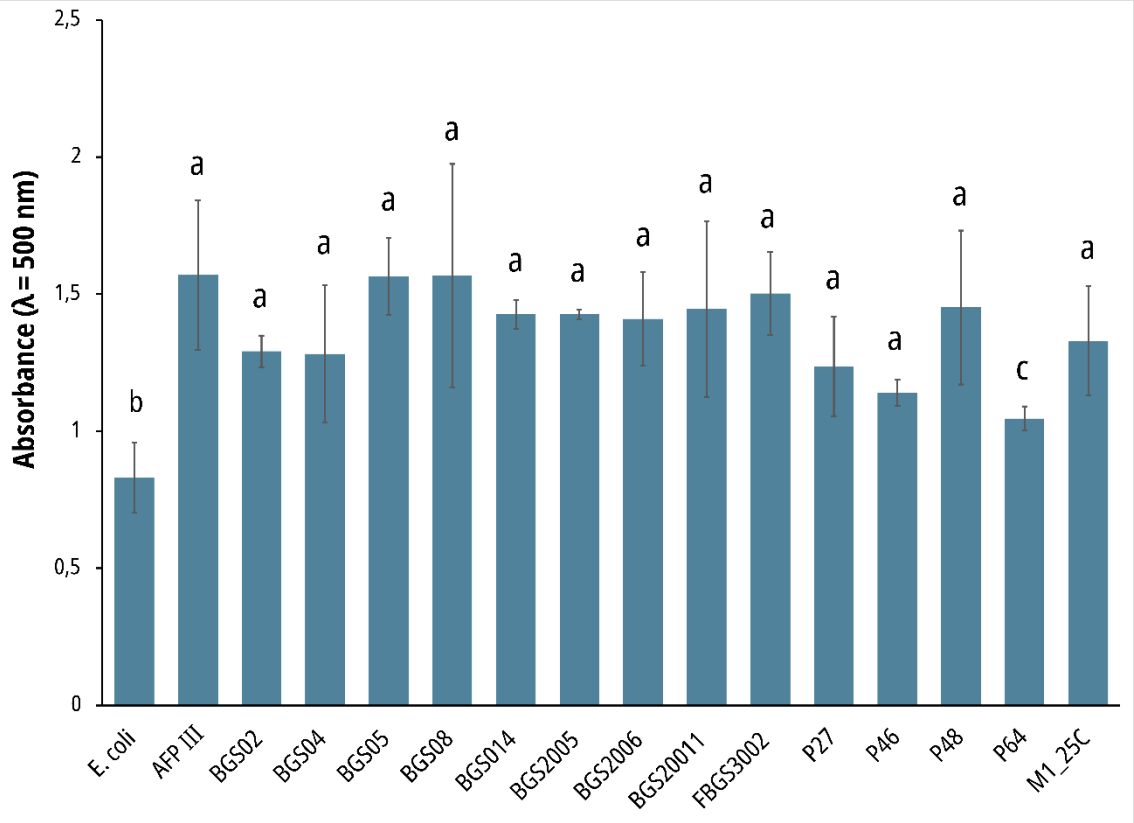

Figure Chapter II 5 - Absorbance reading during the IR test for the negative control (*E. coli* protein extract), positive control (AFP III) and protein extracts of Antarctic isolates. All controls and protein extracts were tested at 0.5 mg.mL^{-1}. The letter above error bars denote statistical diferences ($p<0.05$) after t-test. The experiment was conducted in triplicate.

4.4 DISCUSSION

Antarctica harbors the coldest ecosystems on Earth, and for a long time has been the focus of biotechnological research involving cold-adapted microorganisms. In this study, a total of 215 isolates were obtained from 23 Antarctic samples, including soils exposed by glacier retreat, marine sediment, permafrost and moss-associated communities. Also, our isolation approach employed different culture media and incubation temperatures in order to recover a wider range of microbial species.

The molecular identification of our bacterial isolates revealed a total of 5 phyla (Actinomycetota, Pseudomonadota, Bacillota, Bacteroidota, and Deinococcota) and 28 genera (Table S2). These phyla have been reported in numerous studies of Antarctic ecosystems using cultivation-dependent techniques. For example, García-Echauri *et al.*[53] used a cultivation approach to isolate 260 bacterial strains from different ecosystems around Collins Glacier, such

as the glacier sediment, seaside mud, melted ice, and *Deschampsia antarctica* phyllosphere. These authors used different culture media (PDA, LB, MRS, and YPG) and incubation strategies (aerobic and anaerobic, incubation at 4 °C) compared to our study and identified 70.3% of their isolates belonging to the phylum "Proteobacteria" (Pseudomonadota), while other groups such as "Gram-positive" (Actinomycetota and Bacillota, 16.7%) and "CFB" (Bacteroidota, 13%) were also found. Naturally, the isolates found by these authors differ from our study, since they used more organic-rich culture media and different incubation conditions. In our study, the decision to use organic-poor media intended to better simulate the oligotrophic Antarctic ecosystem. Even though not using anaerobic incubation, our strategy was more efficient to recover a wider range of phyla and genera. Although culture-dependent techniques are not suitable to describe the microbial community compared with metagenomics, the use of culture conditions that better simulate the environment is an interesting approach to recover a wider variety of microorganisms for further biotechnological screenings.

Our study found *Arthrobacter* sp., *Pseudarthrobacter* sp., *Planococcus* sp., and *Psychrobacter* sp. as the most frequent genera identified. Their frequent report in Antarctica likely relates to their ability of coping with the extreme cold environments. For example, *Arthrobacter* sp. is a cosmopolitan Gram-positive soil microorganism, and some Antarctic strains were previously reported to harbor AFP genes [54] while also being freeze resistant [55]. The genus *Pseudarthrobacter* composes Gram-positive psychrotroph aerobes formerly classified as *Arthrobacter* sp [56]. Despite sharing cold adaptations with their original group, the first Antarctic isolate of *Pseudarthrobacter* sp. was described only in 2020 [57], and, to our knowledge, there were no previous reports of AFP studies on this genus. *Planococcus* is a genus comprising both psychrotrophic and psychrophilic species, including *P. halocryophilus* Or1– reported to grow down to -15 °C [58]. Genomic studies on the type-strain *P. halocryophilus* SCU63 revealed that their cold adaptation derives from the synthesis of cold-shock proteins and organic solutes (e.g. betaine, proline, and trehalose), which contribute to maintain its cell osmotic balance under low water activity [59]. *Psychrobacter* is a genus of psychrotrophic and psychrophilic Gram-negative cocco-bacilli reported in dairy products, fermented seafood, fish, and poultry, although more commonly found in cold environments such as Antarctic sea ice, seawater, glacial ice, and permafrost soils [60, 61]. In our study, this genus was found in soils exposed by glacier retreat, marine sediment and permafrost samples (Table S2). Among several adaptations to cold observed in this group [62, 63], an Antarctic isolate of *Psychrobacter* sp.

also showed IR-activity [4], suggesting that Antarctic strains are strong candidates to produce efficient AFPs.

The natural freezing and thawing processes generally shape the existing microbial community in an environment [64, 65]. In our study, a total of 22 bacteria and two yeast isolates were freeze-resistant (Fig. 5). About 58.33% (*n*=14) of the isolates were bacteria obtained from Antarctic permafrost frozen for around 7500 years at King George Island. Freezing-resistant microorganisms are often reported in permafrost samples [66, 67], since they remain viable or dormant at sub-zero temperatures for thousands or millions of years [68]. Ancient microbial communities in permafrost represent a crucial resource for new insights into survival strategies, being an important source of microorganisms for biotechnological applications [69]. Enhancing the recovery of freeze-resistant isolates could also be achieved by using different culture media, at least for the permafrost community (Fig. 3B-C). For instance, in our study, a higher proportion of freeze-resistant bacteria was obtained from R2A plates (9 from 26=34.61%) compared to TSA (3 from 15=20%). Traditionally, the R2A medium is used to obtain oligotrophic microorganisms [70], which are expected to occur in samples with low organic matter content, such as in Antarctic permafrost.

Acinetobacter sp. M1_25C was the only freezing-resistant isolate obtained from the Antarctic moss phyllosphere in this study. *Acinetobacter* is described as a symbiont known to promote plant growth by synthesizing hormones and antioxidants, protecting plants from pathogens and helping with nutrient acquisition [71]. In Antarctica, microbial symbionts also play a role in the freezing survival of their hosts [72, 73]. It has been reported that the freezing resistance of Antarctic mosses is associated with endophytic bacteria producing AFPs that prevent freezing and consequently moss death [74]. While no further investigation was done on the symbiosis between M1_25C and Antarctic mosses, the freezingresistance of this strain could play an important role on the moss survival under sub-zero conditions.

Yeasts exhibiting freezing-resistance have been identified in many cold environments [73, 75]. The two yeasts C0-1 and C100-1 identified as *Rhodotorula* sp. in our work showed freezing-resistance of 93.75% and 100%, respectively. AFP producing yeasts were already discovered in Antarctica [10, 35, 36], but other freezing survival mechanisms are also present in Antarctic yeasts, such as the production of exopolysaccharides [76,77] that improve plasmatic membrane fluidity and prevent freezing [78]. A cold-adapted strain of *Rhodotorula mucilaginosa* isolated from Victoria Land (East Antarctica) showed almost 100% survival rate to desiccation after 27 days in dry conditions [79]. Frozen and dry environments share the lack

of liquid water, and this stress may select microorganisms capable of maintaining water molecules in the vicinity of their cells. In this scenario, organic molecules such as AFPs and exopolysaccharides could hold nearby water in liquid state [80]. It is unclear whether the freezing resistance in *Rhodotorula* sp. isolates C0-1 and C100-1 is linked to the production of exopolysaccharides, AFPs, or both. Further investigation of these yeasts is required to elucidate this aspect.

The inhibition of recrystallization (IR) test is a reliable method to detect antifreeze activity in both crude extracts or purified proteins [21,26,30]. This method has been successfully applied to detect AFP in Antarctic microorganisms isolated from lake sediments [4], sea ice [24], soils [22], and *Deschampsia antarctica* phyllosphere [28]. In our study,14 isolates were positively screened for AFP activity through the IR test,which corresponds to 10.68% of all tested microorganisms (Fig. 5). Our results are comparable to those of Cid *et al*.[28], where 12.07% of Antarctic microorganisms (32 out of 265) tested positive for the IR test. Using a similar method, Gilbert *et al*.[4] identified 21.59% of IR positive Antarctic microorganisms (187 out of 866). In contrast, a lower proportion of positive IR isolates was found by Yamashita *et al*.[21], who identified only 4.61% of ice recrystallization-positive Antarctic isolates (6 out of 130). Although slightly different methods were used to test IR activity,it seems that between 4 and 22% of culturable Antarctic microorganisms present some type of mechanism to inhibit ice recrystallization, including the production of AFPs.

In our study, most isolates positive for IR activity were isolated and grown at 25 °C, with the sole exception of the permafrost isolate *Cryobacterium* sp. P64, which was cultivated at 6 °C (Table S2). Cid *et al*.[28] isolated Antarctic microorganisms under 15 °C but underwent a cold acclimatization at 4 °C for one week before testing for IR activity. These authors reported an increase from 3 to 29% in IR-positive results after acclimatizing the cultures. Overall, these results indicate that psychrotolerant microorganisms isolated at room temperature can also produce AFPs under stressful conditions. This may be explained by AFP genes that can be constitutively expressed at intermediate levels, providing an immediate benefit when the protein is needed in bacterial metabolism under extremely cold freezing conditions [81].

