UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL
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DESVENDANDO O MICROBIOMA BACTERIANO DE *SELAGINELLA* (*SELAGINELLACEAE*, *LYCOPODIOPSIDA*) E *DIRINARIA* (*CALICIACEAE*, *ASCOMYCOTA* LIQUENIZADOS)

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Doctor Scientiae

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DESVENDANDO O MICROBIOMA BACTERIANO DE *SELAGINELLA* (*SELAGINELLACEAE*, *LYCOPODIOPSIDA*) E *DIRINARIA* (*CALICIACEAE*, *ASCOMYCOTA* LIQUENIZADOS)

Tese apresentada à Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição da Universidade Federal de Mato Grosso do Sul, como parte das exigências do Programa de Pós-Graduação em Biotecnologia e Biodiversidade, para obtenção do título de *Doctor Scientiae.*

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DEDICATÓRIA

Este trabalho é dedicado aos meus pais, Eder Lopes Zanella e Amélia Pereira de Santana Zanella, e aos meus irmãos, Lucas Santana Zanella e Yara Santana Zanella. Sem o apoio de vocês, este trabalho não teria sido realizado.

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Este é o começo do resto de sua vida!

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RESUMO

ZANELLA, Mayara Santana, Universidade Federal de Mato Grosso do Sul, junho de 2024. **Desvendando o microbioma bacteriano de *Selaginella* (*Selaginellaceae, Lycopodiopsida*) e *Dirinaria* (*Caliciaceae,* Ascomycota liquenizados).** Orientadora: Aline Pedroso Lorenz. Coorientadora: Gecele Matos Paggi.

Com o avanço das técnicas independentes de cultivo, foi possível realizar análises metagenômicas de comunidades microbianas em diversos ambientes. A metagenômica consiste na extração total do DNA de um ambiente, o que permite análises de diversidade, potencial funcional e taxonomia de uma comunidade de microrganismos. Qualquer organismo pode ser estudado pela metagenômica, que tem como objetivo a compreensão da comunidade bacteriana, seus metabólitos e funções. As plantas do gênero *Selaginella* são conhecidas por suas classes estruturais únicas de produtos naturais e sua ampla gama de efeitos biológicos. São amplamente estudadas na farmacologia devido à presença de metabólitos secundários, como flavonoides, ligninas e os análogos de selaginelina. Os fungos liquenizados são conhecidos por suas propriedades simbióticas e pela produção de metabólitos secundários, sendo utilizados como bioindicadores da qualidade do ar. Apesar do potencial biotecnológico desses dois grupos de organismos, pouco se sabe sobre as associações bacterianas e suas funções em plantas do gênero *Selaginella* e em fungos liquenizados. Este trabalho tem por objetivo gerar e discutir informações sobre a composição, riqueza e abundância de bactérias associadas a duas espécies de *Selaginella* (Lycopodiopsida, Selaginellaceae) e duas espécies de fungo liquenizado do gênero Dirinaria (Lecanoromycetes, Caliciaceae). Para tanto, foram selecionadas populações presentes na área rural (Selaginella) e urbana (Dirinaria) de Campo Grande, Mato Grosso do Sul. Os dados metagenômicos foram gerados na Plataforma Ion Torrent (Gene Studio S5) e analisados quanto à sua diversidade com uso do programa Qiime 2. Os principais filos bacterianos encontrados em ambas as espécies de *Selaginella* foram Actinobacteria, Proteobacteria e Chloroflexi. Os principais representantes a nível de família são Kouleothrixaceae, Pseudonocardiaceae e Sphingomonadaceae. As amostras de raízes exibiram maiores índices de diversidade em comparação com as folhas, com Acidobacteria, Chloroflexi e Verrucomicrobia mais prevalentes nas raízes, enquanto Cyanobacteria foram predominantemente encontradas nas folhas. Não foram observadas variações significativas ao examinar o mesmo órgão entre diferentes espécies de

Selaginella. Nas análises de Dirinaria, a composição da comunidade bacteriana entre as duas espécies foi marcada pela predominância de quatro filos: Verrucomicrobia, Proteobacteria, Planctomycetes e Actinobacteria. Além disso, as populações bacterianas que residem nos talos do líquen apresentaram variação em relação às encontradas no substrato, sugerindo um mecanismo seletivo pelo líquen para bactérias específicas, presumivelmente essenciais para seu bem-estar e funcionalidade.

Palavras-chave: Metabarcoding; Planta da ressurreição; Bacterioma; Fungos liquenizados.

ABSTRACT

ZANELLA, Mayara Santana, Universidade Federal de Mato Grosso do Sul, junho de 2024. **Unraveling the bacterial microbiome of *Selaginella* (*Selaginellaceae*, *Lycopodiopsida*) and *Dirinaria* (*Caliciaceae*, Ascomycota lichenized).** Adviser: Aline Pedroso Lorenz. Co-supervisor: Gecele Matos Paggi.

With the advancement of cultivation-independent techniques, it has become possible to conduct metagenomic analyses of microbial communities in various environments. Metagenomics involves the total extraction of DNA from an environment, allowing for analyses of microbial community diversity, functional potential, and taxonomy. Any organism can be studied through metagenomics, with the aim of understanding the bacterial community, its metabolites, and functions. Plants of the genus *Selaginella* are known for their unique structural classes of natural products and their wide range of biological effects. They are extensively studied in pharmacology due to the presence of secondary metabolites such as flavonoids, lignins, and selagelin analogs. Lichenized fungi are known for their symbiotic properties and the production of secondary metabolites, used as bioindicators of air quality. Despite the biotechnological potential of these two groups of organisms, little is known about the bacterial associations and their functions in *Selaginella* plants and lichenized fungi. This work aims to generate and discuss information about the composition, richness, and abundance of bacteria associated with two species of *Selaginella* (*Lycopodiopsida*, *Selaginellaceae*) and two species of lichenized fungi of the genus *Dirinaria* (*Lecanoromycetes*, *Caliciaceae*). To this end, populations from rural (*Selaginella*) and urban (*Dirinaria*) areas of Campo Grande, Mato Grosso do Sul, were selected. Metagenomic data were generated using the Ion Torrent platform (Gene Studio S5) and analyzed for diversity using the Qiime 2 program. The main bacterial phyla found in both species of *Selaginella* were Actinobacteria, Proteobacteria, and Chloroflexi, with the main representatives at the family level being Kouleothrixaceae, Pseudonocardiaceae, and Sphingomonadaceae. Root samples exhibited higher diversity indices compared to leaves, with Acidobacteria, Chloroflexi, and Verrucomicrobia being more prevalent in roots, while Cyanobacteria were predominantly found in leaves. No significant variations were observed when examining the same organ across different Selaginella species. For *Dirinaria* analyses,

the bacterial community composition between the two species was marked by the prevalence of four phyla: Verrucomicrobia, Proteobacteria, Planctomycetes, and Actinobacteria. Additionally, bacterial populations residing on lichen thalli exhibited variance from those found on the substrate, suggesting a selective mechanism by the lichen for specific bacteria, presumably essential for its well-being and functionality.
Keywords: Metabarcoding; Resurrection plant; Bacteriome; lichenized fungi.

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1. INTRODUÇÃO

Plantas e líquenes, assim como todos os organismos vivos, abrigam comunidades microbianas complexas conhecidas como microbioma. Esses microrganismos, incluindo bactérias e fungos, desempenham um papel crucial na saúde das plantas e no bem-estar ambiental [1]. Nas plantas, o microbioma coloniza as raízes, folhas e até mesmo os tecidos internos, influenciando a aquisição de nutrientes, a tolerância ao estresse e a defesa contra patógenos [2]. Nos líquenes, podem-se encontrar microbiomas únicos que contribuem para sua notável resiliência em ambientes adversos [3]. Um microbioma saudável pode melhorar a fertilidade e a estabilidade do organismo, promovendo o crescimento e aumentando a resistência aos fatores de estresse ambiental [4].

O termo simbiose foi usado pela primeira vez por Albert Bernhard Frank no final do século XVIII para descrever o mutualismo presente nos líquenes. O termo vem do grego e significa “viver junto” (sim- junto; bio- viver; sis- processo), e é muito utilizado para descrever a relação de dois organismos agindo juntos para o benefício de ambos [5]. A evolução da simbiose requer uma forte coordenação fisiológica, estrutural e de ciclos de vida entre os organismos parceiros [6]. Nesse contexto, observa-se que as bactérias são organismos conhecidos por realizar simbiose com diversas espécies de plantas, fungos, animais, entre outros [7-10].

Nas associações com as plantas, destacam-se as bactérias endofíticas, que colonizam os tecidos internos sem mostrar nenhum sinal externo de infecção ou efeito negativo em seu hospedeiro [11, 12]. Essas bactérias existem em uma variedade de tipos de tecidos dentro de várias espécies de plantas, sugerindo uma existência onipresente na maioria, senão em todas as plantas superiores [13]. Quando associadas às plantas, as bactérias podem desempenhar diversas funções, como controle de patógenos, promoção de crescimento, produção direta e indireta de diferentes fitohormônios, mineralização e decomposição de matéria orgânica, além de melhorar a biodisponibilidade de diferentes nutrientes minerais, como ferro e fósforo [14,15].

A interação planta-microbioma é crucial não apenas para o desenvolvimento vegetal, mas também para a existência em uma grande variedade de habitats naturais, por auxiliar na germinação e vigor de sementes, desenvolvimento celular, tolerância ao estresse, absorção de nutrientes, produtividade melhorada e no controle do metabolismo

vegetal responsável pela produção dos metabólitos primários e secundários [16-18]. Os metabólitos secundários das plantas (MSP) atuam na defesa contra patógenos, pragas e herbívoros, na resposta a estresses ambientais e nas mediações de interações com outros organismos, diferente dos metabólitos primários, que atuam mais na reprodução e no crescimento vegetal [19,20]. Além disso, muitos MSP têm efeitos benéficos na saúde humana e na produção agrícola, contribuindo significativamente para a economia [21,22].

Selaginella P. Beauv. pertence à família *Selaginellaceae,* conhecida por suas classes estruturais únicas de produtos naturais e sua ampla gama de efeitos biológicos. Atualmente, apenas um gênero é reconhecido na família *Selaginellaceae,* mas este gênero é cosmopolita e contém aproximadamente 700 espécies, incluindo espécies em climas temperados, tropicais, tolerantes ao gelo do Ártico e tolerantes à seca do [23]. Valdespino et al. (2015) [24] descreveram sete novas espécies de *Selaginella* do Brasil, elevando o número de espécies nativas conhecidas no país para 58. Muitas espécies de *Selaginella* têm importância medicinal, sendo fontes de compostos bioativos para o tratamento de várias doenças e seus sintomas, incluindo inflamação, dismenorreia, hepatite crônica e hiperglicemia [25-28]. Resistir à dessecação, recuperar o funcionamento metabólico e crescer rapidamente após longos períodos de desidratação são outras características muito citadas para algumas espécies de *Selaginella* [29]. Os metabólitos secundários presentes neste gênero são amplamente discutidos quanto à sua importância farmacêutica. Seus componentes químicos de destaque são compostos classificados como dímeros de flavonoides, biflavonoides, ligninas e análogos de selaginelina [30-32].

Liquens são o resultado da interação simbiótica envolvendo fungos (micobionte) e um ou mais fotobiontes, que podem ser uma alga verde e/ou uma cianobactéria [33], além de microrganismos associados [34-36]. Os liquens são encontrados em diversos ambientes, desde regiões subpolares até as florestas tropicais, e são capazes de crescer em diversos substratos, às vezes sob condições ecológicas extremas [37,38]. Amplamente estudados por suas propriedades simbióticas e pela produção de metabólitos secundários (mais de 800 descritos), são considerados possíveis reservatórios de bactérias com potencial biotecnológico.

O sequenciamento de alto rendimento (*high throughput sequencing, next-generation sequencing)* é utilizado para identificar a diversidade de microrganismos, e a metabolômica para identificar metabólitos secundários e vias metabólicas, ajudando a fornecer perspectivas abrangentes sobre como as redes metabólicas dessas plantas são

reguladas ou impactadas pelas bactérias associadas [20]. Apesar de haver uma vasta literatura sobre resistência à dessecação para algumas espécies desse gênero, compostos secundários e suas utilizações na indústria, pouco se sabe sobre a interação de espécies de *Selaginella* e seus microrganismos associados.

A partir de análises metagenômicas, a identificação da microbiota bacteriana associada a liquens, que é altamente diversificada, tornou-se possível, mostrando que a estrutura taxonômica do complexo bacteriano presente nos liquens é semelhante à estrutura presente em solos, pântanos e ecossistemas de água doce com oferta limitada de nitrogênio e minerais [39]. Essas bactérias, assim como as encontradas associadas às plantas, apresentam potencial biotecnológico, como a síntese de vitaminas B12, B7, B9, B11 e B5 [40,41], fixação de nitrogênio [42], formação de biofilme [43], entre outros.

Estudos feitos a partir de técnicas independentes de meios de cultura, como o *fingerprinting* de genes de rRNA, hibridização *in situ* de fluorescência (FISH), sequenciamento de populações microbianas mistas, DGGE (do inglês Denaturing Gradient Gel Electrophoresis), SSCP (*Single-strand conformational polymorphism*) e sequenciamento de alto rendimento, tornaram-se populares devido à sua importância na elucidação da comunidade microbiana em diversos organismos. A aplicação bem-sucedida da metagenômica em estudos de comunidades microbianas pode nos levar a compreender sua composição e seu papel ecológico e simbiótico. Dessa forma, este trabalho tem por objetivo gerar e discutir informações sobre a composição, riqueza e abundância de bactérias associadas a duas espécies de *Selaginella* e a duas espécies de fungos liquenizados.

2. REVISÃO BIBLIOGRÁFICA

2.1. *Selaginella*

Selaginella pertence à família *Selaginellaceae*, uma das maiores famílias de licopódios, com distribuição cosmopolita e aproximadamente 750 espécies, das quais 61 são descritas para o Brasil [44-46, 24]. Os licopódios são um grupo de plantas vasculares que pertencem à divisão Lycopodiophyta, também conhecida como Lycopodiopsida ou Lycophyta. *Selaginella* é caracterizada pela presença de lígula (pequena estrutura em forma de escama localizada na base da folha, com função de proteção e absorção de água), rizóforos, heterosporia e esporângios adaxiais e reniformes. Este gênero possui o único

registro fóssil conhecido, datando do final do Carbonífero [47,48]. *Selaginella* tem uma ampla distribuição em regiões tropicais e subtropicais, com maior diversidade de espécies em florestas tropicais e encostas sombreadas, embora algumas espécies também possam prosperar em condições xerofíticas [49].

As espécies de *Selaginella* são conhecidas por suas estruturas únicas de produtos naturais e pelos diversos efeitos biológicos que apresentam. Elas são utilizadas na medicina tradicional para o tratamento de várias doenças, incluindo inflamação, dismenorreia, hepatite crônica e hiperglicemia [25-28]. Outra característica importante desse gênero é a capacidade de algumas espécies de resistir à dessecação, recuperar o funcionamento metabólico e crescer rapidamente após longos períodos de desidratação [29]. Devido à variedade de moléculas exclusivas e metabólitos secundários, este gênero é extensivamente estudado devido à sua importância farmacêutica. Entre os produtos naturais específicos do gênero estão as selaginelinas, análogos de selaginelina, flavonoides e ligninas [25, 31-32].

2.2. *Selaginella convoluta* (Arn.) Spring e *Selaginella sellowii* Hieron

Encontrados geralmente na América Tropical, os espécimes de *Selaginella convoluta* (Figuras 1 e 2) são facilmente identificados por seus ramos em rosetas, que se enrolam quando secos, e folhas peltadas. Apresentam uma grande variação morfológica devido à sua ampla distribuição geográfica e são encontrados em locais secos e expostos ao sol [50]. Também na América Tropical, *Selaginella sellowii* pode ser reconhecida por suas folhas aciculares, dispostas em espiral, cada uma com uma cerda apical esbranquiçada que às vezes é caducifólia [50]. É uma licófita tolerante à dessecação que cresce em afloramentos rochosos [51].

Em uma revisão de literatura realizada em março de 2022, utilizando o site *Scientific Electronic Library* (Scielo) e Google Acadêmico com os termos ("*Selaginella*" OR "*Selaginellaceae*") AND ("Microbioma" OR "Bactérias associadas" OR "Microbiome"), apenas um estudo focado em bactérias associadas a *Selaginella* foi encontrado. Com o objetivo de encontrar potenciais agentes de biocontrole para espécies invasoras na Nova Zelândia, Dang et al. (2019) [52] utilizaram análises de metagenômica em amostras de *Selaginella kraussiana*.

Figura 1. *Selaginella convoluta*, fevereiro de 2021, Morro do Ernesto, Campo Grande (MS).

Fonte: Elaborada pela autora.

Figura 2. *Selaginella convoluta* e alguns ramos de *S. sellowii*, agosto de 2021, Morro do Ernesto, Campo Grande (MS)

Fonte: Elaborada pela autora.

2.3 Liquens

Em 1876, Albert Bernhard Frank utilizou, pela primeira vez, cinco liquens crostosos como exemplo de simbiose autossustentável, através de estudos microscópicos. A estrutura conhecida como talo liquênico é formada pela produção de energia do fotobionte via fixação de dióxido de carbono, potencializada pelas estruturas de abrigo do parceiro fúngico. Décadas depois, com o avanço das técnicas laboratoriais, [37] cultivaram outros fungos a partir de talos liquênicos macerados, denominando-os 'endoliquênicos'. Apesar da presença de outros fungos hospedeiros, a arquitetura geral do líquen em todos os casos permanece determinada pelo micobionte dominante [37].

A ideia de um papel unitário do parceiro fúngico na determinação dos caracteres de um líquen foi desafiada por [33]. Utilizando técnicas de PCR com primers específicos, eles concluíram que os basidiomicetos, como Cystobasidiomycetes e Pucciniomycotina, representavam um componente integral do córtex superior dos liquens. A presença desses basidiomicetos foi associada à cor amarela presente nos ramos de *Bryoria fremontii* (Tuck.) Brodo & D. Hawksw., alterando a antiga definição de líquen como "a simbiose

entre fungos (micobionte) e cianobactérias ou algas (fotobionte), juntos esses organismos formam uma estrutura denominada talo liquênico" [33].

Com o avanço das técnicas independentes de cultura e da metagenômica, foi constatado que além dos fungos "adicionais", outros microrganismos fazem parte da estrutura liquênica denominada talo, como protistas e vírus [53-55]. O sucesso dessa relação simbiótica é evidenciado pelas mais de 19.000 espécies de fungos liquenizados, encontradas em quase todos os tipos de ambientes, desde zonas climáticas tropicais até polares, e habitats costeiros até de alta altitude [56]. Esses microrganismos desempenham diferentes papéis tróficos e estão relacionados ao processo evolutivo dos liquens, realizando funções como trocas gasosas e ciclagem de nutrientes [57,39].

