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Research Article

Lichen-associated microbial members are prevalent in the snow microbiome of a sub-arctic alpine tundra

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Abstract

Snow is the largest component of the cryosphere, with its cover and distribution rapidly decreasing over the last decade due to climate warming. It is imperative to characterize the snow (nival) microbial communities to better understand the role of microorganisms inhabiting these rapidly changing environments. Here, we investigated the core nival microbiome, the cultivable microbial members, and the microbial functional diversity of the remote *Uapishka* mountain range, a massif of alpine sub-arctic tundra and boreal forest. Snow samples were taken over a two-month interval along an altitude gradient with varying degree of anthropogenic traffic and vegetation cover. The core snow alpine tundra/boreal microbiome, which was present across all samples, constituted of *Acetobacterales*, *Rhizobiales* and *Acidobacteriales* bacterial orders, and of *Mycosphaerellales* and *Lecanorales* fungal orders, with the dominant fungal taxa being associated with lichens. The snow samples had low active functional diversity, with Richness values ranging from 0 to 19.5. The culture-based viable microbial enumeration ranged from 0 to 8.05×10^3 CFUs/mL. We isolated and whole-genome sequenced five microorganisms which included three fungi, one alga, and one potentially novel bacterium of the *Lichenihabitans* genus; all of which appear to be part of lichen-associated taxonomic clades.

Keywords: alpine tundra; bacteria; boreal forest; fungi; lichen; snow microbiome

Introduction

Snow represents the largest component of the cryosphere, with the majority of it found seasonally in the Northern Hemisphere (Fountain et al. 2012). This snow cover substantially decreased over the last decade at a faster rate than previously predicted (Derksen and Brown 2012, Pulliainen et al. 2020). Snow plays a major role in the global climate and is known to harbour abundant and diverse microbial communities with implications for global biogeochemical cycles (Maccario et al. 2015, Antony et al. 2016). Nival (snow) ecosystems represent a climatically sensitive transition zone between the atmosphere and underneath substrates (Kuhn 2001), and are principally seeded by the surrounding environment (though long distance transport is also documented), which allows unique microbial communities to develop within the snow (Tyagi et al. 2015, Malard et al. 2019, Els et al. 2020). Despite an increasing interest in snow algae blooms (Tucker and Brown 2022, Yakimovich and Quarmby 2022) and snow microbiomes of Arctic and the Antarctic environments (Larose et al. 2013, Maccario et al. 2014, Michaud et al. 2014, Maccario et al. 2019, Malard et al. 2019, Zhu et al. 2020), microbial diversity from pristine snow in alpine ecosystems is still poorly characterized. This is especially true for remote areas with low human disturbance, as the few published alpine snow microbiome studies mainly focused on mid-latitude mountain in high trafficked areas (Wunderlin et al. 2016, Azzoni et al. 2018, Brown and Jumpponen 2019).

Lichens are prevalent and widespread in polar and alpine environments (Printzen et al. 2012, Armstrong 2017). They are complex holobionts, defined as a symbiotic association of fungal mycobionts, algal/cyanobacterial photobionts, and often with a non-photosynthetic microbial community (Miral et al. 2022). These complex microbial associations make lichens highly adapted to hostile conditions of cold habitats (Printzen et al. 2012). Alpine tundra and boreal forests harbor a large diversity and abundance of lichen-associated taxa living on different substrata such as soil, plants, and rocks (Printzen et al. 2012, Armstrong 2017). However, their presence, diversity, and viability in context of seasonal snow microbiomes has not been directly investigated; though some lichen-associated sequences have been incidentally reported in snow (de Menezes et al. 2019, Pankratov et al. 2020, Rosa et al. 2020).

Alpine environments are attractive areas for tourism, which can cause negative impacts on nival ecosystems, such as changes in the snow microbiome (Sanchez-Cid et al. 2022). Snowmobiles in particular are known to disturb fauna and flora (Seip et al. 2007, Fuglei et al. 2017, Mullet and Morton 2021) and contributes to air and hydrocarbon pollution (Buckley 2002, Reimann et al. 2009, Drotikova et al. 2021). Due to climate change, the snowmobiling season is expected to decrease and shift to more northern latitudes (Scott 2003). This phenomenon has been observed at the *Uapishka* mountain range (Northern Quebec), with over 10% yearly increase in snowmobile traffic since 2015 (Lachapelle

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2020). *Uapishka* translates to 'Snowy Rocky Peaks' in native Innuaimun and are named as such due to their prolonged seasonal snow cover (UNESCO 2022). This mountain range forms a massif of alpine sub-arctic tundra and boreal forests (Jones and Willey 2012), and is a protected area of the *Uapishka* Biodiversity Reserve, which lies within the 54 800 km^2 *Manicouagan-Uapishka* World Biosphere Reserve (Kermagoret and Dupras 2018, UNESCO 2022). Even though snowmobiling is forbidden above 800 meters above sea level (a.s.l) in the reserve, this regulation is often not respected and is difficult to enforce (Champagne 2018).

Little is known regarding the microbial biodiversity in relatively pristine natural alpine tundra/boreal ecosystems which are rapidly increasing in anthropogenic activities. In this study, our objective was to determine the core nival microbiome common across different sub-environments in the *Uapishka* alpine tundra environment. To this end, we collected snow replicates from the *Uapishka* Mountain range along an altitude gradient (457 to 1058 m a.s.l) with varying degree of anthropogenic traffic and vegetation cover. We first determined the snow's bacterial, archaeal, and fungal diversity via 16S rRNA/ITS amplicon sequencing and microbial isolation, as well as microbial functional diversity of the snow via phenotypic microarray assays, and the snow's quality via chemical analysis (hydrocarbon, metals, nitrites, nitrates, total solids). In addition, whole-genome sequencing of microorganisms isolated (bacterium, fungi, alga) from this protected reserve was performed to better characterize the viable microorganisms of the area.

Methods

Sampling site description, sample collection, and chemical analysis

The *Uapishka* Biodiversity Reserve study site (Fig. 1) is located on the Québec's region of *Côte-Nord*, in Canada (between 51°21'N–51°48'N and between 67°34'W–68°21'W) and is characterized by a cold subpolar and subhumid continental climate featuring a short growth season, with average annual temperatures between −5.0°C and −3.1°C (Gerardin and McKenney 2001, Gouvernement du Québec 2009). For this study, snow replicates from different sub-environments of the *Uapishka* mountain range was sampled along an altitude gradient (Fig. 1), from the R-389 access road (457 m a.s.l), to the alpine tundra of the summit of Mont Jauffret (1058 m a.s.l) and included sampling replicates above and below the boreal treeline, and under different densities of tree cover (Figure S1). These included samples from the tundra (T), boreal forest (BF), near a road (R), a road closer to a boreal forest (RBF), and a snowmobile trail (ST). Two sets of samples were collected in the winter season 2018–2019. A first set of samples was collected between December 17th–20th 2018 (samples denoted with -D), and a second set of samples were collected between February 1st–3rd 2019 (samples denoted with -F). All snow samples consisted of surface powder snow, apart from the highest elevation T1 tundra sample which was compacted due to high wind exposure. Surface snow was collected directly into sterile Whirl-Pak bags and kept frozen (−20°C) during transport to the laboratory.

