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TAXONOMIC AND FUNCTIONAL ANALYSIS OF MICROBIAL ICE COMMUNITIES FROM ANTARCTICA

Florianópolis
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Dissertação submetida ao Programa de Pós-Graduação em Biotecnologia e Biociências da Universidade Federal de Santa Catarina para a obtenção do título de Mestre em Biotecnologia e Biociências.

Orientador: Prof. Rubens Tadeu Delgado Duarte, Dr.

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Camila Tomazini Kinasz

TAXONOMIC AND FUNCTIONAL ANALYSIS OF MICROBIAL ICE COMMUNITIES FROM ANTARCTICA

O presente trabalho em nível de mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Prof.Rubens Tadeu Delgado Duarte, Dr.

Orientador

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Dedicated to my parents

ABSTRACT

The Antarctic region has an important role in global climate processes, the continent is home of the largest single piece of ice on Earth and has nineteen known holocene volcanoes, and at least nine of them are still active. The active volcanoes can disperse pyroclastic minerals at long distances, transporting nutrients and microorganisms to the surrounding glacial environment. The sedimented volcanic materials – called tephras – may interact with glacier ice and produce a unique environment for microbial life. In addition to these environments, between the extremes, ice caves are one of the least investigated cold habitats regarding the diversity and ecological role of ice-contained microbiomes. The variability in temperature characterizes the ice caves, ranging from 0°C down to −14°C, absence of light, high humidity, and reduced availability of food resources. This study aimed to describe the microbial community structure of Antarctic glacier ice with tephra layers and ice samples from an antarctic cave in terms of its taxonomic and functional diversity. Ice samples from Collins Glacier (King George Island) containing tephra layers of Deception Island volcano and ice samples from a cave located in this glacier were analyzed by a whole shotgun metagenomic approach and 16S rRNA pyrosequencing. Taxonomic analysis of ice-tephra revealed a highly diverse community dominated by phylum Bacteroidetes, Cyanobacteria and Proteobacteria. The dominant genera were *Chitinophaga* (13%), *Acidobacterium* (8%), and *Cyanothece* (4%), being all of these known to include psychrotolerant and psychrophilic strains. Functional diversity analysis revealed almost complete carbon, nitrogen and sulfur biogeochemical cycles. Carbohydrate metabolism of the ice-tephra community uses both organic and inorganic carbon inputs, where photosynthesis plays an important role through CO2 fixation. Our results also demonstrate a biotechnological potential for this glacial community, with functional annotations for styrene degradation and carotenoid pigment genes. The metagenome assembly presented genera like *Dyadobacter, Ferruginibacter* and *Solitalae* described previously in cold environments with less than 70% completeness. And in addition the 92.81% MAG for the cyanobacterium *Kovacikia*, previously described in tropical environments. Taxonomic analysis for the ice cave showed Actinobacteria, Acidobacteria, Firmicutes and Proteobacteria. With predominant psychrophile genera with more equity, *Chitinophaga* (6%), *Pseudomonas* (6%), and *Flavobacterium* (5%). Carbohydrates had the most reads for functional diversity and only few reads for photosynthesis. As for biotechnological potential, *Chitinophaga* genera is a candidate for biocontrol. A well formed Phenylacetyl-CoA catabolic pathway showed a capability also for biodegradation of xenobiotics. Future metatranscriptomic studies shall further reveal the active strategies and the biotechnology potential of extremophiles from these unique ice environments and microbial communities.

Keywords: tephra-ice; ice cave; Antarctica; 16S rRNA metabarcoding; glacier ice, metagenomics; Metagenomic-assembled genomes

RESUMO

A região antártica tem um papel importante nos processos climáticos globais, o continente abriga o maior pedaço de gelo da Terra e possui dezenove vulcões holocênicos conhecidos, sendo que pelo menos nove deles ainda estão ativos. Os vulcões ativos podem dispersar minerais piroclásticos a longas distâncias, transportando nutrientes e microrganismos para o ambiente glacial circundante. Os materiais vulcânicos sedimentados – chamados tefras – podem interagir com o gelo das geleiras e produzir um ambiente único para a vida microbiana. Além desse ambiente, entre os extremos, as cavernas de gelo são um dos habitats frios menos investigados em relação à diversidade e papel ecológico dos microbiomas contidos no gelo. Este estudo teve como objetivo descrever a estrutura da comunidade microbiana do gelo da geleira antártica com camadas de tefra e amostras de gelo de uma caverna antártica em termos de sua diversidade taxonômica e funcional. Amostras de gelo da geleira Collins (King George Island) contendo camadas de tefra do vulcão Deception Island e amostras de gelo de uma caverna localizada na mesma geleira foram analisadas por uma abordagem metagenômica completa e pirosequenciamento de 16S rRNA. A análise taxonômica de gelo com tefras revelou uma comunidade altamente diversificada dominada pelos filos Bacteroidetes, Cyanobacteria e Proteobacteria. Os gêneros dominantes foram *Chitinophaga* (13%), *Acidobacterium* (8%) e *Cyanothece* (4%), sendo todos conhecidos por incluir cepas psicrotolerantes e psicrofílicas. A análise da diversidade funcional revelou ciclos biogeoquímicos de carbono, nitrogênio e enxofre quase completos. O metabolismo de carboidratos da comunidade de gelo de tefra usa insumos de carbono orgânico e inorgânico, onde a fotossíntese desempenha um papel importante através da fixação de CO2. Nossos resultados também demonstram um potencial biotecnológico para esta comunidade glacial, com anotações funcionais para degradação de estireno e genes de pigmentos carotenóides. A montagem do metagenoma apresentou gêneros como *Dyadobacter*, *Ferruginibacter* e *Solitalae* descritos anteriormente em ambientes frios com menos de 70% de completude. E ainda o MAG de 92,81% para a cianobactéria *Kovacikia*, previamente descrita em ambientes tropicais. A análise taxonômica para a caverna de gelo mostrou Actinobacteria, Acidobacteria, Firmicutes e Proteobacteria. Com gêneros psicrófilos predominantes com maior equidade, *Chitinophaga* (6%), *Pseudomonas* (6%) e *Flavobacterium* (5%). Carboidratos tiveram mais leituras para diversidade funcional e apenas poucas leituras para fotossíntese. Quanto ao potencial biotecnológico, o gênero *Chitinophaga* é um candidato para biocontrole. Uma via catabólica de Fenilacetil-CoA bem formada mostrou uma capacidade também de biodegradação de xenobióticos. Futuros estudos metatranscriptômicos devem revelar ainda mais as estratégias ativas e o potencial biotecnológico dos extremófilos desses ambientes de gelo únicos e comunidades microbianas.

Palavras-chave: gelo vulcânico; caverna de gelo; Antártica; RNAr 16S metabarcoding; gelo glaciar; metagenômica; MAG

RESUMO EXPANDIDO

Introdução

A Antártica é o lugar mais frio da Terra e também é um deserto. O continente abriga a maior quantidade de gelo da Terra. A região tem um papel importante nos processos climáticos globais. Alguns dos organismos encontrados nas diferentes partes do continente incluem Proteobacteria (como *Colwellia*, *Glaciecola*, *Psychrobacter*, *Halorubrum*, *Methanococcoides*, *Haloplanktis*, *Octadecabacter*, *Polaribacter* e *Pseudoalteromona*), além de Actinobacteriota, Acidobacteriota, Chloroflexota e Proteobacteria. Todas as ilhas do oeste da Antártica são cobertas por geleiras e algumas apresentam atividade vulcânica no presente ou no passado. A ilha Deception tem o vulcão mais ativo no grupo das Ilhas Shetlands do Sul. Termófilos (adaptados a altas temperaturas) e psicrófilos (adaptados a baixas temperaturas) cultiváveis foram recuperados entre o gradiente de temperatura extrema do vulcão de Deception. As erupções mais recentes ocorreram no fim dos anos 70, espalhando tefras (fragmentos ejetados no ar por vulcões em erupção) por mais de 150 km. O estudo dessas tefras é utilizado como ferramenta para correlações entre registros atmosféricos, marinhos e terrestres de mudanças globais. Entre os ambientes extremos frios, as cavernas de gelo são um dos biomas menos explorados. O derretimento do gelo em todo o mundo torna essa investigação urgente. Os achados de trabalhos em cavernas de gelo mostraram membros de Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, incluindo Caldiserica e Verrucomicrobia, como membros de *Cryobacterium, Lysinomonas, Pedobacter, Aeromicrobium, Clostridium, Pseudomonas, Janthinobacterium, Stenotrophomonas,* e *Massilia.* O estudo dessas comunidades pode prover novas enzimas e metabólitos com potencial para aplicações médicas, farmacêuticas e biotecnológicas. Para a Astrobiologia, esses microrganismos nos indicam como condições limitantes à vida como a conhecemos.

Objetivos

Caracterizar a diversidade taxonômica e funcional de dois ambientes antárticos (tefras em gelo e caverna de gelo) usando métodos baseados em sequenciamento de metagenoma e metabarcoding.

Material e Métodos

As amostras de gelo utilizadas no trabalho foram coletadas em 2009 pelo Prof Dr Rubens T. D. Duarte durante o Programa Antártico Brasileiro em parceria com o Laboratório de Ecologia Microbiana da USP-São Paulo. A descontaminação foi feita usando uma sonda de 1000 W aquecida eletricamente para remover contaminantes da superfície externa do gelo. As porções descontaminadas de gelo foram imersas em hipoclorito de sódio 5% (v/v) gelado, seguido de lavagens com água estéril MilliQ gelada. A extração de DNA total foi feita com o kit DNA UltraClean Water. A biblioteca metagenômica foi preparada usando o kit Nextera DNA Sample Preparation (Illumina Inc.) e o sequenciamento foi realizado usando Illumina HiSeq 2500. Parte do gene RNAr 16S das amostras foi amplificada por PCR utilizando os primers U519F e U1068R. As sequências de DNA foram inicialmente analisadas quanto a qualidade utilizando o software Mothur v.1.44.3. O metagenoma shotgun das amostras de gelo foi avaliado de acordo com a diversidade taxonômica e filogenética através do servidor MG-RAST. O processo de montagem de metagenomas utilizou o protocolo do anvi'o v.5.

Resultados e Discussão

Os resultados da amostra AI WGS estão no manuscrito publicado nos Anais da Academia Brasileira de Ciências (https://doi.org/10.1590/0001-3765202220210621) em 8 de outubro de 2021. Os Filos encontrados no sequenciamento do RNAr 16S para a ice-tephra (AI) foram predominantemente Cyanobacteria, Bacteroidetes e Proteobacteria. Dentro dos gêneros mais predominantes estão *Alkalinema* (58,07%), *Flavihumibacter* (12,31%) e *Ferruginibacter* (8,21%). Dentro dos resultados de Bactérias para o metagenoma AI, o Filo Bacteroidetes (36%) foi o mais abundante, seguido por Cyanobacteria (33,4%), Proteobacteria (11,7%), Acidobacteria (8,1%) e Actinobacteria (2%). *Chitinophaga* (13%) foi o gênero dominante, seguido por *Acidobacterium* (8%) e *Cyanothece* (4%). A alta abundância de Bacteroidetes nas amostras pode refletir a presença de fontes de carbono simples dentro deste ecossistema glacial. Quanto ao perfil funcional no ciclo do Nitrogênio, as bactérias oxidantes de amônio *Nitrosococcus* (11.150 reads), *Nitrosomonas* (8.057 reads) e *Nitrosospira* (5.567 reads) dominaram a comunidade. Genes para o metabolismo do enxofre (3.255 reads) também estavam presentes. As vias relacionadas à fotossíntese estavam quase completas nas amostras, com a maioria para os fotossistemas I e II, complexo citocromo b, transporte de elétrons fotossintético e ATPase, além do complexo de clorofila captador de luz e pigmentos secundários do complexo de antena. Os carotenóides (pigmentos tetraterpenóides lipossolúveis com função fotoprotetora) apresentaram via de biossíntese quase completa, exceto por enzimas específicas como a 15-cis-fitoeno sintase. Um total de 1 MAG e 21 Bins foram recuperados de sequências da amostra de ice-tephra (AI). O software Prodigal identificou 29.404 genes nos MAG e Bins. O MAG001 foi atribuído ao gênero *Kovacikia* (Cyanobacteria) com 92,80% de cobertura. A amostra da caverna de gelo (IC) mostrou mais equidade nos resultados de RNAr 16S comparado à amostra de ice-tephra. Os filos dominantes apresentaram Actinobacteria, Acidobacteria, Firmicutes e Proteobacteria como os mais abundantes. Quanto aos gêneros, os mais encontrados foram *Gaiella* (31,18%), *Nocardioides* (22,41%) e *Perlucidibaca* (7,17%), gêneros já encontrados na Antártica ou no gelo. Os Filos dominantes no sequenciamento shotgun foram Bacteroidetes (42,53%), Proteobacteria (36,12%), Cyanobacteria (5,46%), Acidobacteria (1,82%). Os gêneros bacterianos mais abundantes em IC foram *Chitinophaga* (3.961 leituras), *Pseudomonas* (3.931 leituras), *Flavobacterium* (3.287 leituras), *Pedobacter* (2.297 leituras), *Bacteroides* (1.669 leituras) e *Cytophaga* (1.387 leituras), alguns dos quais já descritos na Antártica antes. Quanto ao perfil funcional, os carboidratos tiveram o maior número de reads (2.976), seguidos por subsistemas baseados em agrupamento (2.976 reads). A via catabólica do fenilacetil-CoA (71 reads) e degradação do benzoato (39 reads) se destacaram por seu potencial uso em biotecnologia.