Portanto, diante das atuais discussões acadêmicas sobre a definição de liquens, esses são resultado de uma interação simbiótica complexa e autossustentável envolvendo fungos (micobionte), algas verdes e/ou cianobactérias (fotobiontes) [33], além de microrganismos associados [36].

2.4. *Dirinaria*

Comumente encontrado em países tropicais, *Dirinaria* (Tuck.) Clem. (*Caliciaceae*), representada por 25 espécies e quatro variedades [58] é um gênero cosmopolita, com exceção das regiões boreais e árticas. Por ser um gênero pantropical a subtropical, apresenta uma vasta diversidade no Brasil, e do total de espécies e variedades encontradas, 13 espécies e uma variedade foram relatadas para o estado do Mato Grosso do Sul: *Dirinaria aegialita* (Afzel. *in* Ach.) B.J. Moore; *D. africana* (Mull. Arg.) D.D. Awasthi; *D. applanata* (Fée) D.D. Awasthi; *D. confluens* (Fr.) D.D. Awasthi; *D. confluens* var. *coccinea* (Lynge) D.D. Awasthi; *D. consimilis* (Stirt.) D.D. Awasthi; *D. leopoldii* (Stein) D.D. Awasthi; *D. maracajuensis* T.D. Barbosa & A.A. Spielmann; *D. melanocarpa* (Mull. Arg.) C.W. Dodge; *D. melanoclina* (C. Knight) D.D. Awasthi; *D. papillulifera* (Nyl.) D.D. Awasthi; *D. picta* (Sw.) Clem. & Shear e *D. pruinosa* Kalb), e a variedade *D. purpurascens* (Vain.) B.J. Moore.

As principais características morfológicas do gênero incluem talos foliosos estreitamente aderidos ao substrato, com lacínias radiantes dicotômicas, subdicotômicas a palmatífidas ramificadas. Os ápices variam de flabelados, levemente flabelados a discretos, e a superfície do talo pode ser lisa ou rugosa. A superfície inferior geralmente

é preta, podendo ser marrom escura ou marrom claro/bege, enquanto a medula pode ser branca, alaranjada ou vermelha [59].

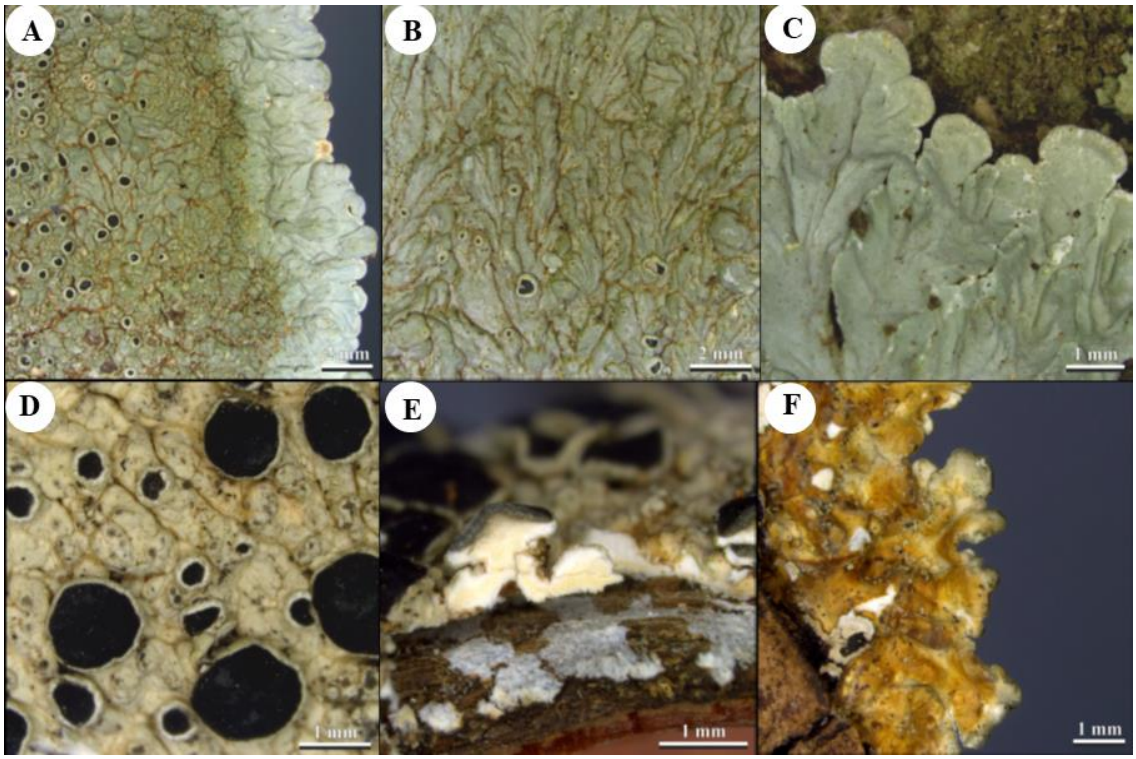
Os principais componentes químicos são a atranorina, o ácido divaricático e o ácido sequicáico. Todas as espécies têm a atranorina, enquanto o ácido sequicáico está presente em quatro espécies, *Dirinaria confusa* D.D. Awasthi (1975), *D. consimilis*, *D. minuta* Kalb (2001) e *D. sekikaica* Elix (2008); já o ácido divaricático está presente nas outras dezoito espécies do gênero [58].

2.5. *Dirinaria melanocarpa* (Müll. Arg.) C. W. Dodge

Esta espécie é exclusivamente neotropical, com distribuição restrita à região central da América do Sul, sendo encontrada na Bolívia, Brasil, Colômbia e Paraguai [59]. Caracterizada por um talo plicado, lacínea confluentes, apotécio sésseis de base constrita, disco plano a convexo, negro, epruinoso (Figura 3). Tem como habitat cascas de árvores, e é distinguida pela superfície inferior branco amarelada, sub-himênio de amarelo a incolor, e pela presença de atranorina e ácido divaricático. A espécie já foi reportada para Mato Grosso do Sul [58, 60] estando presente em oito municípios, Alcinópolis, Aquidauana, Campo Grande, Corguinho, Corumbá, Costa Rica, Jaraguari, Jardim, Nova Andradina e Porto Murtinho.

Figura 3. A. Dirinaria melanocarpa, morfologia do talo (T.D. Barbosa 1898). **B.** Plicação longitudinal na superfície superior proximal (T.D. Barbosa 1898). **C.** Lacinias com ápices flabelados e máculas lineares (T.D. Barbosa 1898). **D.** Apotécios com margem lisa e pruína branca (A.A. Spielmann 11982). **E.** Estipe interno com medula amarelada (A.A.

Spielmann 11982). **F.** Superfície inferior marginal (T.D. Barbosa 1785). (Retirado de Barbosa, 2019).



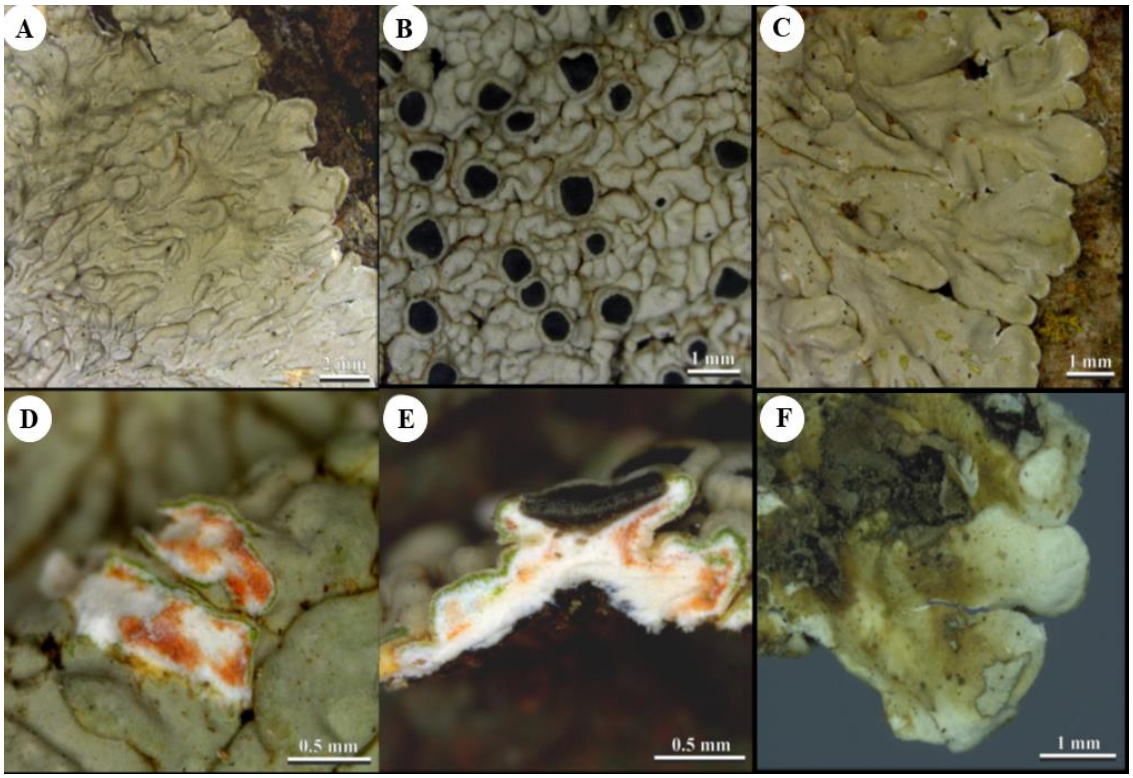
Fonte: Elaborada pela autora.

2.6. *Dirinaria rhodocladonica* Kalb, Schumm & Elix

Encontrada em países tropicais e subtropicais como a Ásia, Américas e Austrália, *D. confluens*, reclassificada como nova espécie com nome de *Dirinaria rhodocladonica* Kalb, Schumm & Elix [61], (Figura 4) é caracterizada morfologicamente por um talo fortemente plicado, lacíneas flabeladas, apotécios abundantes com disco plano a convexo e pruína branca, quimicamente apresenta atranorina e ácido divaricático [59]. Tem como habitat cascas de árvores podendo, também, ser encontrada em superfícies rochosas. A espécie foi reportada no Brasil em 1975 por Awasthi e no estado do Mato Grosso do Sul por [58], estando presente em pelo menos nove municípios, Alcinópolis, Aquidauana, Bodoquena, Campo Grande, Corguinho, Corumbá, Jaraguari, Nova Andradina e Porto Murtinho, e no distrito de Piraputanga.

Figura 4. A. Dirinaria rhodocladonica, morfologia do talo (J.M. Torres 546). B. Plicação irregular, superfície superior proximal verrucosa e apotécios (T.D. Barbosa 1732). C. Lacinias com ápices flabelados e máculas lineares (J.M. Torres 546). D. Pigmento coccíneo na camada superior da medula (M.J. Kitaura 4306). E. Pigmento coccíneo no

estipe interno (M.J. Kitaura 4306). F. Superfície inferior marginal (J.-M. Torres 546). (Retirado de Barbosa, 2019).



Fonte: Elaborada pela autora.

2.7. Diversidade do Microbioma Bacteriano de Liquens

O talo liquênico abriga diversos microrganismos, em particular as bactérias, formando um micro-habitat considerado por alguns autores como o terceiro componente da simbiose em liquens [33,34;62,63]. Os primeiros gêneros de bactérias associadas aos liquens relatados foram Azotobacter [64,65], Pseudomonas [66], Beijerinckia [65], Clostridium [64] e Bacillus [66]. Essas bactérias foram descritas a partir da utilização de meios de culturas quimicamente definidos e complexos.

Com a utilização de análises metagenômicas, a identificação da microbiota bacteriana associada aos liquens, que é altamente diversificada, tornou-se possível, mostrando que a estrutura taxonômica do complexo bacteriano presente é semelhante àquela encontrada em solos, pântanos e ecossistemas de água doce com oferta limitada de nitrogênio e minerais [39]. As comunidades microbianas encontradas nos liquens são consideradas 'flora normal' [67] e participam do ciclo de alguns nutrientes como o nitrogênio e fósforo, além de atuarem na produção de hormônios [39]. Acredita-se que elas também desempenhem papéis funcionais, influenciando na adaptabilidade e

versatilidade da simbiose, contribuindo para a proteção ou regulação do crescimento dos liquens [68].

Diversos autores demonstraram que o grupo de bactérias que se destaca no microbioma associado a liquens é o da classe *Alphaproteobacteria*, do filo das proteobactérias [56,41,68] que é comumente encontrado associada às raízes de plantas promovendo seu crescimento e nutrição [69]. Uma linhagem de *Alphaproteobacteria* ('LAR1' pertencente à ordem *Rhizobiales*) está sempre presente em liquens que possuem como fotobiontes as algas verdes, sugerindo que ela seja uma contribuinte para a simbiose desses liquens. O filo *Bacteroidetes* já foi descrito como comumente encontrado em amostras de liquens marinhos ou litorâneos [70-74]. Bactérias da ordem *Rhizobiales* são predominantes quando associadas às plantas, fornecendo as mesmas vários nutrientes, fitohormônios e precursores de metabólitos essenciais [75,40], assim, acredita-se que elas exerçam funções semelhantes quando associadas aos liquens. Representantes de *Acidobacteriaceae, Acetobacteraceae* e *Brucellaceae* também são encontrados e em alguns casos e formam colônias produtoras de biofilme [71,76].

Apesar dos avanços nos estudos de microbiomas associados a fungos liquenizados, não se pode afirmar se o micobionte realiza a seleção das bactérias ou se elas estão presentes no líquen de forma oportunista [38,39]. O mecanismo que controla a abundância e a diversidade bacteriana nos liquens permanece desconhecido, porém, acredita-se que essas bactérias desempenhem o papel de proteção contra a invasão de grupos bacterianos patogênicos através da produção de metabólitos secundários com atividade antibacteriana [77].

Pankratov et al. [39] sugerem que alguns grupos bacterianos que coabitam diferentes espécies de liquens sejam obrigatórios e outros facultativos, influenciados pelas características do nicho ecológico onde o talo primário se forma. Também podem ser influenciados pela fração considerável de metabólitos secundários com atividades antimicrobianas que exercem uma pressão seletiva sobre as bactérias colonizadoras de liquens [78,3]. A comunidade bacteriana associada aos liquens desempenha funções estruturais e funcionais, participando tanto no metabolismo liquênico quanto na regulação das atividades da simbiose como um todo [79,80].

A composição das comunidades bacterianas é independente da variação sazonal e tem taxas de sobrevivência potencialmente altas no talo liquênico, sendo a abundância relativa das comunidades bacterianas dependente da disponibilidade de substratos, do pH

do ambiente, da concentração de oxigênio e da presença de inibidores da atividade enzimática [79-81]. Apesar dos novos dados sobre a estrutura e função das bactérias associadas ao líquen, pouco se sabe sobre a variação intraespecífica da composição do microbioma e como os liquens adquirem suas comunidades bacterianas específicas.

2.8. Avaliando a diversidade microbiana: técnicas independentes de cultura

A alta diversidade de bactérias, não cultiváveis, associadas aos liquens começou a ser desvendada por [82] que, ao utilizarem técnicas de genética molecular com primers específicos e microscopia confocal de varredura, visualizaram uma composição bacteriana diferente dos resultados obtidos por isolamento em meios de cultura. Os resultados demonstraram a presença de comunidades formadoras de biofilme, dominadas por *Alphaproteobacteria* (60%), seguida de *Actinobacteria* (10%), *Betaproteobacteria* (10%) e *Firmicutes* (<10%). A partir daí, e com o avanço das técnicas de biologia molecular, o estudo do potencial bacteriano associado aos liquens, sua composição e abundância tornou-se importante para a compreensão da simbiose.

Várias técnicas, incluindo *fingerprinting* de genes de rRNA, hibridização *in situ* de fluorescência (FISH), sequenciamento de populações microbianas mistas, DGGE (do inglês Denaturing Gradient Gel Electrophoresis), SSCP (do inglês Single-strand conformational polymorphism) e sequenciamento de nova geração, mostraram a diferença na abundância e diversidade das comunidades bacterianas cultiváveis e não cultiváveis, mostrando que as bactérias são mais diversificadas nos liquens do que havia sido previamente sugerido [82,34,41]. Com esses estudos foi possível notar que a comunidade microbiana presente na superfície do líquen é diferente do presente na parte interna do talo, sendo considerada oportunista, quando associada a superfície e específica, quando associada a medula do líquen.

As comunidades bacterianas dos liquens estudados, até o momento, predominantemente apresentam bactérias pertencentes a classe *Alphaproteobacteria* do filo *Proteobacteria*, e outros filos como o *Bacteroidete*, *Actinobacteria*, *Firmicutes* e *Verrucomicrobia* [34,80-84]. Hodkinson & Lutzoni (2009) [70] utilizando técnicas de PCR encontraram bactérias pertencentes às ordens *Rhizobiales* e *Rhodospirillales*, e às famílias *Acetobacteriaceae*, *Acidobacteriaceae*, *Beijerinckiaceae*, *Methylobacteriaceae*, *Xanthobacteriaceae*, *Hyphomicrobiaceae* e *Bradyrhizobiaceae*. Algumas sequências não foram identificadas, em nenhum nível taxonômico, e os autores sugeriram que elas

pertencem a uma nova ordem dentro das *Alphaproteobacteria*, que são especificamente adaptadas para associações com liquens.

Visando compreender a distribuição das bactérias no talo liquênico, [83] realizaram análises de duas regiões do talo, região central e região periférica. Para a região central os grupos descritos pertencem à *Rhizobiales*, *Rhodospirillales* com a predominância de *Acidobacteria*, para a região periférica a maior diversidade foi observada em sequências não classificadas. Os autores levantaram a hipótese de que elas auxiliam na parte metabólica do líquen uma vez que se encontram nas zonas de crescimento, reforçando assim a sugestão feita por [70] de que algumas espécies bacterianas podem ser obrigatórias na associação com os liquens. Ao identificar genes e proteínas relacionadas à biossíntese da auxina em *Lobaria pulmonaria*, [34] sugeriram que a mobilização e reciclagem de nutrientes presentes na parte senescente do talo ocorrem a partir da associação dos liquens com as bactérias.

Com o avanço do conhecimento sobre a composição da comunidade bacteriana nos liquens, surgem novas questões sobre os mecanismos pelos quais essas comunidades são estruturadas, transmitidas e adquiridas ao longo das gerações. Essa questão pode ser crucial para entender como funciona a relação entre liquens e bactérias [80].

3. OBJETIVOS

3.1. GERAL

Avaliar a riqueza e a abundância bacteriana única dos microbiomas associados às licófitas *Selaginella sellowii* e *S. convoluta* e aos fungos liquenizados *Dirinaria melanocarpa* e *D. rhodocladonica*, focando em seus potenciais papéis nas funções fisiológicas e adaptações ecológicas dessas espécies comuns no Cerrado de Mato Grosso do Sul.