Chemical analysis of subset of snow replicate samples was performed by melting the snow to obtain liquid samples. The samples were then analysed for total solids (via ILCE-043), total nitrates & nitrites (ILCE-060), polycyclic aromatic hydrocarbons (CHM36/ILCE61), petroleum hydrocarbons C10-C50 (CHM38/ILCE36), and metals via inductively coupled plasma mass spectrometry (ICP-MS) metal scanning at the Eurofins EnvironeX laboratory (Longueuil, Quebec, CA).

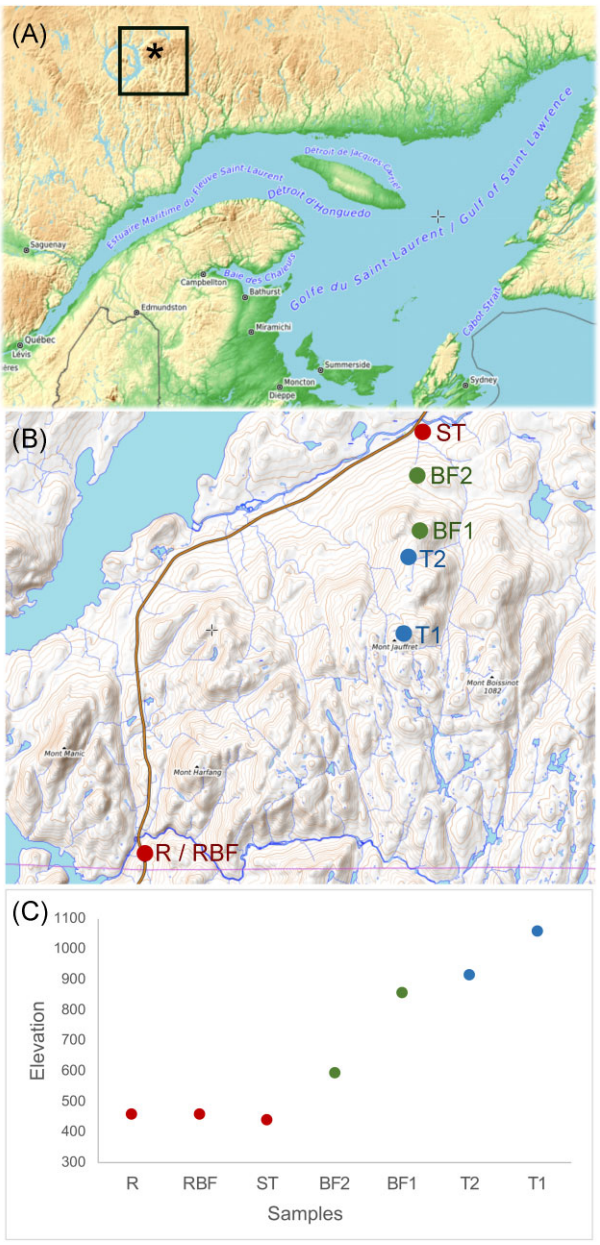

Figure 1. Sampling locations. The study was performed in the *Uapishka* Biodiversity Reserve (A), where replicate snow samples (B) were collected along an altitude gradient (C). T1, T2: tundra; BF1, BF2: boreal forest; RBF: road 389 with boreal forest; R: road 389; ST: snowmobile trail.

Cell enumeration, cultivation and isolation

The viable microbial biomass of the different snow samples was performed using December 2018 samples via an aerobic heterotrophic plate count method (Chan-Yam et al. 2019, Touchette et al. 2022b), using 1/4 Reasoner's 2A (R2A) with 1.5% gellan plates. For each of these samples, snow was melted and immediately spread on three sets of triplicate plates. Each set was incubated at one of three temperatures, 23°C for 12 weeks, and 10°C and 0°C for 14 weeks (3.5 months) to ensure that slow growing microorganisms were recovered, before counting the colony-forming units (CFUs). Specifically, 250 µl of melted snow was spread on

each plate, with the exception of 100 μl used for the BF2-D sample incubated at 23°C, and 500 μl used for the T1-D sample incubated at 0°C to ensure proper CFU counts. For certain samples/temperatures, an additional 1 : 5 dilution (in sterile $Na_4P_2O_7$) of the melted snow was used to have a better resolution of the viable microbial biomass. These volumes and dilutions were taken into account when calculating CFU numbers. Following the viable cell enumeration of the plates, five unique colonies, based on morphology, were transferred from the 0°C and 10°C plates to an individual 1/3 R2A 1.5% gellan plates and incubated at 15°C to isolate individual microbes. After growth, the colonies were transferred again to new 1/3 R2A 1.5% gellan plates to ensure pure isolation prior to whole-genome sequencing.

Community-level physiological profiling

The microbial function and metabolic diversity of snow from the *Uapishka* Biodiversity Reserve was assessed using EcoPlates (BIOLOG, Hayward, CA, United States, R#1506), which consisted of 96-well plates with a triplicate panel of 31 different carbon sources and a carbon-free control well. Each snow sample was melted, mixed for 2 min by vortexing, and settled for 30 min to remove particulate matter. 100 μl of the processed samples were added to each well of the EcoPlate. For each snow sample, one EcoPlate was incubated at 10°C, and one was incubated at 0 °C. Well colour change was monitored by measuring optical density at 590 nm (OD_{590}) on a SpectraMax M2e microplate reader (Molecular Devices, San Jose, CA, United States) every other day for a week, followed with weekly measurements for a month, and then monthly until 4 months. The measurements were normalized by the subtraction of the time-zero, and the averaged carbon-free negative control. Absorbance values > 0.2 were considered as positive values, as per (Rico and Preston 2008, Touchette et al. 2022b). Average well-color development (AWCD), and substrate richness (R) based on carbon substrate utilization were calculated as per (Iţcuş et al. 2016, O'Connor et al. 2021, Touchette et al. 2022b). The R index was calculated once the AWCD reached a plateau phase (i.e. maximum metabolism) in the majority of the samples (Pessi et al. 2009, O'Connor et al. 2021, Touchette et al. 2022b), for our samples this was after 112 days incubation period.

DNA extraction, 16S and ITS rRNA sequencing

DNA from snow sampled was extracted using the DNeasy PowerSoil Pro kit, according to manufacturer's instructions (QIAGEN, Hilden, Germany), using 250 μl of melted snow as input material, with the exception of the T1-D, T2-D, and ST-D samples which required additional steps due to low microbial biomass. For these specific samples 15 ml of melted snow was filtered on a 0.2 μm membrane filter (Whatman, Maidstone, UK), the membranes were used as input material. All samples were homogenized by a 30 s bead beating step (at DNeasy PowerSoil Pro step 2), using the MiniBeadBeater-24 (Biospec Products, Bartlesville, OK, USA).

16S rRNA gene (bacterial) and Internal Transcribed Spacer region (ITS; fungi) amplicon libraries were made following the Illumina's 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA), using the 515f-Y (5′ GTGYCAGCMGCCGCGGTAA 3′) and 926R (5′ CCGYCAATTYMTTTRAGTTT 3′) primers (Parada et al. 2016) for 16S rRNA gene, and the ITS1f (5′ CTTGGTCATTTAGAGGAAGTAA 3′) and ITS2 (5′ GCTGCGTTCTTCATCGATGC 3′) primers for the ITS region (Walters et al. 2016). Amplicons were indexed, pooled, and sequenced with an Illumina MiSeq using a 600-cycle MiSeq Reagent Kit V3 with a 2×250 paired-end configuration. All the amplicon reads are available under the NCBI Bioproject PRJNA948675.