Conclusão

Até o momento, esta é a primeira descrição da diversidade taxonômica e funcional de uma ice-tephra e a primeira comunidade de cavernas de gelo da Antártica usando metagenômica. A montagem do metagenoma mostrou o gênero *Kovacikia* nunca antes encontrado em ambiente frio. A biodiversidade microbiana em amostras de gelo da Antártica tem potencial para o desenvolvimento de novas biotecnologias, sobretudo na degradação de moléculas orgânicas. A comunidade do gelo da Antártica apresenta microrganismos extremófilos de interesse para a Astrobiologia.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

℃ - Graus Celsius

16S rRNA - 16S Ribosomal ribonucleic acid

AI - ash ice or ice-tephra

bp - base pair

CBSS - cloud based system

CFU - colony forming unit

CNPq - National Council for Scientific and Technological Development

DNA - deoxyribonucleic acid

HDPE - high ‑density polyethylene

HMM - hidden Markov models

IC - ice cave

kg - kilograms

km - kilometer

LECOM - Microbial Ecology Laboratory

MAG - metagenome-assembled genome

mL - milliliter

NApOc - Oceanographic and Supply Ship “Ary Rongel”

ng - nanograms

nm - nanometre

PCR - Polymerase chain reaction

PROANTAR - Brazilian Antarctic Program

RDP - ribosomal database project

RNA - ribonucleic acid

S - South

UV - Ultravioleta

W - watts

W - west

WGS - whole-sequencing genome

µL - microlite

µm - micrometer

TABLE OF CONTENTS

1. INTRODUCTION

Earth's biosphere is considered a cold environment, its surface is covered with deep oceans, permafrost, alpine regions, glaciers, marine ice, and snow; more than 80% of the biosphere is permanently below 5°C (CAVICCHIOLI et al., 2000). Due to a great part of the Earth being cold, it is no surprise, a wide range of organisms are able to proliferate in these cold environments, including bacteria, viruses, archaea, yeast and algae. In extreme and isolated cold environments such as the Arctic, Antarctica and alpine regions, autochthonous microorganisms are found, which favors the arising of endemic species (JUNGBLUT, 2010). Although the ice can present challenging conditions for life such as low temperature, desiccation, nutrient shortage, high incidence of ultraviolet radiation, long periods of absence or presence of light (DUARTE, 2010). Low temperatures are also believed to be ideal for long-term preservation of microorganisms and molecules (WILLERSLEV et al., 2004).

The organisms capable of thriving in extreme environments are called extremophiles. Their resistance is specific to physical, chemical and/or biological factors, including temperature (thermophiles and psychrophiles), pH (alkaliphile and acidophile), water availability (xerophile), radioactivity, pressure (piezophiles) energy, nutrients, and others. Within these exceptional organisms, psychrophiles are the ones with optimum growth around 15° C or below. Psychrotolerant or psychrotrophs have the ability to grow at 0° C, but its optimum temperature exceeds 15° C (GARCÍA-LÓPEZ et al., 2016). To be able to live in these extreme conditions, the microorganisms developed adaptations to these unic physic-geochemical conditions (WADHAM et al., 2019). One of the many adaptations found in psychrophiles is the composition of lipids and enzymes that maintain the fluidity of the membrane (FELLER & GERDAY, 2003) and the presence of extracellular polymeric substance or EPS, this molecule can reduce water solidification point (BOETIUS et al., 2015). Moreover, pigments also help the protection against high levels of UV radiation (SAJJAD et al., 2020). The study of psychrophiles is becoming relevant as evidence shows ice in other places in our solar system and Space exploration is increasing in the next few years driven by improvement of technology and the entry of the private sector (COCKELL, 2022).

According to Willerslev et al., (2004), a great number of works that reported recovery

of DNA and/or RNA from ice and permafrost presented conflicting results, some due differences in extraction methodology, others resulting from contamination. However, later studies affirm the existence of a great diversity of microorganisms in cold environments, including bacteria, archaea, fungi and algae (YAO et al., 2006; STEVEN et al., 2006; MARGESIN & MITEVA, 2011; TAŞ et al., 2014).

1.1. ANTARCTICA

Antarctica is the coldest and driest place on Earth. The continent covers approximately 20% of the Southern Hemisphere and includes island territories within the Antarctic Convergence. The islands of the region are: South Orkney Islands, South Shetland Islands, South Georgia, and the South Sandwich Islands, all claimed by the United Kingdom; Peter I Island and Bouvet Island, claimed by Norway; Heard and McDonald islands, claimed by Australia; and Scott Island and the Balleny Islands, claimed by New Zealand. The continent is home of the largest single piece of ice on Earth, this ice sheet can extend beyond the continent when snow and ice are at their most extreme (TURNER et al., 2009).

Microorganisms can be found in all Antarctic ecosystems. In the cold deep seawater, Proteobacteria is the most common group found, specially Gammaproteobacteria like *Colwellia, Glaciecola,* and *Psychrobacter*. On the other hand, deep lake sediments present both Archaea (mostly from Phyla Euryarchaeota and Thaumarchaeota) and Bacteria (Phyla Proteobacteria, Bacteroidetes and Acidobacteria). The sea ice ecosystem has also shown an extensive diversity, including several bacteria found in the Antarctic marine water like *Antarcticus, Haloplanktis, Octadecabacter, Polaribacter, Pseudoalteromonas* (SUYAL, 2021). Antarctic soil communities present a microbial diversity comparable to mesophilic soils at the Phylum level, with Actinobacteriota, Acidobacteriota, Chloroflexota and Proteobacteria often found in ice-free areas of the continent. Glacier ice contains a vast microbial diversity, not only due to the massive ice cover in Antarctica, but also for the conservative characteristics of their subzero temperatures. Accordingly, glacier ice microbes potentially represent the microbes in the atmosphere at the time of their deposition. Culturable bacterial abundance in the ice core varies from 0.02 to 5.8×10^3 CFU mL^{-1} (SHIVAJI et al., 2013). The Antarctic ice shelves can be fertilized by marine benthic debris, following basal

refreezing and surface sublimation (HODSON et al., 2008). Glaciers at different sampling sites from Antarctica showed representatives of Proteobacteria, Actinobacteriota, Bacteroidetes, Cyanobacteria, Firmicutes and Verrucomicrobia (BRINKMEYER et al., 2003; ORTIZ, 2020).

1.2. GLACIER

The formation of glaciers occurs from the precipitation of snow, which undergoes compression and recrystallization. Snow is made up of 90% air and 10% water. With the accumulation of snow over time, the deep layers of snow are compressed, expelling the air and creating a structure called firn (50% air, 50% water), the firn undergoes further compression and recrystallization, forming glacial ice (10% air, 90% water) (Figure 1). The entire process takes around 100 years, depending on snowfall rate (MA et al., 2005). The air trapped inside glacial ice is an important record for glaciology and paleoclimatology, as it contains the atmospheric gas composition of the ice formation period (HUBBARD & GLASSER, 2005).

The ice found in these areas can provide us with paleontological records of temperature, chemical composition of the atmosphere, volcanic eruptions, solar variability, ocean surface productivity, anthropogenic emissions, and an enormous variability of climatic indicators (CHRISTNER, 2005).

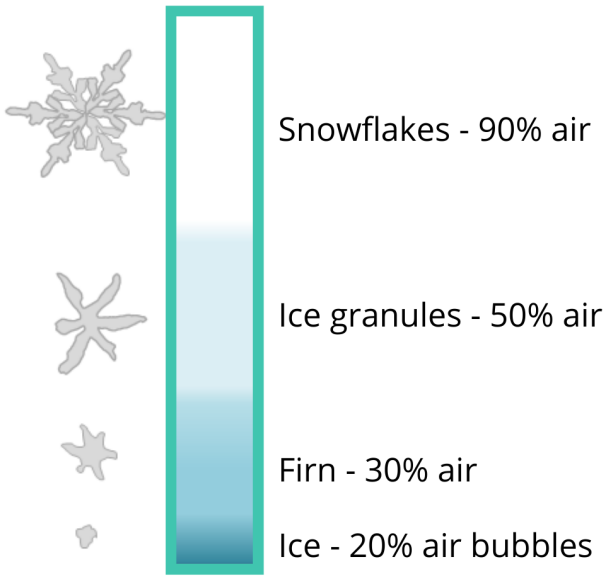


Figure 1. Steps in the process of formation of glacial ice from snow, granules, and firn. Source: EARLE, 2015.

Glacial environments like supraglacial, englacial, and subglacial may differ vastly in terms of their water content, nutrient abundance, redox potential, ionic strength, rock-water contact, pressure, solar radiation, and pH conditions (HODSON, et al., 2008). An important habitat in the supraglacial ecosystem are the cryoconite holes, a common feature to all glacial environments where surface melting occurs. They are formed following deposition onto the glacier surface by aeolian, fluvial, mass movement and melting processes. The holes are unstable, and when perturbed, are able to form new holes following dispersal, providing critical feedback within the ecosystem. The cryoconite holes are home to cyanobacteria, algal cells, some eukaryotes, bacteria and viruses. As for englacial habitats, these not only include deep, buried environments, but also the vertical walls of crevasses and moulins, which can convey water, nutrients, atmospheric gases, and biota into the glacier. The subglacial environments include soil moraines and lakes. At least 145 subglacial lakes are known to lie beneath the Antarctic Ice Sheet, including Lake Vostok, one of the largest freshwater lakes on Earth and the largest of the Antarctic subglacial lakes identified to date (HODSON, et al., 2008).

1.3. VOLCANISM IN ANTARCTICA

The majority of the islands and archipelagos of Lesser Antarctica are volcanic and

heavily glaciated. Antarctica has nineteen known Holocene volcanoes, and at least nine of them are reported active (SIEBERT, 2002). The volcanic explosions can disperse fine ash and volcanic aerosols, which can pose a hazard to human health, infrastructures, and also to the environment (GEYER, et al., 2017). Volcanic sites are challenging as they encompass several extreme characteristics like high temperatures, heavy metal contents, extreme acidity and others. From an ecological perspective this highly dynamic system of volcanoes and glaciers presents a very hostile environment, acting against the survival of microbial life (PEÑA-OCAÑA et al, 2022).

Deception Island has the most active volcano in the South Shetlands Islands group (Figure 2). Culturable thermophiles and psychrophiles have been recovered among the extreme temperature gradient in Deception volcano, which indicates that these extremophiles remain alive even when the conditions do not comprise their growth range. These findings include thermophiles members of genera *Geobacillus*, related to *Brevibacillus thermoruber*, *Anoxybacillus kestanbolensis*, *Thermus thermophilus* and psychrophiles *Arthrobacter*, *Psychrobacter*, *Flavobacterium*, *Pseudomonas*, and *Sphingomonas* (BENDIA et al., 2018).

The most recent eruptions at Deception Island took place in 1967, 1969 and 1970 (PALLÀS et al., 2001). As a result of these eruptions, volcanic dust and pyroclastic materials were dispersed by wind and eventually deposited into the surrounding glacial ice, forming ash layers known as tephras, which represent evidence of past volcanic activities (GEYER et al., 2017). For instance, during an expedition to Collins Glacier at King George Island (about 120 km from Deception Island), Chinese researchers identified “dirty” bands within several ice cores collected at 80.2 m (JIANKANG et al,. 1999). Analysis on the mineralogy and microstructure of these bands revealed characteristics with significant correlation to volcanic ashes dating to the end of 1970 (JIANKANG et al., 1999). The study of tephras is a tool for correlations between atmospheric, marine and terrestrial records of global change (SMELLIE, 1999).

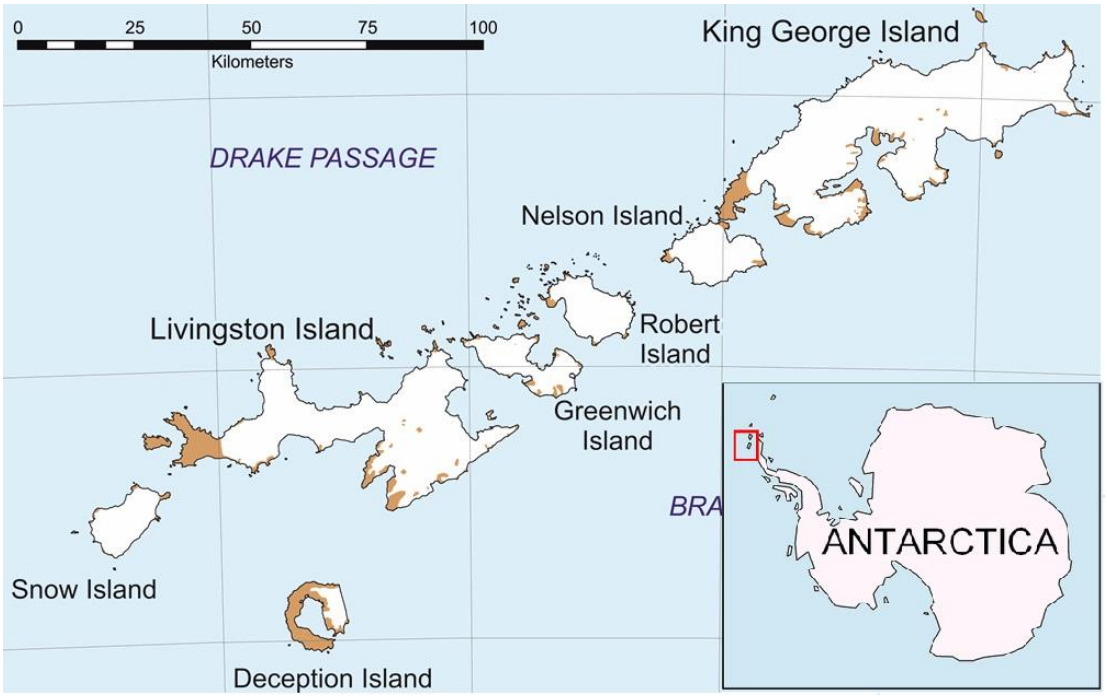


Figure 2. About 75 miles north of the Antarctic Peninsula are the South Shetland Islands. These include Deception Island and King George Island. Source: adapted from the British Antarctic Survey

1.4. ICE CAVE

Among extreme environments, ice caves are one of the least investigated cold habitats regarding the diversity and ecological role of ice-contained microbiomes (ICTUS et al., 2018). Ice caves differ from glacier caves in terms of formation. Glacier caves are often carved out by water running through or under the glacier's ice, while ice caves are permanent caves in rock formations, in which ice forms and remains far into the summer or throughout the year (MCKENZIE, 1969). The environmental conditions in ice caves are characterized by the variability in temperature, ranging from 0 °C down to −14 °C, absence of light, and reduced availability of food resources. Ice caves are found in various climatic regions on Earth, from high latitude subpolar regions in Canada (Yukon territory), Norway and Siberia to Ross Island in the Antarctic region, high altitudes in the Altai Mountains in Russia (often under permafrost conditions), and the Spanish Pyrenees and Picos de Europa in the Mediterranean region (IEPURE, 2018). The melting of ice around the world makes the investigation of ice caves urgent, it can provide climatic and geological data on the life associated with these risky decomposition and disaster environments. The findings of works in ice caves showed members of Phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, including Caldiserica and Verrucomicrobia (ICTUS et al., 2018), as members

of genera *Cryobacterium, Lysinomonas, Pedobacter*, *Aeromicrobium*, *Clostridium, Pseudomonas, Janthinobacterium, Stenotrophomonas*, and *Massilia* (PAUN et al., 2019).

