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SEQUENCIAMENTO POR ION TORRENT REVELA PADRÕES DE INTERAÇÃO E DISTRIBUIÇÃO DE COMUNIDADES MICROBIANAS EM UM PERFIL DE SOLO ORNITOGÊNICO DA ILHA SEYMOUR, PENÍNSULA ANTÁRTICA

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Dissertação apresentada ao programa de Pós-Graduação *Stricto Sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Luiz Fernando Wurdig Roesch

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RESUMO

Neste estudo, foram analisadas e comparadas comunidades bacterianas do solo de uma pinguineira da Ilha Seymour (Península Antártica) em termos de abundância, estrutura, diversidade e rede de interações, a fim de se identificar padrões de interação entre os vários grupos de bactérias presentes em solos ornitogênicos em diferentes profundidades (camadas). A análise das sequências revelou a presença de oito filos distribuídos em diferentes proporções entre as Camadas 1 (0-8 cm), 2 (20-25 cm) e 3 (35-40 cm). De acordo com os índices de diversidade, a Camada 3 apresentou os maiores valores de riqueza, diversidade e uniformidade quando comparado com as Camadas 1 e 2. Em termos de estrutura da comunidade microbiana, a análise UniFrac mostrou que as comunidades microbianas das três camadas foram muito diferentes umas das outras. A análise de redes revelou a existência de um padrão único de interações no qual a rede microbiana formou uma topologia de agrupamento, mas não estruturado em módulos, como de costume em comunidades biológicas. Da mesma forma, através da utilização de análise de redes, foi possível identificar táxons específicos como sendo potencialmente importantes para a estruturação e funcionamento da comunidade microbiana. Além disso, as análises de simulação indicaram que a perda de grupos importantes de microorganismos pode alterar significativamente os padrões de interação dentro da comunidade microbiana. Estes resultados fornecem novos *insights* sobre as interações bacterianas e ecologia microbiana desse importante, mas ameaçado ambiente.

Palavras-chave: Ion Torrent. co-ocorrência, análise de redes. comunidades microbianas. ecologia microbiana. 16S rRNA. Antártica.

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1. INTRODUÇÃO

1.1 Estudos envolvendo comunidades microbianas complexas

Um ecossistema é um sistema complexo em que várias espécies interagem umas com as outras formando redes complexas (Chapin et al., 2012). Através destas redes de interações, o ecossistema é capaz de realizar funções (como, por exemplo, a ciclagem de nutrientes e a estabilidade do ecossistema) que não seriam possíveis considerando-se apenas pequenos grupos de indivíduos (Chapin et al., 2012). Dessa forma, entender estas estruturas complexas é parte essencial da ecologia.

As pesquisas envolvendo comunidades microbianas complexas têm avançado consideravelmente nos últimos anos, principalmente devido ao desenvolvimento de novas metodologias, como o sequenciamento de DNA de alto rendimento que pode gerar uma quantidade sem precedentes de informação genética (MacLean et al., 2009). Uma grande variedade de técnicas pode ser utilizada para se analisar os dados de seqüência genética gerados pelo seqüenciamento de alto rendimento, a fim de descrever a composição das comunidades microbianas, a sua diversidade e como estas comunidades podem mudar ao longo do espaço, tempo ou tratamentos experimentais (Roh et al., 2010).

No entanto, a maior parte das técnicas utilizadas concentra-se em análises limitadas das comunidades em estudo. Por exemplo, estudos descrevendo e comparando a estrutura das comunidades microbianas geralmente focam no número de táxons encontrados em amostras individuais (isto é, alfa-diversidade), na abundância relativa de táxons individuais e na extensão da sobreposição filogenética ou taxonômica entre as comunidades (isto é, beta-diversidade) (Barberán et al., 2012). Medidas de alfa-diversidade (como a riqueza de espécies e curvas de rarefação) geram estimativas da diversidade microbiana e seus limites em ambientes diferentes. Da mesma forma, as análises estatísticas multivariadas possibilitam descrever padrões de beta-diversidade, revelando como as variáveis bióticas e abióticas controlam a composição da comunidade microbiana.

Por outro lado, pouca atenção tem sido dada à utilização de dados de seqüência genômicas para o estudo de interações entre táxons microbianos que

coexistem em amostras ambientais (Chaffron et al., 2010). Estes tipos de estudos associados com as novas tecnologias de seqüenciamento de alto rendimento permitem explorar aspectos da ecologia microbiana que vão além das análises rotineiras de padrões de alfa e beta diversidade.

Ferramentas de análise de rede têm sido amplamente utilizadas por biólogos, matemáticos, sociólogos e cientistas da computação para explorar as interações entre pontos, sejam esses pontos indivíduos em uma comunidade, espécies de uma cadeia alimentar, nós em uma rede de computadores, ou proteínas em vias metabólicas (Junker & Schreiber, 2008). Estas análises de redes são usadas para explorar propriedades estatísticas e estruturais de um conjunto de pontos (nós) e as conexões entre eles. Se aplicadas corretamente em estudos de ecologia microbiana, estas novas metodologias podem fornecer uma nova visão sobre a estrutura e interação de comunidades complexas. Tais informações são particularmente valiosas em ambientes onde a ecologia básica de muitos táxons microbianos permanece desconhecida, como a Antártica.

1.2. Ilha Seymour, Península Antártica

A ilha Seymour (Marambio) está localizada na porção setentrional do Mar de Weddell, entre as coordenadas geográficas 56°43’ (longitude oeste) e 64°14’ (latitude sul), sendo parte do denominado grupo de ilhas de James Ross. Está situada a 100 km a sudeste do extremo norte da Península Antártica, no Mar de Weddell.

O clima da ilha é subpolar, semiárido, com temperaturas médias anuais que oscilam entre -5°C e -10°C (Souza, 2011). Sob o ponto de vista morfodinâmico, a ilha se encontra em ambiente periglacial, sujeita, portanto, a processos ligados ao congelamento e descongelamento (Souza, 2011). Durante o inverno, o solo fica coberto de neve e congelado até a superfície, porém, durante o verão a superfície do terreno se funde, constituindo a camada ativa. Geralmente este descongelamento estacional começa em novembro, coincidindo com o derretimento das acumulações de neve do inverno, estendendo-se até o final de fevereiro.

A superfície da ilha Seymour é quase completamente desprovida de vegetação e apresenta baixos níveis de pedogênese e intemperismo (Michel et al.,

2006), o que sugere que as comunidades microbianas desenvolvidas neste ambiente são mais estáveis do que em outros locais, onde a crioturbação é comum. Dentre os tipos de solos encontrados na Ilha, encontram-se os solos ornitogênicos, que são descritos a seguir.

1.3 Caracterização dos solos ornitogênicos da Península Antártica

Em geral, os solos no continente Antártico são descritos como pouco desenvolvidos e pobres em relação aos nutrientes, o que aliado às condições climáticas, agrava as condições para sobrevivência de plantas e habitats para organismos (Ugolinia e Bockheimb, 2008; Bölter, 2011). Porém, a zona costeira da Antártica com áreas de degelo apresenta formação de solos ornitogênicos (Simas et al., 2007), originados a partir do acúmulo de excrementos de aves, principalmente pinguins. Estes, por sua vez, são ricos em material orgânico de fácil decomposição, podendo apresentar de 10 a 100 vezes maiores teores de carbono orgânico e nitrogênio que os solos não ornitogênicos (Michel et al., 2006; Aislabie et al., 2008).

Estas condições peculiares envolvendo riqueza de material orgânico de fácil decomposição associadas a um ambiente estável (i.e. baixos níveis de pedogênese e intemperismo) tornam os ecossistemas dos solos ornitogênicos da Ilha Seymour modelos adequados para se estudar aspectos relacionados à ecologia microbiana.

2. OBJETIVO

Neste contexto, o objetivo deste trabalho foi identificar e analisar as comunidades bacterianas do solo de uma pinguineira encontrada na Ilha Seymour (Península Antártica) em termos de abundância, estrutura, diversidade e interações de rede, a fim de identificar padrões entre os vários grupos de bactérias presentes em solos ornitogênicos.

3. APRESENTAÇÃO DO MANUSCRITO

O presente trabalho será apresentado na forma de artigo, com formatação conforme as normas da revista The ISME Journal (ISSN: 1751-7362). O manuscrito está dividido em Introdução, Material e métodos, Resultados, Discussão e Conclusões.

4. MANUSCRITO

Post-light based sequencing technology reveals distribution and interaction patterns of bacterial communities in an ornithogenic soil profile of Seymour Island, Antarctic Peninsula

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Abstract

In this study, we analyzed and compared the soil bacterial communities from a penguin rookery site at Seymour Island (Antarctic Peninsula) in terms of abundance, structure, diversity and interaction network in order to identify interaction patterns among the various groups of bacteria presented in an ornithogenic site at three depths (layers). The analysis of the sequences revealed the presence of 8 phyla distributed in different proportions among the Layers 1 (0-8 cm), 2 (20-25 cm) and 3 (35-40 cm). According to the diversity indexes, Layer 3 presented the highest values of richness, diversity and evenness when compared to Layers 1 and 2. In terms of bacterial community structure, the unweighted and weighted UniFrac analysis showed that the soil bacterial communities from the three layers were quite different from each other. Network analysis revealed the existence of a unique pattern of interactions in which the soil microbial network formed a clustered topology, but not structured in modules, as usual in biological communities. In addition, through the use of network analysis, it was possible to identify specific taxa as potentially important for the structuring and functioning of the microbial community. Furthermore, simulation analyzes indicated that the loss of potential keystone groups of microorganisms may significantly alter the patterns of interactions within the microbial communityThese findings provide new insights into the bacterial interactions and microbial ecology of this important, but threatened environment.