3.2 ESPECÍFICOS

- Identificar as diferenças e similaridades entre os microbiomas de plantas simpátricas de *Selaginella sellowii* e *S. convoluta*;
- Associar o microbioma presente em *Selaginella convoluta* e *S. sellowii* às suas funções fisiológicas potenciais, como a fixação de nitrogênio, absorção de nutrientes, resistência a patógenos, entre outros;
- Comparar o microbioma presente nas folhas e raízes de *S. convoluta* e *S. sellowii,* identificando as particularidades das bactérias presentes nos diferentes órgãos e nas diferentes espécies de plantas, inferindo como esses microbiomas podem contribuir para as funções específicas de cada órgão;
- Avaliar quais componentes do microbioma de *S. convoluta* e *S. sellowii* podem estar associados às variáveis climáticas do final da estação seca, buscando grupos de bactérias que potencialmente contribuem para a sobrevivência em ambientes de extremo déficit hídrico;
- Comparar a composição do microbioma de espécimes de *Dirinaria melanocarpa* e *D. rhodocladonica* coletados nos mesmos forófitos de um parque na área urbana de Campo Grande/MS, para entender como a distribuição espacial influencia a comunidade microbiana associada;
- Diferenciar o microbioma presente no substrato (forófito) do intrinsicamente associado a *D. melanocarpa* e *D. rhodocladonica;*
- Investigar as potenciais funções dos grupos de bactérias intrinsicamente associados a *D. melanocarpa* e *D. rhodocladonica*, levantando suas possíveis contribuições em processos metabólicos do talo liquênico, como fixação de nitrogênio e produção de compostos bioativos.

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CAPÍTULO 1

Bacteriome diversity of the desiccation-tolerant *Selaginella convoluta* and *S. sellowii* in the Brazilian Cerrado

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Bacteriome diversity of the desiccation-tolerant *Selaginella convoluta* and *S. sellowii* in the Brazilian Cerrado

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ABSTRACT

Plants have evolved various adaptive strategies to combat dehydration stress, including forming associations with microbial communities. Some bacteria can help plants cope with environmental stressors like drought by altering plant hormone levels, enhancing antioxidant systems, or improving water uptake. The resurrection plants of the *Selaginella* genus are capable of enduring prolonged desiccation and being revitalized with just a few hours of rehydration. The Cerrado (Brazilian savanna), one of the world's biodiversity hotspots located in Central-West Brazil, experiences markedly wet and dry seasons. In this dynamic environment, we investigated the bacteriome of leaves and roots of sympatric plants of *Selaginella convoluta* and *S. sellowii* inhabiting rocky outcrops. We analyzed 16S rRNA (V4 region) bacterial sequences generated on the Ion Torrent platform from 74 samples: 16 leaves of *S. convoluta*, 22 leaves of *S. sellowii*, 16 roots of *S. convoluta*, and 20 roots of *S. sellowii*. In both species, Actinobacteria, Proteobacteria, and Chloroflexi were the dominant phyla, with Kouleothrixaceae, Pseudonocardiaceae, and Sphingomonadaceae being the most prevalent bacterial families. The roots exhibited an increased relative abundance of Acidobacteria, Chloroflexi, and Verrucomicrobia, whereas Cyanobacteria were more common in the leaves. Root samples displayed higher diversity indices than leaves, but no significant differences were observed when comparing the same organ between *Selaginella* species. Differential abundance and heatmap analyses highlighted the main differences between species and organs, suggesting some specialized microbial communities. In both species, the identified bacterial families were beneficial for nutrient acquisition, growth, and fungal regulation at the root interface, contributing to plant health in nutrient-poor, shallow, and acidic soils characteristic of the Cerrado. This study highlights the significance of comprehending the intricate interplay between plants and their microbiomes in harsh environments, particularly in extreme drought conditions. Given the exceptional tolerance of *Selaginella* species, studying their microbiome

is crucial to identify the external factors that potentially enable them to thrive under challenging conditions.

Keywords: 16S rRNA, Abiotic stress, Cerrado, Metabarcoding, Resurrection plant

1. Introduction

Plants of *Selaginella* (*Selaginellaceae*, spike mosses) biosynthesize rare and specialized secondary metabolites, including selaginellins, bioflavonoids, and high-molecular-weight phenylpropanoid derivatives, which exhibit a wide range of biological effects [1,2]. Several *Selaginella* species are important in traditional medicine for treating various disorders and diseases, such as inflammation, dysmenorrhea, chronic hepatitis, and hyperglycemia [2,3-6]. The genus exhibits its highest species diversity within tropical and subtropical forest regions, often thriving on shaded slopes. Notably, some species can withstand environments that are permanently or temporarily subject to severe drought [7]. *Selaginella convoluta* has a broad distribution across the Americas, ranging from the Greater Antilles and Central America to South America, encompassing northwestern Argentina, Bolivia, Brazil, French Guiana, and Paraguay [8]. It occupies biomes with varying degrees of seasonality in Brazil, particularly the Cerrado (Brazilian Savanna) and the Caatinga [9,10]. In its most severe dehydrated state, *S. convoluta* exhibits tightly curled microphylls that resemble compact spheres, which can fully recover upon rehydration [11]. Another desiccation-tolerant lycophyte in tropical America is *S. sellowii*, which occurs in Argentina, Bolivia, Brazil, Colombia, Cuba, Ecuador, Mexico, Paraguay, Peru, Uruguay, and Venezuela. This species occurs on rocky outcrops throughout Cerrado, and its spirally arranged acicular leaves distinguish it, each tipped with a whitish apical bristle that sometimes detaches [12,13].

The Cerrado ecosystem is highly diverse, with a significant degree of endemism and pronounced seasonality [14]. The rainy season extends from November to March, whereas the dry season, characterized by arid conditions and elevated temperatures, lasts from April to October [15]. This seasonality creates a dynamic and challenging environment for the species inhabiting the Cerrado, demonstrating their resilience and the necessity to adapt to contrasting and adverse conditions throughout the year. Desiccation tolerance is a trait that enables plants to endure cycles of severe dehydration and rehydration without losing viability, earning them the designation of resurrection plants [16]. Several *Selaginella* species can rapidly restore metabolic function and resume growth after prolonged desiccation [17]. Some *Selaginella* genes have been associated with desiccation tolerance, including ROS (including ROS scavenger genes) scavenger genes and genes related to abscisic acid synthesis, both of which are overexpressed during desiccation [18]. Furthermore, metabolic profiling studies have shown that *Selaginella* species maintain elevated levels of amino acids, sugar alcohols, and polysaccharides under drought conditions, which may contribute to their robust desiccation tolerance [19].

In addition to metabolic adaptation, plants can employ other survival mechanisms under drought conditions, such as leveraging their microbiome. Many plant species selectively recruit their microbiome in the phyllosphere, rhizosphere, and roots [20]. This plant-microbiome interaction is pivotal for various plant development stages, aiding in seed germination and vigor, cell development, stress tolerance, nutrient uptake, productivity enhancement, and regulation of plant metabolism [21-23]. A particularly important group within the plant microbiome is endophytic bacteria, which colonize internal tissues without showing external signs of infection or exerting negative effects on their host [24]. These bacteria are found within tissues across various plant species, indicating their ubiquitous presence in most higher plants [25]. The interaction is highly specific, as plant-associated microbial communities demonstrate

structured organization and exhibit a defined phylogenetic arrangement during community assembly [26].

Complex interactions between the environment and plant hosts shape plant-microbiome associations. The phyla Proteobacteria and Firmicutes are highly represented in endophytic communities, whereas Bacteroidetes, Firmicutes, Actinomycetes, and Proteobacteria are more abundant in phyllosphere communities [20,27]. Notably, Proteobacteria comprise approximately 50% of the phyllosphere community [20]. Although most Proteobacteria are free-living, certain members, such as rhizobia, form symbiotic relationships with specific leguminous plants to fix nitrogen. These bacteria perform various plant-related functions, including enhancing soil fertility, nitrogen fixation, acting as biopesticides, and promoting plant growth [28,29]. Studies have revealed genes that directly or indirectly contribute to established plant growth-promoting effects [28,29].

The present study investigated the bacteriome diversity within *S. convoluta* and *S. sellowii*, two desiccation-tolerant species commonly found in Cerrado, Brazil. We evaluated the hypothesis that these desiccation-tolerant plants exhibit similarities in their leaves and roots bacteriomes. Although considerable knowledge exists regarding the metabolic adaptations of certain *Selaginella* species to drought conditions, the bacteriomes of *S. convoluta* and *S. sellowii* remain unexplored. This knowledge may enhance our understanding of the ecological processes that underpin the extraordinary ability of *S. convoluta* and *S. sellowii* to thrive during the severe seasonal droughts that affect the Cerrado ecosystems.

2. Material and Methods

2.1 Studied area and species

Selaginella convoluta and *Selaginella sellowii* coexist along rocky outcrops within the Cerrado vegetation in Midwest Brazil. Samples were collected in Mato Grosso do Sul state (-20.339703/-54.695883, approximately 410 m a.s.l.), in August 2020, following 35 days of rainfall shortage (Figure 1). The plants, preferably in pairs of species, were at least 4 m from each other, totaling 16 specimens from *S. convoluta* and 22 from *S. sellowii*. Voucher specimens were deposited in the CGMS Herbarium at Universidade Federal de Mato Grosso do Sul, Brazil, and the project is registered in the National Genetic Heritage Management System (SisGen) under the number A63DB37. Each plant leaf and root samples were stored in separated falcon tubes using sterilized tweezers. The samples were kept in ice during the transport to the laboratory, washed with ultrapure water on the same day, and then stored at -80°C.

2.2 DNA extraction and 16S rRNA sequencing

The DNA was extracted with MagMax™ Microbiome Ultra Nucleic Acid Isolation (Thermo Fisher Scientific, Waltham, MA), using 0.25 g of each sample, according to the manufacturer's instructions. The 16S rRNA region was amplified in triplicate, using the primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) [30] to target the V4 region. The PCR mix consisted of 22.5 µL of Platinum PCR SuperMix High Fidelity (Invitrogen™), 2 µL of genomic DNA, and 0.5 µL of 10 µM primer. The initial step of PCR was 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, primers' annealing at 60°C for 30 seconds, and extension at 68°C for 1 minute. The triplicates were united and purified in two rounds using the AMPure® XP reagent (Beckman Coulter, Indianapolis, IN, USA). The samples were quantified in a Qubit® instrument and then diluted

to 40pM. The amplicon pool was loaded onto the Ion Chef™ System (Thermo Fisher Scientific) for emulsion PCR, enrichment, and loading onto an Ion S5 530 chip. Following templating and chip loading, samples were sequenced using 850 flows in the Ion GeneStudio S5 System, following the manufacturer's instructions (Life Technologies).

2.3 Sequences treatment and diversity analyses

Bioinformatic analyses were performed with QIIME 2 2023.7 [31]. Raw sequence data were quality filtered using the q2-demux plugin, followed by denoising with DADA2 [32]. All amplicon sequence variants (ASVs) were aligned with MAFFT [33] and used to construct a phylogeny with FastTree 2 [34]. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier [35] against the Greengenes 13_8 99% OTUs reference sequences [36]. We filtered all samples to exclude sequences: 1) with less than ten copies; 2) from mitochondria and chloroplast; 3) identified only at the phyllo level.

Alpha-diversity metrics (observed amplicon sequence variants – ASVs – and Faith's Phylogenetic Diversity; [37]), beta-diversity metrics (weighted UniFrac, [38]), unweighted UniFrac [39], Jaccard distance, Bray-Curtis dissimilarity, and Principle Coordinate Analysis (PcoA) were estimated after rarefaction of 8.000 sequences per sample. The Kruskal-Wallis test compared alpha diversity metrics from organ and species samples. PERMANOVA [40] analysis on beta diversity metrics using 999 permutations was used to compare group distances. The fractions of shared and exclusive variants at the family level were visualized using Venn diagrams in the InteractiVenn web platform (http://www.interactivenn.net/; [41]).

Differential abundance was assessed using ANCOM [42] implemented in QIIME 2 2023.7 software. We compared the organs within each species, *S. convoluta* and *S. sellowii*, focusing on the differential abundance of the bacteriome in leaves and roots. Additionally, we compared the species, examining the differential abundance of the bacteriome in leaves and roots separately. For better visualization, we also generated a heatmap of the bacteriome abundance

for the 50 most significant bacteria in leaves and roots and compared species using QIIME 2 2023.7 software [43,44].

3. Results

3.1 Bacteriome composition

After filtering, we obtained 1,765,957 sequences from 74 samples, including 16 leaves of *S. convoluta*, 22 leaves of *S. sellowii*, 16 roots of *S. convoluta*, and 20 roots of *S. sellowii*. We identified 8,487 and 9,404 ASVs for *S. convoluta* and *S. sellowii*, respectively, spanning 34 bacterial phyla. For both *Selaginella* species, the dominant phyla were Actinobacteria, Proteobacteria, and Chloroflexi (Fig. 2 and Supplementary Fig. 1). Upon closer examination, the most prevalent bacterial families for both species were Kouleothrixaceae, Pseudonocardiaceae, and Sphingomonadaceae (Fig. 2 and Supplementary Fig. 1). Comparing leaf and root bacteriome composition, both presented Actinobacteria and Proteobacteria as the main phyla; however, there was an increase in the relative abundance of Acidobacteria, Chloroflexi, and Verrucomicrobia in the roots, while Cyanobacteria was more frequent in the leaves.

Alpha rarefaction plotting revealed no differences in the maximum values of the Shannon index when comparing species (Supplementary Fig. 2A). However, a comparison of plant organs showed that the lines approached a zero slope at different Shannon index values for root and leaf samples (approximately 8 and 6, respectively) (Supplementary Fig. 2B), suggesting greater heterogeneity in root samples as the Shannon index accounts for relative abundance. No significant differences were observed in the alpha diversity metrics of species samples ($p > 0.05$). In contrast, significant differences were noted in all alpha diversity metrics (observed

ASVs, Shannon index, Faith's phylogenetic diversity, and Pielou's evenness) between organs, with root samples consistently exhibiting higher levels (data not shown).

3.2 Taxon exclusivity and sharing patterns

The PCoA plots for each beta diversity metric revealed the clustering of organ and species samples. Leaf and root samples were differentiated along Axis 1, whereas species samples were distinguished along Axis 2 (Fig. 3; Supplementary Table 1, PERMANOVA test statistic = 1.83832; $p = 0.001$). Both organs and species significantly explained the unweighted UniFrac distance (Fig. 4; Supplementary Table 2, PERMANOVA test statistic = 8.56; $p = 0.001$). We compared samples from the same organ and species collected at different points (plants were collected in pairs) and detected no statistical differences in microbiome composition (data not shown; $p > 0.05$). This finding underscores that the influence of organs and species on the bacterial community composition is more pronounced than spatial proximity (Supplementary Tables 1 and 2).

The Venn diagram, derived from barplot data, illustrates the number of unique and shared bacterial taxa at the family level among the sampled species and organs (Fig. 5). The results indicate that *S. convoluta* and *S. sellowii* shared 12.9% of bacterial taxa (117 out of a total of 902; Fig. 5). In contrast, 3.8% were specific to S. convoluta, and 4.1% were specific to S. sellowii. The roots exhibited the highest number of unique taxa at 5.4%, whereas the leaves contained only 1.3%. Selaginella convoluta and S. sellowii shared only one bacteria taxa in the leaves; the bacteriome specific to the S. convoluta leaf was 0.99%, and for S. sellowii, it was a mere 0.33%. Finally, the species share 7.4% of bacterial taxa at roots.

3.3 Bacteriome differential abundance

The ANCOM results for the bacteriome of leaves indicated significantly different ASV abundances between *S. convoluta* and *S. sellowii* (Fig. 6A; W >191; Supplementary Table 3). Two ASVs from the order Actinomycetales and one ASV from the phylum Proteobacteria were highly abundant in *S. convoluta*. In contrast, *S. sellowii* exhibited only one differentially abundant ASV from the order Burkholderiales. Similarly, the bacteriome of roots revealed significant differences in ASV abundance between species (Fig. 6B; W > 380; Supplementary Table 3). The orders Pseudanabaenales, Actinomycetales, and Anaeroplasmatales (one ASV each), along with the phyla Proteobacteria and Cyanobacteria (one ASV each), showed high differential abundance. The ASVs from Pseudanabaenales and Cyanobacteria were more abundant in *S. convoluta*, whereas those from Actinomycetales, Anaeroplasmatales, and Proteobacteria were more abundant in *S. sellowii*.

Results of ANCOM revealed significant differences between the root and leaf tissues of the species. In *S. convoluta*, orders such as Actinomycetales, Rhizobiales, Cytophagales, and Sphingomonadales exhibited high differential abundance (Fig. 7A; W > 279; Supplementary Table 4), predominantly in the leaf. For *S. sellowii*, ANCOM results also indicated significant differences between organs (Fig. 7B; W > 284; Supplementary Table 4). Orders with high differential abundance included Roseiflexales, Actinomycetales, Gaiellales, Rhizobiales, Burkholderiales, Myxococcales, Cytophagales, Pseudomonadales, Gemmatales, Solirubrobacterales, Nitrososphaerales, and Caldilineales. Most orders were more abundant in the root, except for Burkholderiales, Cytophagales, and Rhizobiales (Methylobacteriaceae, *Methylobacterium*), which were more abundant in the leaf.

The heatmap analysis depicted the ASVs (rows) common to each sample and the frequency of their shared occurrence (Fig. 8). In the root samples, a similarity of 97% showed the presence of 50 ASVs, of which 94% were shared between *S. convoluta* and *S. sellowii*. Additionally, the

analysis identified a unique subset of ASVs in the roots of *S. convoluta* and *S. sellowii*, accounting for 4% and 2% of the total, respectively. In the leaf samples, a similarity of 97% and occurrence of 50 ASVs was observed, with 58% shared between the two species. The analysis also highlighted the ASVs predominantly more abundant in *S. convoluta* (ca. 30%) that were not shared with *S. sellowii*.

4. Discussion

Plants are intimately associated with a taxonomically diverse community of microorganisms that can establish complex and dynamic interactions with the microbiota. Influenced by both the environment and host genotype, these interactions can enhance plant resilience to biotic and abiotic factors, such as diseases, drought, life cycle phenology, and environmental stresses [45]. Plants have evolved a multilayered microbial management system to incorporate the most beneficial microbes [46]. The present study revealed variations in plant-associated bacterial communities between the leaf and root organs of two desiccation-tolerant *Selaginella* species (*S. convoluta* and *S. sellowii*) (Fig. 2). These variations in microbial communities suggest a significant selective force within plant compartments that shapes the plant-associated bacterial communities, including epiphytes and endophytes [47].