1.5. METAGENOMIC ANALYSIS

Prokaryotes in the environment can be cultivated in the laboratory, but only fewer than 1%, meaning that most microbial life could not be studied (SCHLOSS & HANDELSMAN, 2005). Metagenomics is the culture-independent analysis of microbial genomes using an approach based either on expression or on sequencing (SCHLOSS & HANDELSMAN, 2003). Molecular technology is making it possible to predict the dominant microbial groups within an ecosystem, and their functional role (PEARCE, 2008). Assembly of short metagenomic reads into contiguous segments of DNA is a computationally intensive task, and its effectiveness often depends on the complexity of the environment. The computational work provides the basic data types for many subsequent analyses, including phylogenetic comparisons, functional annotations, binning of sequences, phylogenomic profiling, metabolic reconstructions and metagenome assembly (SHARON & BANFIELD 2013).

The recent development of shotgun metagenomics has provided the opportunity to investigate the taxonomic and functional diversity of microbial communities, further expanding our knowledge on metabolic pathways and the adaptation mechanisms required to thrive in extreme conditions (GÓMEZ-SILVA et al. 2019). The 16S ribosomal RNA sequencing is still relevant to phylogeny because of its evolutionary and ecological properties, such as the ubiquity, extreme sequence conservation and domain structure with variable evolutionary rate, as for also infer functional diversity (TRINGE & HUGENHOLTZ, 2008).

Metagenome-Assembled Genome (MAG) is a single-taxon assembly based on one or more binned metagenomes that has been asserted to be a close representation to an actual individual genome (JÉGOUSSE et al., 2021). For this reason, sequence contamination is most likely to occur from closely related genomes as they share more similar sequence compositions (LI, 2022) (Figure 3). The reconstruction of genomes from metagenomes is a difficult task due to greater diversity of genomes present and the introduction of intergenomic repeats; similar repetitive regions from different genomes (MAGGIORI et al., 2021). Also the

recovery of MAGs are particularly valuable for lineages as they reveal clues about trophic interactions and ecology within the environment; the uncultivated organisms can be quite distantly related to any isolated species (CROSS, et al., 2019).

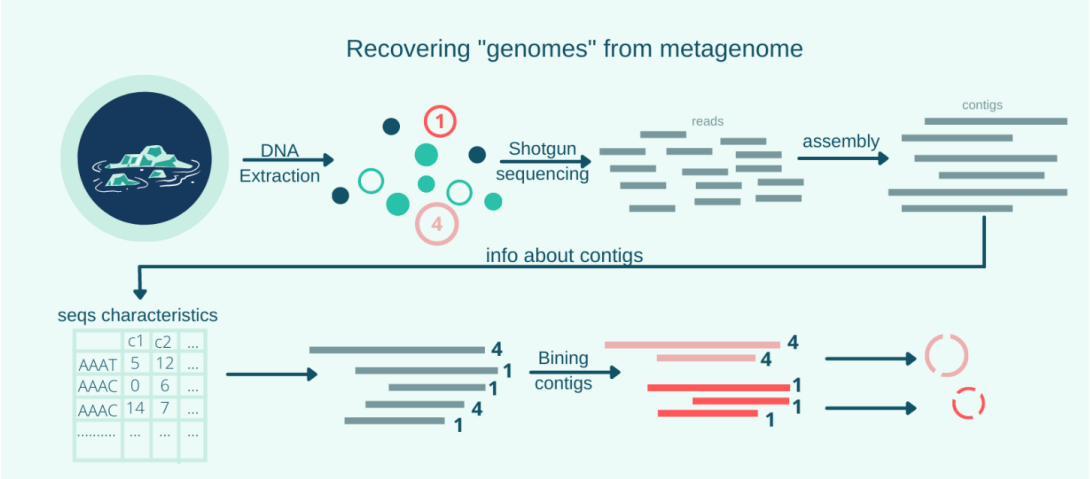


Figure 3. Recovering genomes from metagenome - MAG - Metagenome assembled-Genome. Source: Adapted from LEE, M. D., 2022

1.6. JUSTIFICATION

Additionally, the study of these communities may also reveal novel enzymes and metabolites with potential for medical, pharmaceutical and biotechnological applications. Biotechnology takes advantage of these microorganisms due to their efficiency of enzymatic reactions and catalytic elements with high potential for application in the context of industry (MUÑOZ et at., 2017). These are based on the use of their enzymes, which can be used in cooling systems as additives in washing detergents, in the food industry, in bioremediation, among others. For Astrobiology, these microorganisms reveal the limiting conditions to life as we know it. Furthermore, ice sampling in some places in Antarctica, such as ice caves and volcanoes, presents similar challenges to exploring extreme extraterrestrial environments (BULAT et al., 2011). Therefore, this study aims to analyze the taxonomic and functional diversity of microbial communities from ice-tephra samples and ice from a cave collected from King George Island, Antarctica.

1.7. HYPOTHESIS

Considering the literature and data stated above, the hypothesis for this work is that communities isolated from Antarctic locations, including an ice cave and an ice tephras, are constituted from microorganisms with genes capable of resistance to extreme environments including cold, volcanic related reactions and UV resistance. It may also present a very distinct diversity, since Antarctica is an isolated continent with its own particularities.

2. AIM AND OBJECTIVES

2.1. AIM

To characterize the diversity of two Antarctic environments (ice-tephra and ice cave).

2.2. OBJECTIVES

- To characterize in silico the taxonomic diversity of the samples by sequencing the 16S rRNA gene.
- To characterize in silico the functional and taxonomic diversity and present in samples by sequencing the metagenome.
- To assemble the microbial genomes from the ice-tephra metagenome data.

3. METHODOLOGY

3.1. ICE SAMPLING

Ice samples were carried out during the Brazilian Antarctic Program (PROANTAR / CNPq) in partnership with the Microbial Ecology Laboratory of Universidade de São Paulo (LECOM). The samples called AI (ash ice); later called Ice-tephra, were collected at the terminus of Collins Glacier, King George Island (62°10'4"S; 58°51'11"W) (Figures 4 and 5).

Samples named Ice Cave (IC) (Figure 6) were collected in a cave located at 62°10’23.7”S; 58° 29’24.699”W, an ice cave located near the Henryk Arctowski Polish Antarctic Station, in January 2009 (Table I). Using a new ice pick, decontaminated with a 70% ethanol solution before use, the retrieved ice samples were packed in autoclaved high‑density polyethylene (HDPE) sacks and stored at -20 °C. Samples were transported to Brazil in deep-freezers at -20 °C aboard the Oceanographic and Supply Ship (NApOc) Ary Rongel. Upon arrival, all samples were inspected for cracks, microfractures, melting, and other damages that may occur during transportation. Damaged ice samples were discarded and the remaining samples were used in this study.

Ice samples (ice pieces of 6 to 8 kg each) were decontaminated before the DNA extraction using a modified protocol based on Rogers et al. (2005). Briefly, a 1000 W electric-heated rod was used to remove contaminants from the outer ice surface. The remaining pieces of ice were immersed in a cold 5% sodium hypochlorite solution for 10 s, followed by three 200 mL rinses with cold sterile MilliQ water. The now surface-sterile ice samples were placed inside a new sterile HDPE sack and melted at 4 °C overnight. The melted ice samples were filtered in sterile 0.22 µm membranes (Millipore, USA), and stored at -20 °C until used for DNA extraction.

Table I. Sample coordinates in Antártica DATUM:WGS84

Code[1]	Sample type	Latitude	Longitude
AI	Ice-tephra	62° 10’ 4.001”S	58° 51’ 11.001”W
IC	Ice cave	62° 10’ 23.7”S	58° 29’ 24.699”W

1 Sample code used in the LEMEx sample collection.

Figure 4. Colling Glacier showing ice-tephra (darker lines on ice), King George Island. Source: Rubens Duarte

Figure 5. Sample extraction, Collins Glacier, King George Island. Source: Rubens Duarte

Figure 6. Ice cave, King George Island, Fildes Peninsula, Antarctica. Location close to Arctowski Station. Source: Rubens Duarte

3.2. TOTAL DNA EXTRACTION AND METAGENOMIC SEQUENCING

The ice total DNA was extracted using the UltraClean Water DNA Isolation Kit (Mo Bio, USA) with the following modifications: the original filtering membrane from the kit was replaced by the 0.22 μm membranes used to filter the melted ice; the kit WD5 solution (10 mM Tris) was replaced by autoclaved MilliQ water pre-heated to 60 °C before use. These modifications were applied to optimize the final DNA yield. After extraction, the total DNA was purified and concentrated to a final volume of 10 μL with DNA Clean & Concentrator Kit (Zymo Research, USA) following the manufacturer's protocol. The final DNA concentration and purity were determined with Qubit dsDNA BR kit (Thermo Fisher Scientific, USA). Due to the small cell concentration in the ice samples, a low DNA concentration (~3.7 ng μL^{-1}) was obtained for metagenomic sequencing. Therefore, the total DNA was uniformly amplified with Illustra GenomiPhi V2 DNA Amplification Kit (Cytiva, USA), which uses an isothermal strand displacement amplification approach. Ice metagenome

was sequenced at Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP), Brazil. The metagenomic library was prepared using Nextera DNA Sample Preparation kit and sequencing reactions were carried out using Illumina HiSeq 2500 (paired-end 2x100 bp). For the AI sample, the sequencing was done twice, creating AI sequencing data named "old" (1st sequencing) and "new" (2nd sequencing).

DNA from samples AI and IC used for taxonomic diversity analysis with the help of Mothur v.1.44.3 used the 16S rRNA gene amplified by PCR using primers U519F and U1068R (WANG and QIAN, 2009). The amplification product was sequenced on the 454 Pyrosequencing platform at the Advanced Center for Genomic Technologies - CATG, of the Institute of Chemistry at USP.

3.3. METABARCODING (16S rRNA)

3.3.1. PRE-PROCESSING

Pre-processing of the reads from the pyrosequencing AI and IC samples were processed through Mothur v.1.44.3 (SCHLOSS et al., 2009). Initially, the paired-end raw reads were converted into contigs using Mothur default parameters. After that, contigs were quality filtered by removing all sequences with an average Phred score < 20. Chimeric sequences were removed with ChimeraSlayer almorithm on Mothur.

3.3.2. TAXONOMIC DIVERSITY

Diversity indexes were obtained through Mothur software following the methods described in Duarte (2010). The 16S rRNA sequences were identified according to the Ribosomal Database Project (RDP - Michigan State University). For OTU calculation, values of 75% for the taxonomic level of Phylum, 85% for Class and 95% for Gender are used as cutoff limits. This approach has the advantage of flexibility in parameters and suitability of tools for the specific analysis of the samples in this work.

3.4. SHOTGUN METAGENOMIC

3.4.1. ANNOTATION OF METAGENOME SEQUENCES AND POST PROCESSING

The shotgun metagenome of the ice samples were evaluated through MG-RAST pipeline. The sequences obtained by the WGS were sent in a FASTA indepent file to the automatic server. The pipeline offers quality control, annotation, benchmarking, and storage services for metagenomics and amplified sequences. It also has the function of analyzing metagenomic data, automatically suggesting phylogeny and functional analysis. In addition, its algorithms were designed for the annotation of short reads (~50 bp), as is the case with sequencing on the Illumina HiSeq platform (KEEGAN et al., 2016).

3.5. METAGENOMIC ASSEMBLY AND GENOMIC RECONSTRUCTION

The sample used for this assembly was tephra-ice (AI) submitted to the MG-RAST shotgun analysis called “new” for this part of the study, plus a first sequencing done with the same sample called “old”. Metagenomic data was processed with the help of anvi’o v.5 according to Eren et al. (2015) (Figure 7).