Keywords: Post-Light™ Ion Semiconductor Sequencing; co-occurrence; network analysis; bacterial communities; microbial ecology; 16S rRNA gene; microbiome; Antarctica.

Introduction

The advent of high-throughput sequencing has allowed an unprecedented accurate description of microbial communities, opening a new era in microbiology. These advanced new techniques have been used intensively during recent years to improve our understanding of how these communities assemble, evolve, and function (Roesch et al., 2007; MacLean et al., 2009; Logares et al., 2012). Most of the studies so far have focused on the abundance and structure of microbial communities to access their diversity across different environments (Lauber et al., 2009; Nacke et al., 2011). Nevertheless, biodiversity includes not only the number of species and their abundance but also the complex interactions among different species. In each environment, various species interact with each other, forming complex ecological networks. Through such network interactions, the ecosystem is capable of perform systems level functions (e.g., nutrient cycling, ecosystem stability) which could not be achieved by individual populations (Zhou et al., 2010; Faust & Raes, 2012). For this reason, exploring such ecological networks is essential to our better understanding of microbial ecology.

Exploring the direct or indirect interactions between microbial taxa that coexist in environmental samples is a significant challenge due the vast diversity and the uncultivated status of most microbial species (Delmont et al., 2011), especially in soils, considered the most complex and diverse environment on Earth (Lombard et al., 2011; Rampelotto et al., 2013). Furthermore, quite detailed information on the microbial taxa present in the samples under study is essential to determine whether or not the interaction patterns found are statistically significant (Deng et al., 2012). In this context, the massive amount of high quality data generated by the Ion Torrent technology offers an unprecedented opportunity to examine network interactions among different microbial species. The association of this new high-throughput sequencing methodology with new analytical techniques may shed new lights in our understanding of microbial ecology and environmental microbiology.

Such information is particularly valuable in Antarctica, where the basic ecology and evolution of most microbial taxa remain unknown. Furthermore, Antarctica provides the best models of ecosystems to study microbial ecology due to the low complexity of its microbial network in comparison with other ecosystems and due to

reduced influence of humans, plants and animals (other than penguins) (Rampelotto, 2014). Using Antarctica as a simple ecosystem model with low external influence, we can better analyze how various microbial taxa coexist and interact with each other and determine the overall patterns of soil microbial diversity. Nevertheless, despite these advantages, the Antarctic soils are usually poor in organic material and present low levels of weathering and leaching (Ugolini & Bockheim, 2008), which difficult the development of microbial communities. Ornithogenic soils are of particular interest because they present constant incoming of organic and biological material due to the penguins nesting activity. Indeed, ornithogenic sites constitute the most important reservoirs of organic carbon in antarctic terrestrial ecosystems (Simas et al., 2007). Recent studies indicate that the bacterial community in penguin guano is not only one of the richest in Antarctica, but is extremely diverse, both phylogenetically and morpho-physiologically (Aislabie et al., 2007; Kim et al., 2012).

In this work, samples from a rookery site at different depths were collected in order to identify and analyze the soil bacterial communities from a penguin rookery site at Seymour Island (Antarctic Peninsula) in terms of abundance, structure, diversity and network interactions in order to identify patterns among the various groups of bacteria presented in ornithogenic sites. For this purpose, Ion Torrent PGM sequencing of 16S rRNA genes was used.

Material and methods

Site description, soil sampling and physicochemical analysis

A penguin rookery site (64°17′37″ S, 51°49′48″, 8m a.s.l.) at Seymour Island, an ice−free island located near the northern tip of the Antarctic Peninsula, was selected due its ideal characteristics for our study: constant incoming of organic/biological material and lack of vegetative cover. The climate of Seymour is subpolar-semiarid, with an annual mean temperature between -5°C and -10°C. Soil texture is sandy loam and classified as Ornithic-Oxyaquic Cryosol (Soil Taxonomy, USA) or Ornithic-Salic Leptosol (WRB/FAO). The samples were collected using a clean sterilized stainless steel scoop from the single penguin rookery site at three depths: Layer 1 (0-8 cm, actively penguin-colonized soil), Layer 2 (20-25 cm, at the

beginning of the old rookery site), and Layer 3 (35-40 cm, at the beginning of the old beach). Sampling strategy was described in Souza, 2011. All soil samples were placed into sterile plastic bags and stored in ice chests upon collection and transported to the laboratory for DNA extraction and physicochemical characterization. The physicochemical properties (Table 1) were determined as described in Souza, 2011.

DNA extraction, short amplicon libraries preparation and sequencing

Microbial DNA was extracted from 1 gram of each soil sample with the PowerSoil® DNA Kit (MoBio, USA) according to the manufacturer's instructions. DNA concentrations were determined using NanoVue™spectrophotometer (GE Healthcare, USA) and all DNA samples were stored at -20°C. Three independent PCR reactions (technical replicates) were performed for each of the three soil samples collected in each layer (biological replicates) with the primers 917F and 1046R for the amplification of 129 base pairs of the V6 region of the 16S rRNA gene. PCR was performed with the High Fidelity PCR Enzyme Mix (Thermo Scientific, USA). The mixtures contained 5µl of 10X high fidelity PCR buffer with 15 mM $MgCl_2$, 0.2 mM of each dNTPs, 100 mM of each primer, 2.5 U of high fidelity PCR enzyme mix and approximately 100 ng of DNA template in a final volume of 50 µl. The PCR conditions were 94°C for 2 minutes, 25 cycles of 94°C for 45s; 56°C for 30s; and 72°C for 30s extension; followed by 72°C for 4 minutes.

Prior to Ion Torrent PGM sequencing, the short amplicon libraries were processed in order to add the barcoded adaptors A and P1 necessary for sequencing. The adaptors were added to the amplicons using the Ion Plus Fragment Library Kit and the Ion Xpress™ Barcode Adapters (Life Technologies, USA). The reactions were performed based on the user bulletin MAN0006846 revision 3.0 available at http://ioncommunity.lifetechnologies.com with minor modifications during the amplicon purification step as follow: the bead suspension with the DNA was incubated with the Agencourt® AMPure® XP Reagent (Beckman Coulter, USA) (2x sample volume) at room temperature for 10 min. and all washing steps were performed with 500 µl of freshly prepared 80% ethanol during 30s. All the other steps for preparing short amplicon libraries (end-repair, barcoded adaptors ligation and nik-

repair) were performed according to the user bulletin mentioned above. The following barcodes TTCCGATAAC, TGAGCGGAAC, CTGACCGAAC, TCCTCGAATC, TAGGTGGTTC, TCTAACGGAC, TTGGAGTGTC, TCTAGAGGTC and TCTGGATGAC were added to the short amplicons from soil samples 008, 009, 010 (0 - 8 cm deep), 011, 012, 013 (20 - 22 cm deep) and 014, 015, 016 (35 - 40 cm deep) respectively.

All barcoded amplicons were quantified by quantitative real-time PCR using Ion Library Quantitation Kit (following the user guide Ion Library Quantitation Kit TaqMan® assay quantitation of Ion Torrent libraries Publication Part Number 4468986 Rev. A) and the Applied Biosystems® 7500 Fast Real-Time PCR System according to the manufacturer's instructions. The samples were adjusted to 15 x 10^6 molecules per microliter and mixed in equal amounts to obtain an equimolar pool of amplicons that was used for template amplification onto Ion Sphere™ Particles (ISPs).

The template-positive ISPs containing clonally amplified 16S rRNA genes were prepared with the Ion OneTouchTM System using the Ion OneTouchTM 200 Template Kit v2 following the user guide Publication Number 4478372 Revision B (available at http://ioncommunity.lifetechnologies.com). The resulting ISPs were sequenced on Ion 316TM micro-chip using the Ion Torrent Personal Genome Machine (Life Technologies, USA) and the Ion PGMTM 200 Sequencing Kit following the workflow from the user guide Part Number 4474246. After sequencing, the sequence reads were filtered within the PGM software that removed low quality and polyclonal sequences. All PGM filtered data were exported as FastQ file that was used for the subsequent bioinformatic analysis.

