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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(MICROBIOLOGIA APLICADA)

IN A HOLE IN THE GROUND:
THE MICROBIAL GARDEN OF FUNGUS-GROWING ANTS

MARIANA DE OLIVEIRA BARCOTO

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MARIANA DE OLIVEIRA BARCOTO

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Advisor: Dr. Andre Rodrigues

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Potential impact of this research

“Fungus-growing” or “fungus-farming” ants represent one of the first agricultural practices that evolved on Earth. Attine ants cultivate a basidiomycete fungal crop in the “microbial garden”, a spongy structure where the fungal hyphae grow by degrading the plant substrate brought to the colony to feed the fungus; then, the ants themselves feed on the fungus, to simply put. The impact of this research lays on revealing that there is much more than the cultivated fungus in the garden: it is a complex microbial ecosystem, inhabited by extensive biofilms surrounding the fungus and the substrates brought by the ants. This novel view on fungus-growing ants, a textbook model for studying social organisms and microbial symbiosis, was possible only by employing a comprehensive toolkit composed of solid-state nuclear magnetic resonance, scanning electron microscopy, and metabarcoding. To the best of our knowledge, this is the first report gathering chemical, microbial diversity, and microscopic analysis to investigate the microbial ecosystem of fungus-growing ants. Although our study focused on a specific model, it implements a multidimensional framework for investigating other complex microbial ecosystems. The microbial garden awaits to be studied as a holistic entity responding to the environmental conditions, evolving through time, and likely keeping metabolic routes useful for our future environmental challenges.

Impacto potencial desta pesquisa

As formigas “cultivadoras de fungos” ou “fazendeiras” representam uma das primeiras práticas agrícolas que evoluíram na Terra. As formigas atíneas cultivam um fungo basidiomiceto no “jardim microbiano”, uma estrutura esponjosa onde as hifas fúngicas crescem ao degradar o substrato vegetal trazido à colônia para alimentar o fungo; então, as formigas se alimentam do fungo. O impacto desta pesquisa reside em revelar que há muito mais do que o fungo cultivado no jardim: é um ecossistema microbiano complexo, habitado por extensos biofilmes envolvendo o fungo e os substratos trazidos pelas formigas. Essa nova perspectiva sobre formigas criadoras de fungos, um modelo clássico para estudar organismos sociais e simbiose microbiana, só foi possível com o emprego de um conjunto de ferramentas abrangente, composto de ressonância magnética nuclear de estado sólido, microscopia eletrônica de varredura e *metabarcoding*. Até onde sabemos, este é o primeiro relato reunindo análises químicas, de diversidade microbiana, microscópicas e para investigar o ecossistema microbiano das formigas cultivadoras de fungo. Embora nosso estudo tenha se concentrado em um modelo específico, ele implementa uma estrutura multidimensional para investigar outros ecossistemas microbianos complexos. O jardim microbiano aguarda para ser estudado como uma entidade holística que responde às condições ambientais, evoluindo ao longo do tempo e, provavelmente, revelando rotas metabólicas úteis para nossos futuros desafios ambientais.

Impacto potencial de esta investigación

El cultivo de hongos, o “agricultura de hongos”, practicado por las hormigas representa una de las primeras prácticas agrícolas que evolucionaron en la Tierra. Las hormigas de la tribu Attini cultivan un hongo basidiomiceto en el “jardín microbiano”, una estructura esponjosa donde las hifas del hongo crecen al degradar el sustrato vegetal que las hormigas recolectan y llevan a la colonia para alimentar al hongo. En términos más sencillos, las hormigas se alimentan del hongo que cultivan. El impacto de esta investigación radica en revelar que hay mucho más que el hongo cultivado en el jardín: es un ecosistema microbiano complejo, habitado por extensos biofilms que rodean al hongo y a los sustratos traídos por las hormigas. Esta novedosa visión de las hormigas cultivadoras de hongos - un modelo clásico para estudiar organismos sociales y la simbiosis microbiana - solo fue posible gracias al empleo de un conjunto diverso de herramientas, incluyendo resonancia magnética nuclear de estado sólido, microscopía electrónica de barrido y metabarcoding. Hasta donde sabemos, este es el primer informe que reúne análisis químicos, de diversidad microbiana y microscópicos para investigar el ecosistema microbiano de las hormigas cultivadoras de hongos. Aunque nuestro estudio se centró en un modelo específico, implementa un marco multidimensional para investigar otros ecosistemas microbianos complejos. El jardín microbiano espera ser estudiado como una entidad holística que responde a las condiciones ambientales, evoluciona a través del tiempo y, probablemente, mantiene rutas metabólicas útiles para nuestros futuros desafíos ambientales.