The bacterial richness observed in the roots of *S. convoluta* and *S. sellowii* was greater than that in the leaves. We sampled *Selaginella sp*. during a 35-day period of reduced rainfall. Some Cerrado plants lose all or part of their leaves during the dry season, consequently increasing soil biomass and biogeochemical processes in the rainy season [48]. This leads us to suggest that the low abundance of bacterial groups on the leaves may be related to the lack of environmental humidity. On the other hand, the presence of bacterial groups in the root is

related to the protection that the soil offers to this plant structure, which allows the survival of bacteria in the long term by regulating the balance of microorganisms in the root environment to promote the health and survival of both [49]. Noteworthily, the leaf bacteriome was relatively consistent at the phylum level; however, remarkable differences were evident at the family level. This demonstrates that microbial community profiling reveals significant differences even among plants of different species growing adjacent to one another [50].

The bacterial community composition at the family level on *Selaginella* was enriched with Pseudonocardiaceae, Nocardioidaceae, Comamonadaceae, and Nakamurellaceae (Fig. 6A). This enrichment may be because of the robust ability of these bacteria to produce siderophores (compounds that aid plants to acquire iron from the soil), their ability to grow under specific soil conditions such as rocky outcrops, and their capacity to regulate the abundance and diversity of fungi at the root interface, thus ensuring the overall health of the plant [51,52]. The families identified in *Selaginella* leaves are commonly associated with soil, suggesting that their presence in the leaves is linked to the structural characteristics of *Selaginella*. As a low-lying plant, its leaves are in constant contact with the soil, promoting interactions between the leaf-associated and soil bacteria.

The bacterial composition of *Selaginella* roots primarily comprised members from the Streptosporangiaceae, Anaeroplasmataceae, Pseudanabaenaceae, and Methylobacteriaceae families (Fig. 6B). These bacteria are crucial for maintaining plant health and fitness, as they promote growth, enhance stress resistance, and facilitate the mobilization, transport, and uptake of nutrients [52-54]. The presence of these bacteria may be linked to the climatic conditions of the Brazilian savanna and the nutrient-poor, shallow, and acidic soils of the rocky outcrops [55]. Additionally, members of the phylum Actinobacteria, encountered in abundance on roots in the present study, were significantly enriched during the dry season [56]. Consequently, plant

adaptations include the association with endophytic bacteria that can positively influence the nutrient status in plants, provide protection against pathogens and pests, and improve stress tolerance, all while modulating plant development [57].

The plant-bacteria relationship is crucial for nutritional adaptations in environments such as the Cerrado. The monthly average precipitation exhibits marked seasonality, peaking in spring and summer (October to March), which corresponds to the rainy season. However, from May to September, monthly precipitation levels decrease significantly, potentially reaching zero [58]. These relationships play essential roles in influencing host responses to both biotic and abiotic stresses and modulate plant phenotypic plasticity [59-61]. The present study underscores the significance of bacteria in supporting the growth, maintenance, and survival of *Selaginella*. It also emphasizes that plants should not be studied in isolation but as units comprising hosts and their associated microbiota.

Additional initiatives are necessary to gain in-depth and potentially predictive insights into the interactions between plants and their microbiomes, as these dynamics and biodiversity remain underexplored. Understanding these relationships is crucial for predicting how beneficial plant-microbiome interactions will respond to environmental stresses. Leveraging this knowledge will facilitate the prediction of climate change impacts on plant-associated microbiomes, creating opportunities for novel research that enhances our understanding of plant-microbiome interactions and improves the climate resilience of plant communities.

Data accessibility

The 16S rRNA data are available in the NCBI SRA under the accession number PRJNA1063726. Metadata are available upon request.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

The supplementary data for this article are available online at _____.

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Subtitle of Figures

Fig. 1. (A) Collection area of the Selaginella samples. (B) *Selaginella convoluta*, February 2021, Morro do Ernesto, Campo Grande (MS). (C) *Selaginella convoluta* and some branches of *S. sellowii*, August 2021, Morro do Ernesto, Campo Grande (MS), Brazil.

Fig. 2. Bacteriome diversity composition at bacterium family level, when possible, or the lowest taxonomy level identified by Qiime2. Data are presented for the (A) *Selaginella convoluta* and (B) *Selaginella sellowii* species and samples organized by organ, leaf and root. The stacked bars are representations of each sample and colored fragments represent the fraction of each sample assigned to each family. The complete legend of each taxon can be found in the Supplementary Fig. S1A and S1B.

Fig. 3. Similarities among bacterial communities from the leaf (red) and root (blue) of *Selaginella convoluta* and leaf (yellow) and root (green) of *Selaginella sellowii*. The similarities were calculated by Jaccard distance (test statistic = 1.83832; $p = 0.001$), using the Qiime2 software (version 2023.7).

Fig. 4. Beta diversity metrics for each species and organ of *Selaginella* (from Qiime2 software, version 2023.7). Beta diversity group significance using unweighted UniFrac distance, based on the ASV count (test statistic = 8.56; $p = 0.001$). Distance values of: (A) leaf of *S. convoluta*; (B) root of *S. convoluta*; (C) leaf of *S. sellowii*; and (D) root of *S. sellowii*.

Fig. 5. Venn diagrams for each species and organ of *Selaginella*. Together with the name of the subgroup, the total number of variants represented is indicated in parentheses. The number of variants that are shared between the subgroups is shown at the intersections of the sets.

Fig. 6. Differential abundance analysis using ANCOM on Qiime2 software (version 2023.7), comparing species, *S. convoluta* and *S. sellowii*, by organs: (A) Leaf; (B) Root.

Fig. 7. Differential abundance analysis using ANCOM on Qiime2 software (version 2023.7) comparing samples of leaf and root by species: (A) *S. convoluta*; (B) *S. sellowii*.

Fig. 8. Heatmap based on the distance matrix of UniFrac dissimilarity of the *S. convoluta* and *S. sellowii* organs bacteriome. Annotations on top of the heatmap show the species and on the bottom the organ.

Figure S1. Links for the complete barplot subtitles. (A) for *Selaginella convoluta*: https://drive.google.com/file/d/1ubtI0XJmQoo_1G9opkTl5vdJPSeU2C13/view?usp=sharing; (B) for *Selaginella sellowii*: https://drive.google.com/file/d/1QWAXwi7XMtLmYWG9U1BvdAroEPbosFUy/view?usp=sharing

Figure S2. Alpha rarefaction plotting considering sequences obtained from (A) *Sellaginella* species and (B) organs.

Fig. 1

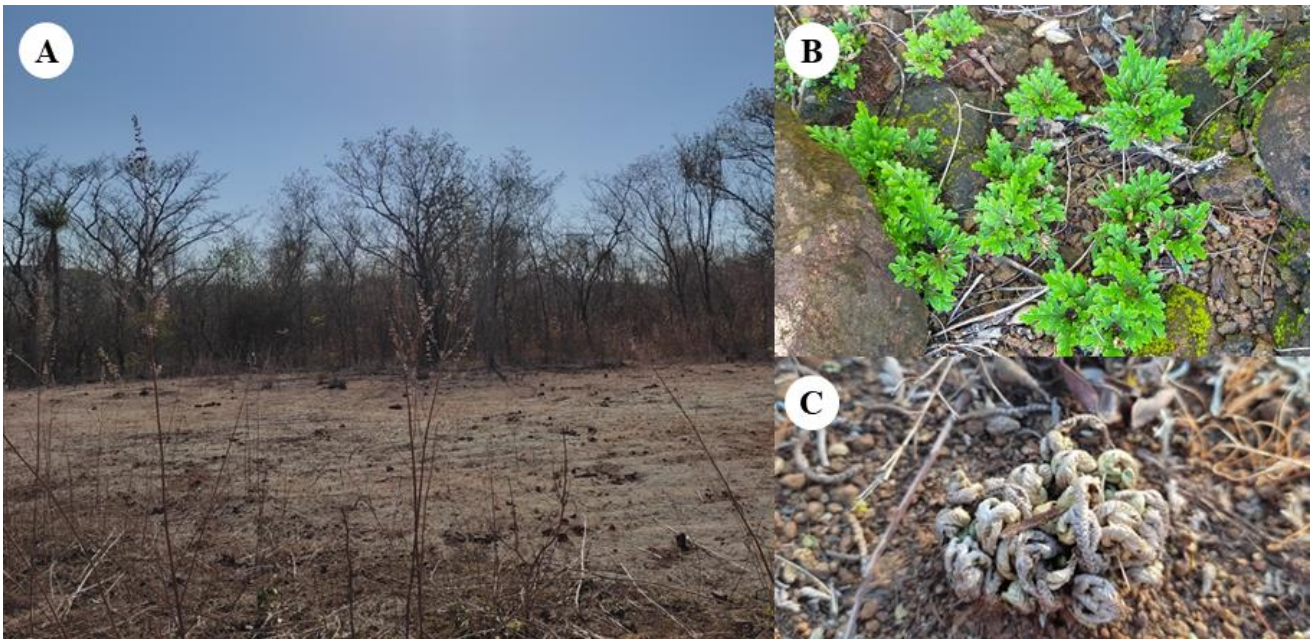
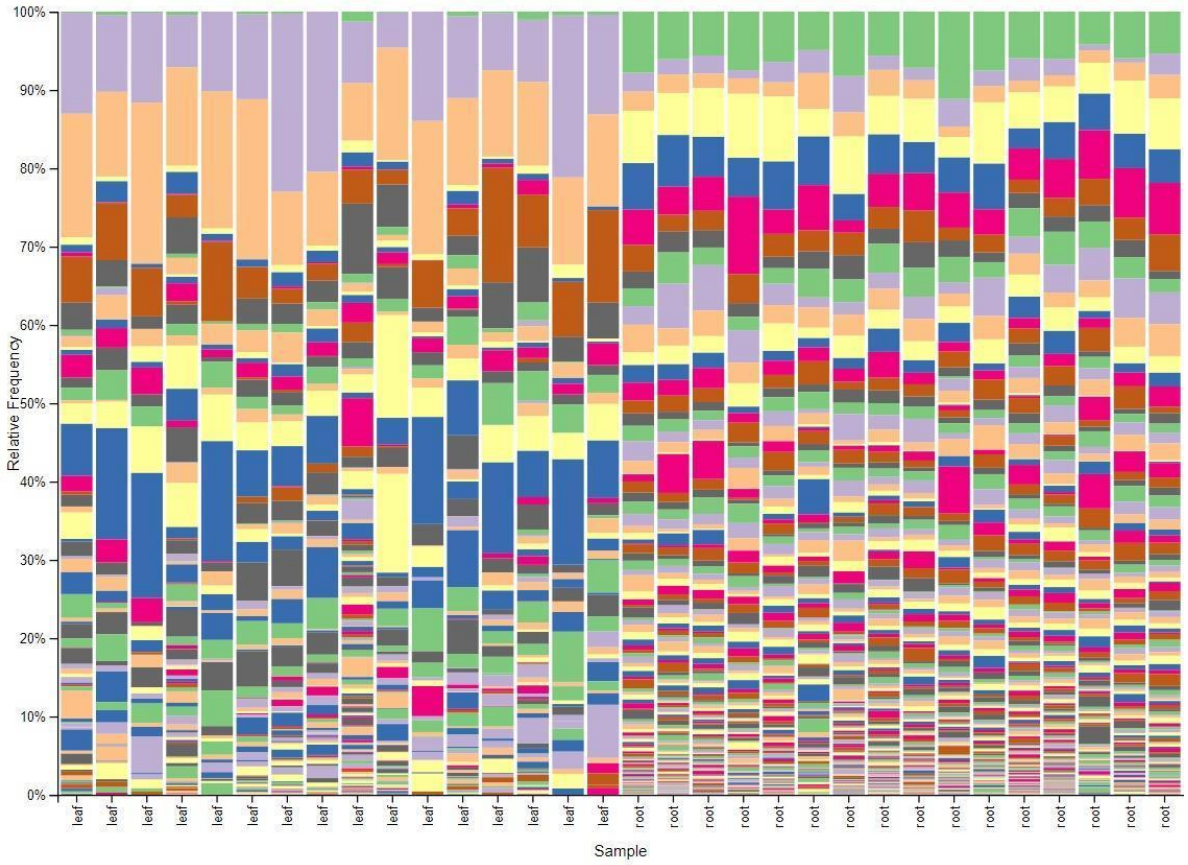
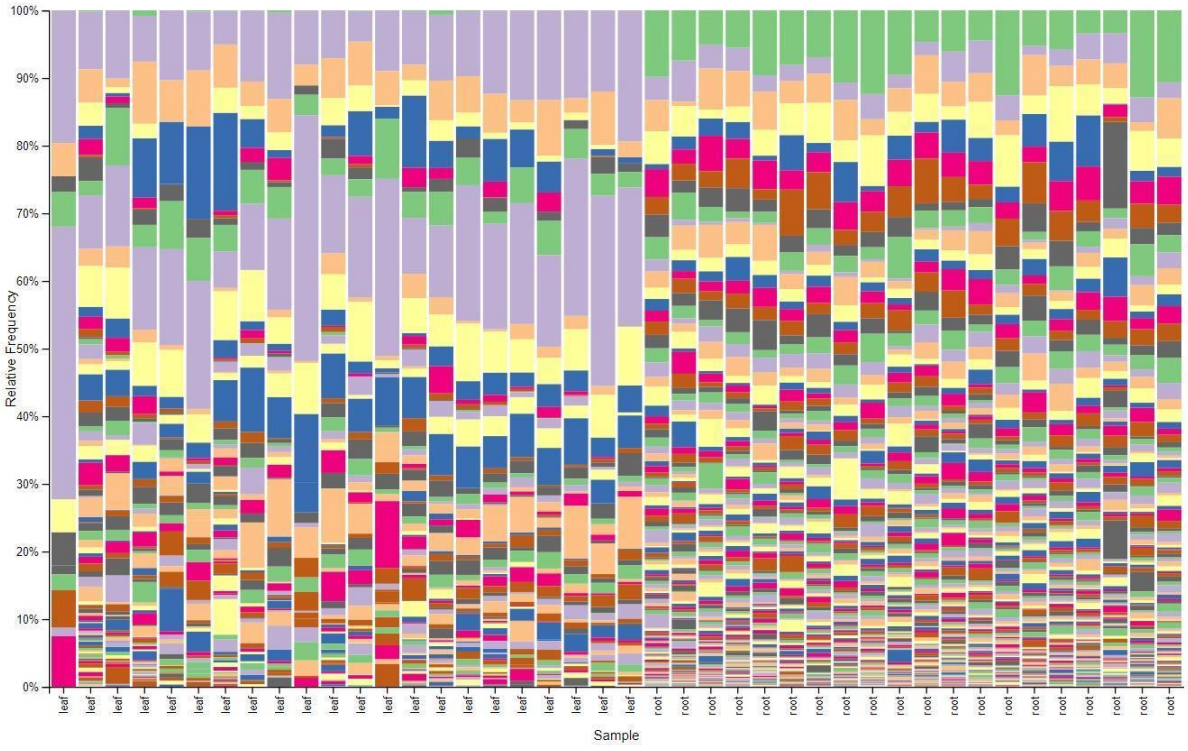

A
B
C

Fig. 2

A


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
leaf
root
Sample

B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Relative Frequency
leaf
root
Sample

Fig. 3

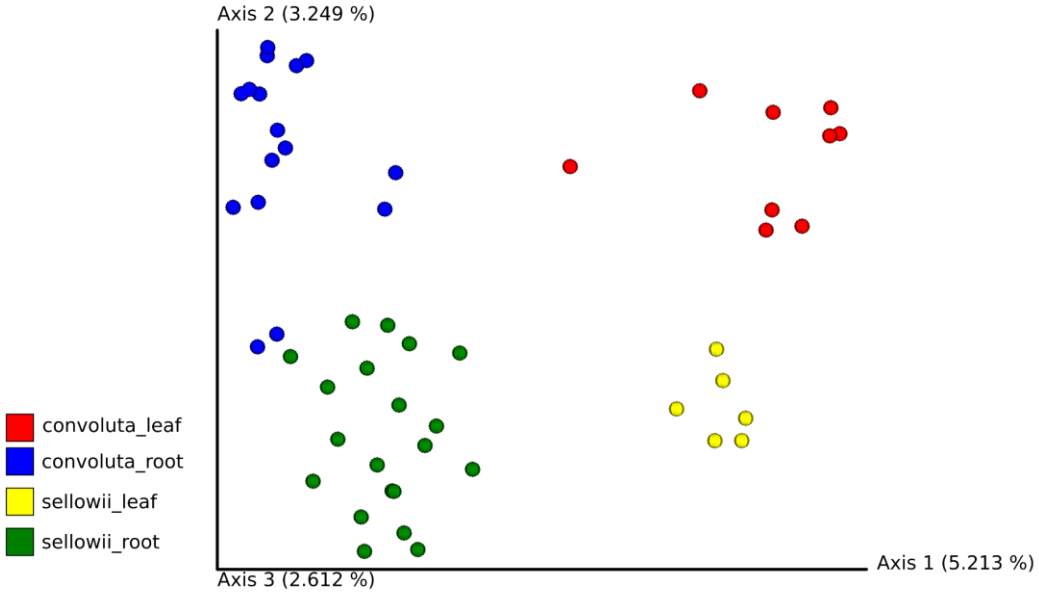

Axis 2 (3.249 %)
convoluta_leaf
convoluta_root
sellowii_leaf
sellowii_root
Axis 1 (5.213 %)
Axis 3 (2.612 %)