The amplicon sequences were analysed using QIIME2 2022.8 with default parameters (Bolyen et al. 2019): raw sequence data were demultiplexed and filtered based on quality using the q2-demux plugin and were denoised with DADA2 via q2-demux (Callahan et al. 2016). Taxonomy was assigned to the amplicon sequence variants (ASVs) using the q2-feature-classifier against the latest SILVA database v138.1 (Quast et al. 2013) trained on the V3-V4 region for the 16S rRNA gene and against the latest UNITE database version 9 (all eukaryotes) (Nilsson et al. 2019) for the ITS amplicons. Community composition analysis and alpha diversity estimations were performed using the R package "phyloseq" v.3.16 (McMurdie and Holmes 2013). Non-bacterial/archaeal ASVs (chloroplasts, mitochondria, eukaryotes) were removed from the 16S amplicon dataset and non-fungal at the Kingdom level ASVs were removed from the ITS amplicon dataset for downstream analysis. In addition, only taxa with more than 1 read in at least two samples were kept. Rarefaction curves were performed with the R package "ranacapa" to ensure each sample reached the maximum ASVs diversity (Figure S2). Shannon alpha-diversity index data was tested for normality using Shapiro-Wilk test, and the effect of "Disturbance", "Altitude", "Month", and "Vegetation" on the Shannon index were tested using Kruskal-Wallis Rank Sum Test, both from the R package "stats" (4.3.0). Data visualization was performed using the R package "microViz" (Barnett et al. 2021) and ggplot2 (Wickham 2016).

Whole-genome sequencing

DNA from the isolates was extracted using the ZymoBIOMICS DNA/RNA miniprep kit (Zymo Research, Seattle, WA, USA) according to the manufacturer's instructions, and whole-genome sequencing libraries were prepared following the Illumina's NEXTERA XT DNA Library Prep kit protocol, with the exception that Sera-Mag Select™ beads (Cytiva, Washington, DC, USA) were used instead of AMPure XP beads during the clean-up step and the Monarch® PCR and DNA Cleanup kit (New England Biolads, Ipswich, MA, USA) was used to concentrate the pooled sequencing library. Sequencing was performed on a NovaSeq 6000 at The Center for Applied Genomics (Toronto, ON, Canada), with a 2×100 paired-end configuration.

For the assembly of the fungal and algal genomes, the raw reads were first trimmed with sickle 1.33 using default settings (Joshi and Fass 2011), and then assembled with SPAdes 3.15.2 with the option "–careful" (Prjibelski et al. 2020). Assembly statistics were calculated with QUAST 5.2.0 (Mikheenko et al. 2018). All contigs shorter than 1000 bp were removed with Seqkit 2.2.0 (Shen et al. 2016). Genome sequences were softmasked with RepeatMasker 4.0.9 (Smit 2015), using "-species fungi" for the fungal genomes and "-species viridiplantae" for the alga. Masked genomes were then annotated with Augustus 3.3.2 (Stanke et al. 2008). Fungal or plant proteins from the OrthoDB 10 (Kriventseva et al. 2019) were used as hints for the annotation and were aligned to the genomes with ProtHint 2.4.0 (Brůna et al. 2020). The training models used as provided in Augustus were "neoformans_B" for genomes 1 and 3, "neurospora_crassa" for genome 2 and "chlamy2011" for genome 4 (Table 1; Table S2). These four genomes were annotated with KofamKOALA (Aramaki et al. 2020), to obtain the KEGG/KO gene classifications (Kanehisa et al. 2023). Genome quality analysis of the eukaryotic isolates was performed using BUSCO 5.4.7 (Manni et al. 2021). The datasets used were "basidiomycota_odb10" for genomes 1 and 3, "ascomycota_odb10" for genome 2 and "chloro-

Table 1. Whole-genome sequencing details of the *Uapishka* microbial isolates.

Uapiskha isolate genome	Source sample	Temperature of plate incubation	Colony color	Genome Completeness	[1]Duplicates/ Contamination	Genome size (bp)	GC content (%)	Protein coding genes (#)	Marker (database used)	Taxonomic classification	[2]Closest relative (% identity)
1	BF1-D	0°C	Light pink	93.5%	0.1%	17 889 413	54.82	6715	ITS (GenBank)	Fungi (Basidiomycota) Genus *Phaeotremella*	ON007423 *Phaeotremella* sp. (98.28%)
2	T2-D	10°C	Pink	92.1%	0.2%	28 402 931	48.49	6566	ITS (GenBank)	Fungi (Ascomycota) Genus *Lecanoraceae* gen. sp.	AY456689 *Miriquidica garovaglii* (95.69%) (synonyms: *Lecidea garovaglii*, *Psora garovaglii*)
3	BF1-D	0°C	Light beige	80.2%	0.6%	32 924 126	52.49	9530	ITS (GenBank)	Fungi (Basidiomycota) Genus *Phenoliferia*	ON007460 *Phenoliferia* sp. (100%)
4	R-D	10°C	Green	81.6%	0.6%	54 386 886	57.90	12 286	ITS (GenBank) & chloroplast 16S (GenBank)	Plantae (*Chlorophyta*) Genus *Asterochloris*	OM914235 *Asterochloris irregularis* (99.77%; ITS) & GU191846.1 *Asterochloris* sp. (100%; chloroplast 16S)
5	BF1-D	10°C	Pink	97.75%	1.1%	4 780 097	66.92	4553	16S (GTDB)	Bacteria (*Proteobacteria*) Genus *Lichenihabitans*	GCF_004323635 *Lichenihabitans psoromatis* (77.92% ANI, 92.34% 16S)

BF: boreal forest, T: tundra, R: road 389, -D: December, ANI: average nucleotide identity
[1]Single-Copy Orthologue Duplicates in the fungal and algae genomes is reported as a percentage based on BUSCO "Duplicates" score, while Contamination in the bacterial isolate genome is based on CheckM score.
[2]Closest relative match is based on the ITS phylogenetic distance (see Material and Methods section), which was corroborated with NCBI BLAST for percent identity. For the genome of algal Uapishka isolate 4, the identity was further corroborated with NCBI-BLAST of the 16S rRNA of the chloroplast. The genome of bacterial Uapishka isolate 5 was identified via GTDB-TK.
Insert Source Here

phyta_odb10" for genome 4. The absence of bacterial contamination in the eukaryotic isolate genomes was verified using Kraken2 2.1.3 (Wood et al. 2019) and the BUSCO phylogenetic lineage placement option.