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Rio Claro, 27 de junho de 2025

To the memory of Ana, João, and Antônio Barcoto
I seeded you in my dreams
In this land you still live,
In the reminiscences of my days going by.

--

À memória de Ana, João e Antônio Barcoto
Eu os semeei em meus sonhos
Nessa terra vocês ainda vivem
Nas reminiscências dos meus dias passados

--

En memoria de Ana, João y Antônio Barcoto
Los sembré en mis sueños.
En esta tierra todavía viven
En las reminiscencias de mis días pasados

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"What we find, if we listen within us, is no separate single voice, no separated self that simply appears out of nowhere (...). We are a community of selves, and all of our ancestors are within us."

- Thich Nhat Hanh, Silence

In the silence of the void, I only listen to the waves of my thoughts. Sometimes gentle, sometimes tempestuous. Sometimes from the past, sometimes from the future, sometimes from a time that never came to be. Tides of time, tides of history, of so many journeys and so many falls, of so many sunrises and sunsets. Tales of so many words written and so many more erased, of ungerminated ideas and so few that flourished. The academic journey was a long-awaited and long-lasting one. A journey of a family, of a community, and so many that supported me and to whom I owe my thanks.

To my family, for their roots in gentleness and wisdom. For greeting me with the warmest hugs and the sincerest smiles. To the memory of those that couldn't get to the end of this history. To my mother, for cultivating and taking care of my dreams, and to carry me back to my way with all her strength. To my father, for sharing the melancholy of farewells and for standing beside me, no matter what. To my brother, for accompanying the struggles and uncertainties of our tortuous path. To my partner, for walking through my darkest days, creating a family from the bonds that pass through us. To the cat that chose me, shared with me many of the long hours of thesis writing, and left me with the most colorful memories drawn in a sheltered place. To the friends that understood the difficulties and absences, offered their shoulder and their laughs. And as it could not be otherwise, to the fungus-growing ants. I learned so much from their instinctive perception and selfless care. Even from my deepest respect, some I made homeless, some I starved to death. I owe them this work, my academic journey, and everything I learned about Microbiology along these years. I also thank my advisor, Prof. Rodrigues, for all the support and for the help in establishing the connections that made this work possible. I thank my co advisor, Prof. Bacci, and also Prof. Bueno, Prof. Azevedo, Prof. Mendes, and Prof. Coan, collaborators who allowed this research to become more robust and multidisciplinary. I thank Prof. Kitajima for inspiring my work and for the right words, at the right time. To the lab friends who made the journey less lonely and more lightful. This study was financed, in part, by the São Paulo Research Foundation (FAPESP), Brazil. Process Number 2021/08013-0. The opinions, hypotheses and conclusions or recommendations expressed in this material are the responsibility of the author(s) and do not necessarily reflect the

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“Art and nature shall always be wrestling until they eventually conquer one another so that the victory is the same stroke and line: that which is conquered, conquers at the same time.”

- Maria Sibylla Merian

“Nature does not hurry, yet everything is accomplished.”

- Lao Tzu

Abstract

By cultivating basidiomycete fungi as a nutritional resource, fungus-growing ants are part of the first agricultural practices that evolved on Earth. Fungus-growing ants forage for diverse substrates (mostly plant-based) to nourish their fungal crop, whose growth forms an intertwined mesh of hyphae, scaffolding a microbiota that colonizes both the hyphae and the plant substrate. When metabolizing added substrates, the fungal crop not only provides nitrogen, lipids, and cellulose-derived carbon to the ants, but also opens niches for a characteristic and adapted microbiota to establish. In this work, we compile data demonstrating that there is much more than the cultivated fungus in the garden; there are exuberant microbial biofilms surrounding the fungus and the substrates brought by the ants. They form together an interkingdom microbial ecosystem known as "microbial garden", from which would emerge properties way beyond the sum of the fungal crop and the microbiota individual capacities. Here, we first review how the same metabolic arsenal allowing microbial plant degradation may lead to the degradation of environmental pollutants. Second, we assessed the bacterial dimension of the garden spatial organization, detailing microbiota-substrate and microbiota-hyphae physical interactions, as well as unveiling biofilm spatial distribution. These data placed the substrate as a keystone of the garden spatial structure, providing the framework for structuring fungal growth patterns and microbiota colonization. Then, in third, we investigated how different plant substrates influence the garden's chemical profile and whether the taxonomic composition and spatial distribution of the microbiota respond to these changes. In forth, we attempted another way to investigate the substrate influence in the microbial garden: by looking into the colony of diverse fungus-growing ants, which have distinct colony size, garden maintenance practices, and, most interesting, the range of foraged substrates. In fifth, we returned to the ideas presented in the first chapter, investigating whether the microbial garden biofilm could colonize highly recalcitrant substrates. From this work, we conclude that the microbial garden is a holistic entity responding to the environmental conditions, evolving through time, and likely, keeping metabolic routes useful for our future environmental challenges.