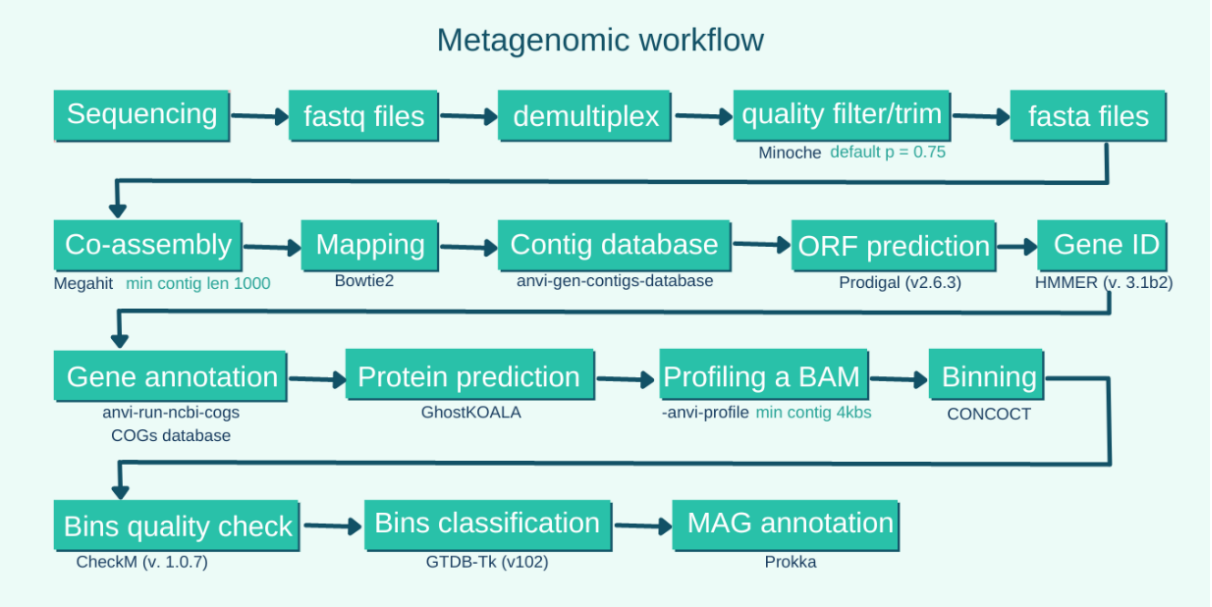


Figure 7. Anvi'o Metagenomic workflow. Source: Author

4. RESULTS AND DISCUSSION

4.1. ICE-TEPHRA (AI) SAMPLES

The results for sample AI shotgun metagenomic are in the manuscript received on April 20, 2021; accepted for publication on October 8, 2021 at Anais da Academia Brasileira de Ciências (ISSN:0001-3765) (KINASZ et al., 2022).

Volcanic eruptions are known for the dispersion and deposition of microorganisms, volcanic ash, heavy metals, and other crystalline particles (WITT et al., 2016). Regardless of being a challenging environment for the maintenance of life, Antarctic soils are inhabited by a distinct array of microorganisms, well-adapted to their demanding physicochemical conditions (ZAIKOVA et al., 2019). Likewise, Antarctic ice can also consist of rich microbial communities, as demonstrated by our metagenomic data. The DNA extraction resulted in 37 ng of DNA from about 1,800 mL of melted glacier ice (or 20.5 ng L^{-1}). This result is expected for a low density microbial environment such as glacier ice (MITEVA, 2008) and contrasts the much higher DNA yield from other Antarctic environments such as a pond of glacial meltwater (600 ng L^{-1}), glacier forefield soils (1,400 ng kg^{-1}), and lake sediments (2,810 ng kg^{-1}) (FERRÉS et al. 2015; MUANGCHINDA et al., 2015; STRAUSS et al., 2012). The

results of treatment done by Mothur v.1.44.3 software presented a low number of sequences due to the reduced concentration of extracted DNA, which uses 16S rRNA. As mentioned before, ice environments are typically poor in nutrients and present very low temperatures. A total of 720 OTUs (95% cutoff) were obtained from ice-tephra. The mean size of sequences was 368 pb.

For the Shotgun metagenomic a total of 16,746,302 reads were generated with an average length of 101±5 bp. The quality filtering validated 14,186,417 (84%) sequences, which were distributed into 16,823 (0.12%) ribosomal RNA sequences, 6,914,275 (48.74%) predicted proteins with known function, and 7,255,319 (51.14%) proteins with unknown function.

4.1.1. TAXONOMY

The Phyla found by the 16S rRNA sequencing for the ice-tephra (AI) was predominantly Cyanobacteria, Bacteroidetes and Proteobacteria (Figure 8). The most predominant 11 genera (Figure 9) include *Alkalinema* (58.07%), *Flavihumibacter* (12.31%), and *Ferruginibacter* (8.21%). The genus *Flavihumibacter* is a member of the family Chitinophagaceae, the genus included five validly published species that have been isolated from soil or sediment soil and still no ice or volcanic environments (SEO et al., 2020). The *Alkalinema* genus means a filament from alkaline lakes. In nature, known strains grow in saline–alkaline lakes in the Brazilian Pantanal wetland (VAZ et al., 2015). Other genera representing the 6.66% of the sample include *Granulicella, Phenylobacterium, Polymorphobacter, Nguyenibacter, Psychrobacter, Dokdonella, Rhizobacter, Flavilitoribacter, Hymenobacter* and *Pedobacter*.

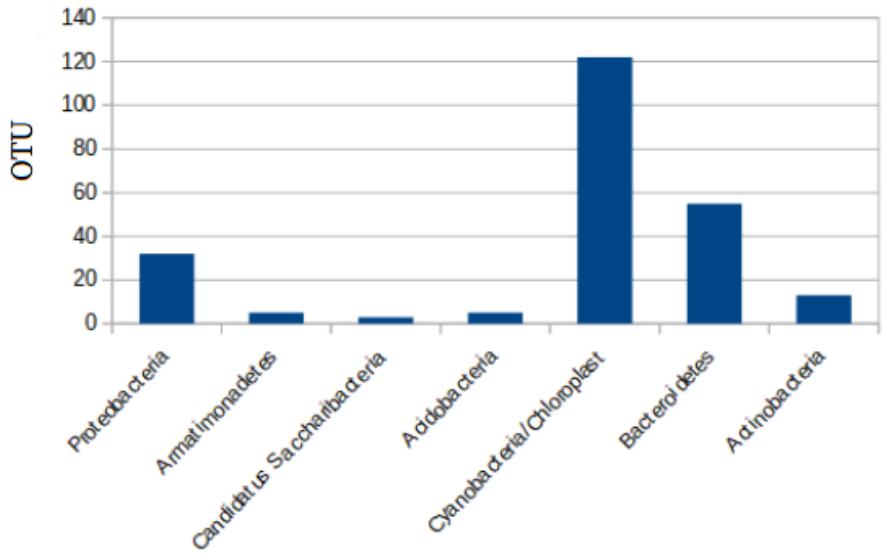


Figure 8. Taxonomic distribution of Phyla in the ice-tephra (AI) 16S rRNA sample according to the results obtained by the Mothur software. Y axis represent taxonomic abundance of OTUs at 95% distance. Graphic: LibreOffice Calc.

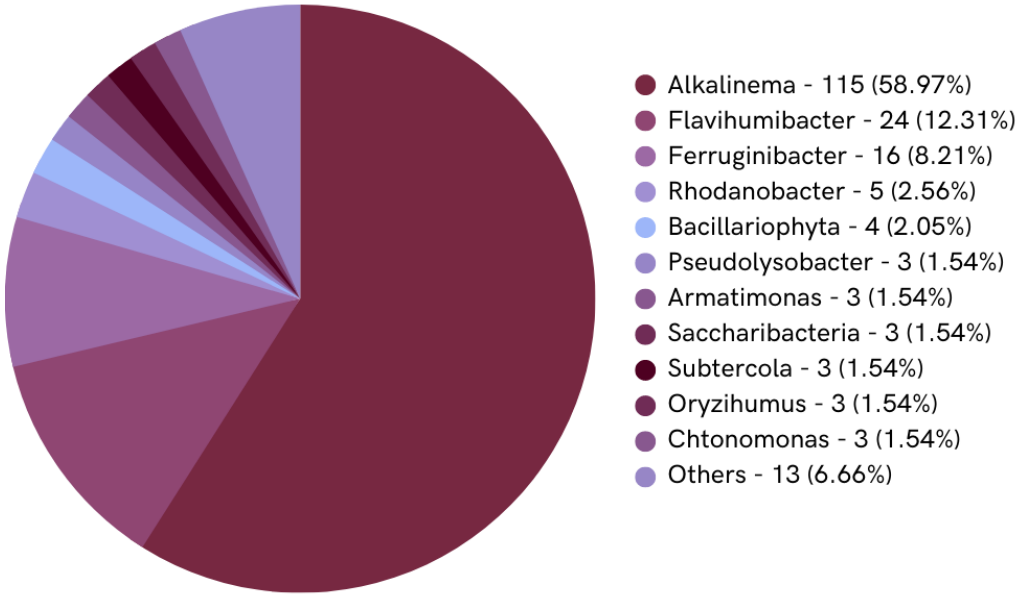


Figure 9. Distribution of microbial groups found in the glacier ice-tephra sample (16S rRNA). Genus absolute abundance, where only the top 11 most abundant OTUs are shown.Graphic: Canva

The Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) taxonomic analysis of the ice-tephra samples revealed a microbial community primarily composed of Bacteria (96%), followed by small fractions of Eukaryota (3%), Archaea (0.14%), and Viruses (0.01%). Within Bacteria (Figure 10a), Phylum Bacteroidetes (36%)

was the most abundant, followed by Cyanobacteria (33.4%), Proteobacteria (11.7%), Acidobacteria (8.1%), and Actinobacteria (2%). These Phyla are well known as widespread organisms with important functions in Antarctic soils (COWAN et al., 2010). Proteobacteria (α, β, and γ), Cyanobacteria, Bacteroidetes, Actinobacteria, and Acidobacteria are also described as major phyla in other glacial ecosystems (BULAT et al., 2004, MITEVA et al., 2008). Our metagenomic data also revealed a high abundance of classes Sphingobacteria (15%), Acidobacteria (7%), and Cytophagia (5%), while *Chitinophaga* (13%) was the dominant genus, followed by *Acidobacterium* (8%), and *Cyanothece* (4%) (Figure 10b).

Bacteroidetes are composed of chemoorganotrophic bacteria, contributing to the carbon cycle mostly as polymeric carbon degraders (AISLABIE et al., 2006). These phyla can use a wide variety of organic (mono-, di-,and polysaccharides) and inorganic (CO_2) compounds as energy sources, as well as ammonia (NH_3) and sulfide (H_2S) as nitrogen and sulfur sources, respectively (SMITH et al., 2006). The high abundance of Bacteroidetes in our sample may reflect the presence of such simple carbon sources within this glacial ecosystem (RIME et al., 2016). The dominant genus, *Chitinophaga*, includes over 37 species (LEE et al. 2020) of chitinolytic bacteria able to degrade chitin and other complex carbon sources such as casein and gelatin (PANKRATOV et al., 2006, COWAN et al., 2010). Several *Chitinophaga* strains are psychrotolerant, able to grow at minimum temperatures of 4 °C to 10 °C (PANKRATOV et al., 2006; LI et al., 2017; JIN et al., 2018). Additionally, some *Chitinophaga* species have a dormant stage that would also allow for survival in periglacial habitats (VIMERCATI et al., 2019). *Acidobacterium*, the second most abundant genus in the ice community, is characterized by growth in acid environments within the range pH 2-6 (KIELAK et al., 2016). *Acidobacterium* species are commonly associated with the dry soils of Antarctica and probably encompassing a role in biogeochemical cycles (LEE et al., 2008; COWAN et al., 2010), able to use several carbon sources including mono- (arabinose, dextrose, and xylose) and polysaccharides (xylan and agar), even in low concentrations (DE CASTRO et al., 2013). The high abundance of *Acidobacterium* in our samples suggest the ice-tephra from King George Island is a relatively acidic environment. Indeed, glacial ice has liquid water veins, described as acidic and oligotrophic, that sustain microbial communities (Price 2000).

The cold-desert characteristics of Antarctica provides biological support for a wide variety of endolithic communities (YUNG et al., 2014), with Cyanobacteria comprising a

substantial part of biomass in such extreme habitats (DE LOS RÍOS et al., 2007). With the main role on carbon and nitrogen fixation (DE LA TORRE et al., 2003), Cyanobacteria add structural biomass to the community (COWAN et al., 2010), and have been identified from nearly all endolithic ecosystems (DE LOS RÍOS et al., 2007). García-Lopez et al., (2021) found several genera of endolithic-colonizers cyanobacteria in volcanic rocks from glacier ice samples in the South Shetland Islands. In our samples, *Cyanothece*, *Nostoc*, *Synechococcus*, and *Oscillatoria* represented the main genera within the Phylum Cyanobacteria. These genera are present in most Antarctic habitats (PANDEY et al., 2004) and are well-known for their capacity for colonizing endolithic communities (DE LOS RÍOS et al. 2007; YUNG et al. 2014), constituting great targets for the study of photosynthesizing organisms with psychrotroph behavior (TANG et al., 2019).

The presence of Cyanobacteria in soils that are otherwise poor in nutritional content reflects in a more suitable environment for other phyla to grow (NIEDERBERGER et al., 2008), although the high content of cyanobacterial biomass may also derive from aerial distribution from water systems, i.e., Antarctic lakes (ADAMS et al., 2006; AISLABIE et al. 2006). This Phylum has a widespread distribution in Antarctica, with some endemic genera (LEE et al. 2012). In this study, we obtained 225,141 reads (11.73%) from Proteobacteria, mostly from Alpha (3.56%), Beta (3.8%), and Gamma (2.45%) classes. The most abundant genera within the Alphaproteobacteria were *Bradyrhizobium* (1.78% of all Proteobacteria), *Rhodopseudomonas* (1.64%), and *Methylobacterium* (1.52%). In the Betaproteobacteria, genera *Burkholderia* (4.4%), and *Polaromonas* (2.69%) ranked as more abundant, while in the Gammaproteobacteria genera *Pseudomonas* (3.2%), and *Xanthomonas* (2.3%) were found in higher proportion. *Polaromonas*, *Gemmatinonas*, *Burkholderiales*, and *Xanthomonas* are predominant genera in Antarctic soil (NIEDERBERGER et al. 2008, WANG et al. 2015). The psychrophilic genera *Polaromonas* and *Acidithiobacillus* are also found in pioneer communities exposed to volcanic activity (FUJIMURA et al. 2016).