To avoid problems with the fragmentation or absence of the ribosomal loci in the genome assemblies, and since their assembly as part of the whole-genome is often suboptimal, ITS and 16S regions for taxonomic assignment were assembled separately from the whole-genome assemblies. Reads putatively belonging to these regions were identified by mapping the sequencing reads to the entire UNITE (ITS) and SILVA (16S) databases using bwa 0.7.17 (Li and Durbin 2009) and extracting the mapped reads with samtools 1.9 (Danecek et al. 2021). Assembly was performed with tadpole, part of BBmap (sourceforge.net/projects/bbmap/), using error read correction and discarding kmers with frequency below 100. If the assembly produced more than one contig, all contigs were compared to the GenBank non-redundant nucleotide database and the contigs with only partial matches were discarded, leaving only one contig per genome. These contigs were then again searched against the non-redundant nucleotide GenBank database (Johnson et al. 2008) to recover up to 200 sequences per query (with e-value cut-off 1e-20). The queries and subject sequences were aligned with Mafft 7.475 (Katoh and Standley 2013) and the alignment was trimmed with TrimAl 1.4.rev15 (Capella-Gutiérrez et al. 2009) with the option '-automated1'. The alignment ends were additionally manually trimmed to either the length of the unknown (query) sequence or (where the unknown sequence was longer than others) to the length of the majority of sequences in the alignment. The resulting alignments were used to construct phylogenetic trees with automatic selection of the best substitution model and SH-like approximate likelihood ratio test (1000 replicates) using IQ-TREE (Schmidt et al. 2014). The phylogenetic distances of the unknown sequence to its matches from GenBank were calculated with the Newick Utilities suite (Junier and Zdobnov 2010), and the ITS phylogenetic trees for each eukaryotic genome reported here are available in the Supplementary material (Figure S3). To obtain a more revolved ITS phylogenetic tree for genome 2, we have supplemented additional sequences from genera of the same family, *Lecanoraceae* (*Lecanora*, *Lecidea*, *Lecidella*, *Ramboldia*, *Scoliciosporum*, and *Rhizoplaca*) into the phylogenetic reconstruction (Zhao et al. 2016).

Moreover, additional phylogenetic trees were constructed on other (non-ITS) standard phylogenetic markers to confirm taxonomies. The markers used were genes for actin (*Act*), beta-tubulin (*Btub*), elongation factor 1a (*Ef1A*), DNA replication regulation protein (*Mcm7*), and RNA polymerase genes (*Rpb1* and *Rpb2*). The markers were recovered from the genomes using a standalone 'blastn', extracted using 'blastdbcmd', and used as 'blastn' queries against the GenBank non-redundant nucleotide database before performing the alignment and phylogenetic analysis of the maximum 200 best hits, as described for the ITS phylogenetic markers. To further support our taxonomy assignment of the algal isolate, we also used Magic-BLAST (Boratyn et al. 2019) to make a custom BLAST database with 16S chloroplast amplicon ASVs from our dataset, blasted the algal isolate's 16S rRNA sequences (in tadpole contigs) against the custom database to identify the 16S rRNA gene of the algal chloroplast. The lowest e-value hit was then also cross-referenced with BLAST against the NCBI-GenBank database.

The assembly of the bacterial isolate (genome 5) was performed using SPAdes genome assembler v3.15.4 (Prjibelski et al. 2020) via Kbase (Arkin et al. 2018) with default settings. GTDBtk v1.7.0 (Chaumeil et al. 2020) was used to assign taxonomy of the isolate and CheckM v1.0.18 (Parks et al. 2015) was used to check for quality of the genome. The annotation of the bacterial isolate was performed with RASTtk v1.073 (Aziz et al. 2008) and DRAM v0.1.2 (Shaffer et al. 2020) on Kbase with default settings. All the whole-genome reads and assemblies are available under the NCBI Bioproject PRJNA948675.

Results

Microbial viable cell enumeration and functional metabolic diversity

Viable microbial enumeration was performed on the December 2018 samples (Fig. 2). Overall, an increase in CFUs was noted for the 10°C incubation compared with the 0°C and 23°C incubations. For each temperature, the boreal forest snow samples (BF1-D, BF2-D) had the highest CFU counts, while the tundra (T1-D, T2-D) and snowmobile trail (ST-D) samples overall had lower CFU counts (Fig. 2, Table S1). An increase in CFUs was noticed with the decrease in altitude for the "undisturbed" environments (T1-D, T2-D, BF1-D, BF2-D). On the contrary, snow samples collected near motorized traffic (ST-D, R-D, RBF-D) had significantly lower viable biomass at 0°C and 10°C ($p \leq 0.05$) while compared to the "undisturbed" sample of closest altitude (BF2-D). However, extremely low microbial biomass was noted for all the samples, ranging from 0 CFUs/mL to a maximum of 8.05×10^3 CFUs/mL (Fig. 2, Table S1).

The microbial functional diversity substrate Richness (R) index was assessed via Biolog EcoPlates (Fig. 2) at 10°C and 0°C incubations. As the average well color development (AWCD) was low and R equaled to 0 for a portion of the samples, Shannon diversity index (H) and Shannon substrate evenness (E), often use in EcoPlate data interpretation, could not be calculated. Overall, substrate utilization richness was significantly higher ($p \leq 0.05$) at 10°C compared to 0°C in the boreal forest and snowmobile trail samples (BF1-F, BF2-D, BF2-F, ST-F), which correlated with the increase on CFU counts from 0°C to 10°C (Fig. S4). However, substrate utilization richness was significantly higher ($p \leq 0.05$) at 0°C compared to 10°C in the tundra and road samples (T2-D, T2-F, RBF-F).

Microbial community composition

16S amplicon sequencing generated 394 unique ASVs, after processing (Figs 2 and 3, Supplemental File S2). The prokaryotic community was composed exclusively of bacteria. The dominant bacterial phylum (63% on average) was *Proteobacteria* (recently renamed *Pseudomonadota*; (Oren and Garrity 2021), followed by *Firmicutes* (renamed *Bacillota*) (13.5%), and *Acidobacteria* (11.5%). Only the *Proteobacteria* and *Acidobacteria* phyla were present in all samples. Overall, the proportion of taxa was similar between samples, with the following exceptions: *Actinobacteria* (renamed *Actinomycetota*) represented 21% of the R-D sample, and *Deinococcota* represented 43% of the BF2-D sample. At the order level, the dominant taxon was *Acetobacterales* (26%), followed by *Rhizobiales* (20%), *Acidobacteriales* (11%), *Lactobacillales* (10%), and *Pseudomonadales* (9%). In addition, *Bacillales* composed 26% and 6% of RBF-F and R-F respectively, and the R-D snow sample was composed by 20% *Micrococcales* of and 10% of *Sphingomonadales*. Shannon alpha-diversity index varied between 1.7 and 4.5, but was not correlated with the vegetation density, the month of sample collection, the altitude, nor the level of "disturbance".