Fig. 4

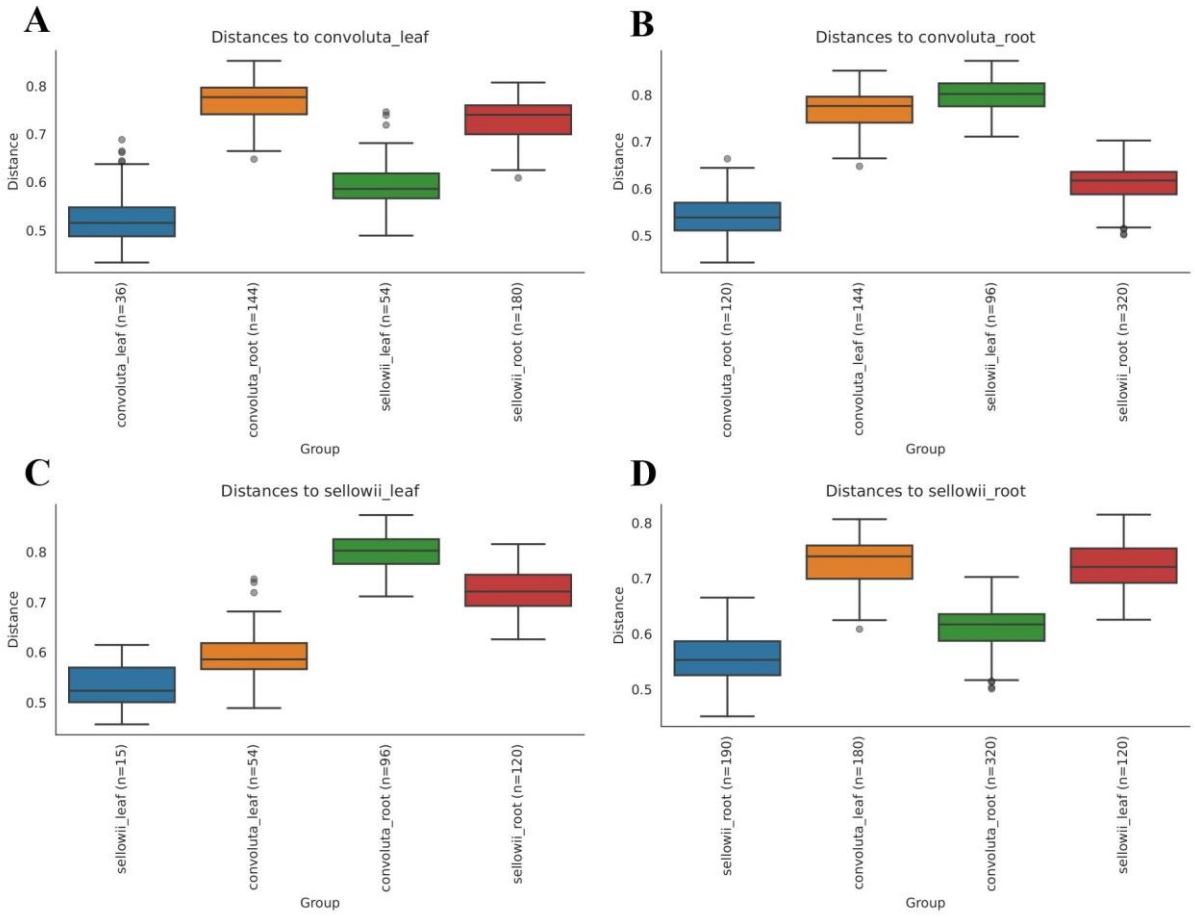
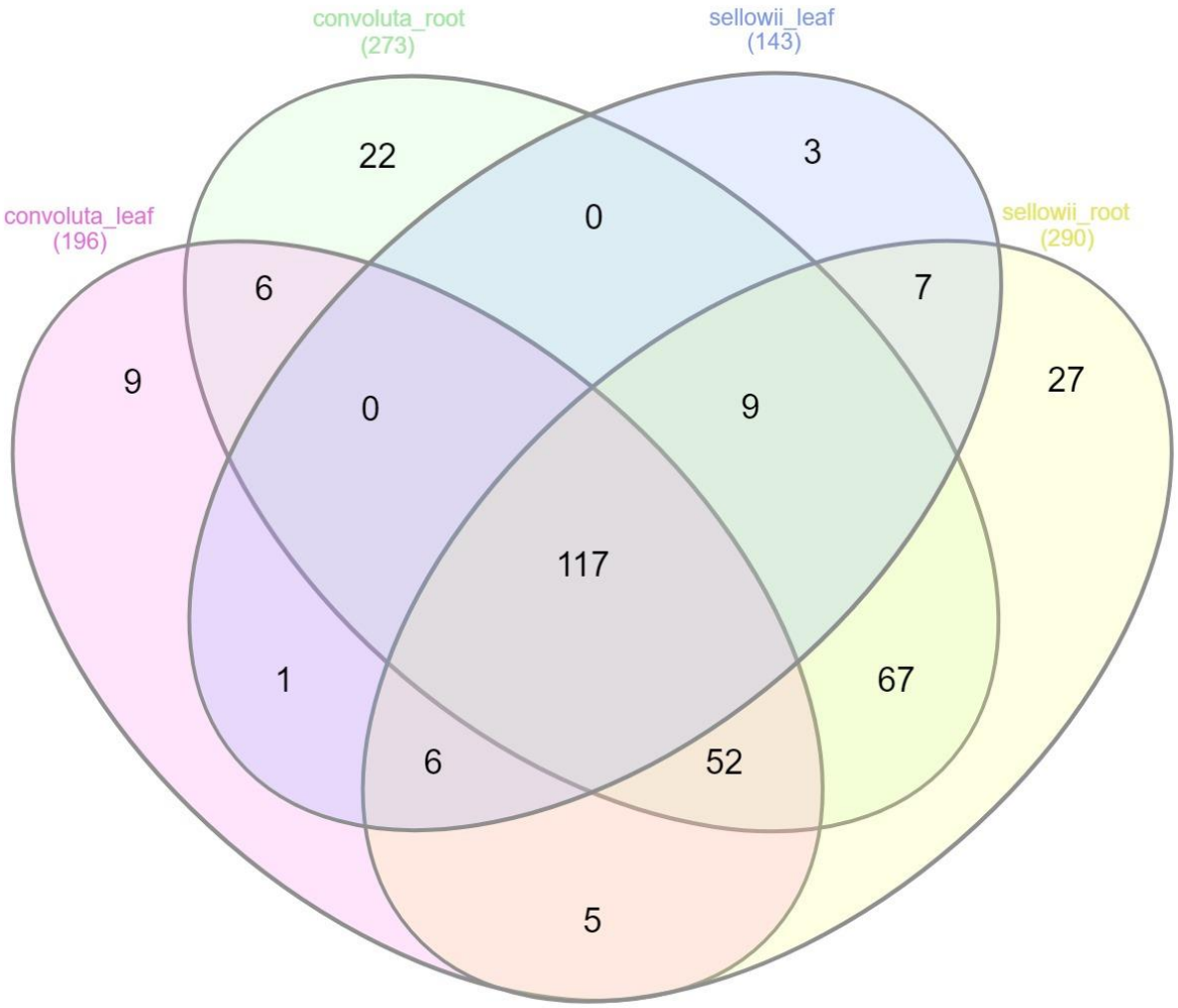

A
Distances to convoluta_leaf
Distance
0.8
0.7
0.6
0.5
convoluta_leaf (n=36)
convoluta_root (n=144)
sellowii_leaf (n=54)
sellowii_root (n=180)
Group
B
Distances to convoluta_root
Distance
0.8
0.7
0.6
0.5
convoluta_root (n=120)
convoluta_leaf (n=144)
sellowii_leaf (n=96)
sellowii_root (n=320)
Group
C
Distances to sellowii_leaf
Distance
0.8
0.7
0.6
0.5
sellowii_leaf (n=15)
convoluta_leaf (n=54)
convoluta_root (n=96)
sellowii_root (n=120)
Group
D
Distances to sellowii_root
Distance
0.8
0.7
0.6
0.5
sellowii_root (n=190)
convoluta_leaf (n=180)
convoluta_root (n=320)
sellowii_leaf (n=120)
Group

Fig. 5


convoluta_root
(273)
sellowii_leaf
(143)
convoluta_leaf
(196)
sellowii_root
(290)
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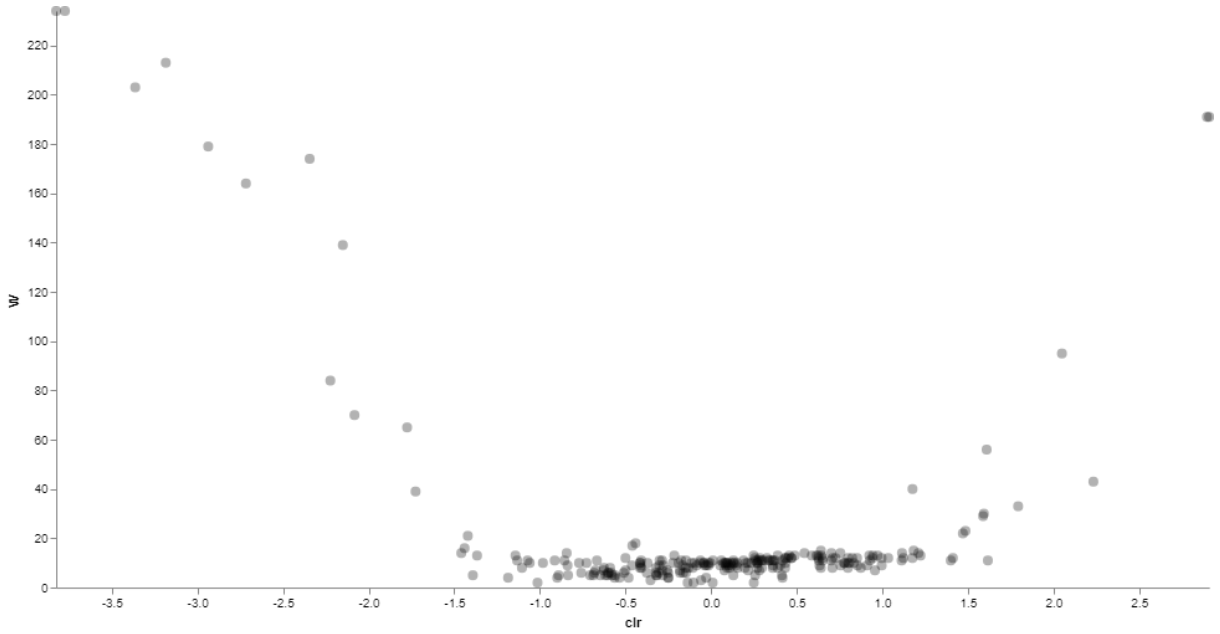
0
6
7
9
0
9
27
117
1
67
6
52
5

Fig. 6

A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200
180
160
140
120
100
80
60
40
20
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-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
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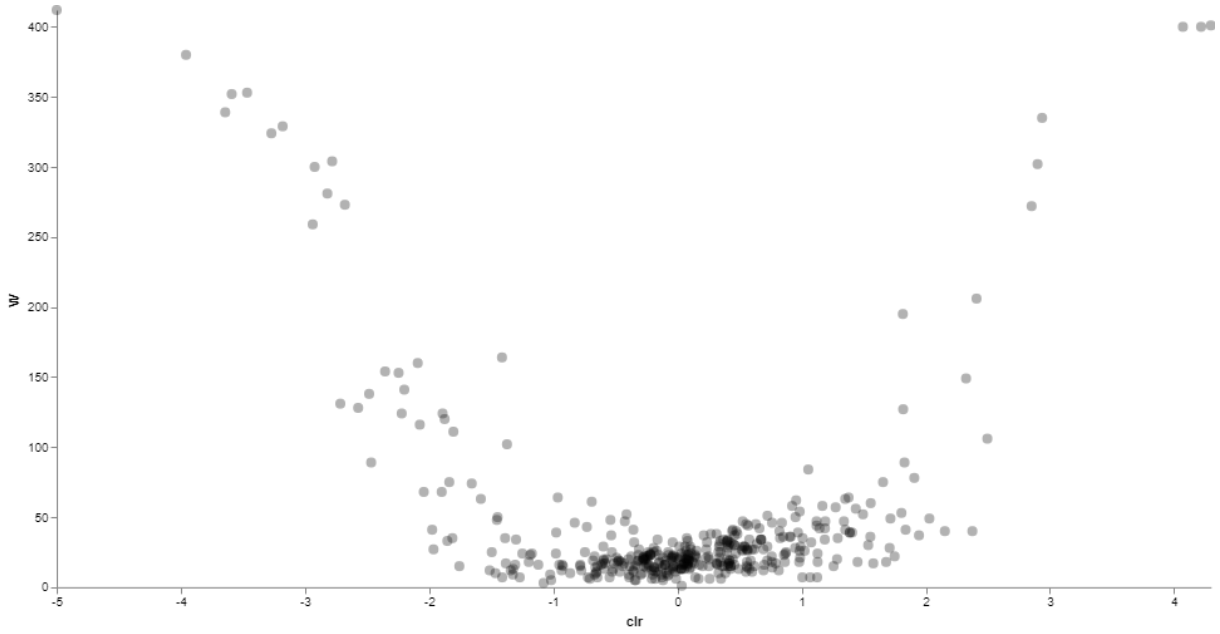
0.5
1.0
1.5
2.0
2.5

B


vr
clr
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350
300
250
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150
100
50
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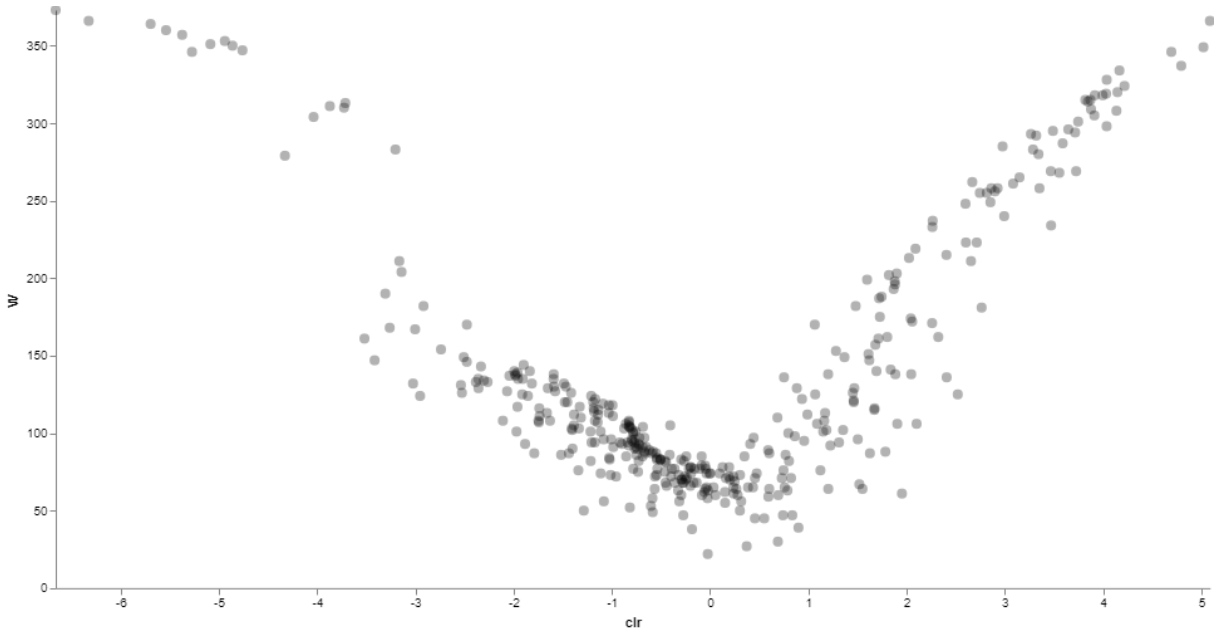
-5
-4
-3
-2
-1
0
1
2
3
4

Fig. 7

A


W
clr
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50
100
150
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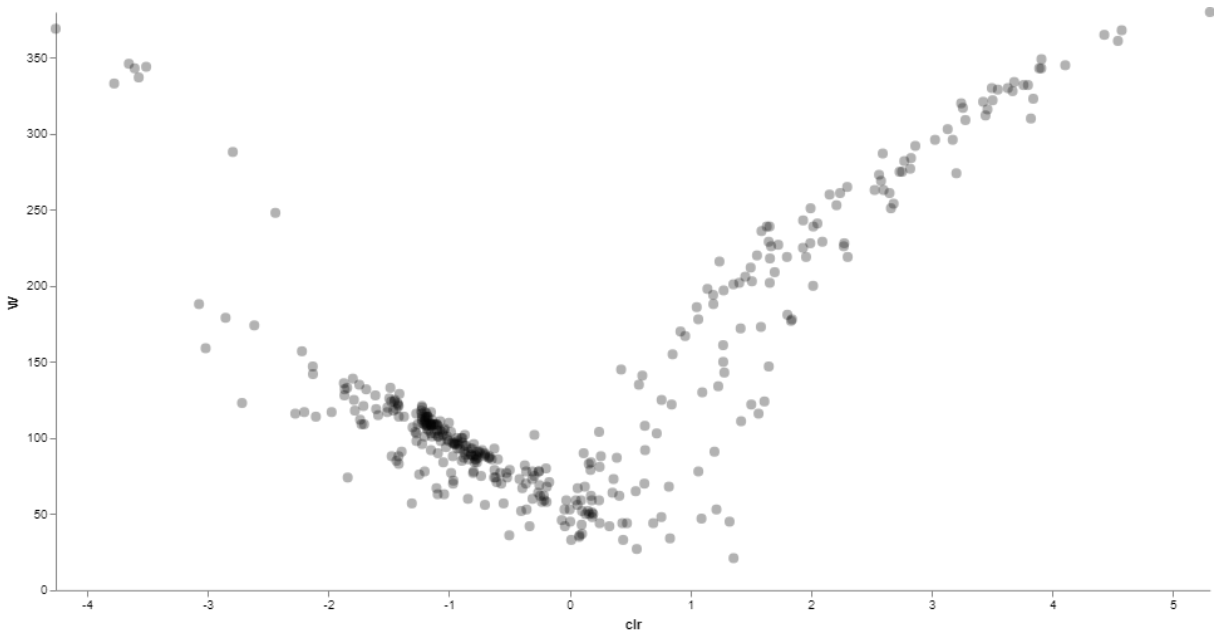
-5
-4
-3
-2
-1
0
1
2
3
4
5

B


W
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-4
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Fig. 8