16S rRNA reads processing for downstream analyses

A fundamental problem with the use of next generation sequencing for single read analysis is the number of artifacts that might exacerbate biases via the presence of chimeric sequences and sequence errors (Schloss et al., 2011). In attempt to reduce the sequencing error we applied a stringent quality-filtering to improve our downstream analysis. The FastQ file exported from the Ion PGMTM System was processed using Mothur v.1.30.2 (Schloss et al., 2009). The multiplexed

reads were first filtered for quality and assigned to the starting soil samples. The filtering criteria removed any sequence where the longest homopolymer was greater than 8 nucleotides, that contained any ambiguous base call, that had more than one mismatch to the barcode sequence and that were smaller than 100 bases in length . Also, the sequences were trimmed by using a moving window that was 50 bases long and requiring that the average quality score over the region not drop below 25. Following this first step, the dataset was simplified by obtaining a non-redundant set of sequences that were further aligned against the SILVA reference alignment (http://www.arb-silva.de/). As we added the barcodes after the PCR reaction we expect to obtain sequences in both directions (forward and reverse) than we applied the flip parameter, that reverse complement sequences when 50% of the bases are removed in the alignment, to produce a better alignment and keep all sequences in the same direction. To maximize the number of sequences that overlap over the longest span, any sequence that starts after the position that 85% of the sequences do, or ends before the position that 85% of the sequences do were removed from the alignment. The alignment was then trimmed since we need they overlap in the same alignment space. This step allowed us to compare only those sequences that overlap the same region. Finally, to reduce sequencing noise a pre-clustering step (Huse et al., 2010) was applied and the chimeric sequences were checked by using chimera.slayer command.

Alpha and Beta diversity analysis

To compare the (di) similarity between bacterial communities from the soil samples we estimated the community diversity, community evenness and sequence coverage at 97% similarity cutoff for Operational Taxonomic Unit (OTU) definition. The alpha calculators were implemented in Mothur v.1.30.2 (Schloss et al., 2009) and according to the recommendations of Lemos et al. (2011) all calculations were performed using a subsample of 7,336 sequences (the size of the smallest library). Briefly, the high quality sequences obtained after database processing were used to generate a distance matrix by calculating the uncorrected pairwise distances between aligned DNA sequences. The distances were used to assign the sequences to OTUs at 97% similarity cutoff using the cluster command with the average

neighbor algorithm. Finally the output was used for building a table with the OTU abundance of each sample and these abundances were used to calculate the alpha diversity estimators.

Beta diversity was analyzed by using Principal Coordinates Analysis (PCoA). The calculations were performed within the QIIME pipeline (Caporaso et al., 2010). A matrix using the UniFrac metric (weighted and unweighted) for each pair of environments was calculated. The distances were turned into points in space with the number of dimensions one less than the number of samples. The first three principal dimensions were used to plot a three-dimensional graph that was visualized using KING (Chen et al., 2009). To test whether the results were robust to sample size we used a sequence-jackknifing technique in which the PCoA clusters were regenerated using a subset of 5,000 sequences (corresponding to about 70% of the total number of sequences obtained in the sample with the smaller number of sequences) randomly selected from each soil for 100 replicate trials. In addition, to see which taxa were more prevalent in different areas of the PCoA plot, the ten most abundant class-level taxa were added to the PCoA plots.

Interaction network analysis

The interaction network analysis was performed at 97% similarity cutoff for Operational Taxonomic Unit (OTU) definition. The analysis at the order level reduced the network complexity and allowed the visualization of the microbial interactions (positive and/or the negative). To test for co-occurrence patterns we first detected the taxon that were present in all nine samples and removed the poorly represented OTUs (OTUs with less than five sequences). For network inference, we calculated all possible Spearman's rank correlations between shared OTUs. Co-occurrence was considered robust when the Spearman's correlation coefficient was both > 0.8 and statistically significant (p-value < 0.05). All statistical analyses were carried out using Mothur v.1.30.2 (Schloss et al., 2009). The network was explored and visualized with the interactive platform gephi (Bastian et al., 2009). To determine whether our webs were not random networks and really represented the actual bacterial interactions in soil, we compared random networks of equal size (same number of nodes and edges) to the networks obtained by this study. The random networks were calculated

by the Erdös–Rényi model (G(n,m)) using the Complex Generator plugin from Gephi (Bastian et al., 2009). This approach is based on using a fixed number of links to connect randomly chosen nodes and serves as point of reference against which our real biological networks might be compared (Vick-Majors et al., 2014).

Results

Soil Physicochemical Properties

The physicochemical properties of soil from the three layers are presented in Table 1. Overall, significant differences were observed in Layer 1 and 2 when compared with Layer 3. Layer 1 and 2 presented high levels of P, K, Na, and Mg, but low concentration of Ca. These layers also presented high acidity potential (H + Al), from the organic matter in the surface/sub-surface, as indicated by the high levels of soil organic carbon. Furthermore, the neutral pH observed indicates that the organic materials deposited by penguins are poorly decomposed, which have not released yet much acidity to the environment, as a result of the dry weather. Recent deposits of guano usually have neutral to alkaline pH, becoming progressively more acidic with the microbial transformation of these materials, which release strong acids in the soil as nitric acid and sulfuric acid.

Composition and distribution of bacterial communities

The analysis of the sequences derived from the 9 libraries (three for each layer) revealed the presence of eight phyla (Table 2). The dominant phyla within the samples were Actinobacteria (29.5±6.8 %), Proteobacteria (25.3±14.2 %), Firmicutes (16.3±6.7 %) and Bacteriodetes (11.7±5.6 %). The four phyla presented in relative low abundance were Verrucomicrobia (0.3±0.3 %), TM7 (<0.1±0.0 %), Fusobacteria(<0.1±0.0 %) and Aquificae (<0.1±0.0 %). Interestingly, significant differences in the distribution of these phyla among the three layers were observed. Actinobacteria, the most abundant phylum, was found in high concentration at the surface layer (38.5±0.4 %), when compared to Layer 2 and Layer 3 (25.2±2.2 % and

24.9±0.9 %, respectively). Actinomycetales, was the most abundant Order within this phylum. On the contrary, Proteobacteria, the second most abundant phylum, was found in low concentration at the surface layer (7.6±1.6 %), but in significant abundance at layer 2 (39.8±1.6 %). Gammaproteobacteria was the dominant group within this phylum. Firmicutes presented high concentration at the surface layer (23.7±5.8 %) and low concentration at layer 3 (10.6±1.5 %); and the phylum Bacteroidetes, specially the Order Flavobacteriales, presented high concentration at the surface layer (17.8±4.2 %) and low concentration at layer 2 (6.3±2.1 %). Approximately 16.7±5.5% of the sequences remained unclassified (unclassified bacteria) however, they were more abundant in the Layer 3 (23.8±2.3 %) compared to the surface layer and layer 2 (12.4±1.0 % and 13.9±0.8 %, respectively).

Diversity and structure of bacterial communities

To analyze how well each sample was representative of the bacterial community in the environment, sequence coverage was calculated (Table 3). Even in the sample with the lowest number of sequences (i.e. 7,336 sequences), it was possible to achieve more than 93% coverage using a grouping criteria of 3% dissimilarity. The datasets analyzed presented reasonable coverage indicating that most of the OTUs were detected, therefore the following analyses were considered to be sensitive enough to be biologically meaningful.

The diversity indexes at 3% dissimilarity cutoff indicate that the samples presented different degrees of richness, diversity and evenness (Table 3). Layer 3 presented the highest values of richness, diversity and evenness when compared to Layers 1 and 2. In terms of bacterial community structure, the unweighted and weighted UniFrac analysis showed that the soil bacterial communities from the three layers were quite different from each other (Figure 1). We find that relatively low variation (52.0%) was explained by the first three axes with Jackknifed unweighted PCoA. On the other hand, the first three axis of the weighted Jackknifed PCoA accounted for 93.7% of the variation, indicating that the overall differences between the clusters were more related to the abundance of specific OTUs than to their presence or absence. According to the PCoA analysis, Lactobacillales (Firmicutes), Desulfuromonadales (Proteobacteria) and Fusobacteriales (Fusobacteria) were the

main groups responsible for the specificity of Layer 1. On the other hand, Solirubrobacterales (Actinobacteria), Xanthomonadales and Enterobacteriales (Proteobacteria) were the main groups responsible for the specificity of Layer 3. These results are in agreement with those presented in Table 4 for generalists/specialists. For both unweighted and weighted PCoA, Layers 2 and 3 were more associated to each other than Layer 1.

In the subsequent analysis, the OTUs at the Order level represented by more than one sequence in the set of taxa were divided in three general categories: specialists (i.e. bacterial taxa ~10 times more abundant in one of the three layers), mid-specialists (i.e. bacterial taxa ~3-4 times more abundant in one of the three layers) and generalists (bacterial taxa broadly distributed in the three layers). Under this criterion, 6 groups were classified as specialists, 4 as mid-specialists, and 14 as generalists (Table 4). Among the specialists, 3 groups (Lactobacillales (Firmicutes), Fusobacteriales (Fusobacteria) and Desulfuromonadales (Deltaproteobacteria)) were abundant in the superficial layer and 3 groups (Solirubrobacterales (Actinobacteria), Enterobacteriales and Xanthomonadales (Gammaproteobacteria)) in layer 3. Some groups of Actinobacteria and Gammaproteobacteria presented a high number of interactions, i.e. more than 8 (Table 4). Some groups of low abundance presented a significant number of interactions, i.e. above the means [e.g. Aquificales (Aquificae) and Pseudomonadales (Gammaproteobacteria)].