Interestingly, four of our isolates demonstrated both freeze-resistance and IR activity: *Arthrobacter* sp. BGS04 and *Pseudomonas* sp. BGS05 isolated from Baranowski Glacier soils, *Cryobacterium* sp. P64 isolated from the permafrost, and *Acinetobacter* sp. M1_25C isolated from the moss phyllosphere collected at Collins Glacier. These bacteria are strong candidates for highly active AFPs and shall be the focus of future investigations in our research group.

4.5 CONCLUSIONS

In summary, our study successfully identified 24 freezing-resistant and 14 IR-active isolates from different Antarctic ecosystems, including four isolates exhibiting resistance to freezing alongside with the ability of hindering ice recrystallization. We emphasize the potential of using diverse culture media and isolation temperatures along with multiple sample sources, thereby enhancing the overall diversity potential of recovered microorganisms. Furthermore, the combination of freeze-resistance and IR activity tests has shown to be effective and cost-effective for preliminary AFP screening. Further studies on the Antarctic isolates are warranted due to their potential in cryopreservation and other biotechnological applications.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s42770- 024-01345-7.

Supplementary Material 1: Fig. S1

Dendrogram based on BOX-PCR band patterns for isolates from Antarctic marine sediment, permafrost, soils, and moss. The Bray-Curtis index was used to calculate the similarity between BOX-PCR patterns.

Supplementary Material 2: Table S1

List of environmental samples used as source for microbial isolation, including GPS location and sample type.

Supplementary Material 3: Table S2

List of all microbial isolates in the study, showing the GPS location and type of sample used as isolation source, the temperature and culture media used for isolation, gram stain test, and the taxonomical identification for selected isolates.

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5. CHAPTER III

ANTIFREEZING PROTECTION: ANALYSIS OF PROTEIN EFFICACY IN CELL AND VIRUS PRESERVATION

This article will be submitted to an A1-ranked journal in the field of biotechnology.

LOPES, J.C.; BONIN, I. T.; BRUNA-ROMERO, O.; DUARTE, R. T. D. Antifreezing Protection: Analysis of Protein Efficacy in Cell and Virus Preservation.

4.6 ABSTRACT

Antarctic microorganisms, adapted to extreme environments, often produce cryoprotective substances. This study evaluated the freezing resistance of 21 isolates at -80 °C and analyzed them using scanning electron microscopy. Four microorganisms—*Rhodotorula* sp. C01, *Pedobacter* sp. BGS4005, *Psychrobacter* sp. P61, and *Salinibacterium* sp. P45—were selected for intracellular and extracellular protein extraction. These extracts were applied to *Escherichia coli*, HEK293 cells, and adenovirus to evaluate their cryoprotective effects and viral stability. Notably, *Psychrobacter* sp. P53 maintained cell viability after freezing at -80 °C, indicating high resilience. Extracellular extracts from *Pedobacter* sp. and *Psychrobacter* sp. improved *E. coli* viability by 5% and 4%, respectively, compared to a 10% improvement with Type III AFP. For HEK293 cells, intracellular extracts achieved viability similar to DMSO (10-15%), while Type III AFP reached 50%. Adenovirus suspensions treated with protein extracts maintained their titers over 60 days at 4 °C. The extract from *Psychrobacter* sp. P61 and Type III AFP effectively preserved viral stability, showing no significant loss in titers after 60 days. These findings underscore the potential of Antarctic microorganisms for biotechnological applications in cryopreservation and viral stability.

Keywords: *cryoprotection*, *Antarctica*, *cell viability*, *viral stability*, *antifreeze proteins*.

5.1 INTRODUCTION

Antarctica is one of the coldest environments on Earth, hosting a microbiota highly adapted to its extreme conditions (Liang *et al.*, 2022; Christner, 2010). Bacteria and fungi in this ecosystem exhibit various cold adaptations, such as highly efficient enzymes at low temperatures, structural changes in cell membranes, and the production of cryoprotective substances, including exopolysaccharides and antifreeze proteins (Gonçalves *et al.*, 2024; Joseph; Kumar; Ramteke, 2019; Ramasamy *et al.*, 2023; Uko *et al.*, 2024). Antifreeze proteins (AFPs) play a crucial role in inhibiting intra- and extracellular ice formation, protecting organisms from the lethal effects of freezing (Lopes *et al.*, 2024b; Eskandari *et al.*, 2020; Davies; Sykes, 1997).

Two essential properties have been observed in AFPs. The first is ice recrystallization inhibition (IRI) activity, which prevents smaller ice crystals from absorbing water molecules and merging into larger crystals during recrystallization (Knight; Duman, 1986; Rahman *et al.*, 2019). The second is the thermal hysteresis (TH) activity, which raises the freezing point, thereby preventing ice formation. Both properties are fundamental for the survival of microorganisms in polar regions (Barrett, 2001; Guo *et al.*, 2012).

In addition to their ecological role in Antarctica, AFPs hold great biotechnological potential, particularly in health, industry, and agriculture. Studies have demonstrated the benefits of AFPs in preserving cells, organs, gametes, embryos, and food subjected to freezing, as well as in the production of genetically modified plants with AFP genes, conferring cold resistance. AFPs can also be used in materials engineering, such as antifreeze surfaces to prevent the freezing of equipment and pavements (Short *et al.*, 2024; Meng *et al.*, 2024; Gwak *et al.*, 2015).

Currently, in cell cryopreservation, DMSO is the primary cryoprotectant used due to its efficiency. However, DMSO is also known for its cellular toxicity (Verheijen *et al.*, 2019). Additionally, chemical reagents are often used on frozen surfaces, contributing to environmental pollution. These substances can seep into the soil and water systems, compromising groundwater quality and harming aquatic ecosystems. In this context, AFPs emerge as a less toxic alternative, offering not only a lower environmental impact but also promising results in terms of cell preservation. Recent studies show that AFPs can enhance cell viability after freezing, overcoming the limitations associated with traditional cryoprotectants (Sirotinskaya *et al.*, 2024). Moreover, AFPs applied to surfaces have demonstrated effectiveness in preventing freezing. Recent research indicates that these proteins offer a promising alternative to conventional chemical reagents, contributing to the development of safer and more environmentally friendly antifreeze surfaces (Short *et al.*, 2024; Meng *et al.*, 2024).

Currently, the commercial usage of AFPs is focused on marine proteins, primarily from fish types I, II, and III (Bogan *et al.*, 2024; Davies; Hew, 1990; Devries, 2020; Leal *et al.*, 2024). However, the exploration of AFPs from microorganisms, including bacteria, fungi, and algae from polar regions, is gaining attention (Batista *et al.*, 2020; Raymond; Remias, 2019; Singh *et al.*, 2021). Although many AFP-producing microorganisms have been described in Antarctica and the Arctic, most studies focus on bioprospecting and protein characterization, leaving gaps in research on their biotechnological applications (Munoz *et al.*, 2017; Khan *et al.*, 2019; Gwak *et al.*, 2015; Kim *et al.*, 2017; Lee *et al.*, 2015; Liu *et al.*, 2021; Nam *et al.*, 2024).

Few studies have investigated the effect of AFPs from Antarctic microorganisms on the cryopreservation of prokaryotic and eukaryotic cells. Preliminary studies with prokaryotic and eukaryotic cells using fish AFP types I and III have demonstrated the potential of these proteins in cell cryopreservation (Kawahara *et al.*, 2009; Sreter; Foxall; Varga, 2022).

However, no research has yet explored the use of these proteins to assess viral stability, an important gap in understanding the effect of these proteins on virus-based vaccines. Vaccine stability during transport is one of the main challenges in global distribution, particularly in regions subject to extreme temperature variations, which can compromise efficacy (Maheshwari *et al.*, 2004; Rexroad *et al.*, 2002; Tuladhar *et al.*, 2012). While research is underway to develop liquid vaccines that are stable at room temperature, the application of AFPs for this purpose has not yet been explored (Berg *et al.*, 2021).

Given the challenges of cell cryopreservation and viral stability, this study aims to evaluate the cryoprotective effect of total proteins obtained from Antarctic bacteria and fungi on prokaryotic and eukaryotic cells subjected to freezing at -18 ºC, as well as on adenovirus stability stored at 4 ºC.

5.2 MATERIALS AND METHODS

5.2.1 Antarctic Isolates

The experiments were conducted in the Laboratory of Molecular Ecology and Extremophiles (LEMEX), Department of Microbiology, Parasitology, and Immunology at the Federal University of Santa Catarina - UFSC. A total of 21 isolates obtained from previous LEMEX projects were used in the experiments (**Table 1**), all of them previously obtained from soil, moss, and permafrost samples collected in the Antarctic Peninsula (Lopes *et al.*, 2024a).

Table 1 - Antarctic isolates used in this study, including the taxonomic identification (16S rRNA and ITS region sequencing for bacteria and fungi, respectively), the GenBank accession number, and the source of isolation (Lopes *et al.* 2024a).

Code	Identification	Genbank Number	Source
C01	*Rhodotorula* sp.	OR237961	Collins Glacier Soil - West Face
C1001	*Rhodotorula* sp.	OR339876	Collins Glacier Soil - West Face
P6	*Planococcus* sp.	OR485314	Permafrost
P9	*Planococcus* sp.	OR485316	Permafrost
P11	*Planococcus* sp.	OR485317	Permafrost
P18	*Planococcus* sp.	OR485318	Permafrost
P25	*Planococcus* sp.	OR167740	Permafrost
P7	*Planococcus* sp.	OR485315	Permafrost
P26	*Psychrobacter* sp.	OR167755	Marine Sediment
P45	*Salinibacterium* sp.	PP534555	Marine Sediment
P49	*Salinibacterium* sp.	OR167753	Marine Sediment
P53	*Psychrobacter* sp.	OR485319	Permafrost
P61	*Psychrobacter* sp.	OR167757	Marine Sediment
P64	*Cryobacterium* sp.	OR167749	Marine Sediment
M1_25C	*Acinetobacter* sp.	OR485312	Collins Glacier Moss - West Face
BGS04	*Arthrobacter* sp.	OR167690	Baranowski Glacier Soil
BGS05	*Pseudomonas* sp.	OR167691	Baranowski Glacier Soil
BGS2009L	*Rhodococcus* sp.	OR485330	Baranowski Glacier Soil
BGS3001	*Arthrobacter* sp.	OR485331	Baranowski Glacier Soil
BGS4005	*Pedobacter* sp.	OR167708	Baranowski Glacier Soil
PSC253	*Psychrobacter* sp.	OR485320	Collins Glacier Soil – Southeast Face

5.2.2 Assessment of Freezing Resistance at -80°C

To select freezing-resistant isolates, a total of 21 microorganisms were subjected to freeze-thawing cycles. For the -80 °C freezing test, the 21 isolates were cultivated in R2A medium, composed of 18 g Agar, 0.5 g Yeast Extract, 0.5 g Hydrolyzed Casein, 0.5 g Glucose, 0.5 g Soluble Starch, 0.3 g K_2HPO_4, 0.3 g Sodium Pyruvate, 0.25 g Peptone, and 0.05 g $MgSO_4$ heptahydrate per 1000 mL of distilled water at 25 °C, to obtain isolated colonies. Subsequently, one colony from each plate was collected using a platinum loop and inoculated into 25 mL of liquid R2B medium in a 50 mL Falcon tube. The inoculum was incubated at 6 °C for two weeks in the dark, without shaking. From this growth, 1 mL of each culture was transferred to an Eppendorf tube, which in turn was centrifuged at 10.000 rpm for 3 minutes. The supernatant was discarded, and 1000 µL of PBS was added. After homogenization, serial dilutions up to 10^{-6} were prepared on a 96-well plate for initial colony-forming unit (CFU) count (N_0). Each well of the plate was filled with 90 µL of PBS and 10 µL of the culture, in triplicate. The initial cell suspension (N_0) was frozen at -80 °C for 24 hours, thawed at room temperature for 30 minutes, and again serially diluted to obtain N_1. Aliquots of 10 µL from the N_0 and N_1 dilutions were inoculated into R2A medium for CFU counting, in triplicate.