ITS amplicon sequencing generated 228 unique ASVs, after processing (Figs 2 and 3, Supplemental File S3). The fungal community was primarily represented by two phyla, with Ascomycota representing 81% on average across samples, followed by Basid-

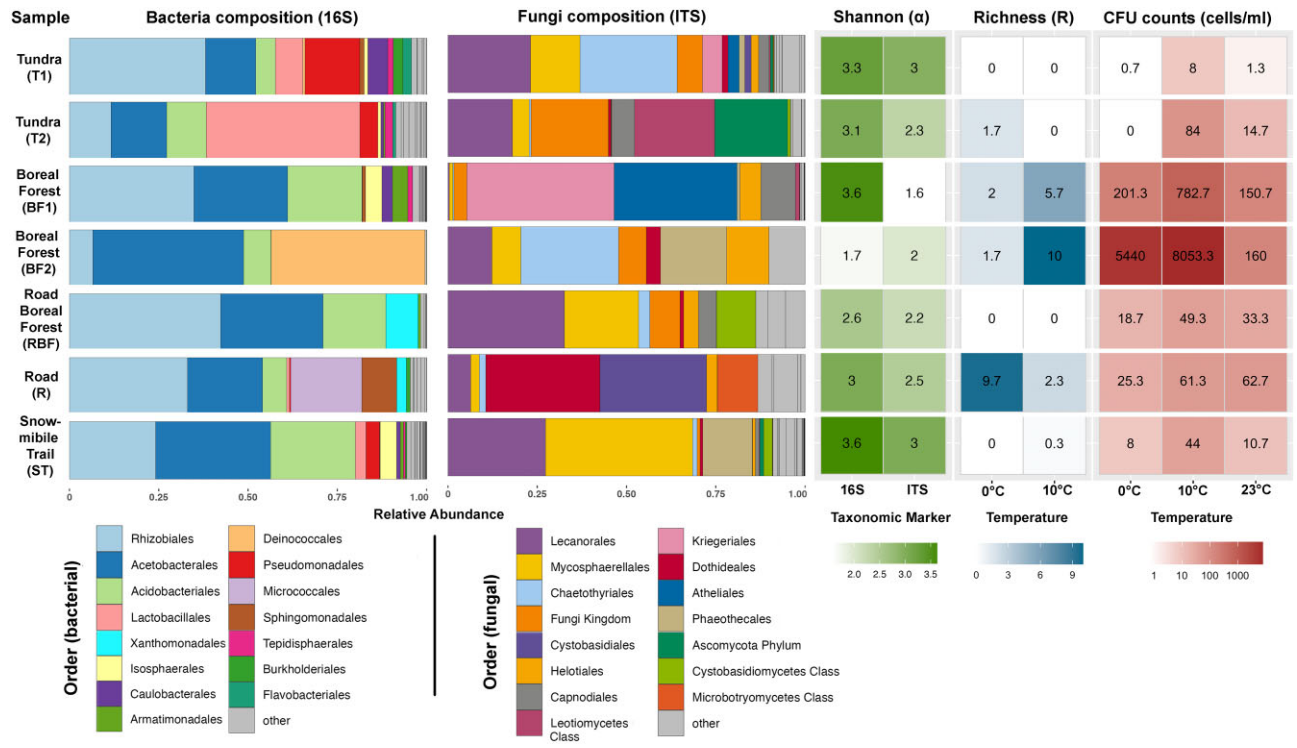

Figure 2. Microbial and metabolic diversity of *Uapishka* snow samples collected in December 2018. For each sample, the bacterial and fungal compositions, the Shannon index, substrate richness and colony-forming unit counts are given. The bacterial and fungal compositions are represented by the top 15 taxa, at the order level. The Shannon index represent the alpha diversity of the communities, for both bacteria (16S) and fungi (ITS). The substrate richness (R) represents the number substrates triggering metabolic activity, at 0°C and 10°C. The colony-forming unit (CFU) represents the viable biomass, in cells per milliliter of melted snow, that can grow at 0°C, 10°C or 23°C.

iomycota at 13%. In addition, on average, 6% of the ASVs were identified as fungi but were not characterized to a specific phylum (Supplemental File S3). The BF2-D snow replicate sample was the only one without any Basidiomycota members. At the order level, only *Mycosphaerellales* and *Lecanorales* taxa were found consistently in each sample, representing on average 27% and 16% of the fungal community respectively. The *Chaetothyriales* (6%), *Dothideales* (5%), *Capnodiales* (4%), and *Phaeothecales* (4%) orders were also well represented across the samples (Figs 2 and 3). The *Kriegeriales* order represented 41% of the BF1-D sample. Shannon alpha-diversity index varied between 1.6 to 3.7, and did not correlate with any parameters.

Isolated microorganisms

The sequenced isolates encompassed three fungal isolates, one algal isolate, and one bacterial isolate (Table 1). As the ITS region of genomes is repetitive with multiple copies, it is often fragmented or absent from whole-genome assemblies. Our ITS-based taxonomic assignments of the fungal isolates was complemented with other taxonomic gene markers (*Act*, *EF1a*, *Rpd1*, *Rdp2*, *Mcm7*, *Btub*), though an in-depth taxonomic assessment would be required to fully denote the taxonomic placement of the fungal and algal isolates; this is outside the scope of the current study. The fungal isolate 1 is of the *Tremellomycetes* class based on all phylogenetic markers, potentially of the *Phaeotremella* genus based on its ITS sequence (Table 1; Table S2). Fungal isolate 2 is of the *Lecanoraceae* family, and likely a lichen member of the *Miriquidica* genus (ITS corroborated with *Ef1a* marker, Table 1; Table S2), originally referred to by its basionym genus *Lecidea* (NCBI: txid1709439). Fungal isolate 3 is of the *Phenoliferia* genus (ITS corroborated with the *Ef1a* and *Rdp1* markers, Table 1; Table S2). The algal genome (isolate 4) is of the *Asterochloris* genus (based on ITS and chloroplast 16S rRNA; corroborated with the *Act* and *Ef1a* markers, Table 1; Table S2). Based on its genome, the algal isolate is capable of carbon fixation and comprises multiple genes associated with cilia and flagella, such as multiple *IFT* and *CFAP* genes (see NCBI Bioproject PRJNA948675).

The bacterial isolate (genome 5) was identified via GTDBtk as belonging to the *Lichenihabitans* genus with the closest taxa at 77.92% average nucleotide identity (ANI) identity as *Lichenihabitans psoromatis*, with the 16S rRNA at 92.34% identity to the *Uapishka* bacterial isolate in this study (NCBI-GenBank BLAST). CheckM of the isolate determined that the genome was 97.75% complete with only 1.1% contamination (based on duplicate supposed single copy genes—though only one colony was sequenced). Based on its genome, the isolate is likely a heterotroph, with genes for flagellar assembly and chemotaxis (*FlaA*, *FliLOPQKGNJ*, *FlgA-L*, *FlhAB*, *MotAB*, *CheDRBW*), stress response (oxidative—superoxide dismutases, hydroperoxide resistance genes, etc; heat shock—chaperone proteins *DnaJK*, heat shock protein *GrpE*, etc), ammonia assimilation (ammonium transporter, glutamate synthase, etc), and multiple copies of alcohol dehydrogenase (*Adh*) and other fermentative metabolism genes (NCBI Bioproject PRJNA948675).