Interaction network *analysis*

In order to identify interaction patterns among the various groups of bacteria presented in ornithogenic sites, the network analysis was performed at the 3% dissimilarity cutoff. The resulting soil microbial network (Figure 2) consisted of 78 nodes (OTUs, TableS2), represented by sequences presented in the three layers, and 708 edges (with a mean of 10.4 edges per node). The clustering coefficient (that is, how nodes are embedded in their neighborhood and, thus, the degree to which they tend to cluster together) was 0.372 and the modularity index was 0.375 (values >0.4 suggest that the network has a modular structure). To verify if our microbial network was not random, we contracted an identically sized random network (Table S1) for each analysis performed. As it is possible to observe, the values of both

networks were quite different, which ratify the validity of our microbial network. Based on these results, it is possible to infer that the soil microbial network was comprised of highly connected OTUs, forming a clustered topology, but not structured among densely connected groups of nodes (i.e. modules). From the 708 interactions, 421 (59.5%) were positive and 287 (40.5%) were negative.

Aiming to identify different interaction patterns when the microbial community is disrupted by the loss of species (in this case, OTUs), we explored the resulting soil microbial network in more details. Firstly, we tried to identify which OTUs would be the most important ones, representing keystone species. For that, 4 parameters generally used to identify important nodes in network studies (representing key OTUs in our study) were selected: Closeness Centrality (CC), Betweenness Centrality (BC), Strongly-Connected ID (SC-ID), and Eigenvector Centrality (EC). As presented in table 5, the results (ranking OTUs) were different for each parameter, which was expected considering that they were calculated in different ways. Betweenness Centrality and Eigenvector Centrality were the parameters that presented significant values for the first 5 OTUs, which indicates that these OTUs may represent keystone species. For this reason, BC and EC were selected for subsequent analysis.

To study the influence of the potential keystone species over the microbial network, the first 5 OTUs ranked by BC and EC were removed one by one and the network parameters Average Degree, Graph Density, Clustering Coefficient and Modularity Index were recalculated. None of the removed OTUs by themselves were able to cause changes in the network parameters (data not shown), which indicate that, although these OTUs may be important (and even considered keystone species) within the microbial community in terms of interactions with other OTUs, if separately removed, they don’t alter the general pattern of the microbial network.

Our next step was to remove the OTUs in groups of 5 (until 20 OTUs, which represents ~25% of the microbial community) and recalculate the network parameters. The simulations represent a scenario of disturbance in the microbial community, caused for example by climatic changes or human activity, with the consequent loss of species. The results are presented in Table 6.

The first analysis was performed with the first 5 OTUs ranked by BC and EC removed altogether. For Average Degree and Graph Density, the three parameters were lower than the observed in the total network. Furthermore, the values of BC and EC were lower than RM (especially for EC). For Clustering Coefficient, although the

value of BC was different in comparison with EC, RM and the Total Network, the result was not significant considering its tendency (ranging from 0.375 to 0.386), which presented values similar to the Total Network. For Modularity Index, only EC presented significant difference when compared to BC, RM and the Total Network. Altogether, these results indicate that EC was better than BC to analyze the influence of potential keystone species in the microbial community.

In the subsequent analysis, the OTUs were removed in groups of 10, 15 and 20, following the ranking presented in Table 5, and the tendency of the network parameters were analyzed (Table 6). For Average Degree, while RM did not present differences, EC and BC presented a similar tendency of decrease. For Graph Density, while RM presented a tendency of increase, EC and BC presented a tendency of decrease. For Clustering Coefficient, while BC and RM did not present differences, EC presented a tendency of decrease. For Modularity Index, while RM presented a tendency of decrease, EC and BC presented a tendency of increase. The total number of interactions for EC and BC decrease significantly when compared with RM. In terms of percentage of positive/negative interactions, the percentage remained constant for BC (60.8±1.5%) and RM (61.8±0.7%), while for EC there was a tendency of increase (from 63.0% to 70.1%). These results indicate that EC was better than BC to analyze the lost of species in the microbial community.

Discussion

In general, our study reported the differences and similarities among soil bacterial communities in three different depths of a penguin rockery site at Seymour Island (Antarctic Peninsula) using Ion Torrent PGM sequencing of 16S rRNA genes. Through network analysis, we analyzed in more details how the core microorganisms, presented in the three layers, may be interacting with each other. The experimental design of this kind of work involving metagenomics studies is difficult due to the complexity of soil matrix and its physicochemical properties (Delmont et al., 2011; Lombard et al., 2011), especially considering soils of an extreme environment. These particular properties and additional methodological challenges confer several critical issues, which were carefully taken into account

during the development of this work. Considering the pioneer nature of our work, special attention was paid to the sampling site and the choice of primers.

An ornithogenic soil was ideal for our purpose because it presents constant incoming of organic and biological material due to the penguins nesting activity; and soils of Seymour Island (Antarctic Peninsula) are of particular interest due the absence of plants and low levels of pedogenesis and intemperism (e.g. leaching impediment imposed by the dry climate) (Feldmann & Woodbume, 1988), which indicate that the microbial communities developed under this environment are more stable than in other locals, where cryoturbation is common. These conditions make the ornithogenic soil ecosystem of Seymour Island a highly tractable model for resolving the roles of abiotic factors in structuring soil microbiota.

The choice of primers is still one of the most critical steps for metagenomics analysis. The use of inappropriate primers can compromise the interpretation of the PCR results, and consequently, lead to questionable biological conclusions (Sipos et al., 2007; Frank et al., 2008). In order to compare the results of different studies, research groups have tended to use the same primers. In several studies of microbial communities, primers 967F and 1046R have been used to amplify the hypervariable V6 region of 16S rRNA to avoid the bias caused by primer selection as well as possible selection of artifacts due to biases in amplifying longer PCR products (Quince et al., 2009; Huse et al., 2010). The V6 region is a common choice for this kind of analysis as it has been proven to yield results similar than those obtained by the whole 16S sequences (Huse et al., 2008; Youssef et al., 2009; Schloss, 2010), and is a sensible choice for short-length sequencing approaches (Degnan & Ochman, 2012). In our study, we focused on this short hypervariable region of 16S rRNA and analyzed a large number of PCR amplicons using the Ion Torrent (PGM) Platform. This sequencing approach provides a much better breadth and depth of sampling.

In addition, using the advanced post-light based sequencing technology and the subsequent use of bioinformatics filtering and processing data analysis, we were able to obtain high quality results with low variation among replicates from each sample. With high coverage (i.e. more than 93% using a grouping criteria of 3% dissimilarity), the analyses performed in this study were adequate and sensitive enough to be biologically meaningful.

Our deep sequencing analysis revealed that only four phyla were present in high abundance and most of the identified microorganisms were members of the Actinobacteria and Proteobacteria phyla, which together constituted approximately 55% of the total community. Conversely, relatively few sequences were associated with Verrucomicrobia, Fusobacteria, Aquificae, and TM7, which together contributed with less than 1% to the total community. Therefore, at the phylum level, the community composition of ornithogenic soils at Seymour Island (Antarctic Peninsula) seems to be less diverse than other regions of the globe, despite the constant input of organic material. Nevertheless, the phylum distribution we found in the bacterial communities was similar to other in the Antarctic Peninsula. Yergeau et al. (2007) studied bacterial communities across a latitudinal gradient in the maritime Antarctica and found that Proteobacteria and Actinobacteria were the dominant phyla in their 16S rRNA clone libraries, while members of Bacteroidetes and Firmicutes were generally less abundant.

The dominance of Actinobacteria in Layer 1 may be explained by the high resistance to adverse conditions (e.g. UV radiation, heat and desiccation) presented by members of this phylum (Le Blanc et al., 2008; Essoussi et al., 2011). Furthermore, due to the production of antibiotics and other secondary metabolites by several groups of Actinobacteria (Mahajan & Balachandran, 2012), they could be able to exclude other bacteria by releasing chemical compounds. Among all taxa of this study, Actinomycetales (Actinobacteria) was the most abundant in the three layers. Considering the relevance of this group in the decomposition of organic matter (Ventura et al., 2007), this result has significant implication on the carbon cycle in ornithogenic soils of Antarctica. Bacteroidetes and Firmicutes also presented a similar tendency of decrease in abundance with depth. Some lines of evidence suggest that soil Bacteroidetes are typically copiotrophic and are most abundant in soils that have relatively large amounts of organic carbon (Acosta-Martínez et al., 2008). Thus, the higher abundance of Bacteroidetes in the surface layer may be associated with their notable ability in exploiting organic matter. On the other hand, the higher abundances of Firmicutes in the surface layer could be related to the high resistance presented by members of this phylum that are known to form spores (e.g., *Bacillus* and *Clostridium*) (Rampelotto et al., 2013). Changes in the relative abundance of Proteobacteria with depth were particularly striking, as this phylum was the less abundant among the dominant groups in the surface layer while the most

abundant in deeper depths. This opposite general tendency in comparison to the other phyla suggests that Proteobacteria may not be adapted to the changing environment of the Antarctic soil surface or that members of this phylum lack the ability to compete with other groups of bacteria. Considering the importance of Proteobacteria to global carbon, nitrogen, and sulfur cycling (Spain et al., 2009), these results are relevant to the geochemical process of Antarctic soils.