Survival rate was calculated using an equation presented by Kwon *et al.* (2018):

$$S\ (\%)\ = \frac{N1}{N0} \times 100$$

where “S” is the Survival Rate Freeze-Thaw (FT) expressed as a percentage (%); “N1” is the viable cell density after thawing, and “N0” is the initial cell density.

5.2.3 Morphological Characterization of Antarctic Bacteria and Yeasts by Scanning Electron Microscopy

For the morphological characterization of the 21 Antarctic isolates, cultivation was performed on R2A medium until colonies appeared. The cells were scraped off and transferred to a tube containing 100 µL of 0.1 M sodium cacodylate buffer (CH3)2AsO2H, homogenized, and centrifuged at 10.000 rpm for 3 minutes. Next, 50 µL of 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer was added and incubated for 24 hours. After incubation, the supernatant was discarded, and 50 µL of 0.1 M sodium cacodylate buffer was added, homogenized, and incubated for 15 minutes, repeating the process three times. The samples

were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. To do this, 25 µL of 1% osmium tetroxide solution in 0.1 M sodium cacodylate buffer was added to the tube containing the sample, and the samples were then incubated for 2 hours. Next, dehydration was performed with absolute ethanol at concentrations of 30%, 50%, 70%, 90%, and 100%. After dehydration, the samples were chemically dried with HMDS (hexamethyldisilazane) solution and fixed with double-sided tape on metal stubs. Metallization was carried out by depositing a thin layer of gold (10 nm) onto the samples, which were then analyzed by SEM at magnifications of 100x, 1000x, 3000x, 5000x, and 10, 000x, using an accelerating voltage of 15 kV.

5.2.4 Protein Extraction from Antarctic Bacteria and Yeasts

After selecting freezing-resistant isolates from -80 °C, total protein extraction was performed as described by Gilbert *et al.* (2004). The isolates were cultivated in R2B broth for two weeks at 15 °C and 4 °C with shaking at 200 rpm. After growth, the cultures were centrifuged at 5000 g at 4 °C for 15 minutes and then frozen at -18 °C to facilitate protein extraction. Cells were thawed and the biomass was standardized to 1 gram, followed by a treatment with 4 mL of Thermo Fisher B-PER kit, 8 µL of lysozyme at 50 mg/mL, and 8 µL of DNase at ≥ 2.500 units/mL. Samples were homogenized, incubated for 15 minutes at room temperature, centrifuged at 5.000 g at 4 °C for 15 minutes, and the supernatant was stored at -18 °C.

For yeast total protein extraction, the method described by Mouro (2016) was used. Yeasts were cultivated in 1 liter of R2B broth for two weeks at 15 °C and 4 °C, with constant shaking at 200 rpm. After growth, cultures were centrifuged at 5000 g and 4 °C for 15 minutes and then frozen to facilitate protein extraction. Thawed cells were standardized to 1 gram and washed with 500 µL of buffer A (100 mM MOPS-NaOH, pH 6.8). Cells were then centrifuged again at 3000 g for 5 minutes and resuspended in 200 µL of buffer B (100 mM MOPS, pH 6.8, containing 20% glycerol, 0.5 mM EDTA, 0.5 mM EDTA e 0.5 M DTT), 200 µL of glass beads were added. The mixture was vortexed and then centrifuged at 12, 000 g for 5 minutes (Mouro, 2016). The resulting supernatant was collected and stored at -18 °C.

Protein extracts were quantified using the Bradford method for subsequent standardization of the concentrations tested in the cryopreservation of prokaryotic and eukaryotic cells and in viral stability.

5.2.5 Characterization of the Antifreeze Activity of Microbial Proteins in the Cryopreservation of Prokaryotic Cells

To evaluate the antifreeze activity of microbial proteins in the cryopreservation of prokaryotic cells, the *Escherichia coli* ATCC 25922 strain was used. *E. coli* was cultured at 37 °C on TSA medium until colonies appeared, and an entire colony was then inoculated into 100 mL of TSB medium for 24 hours at 200 rpm and 25 °C. After this period, 1 mL of the culture was transferred in triplicates to Erlenmeyer flasks containing 100 mL of TSB medium, incubated at 200 rpm and 25 °C until an OD600 of 0.8 to 1 was reached. Three 1 mL aliquots of each culture were centrifuged at 10.000 rpm for 3 minutes, and the supernatant was discarded. The cells were washed twice with PBS, homogenized, and centrifuged again. Subsequently, the *E. coli* cells were resuspended in 1 mL of intracellular protein extracts at concentrations of 1 $mg \cdot mL^{-1}$ and 0.1 $mg \cdot mL^{-1}$. For the extracellular extracts (supernatant of centrifuged liquid culture medium), 1 mL was resuspended in *E. coli* cells. After homogenization, the serial dilution process described earlier was repeated, with the freezing temperature changed to -18 °C for 24 hours. The plates were prepared in triplicate, and the counting was performed using the previously mentioned formula. To confirm the protein's role in the cryopreservation of *E. coli*, two treatments were tested: one with proteinase K at 2.75 $mg.mL^{-1}$ at 25 ºC for 30 minutes, and the other involving heat treatment at 100 ° for 30 minutes.

5.2.6 Characterization of the Antifreeze Activity of Microbial Proteins in the Cryopreservation of Eukaryotic Cells

HEK293 cells were maintained in DMEM medium supplemented with 10% v/v fetal bovine serum and incubated at 37 °C with 5% CO_2. Once approximately 90% confluency was reached, the cells were washed with PBS and dissociated using trypsin. Cell viability was then assessed using 0.4% Trypan Blue staining and counting with a hemocytometer.

For cryopreservation, cells were evaluated for viability before freezing, selecting those with 90% confluency. The following cryopreservation conditions were tested for HEK293 cells (**Table 2**).

Table 2 - Stabilizers used in the study for the cryopreservation of HEK293 cells.

Stabilizer	Concentration
DMSO	10%
AFP III	1 mg·mL^{-1}
Rhodotorula sp. C01	1 mg·mL^{-1}
Pedobacter sp. BGS4005	1 mg·mL^{-1}
Psychrobacter sp. P61	1 mg·mL^{-1}
Salinibacterium sp. P45	1 mg·mL^{-1}
Fetal Bovine Serum (negative control)	40%

The tubes containing the cells and cryopreservation solutions were frozen at -18 °C for 30 days. After this period, cell viability was re-evaluated using 0.4% Trypan Blue staining.

5.2.7 Characterization of the Antifreeze Activity of Microbial Proteins in Adenovirus Stability

HEK293 cells were cultured in DMEM medium supplemented with 10% v/v fetal bovine serum and incubated at 37 °C with 5% CO_2 until approximately 90% confluency was reached. The cells were then infected with the non-replicative recombinant adenovirus RAdCMV-CE1 (Bruna-Romero *et al.*, 1997). After about 48 hours of infection, the cells were collected and centrifuged at 1.200 rpm and 4 °C in 50 mL tubes, with the supernatant discarded. The samples were frozen for subsequent Adenovirus extraction.

The samples were thawed and treated with 0.01 M Tris HCl and 5% sodium deoxycholate, followed by homogenization. After homogenization, the suspension was centrifuged, and the supernatants were collected and transferred to a 25 mL tube. The volume was adjusted to 15 mL with 0.01 M Tris HCl and centrifuged again. After additional washings, the reservoir volume was recovered, diluted in 0.01 M Tris HCl pH 8.0, and frozen.

Seven stabilizing solutions were prepared from the total protein extracts of Antarctic bacteria and yeasts (**Table 3**).

Table 3 - Stabilizers used in Adenovirus viral suspensions.

Stabilizer	Concentration
Glycerol	2.5%
AFP III	1 mg·mL^{-1}
Rhodotorula sp. C01	1 mg·mL^{-1}
Pedobacter sp. BGS4005	1 mg·mL^{-1}
Psychrobacter sp. P61	1 mg·mL^{-1}
Salinibacterium sp. P45	1 mg·mL^{-1}
Negative Control	0.01 M Tris HCl

Each stabilizing solution was added together with the viral stock at a 1:1 ratio (50 µL of viral stock and 50 µL of the stabilizing solution).

The stabilizing formulations were stored at 4 °C for 60 days. Viral thermal stability was assessed using viral titration by limiting dilution at five different time points (0, 15, 30, 45, and 60 days). Titers of recombinant adenoviruses were performed by serial dilutions in 5% DMEM medium and incubation with HEK293 cells. Plaque-forming units (PFU) counting was used to determine viral concentration in each condition.

5.2.8 Statistical Analysis

To evaluate the isolates resistant to -80 °C and the viability of *E. coli* subjected to treatments with intracellular and extracellular proteins, the survival rate (S) of each isolate was determined and compared to the negative control (*E. coli*). Initially, data were assessed for normality using the Kolmogorov-Smirnov test and for homogeneity using the Cochran test. An Analysis of Variance (ANOVA) was then used to identify significant differences in population density before and after freezing cycles. To identify differences between means, the post hoc Student-Newman-Keuls (SNK) test was applied, with a significance level of $p > 0.05$. In the analysis of HEK293 cell viability, statistical tests were applied to compare the percentage of survival among different treatments, providing a detailed understanding of the effects of each treatment on cell viability. For the analysis of viral stability, ANOVA was also used, allowing examination of statistical differences in viral titers between different experimental groups.

5.3 RESULTS

5.3.1 Evaluation of Freeze Resistance at -80°C

Twenty-one isolates were tested for freeze resistance at -80 °C. The results of the survival rates of the isolates showed significant variations (**Figure Chapter III 1**). Among the isolates with low survival rates, less than 10%, are *Rhodotorula sp.* C1001 (0.8%), *Planococcus sp.* P6 (7.20%), *Planococcus sp.* P18 (9.03%), *Arthrobacter sp.* BGS3001 (7.86%), *Cryobacterium sp.* P64 (1.04%), *Psychrobacter sp.* P61 (0.97%), *Pseudomonas sp.* BGS05 (0.95%), and *Planococcus sp.* P7 (6.15%). The isolates with intermediate survival rates (from 10% to 40%) include *Acinetobacter sp.* M1 25C (20.91%), *Rhodotorula sp.* C01 (12.84%), *Planococcus sp.* P9 (17.38%), *Arthrobacter sp.* BGS04 (23.33%), *Rhodococcus* sp. BGS2009L (24.88%), *Psychrobacter sp.* P26 (29.56%), *Psychrobacter sp.* PSC253 (34.04%), and *Planococcus sp.* P25 (12.74%). These values indicate a moderate resistance to the treatments applied at -80 °C. On the other hand, isolates with high survival rates, greater than 40%, include *Psychrobacter sp.* P53 (79.64%), *Pedobacter sp.* BGS4005 (49.87%), *Salinibacterium sp.* P45 (42.67%), *Salinibacterium sp.* P49 (52.08%), and *Planococcus sp.* P11 (43.52%). Notably, *Psychrobacter* sp. P53 maintained the same number of cells before and after freezing, with no significant difference ($p > 0.05$).

