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Intrathalline Fungal and Bacterial Diversity Is Uncovered in Antarctic Lichen Symbioses

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ABSTRACT

Although the Antarctic continent represents one of the most hostile environments on earth, microbial life has adapted to cope with these extreme conditions. Lichens are one of the most successful groups of organisms in Antarctica, where they serve as unique niches for microbial diversification. We have selected eight epilithic lichen species growing in Victoria Land (three cosmopolitan and five endemic to Antarctica) to describe with amplicon sequencing the diversity of the associated fungal and bacterial communities. The lichen mycobiota is predominantly composed of *Ascomycota* belonging to the classes *Chaetothyriomycetes* and *Dothideomycetes*, while a few key representative taxa were recognised as basidiomycetous yeasts. Bacteria associated with lichens were represented by *Pseudomonadota*, *Cyanobacteria*, and *Bacteroidota* in which psychrophilic genera were identified. The microbiota was diverse among the lichen species, and their variation was driven by the lichen species itself and their endemic or cosmopolitan distribution. There was a strong association of the microbial communities linked to the lichen itself, rather than to the specific characteristics of the collecting site. The lichen thallus, thus, plays an important role in microbial diversification and may potentially act as a selective biodiversity filter in which different fungal and bacterial communities thrive in it.

1 | Introduction

Antarctica represents one of the most hostile environments on Earth (Øvstedal and Lewis Smith 2001; Selbmann et al. 2013). Here, vascular plants do not settle and are replaced by cryptogamic communities composed of mosses and, above all, lichens. Mosses and lichens spread along the coasts on rocks and soil, but lichens are the only ones able to settle even throughout the interior areas of the continent. As a result, lichens represent an important group of organisms in this extreme environment, with a high percentage of endemism (33%–50% in the continental

Antarctica): 130 endemic lichen species have been reported so far from a total Antarctic flora of 393 species (Øvstedal and Lewis Smith 2001; Castello and Nimis 1997, 2000).

Lichens are indeed notable examples of self-sustaining, long-living, symbiotic systems that derive from the mutualistic associations between biotrophic fungi (the mycobionts) and phototrophic green microalgae or cyanobacteria (the photobionts, i.e. chlorobionts and the cyanobionts, respectively; Hawksworth and Honegger 1994). In addition, lichen thalli host a multiplicity of other microorganisms (Honegger 2012;

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Hawksworth and Grube 2020). The two main lichen symbionts have co-evolved into peculiar phenotypes—building up three-dimensional structures, that is, the lichen thalli—and have been able to adapt to the harshest environments on Earth (Lutzoni et al. 2001; Onofri et al. 2007). Despite the ecological roles of the lichen-inhabiting fungi (i.e., the lichen mycobiota; Fernández-Mendoza et al. 2017) and bacteria being still largely unknown, their presence has been correlated with the growth form and the ecology of the lichen hosts (Harutyunyan et al. 2008; Arnold et al. 2009; U'Ren et al. 2010, 2012, 2014; Muggia et al. 2016; Fernández-Mendoza et al. 2017; Muggia and Grube 2018; Smith et al. 2020; Spribille 2018). So far, these lichen-associated microorganisms were supposed to be active players for the ecological success of lichen symbioses, also in extreme environments (Grube et al. 2009, 2015; Grube and Berg 2009; Muggia and Grube 2018), but still, it has not been possible to report on the practical evidence of their potential roles.

To date, studies have mainly focused on non-lichenized fungi and bacteria associated with the endolithic communities and were based on culture isolation and genetic identification of the grown strains (i.e., de la Torre et al. 2003; Selbmann et al. 2005, 2008; Egidi et al. 2014). More recently, thanks to the advent of high-throughput sequencing techniques (HTS; Greco et al. 2021; Ji et al. 2013; Smith and Peay 2014; Tedersoo et al. 2015), a more accurate census of the viral, eukaryotic, and prokaryotic assemblages of Antarctic microbial communities has been reached (de la Torre et al. 2003; Archer et al. 2017; Coleine et al. 2018a, 2018b, 2019, 2021; Coleine, Gevi et al. 2020; Coleine, Masonjones et al. 2020; Albanese et al. 2021; Mezzasoma et al. 2022; Ettinger et al. 2023). On the other hand, only two studies have focused on microbial diversity from epilithic lichens in Antarctica. These correspond to the first pioneer study from Selbmann et al. (2013), who considered 13 species of epilithic lichens collected in Northern and Southern Victoria Land. Later, Santiago et al. (2015) reported ascomycetes, basidiomycetes, and taxa formerly recognised as zygomycetes from multiple thalli of the two endemic macrolichen species *Usnea antarctica* and *U. aurantiaco-atra* from the Antarctic Peninsula.

To address this knowledge gap, in the present study we collected Antarctic epilithic lichens, both endemic and cosmopolitan, with the main aim to characterise by HTS their inhabiting microbial diversity comprising microfungi and bacteria. In particular, we studied five Antarctic endemic and three worldwide distributed (hereon referred to as ‘cosmopolitan’) lichen species from 14 localities in the Victoria Land to: (i) define the fungal and bacterial diversity associated with the lichen thalli; (ii) verify which factor is determinant for shaping the associated assemblages; (iii) determine the presence of a potential group of taxa that are uniquely associated either with cosmopolitan or endemic lichen species.

2 | Material and Methods

2.1 | Study Area and Sampling

The sampling campaign took place during the 37° Antarctic expedition, November 2021–February 2022. In this campaign, 14 selected localities have been visited in Northern Victoria Land,

within the south latitudinal range from the 72° to 77° parallel (Figure 1, Table 1). In each locality, when present and feasible to collect, at least three individuals of each selected lichen species were sampled (Table 1). The variability of lichen species and thalli collected in each locality is primarily due to the availability of lichen specimens in the field and the sampling conditions in which the operator (i.e., one of the authors) had to collect. Sampling conditions are determined by the extreme environmental conditions; the logistic and climatic constraints often limit the time available for searching, identifying, and collecting samples at the localities. Thus, a homogeneity of the number of samples, the same number and type of specimens in all sampling sites could not be guaranteed. Indeed, there are cases in which the lichens were extremely rare, and thus less than three thalli for each occurring species were collected and used for the molecular analyses (Table 1; for example in Key Island only one thallus of *Rhizoplaca melanophthalma* was collected and used for the amplicon sequences analyses). The samples were aseptically collected with the rock substrate using a geological hammer, placed into sterile plastic bags, and stored dry at -20°C , also during the transportation to the laboratories, until downstream analyses.

The collected and analysed lichen species are all chlorolichens (i.e., forming symbiosis with green trebouxioid photobionts): the endemic Antarctic *Acarospora flavocordia*, *Buellia frigida*, *Lecanora fuscobrunnea*, *Lecanora physciella*, and *Lecidea cancriformis*, and the cosmopolitan *Pleopsidium chlorophanum*, *Rhizoplaca melanophthalma*, and *Rusavskia elegans*. The lichen species were chosen based on literature knowledge and their availability at the sampling sites.

2.2 | DNA Extraction, Amplification and Sequencing of Fungal and Bacterial Communities

For the amplicon sequencing analyses, up to three thalli of each species from each locality were used. Thus, the amplicon sequencing analysis was performed on a total of 140 environmental samples belonging to the eight lichen species (*A. flavocordia*, *B. frigida*, *L. fuscobrunnea*, *L. physciella*, *L. cancriformis*, *P. chlorophanum*, *R. melanophthalma*, *R. elegans*; Table 1). Metagenomic DNA extraction was performed on a 0.5 cm² fragment of the thallus, devoid of any symptoms of external infection or damage, which was removed from the substrate using a sterile razor blade and transferred into a 1.5 mL reaction tube. The fragments underwent a series of washes: they were rinsed three times for 15 min with sterile water, followed by a 30 min cleaning step using a 2% Tween 80 solution. A final wash was performed for 15 min with sterile water. The DNA extraction from the cleaned fragments followed the CTAB protocol of Cubero et al. (1999), with minor adjustments. Potential contaminants during the DNA extraction process were checked by establishing negative control samples for each lichen species. These negative controls consisted of a 1.5 mL tube kept open during the whole extraction process and processed in the same way as the lichen samples for the amplification and sequencing steps.