Key words: Fungiculture. Microbiota. Biofilm. Metabolic flexibility. Microbial ecology. Bioremediation.

Resumo

Ao cultivar fungos basidiomicetos como recurso nutricional, formigas cultivadoras de fungos constituem uma das primeiras práticas de agricultura que evoluíram na Terra. Formiga cultivadoras de fungos forrageiam por diversos substratos (em sua maioria derivados de plantas) para nutrir seu cultivo fúngico, o qual cresce formando uma rede interconectada de hifas, sustentando uma microbiota que coloniza tanto as hifas quanto o substrato. Ao metabolizar os substratos adicionados, o fungo cultivado não somente fornece nitrogênio, lipídeos e carbono derivado de celulose às formigas, mas também abre nichos para que uma microbiota característica e adaptada se estabeleça. Neste trabalho, compilamos dados demonstrando que existe muito mais do que o fungo cultivado no jardim: existem exuberantes biofilmes microbianos ao redor das hifas e sobre o substrato trazido pelas formigas. Tais microrganismos formam, em conjunto, um ecossistema microbiano inter-reino conhecido como "jardim microbiano", a partir do qual emergiram propriedades muito além da soma das capacidades individuais do fungo cultivado e da microbiota. Aqui, em primeiro lugar, nós revisamos como um mesmo arsenal metabólico por trás da degradação microbiana de plantas pode permitir a degradação de poluentes ambientais. Em segundo lugar, acessamos a dimensão bacteriana da organização espacial do jardim, detalhando interações físicas entre microbiota-substrato e microbiota-hifa, assim como revelando a distribuição espacial do biofilme. Esses dados posicionaram o substrato como peça chave na organização espacial do jardim, fornecendo a base para que padrões de crescimento fúngico e colonização da microbiota sejam estruturados. Em terceiro lugar, investigamos como diferentes substratos vegetais influenciam o perfil químico do jardim, avaliando se a composição taxonômica e a distribuição espacial da microbiota respondem a essas mudanças. Em quarto lugar, tentamos outra maneira de investigar a influência do substrato no jardim microbiano: examinando o jardim de diversas formigas cultivadoras de fungos, que apresentam distintos tamanhos de colônia, práticas de manutenção do jardim e, mais interessante, forrageiam por diferentes substratos. Em quinto lugar, retornamos às ideias apresentadas no primeiro capítulo, investigando se o biofilme do jardim microbiano poderia colonizar substratos altamente recalcitrantes. A partir deste trabalho, concluímos que o jardim microbiano é uma entidade holística que responde às condições ambientais, evolui ao longo do tempo e, provavelmente, apresenta rotas metabólicas úteis para nossos futuros desafios ambientais.

Palavras-chave: Fungicultura. Microbiota. Biofilme. Flexibilidade metabólica. Ecologia microbiana. Biorremediação.

Resumen

Al cultivar hongos basidiomicetos como recurso nutricional, las hormigas cultivadoras de hongos son parte de las primeras prácticas agrícolas que evolucionaron en la Tierra. Las hormigas cultivadoras de hongos buscan diversos sustratos (en su mayoría de origen vegetal) para nutrir su cultivo de hongo, cuyo crecimiento forma una red entrelazada de hifas, sosteniendo una microbiota que coloniza tanto las hifas como el sustrato vegetal. Al metabolizar los sustratos añadidos, el hongo cultivado no sólo aporta nitrógeno, lípidos y carbono derivado de la celulosa a las hormigas, sino que también abre nichos para que se establezca una microbiota característica y adaptada. En este trabajo, recopilamos datos que demuestran que en el jardín hay mucho más que el hongo cultivado: hay biofilms microbianos exuberantes que rodean al hongo y los sustratos traídos por las hormigas. Juntos forman un ecosistema microbiano interreino, conocido como "jardín microbiano", del cual surgirían propiedades que van más allá de la suma de las capacidades del hongo cultivado y de la microbiota individual. Aquí, primero revisamos cómo el mismo arsenal metabólico que permite la degradación de las plantas por parte de los microorganismos puede conducir también a la degradación de contaminantes ambientales. En segundo lugar, evaluamos la dimensión bacteriana de la estructura espacial del jardín, detallando las interacciones físicas microbiota-sustrato y microbiota-hifas, además de revelar la distribución espacial del biofilm. Estos datos sitúan al sustrato como una pieza clave de la estructura espacial del jardín, proporcionando la base para estructurar los patrones de crecimiento de los hongos y la colonización de la microbiota. Luego, en tercer lugar, investigamos cómo los diferentes sustratos vegetales influyen en el perfil químico del jardín, y si la composición taxonómica y la distribución espacial de la microbiota responden a estos cambios. En cuarto lugar, intentamos otra forma de investigar la influencia del sustrato en el jardín microbiano: analizando la colonia de diversas especies de hormigas cultivadoras de hongos, que varían en tamaño, prácticas de mantenimiento del jardín y, lo más interesante, en los tipos de sustratos que recolectan. En quinto lugar, retomamos las ideas presentadas en el primer capítulo e investigamos si el biofilm del jardín microbiano podría colonizar sustratos altamente recalcitrantes. De este trabajo concluimos que el jardín microbiano es una entidad holística que responde a las condiciones ambientales, evoluciona lo largo del tiempo y, probablemente, mantiene rutas metabólicas útiles para nuestros desafíos ambientales futuros.