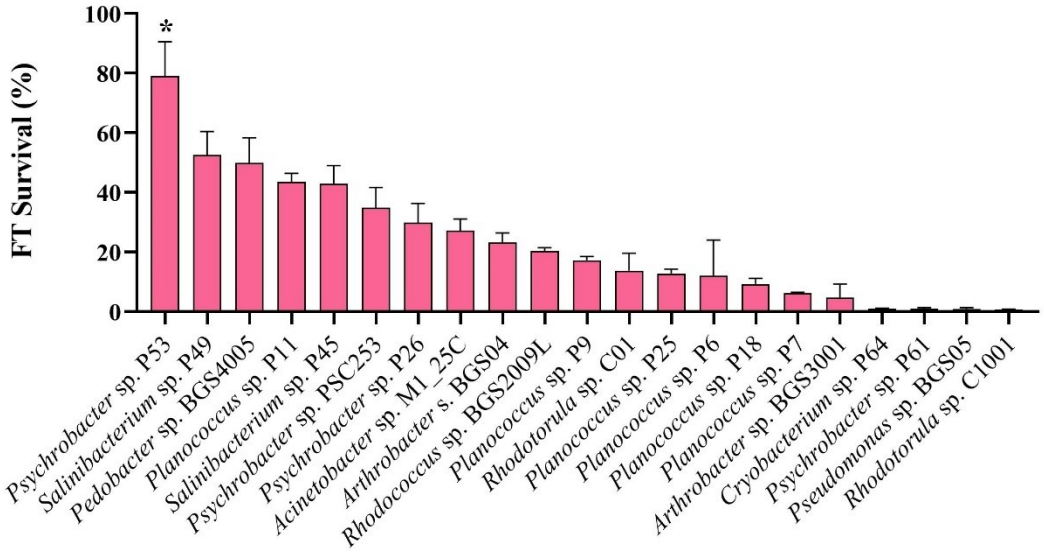
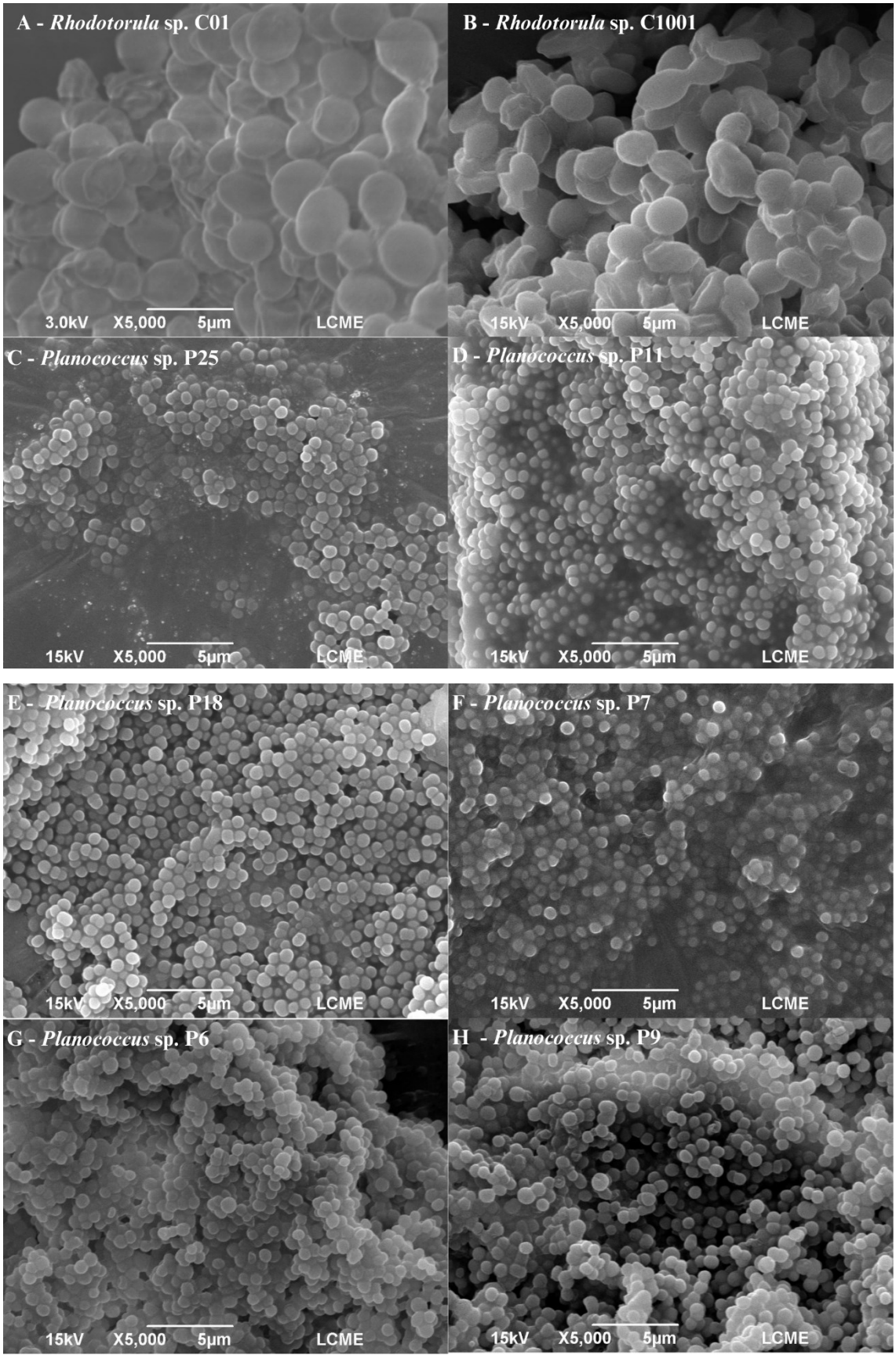
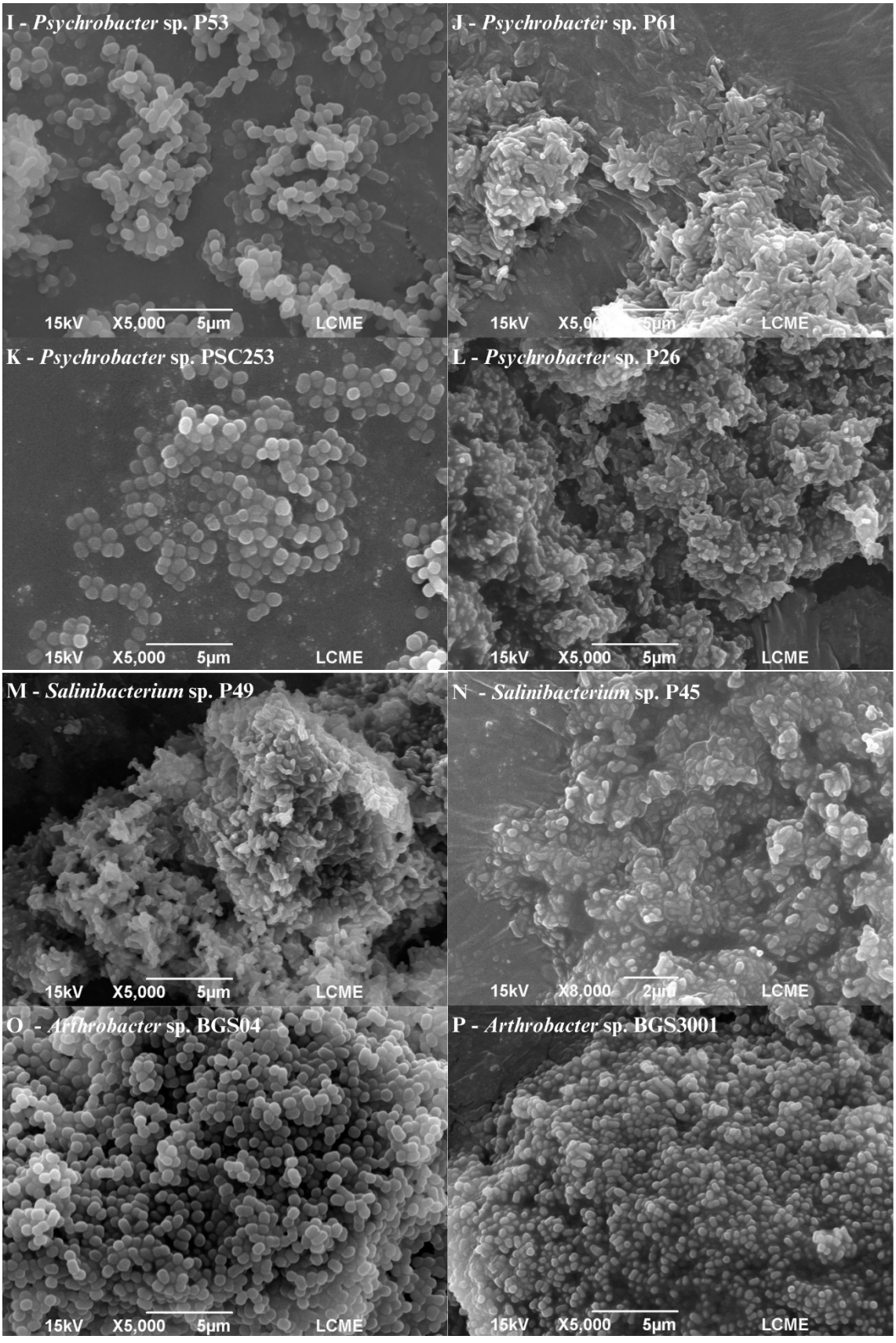


Figure Chapter III 1 - Freeze-Thaw (FT) survival rate at -80 °C of Antarctic isolates. The experiments were conducted in triplicate. An asterisk (*) indicates no differences on cell viability before and after freezing-thawing cycles.

5.3.2 Morphological Characterization of Antarctic Bacteria and Yeasts by Scanning Electron Microscopy

The results obtained from electron microscopy revealed various shapes for bacteria and yeasts (**Figure Chapter III 2**). In terms of bacteria, different forms were observed, including cocci, cocobacilli, and rods. Specifically, the genera *Planococcus* and *Arthrobacter* exhibited a cocci shape, *Psychrobacter* showed both cocobacilli and rods, *Acinetobacter* presented as cocobacilli, while *Salinibacterium*, *Pedobacter*, *Cryobacterium*, and *Pseudomonas* were identified as rods. The proportions of these bacterial forms were 55% cocci, 38% rods, and 5% cocobacilli, respectively.