To study the diversity of fungal communities, the nuclear ribosomal internal transcribed spacer 1 region (ITS1) was

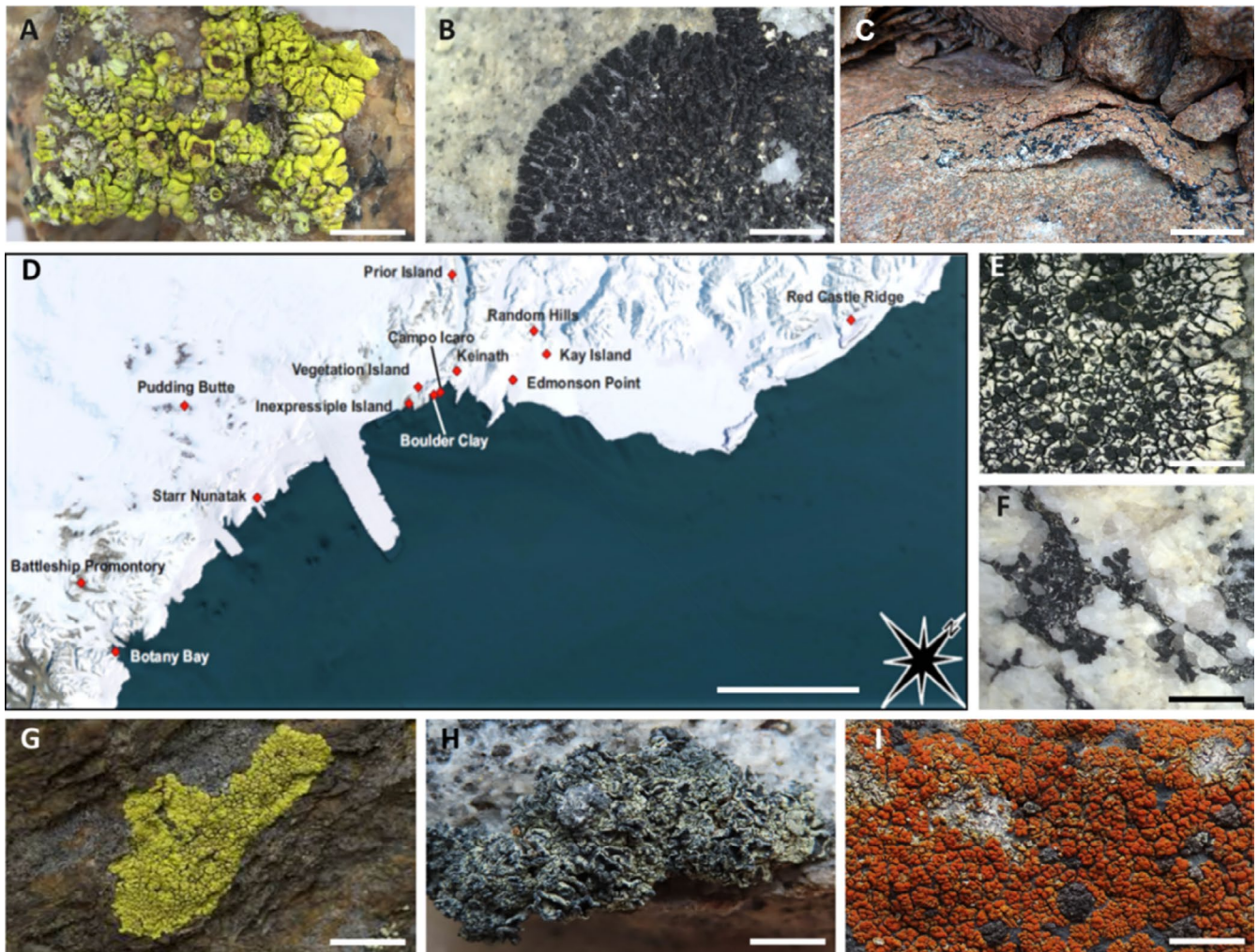


FIGURE 1 | Lichen species and sampling area. (A) *A. flavocordia*; (B) *B. frigida*; (C) *L. fuscobrunnea*, (D) collecting sites in Victoria Land (Antarctica) visited for the sampling of the eight lichen species here analysed; (E) *L. physciella*; (F) *L. cancriformis*; (G) *P. chlorophanum*; (H) *R. melanophthalma*; (I) *R. elegans*. Scale bars: (A–C, E–H) 1 cm; (I) 2 cm.

targeted. The ITS1 region was amplified using the barcoded primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC; White et al. 1990; Smith and Peay 2014). The PCR amplification protocol for the ITS1 fragments was run with the following conditions: an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, and a final extension step at 72°C for 5 min.

The V4 variable region of the small ribosomal subunit 16S rDNA was used to assess bacterial diversity. This variable region was amplified using the primers F515 (GTGCCAGCMGCCGCGGTAA) and R806 (GGACTACHVGGGTWTCTAAT) as described by Caporaso et al. (2012). The PCR amplification of the V4 variable region was performed with the following protocol: an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, extension at 72°C for 90 s, and a final extension step at 72°C for 10 min.

Samples were then sequenced in paired ends (2 × 300 bp) using the Illumina MiSeq platform at the Edmund Mach Foundation (San Michele all'Adige, Italy).

2.3 | Bioinformatics Analyses

The samples were processed after checking for extraction contamination with Decontam (Davis et al. 2018). Raw reads were analysed using the Amplicon ToolKit (AMPTk) for Next Generation Sequence (NGS) v1.2.1 (Palmer et al. 2018), USEARCH (Edgar 2010) and VSEARCH (Rognes et al. 2016). The reads were trimmed, resulting in sequences with a length of 250 bp; reads shorter than 100 bp were discarded, and chimera removal was performed using USEARCH v. 9.2.64 with default parameters. Sequence quality filtering was performed with the expected error parameter of < 1.0 (Palmer et al. 2018). The dataset was clustered with DADA2 v1.6.0 using a 99% identity parameter to generate the Amplicon Sequence Variants (ASVs). Further filtering was performed in which rare ASVs (i.e., ASVs having less than five reads), singletons, and chimeras were discarded and not considered for the subsequent analyses according to Lindahl et al. (2013). The taxonomy assignment was performed via the UNITE v10.5 (2023) and RDP release 11.7.2023 (Cole et al. 2014) database, which uses the hybrid SYNTAX algorithm (Abarenkov et al. 2020; Edgar 2010); the sequences were aligned, and the taxonomy was assigned to the corresponding ASVs of the ITS and

TABLE 1 | Sampling information of localities and the collected lichen species.