Interesting was to observe that approximately 17% of the sequences remained unclassified (unclassified bacteria), which indicate that these cold soil habitats harbor a yet undescribed part of the microbial community with currently unknown physiological and ecological functions. Furthermore, no members of the Acidobacteria phylum were found in our study, which differs from the patterns found in other studies (Niederberger et al., 2008) were Acidobacteria is usually one of the dominant groups. These contrasting results may be due to the use in previous studies of different primers (Kim et al., 2012) or limited approaches like fingerprinting techniques (Aislabie et al., 2009; Ganzert et al., 2011). Our result seems consistent considering the advanced sequencing technology used as well as the good coverage of the primers for Acidobacteria (a simple search using Probe Match indicated that the primers used might be able to amplify many representatives of this group, http://rdp.cme.msu.edu/probematch/search.jsp), and thus the absence of this phylum in ornithogenic soils of Antartica Peninsula may have ecological significance.

In the subsequent analysis, we observed that the bacterial community structures from Layer 2 and 3 were more associated to each other than Layer 1, despite the differences in soil composition which showed that Layer 1 and 2 are physicochemically more similar to each other than Layer 3 (Table 2). We hypothesized that in addition to the physicochemical parameters, broadly considered the main factors on the development of soil microbiology, other environmental conditions may also have a strong influence on the structuring of soil bacterial communities, e.g. the stabilizing environment provided by the subsurface, in contrast to the changing environment of the surface. Our hypothesis is supported by other studies in Antarctic Peninsula showing the dissociation between soil bacterial community structure and physicochemical parameters (Teixeira et al., 2010).

In terms of diversity, Layer 3 presented the highest values of richness and evenness when compared to Layers 1 and 2. Apparently, the bacterial community in the anoxic subsurface layer had not only greater species richness but also was more

evenly structured than the community in the oxic surface layer. Furthermore, the relative sequence abundance of the core phyla among the three layers was clearly different for several groups. Layer 1 presented several taxa in higher abundance, here classified as specialists or mid-specialist (Table 4), which suggests that these bacterial groups are adapted to environments in Antarctica with high concentration of nutrients where they dominate the microbial ecosystem. Most of these specialists and mid-specialist related to Layer 1 belong to the phylum Actinobacteria, Bacteroidetes and Firmicutes, which as previously highlighted, are particularly more abundant at the surface (most probably due increased organic C availability).

In Layer 3, due the constraints imposed by abiotic factors characteristics from the depth-subsurface, the number of microorganisms may be low, diminishing the effects of competition, which reflects in a low level of dominance and higher diversity. Only three taxa of specialists were found in Layer 3. In most studies, Gammaproteobacteria is reported to decrease with depth (which differs from our results). This is caused because the alphaproteobacterial order Rhizobiales is usually found in high abundance in most soils and present a decreasing profile with depth (Sessitsch et al., 2002). This order includes the genera *Rhizobium* and *Bradyrhizobium*, which comprise members that are able to fix nitrogen and are associated with roots of plants (Sessitsch et al., 2002). Since plant root are abundant near the surface and decrease with depth, the same profile is observed to the microbial groups associated with them. In our study, due to the complete absence of plants, no members of the order Rhizobiales were observed and all the other orders of Gammaproteobacteria identified were present in higher abundance in Layer 3, including two specialists (Xanthomonadales and Enterobacteriales). The third specialist taxa identified inlayer 3 was the order Solirubrobacterales (Actinobacteria). Although Solirubrobacterales has not been extensively studied, recent studies have shown that their members have the ability to colonize different ecosystems including desert and Antarctic soils (Chong et al., 2012), where the levels of soil organic carbon is very low.

In general, these results bring new information to the limited literature on the bacterial composition of ornithogenic soils. The few available studies have focused on the diversity and abundance of dominant groups presented in ornithogenic soils (Zdanowski et al. 2004; Aislabie et al. 2009; Kim et al., 2012), and some novel species have been isolated (Bowman et al., 1996; Gupta et al., 2004). Nevertheless,

many aspects related to the bacterial communities of these special sites need to be understood in more details.

Usually, studies in microbial ecology consider just the abundance and diversity of species, and ignore the interactions among the different microorganisms, especially in Antarctica, where our knowledge on this subject remains quite limited. However, the understanding of how different species may interact within a community and their responses to disturbances may provide new insight into the soil microbial ecology. For this reason, once we have identified differences and similarities among the soil bacterial communities in terms of abundance, structure, diversity, our next step was to study patterns of interaction among the various groups of bacteria.

Based on the high throughput metagenomic data provided by Post-Light™ Ion Semiconductor Sequencing, we constructed networks for microbial species shared in the three layers of the ornithogenic site and were able to identify a general pattern of interactions. The soil microbial network was comprised of highly connected OTUs, forming a clustered topology, but not structured among densely connected groups of nodes (i.e. modules). Interestingly in this pattern was the lack of a modular structure, which is usually found in biological networks, including microbial networks from other environments (Barberán et al., 2012). The result indicates that the microbial community in Antarctica may be subjected to a prevalent factor; in this case, the cold environment may be the main factor influencing the development of microbial communities, with the selection of cold adapted and tolerant microorganisms.

The identification of keystone species is a critical issue in such ecological studies (Faust & Raes, 2012). A keystone species is a species that plays a critical role in maintaining the structure of an ecological community and whose impact on the community is greater than would be expected based on its relative abundance or total biomass (Cottee-Jones & Whittaker, 2012). Nevertheless, it is very difficult to find keystone species , especially in soil microbial communities given their extreme complexity, high diversity, and uncultivated status. Trying to overcome this problem, we have analyzed different node parameters from the network in order to identify potential keystone taxa (a better term for this study, instead of keystone species). First, we used Closeness Centrality (CC), which is defined as the inverse of the sum of the shortest distances between each node and every other node in the network (Wassermann & Faust, 1994). According to this measure, a node is more central if it

is more near to all the other nodes of the network. Second, we utilized Betweenness Centrality (BC), which indicates the relevance of a node as capable of holding together communicating nodes: the higher the value the higher the relevance of the node as an organizing regulatory node (Deng et al., 2012). Third, we used Strongly-Connected ID (SC-ID), on which each node can reach every other node by following directed edges (Wassermann & Faust, 1994). Fourth, we used Eigenvector Centrality (EC), which measure the importance of a node by the number of important nodes the node links to (Deng et al., 2012). It assigns relative scores to all nodes in the network based on the concept that connections to high-scoring nodes contribute more to the score of the node in question than equal connections to low-scoring nodes.

Among these parameters, Eigenvector Centrality presented as the best parameter to identify potential keystone taxa. By using this kind of analysis we have targeted specific taxa as potentially important, which simplify the analysis of the microbial community. For this reason, the applications of network approaches to microbial communities could provide new ways to answer fundamental questions such as: "*What are the most important taxa for the structuring and functioning of the microbial community*?". This question is not relevant only within the Antarctic context, but is a fundamental theme for microbial ecology.

Considering the recent evidences of the impact of climatic changes and human activity in Antarctic ecosystems, a fundamental issue in current Antarctic researches is to understand how different biological communities will respond to such alterations. In this context, through the applications of network approaches, we were able to simulate the consequent loss of species in the microbial community under study. The results indicate that the loss of potential keystone groups of microorganisms may significantly alter the patterns of interactions within the microbial community. These results demonstrate that the applications of network approaches to microbial communities could provide a general framework for assessing the consequences of environmental disturbances at the whole-community level, which can serve as the first step toward a predictive microbial ecology within the context of global environmental change and anthropogenic impact in the threatened environment of Antarctica.

Conclusions

This work involving network analysis in Antarctica revealed the existence of spatial patterns among soil bacteria at small scales, i.e. less than 0.5m depth. Although our sequencing effort was intended to identify dominant bacterial species, the results have shown that microbial communities in Antarctica appear to be highly localized and that selection by local abiotic factors so far not taken into account may be much stronger than previously expected. It was possible to observe a unique pattern of interactions in which the soil microbial network formed a clustered topology, but not structured in modules, as usual in biological communities. In addition, specific bacterial taxa were identified as potentially important for the structuring and functioning of the microbial community. Simulation analyzes indicate that the loss of potential keystone groups of microorganisms may significantly alter the patterns of interactions within the microbial community. These findings provide new insights into the bacterial interactions and microbial ecology of this important, but threatened environment. Such studies involving habitats in Peninsula Antarctica with high nutrient input is of primary importance since in future scenarios, the predicted increase of the mean annual air temperatures in the maritime Antarctic could result in an increase in plant coverage together with a higher nutrient input and could therefore possibly lead to a significant change in the soil microbial community.

