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DEPARTMENT OF GENERAL AND APPLIED BIOLOGY

GRADUATE PROGRAM IN BIOLOGICAL SCIENCES
(APPLIED MICROBIOLOGY)

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FUNGAL-HELPER-BACTERIA COMMUNITIES IN HIGHER ATTINE ANT GARDENS

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This thesis is presented to the Institute of Biosciences at Rio Claro, from the São Paulo State University (UNESP), as part of the requirements to obtain the title of Doctor of Philosophy in Biological Sciences (Applied Microbiology).

Advisor: Dr. Andre Rodrigues

Co-advisor: Dra. Laura Victoria Flórez Patino

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POTENTIAL IMPACT OF THIS RESEARCH

This study was focused on the study of the leafcutter ant´s system. These insects are important for the economy of the American continent because they are pest of many agricultural crops. For science fungus-growing ants is a classic example of symbiosis and evolution. This system owes its evolutionary success to the symbiotic relationships they maintain with microorganisms. The tripartite mutualism between ants, mutualistic fungi and bacteria, was suggested as one of the key symbioses that led to the evolutionary success of this system. Recent studies have shown a great diversity of bacteria living within ant colonies. However, little was known about the symbiotic relationships between these microorganisms and the ants' cultivars. Here we explored bacteria-fungus interaction inside of the fungus garden of *Acromyrmex coronatus* and *Atta sexdens*. Our hypothesis suggested the existence of bacterial communities closely associated with the hyphae of the attine cultivars. Using different approaches like molecular and microscopic techniques, we showed that bacteria can interact with the fungus mutualists on their surface (forming biofilms) and inside of the hyphae (as an endobacteria), comprising the fungus mutualist` microbiota. That supports the idea the microbiome of attine ants can be used as a model system of interkingdom interactions. The results of this study allow us to have a broader idea on the microbial symbiosis within the attine system, as well as the importance of these relationships in the evolutionary success of these social insects. In addition, this study may help researchers to discover new pathways for the biological control of these insects, as well as, for the discovery of new application of this intertwining net of microorganisms. Finally, this study proves that only multidisciplinary approaches like the one we develop in collaboration with L'Institut National de la Recherche Agronomique (INRAe, France) and the University of Copenhagen (Denmark), will help scientists to reveal the importance of the microorganisms and their symbiotic relationships in the evolutionary success of the life.

IMPACTO POTENCIAL DA PESQUISA

A presente tese de doutorado teve como alvo de estudo o sistema das formigas cortadeiras. Estes insetos são importantes para a economia do continente americano, por serem pragas de diversas culturas agrícolas. Para a Ciência, o sistema das formigas cultivadoras de fungos é um exemplo clássico para estudos de simbiose e evolução. Esse sistema deve seu sucesso evolutivo às relações simbióticas que as formigas mantêm com microrganismos. A interação tripartida entre formigas, fungos mutualísticos e bactérias, por exemplo, foi sugerida como uma das principais simbioses que levaram ao sucesso evolutivo do sistema. Estudos recentes revelaram uma grande diversidade de bactérias nas colônias dessas formigas. No entanto, nada se sabia sobre as relações simbióticas entre esses microrganismos e os cultivares das formigas. Nesta tese de doutorado exploramos a interação bactéria-fungo nos dos jardins de fungo de *Acromyrmex coronatus* e *Atta sexdens*. Nossa hipótese sugere a existência de comunidades bacterianas intimamente associadas às hifas dos cultivares das formigas atíneas. Utilizando diferentes abordagens, como técnicas moleculares e microscópicas, mostramos que as bactérias podem interagir com os mutualistas do fungo na superfície (formando biofilme) e dentro da hifa (como uma endobactéria), formando a microbiota do fungo mutualista. Isso apoia a ideia de que o microbioma das formigas atíneas pode ser usado como um sistema modelo de interações entre reinos. Nossos resultados permitiram ter uma ideia mais ampla sobre a simbiose microbiana no sistema das atíneas, bem como a importância destas relações no sucesso evolutivo desses insetos sociais. Além disso, este estudo ajudará a pesquisadores a descobrir novos caminhos para o controle biológico desses insetos, bem como, para a descoberta de novas aplicações dessa rede interconectada de microrganismos. Finalmente, este estudo prova que só abordagens multidisciplinares, como a que desenvolvemos em colaboração com o L'Institut National de la Recherche Agronomique (INRAe, França) e da Universidade de Copenhagen (Dinamarca), ajudarão os cientistas a revelar a importância dos microrganismos e suas relações simbióticas no sucesso evolutivo da vida.