Locality	Coordinates	Endemic lichens	Cosmopolitan lichens	Samples used for amplicon sequencing analyses (endemics; cosmopolitan)
Botany Bay	77°00'00"S 162°35'00" E	<i>B. frigida</i> (6), <i>L. cancriformis</i> (6)	<i>R. elegans</i> (4)	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3); <i>R. elegans</i> (3)
Inexpressible Island	74°53'21.5"S 163°44'43.8" E	<i>B. frigida</i> (8), <i>L. cancriformis</i> (5)	<i>R. elegans</i> (6)	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3); <i>R. elegans</i> (3)
Vegetation Island	74°47'03" S 163°39'35" E	<i>B. frigida</i> (5), <i>L. physciella</i> (3)	<i>P. chlorocephalum</i> (3), <i>R. elegans</i> (6)	<i>B. frigida</i> (3), <i>L. physciella</i> (3); <i>P. chlorocephalum</i> (3), <i>R. elegans</i> (3)
Prior Island	75°41'30" S 162°52'43" E	<i>B. frigida</i> (6), <i>L. cancriformis</i> (5)	—	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3)
Key Island	74°04'13" S 165°18'57" E	<i>A. flavocordia</i> (12), <i>B. frigida</i> (5), <i>L. cancriformis</i> (7), <i>L. physciella</i> (7)	<i>R. elegans</i> (7), <i>R. melanophthalma</i> (1)	<i>A. flavocordia</i> (3), <i>B. frigida</i> (3), <i>L. cancriformis</i> (3), <i>L. physciella</i> (3); <i>R. elegans</i> (3), <i>R. melanophthalma</i> (1)
Starr Nunatak	75°53'54.7" S 162°35'34.4" E	<i>B. frigida</i> (11), <i>L. cancriformis</i> (15)	<i>R. elegans</i> (12)	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3); <i>R. elegans</i> (3)
Mt Keimath	74°55' S 164°00' E	<i>L. cancriformis</i> (1)	<i>P. chlorocephalum</i> (6)	<i>L. cancriformis</i> (1); <i>P. chlorocephalum</i> (3)
Battleship Promontory (McMurdo Dry Valleys)	76°54' S 160°50'00.0" E	<i>L. fuscobrunnea</i> (7)	—	<i>L. fuscobrunnea</i> (3)
Edmonson Point	74°19.902' S 165°08.223' E	—	<i>R. elegans</i> (6)	<i>R. elegans</i> (3)
Random Hills site 2	74°0.1'0.15" S 164°21'23.4" E	<i>A. flavocordia</i> (8), <i>B. frigida</i> (4), <i>L. cancriformis</i> (6)	<i>R. elegans</i> (5)	<i>A. flavocordia</i> (3), <i>B. frigida</i> (3), <i>L. cancriformis</i> (3); <i>R. elegans</i> (3)
Random Hills site 3	74°0.0'10.9" S 164°28'56.4" E	<i>A. flavocordia</i> (1), <i>B. frigida</i> (6)	<i>P. chlorocephalum</i> (7), <i>R. elegans</i> (6), <i>R. melanophthalma</i> (2)	<i>A. flavocordia</i> (1), <i>B. frigida</i> (3); <i>P. chlorocephalum</i> (3), <i>R. elegans</i> (3)
Random Hills site 4	74°0.1'42.2" S 164°44'44.5" E	<i>A. flavocordia</i> (11), <i>B. frigida</i> (5), <i>L. physciella</i> (8)	<i>R. elegans</i> (6), <i>R. melanophthalma</i> (21)	<i>A. flavocordia</i> (3), <i>B. frigida</i> (3), <i>L. physciella</i> (3); <i>R. elegans</i> (3), <i>R. melanophthalma</i> (3)
Random Hills site 5	74°0.4'33.4" S 164°48'32.5" E	<i>A. flavocordia</i> (8), <i>L. physciella</i> (15)	<i>R. melanophthalma</i> (3)	<i>A. flavocordia</i> (3), <i>L. physciella</i> (3); <i>R. melanophthalma</i> (3)
Pudding Butte	75°51'30.2" S 159°58'25.7" E	<i>L. fuscobrunnea</i> (2)	—	<i>L. fuscobrunnea</i> (2)
Red Castle Ridge	72°26'00" S 169°57'00" E	<i>B. frigida</i> (5), <i>L. cancriformis</i> (5)	<i>R. elegans</i> (7)	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3); <i>R. elegans</i> (3)
Campo Icaro	74°42'33.5" S 164°05'45.9" E	<i>B. frigida</i> (6), <i>L. cancriformis</i> (6)	—	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3)
Boulder Clay	74°44'6.8" S 164°02'22.50" E	<i>L. fuscobrunnea</i> (1)	<i>P. chlorocephalum</i> (16)	<i>L. fuscobrunnea</i> (1); <i>P. chlorocephalum</i> (3)

Note: The number of thalli collected and used for amplicon sequencing analyses from each site and lichen species is shown in parentheses.

16S. Regarding the bacteria dataset, we excluded both mitochondria and chloroplasts from the analysis.

2.4 | Statistical Analyses

To test the effectiveness of the sampling effort of the overall lichen-associated fungal and bacterial diversity in the studied area, species accumulation curves were calculated using the species accumulation function in the ‘vegan’ package (Oksanen et al. 2013). Because of the dishomogeneous number of species and samples collected for each species in each locality, we calculated species accumulation curves for bacterial and fungal reads both for the entire dataset and for individual species.

The Linear discriminant Effect Size (LEfSe) analysis was performed to identify those taxa that explain the differences between endemic and cosmopolitan lichens myco- and microbiota (Segata et al. 2011).

To explore fungal and bacterial diversity, the analyses were done by clustering the ASVs by lichen mycobiont species (eight species) and by their ecological distribution (i.e., Antarctic endemic vs. cosmopolitan). Alpha diversity was calculated with the Chao1 index, beta diversity was calculated using the Bray–Curtis dissimilarity index, and PCoA analyses were performed and tested with PERMANOVA. All analyses were performed using R packages *microeco* (Liu et al. 2021) and *phyloseq* (McMurdie and Holmes 2013). Kruskal–Wallis and Wilcoxon tests (Kruskal and Wallis 1952; Wilcoxon 1945) were used to assess significant diversity in differences among lichen species and between groups with different ecological distributions. To explore the correlation in terms of Shannon diversity between fungi and bacteria, we conducted a Linear Regression Analysis calculated with the ‘vegan’ package (Oksanen et al. 2013). The sequencing depth, defined as the total number of reads per sample after quality filtering, was calculated using the *phyloseq* package (McMurdie and Holmes 2013) in R for both individual samples (for each species) and types of ecological groups (endemic and cosmopolitan).

3 | Results

3.1 | Taxonomic Composition of Myco- and Microbiota

The outcomes of sequencing returned 10,603,745 reads for fungi and 18,438,804 for bacteria, which were clustered at 99% identity and collapsed into 2073 and 24,784 ASVs, respectively. To specifically investigate the lichen mycobiota, a further filtering was applied: all ASVs matching any lichen-forming fungi (i.e., any lichen mycobionts known) were excluded from the analysis (Table S1). This resulted in a final dataset that exclusively comprised fungal taxa classified as not lichen-forming fungi. The complete datasets for fungi and bacteria were finally obtained by removing chimeras, singletons, contaminants, and archaeal sequences, resulting in a total of 359 fungal and 5835 bacterial ASVs. Unassigned ASVs were not included in the analyses, but they are presented and available in the (Tables S7 and

S8, Supporting Informations 1 and 2). The sequencing depth showed no significant differences between the endemic and cosmopolitan samples analyzed for both fungal and bacterial data, p value > 0.05 (Table S9).

We performed accumulation curves for bacteria and fungal reads both for the entire dataset and for individual species (Figures S1 and S2). When examining individual lichen species, the species accumulation curves for fungi and bacteria level off for only a few lichen species (Figures S2A,B). This suggests that individual lichen species alone do not account for the full diversity observed in the dataset. Conversely, when considering all species collectively (Figures S1), we observe that the species accumulation curves gradually levelled off for both fungi and bacteria, indicating that the number of samples analysed approaches an adequate number to encompass the biodiversity of the studied communities (Figure 1).