The genera presented in the different sequencing methods showed 3 mutual genera. For the shotgun genera 650 were unique and 19 for metabarcoding (Figure 11).

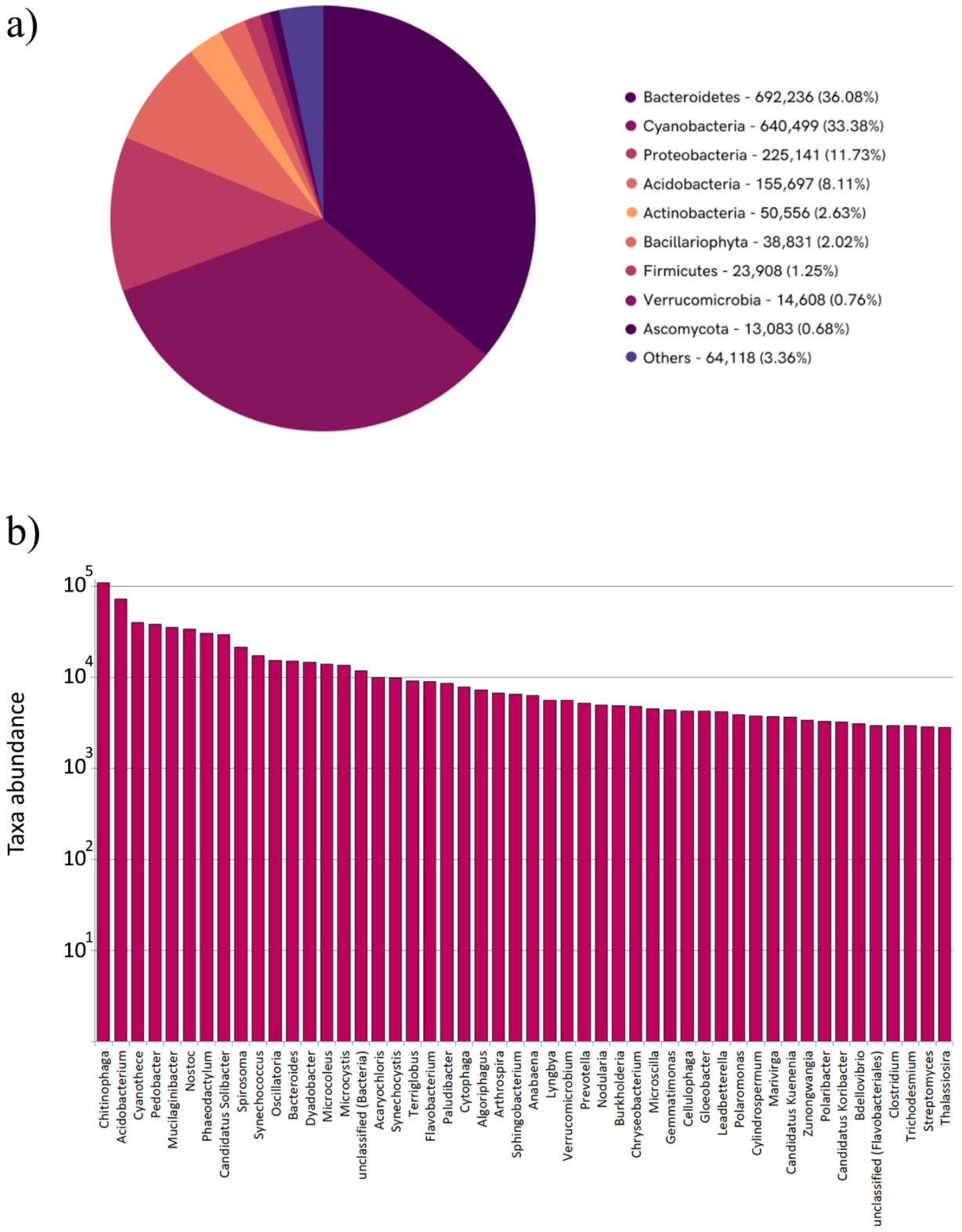


Figure 10. Distribution of microbial groups found in the glacier ice-tephra sample. a) Phyla abundance. b) Genus abundance, where only the top 50 most abundant are shown. The y-axis plots the abundances of annotations on a log scale. Graphic: Canva

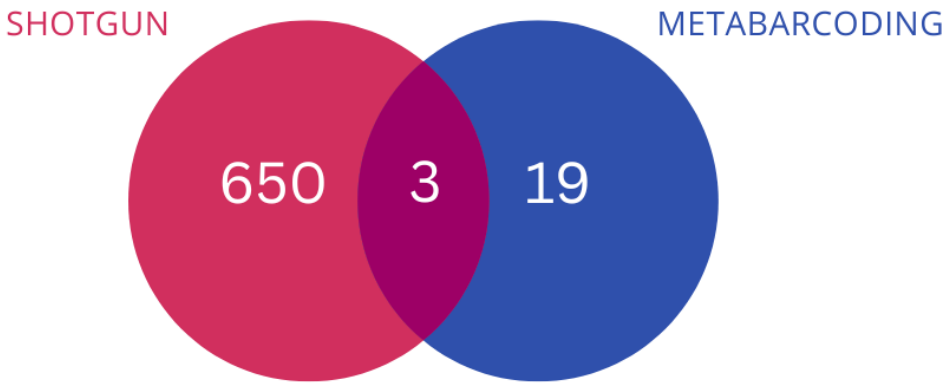


Figure 11. Venn diagram showing bacteria genera detected in both methods of sequencing (WGS and pyrosequencing) for the tephra ice sample, highlighting those detected in both methods. Graphic: Canva

4.1.2. FUNCTIONAL PROFILING

Nitrogen cycling is an important biogeochemical process in glacier ecosystems (BENDIA et al., 2018). Genes related to several pathways of the nitrogen cycle were found in the ice-tephra samples, including nitrogen fixation, nitrification, and denitrification. However, the low number of reads (161) and genes (12) relating to nitrogen fixation suggests that this microbial community may use other inorganic molecules (ammonium or nitrate) or organic nitrogen compounds as nitrogen sources. As for nitrification, the ammonium-oxidizing bacteria *Nitrosococcus* (11,150 reads), *Nitrosomonas* (8,057 reads), and *Nitrosospira* (5,567 reads) dominated the community. These nitrifying microorganisms are widespread in different aerobic ecosystems, including psychrotolerant species such as *Nitrosospira* lacus (URAKAWA et al., 2015). Interestingly, sequences annotated for ammonium-oxidizing archaea were also found (*Nitrosopumilus* sp., 334 reads), indicating their support in the nitrification process. Additionally, nitrite oxidation genes from *Nitrobacter* (7,974 reads), *Nitrospira* (2,707 reads), and *Nitrococcus* (1,562 reads) were present and suggest the complete potential of ammonium to nitrate oxidation in the ice-tephra environment. On the anaerobic route of the nitrogen cycle, cold adapted denitrifying bacteria were found with higher abundance of *Dyadobacter* (63,180 reads), *Gramella* (40,774 reads), and *Pseudomonas* (24,021 reads). *Dyadobacter* and *Gramella* were reported in other cold ecosystems like Tibetan Glacier ice (SHEN et al., 2013) and Antarctic marine sediments (LI et al., 2018),

while genus *Pseudomonas* is commonly distributed worldwide and include several cold-adapted strains (REDDY et al., 2004). Genes for copper-containing nitrite reductase (84 reads), nitrous-oxide reductase (84 reads), and nitric-oxide reductase (80 reads) were found in the metagenome, suggesting the possible conversion of nitrate to dinitrogen by the ice community. Members of the phylum Planctomycetes were present in the metagenome (0.61% of total Bacteria), however, no anammox genes were found. Similarly, cryoconite holes from Asian glaciers hold a relatively diverse community of nitrifiers and denitrifiers, but no anammox microorganisms are present (SEGAWA et al., 2014).

Genes for sulfur metabolism (3,255 reads) were also present in the ice-tephra samples. Microorganisms related to the sulfur cycle are those that best fit volcanic gradients, with a function on both oxidation and reduction of sulfur compounds. Oxidation of hydrogen sulfide (H_2S) under aerobic or anaerobic conditions generates key elements — elemental sulfur (S_0) and sulfate (SO_4^{2-}) — for the growth of photosynthesizing microorganisms (GARCÍA-LOPEZ et al., 2021). Sulfate is also used by sulfur reducing bacteria as a terminal electron acceptor during anaerobic respiration, which generates H_2S under strictly anaerobic conditions. Analysis of the sulfur cycle in the ice-tephra samples showed a relatively higher number of sequences related to sulfur assimilation (69.4%), rather than sulfur oxidation (16.8%) or sulfate reduction (13.8%). Interestingly, the balance of sulfur oxidation and sulfate reduction genes was also observed at the taxonomic level, with the main genera associated with sulfur oxidation (e.g Proteobacteria *Paracoccus*, *Thiomonas*, and *Thiobacillus*) were as abundant as those related to sulfate reduction (e.g., *Desulfotomaculum*, *Desulfomicrobium*, and *Desulfococcus*). One exception was *Desulfovibrio*, which outnumbered both groups, while sulfur-oxidizing and sulfate-reducing bacteria were found between 0.02-0.08%. While this is not clear for our samples, the complete sulfur cycle seems to occur in the ice-tephra, registering an important role for the above-mentioned genera. Nonetheless, microorganisms related to sulfur metabolism are believed to be dispersed to long distances as a consequence of explosive volcanic eruptions (GARCÍA-LOPEZ et al. 2021), and should be further investigated to better understand their function in this community.

Cold adaptations in microorganisms include specific proteins for DNA replication, transcription, and translation. The low temperatures provide reduced thermal energy, inducing other physicochemical restrictions such as increased solvent viscosity and solubility of gases and increased osmotic stress (COLLINS & MARGESIN, 2019). As enzymatic activities

decrease in cooler temperatures, the ATP demand is reduced, thus resulting in the formation of reactive oxygen species (ROS) and requiring the activation of antioxidant defenses (MYKYTCZUK et al., 2013). For psychrophiles, the accumulation of solutes like glycine, betaine, and choline is important for overall osmotic balance (GOORDIAL et al., 2016). A higher number of glycolytic proteins in psychrophilic compensate for the low efficiency of glycolytic enzymes in cold environments, including fructose-1,6-bisphosphatase (MYKYTCZUK et al., 2011), found in our study in major quantities (1,197 reads) (Table II).

Table II. Functional Diversity. Abundance of ice-tephra metagenomic reads assigned to the general SEED functional subsystems.

Functional Subsystem	No. of reads
Carbohydrates	237,448
CBSS	206,743
Amino Acids and Derivatives	161,270
Protein Metabolism	152,309
Miscellaneous	117,698
Cofactors, Pigments, Others	111,779
DNA Metabolism	99,246
Respiration	74,311
Cell Wall and Capsule	67,684
RNA Metabolism	64,795
Virulence, Disease and Defense	62,837
Nucleosides and Nucleotides	59,706
Membrane Transport	42,924
Fatty Acids, Lipids, and Isoprenoids	41,937
Stress Response	39,284
Cell Division and Cell Cycle	20,354
Phages, Prophages, Plasmids	19,170
Photosynthesis	17,057
Regulation and Cell signaling	15,535
Potassium, Nitrogen and Phosphorus metabolism	46,027
Metabolism of Aromatic Compounds	14,615
Sulfur Metabolism	12,054
Others (motility, iron and secondary metabolism and sporulation)	25,622

4.1.3. PHOTOSYNTHETIC PIGMENTS AND CAROTENOIDS

Photosynthesis-related pathways were almost complete in our samples, with most of for photosystems I and II, cytochrome b complex, photosynthetic electron transport, and ATPase (Figure 12a), besides the light-harvesting chlorophyll complex and secondary pigments of the antenna complex (Figure 12b) (MACKEY et al., 2013). As the second most abundant Bacteria Phylum in our samples, we can hypothesize that Cyanobacteria are the main microorganisms responsible for photosynthesis in these ice-tephra communities. The possession of antenna complexes can be largely accountable for such important functions, with secondary pigments allophycocyanin, phycocyanin, and phycoerythrin absorbing different wavelengths of the sunlight spectrum and, thus, contributing for an extended range of photosynthetically-active radiation that can be absorbed for photosynthesis (CAMPBELL et al., 1998). Due to the increased incidence of UV radiation, the Antarctic continent represents a hotspot habitat for UV-resistant microorganisms (MARIZCURRENA et al., 2017, MONSALVES et al., 2020), with Antarctic pigmented strains generally presenting higher resistance than their nonpigmented counterparts (DIESER et al., 2010). Besides chlorophyll and the aforementioned secondary pigments, carotenoids also play a major role for photosynthetic microorganisms living in Antarctica.

Carotenoids are liposoluble tetraterpenoid pigments that serve as photoprotective compounds (STAHL & SIES, 2003), reducing the deleterious effects of radiation either directly, absorbing light especially in the spectrum between 400-550 nm, or indirectly, acting as strong antioxidants through the quenching and scavenging of reactive oxygen species (DIESER et al. 2010). Carotenoids may also act in the regulation of membrane fluidity and stability under low temperatures (MOHAMMADI et al., 2012; REIS-MANSUR et al., 2019). In our metagenomic analysis, the pathway for carotenoid biosynthesis is almost complete (Figure 13), except for specific enzymes such as 15-cis-phytoene synthase.