Palabras clave: Cultivo de hongos. Microbiota. Biofilm. Flexibilidad metabólica. Ecología microbiana. Biorremediación.

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Prologue

"In a hole in the ground there lived a hobbit. Not a nasty, dirty, wet hole, filled with the ends of worms and an oozy smell, nor yet a dry, bare, sandy hole with nothing in it to sit down on or to eat: it was a hobbit-hole, and that means comfort."

J.R.R. Tolkien, The Hobbit, 1937

I will respectfully borrow Tolkien's words to tell the reader about the microbial world in which I have dived for the last few years. I will do so because these were the very words coming into my mind the first time I saw, in a hole in the ground, the garden chamber of a fungus-growing ant, the leaf-cutting *Atta sexdens* (*Hymenoptera: Formicidae: Myrmicinae: Attini: Attina*; Ward *et al.,* 2015). Back then, I was mesmerized by the ants' strength to dig so deeply, to build underground chambers and tunnels, and to carry substrates far heavier than their own bodies (Cherret, 1972; Camargo *et al.,* 2011; 2013; Moll *et al.,* 2013; Bruce *et al.,* 2018; Segre; Taylor, 2019). It seemed unlikely that under that amount of rocky soil, which took us hours to excavate, there laid such a delicate and neat spongy structure. At that time, we knew this structure as "fungus garden", since it is where the ants cultivate their food - the fungus *Leucoagaricus gongylophorus* (*Basidiomycota: Agaricales: Agaricaceae;* Weber, 1966; 1972; Chapela et al., 1994; Fisher *et al.,* 1994). Then I will explain why, after four years of research, we think it is more appropriate to refer to this spongy ecosystem as "microbial garden" (Barcoto *et al.,* 2024). This is the main message I hope to convey here, through the following five chapters of this Thesis. There is much more than the cultivated fungus in the garden; there are marvelous microbial biofilms surrounding the fungus and the substrates brought by the ants. From the microbial garden would emerge properties way beyond the sum of the fungal crop and the microbiota individual capacities (Zilber-Rosenberg; Rosenberg, 2008; Gordon *et al*., 2013; Guerrero *et al.,* 2013). The microbial garden awaits to be studied as a holistic entity responding to the environmental conditions, evolving through time, and likely keeping metabolic routes useful for our future environmental challenges.

Introduction and thesis overview

In a hole in the ground there lived a microbial garden. Not a nasty, dirty, wet hole, (...) nor yet a dry, bare, sandy hole with nothing in it (...): it was a garden chamber, and that means care. Fungus-growing ants execute every task with extreme care: from foraging, garden maintenance, to nourishing the offspring and the queen (**Figure 1A**; Hölldobler; Wilson, 1990; Mueller *et al.*, 2005). In that hole, protected by the teamwork of many ant workers, looking like a whitish delicate sponge, laid the microbial garden (Fisher *et al.*, 1994; Schultz, 2022). It came into existence when a queen-to-be left her parental colony, carrying a garden piece inside a pouch of her pharynx, known as infrabuccal pocket (Weber, 1937; 1972). From queen to queen, the garden is a continuation of a culture lineage, the main way by which we know the fungus reproduces. The queen-to-be flies once in a lifetime for mating in the air, then falling to the ground after this nuptial flight. She sheds her wings and looks for a suitable place to begin the nest, excavating the first garden chamber. Digging requires a lot of effort and energy, as it may take about five hours to be completed (Autuori, 1942; Camargo *et al.*, 2013). The *Atta* queen then closes the chamber, places the garden inoculum she carried, and lays the eggs on it. She will not leave the colony for the rest of her life. In this early stage, the queen herself takes care of the garden, nourishing it using her liquid excrement. She produces the progeny using, as energy source, only the contents of her body, largely composed of fat. This is enough to produce offspring over nine weeks, when the first workers begin foraging for leaves and flowers (Autuori, 1942; Weber, 1972; Camargo *et al.*, 2011; 2013).