The relative abundance of the community analyses (Figure 2) of the mycobiota revealed *Ascomycota* and *Basidiomycota* as the dominant phyla (Figure 2A). In seven (e.g., *A. flavocordia*, *B. frigida*, *L. physciella*, *L. cancriformis*, *R. melanophthalma*, *R. elegans*, *L. fuscobrunnea*) out of the eight lichen species, the phylum *Ascomycota* constituted the majority of ASVs, whereas the phylum *Basidiomycota* was predominant only in the thallus of *P. chlorophanum*. Based on relative abundances (Figure 2C), it emerged that the most represented genera were *Antarctolichenia* in *A. flavocordia* (24,3%), *B. frigida* (27,4%), *L. cancriformis* (31,14%), *L. fuscobrunnea* (20,3%), *L. physciella* (48%); *Knufia* in *R. elegans* (40%) and *Tremella* in *P. chlorophanum* (31,5%), *R. melanophthalma* (11,5%).

The analyses of the bacterial communities revealed *Pseudomonadota* as the dominant phylum in all the lichen species (Figure 2B). Looking at the relative abundance of bacteria at the genus level (Figure 2D), we found that most of the genera were: *Hymenobacter* in *R. elegans* (26,3%), *B. frigida* (21%), *L. cancriformis* (16,2%); *Acidisoma* in *R. melanophthalma* (10%) and *L. physciella* (6%); *Armatomonas* in *L. fuscobrunnea* (9,69%) and *P. chlorophanum* (7,64%); and *Staphylococcus* (4,81%) in *A. flavocordia*. Under “Others” we included all the other genera and higher taxonomic ranks that were not among the 15 most frequently recovered ones.

3.2 | Alpha Diversity Analyses

The Chao1 indexes for fungal and bacterial diversity are shown in Figure 3. Mycobiota of *A. flavocordia* (Chao1 = 52) and *L. cancriformis* (Chao1 = 47.43) presented the highest Chao1 values among the lichen species, while *L. fuscobrunnea* displayed the lowest value (Chao1 = 8; Figure 3A). The Kruskal–Wallis Dunn test, conducted for multiple comparisons within lichen species, revealed significant statistical differences between *L. fuscobrunnea* and *A. flavocordia*, *B. frigida*, and *L. cancriformis* (p value < 0.001). Moreover, statistical differences were observed between *P. chlorophanum* and *A. flavocordia* (p value < 0.001), *B. frigida* (p value < 0.01), and *L. cancriformis* (p value < 0.01). *R. elegans* displayed statistical differences with *A. flavocordia* (p value < 0.001), *B. frigida* (p value < 0.001), and *L.*

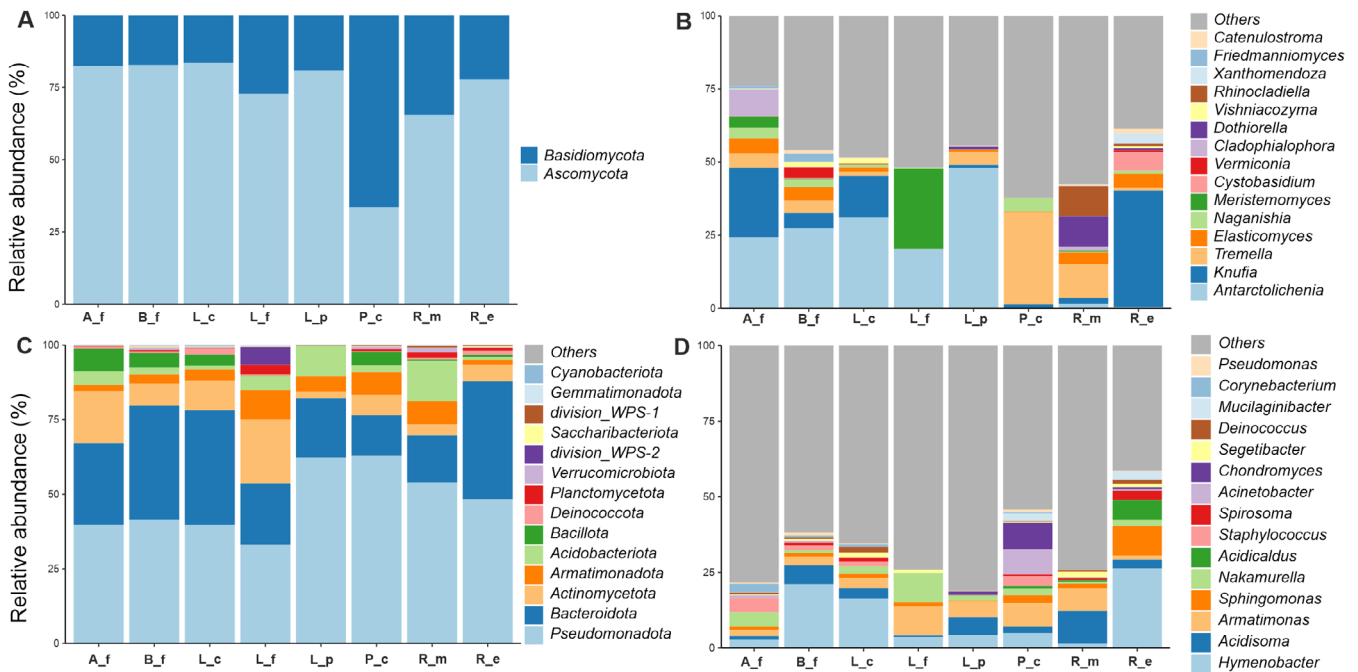


FIGURE 2 | Relative abundance plots of fungi (A, C) and bacteria (B, D) reported for each lichen species; the 15 most frequently recovered genera are shown. Lichen species names were abbreviated as follows: *Acarospora flavocordia* (A_f), *Buellia frigida* (B_f), *Lecidea cancriformis* (L_c), *Lecanora fuscobrunnea* (L_f), *Lecanora physciella* (L_p), *Pleopsidium chlorophanum* (P_c), *Rusavskia elegans* (R_e), *Rhizoplaca melanophthalma* (R_m). Abundance data are shown in percentage. “Others” includes all the other genera and higher taxonomic ranks which were not among the 15 most frequently recovered ones.

cancriformis (p value < 0.001 ; Tables S2–S5 report the detailed statistic values).

Looking at the bacterial assemblages, *R. melanophthalma*, *L. cancriformis*, and *B. frigida* displayed the highest Chao1 values (797, 789, and 765, respectively), while *P. chlorophanum* (Chao1 = 447) the lowest (Figure 3B). *L. cancriformis* showed statistical differences (p value < 0.001) with *A. flavocordia* and *P. chlorophanum*; with *L. fuscobrunnea* and *L. physciella* (p value < 0.05). *B. frigida* shows statistical differences (p value < 0.05) with *A. flavocordia* and *P. chlorophanum*. *R. melanophthalma* showed differences (p value < 0.05) with *A. flavocordia* and *P. chlorophanum*. Also, statistical differences were shown for *R. elegans* against *A. flavocordia* and *P. chlorophanum* (p value < 0.05 ; Figure 3B). Moreover, to understand the relationship between the microbial diversity of bacteria and fungi in our Antarctic epilithic communities, we conducted a linear regression analysis between the Shannon indices of the bacterial and fungal communities (Figure S3). The scatter plot clearly shows the linear relationship between the two indices, indicating that an increase in bacterial diversity is associated with an increase in fungal diversity in our sampled communities (Figure S3).

3.3 | Beta Diversity Analyses

The Bray-Curtis (incidence and relative abundance data) distance was used to assess the beta diversity and the differences in fungal and bacterial assemblages among lichen species. The results were presented through PCoA plots along with PERMANOVA statistical analysis. The effects of the lichen species and ecological

distribution (i.e., endemic vs. cosmopolitan) were found to be significant (p value < 0.001), although the ecological distribution seemed to have a lesser impact on the community ordination than that of the lichen species (Figure 3C,D).