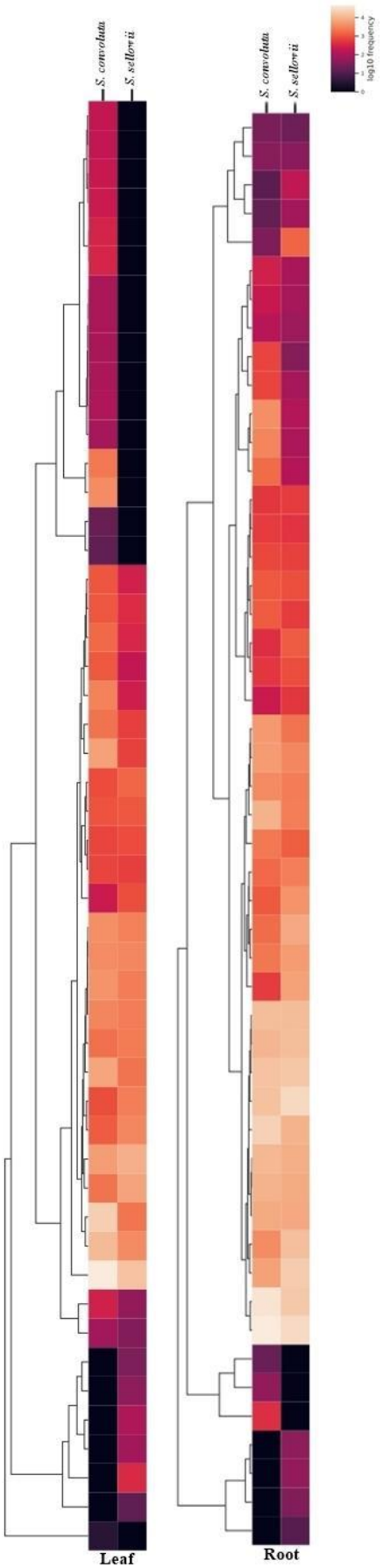
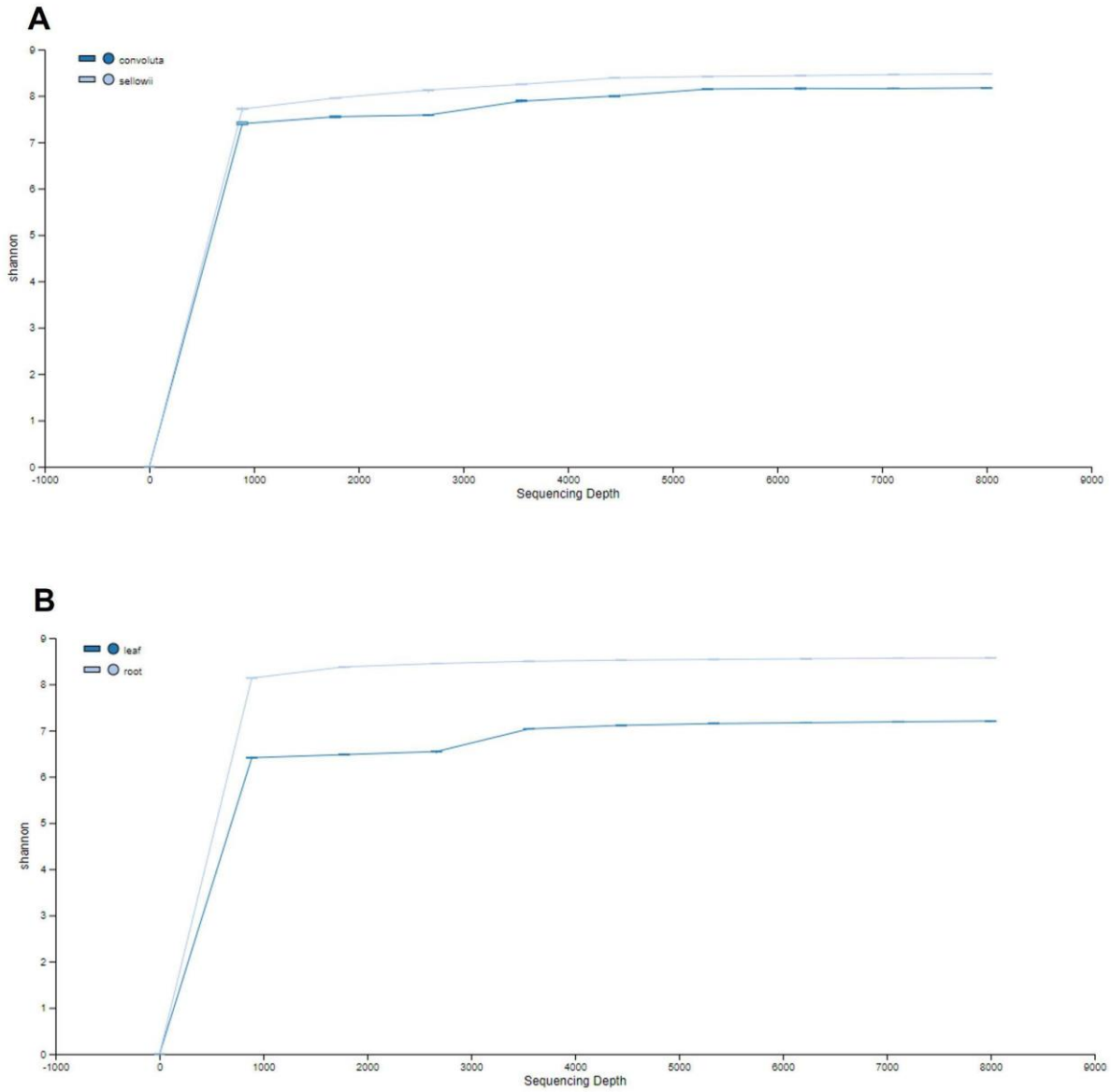

S. convoluta
S. sellowii
log10 frequency
S. convoluta
S. sellowii
Leaf
Root

Fig. S1. Links for the complete barplot subtitles. (A) for Selaginella convoluta: https://drive.google.com/file/d/1ubtI0XJmQoo_1G9opkTl5vdJPSeU2C13/view?usp=sharing; (B) for Selaginella sellowii: https://drive.google.com/file/d/1QWAXwi7XMtLmYWG9U1BvdAroEPbosFUy/view?usp=sharing

Fig. S2



Supplementary Table 1

Beta diversity analysis considering the overview and pairwise PERMANOVA results from Jaccard distance analysis for each species and organ of *Selaginella*. conv: *Selaginella convoluta*; sell: *Selaginella sellowii*.

Overview

Method name	PERMANOVA
Test statistic name	pseudo-F
Sample size	51
Number of groups	4
Test statistic	1.83832
p-value	0.001
Number of permutations	999

Pairwise

Pairwise PERMANOVA results

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
conv-leaf	conv-root	25	999	2.279003	0.001	0.001
	sell-leaf	15	999	1.428483	0.001	0.001
	sell-root	29	999	2.086366	0.001	0.001

	sell-leaf	22	999	1.994651	0.001	0.001
conv-root						
	sell-root	36	999	1.544665	0.001	0.001
sell-leaf	sell-root	26	999	1.654736	0.001	0.001

Supplementary Table 2

Beta diversity analysis considering the overview and pairwise PERMANOVA results from unweighted UniFrac analysis for each species and organ of *Selaginella*. conv: *Selaginella convoluta*; sell: *Selaginella sellowii*.

Overview

Method name	PERMANOVA
Test statistic name	pseudo-F
Sample size	51
Number of groups	4
Test statistic	8.56186
p-value	0.001
Number of permutations	999

Pairwise

Pairwise PERMANOVA results

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value

	conv-root	25	999	12.728975	0.001	0.001
conv-leaf	sell-leaf	15	999	2.727502	0.001	0.001
	sell-root	29	999	10.532591	0.001	0.001
	sell-leaf	22	999	11.428464	0.001	0.001
conv-root	sell-root	36	999	5.426848	0.001	0.001
sell-leaf	sell-root	26	999	7.738903	0.001	0.001

Supplementary Table 3. Statistical data from Ancom, considering species by organs.

https://docs.google.com/spreadsheets/d/1Wmorj50aLGUTJLHwwZcTMw3rKKIZh0Qak4xCRcMRtyE/edit?usp=sharing

Supplementary Table 4. Statistical data from Ancom, considering organs by species.

https://docs.google.com/spreadsheets/d/18iCig5kME9mbe1pAoRUxnRPQxqMKHhl1CfY0QRNT-RQ/edit?usp=sharing

CAPÍTULO 2

Unraveling bacteriome diversity using metabarcoding analysis of two co-occurring lichen species from the Cerrado biome: Dirinaria melanocarpa and D. rhodocladonica

À ser submetido à revista **The Lichenologist**

Unraveling bacteriome diversity using metabarcoding analysis of two co-occurring lichen species from the Cerrado biome: *Dirinaria melanocarpa* and *D. rhodocladonica*

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Abstract

Lichens represent intricate organisms formed through the symbiotic relationship between a fungus, algae or cyanobacteria, and various microorganisms. They serve as crucial components of ecosystems and demonstrate remarkable resilience to environmental changes. This article aims to identify the bacteriome communities associated with two lichen species, *Dirinaria melanocarpa* and *D. rhodocladonica*, inhabiting the Cerrado biome in central Brazil utilizing the 16S rRNA (V4 region) gene. Our analysis revealed significant differences in the bacterial community composition of the two species, with the dominance of four phyla: Verrucomicrobia, Proteobacteria, Planctomycetes, and Actinobacteria. Furthermore, the bacterial communities present on the lichen thalli differed from those observed on the substrate, suggesting a selective process by the lichen for specific bacteria, likely crucial to its health and function. Overall, this study offers valuable insights into the diverse and indispensable bacterial communities associated with lichens, particularly within the context of *Dirinaria* species in the Cerrado biome. These findings underscore the importance of investigating these intricate bacteriome interactions to gain a deeper understanding of lichen ecology and their ecosystem roles.

Keywords: Bacteriome, lichenized fungi, dry season, biodiversity, ecological interactions, Brazilian savanna.

Introduction

The Cerrado savannas extends over 2 million km^2, encompassing 23% of the Brazilian territory. The landscape of this biome exhibits high heterogeneity, thriving under a variable mean annual precipitation regime ranging from 800 to 2000 mm. Over 90% of the area experiences a six-month arid dry season, typically between April and September (Nascimento & Novais 2020; Gamarra *et al.* 2021). Owing to its high species richness and endemism rates, the Cerrado is recognized as a global biodiversity hotspot but faces significant threats due to accelerated agricultural expansion and vegetation conversion (Myers *et al.* 2000; Pompeu *et al.* 2024). The Cerrado plays a critical role in regulating freshwater resources, maintaining biodiversity, and providing essential ecosystem services in Brazil (Overbeck *et al.* 2015).

Widespread across terrestrial habitats, lichens result from symbiotic associations between a fungus (mycobiont) and one or more photosynthetic partners (photobionts), typically green algae or cyanobacteria (Spribille *et al.* 2016), along with associated microorganisms (Grube *et al.* 2009; Grube & Wedin 2016; Hawksworth & Grube 2020). They play a significant role in mineral cycling and global energy flow and can exhibit a diverse array of morphological structures and growth patterns, encompassing brushy, crust-like, or leaf-like forms, among others, representing significant variations in their appearance and growth characteristics (Grimm *et al.* 2021). Lichens are also known for their highly diverse microbial populations, mainly composed of mycobionts (including Basidiomycetes yeasts), photobionts, and complex bacteriomes (Swamy & Gayathri 2021). This makes the lichen thallus a bio-network of both prokaryotes and eukaryotes, forming a miniature ecosystem (Anderson 2014; Hawksworth & Grube 2020).

Several studies have shed light on the diverse roles played by these bacteria in shaping the lichen's ecological niche and enhancing its resilience (Grimm *et al.* 2021). The bacteriome

contributes to multiple essential functions within the lichen system. It facilitates nutrient acquisition by aiding in the uptake and assimilation of various substances like iron, phosphate, sulfur, amino acids, dipeptides, sugar, and xylose (Erlacher *et al.* 2015). Additionally, the bacterial community enhances the lichen's resilience against non-living stressors such as toxic environmental compounds, oxidative or osmotic stress, and also facilitates processes like growth hormone production and nitrogen fixation (Cernava *et al.* 2017).

The Cerrado, a biodiversity hotspot, harbors a wealth of unknown lichen microbiomes. This initial study represents the first comprehensive analysis of *Dirinaria melanocarpa* (Müll. Arg.) C.W. Dodge and *Dirinaria rhodocladonica* (Kalb, Schumm & Elix) bacteriomes in an urban area within the Cerrado biome, providing valuable insights into their bacterial diversity and symbiotic relationships.

Material and Methods

Studied area and species

Dirinaria melanocarpa and *D. rhodocladonica* thalli were individually collected on the bark of *Pachira aquatica* Aubl. (Malvaceae) trees for substrate standardization. Two collections were conducted in the Parque dos Poderes, an urban park in Campo Grande city, Mato Grosso do Sul state (-20.456740/-54.563724). The first collection was at the end of the dry season, with a daily average of 30% relative humidity on August 26th, 2021, when there had been a shortage of rainfall in the city for 35 days. The second collection occurred on August 30th, following a 6 mm rainfall and an 86% relative humidity daily average. We collected 28 samples, which were transported to the laboratory into ice on the same day. The lichen samples were carefully separated from their substrates using sterile tweezers and scalpels, cut into small pieces weighing ± 0.25 g, and transferred to sterile 1.5 mL microtubes before being stored at -80°C.

DNA extraction and 16S rRNA sequencing

DNA was extracted from 0.25 g of each sample using the MagMax™ Microbiome Ultra Nucleic Acid Isolation kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The 16S rRNA region was amplified in triplicate using the primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caropaso *et al.* 2011) to target the V4 region. The PCR (Polymerase Chain Reaction) mixture contained 22.5 µL of Platinum PCR SuperMix High Fidelity (Invitrogen™, Thermo Fisher Scientific Inc.), 2 µL of genomic DNA (~20 ng/µL), and 0.5 µL of each 10 µM primer. The PCR protocol began with an initial step at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 1 min. The triplicate reactions were combined and purified in two rounds using AMPure® XP reagent (Beckman Coulter, Indianapolis, IN, USA). Quantification was performed on a Qubit® instrument, and samples were diluted to 40 pM. The amplicon pool was then processed on the Ion Chef™ System (Thermo Fisher Scientific) for emulsion PCR, enrichment, and loading onto an Ion S5 530 chip. Sequencing was carried out using 850 flows on the Ion GeneStudio S5 System, following the manufacturer's instructions (Life Technologies, Carlsbad, California).

Sequence treatment

Bioinformatic analyses were conducted using QIIME 2 2023.7 (Bolyen *et al.* 2019). The raw sequence data were demultiplexed with the q2-demux plugin and subsequently quality-filtered using DADA2 (Callahan *et al.* 2016). All amplicon sequence variants (ASVs) were aligned with MAFFT (Katoh *et al.* 2002), and the resulting alignment facilitated the construction of a phylogeny with FastTree 2 (Price *et al.* 2010). Taxonomy was assigned to ASVs with the classify-sklearn naive Bayes taxonomy classifier (Bokulich *et al.* 2018a) against the Greengenes 13_8 99% ASVs reference sequences (McDonald *et al.* 2011). We excluded

sequences from all samples that met at least one of the following criteria: 1) fewer than 5 copies, 2) originating from mitochondria or chloroplasts, 3) identified solely at the phylum level.

Diversity analyses

Alpha-diversity metrics were estimated using Shannon diversity index (Shannon 1948), Observed Features, Faith's Phylogenetic Diversity (Faith 1992) and Evenness, along with the beta-diversity metrics, weighted UniFrac (Lozupone *et al.* 2007), unweighted UniFrac (Lozupone *et al.* 2005), Jaccard distance, and Bray–Curtis dissimilarity, were estimated after rarefying to 8000 sequences per sample. Venn diagrams were generated on the InteractiVenn web platform (http://www.interactivenn.net/; Heberle *et al.* 2015), visualizing the fractions of shared and exclusive variants at the family level. We compared the substrates and thallus within each species, *D. melanocarpa,* and *D. rhodocladonica,* focusing on the differential abundance of the bacteriome in species-substrates. Additionally, we compared the samples, examining the differential abundance of the bacteriome between thallus and substrates separately. For enhanced visualization, we generated a heatmap of the bacteriome abundance for the 50 most significant bacteria in species for thallus and substrates and compared species using QIIME 2 2023.7 software (Bokulich *et al.* 2018b; Pedregosa *et al.* 2011).

Results

Taxonomic assignments of bacterial communities and □-Diversity

Eleven phorophytes were selected. During the dry period, a total of 23 samples were collected, of which 12 were from thallus and 11 from substrate. Conversely, during the wet period, 18 samples were obtained, of which 11 were from thallus and 7 from substrate. After filtering, we obtained a total of 726,342 sequences from 28 samples. The frequency of sequences per sample varied from 923 (minimum) 87,926 (maximum), with a mean frequency of 25,940. From all these sequences, we obtained 5,554 ASV's referred to as features. For both *Dirinaria* species, the dominant phyla were Verrucomicrobia, Proteobacteria, Planctomycetes, and Actinobacteria. Within the *Dirinaria* species, there was a higher predominance of the Alphaproteobacteria and Planctomycetia classes. Upon closer examination, we observed that the most dominant orders were Sphingomonadales, Chthonomonadales, Rhodospirillales, Rhizobiales, and Gemmatales (Fig. 1A; Fig. S1). The primary phyla identified on the bacteriome of *D. melanocarpa* was Proteobacteria, and of *D. rhodocladonica* was Verrucomicrobia. Comparing thallus and substrate (not considering the *Dirinaria* species), the main phyla were Verrucomicrobia, Proteobacteria, Planctomycetes, and Armatimonadetes (Fig. 1B; Fig. S1). The differences become evident when we observe the predominant orders for each type. For the thallus, the most prevalent orders were Rhizobiales, Sphingomonadales, and Chthoniobacterales, and for the substrate, Actinomycetales and Deinococcales. By plotting the Shannon's diversity index as a function of sequencing depth among the different species, thallus and substrates, we observed a slight difference in the maximum number of features among them (Fig. S2); the same can be observed with Faith's Phylogenetic Diversity (Fig. S3). This finding implies that samples exhibit greater heterogeneity compared to thallus and substrate samples. Alpha diversity metrics showed no significant differences regarding *Dirinaria* species and thallus/substrate samples (Fig. 2A-D).

-*Diversity, taxon exclusivity, and sharing patterns*

We compared samples from the same species collected at different periods where the humidity ranged from 30% to 82% (plants were collected in pairs - thallus and substrate - on the same phorophyte) and detected no statistical differences in microbiome composition (data not shown; $p > 0.05$). Therefore, humidity was not relevant enough to generate a difference in the microbiome.

Using Jaccard and Bray-Curtis similarity indices, beta diversity metrics, demonstrated a striking dissimilarity ($p = 0.001$) between the bacterial communities of the lichen thallus and the substrate (Fig. 3A & B). The dissimilarity between bacterial communities of lichen thalli (*D. melanocarpa* and *D. rhodocladonica*) and their corresponding substrates was assessed using unweighted (qualitatively) and weighted (quantitatively) UniFrac distances (Fig. 4A & B). These metrics consider the phylogenetic relationships between taxa to assess compositional differences between communities. These results showed that the communities present in the thallus are different from those found in the substrate, disregarding *Dirinaria* species (Supplementary Tables 1 & 2).

The Venn diagram, constructed using family-level bacterial taxa identified from barplot data, demonstrated the distribution of unique and shared taxa between *Dirinaria* species, by thallus and substrate samples (Fig. 5). The results indicate that the samples shared 14.9% of bacterial taxa (in a total of 677). When we analyze the specificities for each thallus species, *D. melanocarpa* has 9.8% of exclusive taxa (in a total of 153) and 4.3% were exclusive for *D. rhodocladonica* (in a total of 163). The substrate of *D. melanocarpa* shares 2.4% of bacterial taxa with thallus, and has 8.3% of exclusive taxa (180). For substrates of *D. rhodocladonica*, 1.7% were shared with thallus, and 12.2% were exclusive taxa (181). The exclusive taxa of each sample can be observed in Tables 1 and 2 at the family level.

Bacteriome differential abundance

The heatmap analysis depicted the ASVs (rows) more common to each sample and the frequency of their shared occurrence (Fig. 6). Among the 50 ASVs, the analysis showed approximately 86% similarity between thallus samples of *D. melanocarpa* and *D. rhodocladonica* which corresponds to 43 ASVs. For thallus-exclusive ASVs, we have 6% in the *D. melanocarpa* and 8% in the *D. rhodocladonic*a. In the substrate, for *D. melanocarpa* and *D. rhodocladonica* samples, the similarity was approximately 94% for the 50 ASVs, of which 47 ASVs were shared. Additionally, the analysis identified a unique subset of ASVs in the *D. melanocarpa* and *D. rhodocladonica* substrate, accounting for approximately 2% and 4% of the total, respectively.