The microbial garden is spongy due to the fungal hyphae that grows by degrading the plant substrate brought to the colony to feed the fungus; then, the ants themselves feed on the fungus, to simply put (Martin; Martin, 1970; Quinlan; Cherrett, 1979; Bass; Cherret, 1995; Moller *et al*., 2011; Grell *et al.*, 2013; Lange; Grell, 2014). That is why they are known as “fungus-growing” or “fungus-farming” ants, being part of the first agricultural practices that evolved on Earth (Mueller *et al.*, 2005; Schultz *et al.*, 2024). Cultivation practices had a little push to evolve after the Chicxulub asteroid impacted the current site of Yucatán Peninsula, about 66 million years ago, when the atmosphere became so dusty that photosynthesis was interrupted. Such a massive event favored the dissemination of fungi, which consume organic matter for getting energy independently of light. It also favored the ancestral of ants in the subtribe *Attina* (the attine ants), to start fungal cultivation (Schultz *et al.*, 2024). Not only attine ants cultivate fungus; indeed, diverse insect lineages independently evolved relationships with fungi (Biedermann; Vega, 2020). We reviewed

some of these interactions in the Perspective Paper **"Lessons from insect fungiculture: From microbial ecology to plastics degradation"**, published at *Frontiers in Microbiology* in 2022, presented as **Chapter 1** (**Figure 1B**). There we pointed out that, by cultivating plant-degrading fungal crops, these symbioses are notorious organic matter decomposers. We also compiled data supporting that the same metabolic arsenal that allows microbial plant degradation may lead to pollutants degradation, turning the attention to plastic bioremediation. Then we suggest that one could find, in plant-degrading systems maintained by insects, promising candidates for degrading synthetic plastics (Barcoto; Rodrigues, 2022).

Carried from the *Atta* parental nest, the first garden piece brings, together with the fungal crop, the garden microbiota (Meirelles *et al*., 2016; Moreira-Soto *et al*., 2017). Being there since the garden foundation, the microbiota taxonomic composition and metabolic capacity suggest their participation in the garden physiology (Aylward *et al.,* 2014; Barcoto *et al.,* 2020; Francoeur *et al.,* 2020). This would not be unexpected, as virtually every being and ecosystem in this planet lives in association with microbes (McFall-Ngai *et al.,* 2013; Flemming; Wuertz, 2019). In the early stages of the colony, even before foragers go outside, the bacterial community is already present, forming biofilms over and amidst the fungal hyphae (**Figure 1A**). Biofilms are multicellular systems, where microorganisms embedded in a self-secreted matrix form social networks (Flemming; Wingender, 2010; Flemming *et al*., 2016; 2023). Both plant degradation and metabolites detoxification are considered to take advantage of their associated microbiota (Barcoto *et al.*, 2020; Francoeur *et al.*, 2020; Martiarena *et al.*, 2023). Supposedly, this microbial community could become more diverse as the ants foraging activities bring plant substrates to feed the fungus. Even though the workers invest many efforts to clean these fragments, to remove the wax layer and superficial microbes, they are not sterilized (Hölldobler; Wilson, 1990; Currie; Stuart, 2001; Garcia *et al.,* 2005). Microbial members may come from various sources, such as the substrate, the soil, and the ant bodies. Some of these microbes are thought to contribute to the garden microbial ecosystem (Ramalho *et al.,* 2020; Vieira *et al.,* 2020; Bringhurst *et al.,* 2023; Innocent *et al.* 2024).

We knew the bacterial community inhabited the garden, though we were not sure about their spatial organization. Far back, Sergey Winogradsky already observed that microbial ecosystems are heterogeneously organized in space, having, as consequence, cohabiting species that are more likely to interact and influence each other's physiology (Winogradsky, 1949; Wimpenny, 1981; McCallum; Tropini, 2024). Assessing the bacterial dimension of the garden spatial structure required a deep look at the garden, at micrometer levels. Visualizing how the

microbial community is organized in the midst of the fungal matrix demanded us to develop a specific Scanning Electron Microscopy approach. The Protocol Paper **"Microbiota of attine ants' gardens: Visualizing a microbial landscape by Scanning Electron Microscopy"**, published at the *Journal of Visualized Experiments (JoVE)* in 2024, is presented as **Chapter 2**. There we describe the methodological steps for imaging the microbial garden in high resolution. We were able to preserve the delicate garden structure, to detail microbiota-substrate and microbiota-hyphae physical interactions, and to unveil biofilm spatial distribution (**Figure 1B**). We perceived the substrate as a keystone of the garden spatial structure, providing the framework for structuring fungal growth patterns and microbiota colonization (Barcoto *et al.*, 2024).