3.4 | Cosmopolitan Versus Endemic Lichen Species

When the lichen species were grouped according to their endemic and cosmopolitan distribution, no significant differences in the relative abundances of fungal phyla were found. For fungi, in both endemic and cosmopolitan lichens, the phylum *Ascomycota* was the dominant one (82% and 65%, respectively), followed by *Basidiomycota* (18% in endemic and 35% in cosmopolitan) (Figure 4A). The genera *Antarctolichenia*, *Meristemomyces*, and *Vermiconidia* were more abundant in endemic lichens, while *Cystobasidium*, *Rhinoctadiella*, *Dothiorella*, and *Knufia* were more abundant in the cosmopolitan lichens (Figure 4C).

Concerning bacteria, the most abundant phyla were *Pseudomonadota* and *Bacteroidota*. Specifically, *Pseudomonadota* was the main represented phylum in both endemic (42%) and cosmopolitan (53%) species. *Bacteroidota* was the second most abundant phylum in cosmopolitan (29%) and in endemic (33%) lichens (Figure 4B). Looking at the genus level, the majority of the fungi are represented by the ascomycetous genera *Antarctolichenia*, *Knufia*, and *Elasticomyces*, followed by the basidiomycetous yeast *Tremella* and *Naganishia*. Among the bacteria, the most abundant genera were *Hymenobacter* (14%), *Armatimonas*

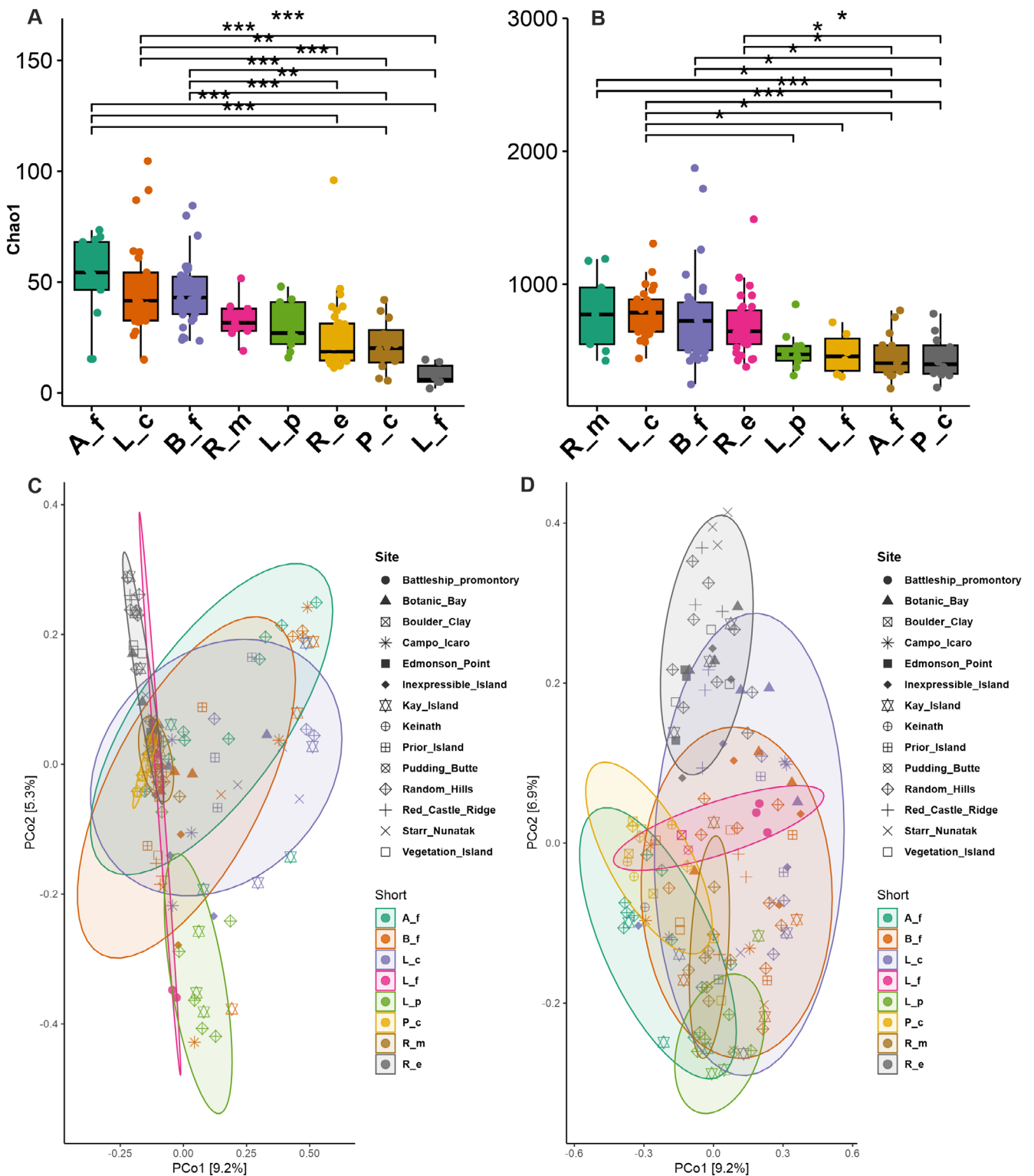


FIGURE 3 | (A, B) Alpha diversity and Chao1 index plots of fungal (A) and bacteria (B) communities. Statistical differences between groups, tested by Kruskal-Wallis test were marked with *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (C, D) Beta diversity and principal component analysis (PCoA) based on Bray-Curtis distance including ellipse of 95% confidence interval of fungal (C) and bacteria (D) communities. On the axes and legends lichen species names were abbreviated as follow: *Acarospora flavocordia* (A_f), *Buellia frigida* (B_f), *Lecidea cancriformis* (L_c), *Lecanora fuscobrunnea* (L_f), *Lecanora physciella* (L_p), *Pleopsidium chlorophanum* (P_c), *Rusavskia elegans* (R_e), *Rhizoplaca melanophthalma* (R_m).