Extremophilic microorganisms have been drawing attention in the last few years as novel sources of bioproducts with distinct properties, and extremophiles-derived carotenoids represent promising environmental-friendly alternatives for the biotechnological industry (ÓRDENES-AENISHANSLINS et al., 2016; REIS-MANSUR et al., 2019). Carotenoids can be used in a vast range of applications, from cosmetology (NÚÑEZ-MONTERO & BARRIENTOS, 2018) to food industry (SINGH et al., 2019). Moreover, the light-harvesting

properties of carotenoids provide for the increasing interest in their application for DyeSensitized Solar Cells (ÓRDENES-AENISHANSLINS et al., 2016). Recent research demonstrating the antimicrobial potential of pigmented Antarctic microorganisms (MOJIB et al., 2010; LEIVA et al., 2015; RAMESH et al.. 2019) has also claimed attention towards their use for pharmacological purposes. Future bioprospection studies shall continue revealing promising bioactive molecules from Antarctic pigmented microorganisms (SILVA et al. 2020), and the ice-tephra communities represent a potential source for the discovery of new metabolites.

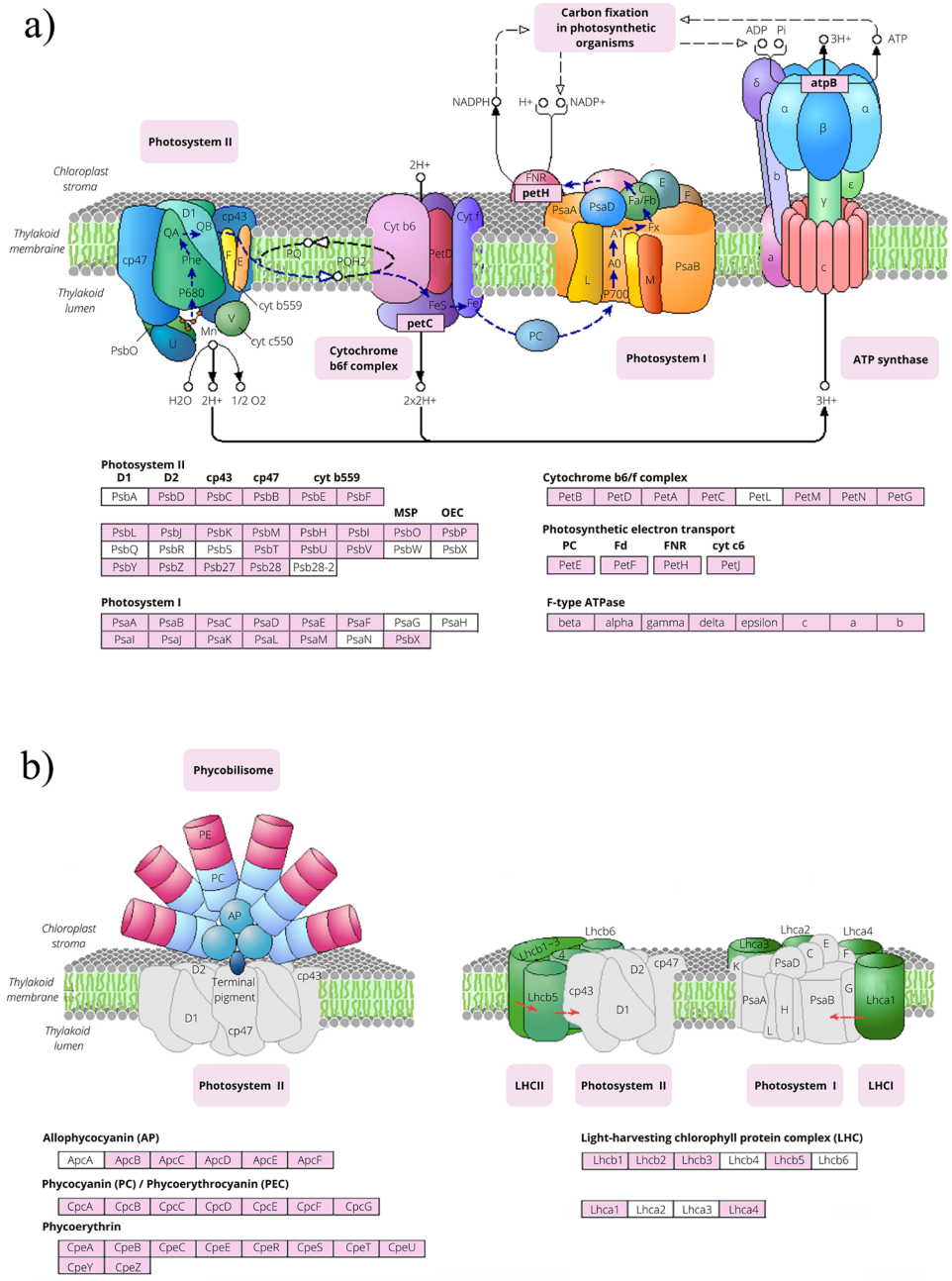


Figure 12. Photosynthetic pathways. a) Photosynthesis. b) Light-harvesting chlorophyll and secondary pigments. KEGG pathway map adapted from Kanehisa Laboratories.

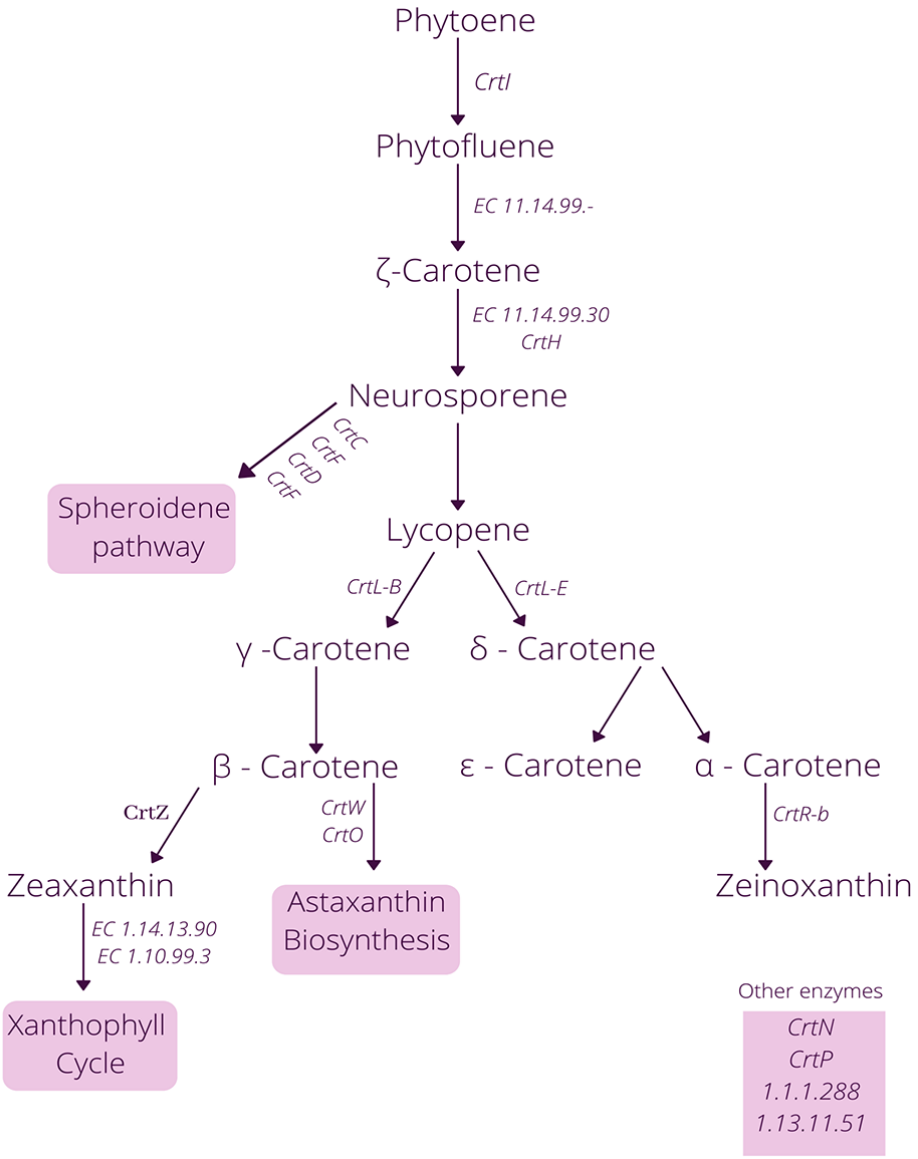


Figure 13. Carotenoid biosynthesis pathway based on KEGG map for the ice-tephra metagenome.

4.1.4. BIOTECHNOLOGY POTENTIAL

Life in the cold Antarctic environments and volcano surroundings may also select for enzymatic pathways with further biotechnological potential (ZAIKOVA et al., 2019). For example, our functional profiling analysis revealed enzymes with potential for bioremediation techniques, with annotations for aerobic and anaerobic degradation of aromatic compounds (e.g., toluene, xylene, methylnaphthalene, and styrene) found in 14,615 reads. Degradation of styrene, an aromatic hydrocarbon present in industrial effluents (MOONEY et al., 2006; TAN

et al., 2015), revealed almost complete pathways, with enzymes such as homogentisate 1,2-dioxygenase (250 reads) (Figure 14). Styrene biodegradation relies especially on Proteobacteria species which oxidize this toxic compound into styrene oxide that is further isomerized into phenylacetaldehyde (MOONEY et al., 2006, RUNYE at al., 2015). Final enzymatic reactions lead the converted compounds into the Citrate Cycle (MOONEY et al. 2006), thus participating in carbon metabolism. Styrene bioremediation processes have attracted attention in the last few years especially for the conceivable bioconversion of toxic wastes into market-valuable compounds such as polyhydroxyalkanoates (PHA), which offer high commercial interest for the pharmaceutical industry (RAI et al., 2011). Xanthobacter and Pseudomonas strains have been widely studied for their potential in styrene bioremediation (MOONEY et al., 2006; TAN et al., 2015). Nonetheless, the isolation and characterization of novel strains with enzymatic capacity for degradation of styrene — as for other aromatic hydrocarbons— is highly appreciated (TAN et al., 2015), and the discovery of enzymes for degradation of these toxic compounds in ice-tephra samples could represent one of the many potentials for biotechnological applications of such exquisite and unknown environment. Further analysis of glacial ice with volcanic sediments using both culture-dependent and independent approaches should reveal more details on such perspectives.

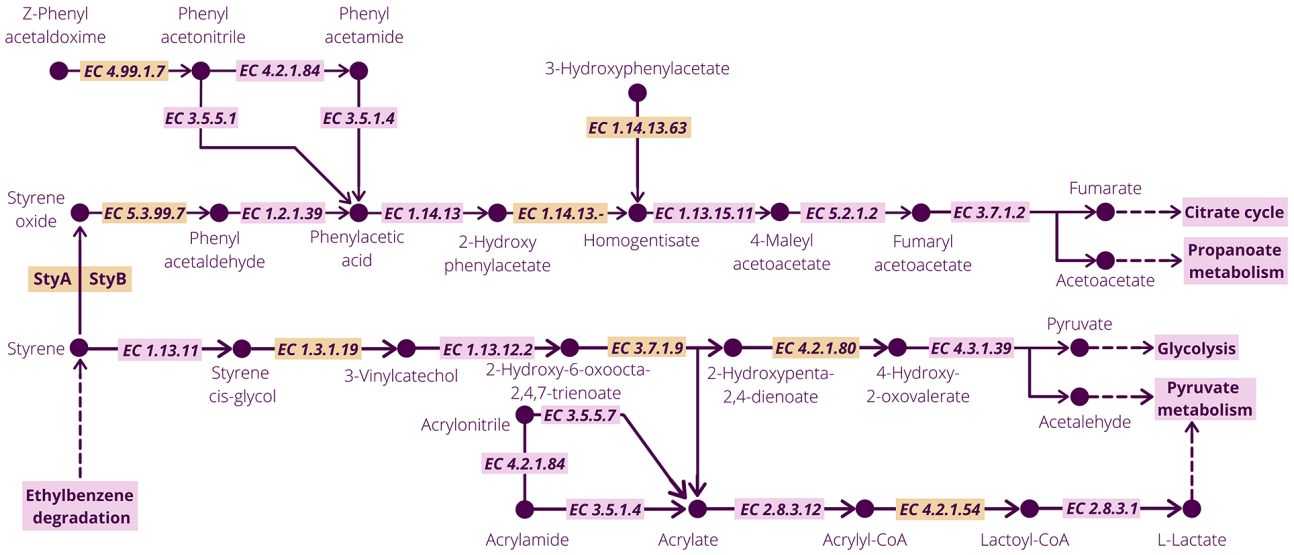


Figure 14. Styrene degradation pathway. Purple boxes represent the enzymes found in the ice-tephra metagenome.

4.1.5. METAGENOMIC ASSEMBLY AND GENOMIC RECONSTRUCTION

Using Anvi'o, a total of 1 MAG and 21 Bins were recovered from Collins ice-tephra (AI) old and new sequences (Table III). Prodigal (v2.6.3) has identified 29404 genes. The bins that were identified in the merged profile database 'SAMPLES MERGED' and stored in the database as "selectedMAGs" collection, describe 22 bins accounting for 19,888,915 nucleotides, which represent 69.39% of all nucleotides stored in the contigs database, and 100.00% of nucleotides stored in the profile database (Figure 15). The merged profile database that was generated with the minimum contig length of 4,000 contained 1,785 contigs, which correspond to 26% of all contigs, and 69% of all nucleotides found in the contigs database.