But what if the plant substrate changes? *Atta* is the most derived genera within an extensive lineage of fungus-growing ants, all practicing fungiculture, though in different configurations. These are currently categorized into five agricultural types: i) the one practiced by the less derived attines; ii) the one that cultivate *Pterulaceae* fungi (known as coral fungi); iii) the one that cultivate *Leucocoprinae* yeasts; iv) the one practiced by higher attines that cultivate *Leucoagaricus* sp.; and v) the one practiced by leaf-cutting higher attines, that cultivate *L. gongylophorus*. Each agricultural type has particularities, such as colony size, garden maintenance practices, and, the most interesting, the range of foraged substrates. For instance, while higher and leaf-cutting attines forage for plant parts, lower attines also include arthropods carcasses and frass to the menu (Chapela *et al.*, 1994; Schultz; Brady, 2008; De Fine Licht; Boomsma, 2010; Ward *et al.*, 2015; Schultz, 2022). Could this render gardens with different chemical profiles and microbiota composition? We delved into this question in the Research Manuscript **"Microbial gardens of fungus-growing ants: Unique chemistry and spatial structure, yet a shared microbiota across the attine phylogeny"**, in preparation and presented as **Chapter 3** (**Figure 1B**). We report that attine microbial gardens are primarily herbivorous, forming a longitudinal continuum of lignocellulosic degradation. The microbial garden exhibits a lignocellulosic nature, and indeed, and plant-derived fragments are the majority of recognizable substrates. Lignocellulose modification seems to be achieved to a greater extent at gardens of lower fungiculture systems than at the higher and leaf-cutting ones. All attine gardens, but *Atta* sp., share a similar taxonomic composition. The fungiculture system of the lower attines *Mycocepurus* sp. and *Mycetophylax* sp. are richer and more diverse, whereas the leaf-cutting ant *Atta* sp. has lower richness and diversity indices. Although each fungiculture has its own characteristic spatial structure, biofilms are ubiquitous components of attine gardens.