Snow chemical properties

The chemical analysis content of a subset of three snow replicates is summarised in Supplemental File S1. Overall, the snows demonstrated an overall low contamination of hydrocarbons and metals, with relatively low total solids and nitrates/nitrite concentrations. Across all three replicates most metals and hydrocarbons

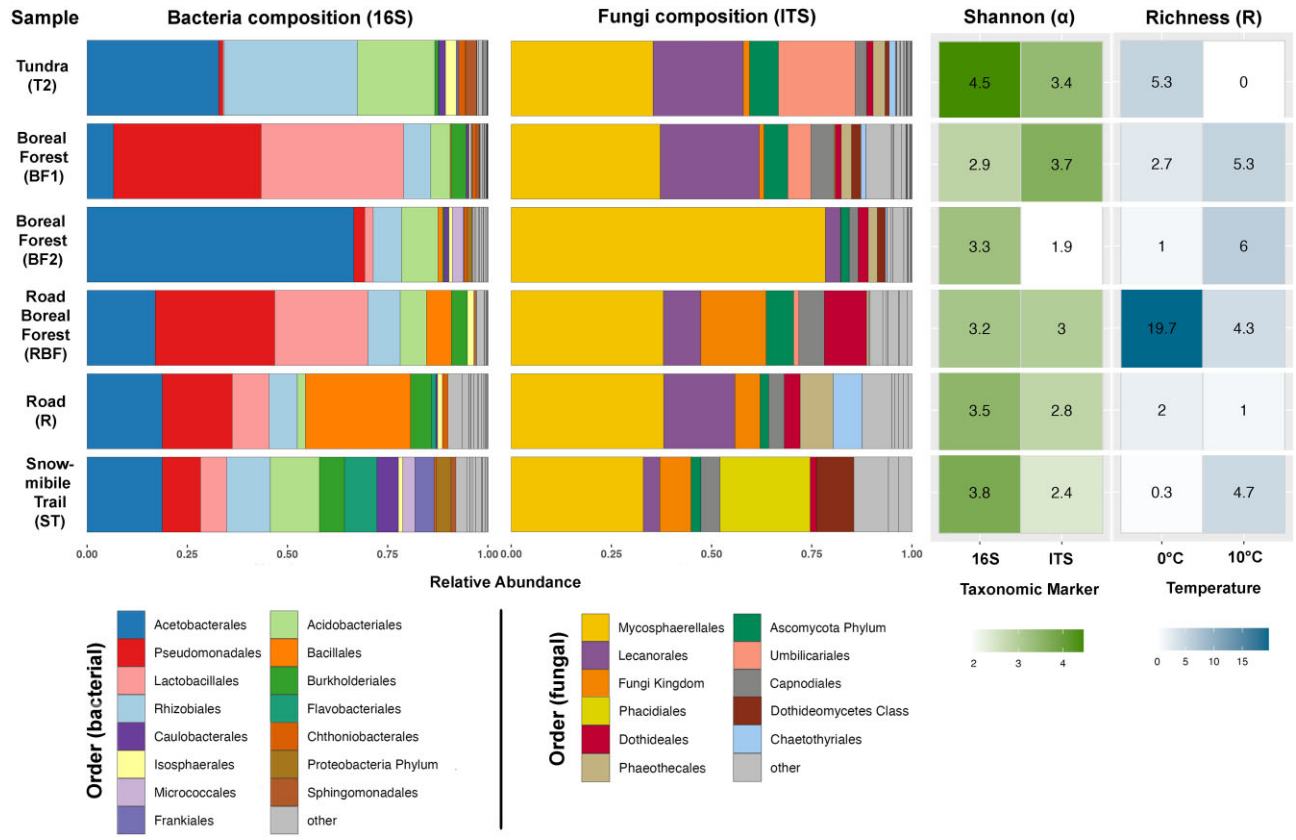

Figure 3. Microbial and metabolic diversity of *Uapishka* snow samples collected in February 2019. For each sample, the bacterial and fungal compositions, the Shannon index, and the substrate richness are given. The bacterial and fungal compositions are represented by the top 15 taxa, at the order level. The Shannon index represent the alpha diversity of the communities, for both bacteria (16S) and fungi (ITS). The substrate richness (R) represents the number substrates triggering metabolic activity, at 0°C and 10°C.

were below the detection limit of <0.10 μg/L (File S1). All three snow replicates contained petroleum hydrocarbons (C10-C50) between 0.2 and 0.1 mg/L, naphthalene at 0.1 μg/L, and zinc between 0.025 and 0.067 mg/L (File S1). The nitrates/nitrites of the three-snow sample replicated ranged between 0.1 and 0.2 mg/L.

Discussion

The snow viable biomass was low across all samples and incubation temperatures, ranging from no cultured microorganism (0 CFUs/mL) to a maximum of 8.05×10^3 CFUs/mL (Fig. 2). This is similar to other cold environments, such as polar snowpacks (Michaud et al. 2014, Maccario et al. 2015, Maccario et al. 2019), glacial cryoconites holes (Margesin et al. 2002, Makowska et al. 2016), and arctic sea water (Touchette et al. 2022b). We observed lower viable cell counts at the 23°C incubation temperature compared to 10°C, suggesting that overall cold-adapted psychrophilic microorganisms inhabit the *Uapishka* snow, as the optimum temperature of psychrophile is below 20°C (Cavicchioli et al. 2002). However, the viable biomass at 10°C was higher than at 0°C, suggesting that the relatively warmer temperature still promoted the growth of the psychrophilic microorganisms. Similarly, the *Uapishka* snow samples had low functional diversity (R value range of 19.7–0; Fig. 2, Fig. 3), which is also similar to other cold habitats, such as other alpine snowpack (Lazzaro et al. 2015), glacial ice (Touchette et al. 2023), cryoconite holes (Grzesiak et al. 2015a, Grzesiak et al. 2015b, Sanyal et al. 2018), and ice caves (Iţcuş et al. 2016). Both viable biomass and functional diversity were higher at 10°C compared to 0°C for the boreal forest and snowmobile trail snow samples (BF1, BF2 and ST, at both sampling months), suggesting a temperature-dependent enhancement of metabolism. Conversely, the less forest-sheltered tundra and road snow samples (T2-D, T2-F, RBF-F, R-D and R-F), had a higher functional diversity at 0°C compared to 10°C, which could be due to these microbial communities being better adapted to cold.

The dominant bacterial phyla across all *Uapishka* snow samples comprised of *Proteobacteria*, *Firmicutes*, and *Acidobacteria*, which is overall consistent with previous studies on nival microbial communities (Amato et al. 2007, Liu et al. 2009, Liu et al. 2011, Møller et al. 2013). However, unlike other nival environments (Larose et al. 2013), our samples were not dominant in the *Cyanobacteria* and *Bacteroidetes* (renamed *Bacteroidota*) phyla, as these phyla were almost completely absent across all of our *Uapishka* snow sample replicates. *Cyanobacteria* are thought to be dominant in surface nival ecosystems as they are capable of primary production, though they tend to occur in blooming patches or mats (Maccario et al. 2019). The dominance of *Cyanobacteria* does not seem to be universal as this phylum was present at very low amounts in our samples (0%–0.1%), this is perhaps due to *Uapishka* snow being sampled early in the winter when the snow is still mostly fresh, powdered, and frozen, while *Cyanobacteria* tend to be more abundant in late season snow (Larose et al. 2010). Low abundance of *Cyanobacteria* could also be partially explained by our filtering of chloroplasts from the 16S dataset as we focused on identifying

the bacterial community. Although chloroplasts are organelles of eukaryotic cells in the *Viridiplantae* kingdom, they are categorized in the 16S SILVA database within the *Cyanobacteria* phyla as they share proteins and components necessary for photosynthesis. Furthermore, *Bacteroidota* are often associated with colored snow (Hoham and Remias 2020, Luo et al. 2020), specifically they favor association with red snow algae (Terashima et al. 2017, Luo et al. 2020). Unlike other nival environments, *Bacteroidota* phylum was not dominant in the *Uapishka* snow samples (1.2% average across all samples), potentially because no coloration of the snow was observed during sampling.