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TÍTULO DA TESE: FUNGAL-HELPER-BACTERIA COMMUNITIES IN HIGHER ATTINE ANT GARDENS

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ORIENTADOR: ANDRÉ RODRIGUES

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To my mother and my best friend, Josefina Sutta Martiarena.

“People born with a seed of curiosity, however environmental condition allows few people keep the childish innocence to ask silly things, that makes the world works.”

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RESUMO

As bactérias desempenham papéis metabólicos fundamentais para a sobrevivência de outros organismos, criando sistemas complexos. Os jardins de fungos das formigas attine (Hymenoptera: Attini: Attina) são exemplos didáticos dessa complexidade, pois esses insetos dependem obrigatoriamente de fungos basidiomicetos para sua alimentação. Embora esse mutualismo seja o cerne das colônias attinas, outros organismos estão envolvidos na manutenção desse microhabitat. Vários estudos mostraram comunidades bacterianas na matriz do jardín de fungo compostas por *Pantoea, Klebsiella, Pseudonocardia*, *Streptomyces* e outros gêneros considerados envolvidos na ciclagem de nutrientes e defesa contra fungos antagônicos que ameaçam o jardín. Por outro lado, a função de grupos bacterianos localizados na hifosfera, região que envolve a hifa fúngica e sob sua influência, não foi abordada em previos estudos. Nesta tese, objetivamos avaliar as comunidades bacterianas da hifosfera de *Leucoagaricus gongylophorus* e seus potenciais efeitos diretos sobre o cultivar das formigas. Isolamos bactérias da superfície da estafila (grupo de hifas inchadas onde os nutrientes do fungo são armazenados) com potenciais funções metabólicas para o desenvolvimento do cultivar e do jardim. Nossos resultados mostraram a presença de comunidades bacterianas como parte da microbiota do fungo. Ensaios *in vitro* mostraram que as bactérias que habitam a hifosfera podem ser benéficas para o fungo, aumentando a produção da biomassa e auxiliando na mineralização de compostos. Análise transcriptômica de *L. gongylophorus* na presença de *Staphylococcus* sp. mostraram vias antimicrobianas down-regulated permitindo o estabelecimento bacteriano nas hifas, bem como transcritos up-regualted relacionados à síntese de lipídios e carboidratos. Além disso, usando técnicas moleculares e microscópicas mostramos evidências de bactérias dentro da hifa de *L. gongylophorus*. Implicações ecológicas dessas bactérias para o fungo e para as formigas são discutidas. Nossos resultados coletivamente, mostram bactérias na superfície da hifa, bem como dentro da hifa, que constituem a microbiota fúngica que sustenta a vida microbiana no jardim de fungos.

Palavras-chave: Bactéria, *Leucoagaricus gongylophorus*, formigas attine, interação.