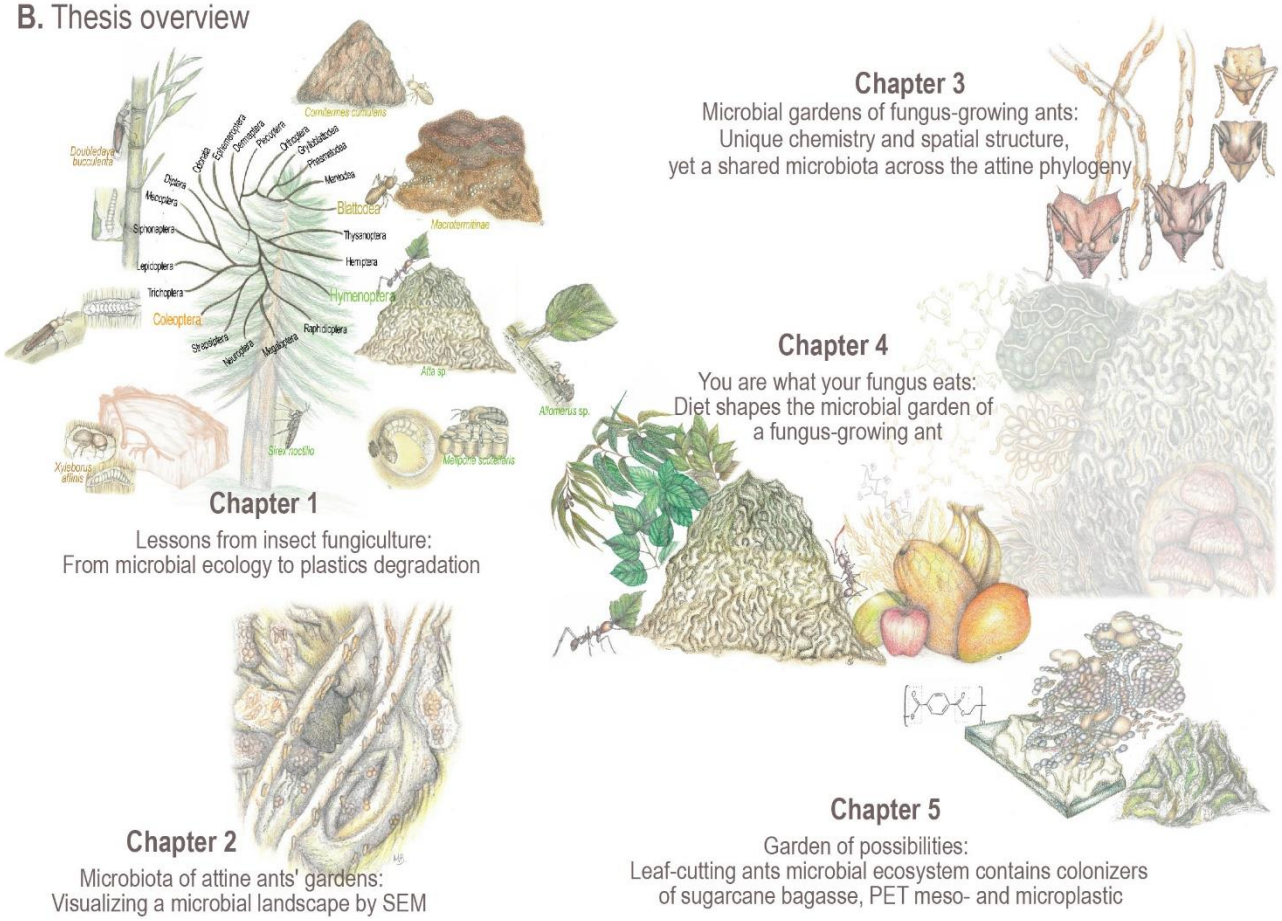
For the gut of several herbivores, when dietary sources are altered, the microbiota composition and spatial distribution respond to the variations (Tropini *et al*., 2017; Wu *et al*., 2017; Hicks *et al*., 2018; Riva *et al*., 2019; Baniel *et al.*, 2021; Huang *et al*., 2022; Li *et al*., 2023). The microbial garden is considered an external gut, due to the efficient digestion of plant biomass and the microbiota functional profile (Pinto-Tomás *et al.,* 2009; Suen *et al.*, 2010; Aylward *et al.*, 2012). Thus, we assumed that a similar response could be elicited when different substrates are brought to nourish the fungal crop. It is not fully comprehended how foragers select the substrates, among the leaves, flowers, fruits, seeds, twigs, and barks they choose to carry to the colony. It is known that they tend to select for the most nutritious and less toxic plant parts, such as young leaves, floral parts, and fruits (Wirth *et al.* 2003; Mundim; Costa; Vasconcelos, 2008; De Fine Licht; Boomsma, 2010; Falcão *et al.* 2011). We supposed that, according to its nutrient content and recalcitrance, the substrate could influence the microbiota composition and colonization patterns. This is the backbone of the Research Manuscript **"You are what your fungus eats: Diet shapes the microbial garden of a fungus-growing ant"**, under review at *npj Biofilms and Microbiomes*, presented as **Chapter 4** (**Figure 1B**). There we report how the garden and the microbiota changes when lab colonies of the leaf-cutting *Atta sexdens* were set up into four different dietary regimens, varying in fiber recalcitrance and nutritional value. We employed a comprehensive toolkit to achieve a multidimensional understanding of the microbial garden plasticity and the microbiota's response to environmental fluctuations. Surprisingly, we found that the diet not only shapes the nutritional landscape for microbial communities but also determines whether *A. sexdens* colonies function healthily. When enriched in sugars and less recalcitrant substrates, the diet induced compositional changes in the microbiota, in parallel to functional changes, what could be interpreted as dysbiosis (Petersen; Round, 2014; Hooks; O'Malley, 2017).

We verified biofilm occurring in gardens from different attine genera, colonizing both the fungal hyphae and the substrate (Barcoto *et al.*, 2024). Thought as a microbial fortress, biofilms enclose a microbial community in a self-secreted matrix of extracellular polymeric substances (Flemming; Wingender, 2010; Flemming *et al*., 2016; 2023). Since attine microbial gardens have a lignocellulolytic nature (Moller *et al*., 2011; Grell *et al.*, 2013; Lange; Grell, 2014), we returned to the ideas presented in the first chapter, investigating whether the attine-derived biofilm could colonize highly recalcitrant substrates. Our findings are compiled in the Research Manuscript **"Garden of possibilities: Leaf-cutting ants microbial ecosystem contains colonizers of sugarcane bagasse, PET meso- and microplastic"**, in preparation and presented as **Chapter 5**