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Discussion

Following the notion that lichens are not single organisms but rather complex symbiotic consortia (Hawksworth & Grube 2020), this study delves into the microbial diversity associated

with *Dirinaria* species. Lichens are poikilohydric organisms lacking the ability to maintain and actively regulate their water content (Grimm *et al.* 2021). This means their water status passively varies with the surrounding environment. Besides, many lichens have evolved extraordinary tolerance to desiccation. This tolerance includes antioxidant and photoprotective mechanisms (Kranner *et al.* 2008), as well as strategies enabling them to cope with mechanical stress under changing hydration conditions. This remarkable desiccation tolerance likely explains why, in this study, the associated microbiota remained relatively unaltered across different humidity periods. Long-lived lichen thalli show stable bacterial communities throughout the seasons, further highlighting the resilience of lichens to environmental fluctuations (Grube *et al.* 2009).

A lichen thallus provides as a habitat for a diverse array of bacteria, potentially hosting over 800 bacterial species (Grimm *et al.* 2021). Analysis of the bacterial communities associated with *Dirinaria* species revealed a dominance of four phyla: Verrucomicrobia, Proteobacteria, Planctomycetes, and Actinobacteria. The increase in Actinobacteria abundance under stressful conditions aligns with research demonstrating their capacity to enhance plant drought tolerance and growth (Bonatelli *et al.* 2021). While not directly tested, given the natural occurrence of water stress periods in the Cerrado region, our study adds weight to the Zhang *et al.* (2023) hypotheses. These suggest similarities in the functioning of this bacterial group, indicating a potential relationship between Actinobacteria and lichen growth, as well as nutrient acquisition. In our analysis, we also identified members of the Sphingomonadales and Rhizobiales orders, known for their facultative photosynthetic abilities and nitrogen-fixing symbiosis with plants, respectively (Long 1989; Erlacher *et al.* 2015). These bacterial partners likely play a role in producing specific secondary metabolites and cycling nutrients within the lichen symbiosis. Their influence directly impacts the growth of the lichen thallus (Grube *et al.* 2015). Thus, we can infer that their association is not random but rather specific to the thallus, as they contribute

to its maintenance. Another factor that reinforces the idea of selectivity in the lichen thallus, besides the tolerance to rapid changes in hydration state (Cernava *et al.* 2018), is the resistance of these bacterial groups to the microscopic extracellular crystals formed by chemical compounds with known antibacterial properties produced by lichens (Boustie *et al.* 2005).

Various lichen species exhibit notable distinctions in the composition of their bacterial communities (Hodkinson *et al.* 2012). This indication suggests that a significant portion of the bacteria associated with lichens are indeed adapted to the microhabitats found in the thalli of different lichens (Leiva *et al.* 2021). In our study, we found a slight difference between *D. melanocarpa* and *D. rhodocladonica*, considering the species occur in the same geographical area; and for the thallus of *D. melanocarpa*, almost twice as many exclusive taxa were observed (Table 1). The most notable differences were observed between the bacteriome from thallus and substrate (Figs 4 & 5). Leiva *et al.* (2021), using the *Peltigera frigida* as a model, showed that the bacterial community in the lichen thallus is different from the substrate, raising the following assumptions: the thallus microbiome differs from that of its growing substrates; and, this divergence may stem from lichen codispersal with specialized bacteria, as well as enrichment from surrounding environments. Thus, we suggest that the lichen thallus positively or negatively selects which bacteria will be part of its microbiome. In contrast, we see a less specific relationship when observing the substrate.

One group that can emphasize this specificity of the thallus microbiome are bacteria belonging to the order Sphingomonadales, found in this study in greater abundance. Bacteria of this order have the ability to decompose organic matter and resilient compounds (Glaeser & Kämpfer 2014). Consequently, it has been suggested that their presence in lichens may be linked to nutrient absorption and the decomposition of aged thallus components (Aschenbrenner *et al.* 2014; Aschenbrenner *et al.* 2017). The bacterial communities in the substrate of *D. melanocarpa* and *D. rhodocladonica* showed a different pattern (Table 2). Evidently, the

substrate exclusively presents the classes: Flavobacteriia, Thermomicrobia, and Gemmatimonadetes. The absence of these bacterial groups in the thallus may indicate that lichens would avoid these bacteria, possibly due to the production of secondary metabolites with antimicrobial activity (Leiva *et al.* 2016), or perhaps these bacteria lack mechanisms adapted to the conditions offered by the thallus.

Considering all these factors collectively, it becomes evident that lichens, with their well-defined, light-exposed thalli, present a valuable model system for studying the structure and variation within microbiomes (Grube *et al.* 2009). Understanding these intricate relationships between lichens and their hosts, essentially a functional microbiome, offers significant potential for new discoveries concerning the ecology and evolution of microbes in the environment. Unraveling the lichen microbiome also opens up a vast horizon of possibilities for discovering ecological processes and adaptation strategies that shape the resilience of the Cerrado in the face of environmental changes.

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Data accessibility. The 16S rRNA data are available in the NCBI SRA under the accession number _____. Metadata are available upon request.

Declaration of competing interest. The authors declare no conflicts of interest.

Supplementary Material. The Supplementary dataMAterial for this article can be found at _____.

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Tables

Table 1. Exclusive taxa for *Dirinaria melanocarpa* (mela-lichen), the substrate under *D. melanocarpa* (mela-subs), *Dirinaria rhodocladonica* (rhodo-lichen), and the substrate under *D. rhodocladonica* (rhodo-subs). k = kingdom; p = phylum; c = class; o = order; f = family.

Species-Type	Exclusive taxa
mela-lich	k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__11-24;f__
	k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__PK29;f__
	k__Bacteria;p__Acidobacteria;c__S035;o__;f__
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Beutenbergiaceae
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Micrococcales;f__
	k__Bacteria;p__Chloroflexi;c__Ellin6529;o__;f__
	k__Bacteria;p__Gemmatimonadetes;c__Gemm-1;o__;f__
	k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bartonellaceae
	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__[Entotheonellales];f__[Entotheonellaceae]
	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__OM27
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__;__
	k__Bacteria;p__Tenericutes;c__Mollicutes;__;__
	k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae
	k__Bacteria;p__Verrucomicrobia;c__[Methylacidiphilae];o__Methylacidiphilales;f__
mela-subs	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dermacoccaceae
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Gordoniaceae
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae

	k__Bacteria;p__Armatimonadetes;__;__;__
	k__Bacteria;p__Bacteroidetes;c__At12OctB3;o__;f__
	k__Bacteria;p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__Rhodothermaceae
	k__Bacteria;p__Chloroflexi;c__Chloroflexi;o__Herpetosiphonales;f__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae
	k__Bacteria;p__Gemmatimonadetes;c__Gemmatimonadetes;o__Gemmatimonadales;f__Gemmatimonadaceae
	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Nannocystaceae
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;__
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Salinisphaerales;f__Salinisphaeraceae
	k__Bacteria;p__TM7;c__;o__;f__
	k__Bacteria;p__TM7;c__TM7-3;o__;f__
rhodo-lich	k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__Ellin7246;f__
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Blattabacteriaceae
	k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;__;__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;__
	k__Bacteria;p__MVP-21;c__;o__;f__
	k__Bacteria;p__OP11;c__;o__;f__
	k__Bacteria;p__TM7;__;__;__
rhodo-subs	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__;f__
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dermabacteraceae
	k__Bacteria;p__Armatimonadetes;c__OS-L;o__;f__
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;__
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]

	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae
	k__Bacteria;p__Chloroflexi;c__Ktedonobacteria;o__Elev-1554;f__
	k__Bacteria;p__Chloroflexi;c__S085;o__;f__
	k__Bacteria;p__Elusimicrobia;c__Elusimicrobia;o__FAC88;f__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae
	k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae
	k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae
	k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__;f__
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae

Table 2. Exclusive taxa for lichen and substrate considering both species, *Dirinaria melanocarpa* and *D. rhodocladonica*. k = kingdom; p = phylum; c = class; o = order; f = family.

Type	Exclusive taxa
Lichen	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Streptosporangiaceae
	k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Rickettsiaceae
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;__
	k__Bacteria;p__Proteobacteria;c__TA18;o__PHOS-HD29;f__
Substrate	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae
	k__Bacteria;p__Armatimonadetes;c__SHA-37;o__;f__
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae]
	k__Bacteria;p__Chloroflexi;c__Thermomicrobia;o__Ellin6537;f__
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae]
	k__Bacteria;p__Gemmatimonadetes;c__Gemmatimonadetes;o__Gemmatimonadales;f__Ellin5301
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae
	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__FAC87;f__

Figures

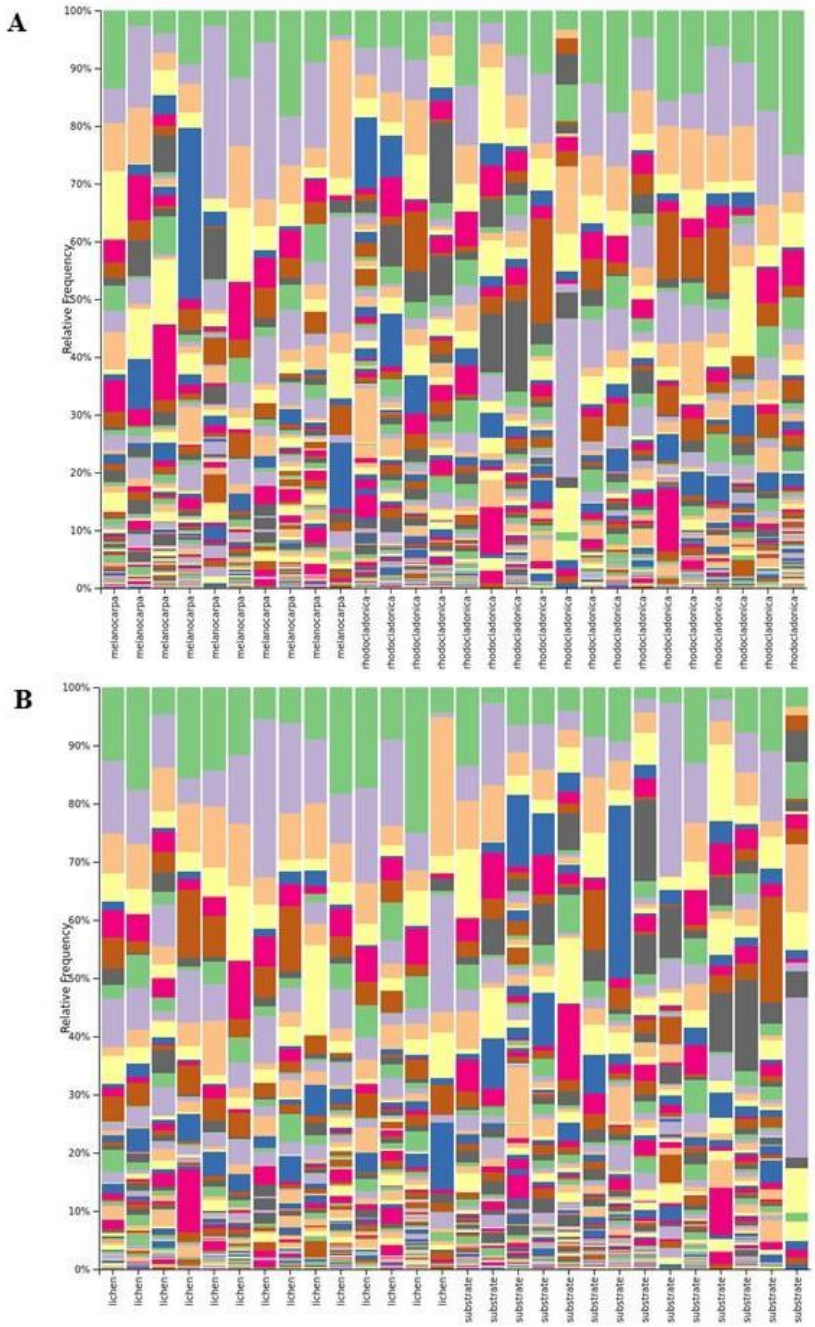

Figure 1. A, Bacteriome composition for *Dirinaria melanocarpa* and *Dirinaria rhodocladonica* samples at the family level. B, Bacteriome composition for *Dirinaria sp.* thallus and substrate samples at family level. The stacked bars represent each sample, and coloured fragments represent the fraction of each sample assigned to each family.

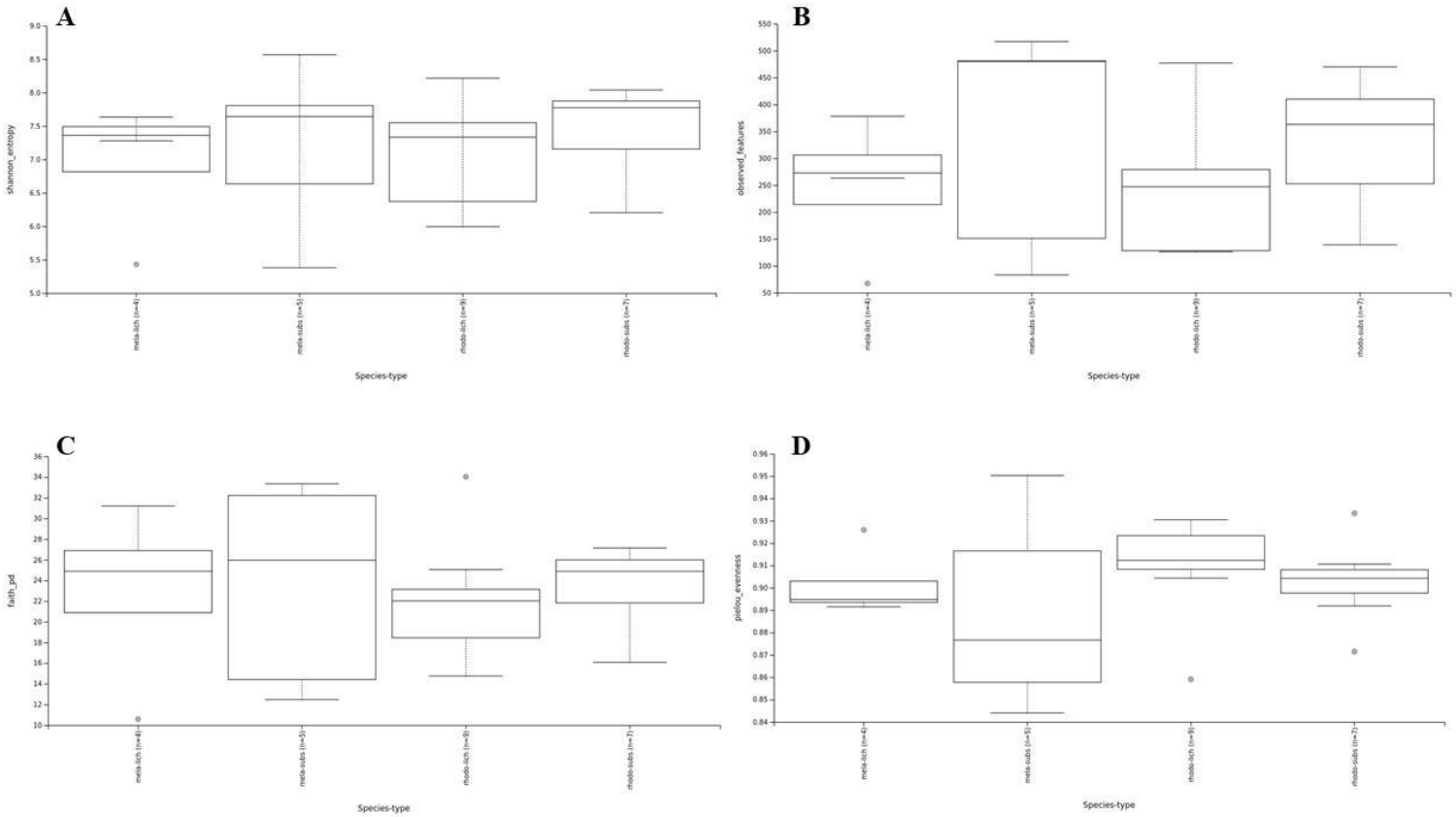

Figure 2. Alpha diversity analysis results for *D. melanocarpa* thallus, *D. melanocarpa* substrate, *D. rhodocladonica* thallus and *D. rhodocladonica* substrate**.** Alpha diversity was calculated using: A, Shannon's diversity (p = 0.5406); B, Observed Features (p = 0.4331); C, Faith's Phylogenetic Diversity (p = 0.8632); and D, Evenness (p = 0.4364), using the Qiime2 software (version 2023.7).

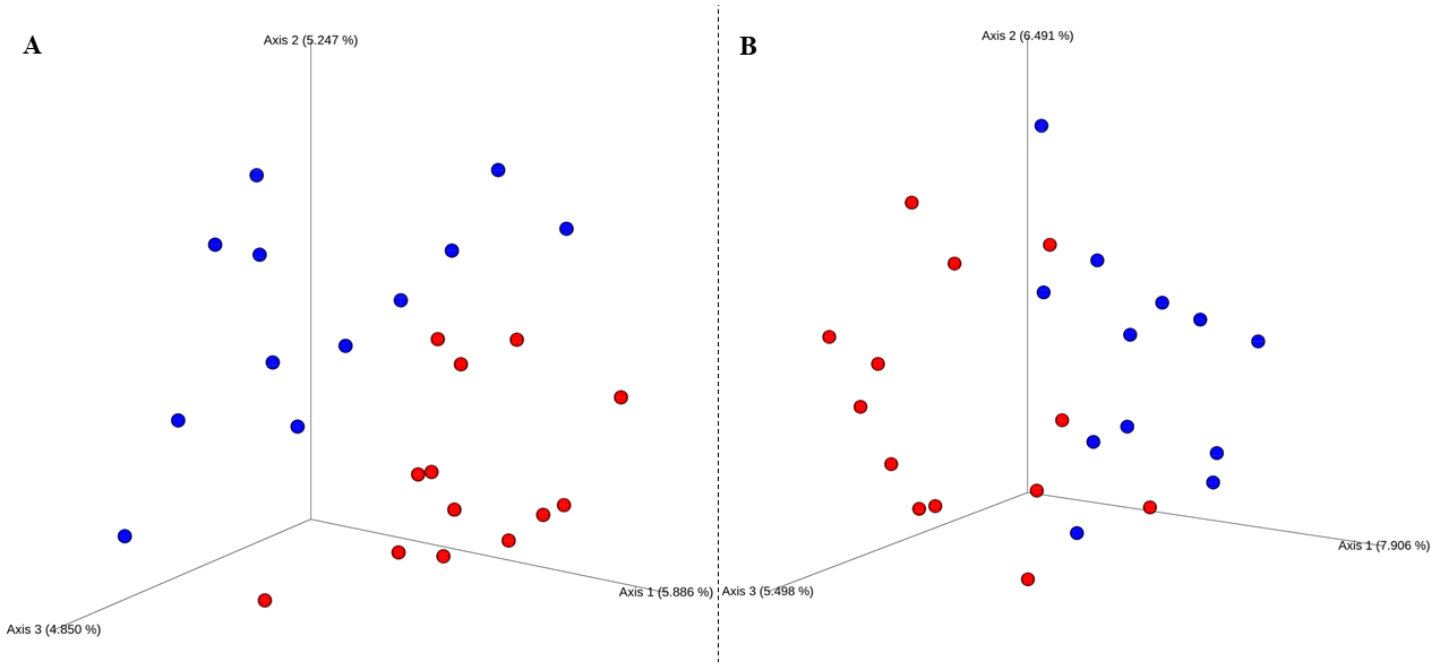

Figure 3. Similarities among bacterial communities from lichen thallus (red) and substrate (blue). The similarities were calculated by: Beta diversity was calculated using: A, Jaccard distance (test statistic = 1.264454; p = 0.001); and B, Bray-Curtis distance (test statistic = 1.666819; p = 0.001), using the Qiime2 software (version 2023.7).