A - *Rhodotorula* sp. C01
3.0kV X5,000 5μm LCME
B - *Rhodotorula* sp. C1001
15kV X5,000 5μm LCME
C - *Planococcus* sp. P25
15kV X5,000 5μm LCME
D - *Planococcus* sp. P11
15kV X5,000 5μm LCME
E - *Planococcus* sp. P18
15kV X5,000 5μm LCME
F - *Planococcus* sp. P7
15kV X5,000 5μm LCME
G - *Planococcus* sp. P6
15kV X5,000 5μm LCME
H - *Planococcus* sp. P9
15kV X5,000 5μm LCME


I - *Psychrobacter* sp. P53
15kV X5,000 5µm LCME
J - *Psychrobacter* sp. P61
15kV X5,000 5µm LCME
K - *Psychrobacter* sp. PSC253
15kV X5,000 5µm LCME
L - *Psychrobacter* sp. P26
15kV X5,000 5µm LCME
M - *Salinibacterium* sp. P49
15kV X5,000 5µm LCME
N - *Salinibacterium* sp. P45
15kV X8,000 2µm LCME
O - *Arthrobacter* sp. BGS04
15kV X5,000 5µm LCME
P - *Arthrobacter* sp. BGS3001
15kV X5,000 5µm LCME

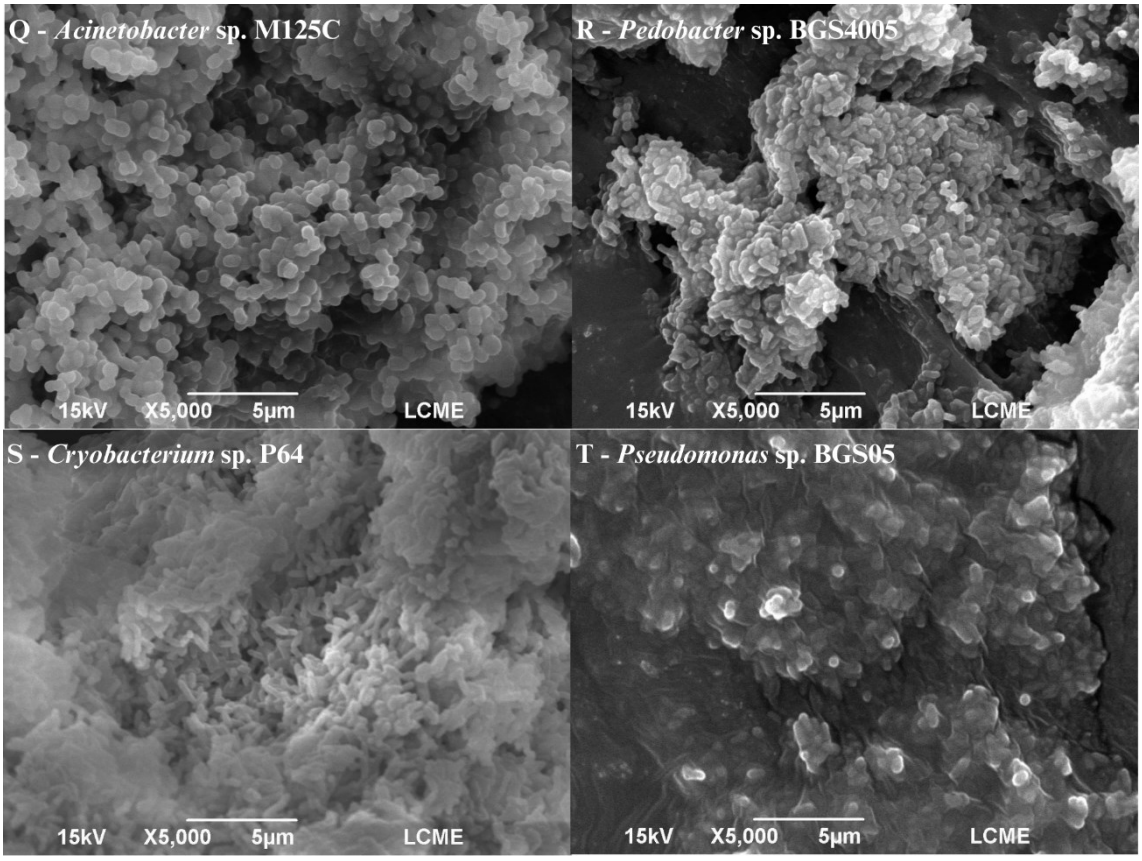


Figure Chapter III 2 - Photos of Antarctic isolates taken by scanning electron microscopy (SEM) at magnifications of 5, 000x and 8, 000x at 15 kV. Panels A-B show yeasts, and panels C-T show bacteria.

5.3.3 Characterization of the Antifreeze Activity of Microbial Proteins in the Cryopreservation of Prokaryotic Cells

To evaluate the cryopreservation of *E. coli* cells, total intracellular protein extracts obtained from *Pedobacter sp.* BGS4005, *Psychrobacter sp.* P61, and *Salinibacterium sp.* P45 were used at concentrations of 0.1 and 1 mg.mL^{-1}. The results obtained from the treatment of *E. coli* showed that treatments at a concentration of 1 mg.mL^{-1} were not effective in cryopreservation at -18 ºC, as all extracts lead to a survival rate of about 0%, with no statistical difference compared to the negative control ($p > 0.05$) (**Figure Chapter III 3A**). In contrast, at a concentration of 0.1 mg.mL^{-1}, the intracellular protein extracts from *Pedobacter sp.* BGS4005, *Psychrobacter sp.* P61, and *Salinibacterium sp.* P45 increased the survival of *E. coli* to 1%, 2.85%, and 3.3%, respectively. When AFP type III was used at 1 mg.mL^{-1}, it was not effective in cryopreserving *E. coli* compared to the 0.1 mg.mL^{-1} concentration of AFP type III, which showed a significant difference compared to the negative control ($p<0.05$) (**Figure Chapter III 3B**).

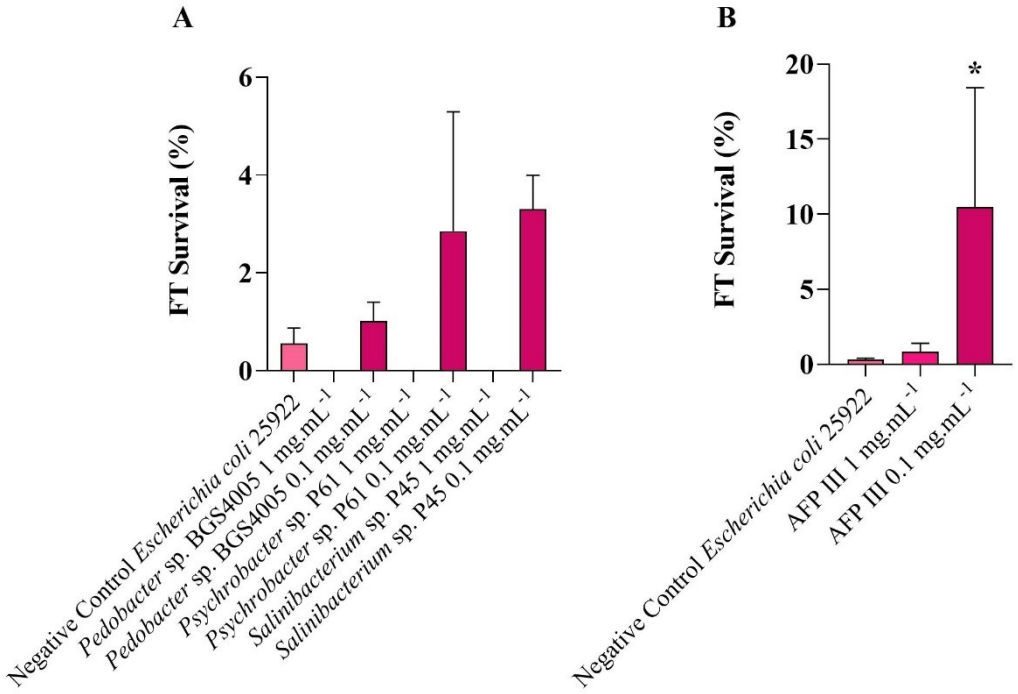


Figure Chapter III 3 - Relationship between the Survival Rate Freeze-Thaw (FT) of *Escherichia coli* after freezing at -18 ºC under different treatments of total intracellular protein extracts from Antarctic isolates (A) and under different concentrations of AFP type III (B). The experiments were conducted in triplicate.

To evaluate the cryopreservation of *Escherichia coli* cells, four extracellular extracts were used: *Pedobacter sp.* BGS4005, *Psychrobacter sp.* P61, *Salinibacterium sp.* P45, and *Rhodotorula sp.* C01 (**Figure Chapter III 4A**). The negative control had a survival rate of 0.33%, while the positive control (AFP III 1 mg.mL^{-1}) showed a slightly higher survival rate of 0.84%. Among the treatments with different isolates, *Psychrobacter sp.* P61 had a survival rate of 4.61%, and *Salinibacterium sp.* P45 showed a survival rate of 2.51%. *Pedobacter sp.* BGS4005 exhibited the highest survival rate at 5.51%, followed by *Rhodotorula sp.* C01, which had a survival rate of 1.64%. These results indicate that treatment with *Pedobacter sp.* BGS4005 was the most effective, resulting in the highest survival rate. The positive control

with AFP III also showed an increased survival rate, although not as significant as the treatments with bacterial and fungal isolates.

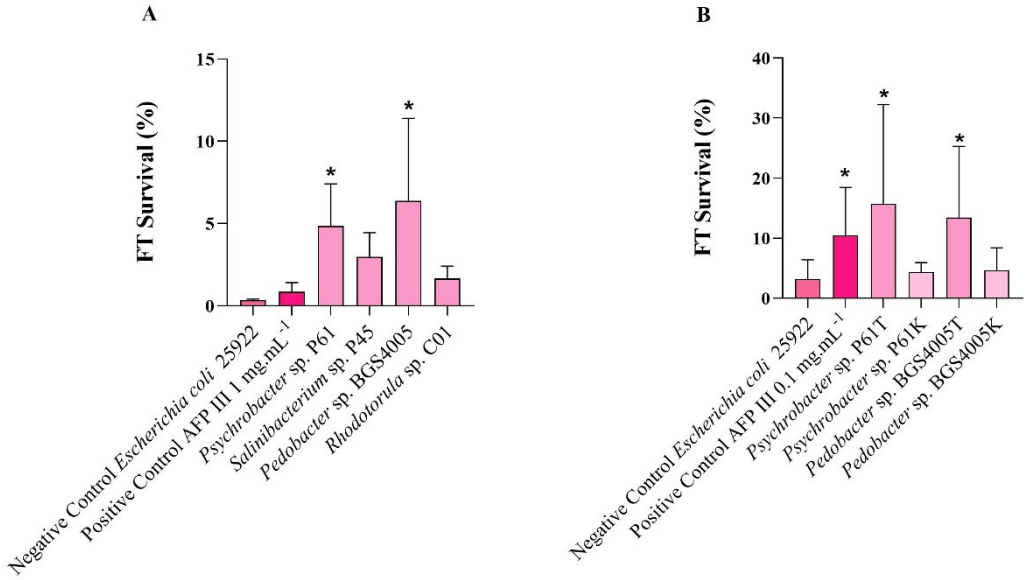


Figure Chapter III 4 - Survival Rate Freeze-Thaw (FT) of *Escherichia coli* to Freezing at -18°C Under Different Treatments with: Extracellular Extracts from Antarctic Isolates (A) and Extracellular Extracts from Antarctic Isolates Subjected to Thermal and Enzymatic Treatment with Proteinase K 2.75 mg.mL^{-1} (B). The experiments were conducted in triplicate. On both figures, an asterisk (*) indicates significant differences between the treatment and negative control.

To confirm whether the cryopreservation effect on *E. coli* cells from the extracellular extracts of *Pedobacter* sp. BGS4005 and *Psychrobacter* sp. P61 is due to the action of proteins contained in the liquid culture medium, two treatments were performed: a thermal treatment where the extract was heated at 100 °C for 30 minutes, and an enzymatic treatment with Proteinase K at 2.75 mg.mL^{-1} at 25 °C for 30 minutes (**Figure Chapter III 4B**).

The analysis of treatment percentages reveals a marked variation in efficacy, emphasizing the distinction between thermal methods and the action of the enzyme Proteinase K. It is notable that the closer the percentage between a treatment and the negative control (0.43%), the greater its efficacy in suppressing the response or activity under evaluation. While thermal treatments, such as *Psychrobacter* sp. P61T (12.45%) and *Pedobacter* sp. BGS4005T (12.13%), showed high percentages ($p < 0.05$), treatments with the enzyme Proteinase K, such as *Psychrobacter* sp. P61K (2.99%) and *Pedobacter* sp. BGS4005K (4.59%), proved to be more efficient. This pattern suggests a greater efficacy of enzymatic treatments in the assessed

activity. Statistical analysis reinforces this observation, indicating a significant difference only in thermal treatments, confirming that the cryopreservation of *E. coli* is associated with extracellularly secreted proteins in the culture medium. Additionally, the AFP III 0.1 mg.mL^{-1} treatment exhibited a considerable percentage of 9.65%.