(Figure 1B). There we report the lignocellulolytic and biofilm-forming microbiota chemically modifying sugarcane bagasse, while colonizing PET mesoplastic without chemical modifications. The biofilm-forming microbiota is mostly defined by the inoculum of origin, more than by the substrate. Fungi isolated from PET microcosmos colonized PET mesoplastics, though did not thrive in PET microplastics. With it, we aim to turn the attention to the vast metabolic - and therefore biotechnological - potential of the attine microbiota. Besides the possibility for biotechnological research, the metabolic diversity and flexibility of microbial gardens raise questions on their eventual role in the attine ant systems. Microbial diverse and flexible responses to diverse substrates could imply them acting as a "buffering" mechanism, allowing the system to cope with environmental perturbations. This would allow colonies to explore novel resources and environments, ultimately extending their evolutionary potential (Kooij *et al.*, 2011; Khadempour *et al.*, 2016; Kolodny; Schulenburg, 2020; Henry *et al.*, 2021). Whether the microbial garden is somehow shaped by the ants, how the microbiota and the fungal crop interacts, and what are the metabolic consequences of their interactions, and so much more questions are waiting for our curiosity to pursue them.

A. *Atta sexdens* microbial garden

B. Thesis overview



Chapter 1

Lessons from insect fungiculture:
From microbial ecology to plastics degradation

Chapter 2

Microbiota of attine ants' gardens:
Visualizing a microbial landscape by SEM

Chapter 3

Microbial gardens of fungus-growing ants:
Unique chemistry and spatial structure,
yet a shared microbiota across the attine phylogeny

Chapter 4

You are what your fungus eats:
Diet shapes the microbial garden of
a fungus-growing ant

Chapter 5

Garden of possibilities:
Leaf-cutting ants microbial ecosystem contains colonizers
of sugarcane bagasse, PET meso- and microplastic

Figure 1 - Entire microbial worlds within attine colonies. A. *Atta sexdens* microbial garden, yet incipient (we estimate it was a few months old), maintained by the queen. As evidenced by scanning electron microscopy, the bacterial community is already present, forming biofilms over and amidst the fungal hyphae. **B.** The following five chapters of this Thesis attempt to convey that there is much more than the cultivated fungus in the garden, making sense referring to it as a "microbial garden". Photo, SEM images, and illustrations by Mariana Barcoto (illustrations adapted from Barcoto; Rodrigues, 2022; Barcoto *et al.*, 2024).

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Conclusions

Fungus-growing ants are part of the first agricultural practices that evolved on Earth, and for more than 150 years have fascinated naturalists, scientists, and students. When we started the project that ended up becoming this PhD Thesis, it was known that a taxonomically convergent and functionally adapted microbiota inhabited ants "fungus-gardens", i.e., the spongy structure where ants cultivate their fungal crop. Results from both culture-dependent and culture-independent methods suggested the microbial community metabolic capacity supported their participation in the garden physiology. Although we knew that a bacterial community inhabited the garden, there were no data available on their spatial organization. Then we figured out that there is much more than the cultivated fungus in the garden; there are exuberant microbial biofilms surrounding the fungus and the substrates brought by the ants (chapter 2). Being there since the garden foundation, biofilms are ubiquitous components of attine gardens, occurring in colonies from different attine genera. So much so that we considered more appropriate to refer to this ecosystem as "microbial garden". We found that attine microbial gardens are primarily herbivorous, forming a continuum of lignocellulosic degradation as seen by NMR analysis in different fungicultures. The microbial garden exhibits a lignocellulosic nature, and indeed, plant-derived fragments are the majority of recognizable substrates (chapter 3). We understood the substrate as a keystone of the garden spatial structure, providing the framework for structuring fungal growth patterns and microbiota colonization (chapter 2). The microbial garden responds to changes in the substrate by shifting microbial taxonomic composition and spatial organization (chapter 4), and some of its members may even colonize highly recalcitrant polymers (chapter 5). This finding supports our assumption that one could find, in fungus-growing ants microbial ecosystems, promising candidates for degrading agroresidues and plastic waste (chapter 1). Our work unveiled the architecture of the fungus-growing ants microbial landscape, but we are aware that we only scratched the (garden) surface. The microbial garden awaits to be studied as a holistic entity responding to the environmental conditions, evolving through time, and likely keeping metabolic routes useful for our future environmental challenges. I gave a few steps in this journey, yet, there are entire microbial worlds to be known, down there in holes in the ground.

"Not all those who wander are lost."

J.R.R. Tolkien, The Fellowship of the Ring