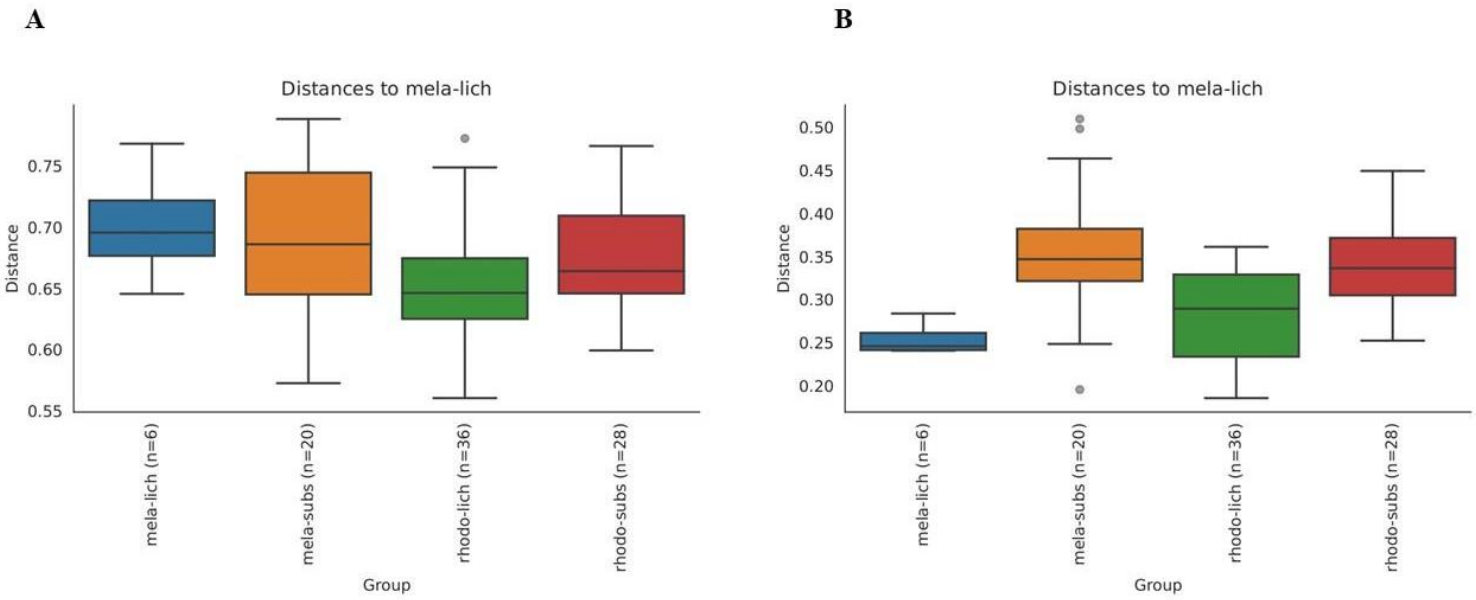

Figure 4. Dissimilarity between bacterial communities from lichen thallus (mela-lich: *D. melanocarpa* thallus; rhodo-lich: *D. rhodocladonica* thallus) and substrate (mela-subs: *D. melanocarpa* substrate; rhodo-subs: *D. rhodocladonica* substrate). Beta diversity was calculated using: A, unweighted UniFrac distance; and B, weighted UniFrac distance, using the Qiime2 software (version 2023.7).

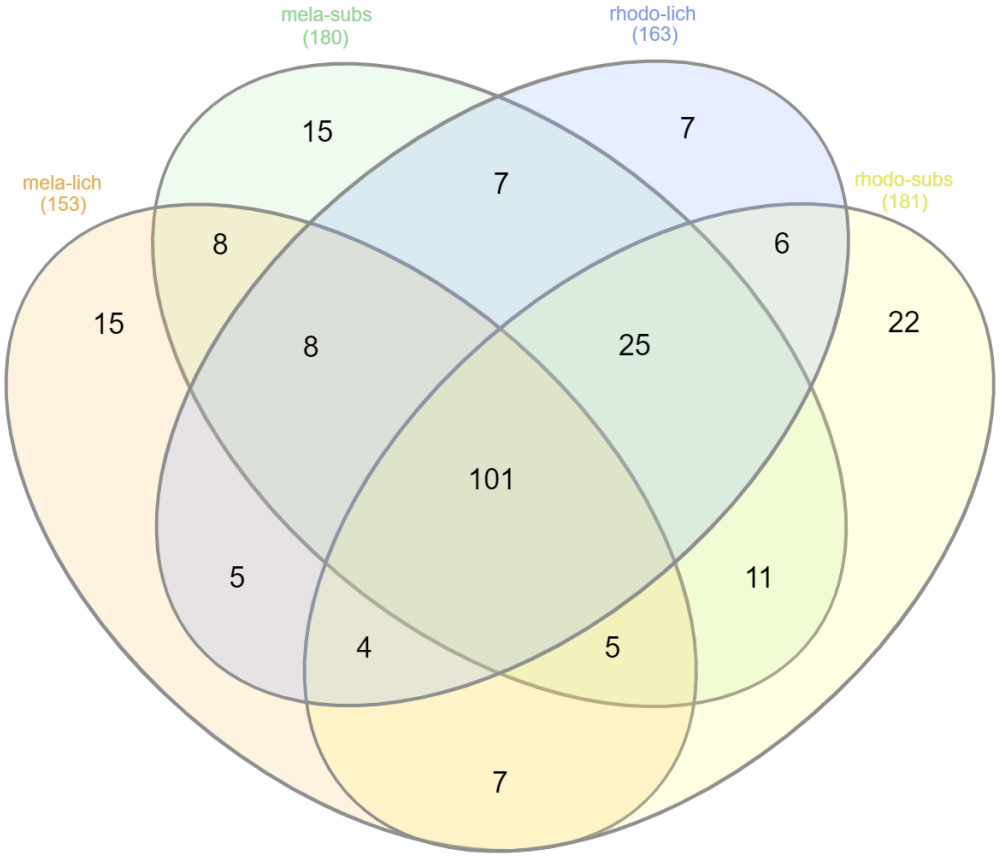

Figure 5. Venn diagram for lichen thallus (mela-lich: *D. melanocarpa* thallus; rhodo-lich: *D. rhodocladonica* thallus) and substrate (mela-subs: *D. melanocarpa* substrate; rhodo-subs: *D. rhodocladonica* substrate). Together with the subgroup's name, the total number of variants represented is indicated in parentheses. The number of variants shared between the subgroups is shown at the intersections of the sets.

Figure 6. Heatmap based on the distance matrix of UniFrac dissimilarity of the lichen thallus (mela-lich: *D. melanocarpa* thallus; rhodo-lich: *D. rhodocladonica* thallus) and substrate (mela-subs: *D. melanocarpa* substrate; rhodo-subs: *D. rhodocladonica* substrate) bacteriome.

Supplementary Material

https://drive.google.com/file/d/1sd_1hw-Ysf-1BmnPWLlUEKYG4NHx_cpY/view?usp=drive_link

Fig S1. Link for the barplot subtitles in the Fig. 1A & B.

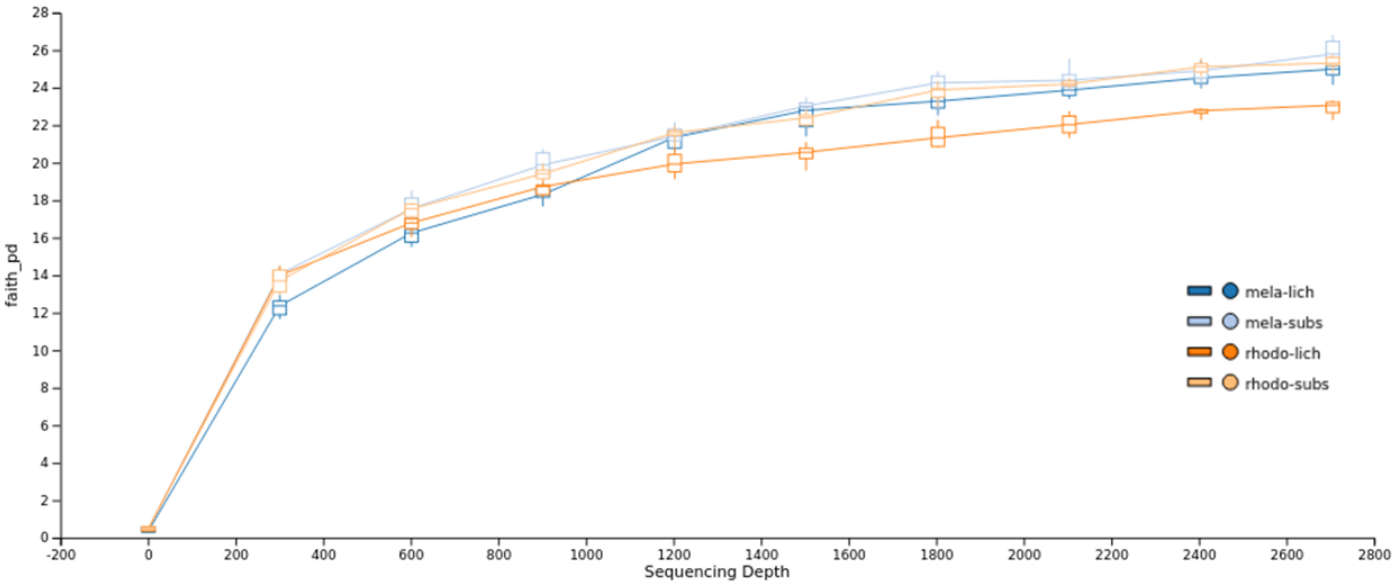

Fig S2. Alpha rarefaction plotting considering sequences obtained from from lichen thallus (mela-lich: *D. melanocarpa* thallus; rhodo-lich: *D. rhodocladonica* thallus) and substrate (mela-subs: *D. melanocarpa* substrate; rhodo-subs: *D. rhodocladonica* substrate), calculated using Shannon's diversity index in Qiime2 software (version 2023.7).

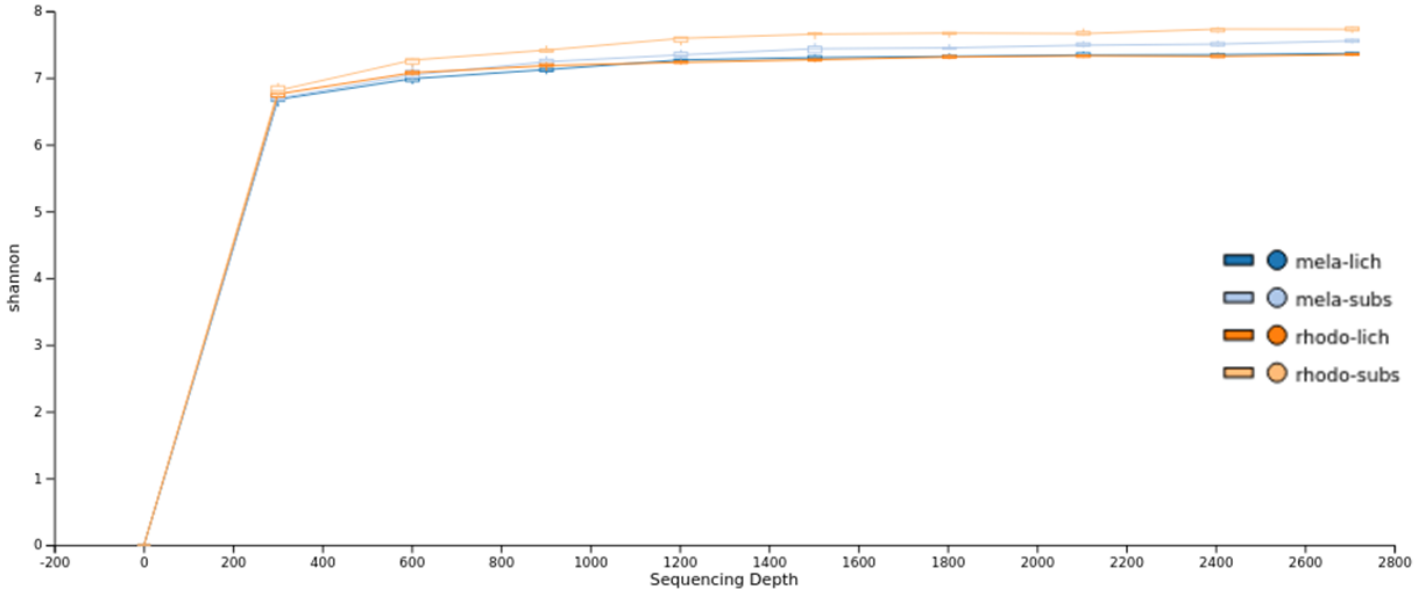

Fig S3. Alpha rarefaction plotting considering sequences obtained from lichen thallus (mela-lich: *D. melanocarpa* thallus; rhodo-lich: *D. rhodocladonica* thallus) and substrate (mela-subs: *D. melanocarpa* substrate; rhodo-subs: *D. rhodocladonica* substrate), calculated using Faith's Phylogenetic Diversity in Qiime2 software (version 2023.7).

Supplementary Table 1. Beta diversity analysis considering the overview and pairwise PERMANOVA results from unweighted UniFrac analysis for lichen thallus (mela-lich: *D. melanocarpa* thallus; rhodo-lich: *D. rhodocladonica* thallus) and substrate (mela-subs: *D. melanocarpa* substrate; rhodo-subs: *D. rhodocladonica* substrate).

Overview

	PERMANOVA results
Method name	PERMANOVA
Test statistic name	pseudo-F
Sample size	25
Number of groups	4
Test statistic	1.20069
p-value	0.056
Number of permutations	999

Pairwise permanova results

		Sample size	**Permutations**	**pseudo-F**	**p-value**	**q-value**
Group 1	**Group 2**					
mela-lich	**mela-subs**	9	999	0.834348	0.785	0.7850
	rhodo-lich	13	999	1.104298	0.212	0.3180
	rhodo-subs	11	999	1.425041	0.023	0.0840
mela-subs	**rhodo-lich**	14	999	1.344678	0.048	0.0960
	rhodo-subs	12	999	1.006673	0.419	0.5028
rhodo-lich	**rhodo-subs**	16	999	1.400597	0.028	0.0840

Supplementary Table 2. Beta diversity analysis considering the overview and pairwise PERMANOVA results from weighted UniFrac analysis for lichen thallus (mela-lich: *D. melanocarpa* thallus; rhodo-lich: *D. rhodocladonica* thallus) and substrate (mela-subs: *D. melanocarpa* substrate; rhodo-subs: *D. rhodocladonica* substrate).

Overview

	PERMANOVA results
Method name	PERMANOVA
Test statistic name	pseudo-F
Sample size	25
Number of groups	4
Test statistic	2.206696
p-value	0.002
Number of permutations	999

Pairwise permanova results

		Sample size	**Permutations**	**pseudo-F**	**p-value**	**q-value**
Group 1	**Group 2**					
mela-lich	**mela-subs**	9	999	2.132085	0.039	0.0585
	rhodo-lich	13	999	1.381082	0.125	0.1500
	rhodo-subs	11	999	2.935428	0.004	0.0080
mela-subs	**rhodo-lich**	14	999	2.949898	0.004	0.0080
	rhodo-subs	12	999	0.758170	0.715	0.7150
rhodo-lich	**rhodo-subs**	16	999	2.998994	0.001	0.0060

Considerações Finais

Os solos do Cerrado abrigam um microbioma extremamente diversificado, composto por uma ampla variedade de bactérias e fungos (Castro et al. 2016; Gnangui et al. 2021). A seleção de bactérias específicas pode desempenhar um papel na mediação da tolerância ao estresse das plantas (Liu et al. 2020), e neste estudo, sugerimos que o mesmo possa ocorrer para os liquens, uma vez que, o ambiente do Cerrado demanda algumas adaptações dos organismos que nele habitam.

A presente tese investigou as interações entre as comunidades bacterianas associadas a dois organismos, utilizando como modelos duas espécies distintas de plantas do gênero *Selaginella* e duas de fungos liquenizados do gênero *Dirinaria* no bioma do Cerrado. A análise do bacterioma revelou padrões na composição e diversidade bacteriana para as espécies de *Selaginella* e *Dirinaria*, onde a presença predominante dos filos Actinobacteria, Proteobacteria e Chloroflexi foi observada para ambas. As interações líquen-bacterioma e planta-bacterioma são cruciais para a adaptação das espécies às duras condições ambientais do Cerrado, como solos ácidos e disponibilidade sazonal de água.

Os estudos de comunidades microbianas, estão se destacando como uma possível estratégia para combater a perda de biodiversidade e fortalecer a resiliência dos ecossistemas (Peixoto et al. 2022). Com isso, a variedade de microrganismos presente no bioma Cerrado é essencial para fortalecer a resiliência desse ecossistema, ao sustentar uma ampla gama de processos ecológicos (Castro et al. 2016), pois os microrganismos desempenham um papel crucial na preservação da estrutura do solo, ao regular a retenção de água e proteger contra perturbações ambientais (Souza & Procopio 2021), desempenhando um papel fundamental na manutenção da estabilidade do ecossistema do Cerrado.

Um dos principais fatores que pode prejudicar a comunidade microbiana no Cerrado são as intensas atividades agrícolas, que a cada ano provocam mudanças substanciais no microbioma, impactando sua diversidade taxonômica e funcional (Souza et al. 2016). A conversão de habitats naturais em áreas agrícolas tem perturbado o equilíbrio delicado do microbioma, expondo dessa forma a necessidade de preservar essa diversidade microbiana para garantir a manutenção do Cerrado a longo prazo. Diante do exposto, os estudos de microbiomas se mostram uma alternativa para revelar uma riqueza de dados, que produzem uma visão aprofundada sobre a natureza das comunidades microbianas, incluindo as suas interações e efeitos, tanto dentro de um hospedeiro como num ambiente externo, classificando-os como parte da comunidade ecológica (Cullen et al. 2020). Sendo assim, a compreensão do papel da microbiota nos ecossistemas pode abrir caminho para o desenvolvimento de novas técnicas de diagnóstico e estratégias de intervenção, que podem auxiliar na preservação do Cerrado.

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