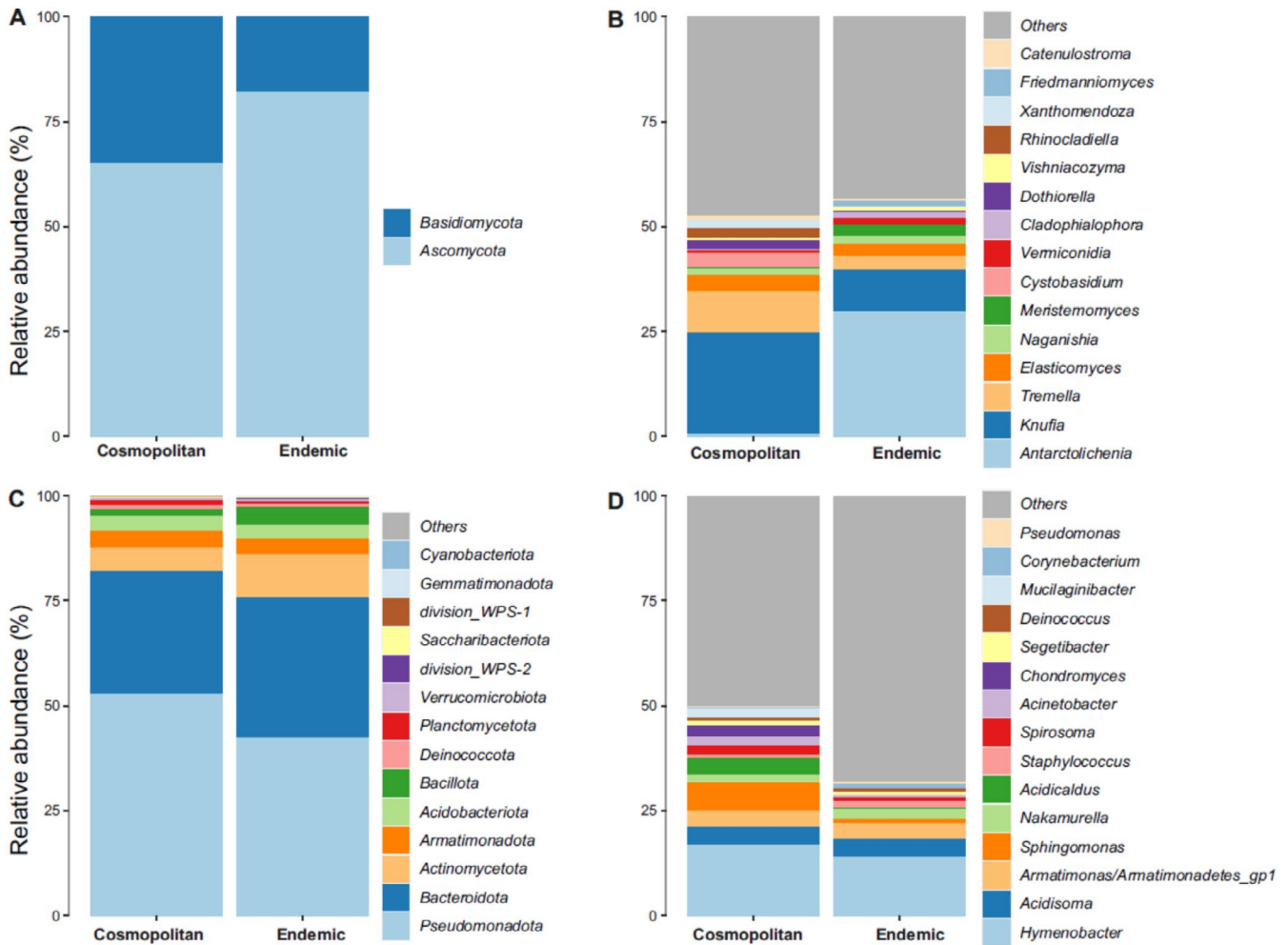


FIGURE 4 | Relative abundance plots according to lichen distribution, that is endemic versus cosmopolitan species: Composition of phyla (A) and genera (C) of fungi; composition of phyla (B) and genera (D) of bacteria. “Others” includes all the other genera and higher taxonomic ranks which were not among the 15 most frequently recovered ones.

(3.65%), *Acidisoma* (4.1%) and *Nakamurella* (2.65%) in endemic lichens, and *Hymenobacter* (17%), *Sphingomonas* (6.66%), *Acidisoma* (4.12%), *Acidicaldus* (4%) and *Armatimonas* (3.86%) in cosmopolitan lichens (Figure 4D).

3.5 | Cosmopolitan Versus Endemic Diversity

The Chao1 index indicated that the fungal and bacterial diversity values were higher for the endemic than for the cosmopolitan lichens (p value < 0.001; Figure 5A). The Wilcoxon test conducted on the analysis did not indicate a significant difference in alpha diversity between the two lichen groups regarding bacterial diversity (Figure 5B).

3.6 | Species Distribution

The effect size of the separation (LefSe) between the endemic and cosmopolitan fungal and bacterial diversity analyses showed taxa with the highest discriminatory power between the two groups of lichen species. The statistically significant results (LDA > 2, p

value < 0.001) were reported in Table S6. The fungal taxa with the highest segregative values were the members of *Arthoniomycetes*, *Lichenostigmatales*, and *Phaeococcomycetaceae* for the endemic lichens, whereas the species *Friedmanniomyces endoliticus* was for cosmopolitan lichens (Figure 6A). Regarding the bacterial diversity, the taxa with the highest segregative values were represented by the *Sphingobacteriales* for endemics and the *Sphingomonadales* for cosmopolitan lichens (Figure 6B).

4 | Discussion

In the present study, we have uncovered the diversity of fungi and bacteria associated with thalli of eight lichen species growing in Victoria Land, which themselves represent peculiar microniches and may act as cradles for the diversification of microbial life (Selbmann et al. 2010, 2013; Arnold et al. 2009; Santiago et al. 2015). While substantial progress has been made in investigating the diversity of fungi and bacteria inhabiting lichen thalli at boreal and arctic latitudes (Cardinale et al. 2006; Liba et al. 2006; Selbmann et al. 2010; Lee et al. 2014; Grube et al. 2009; Hodkinson and Lutzoni 2009; Bates et al. 2011;

Spribille et al. 2020; Timling et al. 2014; Klarenberg et al. 2020), so far there have been fewer possibilities to analyse the intrathallic diversity of lichens from Antarctica (Grimm et al. 2021).

4.1 | The Lichen Mycobiota: Composition and Diversity

In general, the lichen-associated fungal diversity in the investigated species predominantly comprised *Ascomycota* taxa, and these results are in line with previous studies which have either

analysed lichen-dominated endolithic communities (Selbmann et al. 2005, 2008; Egidi et al. 2014; Coleine et al. 2018a) or fruticose lichens growing on soils with different deglaciation times (Beck et al. 2023), as well as the diversity of moss-associated fungi in the ice-free coastal outcrops in Antarctica (Cox et al. 2016; Ji et al. 2016; Yung et al. 2014; Hirose et al. 2016). The prevalence of *Chaetothyriomycetes* and *Dothideomycetes* is another important outcome that supports the frequent occurrence of these groups of fungi in epilithic lichens from dry and cold habitats (Muggia et al. 2016; Muggia and Grube 2018; Cometto et al. 2023, 2024), and as common representatives in lichen-dominated endolithic

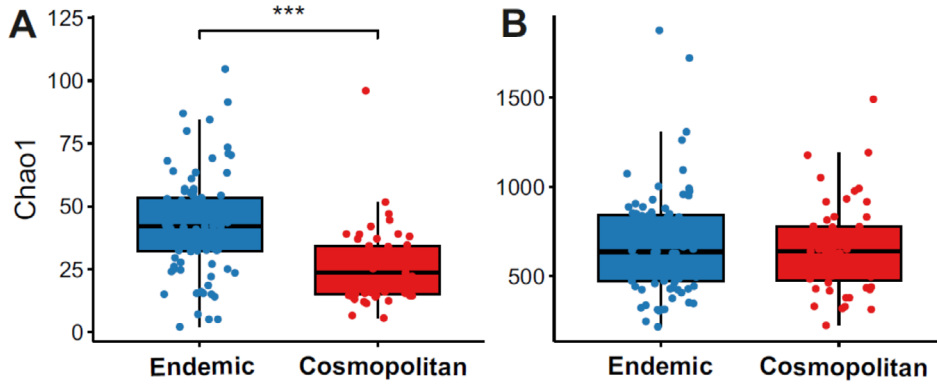


FIGURE 5 | Comparison of alpha diversity using the Chao1 index of fungal (A) and bacterial (B) diversity between endemic and cosmopolitan lichens. The statistical support was tested by the Wilcoxon test and is indicated as *** $p < 0.001$.