5.3.4 Characterization of the Antifreeze Activity of Microbial Proteins in the Cryopreservation of Eukaryotic Cells

The results obtained for the cryopreservation of HEK293 cells show that the proposed treatments – Negative control (no treatment), DMSO 10%, AFP III 1 mg.mL^{-1}, P45 1 mg.mL^{-1}, P61 1 mg.mL^{-1} and BGS4005 1 mg.mL^{-1} – achieved cell viability values of 0%, 15%, 50%, 15%, 15%, and 10%, respectively (**Figure Chapter III 5**).

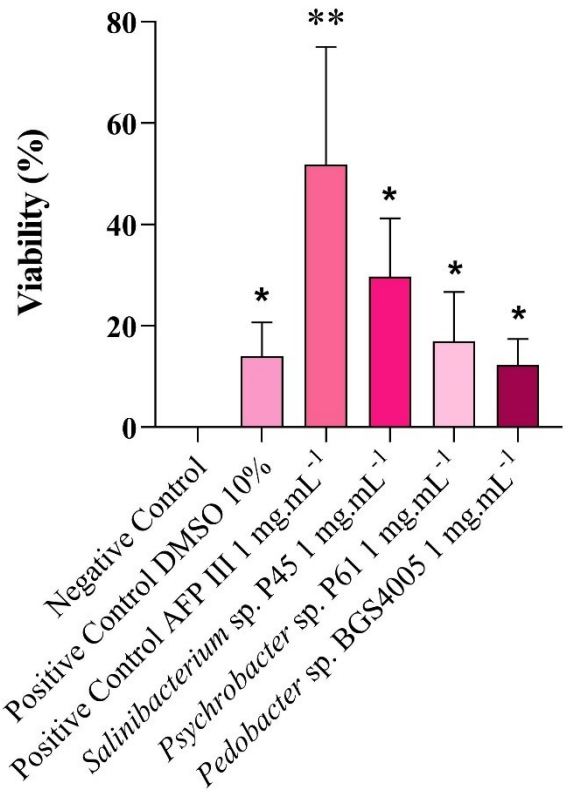


Figure Chapter III 5 - Analysis of cell viability using trypan blue in HEK293 cells after freezing for 30 days at -18°C. A total of 5 x 10^6 cells was cryopreserved with 10% DMSO, 1 mg.mL^{-1} of fish antifreeze protein type III, and protein extracts obtained from Antarctic bacteria. The experiment was conducted in triplicate. An asterisk (*) indicates significant differences compared to the negative control.

Using ANOVA analysis, it was observed that treatment with AFP type III at 1 $mg.mL^{-1}$ showed a significant difference compared to the positive control DMSO 10%, bacterial protein extracts at 1 $mg.mL^{-1}$, and the negative control with 40% fetal bovine serum ($p < 0.05$). Bacterial protein extracts did not show significant differences compared to the positive control with DMSO 10% ($p > 0.05$). The results indicate that AFP III enhances the cryopreservation of HEK293 cells and that bacterial protein extracts provide cryopreservation equal to or equivalent to DMSO in HEK293 cells.

5.3.5 Characterization of the Antifreeze Activity of Microbial Proteins in Adenovirus Stability

To evaluate viral stability, seven treatments were tested over 60 days, with viral titration performed every 15 days (**Figure Chapter III 6**).

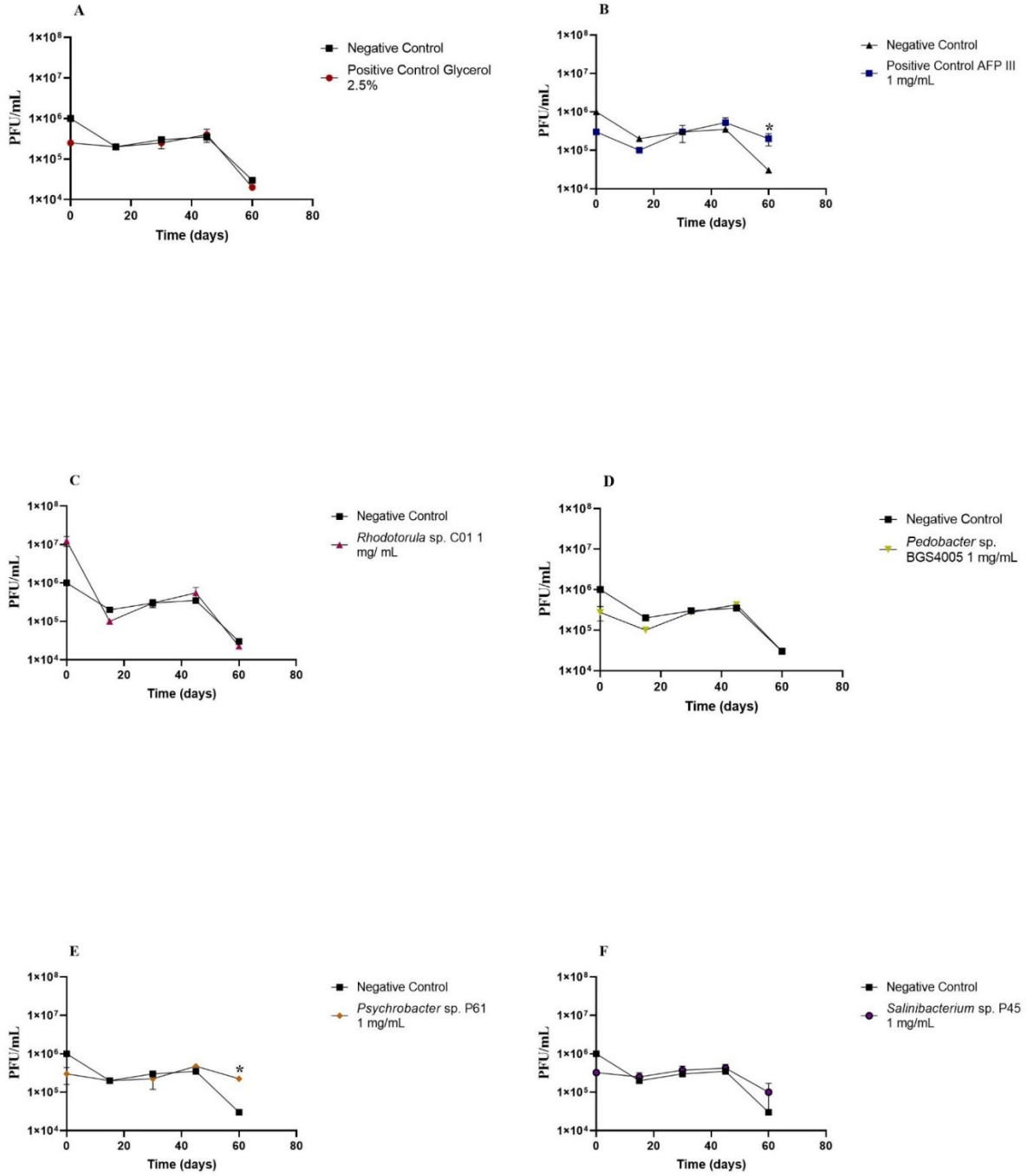


Figure Chapter III 6 - Evaluation of adenovirus stability over 60 days treated with: Glycerol 2.5% (A), AFP type III 1 mg.mL^{-1} (B), *Rhodotorula* sp. C01 1 mg.mL^{-1} (C), *Pedobacter* sp. BGS4005 1 mg.mL^{-1} (D), *Psychrobacter* sp. P61 1 mg.mL^{-1} (E), and *Salinibacterium* sp. P45 1 mg.mL^{-1} (F). The experiment was conducted in duplicate. An asterisk (*) indicates significant differences for the PFU concentration between the treatment and the negative control at the respective time.

The results indicate that, in general, adenoviruses treated with proteins and the negative control maintained similar PFU levels over the first 45 days, with no significant difference ($p > 0.05$). However, after 60 days, a reduction in viral titers was observed for the negative control, the positive control with 2.5% glycerol, and the treatments with *Rhodotorula* sp. C01, *Pedobacter* sp. BGS4005, and *Salinibacterium* sp. P45, indicating that these treatments were not effective in maintaining adenovirus stability. On the other hand, a total protein extract from *Psychrobacter* sp. P61, a microrganism from Antarctica, was effective, showing a significant difference compared to the negative control ($p < 0.05$). The fish AFP type III protein also showed a significant difference compared to the negative control ($p < 0.05$). These findings suggest that proteins from Antarctic microorganisms could potentially be used to improve long-term viral stability.

5.4 DISCUSSION

The potential of Antarctic microorganisms for producing cryoprotective substances, although underexplored, has garnered growing interest due to its significance in biotechnology. These substances, which include antifreeze proteins (AFPs) and exopolysaccharides, are part of the adaptive strategies that enable survival in extreme environments such as Antarctica. In this environment, freezing and thawing cycles, nutrient scarcity, and the absence of liquid water present severe challenges for microbial life.

In our study, resistance to freezing at -80 °C was tested in Antarctic bacteria and yeasts. The results showed that *Rhodotorula* sp. C1001, *Cryobacterium* sp. P64, *Psychrobacter* sp. P61, and *Pseudomonas* sp. BGS05 did not survive at this temperature. On the other hand, strains of *Acinetobacter* sp. M125C, *Rhodotorula* sp. C01, *Planococcus* sp., *Arthrobacter* sp., *Psychrobacter* sp., *Pedobacter* sp., *Rhodococcus* sp. and *Salinibacterium* sp. displayed varying degrees of resistance, with survival rates ranging from 4% to 79%. Notably, *Psychrobacter* sp. P53, *Pedobacter* sp. BGS4005, *Salinibacterium* sp. P45/P49, and *Planococcus* sp. P11 demonstrated high resistance, with survival rates exceeding 40%.

These findings reinforce the results of Moreira *et al.* (2022), who also identified resistance to freezing at -80 °C in isolates from the genera *Arthrobacter*, *Psychrobacter*, and *Rhodococcus*. This suggests that certain microbial genera have more efficient protective mechanisms against freezing, possibly associated with the production of AFPs or other cryoprotective molecules.

However, resistance to freezing is not uniform across different species and strains. For example, while *Psychrobacter* sp. P53 exhibited high survival, other strains of the same genus, such as *Psychrobacter* sp. P61, were not resistant to -80°C. This variation may be related to genomic and metabolic diversity within the genus, resulting in different capacities to produce AFPs or adapt their cell membrane composition to cope with freezing stress.

Previous studies, such as those by Walker *et al.* (2006) and Wilson *et al.* (2006), investigated freezing resistance in Antarctic microorganisms using temperatures like -18°C. These studies identified strains with antifreeze activity, including *Chryseobacterium* sp. and *Pseudomonas borealis*, which exhibited ice recrystallization inhibition, an essential mechanism for preventing damage caused by ice crystals during freezing. Our results complement these studies, particularly regarding the resistance of *Psychrobacter* and *Pedobacter* strains, suggesting that these bacteria may use similar mechanisms to survive extreme freezing.

The resistance observed in our Antarctic yeasts, *Rhodotorula* sp. C01 and C1001, was relatively low, with survival rates of 13.8% and 0.7%, respectively. Previous studies on Antarctic yeasts, such as *Glaciozyma antarctica* and *Rhodotorula svalbardensis*, tested freezing resistance at milder temperatures, such as -25 °C and -1 °C, and reported AFP production that aids survival. The lower resistance of the strains tested in our study may indicate differences in the specific adaptations of these yeasts to environments with less severe freezing cycles.