The "core" snow bacterial microbiota of *Uapishka* mountain range samples (ie. defined as the taxa present across all replicate samples), consisted of three orders (Figs 2 and 3): *Acetobacterales* (ranging from 7% to 67% across the samples), followed by *Rhizobiales* (7%–42%), and *Acidobacteriales* (2%–24%). The *Acetobacterales* order was solely represented by the *Acetobacteraceae* family that consists of strict aerobic chemoorganotrophs which can be found in or on plant material (Robinson 2014). This is in line with our snow samples being collected in a boreal forest area (Jones and Willey 2012), with these genera likely originating from the indigenous forest flora. In our samples, this family was mostly represented by genera found in acidic soil (Christel et al. 2019, Li et al. 2019, Haq et al. 2022), such as *Endobacter* previously found in Northeastern America (Alonso-García and Villarreal A 2022), *Acidocella* also found in Finnish boreal forest (Sun et al. 2016), as well as *Acidiphilium* and *Acidisoma,* both identified in Norway spruce and Finnish springs (Sun et al. 2014, Haas et al. 2018).

Similarly, the *Acidobacteriales* order was only represented by the *Acidobacteriaceae* (subgroup1) family. Within our samples the members of this family mainly consisted of *Bryocella* and *Granulicella* genera, which are both acidophiles also found in acidic sphagnum peats of boreal forests (Oshkin et al. 2019, Mastný et al. 2021). This is likely due to them being seeded from the phyllosphere of the acidic-loving alpine plants and sphagnum mosses representative of the *Uapishka* Biodiversity Reserve (Jones and Willey 2012). Furthermore, both *Granulicella* and *Bryocella* bacterial genera are found in lichens (Grimm et al. 2021, Tagirdzhanova et al. 2023), including in subarctic tundra (Klarenberg et al. 2020).

The *Rhizobiales* order was represented in majority by the *Beijerinckiaceae* family, which in our samples consisted mostly of three genera: *1174–901–12*, *Methylobacterium-Methylorubrum*, *Methylocella*. These genera were previously identify in bark (Aschenbrenner et al. 2017), alpine permafrost (Perez-Mon et al. 2022), and the Swedish boreal fen (White et al. 2022). Specifically, the *1174–901–12* genus identified through the amplicon 16S SILVA database was present in all (13) the snow replicate samples and matched the bacterial isolate (genome 5) we cultured and whole-genome sequenced from the boreal forest sample (BF1-D). Using GTDBtk, this isolate was identified as part of the *Lichenihabitans* genus, with the closest species being *L. psoromatis* at 77.92% ANI identity and 92.34% 16S rRNA identity, thus this isolate is potentially a novel bacterial species of the *Lichenihabitans* genus. The *Lichenihabitans* genus (on GTDB) corresponds to the *1174–901–12* genus in the SILVA database (based on 16S rRNA). The consistent recovery of this isolate's genus from all of our snow sample 16S rRNA amplicons, as well as its recovery through culturing further corroborates that it is part of the *Uapishka* tundra core snow microbiome. *Lichenihabitans psoromatis,* was isolated from an Antarctic lichen (Noh et al. 2019), and the *Lichenihabitans* genus (also known as the Lichen-Associated *Rhizobiales* "LAR1" lineage) is a ubiquitous and abundant group of bacteria in lichens from temperate regions (Noh et al. 2020) and also found in glacial snow (Pankratov et al. 2020). The whole-genome sequencing of the *Uapishka Lichenihabitans* isolate revealed that it contained genes associated with cold adaptation, such as oxidative stress response genes (Raymond-Bouchard and Whyte 2017, Tribelli and López 2018) and alcohol dehydrogenase (Touchette et al. 2022a), which may allow it to thrive in the boreal snow environment. In addition, like other snow microbes that have flagella and demonstrate motility (Uetake et al. 2006, Borer et al. 2022), this isolate has genes coding for flagella assembly and motility. Though this genus is associated and previously isolated from lichens, the presence of flagella and the ubiquitous presence of *Lichenihabitans* across all snow samples may suggest that it also has a free-living stage in snow, potentially for dispersal.

Although less common, members of the *Lactobacillales* order were present in a considerable proportion (in 12 replicate samples; ranging 0.06%–43%) of the snow samples (Figs 2 and 3). Members of this order are often associated to wildlife faeces which was likely the source of these microbes in the *Uapishka* snow samples (Usui and Funck 2018, Jarma et al. 2021, Maraci et al. 2022). *Pseudomonadales* was also commonly recovered (in 10 replicate samples; ranging 0.3%–37%) (Figs 2 and 3). These microorganisms are known to promote plant growth and resistance, and are commonly found in soil and phyllosphere, even in cold adapted plants (Cid et al. 2017).

No archaeal sequences were recovered from any *Uapishka* snow samples. This corresponds to previous studies that report absence of archaea in snow (Maccario et al. 2014, Lazzaro et al. 2015), and when present, archaea are in very low relative abundance and diversity (Hamilton et al. 2013, Maccario et al. 2014, Lutz et al. 2015, Antony et al. 2016). As majority of archaea are anaerobic (Teske 2018) and snow is highly permeable to gas exchanges (Kuhn 2001, Feng et al. 2002), the absence of archaea is likely explained by the surface snow selecting for aerobic microorganisms (Hamilton et al. 2013).

Fungal taxa recovered across all *Uapishka* snow samples belonged to Ascomycota (81% on average) and Basidiomycota (13% on average) phyla. This is consistent with other research on snow fungal communities, where Ascomycota and Basidiomycota dominate and other phyla, such as Chytridiomycota and Zygomycota, typically represent less than 5% of the community (Kuhnert et al. 2012, Brown and Jumpponen 2019, de Menezeset al. 2019, Rosa et al. 2020). The "core" snow fungal community of *Uapishka* mountain range samples (ie. defined as the taxa present across all replicate samples), consisted of two orders (Fig. 2, Fig. 3): *Mycosphaerellales* (ranging from 1%–41% across the samples) and *Lecanorales* (0.4%–27%), both belonging to Ascomycota. In our samples, the *Mycosphaerellales* order was mostly represented by the *Teratosphaeriaceae* family, which comprises many extremotolerant taxa (Muggia and Grube 2018), lichens, and plant pathogens (Hujslová et al. 2013). *Teratosphaeriaceae* are often found in acidic soils (Jimu et al. 2018, Kolařík et al. 2021) and were previously observed in a forest-tundra of Northern Quebec (Matsuoka et al. 2021). The *Lecanorales* order was mainly represented by *Parmeliaceae* (genera *Bryoria* and *Hypogymnia*) and *Tephromelataceae* (genus *Violella*) families, both mycobionts of lichens. Members of the *Bryoria* genus, commonly referred to as the horsehair lichens, are often found in arctic-alpine regions (Myllys et al. 2011, Velmala et al. 2014) and were observed in the forest during our field sampling, while members of *Hypogymnia* lichens also inhabit North American high-elevation and subalpine boreal forest area (Hinds et al. 2009). The *Violella* genus lichens are widely distributed across temperate and polar regions (Spribille et al. 2011). This is in line with our culturing of a fungal isolate (genome 2) of the *Leconorales* order, which is

part of the "core" *Uapishka* snow microbiome. This fungal isolate (2) is part of the lecanoroid lichen *Lecanoraceae* family, potentially of the genus *Miriquidica,* though our phylogeny was not well resolved. This genus was previously classified as part of the *Lecidea* genus, members of which are present in arctic and alpine environments of Québec (Hertel 2014, McMullin et al. 2017, Yakovchenko et al. 2021). To further support this, two ITS ASVs were identified as *Lecidea*, one of which was found in the same snow sample as this isolate (T2-D sample, Supplemental File S3).