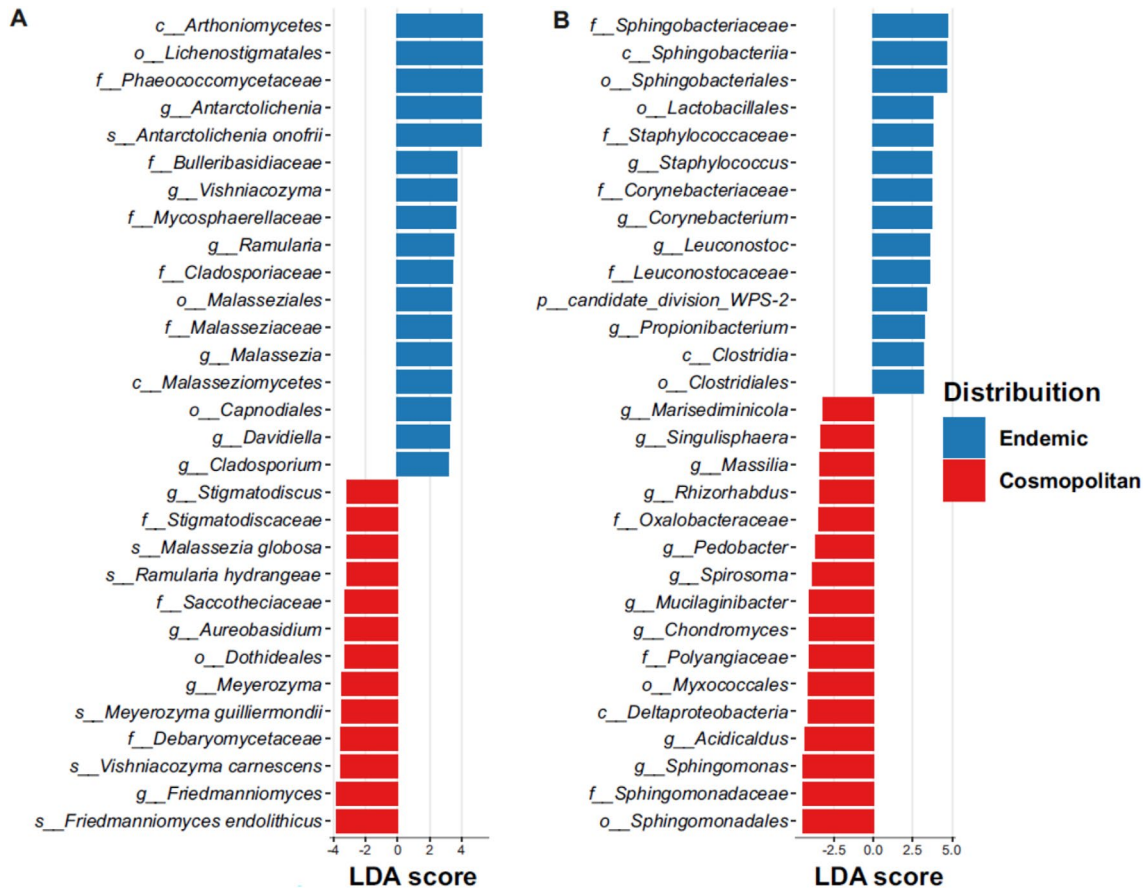


FIGURE 6 | LEfSe analysis between endemic and cosmopolitan lichens relative to the fungal (A) and bacterial diversity (B). The 30 taxa with the highest LDA score are shown.

communities (Selbmann et al. 2005, 2008; Egidi et al. 2014). *Knufia* (*Chaetothyriales*) is reported here as the most abundant genus present in the cosmopolitan lichens, characterised by its capacity to adapt to different ecologies, such as rock-inhabiting fungus in extremophilic conditions (Isola et al. 2016) or as a lichen-associated taxon (Untereiner et al. 2011; Cometto et al. 2023, 2024).

The ASVs analysis at the genus level revealed the presence of a recently described fungal species in the endemic type of distribution, *Antarctolichenia onofrii*, which was originally isolated multiple times from Antarctic rocks as a rock-dwelling fungus (Muggia et al. 2021). This species is a meristematic melanized fungus and was shown to be a peculiar representative of the Antarctic mycobiota. It also emerges here as part of the lichen mycobiota in Antarctica, as it has never been found elsewhere so far (i.e., from rock or lichen thalli from other geographic origins). *A. onofrii*, albeit clearly free-living, still seems to maintain a loose relation to and interdependence with microalgae, as it was isolated together with microalgae and it is not possible to propagate it in axenic culture for a long time (Muggia et al. 2021). Its constant occurrence in nature with lichens (both epilithic and endolithic) supports the hypothesis for a possible link between a free-living (rock associated) and symbiotic lifestyle.

Our findings, however, also unveil a significant presence of *Basidiomycota* in all examined thalli, even though their abundances were, in general, much lower than *Ascomycota*. Notably, only in the case of the lichen *P. chlorophanum* was a higher abundance of *Basidiomycota* detected. *Basidiomycota* were found to be particularly abundant in the Antarctic region only in two studies conducted in the waters of the Antarctic Peninsula (Garmendia et al. 2021) and in snow-ice near the Italian–French station Concordia, respectively (Stoppiello et al. 2023). In lichens from other regions of the world, *Basidiomycota* have also been shown to be quite ubiquitous but in much lower amounts than *Ascomycota*, and they rarely showed specificity for the host lichen (Smith et al. 2020; Lendemmer et al. 2019; Cometto et al. 2022). The three most abundant basidiomycetous yeast genera were recovered in both the Antarctic endemic and the cosmopolitan lichens, that is, *Tremella*, *Cystobasidium*, and *Naganishia*. These three genera are known to be extremophilic yeasts (Buzzini et al. 2018), which are even able to develop pathogenicity toward humans (Rush et al. 2023; Nizovoy et al. 2021). Environmental strains of *Tremella*, *Cystobasidium*, and *Naganishia* were also found in the two cosmopolitan lichens *T. atra* and *R. melanophthalma* collected across the other six continents (Cometto et al. 2022, 2024); however, only *R. melanophthalma* was included in the present study, as it was found in the sampling localities. The genus *Tremella* emerges here because it is a well-known lichen parasite (Millanes et al. 2011), which has been studied for its phenotypic traits on many host lichen thalli (Millanes et al. 2012, 2014, 2015). Here, we present an additional case of its occurrence as cryptic species within the thalli (Tuovinen et al. 2021), as no symptoms of its infection could be found on any of the analysed lichen specimens. Cometto et al. (2022, 2024) already reported *Tremella* as cryptically present in lichens and succeeded in isolating it in culture (Cometto et al. 2022), as we did for samples included in the dataset presented here (De Carolis et al. in preparation).

The high abundance of basidiomycetous yeasts compared to ascomycetous yeasts in Antarctic lichens could be due to the different organisation of the yeast cells. Indeed, the thicker cell wall and presence of capsules in basidiomycetous yeasts may represent an adaptive advantage in the Antarctic environment (Garmendia et al. 2021; Stoppiello et al. 2023), as well as their tendency to reside in the lichen thalli as resting cells/spores.

Eventually, species diversity analysis performed between the mycobiota of endemic and cosmopolitan lichens revealed that a few fungal genera that are endemic to Antarctica are also elements of only the endemic lichen mycobiota. One example is *Friedmanniomyces*, which has been frequently reported as a component of endolithic antarctic communities (Onofri et al. 1999).

4.2 | The Lichen-Associated Bacterial Communities: Composition and Diversity

Pseudomonadota, *Bacteroidota*, and *Actinomycetota* were recovered as the most abundant bacterial phyla. *Pseudomonadota* is in general widely distributed in different habitats on Earth (Nemergut et al. 2011) and in bioaerosols in the atmosphere (Fang et al. 2020; Tang et al. 2018), while *Bacteroidota* were also already found as predominant phyla among bacteria living in Antarctic lakes (Michaud et al. 2012). According to previous studies, it was found that heterotrophic *Pseudomonadota* and *Bacteroidota* coexist with Antarctic cyanobacteria (Cornet et al. 2018) in glaciers and cold deserts in Antarctica, for example in the King George and James Ross Islands, in the Arctic (Alaska and Greenland) and in Asia (Himalaya Mountains, Pamir, Then-Shan and Qilian; Segawa et al. 2017). *Actinomycetota* were isolated from Antarctic soils (Silva et al. 2020; Araujo et al. 2020).