In this work, we have focused our analyzes in an ornithogenic site at different depths to explore the general patterns of distribution, structure, diversity and network interactions presented by the microbial communities of this unique environment. The next logical step is to expand our analyzes of deep sequencing to other types of Antarctic soils in order to compare the results and better understand the development and structuring of bacterial communities in Antarctica.

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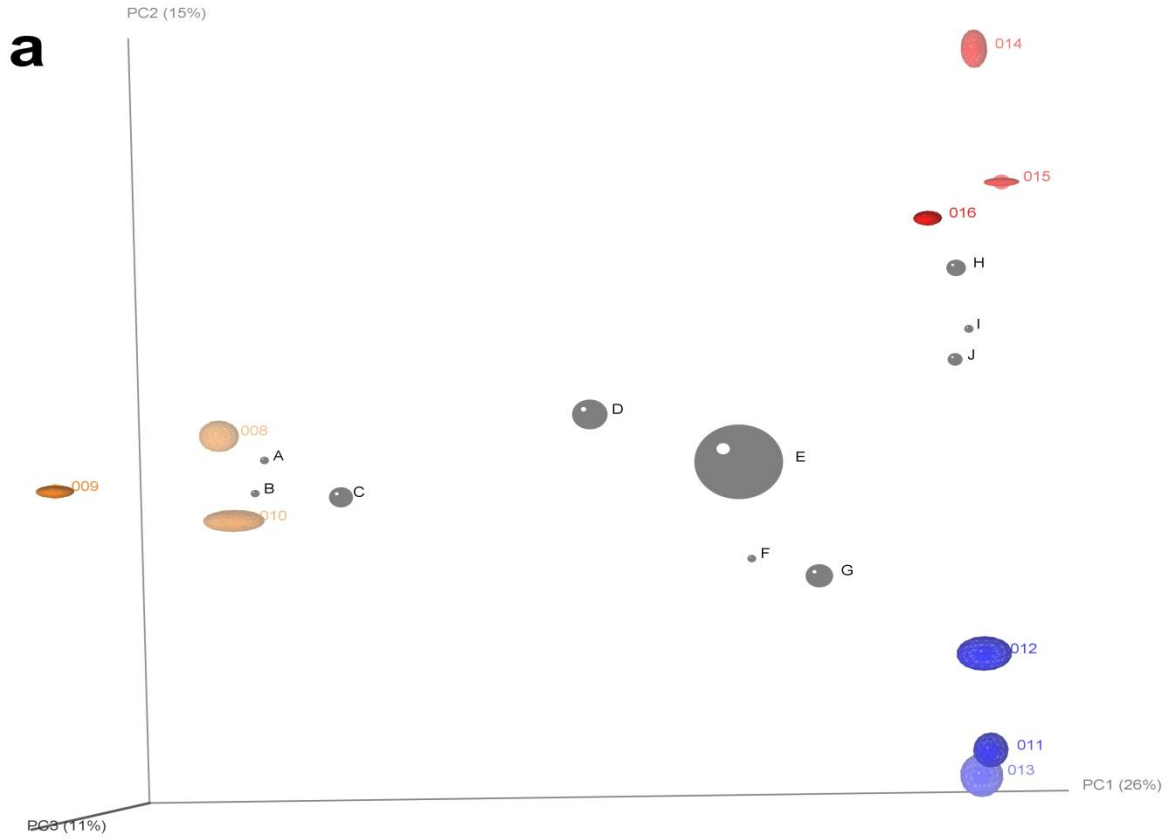

Figure 1 Jackknifed principal coordinates plot (PCoA) depicting the clusters of bacterial communities within the soil sample from each layer. a) unweighted UniFrac distance metric. The colored nodes represent samples from the three layers: 008, 009 and 010 (Layer 1); 011, 012 and 013 (Layer 2); 014, 015 and 016 (Layer 3). The gray nodes presented taxa of bacteria: A (Desulfuromonadales - Proteobacteria), B (Fusobacteriales - Fusobacteria), C (Lactobacillales - Firmicutes), D (Flavobacteriales - Bacteroidetes), E (Actinomycetales - Actinobacteria), F (Firmicutes), G (Clostridiales - Firmicutes), H (Xanthomonadales - Proteobacteria), I (Enterobacteriales - Proteobacteria), J (Solirubrobacterales - Actinobacteria).

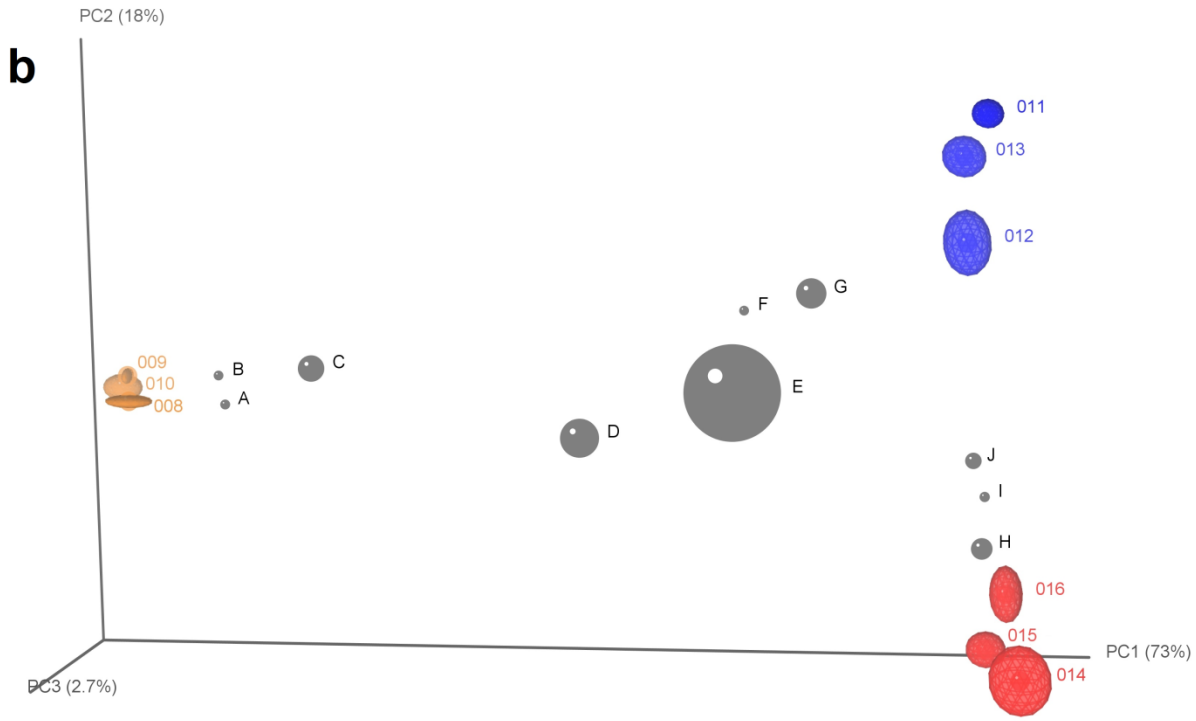

Figure 1 Jackknifed principal coordinates plot (PCoA) depicting the clusters of bacterial communities within the soil sample from each layer. b) Weighted UniFrac distance metrics. The colored nodes represent samples from the three layers: 008, 009 and 010 (Layer 1); 011, 012 and 013 (Layer 2); 014, 015 and 016 (Layer 3). The gray nodes presented taxa of bacteria: A (Desulfuromonadales - Proteobacteria), B (Fusobacteriales - Fusobacteria), C (Lactobacillales - Firmicutes), D (Flavobacteriales - Bacteroidetes), E (Actinomycetales - Actinobacteria), F (Firmicutes), G (Clostridiales - Firmicutes), H (Xanthomonadales - Proteobacteria), I (Enterobacteriales - Proteobacteria), J (Solirubrobacterales - Actinobacteria).