ABSTRACT

Bacteria play fundamental metabolic roles for the survival of other organisms, creating complex systems. The fungus gardens of attine ants (Hymenoptera: Attini: Attina) are textbook examples of such complexity, as these insects obligatory depend on basidiomycetous fungi for food. Although this mutualism is the core of attine colonies, other organisms are involved in the maintenance of this microhabitat. Several studies showed bacterial communities in the fungus gardens matrix composed of *Pantoea, Klebsiella, Pseudonocardia*, *Streptomyces*, and other genera thought to be involved in nutrient cycling and defence against antagonistic fungi that threaten the gardens. In contrast, the function of bacterial groups located in the hyphosphere, the region surrounding the fungal hypha and under their influence, were not tackled in any study. In this thesis, we aimed to assess bacterial communities on and inside of the hyphosphere of *Leucoagaricus gongylophorus* and their direct potential effects on this ant fungal cultivar. We isolated bacteria from the staphyla surface (swollen hypha where fungal nutrients are stored) with putative metabolic functions for the development of the fungus cultivar and the garden. Our findings showed the presence of bacterial communities as part of the fungus microbiota. *In vitro* assays showed that bacteria inhabiting the hyphosphere can be beneficial for the fungus, increasing biomass production and helping in compound mineralization. Transcriptomics analysis of *L. gongylophorus* in presence of *Staphylococcus* sp. showed down-regulated antimicrobial pathways allowing the bacterial establishment on the hyphae, as well as upregulated transcripts related to the synthesis of lipids and carbohydrates. In addition, we showed evidence of bacteria inside *L. gongylophorus* hypha using molecular and microscopic techniques. Ecological implications of these bacteria for the fungus and the ants are discussed. Collectively, bacteria on the hypha surface as well as inside the hypha comprise the fungal microbiota that support microbial life in the fungus garden.

Keywords: Bacteria, *Leucoagaricus gongylophorus*, attine ants, interaction.

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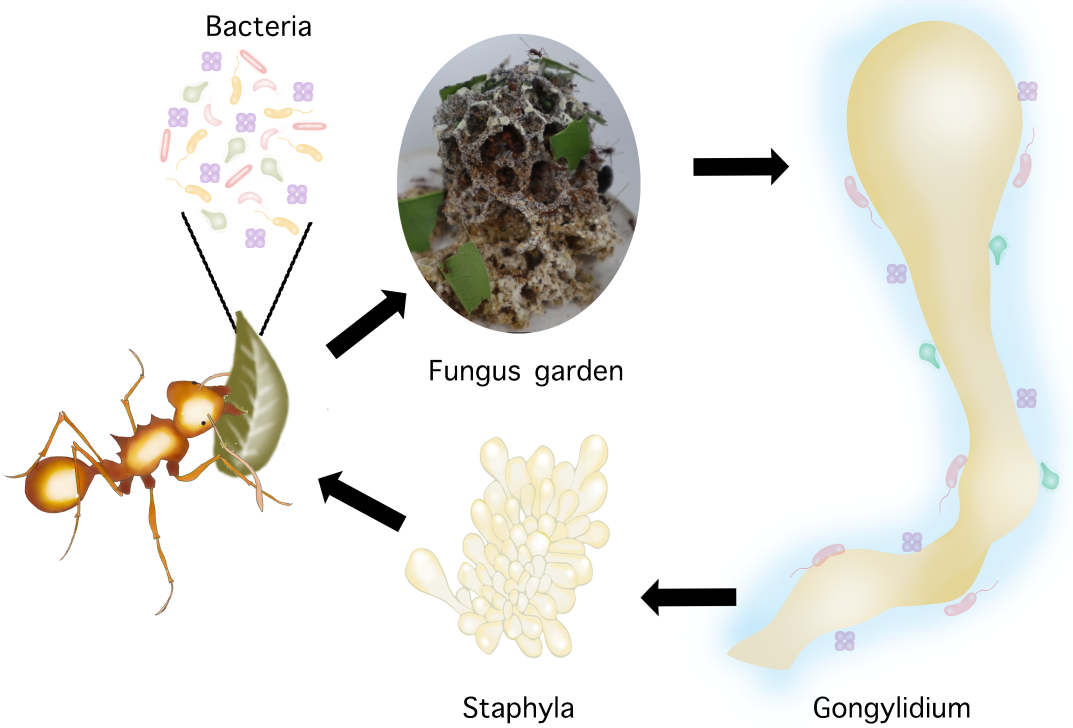
THESIS OVERVIEW