Hymenobacter, *Acidisoma*, *Armatimona/Armatimonadetes*, and *Sphingomonas* were among the most present genera identified in this study, and they are also the most frequently isolated genera from East Antarctica (Lambrechts et al. 2019; Peeters et al. 2011; Benaud et al. 2022; Pudasaini et al. 2017). These bacteria are all presented as chemoheterotrophic, strictly aerobic, and UV-resistant. These findings are consistent with their ability to survive the harsh environmental conditions of the region. Members belonging to *Acidisoma* are among the most abundant, as also evidenced by previous studies of endolithic communities (Coleine et al. 2019). This genus has rarely been observed in the ice-free areas of Victoria Land but is common in cold regions such as the Alps (Nakai et al. 2013), suggesting that it may have evolved unique adaptations for cold resistance. *Acidisoma* species are acidophilic and psychrotolerant heterotrophic bacteria. They can use a wide range of organic substrates, particularly carbohydrates and amino acids (Belova et al. 2009). The predominant genus analysed was *Hymenobacter*, which is found in several Arctic coastal glaciers. Glaciers, indeed, have been identified as a possible vehicle for bacterial transport to downstream marine waters. Several taxa belonging to this group have been retrieved in the McMurdo Dry Valleys in Antarctica (Hirsch et al. 1998). In addition, it has also been identified in coastal ice samples in southern Shetland (Garcia-Lopez et al. 2021). These locations are all oligotrophic, which could suggest that the genus *Hymenobacter* owns the genetic setup to grow using a limited number of carbon

sources, such as some sugars, sugar alcohols, organic acids, and some amino acids (Marizcurrena et al. 2019). On the other hand, the chemoheterotrophic genus *Armatimonas* has not been reported in Antarctic areas but has been found in alpine glaciers (Tolotti et al. 2020). These bacteria are members of the phylum *Armatimonadetes*, which is still poorly studied. Many of the *Armatimonas* bacteria have a wide range of enzymes that can be activated at different temperatures, allowing them to use the available resources (Tamaki et al. 2011).

A taxonomic peculiarity concerns the phylum *Planctomycetota*, which includes bacterial species known for their ability to adapt to extreme environmental conditions, such as low temperatures, high UV radiation, and freeze–thaw cycles, which are common in Antarctic environments (Jiya et al. 2024; Bradley et al. 2023). During this study, they were found to be associated with *L. fusco-brunnea* thalli. The survival capabilities of the *Planctomycetota* bacteria under extreme conditions make this phylum an interesting subject of study for astrobiological research.

4.3 | Lichen Symbioses Select Their Own Fungal and Bacterial Diversity

The low Chao1 values for the mycobiota approached those found in the mycobiota of two lichen species collected worldwide in extreme, high altitudinal environments (*R. melanophthalma* and *T. atra*) by Cometto et al. (2024). On the other hand, Chao1 values were relatively high for the bacteria and showed extremely significant differences in the composition of the communities (e.g., Hodkinson et al. 2012; Bates et al. 2011; Cardinale et al. 2006) suggesting that a considerable proportion of the lichen-associated bacteria are specific to the lichen species. Indeed, the statistically significant differences that we found between the lichen fungal and bacterial diversity among the analysed species suggest that the lichen itself influences most of the diversity associated with the thalli. This is supported further by the PCoA analysis concerning beta-diversity, in which the lichen species mostly explained the community variation. Instead, the ‘site’ factor contributed less to the overall variation in the fungal and bacterial community, contrasting the results reported previously by Coleine et al. (2019). This observation may be explained by the fact that in the lichen symbioses, the mycobionts (more than the photobionts) shape, also in unique ways, the phenotype of the lichen thallus itself, with their morpho-anatomical structures and the production of secondary metabolites. However, our analyses showed that the investigated lichen species, although differing only slightly in their external phenotypes, most of them being crustose epilithic (with the exception of the fruticose *R. melanophthalma*, and the partially foliose *R. elegans*), and either Antarctic endemics or cosmopolitans, shared only part of their fungal and bacterial diversity. Lichens, thus, seem to act as environmental filters for microorganisms in general (Hodkinson et al. 2012; Cardinale et al. 2006; Almendras et al. 2018) but also in Antarctica. Interestingly, the influence of the lichen on the whole microbial diversity does not emerge when only different populations of a single or a few lichen species are compared (Cometto et al. 2024), or when only a few specimens would be investigated for a certain species. It seems, instead, that when a wide spectrum of species is collected in different localities, though under the same (or highly similar) environmental

conditions, the lichen identity, together with the key ecological traits of its thallus, lets emerge the intrathalline fungal and bacterial diversity.

This study clearly highlighted that some fungal and bacterial taxa are more predominant in cosmopolitan or endemic lichen species. There are only a few taxa, for example, *Antarctolichenia* or *Sphingobacteriales*, for which we could assume that their stronger association with endemic lichens would relate to the physical and/or biotic barriers that limit the dispersal of the endemic lichen species outside the continent. This would promote the independent evolution of isolated populations of the lichens themselves and their associated fungi and bacteria (Olden et al. 2004; Armstrong 2017). Regarding cosmopolitan lichens, it is observed that the species *Friedmanniomyces endolithicus* is the most associated with this type of distribution. *F. endolithicus* is exclusively associated with endolithic microbial communities in the ice-free areas of Victoria Land, including the McMurdo Dry Valleys, which are characterised by high UV irradiation, low temperatures, and strict oligotrophy. Among the black meristematic fungi in these communities, *F. endolithicus* is the most widespread and frequently isolated, further supporting its high degree of adaptation to the prohibitive environmental conditions of this area (Selbmann et al. 2015). It may be speculated that also inside the lichen thallus of the cosmopolitan lichens, *F. endolithicus* helps the symbionts in resisting UV irradiation as it does for endolithic communities. Prolonged isolation could be a structuring factor for the biogeography of microorganisms in Antarctica, and this could explain why only a few certain genera are detected as more abundant in endemic than in cosmopolitan lichens.

5 | Conclusions

This study reports in detail the comparison of fungal and bacterial diversity associated with lichen thalli in Antarctica, by considering either Antarctic endemic and cosmopolitan species. We found a higher biodiversity associated with endemic than with cosmopolitan lichen species, representing also some endemic microbial taxa. This could be due to physical or biotic barriers that limit the dispersal of species and thus promote the independent evolution of isolated microbial assemblages. Observing the data, we found two distinct groups within the endemic lichens: one characterised by low diversity and another by high diversity. This variation in diversity among endemic lichens could be attributed to their specific adaptations to the unique microhabitats and environmental conditions present in Antarctic ecosystems. Endemic lichens may have evolved distinct ecological strategies, leading to a greater variability in their microbial communities. In contrast, among cosmopolitan lichens, there is less variability in microbial diversity. This consistent pattern suggests that cosmopolitan lichens might possess more generalised microbial communities, possibly due to their ability to thrive in a wider range of environmental conditions and their broader geographical distribution. The association between fungi and bacteria provides a robust understanding of the intricate associations between these two microbial communities within the lichen thalli. The significant correlation underscores the potential interdependence and co-evolution of bacterial and fungal communities in these unique Antarctic ecosystems.

Author Contributions

Lucia Muggia: conceptualization, methodology, investigation, funding acquisition, project administration, writing – review and editing, writing – original draft, resources, formal analysis, supervision. **Gerardo A. Stoppiello:** methodology, data curation, investigation, visualization, writing – original draft, formal analysis. **Roberto De Carolis:** methodology, data curation, visualization, investigation, writing – original draft, formal analysis. **Claudia Coleine:** conceptualization, methodology, data curation, writing – original draft, formal analysis. **Mauro Tretiach:** conceptualization, supervision, writing – review and editing, resources. **Laura Selbmann:** conceptualization, methodology, investigation, funding acquisition, writing – original draft, writing – review and editing, project administration, resources, formal analysis, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data have been deposited in NCBI under the bioproject accession number PRJNA1042949. It is also available as Supporting Informations 1 and 2.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.