The bins summary includes the values of completeness, contamination, GC content, total reads mapped. The MAG001 was assigned to *Kovacikia* and has 92.80% completion with 4830776 contigs. Bins 5 and 12 were also annotated as *Kovacikia*. Bin 2 had 67.62% of completion for *Solitalea*. Bin 3 and 4, *Fertuginibacter* had 61.51% and 58.27% completion. The other bins presented less than 50% completion. The source of a mismatch may be artificial, such as stochastic sequencing or PCR error; some mismatches may represent ecologically informative variation (EREN et al., 2015).

Table III - Anvi'o analyzed sequences

	Number of pairs analyzed	Total pairs passed
Old	122,929	109,041 **(**88.70% of all pairs**)**
New	8,430,626	7,568,595 (89.78% of all pairs)

Bin	Source	Taxonomy	Total Size	Num Contigs	N50	GC Content	Compl.
DC_MAG_00001	CONCOCT	Kovacikia	4.83 Mb	319	19,831	49.17%	92.81%
DC_Bin_00002	CONCOCT	Solitalea	1.88 Mb	200	10,600	35.82%	67.63%
DC_Bin_00003	CONCOCT	Ferruginibacter	3.70 Mb	350	11,701	37.68%	61.15%
DC_Bin_00004	CONCOCT	Ferruginibacter	4.59 Mb	287	20,076	39.40%	58.27%
DC_Bin_00005	CONCOCT	Kovacikia	3.07 Mb	317	11,251	45.36%	48.20%
DC_Bin_00007	CONCOCT	Unknown	1.11 Mb	195	5,485	37.85%	23.02%
DC_Bin_00006	CONCOCT	Chondrus	33.02 Kb	4	7,704	30.27%	20.86%
DC_Bin_00009	CONCOCT	Unknown	193.57 Kb	32	5,770	52.78%	0.00%
DC_Bin_00011	CONCOCT	Unknown	166.19 Kb	28	6,044	44.08%	0.00%
DC_Bin_00013	CONCOCT	Fragilariopsis	106.72 Kb	17	6,832	45.91%	0.00%
DC_Bin_00014	CONCOCT	Unknown	66.80 Kb	10	5,889	41.70%	0.00%
DC_Bin_00008	CONCOCT	Unknown	38.02 Kb	8	4,712	50.27%	0.00%
DC_Bin_00010	CONCOCT	Panacibacter	25.85 Kb	3	12,954	37.96%	0.00%
DC_Bin_00015	CONCOCT	Tolypothrix	16.47 Kb	3	5,631	42.38%	0.00%
DC_Bin_00012	CONCOCT	Kovacikia	11.20 Kb	2	5,613	45.71%	0.00%
DC_Bin_00016	CONCOCT	Phytophthora	10.73 Kb	2	5,378	42.37%	0.00%
DC_Bin_00021	CONCOCT	Leptolyngbya	10.57 Kb	2	6,199	47.24%	0.00%
DC_Bin_00022	CONCOCT	Dyadobacter	8.51 Kb	2	4,312	28.32%	0.00%
DC_Bin_00020	CONCOCT	Fulvivirga	6.33 Kb	1	6,327	42.41%	0.00%
DC_Bin_00018	CONCOCT	Leptolyngbya	5.93 Kb	1	5,935	48.29%	0.00%
DC_Bin_00019	CONCOCT	Sphingobacteriaceae bacterium GW460-11-11-14-LB5	4.04 Kb	1	4,038	34.42%	0.00%
DC_Bin_00017	CONCOCT	Limnospira	4.00 Kb	1	4,000	46.73%	0.00%

Figure 15. Taxonomic distribution of genera from the ice-tephra sample, according to the results obtained via Anvi'o.

Since the sample represents the same location but different sequencing, the same genera appears. *Dyadobacter* is an aerobic gram-negative that contains several typical fatty acids, able to endure low temperatures (SHEN et al., 2013). *Fulvivirga* is a genus within Bacteroidetes capable of degrading chitin, it was found in extreme environments like a mud volcano (TU et al., 2017) and Antarctic sponge (RUOCCO et al., 2021). Cyanobacteria *Leptolyngbya*, as heterotrophs *Ferruginibacter* and *Solitalae* are typical of snow and were also found in glacier stratovolcano (HAVIG & HAMILTON, 2019).

The genus *Kovacikia* was first described in 2016 by Miscoe, Pietrasiak et Johansen. The samples were collected in Waikapala'e Cave, Kauai, Hawaii, a basaltic sea cave. The island of Kauai is a single shield volcano that is approximately five million years old and 1446 square km in area. The taxonomy was based on morphology, ecology, and phylogenetic analyses of the 16S rRNA gene. Identification of Leptolyngbya-like strains has been controversial because of their simple morphology, lacking significant discrimination (TANG et al., 2021). *K. muscicola* was also found in the geyser of Cisolok hot spring, with water temperature ranging from 90 - 100 °C (PRIHANTINI et al., 2018). The species was also

found in Terrestrial Cave Perama, Greece, the work showed the bacteria's capacity to produce HCN, which could be linked with nitrogen fixation. (PANOU & GKELIS, 2020). In a 2022 study conducted by Shen et al., with isolates from a shaded freshwater pond, a new species of *Kovacikia, K. minuta* was found with similarities of 97.2%–97.4% to the recently reported type species of *K. muscicola* HA7619-LM3. The new species produces Chlorophyll *f*, which enables photosynthesis in far-red light (FRL) (SHEN et al., 2022). Chlorophyll *f*-producing organisms have been isolated from various shaded environments such as microbial mats, soil, and stromatolites; relatively little is known of their full spread in nature (ANTONARU et al., 2020). In this study, although *Kovacikia* MAG001 had a 92.81% completeness, it was not possible to make a phylogenetic analysis based on 16S rRNA, since it was not found in the MAG.

4.2. ICE CAVE (IC) SAMPLE

Over the last decades, the analysis of icy environments has become a major scientific direction to unravel the presence, adaptation mechanisms and role of ice-contained microorganisms. Most ice cave deposits come from water that has percolate, through soil or rocks ponded for days through months before freezing (BRAD et al., 2018).

The analysis of 16S rRNA presented a total of 12,145 reads, in which 3,291 were high quality sequences. These sequences utilized the same parameter as for the ice-tephra (200 e 500 pb mean size, with 2 bases and 3 and 6 homopolymers). The sequences were also identified to the RDP database and classified in Phylum and Genus. Since only 16S rRNA gene sequences were evaluated, it was not possible to know if these psychrophiles would be viable in those conditions. All the results represent an environment with very few studies done, so they are very important for the representation of these habitats.

4.2.1. TAXONOMY

The ice cave (IC) 16S rRNA showed more equity in results than the ice-tephra sample. Dominant phyla presented Actinobacteria, Acidobacteria, Firmicutes and Proteobacteria as

the most abundant (Figure 16). Since caves are known for absence of light, cyanobacteria, although present, were in less quantity. Other studies in Antarctica showed Actinobacteria, Proteobacteria, Cyanobacteria and Acidobacteria as major phyla for the environment (BOETIUS et al., 2015). Proteobacteria (α, β, and γ), Firmicutes, and Actinobacteria and able to degrade organic compounds common in Arctic snow (propionate, acetate, and formate) (HODSON, et al., 2008). As for genera, the 10 abundant most found were *Gaiella* (31.18%), *Nocardioides* (22.41%), and *Perlucidibaca* (7.17%) (Figure 17). *Gaiella, Nocardioides* and are genera already found in glacial environments, including Antarctica (ARAUJO et al., 2020; VASILEVA-TONKOVA et al., 2005, VERGEYNST et al., 2018). For the other 17.41% of the genera in the sample we got *Aeromicrobium, Luteolibacter, Gemmatimonas, Sphingomonas, Hymenobacter, Arenimonas, Baekduia, Stenotrophobacter* and *Albidiferax.*

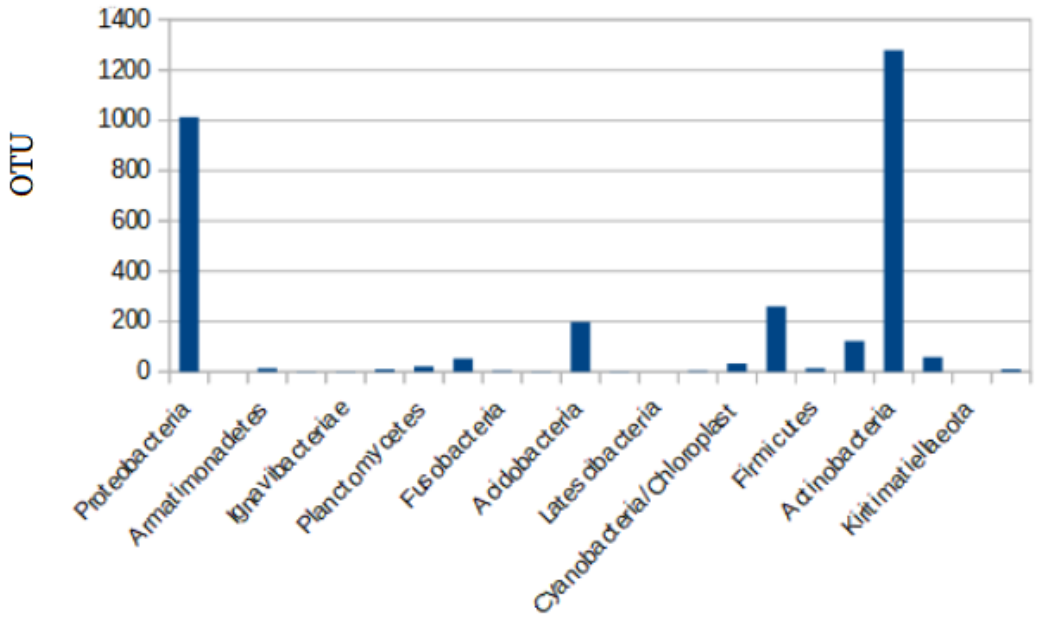


Figure 16. Taxonomic distribution of Phyla in the cave ice (IC) sample, according to the results obtained by the Mothur software. Y axis represent taxonomic abundance of OTUs at 95% cutoff. Graphic: LibreOffice Calc

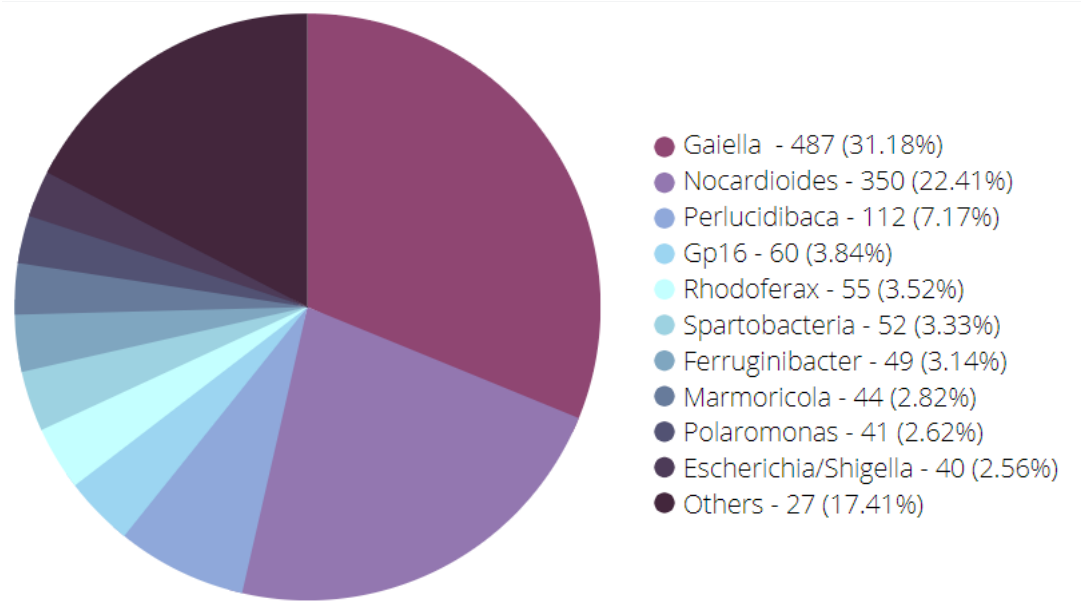


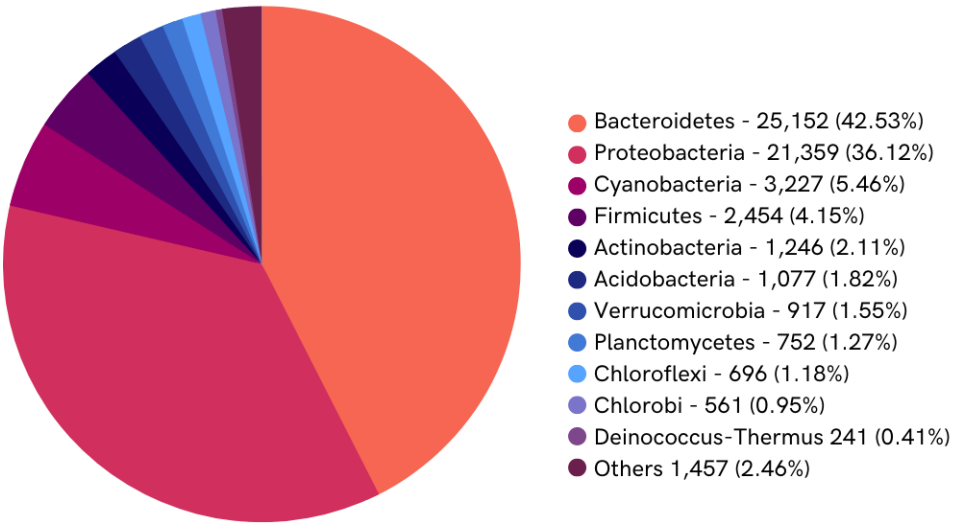
Figure 17. Distribution of microbial groups found in the glacier ice cave sample (rRNA 16S). Genus absolute abundance, where only the top 10 most abundant are shown. Graphic: Canva

The Metagenomic Rapid Annotations using Subsystems Technology (mg-rast) for Ice cave samples showed a similar profile as ice-tephra dominant phyla like Bacteroidetes (42.53%), Proteobacteria (36.12%), Cyanobacteria (5.46%), Acidobacteria (1.82%) (Figure 18a). The Phylum Bacteroidetes was the most predominant, it is usually found in abundance in ice samples, as well as Firmicutes, Proteobacteria and Cyanobacteria (BOETIUS et al, 2015). Bacteroidetes are present in various types of environments such as freshwater, marine environments, soil, and ice. A large number of Bacteroidetes have already been found in glaciers and ice floes (XIAN et al., 2009). As the 16S rRNA already showed, Cyanobacteria are present in less quantities in the ice cave samples, probably due to less solar incidence. The presence of Proteobacteria in the samples could indicate the presence of contaminants, since the Phylum contains bacteria such as Escherichia coli, Salmonella, among others, although it also contains psychrophiles genera such as Polaromonas and Psychrobacter. Among the most abundant bacterial genera assigned to the sample *Chitinophaga* (3,961 reads), *Pseudomonas* (3,931 reads), *Flavobacterium* (3,287 reads), *Pedobacter* (2,297 reads), *Bacteroides* (1,669 reads) *and Cytophaga* (1,387 reads) (Figure 18b). *Chitinophaga, Pseudomonas,* were also found in ice-tephra samples in higher quantities. *Pseudomonas*, *Flavobacterium*, *Pedobacter* and *Bacteroides* are genus found in the Antarctic before (DA et al., 2015; MCCAMMON et

al., 2000; HIRSCH et al., 1998).