Although the Basidiomycota phylum was not represented in the *Uapishka* "core" snow fungal community, we cultured two fungal isolates from this phylum. We isolated a member of the *Tremellales* order (genome 1), this fungal order was scarcely present in our snow sample amplicons (Supplemental File S3), but is known to be associated with boreal bryophytes (Kauserud et al. 2008), Arctic lichens (Zhang et al. 2015), and members of this order are parasites/cohabitants of lichen-forming fungi (Lindgren et al. 2015). We also isolated a fungus of the *Phenoliferia* genus (order *Kriederiales*, genome 3) from the BF1-D snow sample, which is in line with the ITS amplicon results where *Phenoliferia psychrophila* constituted 41% of the fungal composition in this sample (Fig. 2, Supplemental File S3). The *Phenoliferia* genus was previously found in snow and other cold environments (Saito et al. 2018, de Menezes et al. 2019), on rocks near lichens or in association with plants, including bryophytes and microalgae (Ogaki et al. 2020), and it was recently identified as one of the endophytic microbes associated with the lichen *Usnea longissimi* (old man's beard) at high altitudes (Wang et al. 2022), which was also visually observed at the site during field sampling. Furthermore, *P. psychrophila* (formerly *Rhodotorula psychrophila*), was isolated from alpine environments and is known to be a psychrophilic species (Margesin et al. 2007). Our study cannot differentiate if these isolates were actively growing in the snow, were present as a spore or conidium, or if they were present in the snow as a small lichen piece. Although uncommon, lichen mycobionts can be found in a free-living stage in nature (Pichler et al. 2023). Our ability to culture a lichen mycobiont (*Lecanoraceae gen. sp.*; isolate 2) in a pure culture may suggest that it is at least possible for this isolate to have a free-living stage.

Alongside three fungi and one bacterium, we cultured a green alga (*Chlorophyta*, genome 4) of the *Asterochloris* genus (Table 1). This is a common lichen photobiont with members found in cold environments of the Northern Hemisphere, such as Canada, Scandinavia, and Russia (Brown and Jumpponen 2019, Pino-Bodas and Stenroos 2021), and in high altitude ecosystems (Medeiros et al. 2021). *Asterochloris* can also be the photobiont component of a lichen in association with a mycobiont member of the *Lecidea* genus (Meeßen et al. 2013), which would support the taxonomic assignment of isolate 2 to its basionym *Lecidea* genus. Based on its whole-genome annotation, this *Asterochloris* isolate has genes for flagella and cilia assembly. Snow green algae can have cilia and can be motile through snow. This has been demonstrated in the context of glacier snow, where snow algae move from previous year snow and ice, to the top layer of newly deposited snow, where light is more available (Kohshima et al. 2007). In the context of lichen-associated algal symbionts, it is possible that algae in the snow environment can use the free-living stage to migrate through snow pack and spread to phototrophic snow layers, which are more favourable to their growth (Raymond et al. 2022), and to disperse to find new fungal symbiotic partners which can capture free-living photobionts via hyphae (Sanders and Masumoto 2021). This could be the case in snow ecosystems of alpine tundra and boreal environments, such as in the *Uapishka* mountain range.

The chemical analysis of the three snow sample replicates demonstrated that the *Uapishka* mountain range snow's hydrocarbon, nitrogen (nitrite/nitrate), total solid, and metal concentrations were overall comparable with the few previous studies on boreal or low arctic tundra snowpacks (Simonetti et al. 2000, Constant et al. 2007, Pelster et al. 2009, Birks et al. 2017). Hydrocarbon concentrations in the *Uapishka* snow samples were considerably below (e.g. <0.1 mg/L benzopyrene, 0.1 mg/L naphthalene of melted snow; File S1) those reported in areas with high snowmobile traffic (Reimann et al. 2009, Abramova et al. 2016). While the literature in this domain is already limited, less is known regarding the intersection between snow chemical properties (including hydrocarbon contaminants) and microbiota. Though our study did not focus on filling this gap of knowledge, this would be a beneficial avenue for future studies.

Furthermore, the effects of the heterogeneity of the environment across the sample replicates (altitude, chemical properties, vegetation cover) on the microbial community composition is an important topic of discussion, though outside the aim of this study which focused on the core snow microbiome irrespective of biotic/abiotic parameters and environment heterogeneity. However, these parameters were still measured and reported to contextualize the study area. In addition, future studies should also focus on differences between fresh winter powdered snow and springtime warmer melting snow and between the surface and deeper snow layers.

Overall, the nival microbial community in the alpine sub-arctic tundra and boreal forest was characterized by a very low viable biomass, and a low active functional diversity, similar to other environments of the cryosphere, including snow. However, there are key taxonomic differences between dominant taxa in the boreal/tundra alpine snow environment compared to polar snow systems, exhibited by the presence of lichen fungal and algal symbiont taxa and lichen-associated bacteria. This may be due to higher presence of vegetation in the alpine system that may facilitate lichen dispersal through wind. The high representation of lichen-forming fungal taxa across all *Uapishka* snow samples is consistent with our recovery of the lichen-associated bacterial isolate (*Lichenihabitans*) and the algal photobiont isolate (*Asterochloris*). High abundance and ubiquity of lichen-associated taxa in the snow replicate samples regardless of other variables (anthropogenic traffic, altitude, vegetation cover, month) suggest that these taxa are part of the core alpine tundra/boreal snow microbiome. As they were able to maintain viability in the snow environment (as well as having flagellar genes in the algal/bacterial isolates), it is possible that these lichen-associated fungi, algae, and bacteria utilize the snow medium to "mix & swap" their symbiotic partners and to disperse throughout the ecosystem during snowmelt, and potentially to nearby mountain ranges including above tree-line summits during heavy winds and snowstorms. The snow microbiome of the tundra/boreal alpine ecosystem, like the *Uapiskka* mountain range, is uniquely characterised by dominance and ubiquity of lichen-associated microbial community, currently irrespective of anthropogenic disturbances. Isolation of potential novel microbial taxa from this region, supports the need to further protect this Biodiversity Reserve and other alpine ecosystems.

Author contributions

D Touchette (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original

draft, Writing – review & editing), C Gostinčar (Formal analysis, Methodology, Writing – review & editing), LG Whyte (Funding acquisition, Project administration, Writing – review & editing), and I Altshuler (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing)

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Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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