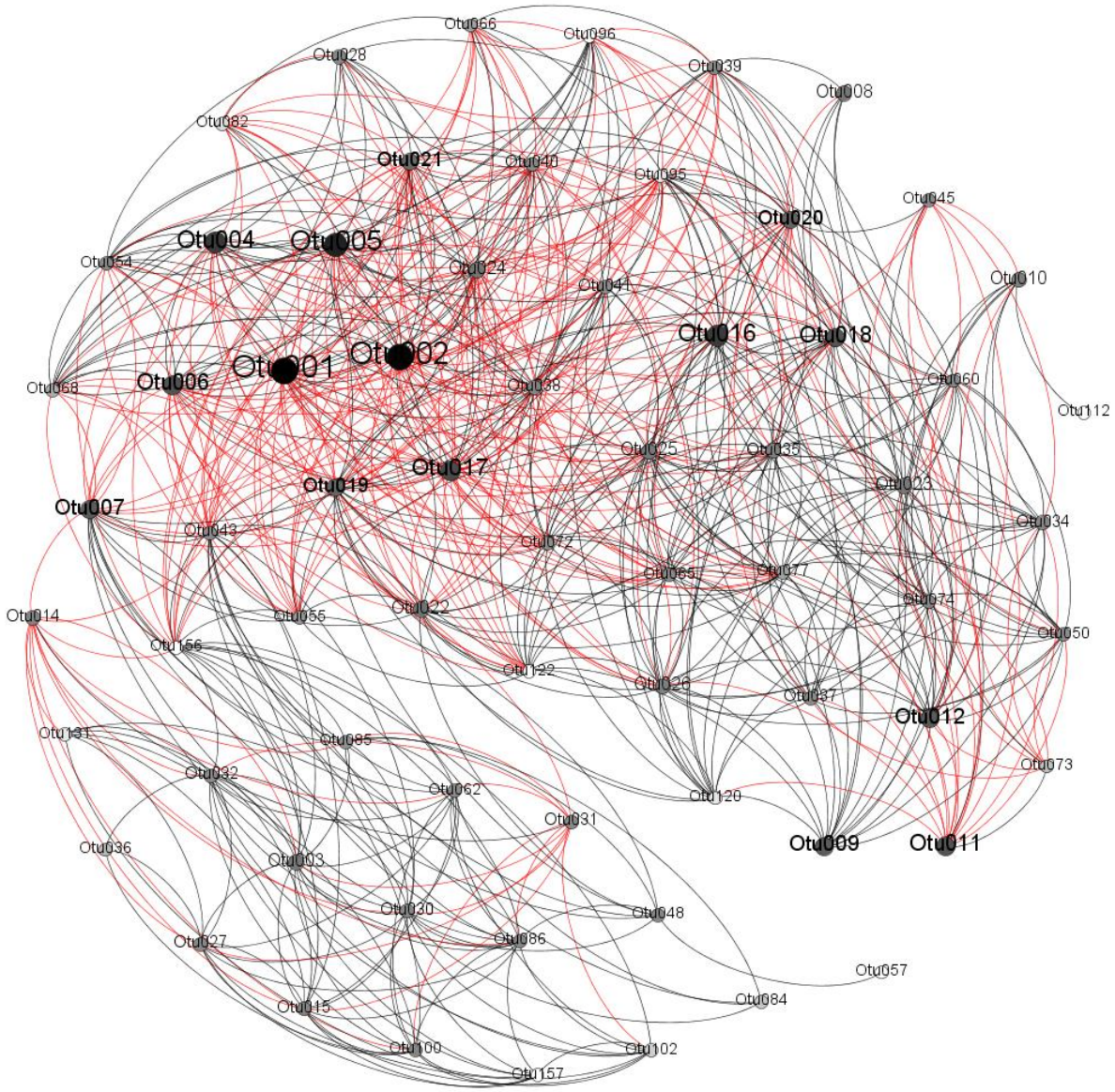

Figure 2 Network analysis of the microbial core presented in the three layers. Nodes represent OTUs at 3% cutoff and are weighted to the values of Eigenvector Centrality (Table 5). The classification of each OTU is presented in TableS2. Red edges represent negative interactions.

Table 1 Physicochemical properties of soil from the three layers.

	Depth (cm)	pH	P	K	Na	Ca^{2}+	Mg^{2}+	Al^{3}+	H+Al	SOC*
Layer 1	0-8	7.01	1927.70	2150.00	2571.00	0.42	5.06	0.00	9.80	9.78
Layer 2	20-22	7.34	1984.60	1501.00	1418.50	1.94	6.75	0.00	4.70	4.56
Layer 3	35	6.04	34.20	877.00	241.00	6.00	0.58	0.00	2.60	0.78

*Soil Organic Carbon

Table 2 Relative abundance of phyla for each layer.

Taxonomy	Total (%)	SD*	Layer 1 (%)	SD*	Layer 2 (%)	SD*	Layer 3 (%)	SD*
Actinobacteria	29.5	6.8	38.5	0.4	25.2	2.2	24.9	0.9
Proteobacteria	25.3	14.2	7.6	1.6	39.8	1.6	28.5	1.6
Firmicutes	16.3	6.7	23.7	5.8	14.6	3.8	10.6	1.5
Bacteroidetes	11.7	5.6	17.8	4.2	6.3	2.5	11.0	1.5
Verrucomicrobia	0.3	0.3	0	0	0.1	0	1	0.1
TM7	<0.1	0	0	0	0.1	0	0.1	0
Fusobacteria	<0.1	0	0	0	0.1	0	0	0
Aquificae	<0.1	0	0	0	0.1	0	0.1	0
Other	16.7	5.5	12.4	1.0	13.9	0.8	23.8	2.6

* Standard Desviation (SD)

Table 3 Coverage and indices of diversity for each layer.

	Coverage	Richness	SD	Shannon	SD	Evenness	SD
Layer 1	0.95	605.99	31.04	3.74	0.11	0.58	0.01
Layer 2	0.94	696.42	36.69	4.04	0.17	0.62	0.02
Layer 3	0.93	821.02	30.03	4.73	0.03	0.70	0.00

* Standard Desviation (SD)

Table 4 Relative abundance of bacterial taxa in each layer. For three groups, it was not possible to classify them at the Order level.

ID	Taxon	Layer 1*	Layer 2*	Layer 3*	Classification	Number of interactions
1	Bacteria;Actinobacteria;Actinobacteria;Acidimicrobiales	0.51	0.33	1.06	generalist	11
2	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales	23.79	3.15	8.33	mid-specialist	10
3	Bacteria;Actinobacteria;Actinobacteria;Solirubrobacterales	0.17	0.22	1.57	specialist	6
4	Bacteria;Aquificae;Aquificae;Aquificales	0.01	0.02	0.04	generalist	6
5	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales	0.21	0.01	0.01	generalist	6
6	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales	10.43	0.54	2.74	mid-specialist	6
7	Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales	1.49	0.44	1.28	generalist	3
8	Bacteria;Firmicutes	0.09	0.02	0.02	mid-specialist	4
9	Bacteria;Firmicutes;Bacilli	0.02	0.00	0.02	generalist	1
10	Bacteria;Firmicutes;Bacilli;Bacillales	3.00	0.87	2.90	generalist	4
11	Bacteria;Firmicutes;Bacilli;Lactobacillales	13.56	0.25	0.25	specialist	4
12	Bacteria;Firmicutes;Clostridia;Clostridiales	4.18	1.18	1.01	mid-specialist	5
13	Bacteria;Firmicutes;Erysipelotrichi;Erysipelotrichales	0.02	0.01	0.04	generalist	4
14	Bacteria;Fusobacteria;Fusobacteria;Fusobacteriales	0.31	0.00	0.00	specialist	5
15	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales	0.12	0.01	0.11	generalist	6
16	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales	0.09	0.59	1.36	generalist	6
17	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales	0.41	0.00	0.01	specialist	5
18	Bacteria;Proteobacteria;Gammaproteobacteria	0.03	0.05	0.09	generalist	5
19	Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales	2.46	3.06	3.57	generalist	5
20	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales	0.01	0.02	0.18	specialist	3
21	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales	0.11	0.16	0.38	generalist	8
22	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales	0.02	0.04	0.11	generalist	8
23	Bacteria;Proteobacteria;Gammaproteobacteria;Thiotrichales	0.00	0.03	0.11	generalist	3
24	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales	0.11	0.23	2.42	specialist	3

* in % of the total

Table 5 Topological parameters of the bacterial network used to identify potential keystone taxa.

OTU	Eigenvector Centrality	Closeness Centrality	Betweenness Centrality	Strongly-Connected ID
Otu001	1.000	0.00	0.00	0
Otu002	0.957	1.00	0.43	1
Otu005	0.762	1.00	4.14	3
Otu004	0.601	1.00	0.13	2
Otu016	0.592	1.25	50.79	9
Otu017	0.477	1.50	26.83	12
Otu006	0.426	1.00	0.13	4
Otu011	0.422	0.00	0.00	7
Otu018	0.407	1.43	50.63	14
Otu012	0.371	1.00	24.35	8
Otu009	0.370	0.00	0.00	5
Otu007	0.365	1.00	91.69	11
Otu019	0.305	1.47	36.95	15
Otu020	0.303	1.53	38.11	16
Otu021	0.262	1.41	29.13	17
Otu022	0.202	1.56	20.32	18
Otu024	0.190	1.37	28.34	19
Otu008	0.176	0.00	0.00	13
Otu003	0.172	0.00	0.00	10
Otu023	0.162	1.81	21.09	20
Otu025	0.162	1.24	52.42	21
Otu010	0.124	0.00	0.00	6
Otu026	0.122	1.36	29.83	22
Otu035	0.100	1.29	52.23	24
Otu014	0.092	1.71	25.25	30
Otu038	0.080	1.38	67.23	26
Otu015	0.076	2.38	0.27	31
Otu034	0.065	1.65	15.11	23
Otu028	0.062	1.70	1.38	25
Otu030	0.057	1.50	25.43	33
Otu040	0.055	1.34	105.52	29
Otu027	0.054	2.22	3.77	32
Otu037	0.043	1.68	7.53	27
Otu039	0.041	1.46	35.21	28
Otu031	0.037	2.00	5.38	34
Otu041	0.034	1.27	28.73	36
Otu043	0.034	1.74	273.41	35
Otu032	0.024	1.92	8.99	41
Otu050	0.020	1.59	6.52	39
Otu045	0.018	2.13	0.60	38
Otu062	0.017	2.43	32.41	42
Otu060	0.016	1.55	15.43	40