Finally, it is important to highlight that freezing resistance in Antarctic microorganisms is directly related to the production of cryoprotective substances and the expression of genes associated with cold tolerance. Further studies investigating the composition of these substances and the molecular mechanisms involved are essential to better understand the adaptations of these microorganisms and explore their biotechnological applications, such as in cell cryopreservation and vaccine stabilization.

The results obtained in the study on the cryopreservation of *Escherichia coli* 25922 cells with intracellular protein extracts at 1 $mg.mL^{-1}$ showed that this concentration was not efficient in maintaining cell survival after freezing at -18 °C. Conversely, extracts at 0.1 $mg.mL^{-1}$ demonstrated an increase in cell survival, with rates 1% to 3.3% higher than the negative control. This observation is consistent with the study by Kawahara *et al.* (2009), which analyzed the effect of fish AFP type I on the cryopreservation of *E. coli* cells subjected to rapid freezing with liquid nitrogen (-196 °C). In that study, a concentration of 0.1 $mg.mL^{-1}$ of AFP type I resulted in an increase in cell survival from 0.73% to 2.96%. However, at higher

concentrations (1 $mg.mL^{-1}$), the cell survival rate drastically decreased to 0.09%, suggesting a bactericidal effect.

In the case of extracellular extracts from *Pedobacter* sp. BGS4005, *Psychrobacter* sp. P61, *Salinibacterium* sp. P45, and *Rhodotorula* sp. C01, the extract from *Pedobacter* sp. BGS4005 showed the highest efficacy, with a survival rate of 5.51%, compared to the negative control (0.33%) and the positive control containing AFP type III (0.84%). The other extracts showed survival rates ranging from 1.64% to 4.61%, indicating that the substances secreted by these Antarctic microorganisms have a cryoprotective effect superior to the controls used.

The literature supports these findings, highlighting that microorganisms from the genera *Pedobacter*, *Psychrobacter*, and *Rhodotorula* are known to produce exopolysaccharides and AFPs that may contribute to freezing resistance (Hamidi *et al.*, 2020; Yu *et al.*, 2016; Wang *et al.*, 2023). Additionally, freezing resistance at -18 °C and -80 °C has already been reported in several isolates from these genera, including *Psychrobacter* (9 isolates), *Pedobacter* sp. BGS4005, *Salinibacterium* sp. P45, *Rhodotorula svalbardensis*, and *Rhodotorula* sp. C01 (Lopes *et al.*, 2024a; Gilbert *et al.*, 2004; Moreira *et al.*, 2022; Singh *et al.*, 2014).

In addition to antifreeze proteins, some isolates, such as those from the genus *Salinibacterium*, have also shown potential for carotenoid production and UV radiation resistance, characteristics that reinforce their adaptive capabilities in extreme environments (Vila *et al.*, 2019; Coppola *et al.*, 2023).

The variation in the efficacy of protein extracts in cell cryopreservation may be related to the concentration of AFPs or other cryoprotective compounds present in the extracts. Higher concentrations do not always guarantee greater efficacy, as demonstrated in the study by Kawahara *et al.* (2009), where an increase in AFP type I concentration resulted in a bactericidal effect. This non-linear relationship between concentration and efficacy may be attributed to potential toxicity at higher concentrations or interference of proteins with normal cellular processes.

Another crucial aspect is the complexity of the crude extracts used in our research. These extracts contain a diversity of proteins and other compounds that may interfere with the results. Purification of the extracts is an essential step in identifying which specific components are responsible for cryopreservation efficacy. Purification not only eliminates compounds that may inhibit AFP activity but also facilitates the detailed characterization of active components.

Cryopreservation is a fundamental technique in biotechnology and medicine, allowing the preservation of cells and tissues for future use in therapies, research, and transplants. This study investigated the efficacy of fish AFP type III and bacterial protein extracts in the cryopreservation of HEK293 cells. The results revealed that AFP type III at a concentration of 1 $mg.mL^{-1}$ significantly increased cell viability after thawing. Additionally, the bacterial protein extracts demonstrated efficacy comparable to DMSO, suggesting their potential as a less toxic alternative in cell cryopreservation. However, as crude extracts contain a diversity of proteins, it is possible that some components interfere with the results. Therefore, purifying these extracts could improve efficacy by removing undesirable proteins and concentrating the most promising components.

The results presented in this study corroborate previous findings on the efficacy of AFP III. Tomás *et al.* (2019) demonstrated that AFP III at concentrations ranging from 0.1 to 1 $mg.mL^{-1}$ increased the recovery of A549 cells subjected to a freezing regimen of -80 ºC followed by -196 ºC. In their study, AFP III at 0.8 $mg.mL^{-1}$ increased viability by 60% in its extracellular form, whereas the intracellular form did not have the same effect. Another study by Sreter *et al.* (2022) with HEK293 cells showed that combining AFP III with DMSO enhanced cell survival, with the highest efficacy when AFP III was used in both forms (intracellular and extracellular). These findings further emphasize the versatility of AFP III across different cell types and experimental conditions.

Moreover, Lee *et al.* (2015) investigated the effect of AFPs (FfIBP, LeIBP, and AFP III) on the cryopreservation of mouse ovarian tissue, finding that LeIBP at 10 $mg.mL^{-1}$ was particularly effective. Compared to other AFPs, AFP III has shown efficiency in various scenarios, standing out as a versatile cryoprotective agent.

In the context of cryopreservation of HEK293 cells, our results complement the data from Sreter *et al.* (2022), suggesting that combining AFP III and DMSO offers robust protection, while bacterial protein extracts may represent promising alternatives to DMSO, particularly in applications where toxicity is a concern. However, purifying these extracts is essential to enhance the effectiveness of cryopreservation, as the presence of non-cryoprotective proteins may reduce overall performance.

Another relevant aspect, not explored in this study, is the supplementation of the freezing medium. Previous studies have shown that adding cryoprotectants like sugars can further improve post-thaw cell survival. Therefore, future studies should consider integrating

different supplements into the freezing medium, exploring synergies between AFPs and other cryoprotectants to optimize cell viability.

Comparative analysis with previous studies also highlights the critical role of AFP III in viral stability, with direct implications for vaccine biotechnology. In our study, protein extracts from *Salinibacterium* sp. P45 and *Psychrobacter* sp. P61 demonstrated the ability to maintain higher viral titers than the negative control in adenovirus type 5 over 60 days, suggesting potential for maintaining vaccine thermostability. Previous studies indicate that the loss of adenovirus infectivity is highly dependent on storage temperature, with the formulation of thermostable vaccines being a central focus of recent research (Berg *et al.*, 2021; Sedik *et al.*, 2024).

Thus, the logical next step in our research would be the purification of bacterial protein extracts to assess their cryoprotective potential more accurately. This would not only allow for the optimization of the concentrations used but also the identification of new cryoprotective compounds with potential applications in cryopreservation, vaccine formulations, and even in the preservation of organs and tissues for transplantation.

5.5 CONCLUSIONS

In conclusion, this study highlights the significant potential of Antarctic microorganisms and their antifreeze proteins for applications in biotechnology. The high survival rates observed in strains such as *Psychrobacter* sp. P53 and *Pedobacter* sp. BGS4005 at -80 ºC, as well as the efficacy of microbial protein extracts in the cryopreservation of prokaryotic cells such *Escherichia coli*, suggest new possibilities for the preservation of microorganisms subjected to freezing. Type III AFP proved particularly effective for the cryopreservation of eukaryotic cells such as HEK293, and its stabilizing properties offer a promising perspective for the long-term conservation of adenovirus-based vaccines. These findings pave the way for the development of new biotechnological strategies and products, such as less toxic cryoprotectants and improved methods for cell and vaccine storage, emphasizing the importance of continuing to explore and apply the unique properties of these microorganisms and their antifreeze proteins in biotechnology.

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6. CHAPTER IV

FINAL CONSIDERATIONS

6.1 SYNTHESIS

The collection of research presented from Chapter 1 to Chapter 3 has made significant advances in the study of Antarctic microorganisms as producers of antifreeze proteins (AFP). Below is a synthesis of the three studies conducted in this thesis. These studies employed a variety of techniques, including the isolation, selection, identification of isolates, and screening of their antifreeze potential, with a focus on freeze-resistance and ice recrystallization inhibition tests. The primary goal was to explore the potential of Antarctic microorganisms in the production of cryoprotective substances, such as antifreeze proteins, for biotechnological applications in products subjected to freezing.

In Chapter 1, an in-depth review of articles published up to 2024 on microorganisms that produce antifreeze proteins (AFP) was conducted. This review was not limited to bacteria but also included fungi, algae, and archaea. The objective was to compile as much information as possible about these microorganisms, covering data on isolation, sample collection sites, molecular identification, the name of the antifreeze protein, evaluation of antifreeze activity (including thermal hysteresis and recrystallization inhibition assays), protein size, and the accession number of the protein or microorganism.

The review identified 174 microorganisms, of which 127 were identified at the genus level. Among the groups, in bacteria, the phylum Proteobacteria was the most abundant, followed by the phylum Basidiomycota in fungi, the class Chlorophyceae in algae, and the phylum Euryarchaeota in archaea. In terms of the most prevalent genera, *Arthrobacter* stood out among bacteria, *Chloromonas* among algae, *Typhula* among fungi, and *Halobacterium* among archaea.

Regarding isolation sources, bacteria exhibited the greatest diversity of sources (n=9), followed by fungi (n=7) and algae (n=4). In the Arctic and Antarctica, the most commonly used sources were soil and the plant *Deschampsia antarctica*. Concerning the measurement of antifreeze activity, 66 microorganisms were tested for recrystallization inhibition (IR) activity, with 34 valid tests; of these, 58% of the isolates were tested at a concentration of 1.5-3.3 $mg.mL^{-1}$, and 38% at a concentration of 0.001-0.5 $mg.mL^{-1}$. For thermal hysteresis (TH) activity, 64 microorganisms were tested, with 31 used in the final results. The data showed that when the TH value was higher, a lower protein concentration was used.

Regarding protein size and TH activity, two groups of proteins were observed: 72% of the proteins had sizes between 22-34 kDa, and 20% between 41-59 kDa. Additionally, smaller proteins exhibited a TH value of 1.16ºC, while larger proteins had a TH value of 1.01ºC, indicating that smaller proteins are more efficient in antifreeze activity.

Antarctica presented a significantly greater number of microorganisms (Bacteria n=110, Fungi n=22, Algae n=8) compared to the Arctic (Bacteria n=10, Fungi n=3, Algae n=1). This difference is due to the varying levels of scientific effort in prospecting for AFP-producing microorganisms in each region, as well as the logistics involved in accessing samples, which can influence the number of studies conducted. In Antarctica, a higher concentration of isolates was observed along the coast, with the South Shetland Islands and McMurdo Valley accounting for about 36% and 25% of the isolates, respectively. In the Arctic, about 62% of the isolates were described on Svalbard Island.

In Chapter 2, a comprehensive study was conducted to isolate, select, identify, and screen Antarctic microorganisms with potential for antifreeze protein production. A total of 215 microorganisms were isolated: 91 from Collins Glacier Soil, 55 from Baranowski Glacier Soil, 52 from Marine Sediment, and 13 from Permafrost.

Regarding cultivation temperature, 102 bacteria and 2 yeasts were isolated at 25 ºC, 15 bacteria at 15 ºC, and 96 bacteria and 3 yeasts at 6 ºC. Concerning the culture media used, R2A at 100% yielded 121 bacteria and 2 yeasts, R2A at 10% yielded 18 bacteria, Soil Extract medium yielded 9 bacteria, and TSA resulted in the isolation of 62 bacteria and 3 yeasts.