The genera presented in the different sequencing methods showed 7 mutual genera. For the shotgun genera 529 were unique and 13 for metabarcoding (Figure 19).

a)



b)

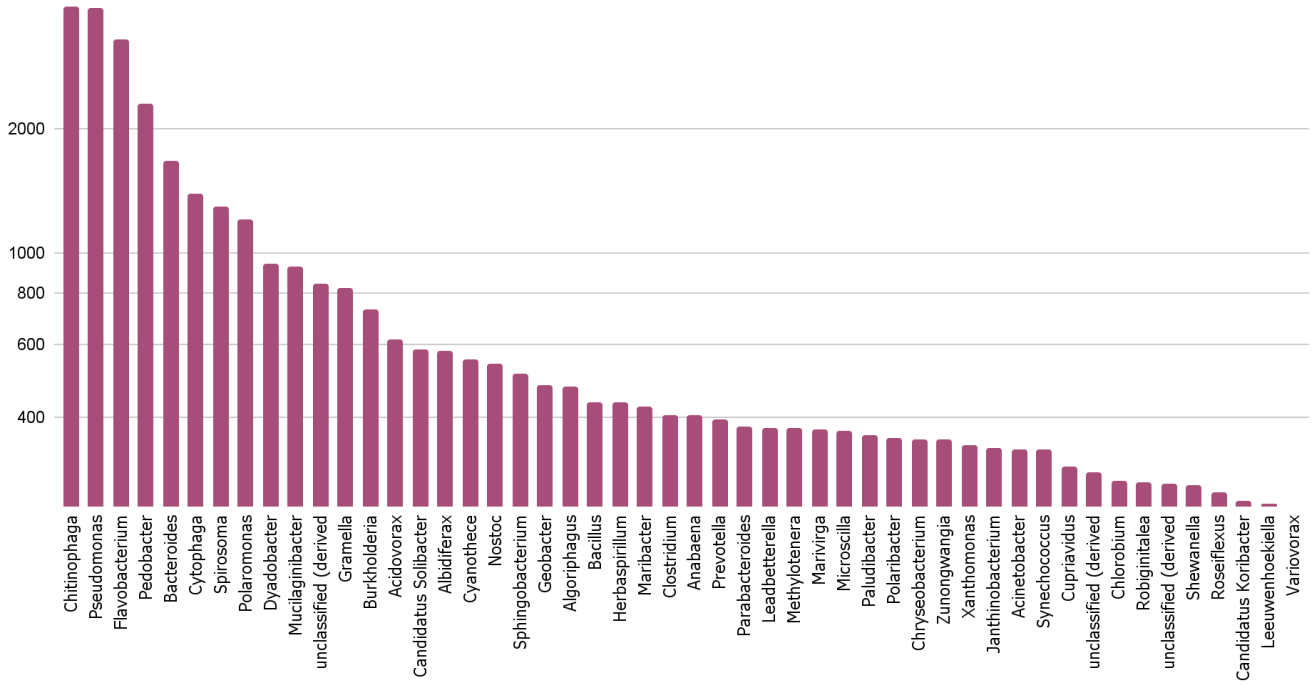


Figure 18. Distribution of microbial groups found in the glacier ice-cave sample. a) Phyla abundance. b) Genus abundance, where only the top 50 most abundant bacteria are shown. The y-axis plots the abundances of annotations on a log scale.

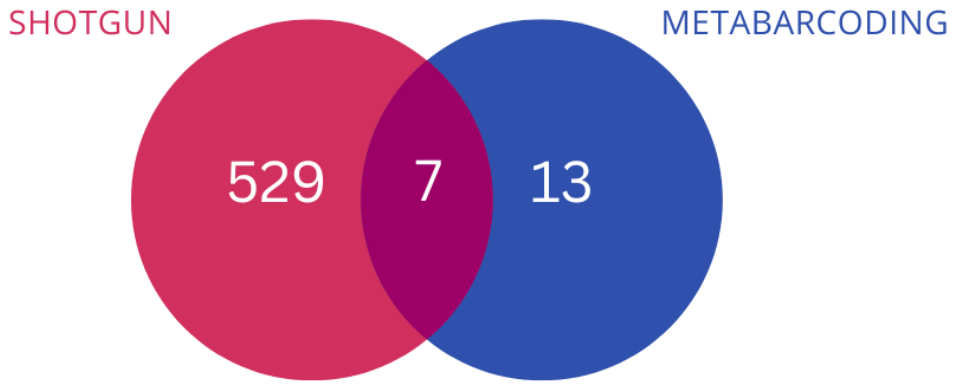


Figure 19. Venn diagram showing bacteria genera detected in both methods of sequencing (shotgun and pyrosequencing) for the ice cave sample, highlighting those detected in both methods. Graphic: Canva

4.2.2. FUNCTIONAL PROFILING

The ice cave also revealed enzymes with potential for bioremediation techniques, with annotations for aerobic and anaerobic degradation of aromatic compounds (e.g., toluene, xylene, methylnaphthalene, and styrene) found in it (Table IV). Carbohydrates had the most reads for functional diversity (2,976), followed by clustering-based subsystems (2,976 reads) and amino acid derivatives (2,258 reads). Only 35 reads for photosynthesis, which corroborates for lower light incidence in caves. Subsystem level 3 for clustering-based showed 204 reads for sugar utilization in, beta-hexosaminidase (EC 3.2.1.51) had the most with 29 reads. Central carbohydrate metabolism had the highest number for TCA cycle (143), with Fumarate hydratase class II (EC 4.2.1.2) (33 reads) highest function. Amino acid derivatives (2,258 reads), presented Lysine, threonine, methionine, and cysteine with 593 reads.

Table IV. Functional Diversity. Abundance of ice cave metagenomic reads assigned to the general SEED functional subsystems.

Functional Subsystem	No. of reads
Carbohydrates	2,976
Clustering-based subsystems	2,914
Amino Acids and Derivatives	2,258
Protein Metabolism	1,764
Miscellaneous	1,434
Cofactors, Vitamins, Prosthetic Groups, Pigments	1,266
Cell Wall and Capsule	1,229
DNA Metabolism	1,210
Respiration	1,049
Virulence, Disease and Defense	997
Nucleosides and Nucleotides	907
RNA Metabolism	890
Membrane Transport	708
Stress Response	589
Fatty Acids, Lipids, and Isoprenoids	573
Metabolism of Aromatic Compounds	343
Phosphorus Metabolism	299
Phages, Prophages, Transposable elements, Plasmids	289
Regulation and Cell signaling	278
Cell Division and Cell Cycle	225
Iron acquisition and metabolism	214
Nitrogen Metabolism	183
Potassium metabolism	168
Sulfur Metabolism	163
Motility and Chemotaxis	128
Secondary Metabolism	94
Dormancy and Sporulation	41
Photosynthesis	35

4.2.3. BIOTECHNOLOGY POTENTIAL

Cave conditions may enhance the production of antibiotic compounds (SADOWAY et al., 2013). The Cobalt zinc cadmium resistance was the second most abundant SEED subsystem with 270 reads for the sample. This resistance can be related to cross-resistance, a selection process in which heavy metals and antibiotic resistance mechanisms are

physiologically coupled (FARIAS et al., 2015). Since this resistance could in fact exist, the mechanisms of evolution may have helped other species in antibiotic production as a competition factor. The ice cave showed *Chitinophaga* as a dominant genera in the sample. This genera is already studied for biocontrol. Due to the lack of chitin in plants and vertebrates, phytopathogenic fungi and phytophagous nematodes can be targeted by chitinases, without any adverse effect on the environment, vertebrates and plants (SHARMA et al., 2020).

4.2.4. XENOBIOTICS BIODEGRADATION AND METABOLISM

Aromatic compounds constitute the second most abundant class of organic substrates and environmental pollutants, a substantial part of which is metabolized by bacteria via phenylacetate (Table V). Phenylacetyl-CoA catabolic pathway showed 71 reads. Still on biodegradation, the wastewater generated during the production of terephthalic acid is done by aerobic technologies. One of the main components in these waste streams include benzoic acid. In anaerobic degradation of terephthalate, benzoate plays a key role (KLEEREBEZEM et al., 1999). The Ice Cave samples present many genes for enzymes of the benzoate degradation with 39 reads for benzoate transport and degradation cluster and 11 for benzoate degradation (Figure 20).Genes covering conversion of benzene to acetyl-CoA, via initial conversion to phenol and then catechol like 2-keto-4-pentenoate hydratase (EC K02554) where found (MADUEÑO et al., 2021). This same gene is also found in the degradation pathway of xylene degradation (BARBATO et al., 2022). The gene is present in Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria.

Table V - Abundance of ice cave metagenomic reads assigned to the general SEED functional subsystems Metabolism of Aromatic Compounds - Level 3.

Metabolism of Aromatic Compounds	No. of reads
Phenylacetyl-CoA catabolic pathway (core)	71
Benzoate transport and degradation cluster	39
Homogentisate pathway of aromatic compound degradation	39
n-Phenylalkanoic acid degradation	38
Anaerobic benzoate metabolism	33
Phenylpropanoid compound degradation	15
Benzoate degradation	11
Salicylate and gentisate catabolism	11
4-Hydroxyphenylacetic acid catabolic pathway	9
Aromatic Amine Catabolism	9
Catechol branch of beta-ketoadipate pathway	8
Central meta-cleavage pathway of aromatic compound degradation	8
Benzoate catabolism	7
Biphenyl Degradation	7
Protocatechuate branch of beta-ketoadipate pathway	6
Carbazol degradation cluster	6
Chloroaromatic degradation pathway	5
Gentisare degradation	5
N-heterocyclic aromatic compound degradation	5
p-Hydroxybenzoate degradation	4

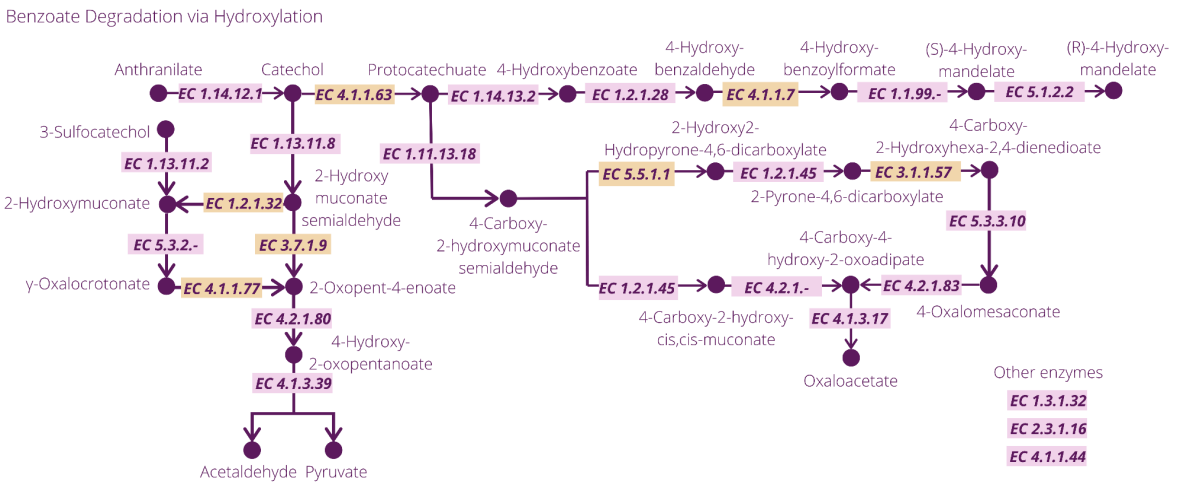


Figure 20. Benzoate Degradation via Hydroxylation pathway based on KEGG. Purple boxes represent the enzymes found in the ice cave metagenome.

5. CONCLUSION

To my knowledge, this is the first description of the taxonomic and functional diversity of an ice-tephra and the first Antarctic ice cave community using metagenomics. The results of both habitats showed these unique sites presenting distinct features of their own, including biotechnological potential. The metagenome assembly showed genus *Kovacikia* never found before in a cold environment.

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