Otu065	0.015	1.29	15.71	49
Otu054	0.014	1.78	27.22	37
Otu048	0.012	2.36	47.94	43
Otu086	0.011	2.32	99.77	46
Otu074	0.007	1.61	10.98	51
Otu100	0.007	3.02	6.33	48
Otu055	0.006	1.67	12.82	54
Otu072	0.006	1.62	17.94	52
Otu085	0.006	2.40	6.48	45
Otu066	0.005	1.61	3.05	62
Otu068	0.005	1.78	11.51	56
Otu077	0.005	1.67	9.19	53
Otu036	0.004	2.69	0.00	47
Otu073	0.003	2.25	0.00	50
Otu084	0.003	2.58	0.00	44
Otu102	0.003	3.02	0.89	55
Otu082	0.002	1.88	0.26	63
Otu095	0.001	1.61	10.32	64
Otu120	0,001	1.93	1.75	58
Otu131	0.001	3.07	0.14	60
Otu057	0.000	3.30	0.00	66
Otu096	0.000	1.87	0.00	65
Otu112	0.000	1.00	0.00	67
Otu122	0.000	1.71	0.00	59
Otu156	0.000	1.65	0.00	57
Otu157	0.000	2.98	0.00	61

Table 6 Topological parameters of the whole-bacterial network, based on BC, RM and EC.

	Number of OTUs	OTUs removed	Total Interactions	Positive Interactions	Negative Interactions	Average Degree	Graph Density	Clustering Coefficient	Modularity Index
TN*	68	0	708	421	287	10.4	0.155	0.372	0.375
BC**	63	-5	580	358	222	9.2	0.148	0.386	0.369
	58	-10	453	268	185	7.9	0.142	0.378	0.454
	53	-15	354	213	141	6.8	0.133	0.385	0.495
	48	-20	259	165	94	5.5	0.120	0.375	0.543
RM***	63	-5	621	378	243	9.8	0.159	0.360	0.350
	58	-10	520	319	201	9.1	0.163	0.364	0.343
	53	-15	456	285	171	8.7	0.172	0.363	0.309
	48	-20	437	274	163	9.3	0.202	0.386	0.287
EC****	63	-5	557	351	206	8.8	0.143	0.363	0.415
	58	-10	450	297	153	7.7	0.136	0.356	0.419
	53	-15	347	242	105	6.5	0.126	0.354	0.456
	48	-20	271	190	81	5.6	0.120	0.347	0.478

* Total Network
** OTUs removed according to the Betweenness Central (BC)
*** (Empirical) OTUs randomly removed (RM)
**** OTUs removed according to the Eigenvector Centrality (EC)

Table S1 Random network analysis for the topological parameters presented in Table 6.

	Number of OTUs	OTUs removed	Total Interactions	Average Degree	Graph Density	Clustering Coefficient	Modularity Index
R-TN*	68	0	708	20.8	0.311	0.302	0.144
R-BC**	63	-5	580	18.4	0.297	0.297	0.154
	58	-10	453	15.9	0.284	0.302	0.185
	53	-15	354	13.6	0.267	0.260	0.170
	48	-20	259	11.0	0.240	0.249	0.216
R-RM***	63	-5	621	19.7	0.318	0.320	0.129
	58	-10	520	18.2	0.326	0.315	0.145
	53	-15	456	17.5	0.344	0.349	0.141
	48	-20	437	18.6	0.404	0.403	0.134
R-EC****	63	-5	557	17.6	0.285	0.296	0.170
	58	-10	450	15.5	0.272	0.274	0.159
	53	-15	347	13.0	0.252	0.259	0.177
	48	-20	271	11.3	0.240	0.253	0.196

* Random Analysis for theTotal Network
** Random Analysis for the Betweenness Central (BC)
*** Random Analysis for the (Empirical) OTUs randomly removed (RM)
**** Random Analysis for the Eigenvector Centrality (EC)

Table S2 Classification of the 78 OTUs from the soil microbial network (Figure 2).

OTU	Classification
Otu001	Bacteria;Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae;Marinilactibacillus
Otu002	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Schumannella
Otu003	Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Marinobacter
Otu004	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Cryomorphaceae;Brumimicrobium
Otu005	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Intrasporangiaceae;Kribbia
Otu006	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Salinimicrobium
Otu007	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Microbacterium
Otu008	Bacteria;Actinobacteria;Actinobacteria;Solirubrobacterales;Conexibacteraceae;Conexibacter
Otu009	Bacteria;Actinobacteria;Actinobacteria;Acidimicrobiales
Otu010	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Salirhabdus
Otu011	Bacteria;Firmicutes;Clostridia;Clostridiales
Otu012	Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Luteimonas
Otu014	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Maribius
Otu015	Bacteria;Firmicutes;Bacilli;Bacillales;Planococcaceae;Paenisporosarcina
Otu016	Bacteria;Deinococcus-Thermus;Deinococci;Deinococcales;Trueperaceae;Truepera
Otu017	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Dermatophilaceae;Dermatophilus
Otu018	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;Microlunatus
Otu019	Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Marinimicrobium
Otu020	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Cellulomonadaceae;Paraoerskovia
Otu021	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Nocardiaceae;Millisia
Otu022	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;Desulfovibrio
Otu023	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;Propionimicrobium
Otu024	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Yaniellaceae;Yaniella
Otu025	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Aequorivita
Otu026	Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Saprospiraceae;Haliscomenobacter
Otu027	Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae;Ammoniphilus
Otu028	Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Saprospiraceae;Aureispira
Otu030	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Leifsonia
Otu031	Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Balneola
Otu032	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium
Otu034	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Micrococcaceae;Arthrobacter
Otu035	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Nakamurellaceae;Saxeibacter
Otu036	Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae_Sedis_XIV;Proteocatella
Otu037	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;Granulicoccus
Otu038	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Paucisalibacillus
Otu039	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Virgibacillus
Otu040	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Micrococcineae
Otu041	Bacteria;Actinobacteria;Actinobacteria;Actinobacteria
Otu043	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Leucobacter
Otu045	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Sanguibacteraceae;Sanguibacter
Otu050	Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae;Luteolibacter
Otu054	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Intrasporangiaceae;Terracoccus
Otu055	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Gelidibacter
Otu057	Bacteria;Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae;Trichococcus
Otu060	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Nocardioidaceae;Aeromicrobium
Otu061	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Cryomorphaceae;Cryomorpha
Otu062	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Hahellaceae;Hahella

Otu066	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Salinibacillus
Otu068	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Cerasibacillus
Otu072	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;Luteococcus
Otu073	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Intrasporangiaceae;Janibacter
Otu074	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;Tessaracoccus
Otu077	Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae;Persicirhabdus
Otu082	Bacteria;Firmicutes;Bacilli;Bacillales;Caryophanaceae;Caryophanon
Otu084	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Halolactibacillus
Otu085	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Glaciibacter
Otu086	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Okibacterium
Otu094	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Dermacoccaceae;Dermacoccus
Otu095	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Marinibacillus
Otu096	Bacteria;Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae;Atopostipes
Otu100	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Thermobrachium
Otu102	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Alcanivoracaceae;Kangiella
Otu112	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;Desulfocurvus
Otu120	Bacteria;Proteobacteria;Gammaproteobacteria;Chromatiales;Halothiobacillaceae;Thiovirga
Otu122	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Pseudospirillum
Otu131	Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae;Pilibacter
Otu156	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Nitrincola
Otu157	Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococcaceae;Desulfonispora

5. CONCLUSÕES

Este trabalho envolvendo análise de redes na Antártida revelou a existência de padrões de distribuição espaciais entre as bactérias do solo em pequenas escalas, ou seja, menores que 0,5 metros de profundidade. Apesar de nosso esforço de seqüenciamento ter sido destinado a identificar espécies de bactérias dominantes, os resultados mostraram que as comunidades microbianas na Antártida parecem ser localizadas e que a seleção por fatores abióticos locais até agora não levadas em consideração pode ser muito mais forte do que o anteriormente esperado. Foi possível observar um padrão único de interações em que a rede microbiana do solo formou uma topologia em agrupamento, mas não estruturada em módulos, como de costume em comunidades biológicas. Análises de simulação indicaram que a perda de "grupos chave" pode alterar significativamente os padrões de interação dentro da comunidade microbiana. Estes resultados fornecem novos *insights* sobre as interações bacterianas e sobre a ecologia microbiana desse importante meio ambiente. Neste trabalho, focamos nossas análises em diferentes profundidades de uma pinguineira para explorar os padrões gerais de distribuição, estrutura, diversidade e interações de rede apresentadas pelas comunidades microbianas deste ambiente único. O próximo passo lógico é expandir nossas análises de seqüenciamento para outros tipos de solos da Antártica, a fim de comparar os resultados e entender de uma melhor forma o desenvolvimento e a estruturação das comunidades bacterianas presentes na Antártida.

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