In molecular identification, 118 isolates were classified into 7 phyla, with Actinomycetota and Pseudomonadota being the most prominent. At 25 ºC, the genera *Pseudarthrobacter*, *Arthrobacter*, and *Pseudomonas* were predominant; at 6 ºC, *Psychrobacter*, *Planococcus*, and *Arthrobacter* stood out; and at 15 ºC, the most common genera were *Arthrobacter*, *Rhodococcus*, and *Pedobacter*.

Forty-six isolates were subjected to freeze-resistance testing, of which 24 were resistant, including 22 bacteria and 2 yeasts. Among the 13 identified genera, *Planococcus* had the highest number of freeze-resistant isolates.

In the ice recrystallization inhibition (IRI) activity test, 131 protein extracts were evaluated, with 81 showing positive results (values above the negative control) and 14 exhibiting significant absorbance ($p < 0.05$). Additionally, four isolates demonstrated both

freeze-resistance and IRI activity: *Arthrobacter* sp. BGS04, *Pseudomonas* sp. BGS05, *Cryobacterium* sp. P64, and *Acinetobacter* sp. M1_25C.

In Chapter 3, a study was conducted to morphologically characterize Antarctic isolates that were tested at -80 ºC, selected for total protein extraction, and evaluated for cryopreservation of prokaryotic cells, such as *Escherichia coli*, eukaryotic cells of the HEK293 lineage, and viral stability of adenovirus. Twenty isolates were characterized by scanning electron microscopy (SEM), revealing cocci, cocobacilli, and rod shapes in bacteria. Twenty-one isolates were tested for freeze-resistance at -80 ºC for 24 hours, of which five showed survival rates above 40%: *Psychrobacter* sp. P53 (79.64%), *Pedobacter* sp. BGS4005 (49.87%), *Salinibacterium* sp. P45 (42.67%), *Salinibacterium* sp. P49 (52.08%), and *Planococcus* sp. P11 (43.52%).

Four microorganisms (*Psychrobacter* sp. P61, *Rhodotorula* sp. C01, *Pedobacter* sp. BGS4005, and *Salinibacterium* sp. P45) were selected for intracellular and extracellular protein extraction. Three intracellular protein extracts and four extracellular extracts were tested in *Escherichia coli* subjected to -18 ºC for 24 hours. The results showed that the intracellular proteins, at the two tested concentrations, were not effective in cryopreserving *E. coli* cells, while the extracellular extracts were effective, with *Pedobacter* sp. BGS4005 and *Psychrobacter* sp. P61 standing out.

Four intracellular protein extracts were tested in HEK293 cells subjected to -18 ºC for 30 days. Of these, three extracts were as effective as DMSO, a commonly used cryoprotectant. Additionally, four intracellular protein extracts were assessed for adenovirus stability over 60 days at 4 ºC. One of these extracts (*Psychrobacter* sp. P61) maintained viral titres above the negative control after 60 days.

6.2 RESEARCH PERSPECTIVES

6.2.1 Large-Scale Production and Biotechnological Application

The large-scale production of microbial antifreeze proteins (AFPs) involves significant challenges, such as selecting model organisms for the heterologous expression of AFP genes and optimizing cultivation conditions to maximize yield and production efficiency. For instance, Lee *et al.* (2013) optimized the production of recombinant ice-binding proteins from *Leucosporidium* in *Pichia pastoris*, determining that the ideal temperature and pH for

production were 25ºC and 6, respectively. Their fed-batch culture system increased the recombinant protein concentration by 100 mg, achieving a yield of 300 mg/L in pilot-scale fermentation, with a thermal hysteresis activity of 0.42 ºC, comparable to other studies (LEE *et al.*, 2013). Similarly, Kim *et al.* (2014) investigated the recombinant production of antifreeze proteins from the Antarctic bacterium *Flavobacterium frigoris* PS1 using a cold shock induction system. Their results showed protein yields of 126 g and 8.4 g at 37 ºC and 15 ºC, respectively, with a thermal hysteresis activity (TH) of 1.53 ºC, which was 3.6 times greater than that of AFP LeIBP produced in *Pichia* (Kim *et al.*, 2014).

The activity of AFPs can also be influenced by pH, which is crucial for optimizing their functionality. Delesky *et al.* (2020) examined the effect of pH on the antifreeze activity of *Marinomonas primoryensis* MpIBP using both recrystallization inhibition and nanoliter osmometry. They found that MpIBP maintained its secondary structure and antifreeze function between pH 6 and pH 10, with no activity observed at pH $\leq$ 4 or pH $\geq$ 13. These results underscore MpIBP's potential for biotechnological applications in diverse pH environments (Delesky *et al.*, 2021).

Microbial AFPs hold promising potential in various biotechnological applications, particularly in cryopreservation. AFPs can enhance cell and tissue viability during freezing and thawing by preventing ice crystal formation, which can cause structural damage. Preliminary trials have highlighted the effectiveness of AFPs in maintaining cellular integrity during freezing (Cho *et al.*, 2019; Gwak *et al.*, 2015; Khan *et al.*, 2020; Kim *et al.*, 2017; Koh *et al.*, 2015; Lee *et al.*, 2015; Lee *et al.*, 2012; Liu *et al.*, 2021; Muñoz *et al.*, 2017; Nam *et al.*, 2024; Sun *et al.*, 1995).

Among the diverse range of AFPs derived from polar microorganisms, *Glaciozyma* sp. AY30 (LeIBP), an Arctic fungal AFP, has been extensively studied for its various applications. In healthcare, LeIBP has proven effective in reducing hemolysis to less than 16% at a concentration of 1 mg/mL (Lee *et al.*, 2012). It has also improved cell viability in several cell lines, including human cervical cancer cells (HeLa), mouse fibroblasts (NIH/3T3), human preosteoblasts (MC3T3-E1), Chinese hamster ovary cells (CHO-K1), and human keratinocytes (HaCaT), with cell viability ranging from 10% to 28% (Kim *et al.*, 2015). Additionally, LeIBP increased the concentration of chlorophyll a in *Phaeodactylum tricornutum* cells (Koh *et al.*, 2015) and showed protective effects in mouse ovarian tissue cryopreservation (Lee *et al.*, 2015).

Glaciozyma antarctica, an Antarctic fungal species, has also been the subject of significant studies. Khan *et al.* (2019) reported that the AFP produced by *Glaciozyma antarctica* (Afp1), at concentrations of 5 and 10 mg/mL, provided better tissue preservation at -10 ºC, highlighting its potential in healthcare, especially for tissue preservation.

In Antarctica, several bacterial AFPs have been explored for their applications in agriculture and industry. *Sphingomonas* sp. (AFP5.1), *Pseudomonas* sp. (GU1.7.1), and *Platibacter* sp. (GU3.1.1) have been effective in enhancing plant cell viability in cucumber and zucchini, with *Platibacter* sp. achieving up to 50% viability at a concentration of 0.1 $mg.mL^{-1}$ (Muñoz *et al.*, 2017). *Chaetoceros neogracile* (Cn-AFP), an Antarctic algal AFP, has shown potential for industrial applications due to its sufficiently low supercooling point, making it suitable for metal surface-coating processes (Gwak *et al.*, 2015). Similarly, *Chloromonas* sp. KNF0032 (CmIBP1), another Arctic algal AFP, has been found to slightly increase freezing tolerance in transgenic plants, suggesting its potential use in agriculture (Cho *et al.*, 2019).

Flavobacterium frigoris (FfIBP), an Antarctic bacterial AFP, has demonstrated efficacy across various applications. It provided protective effects in mouse ovarian tissue cryopreservation at concentrations ranging from 0.1 to 10 $mg.mL^{-1}$ (Lee *et al.*, 2015) and maintained its IR activity even when mixed with commercial cryoprotectants (Nam *et al.*, 2024). FfIBP was also effective in preserving the viability of *Isochrysis galbana* cells (Kim *et al.*, 2017). Finally, *Marinomonas primoryensis* (MPAFP_RIV), another Antarctic bacterial AFP, has shown significant benefits in healthcare applications, particularly in preserving the viability of blood cells (Liu *et al.*, 2021).

Overall, the diverse range of AFPs derived from polar microorganisms highlights their versatile applications and underscores their importance in advancing both scientific research and practical solutions in healthcare, agriculture, and industry. The exploration of these proteins represents a promising frontier in biotechnology, although further research is needed to optimize processes and validate large-scale applications.

6.2.2 Proteomic as a Tool for the Identification and Development of an Antifreeze Protein Database

The identification of new proteins in extremophiles has gained prominence in recent years due to their unique adaptation characteristics to extreme environments. Proteomics, combined with bioinformatics, has become essential for the discovery and classification of these proteins (Brewis; Brennan, 2010; Noor *et al.*, 2020; Yun *et al.*, 2016).

Proteomic analysis begins with sample preparation and protein separation, which are then analyzed using methods such as Edman degradation or mass spectrometry (MS). MS stands out for its sensitivity and precision in protein identification (Yun *et al.*, 2016).

Recently, several bioinformatics tools have been developed for identifying antifreeze proteins (AFPs). Yang *et al.* (2015) created the AFP-Ensemble system, which uses random forest classifiers to improve the accuracy of AFP identification (Yang *et al.*, 2015). Eslami *et al.* (2018) introduced afpCOOL, a method based on support vector machines (SVM), which has proven superior to existing methods, highlighting the importance of physicochemical descriptors (Eslami *et al.*, 2018). Zhang *et al.* (2021) enhanced AFP prediction using the Minimum Redundancy Maximum Relevance (mRMR) method to identify essential features and new motifs for AFP detection (Zhang *et al.*, 2021). Additionally, Pratiwi *et al.* (2017) developed the Cryoprotect platform to classify proteins as antifreeze based on their amino acid sequences (Pratiwi *et al.*, 2017).

Despite these advancements, the number of known AFPs remains limited, underscoring the need for reliable databases and bioinformatics tools to predict new proteins. Recent studies, such as those by Miyata *et al.* (2021) and Usman *et al.* (2022), have demonstrated significant progress in the accuracy and efficiency of AFP identification (Miyata *et al.*, 2021; Usman *et al.*, 2022).

6.3 FINAL CONSIDERATIONS

This thesis provides an in-depth analysis of how Antarctic microorganisms are adapted to extreme cold conditions, with a special focus on antifreeze proteins (AFPs). The three interconnected chapters comprehensively explore the significance of these proteins and their potential for biotechnological applications.

Chapter 1 offered a detailed overview of the adaptive strategies of polar organisms, highlighting the crucial role of AFPs in surviving extreme temperatures. The meta-analysis conducted revealed the widespread presence of these proteins in cold environments and their promising applications in industrial and environmental sectors, demonstrating their biotechnological relevance.

Chapter 2 complemented this analysis by investigating the potential of Antarctic isolates to produce AFPs. The chapter focused on selecting isolates with the highest ice recrystallization inhibition (IRI) efficiency and improved survival rates during freezing, highlighting the specific adaptations that allow microorganisms to endure prolonged freezing periods.

Chapter 3 focused on the practical application of protein extracts from Antarctic microorganisms, assessing their cryoprotective capacity in various biological systems. The results demonstrated the effectiveness of AFPs in preserving cell viability and viral stability under extreme conditions, highlighting their potential for advanced biotechnological innovations.

In summary, this thesis underscores the importance of Antarctic microorganisms biological adaptations and the significant potential of AFPs as cryoprotective biomolecules. The findings provide a solid foundation for future research aimed at optimizing the use of these proteins in diverse biotechnological applications. Further studies are essential to fully explore the potential of AFPs, from their identification to their applications in advanced biotechnological processes.

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