Bacteria are ubiquitous and play fundamental roles in the survival of almost all organisms. In complex systems bacteria can establish multidirectional interactions with different organisms as it occurs in the fungus gardens of fungus-farming ants (Hymenoptera: Formicidae: Attini: Attina, "the attines"). Considered the most derived fungiculture in the subtribe Attina (QUINLAN; CHERRETT, 1979), fungus gardens of higher attine ants comprise the obligatory symbiosis with mutualistic fungi (either *Leucoagaricus gongylophorus* or an unidentified *Leucoagaricus* species) as the main component (MARTIN; STADLER MARTIN, 1970), and a microbial community consisting of bacteria, filamentous fungi, yeasts, and other unknown microbes (CURRIE, 2001). Ants supply the fungus with plant material, and in exchange, they obtain from the cultivar nutrients, stored in a special fungal structure named "gongylidium" (POWELL ROY, 1984, Figure 1). While the symbiosis between the fungal cultivar and the ants is the core of the garden establishment and maintenance; other fundamental interspecific relationships take place in this habitat. Bacterial-fungal interactions are highly recognized in the attine ant colonies, especially those involving Actinobacteria in the genera *Streptomyces* and *Pseudonocardia,* which defend ant nests against antagonistic fungi, such as *Escovopsis* (LITTLE et al., 2006). Several studies focused in the fungus garden matrix, reported a great diversity of bacterial communities, such as *Burkholderia*, *Klebsiella, Pantoea,* and *Pseudomonas* (AYLWARD et al., 2012; BARCOTO et al., 2020; PINTO-TOMÁS et al., 2009). Nevertheless, no studies focused on the bacterial communities that are in direct contact with the cultivar hyphosphere, the surrounding area of the fungal hypha as well as its interior.

In model systems like mycorrhiza-plants, lichens, corals and animal guts, bacteria use the hyphosphere as a source of nutrients, either by living on the surface or inside the hypha (WARGO; HOGAN, 2006). In exchange, specific bacteria influence spore germination, hyphal growth, genetic regulation, nutrition, health, survival and virulence of the fungus (BAREA et al., 2005). Thus, bacteria and fungi can establish tight mutualistic relationships, which are often determinant for the survival of both organisms. Here we aimed to assess the bacteria present in the hyphosphere of the fungal cultivar and understand their putative roles. We assessed the putative metabolic functions of these bacterial groups, how they interact with the fungus in the hyphosphere, as well as determine the presence of intracellular bacterial groups in the hypha of the fungus cultivar. We structured this thesis in three chapters: Chapter I - "The hyphosphere of leaf-cutting ant cultivars is enriched in helper bacteria", Chapter II - "Pairwise transcriptomic analysis reveals signatures of fungal growth promotion and biofilm formation by bacteria in the hyphosphere of *Leucoagaricus gongylophorus*", and Chapter III - "Leafcutter ants' story: In the ants' colony has a garden, in the garden has a fungus, and in the fungus more life".

Figure 1. Mutualism between attines ants and their fungi. Ants cultivate fungi for food in structures named "fungus gardens". Based on published data we suggest the term "Fungal-helper" for the bacterial community in the hyphosphere of higher attine ant gardens. The surface of swollen hyphae (gongylidium) is a nutrient resource for bacterial nutrition; bacterial communities may collaborate for the maintenance of healthy garden.



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OVERALL THESIS CONCLUSION

Bacteria have unpredictable behaviors in different systems, mainly because of their vast metabolic plasticity and sometimes because of their undetectable presence. The field of bacteria–fungal interactions is still in its early stages of knowledge. Leafcutter ants are the perfect model systems to study this kind of interaction because of the vast diversity of microorganisms that support life in the ant colony. This is the case for many poorly addressed interactions found in the fungus garden. Since bacteria originated long before fungi and established associations with all kinds of organisms, it is very likely that the bacteria-fungus cultivar interaction arose from the very origin of the ants` fungiculture. Understanding how this interaction works will pave the road to comprehending the microbial garden of attine ants. We expected that our study ignites new research lines, especially those interested in determining if these bacteria are merely accompanying or are decisive for the success of the leafcutter ant system.