Microbial mediation of herbivory in leaf-cutter ant fungus gardens

By

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Dissertation Abstract

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Most metazoans lack the physiological capacity to use plants as their sole source of energy and nutrients. To compensate for this, metazoans associate with microbial symbionts, which aid their hosts with the breakdown of recalcitrant biomass, remediation of plant defense compounds, and nutrient supplementation. Leaf-cutter ants, dominant herbivores in the Neotropics, are a paradigmatic example of this microbial mediation of herbivory. The ants cut foliar biomass from their surroundings and provide it as a growth substrate to their fungal cultivar *Leucoagaricus gongylophorus*. The cultivar breaks down the plant biomass and provides specialized hyphal swellings called gongylidia, which the ants consume as their primary source of energy. In this dissertation, I explore the relationships between the different types of substrates ants incorporate into their fungus gardens and how both the fungal cultivar and the bacterial community facilitate the ants' breadth in substrate use. I approach these investigations through an ecological and evolutionary lens using contemporary 'omics' tools. In chapter 1, I present the context in which this dissertation work was completed. I describe trends in the microbial mediation of herbivory and review

the current state of understanding of how this relates to leaf-cutter ants. In chapter 2, I use metaproteomics to compare the proteins that the cultivar secretes when provided with different plant substrates. I show that the fungus responds in a flexible, substrate-specific manner to the material that the ants incorporate into their gardens. In chapter 3, I focus on the bacterial community in the fungus gardens of ants and how they may facilitate the ants' transition from using dicots to grasses. Using metagenomics, I show a shift in the bacterial community between these two types of substrate specialization and an associated shift in the functional capacity of the bacteria. In chapter 4, I continue investigating the ants' evolutionary transition to a novel substrate by examining the genomes of the fungal cultivars from the colonies of ants with grass and dicot substrate specializations. In this chapter I present preliminary results that further support the fungus' capacity for biomass degradation and present future directions into the evolution of this fungus, in terms of its transition to symbiosis, its transition to novel substrates, and its polykaryotic life history. In sum, the work presented in this dissertation expands our knowledge into the microbial mediation of herbivory in the leaf-cutter ant system.

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Table of Contents

Chapter 1: Introduction to dissertation

1.1 Microbial mediation of herbivory

Consumption of plant biomass by herbivorous metazoans is a critical component of terrestrial ecosystem functioning. Through feeding on plant biomass, herbivores serve as primary consumers and initiate passage of nutrients from producers through the food chain. Herbivores are also the most abundant animals on earth and are very diverse. They span at least ten phyla of animals, from simple single-celled organisms, through roundworms, mollusks, arthropods to vertebrates, and mammals. The plants that they consume are also diverse, with a multitude of different physiologies and morphologies designed to help avoid or reduce herbivory (Ricklefs & Miller 2000). These anti-herbivory adaptations have, in turn, caused many herbivores to specialize to consume particular plants or plant parts (Schultz 1988). It has become increasingly clear that herbivorous animals rely on microbes to help overcome plant defenses and to utilize this resource for their primary nutrients; herbivores' success is dictated by microbial symbiotic communities (Hume & Warner 1980; Barbosa *et al.* 1991). Understanding the mechanisms of herbivory, including their interactions with symbiotic microbes, is essential to understanding carbon cycling and ecological dynamics.

Plant biomass represents the largest reservoir of stored organic energy in terrestrial ecosystems, however, much of it remains inaccessible to animals occurring in the form of recalcitrant cellulose, hemicellulose and lignin. Generally, cell walls contain crystalline cellulose packed tightly together with hydrogen bonds and a uniform structure; cellulose sheaths are separated by hemicellulose, which is less crystalline and more variable in its structure; finally, lignin, an amorphous compound, keeps these components together, serving as a sort of glue (Himmel *et al.* 2007). All three of these compounds require a suite of

unique enzymes in order to be penetrated and subsequently broken down to serve as an energy source. Cellulose alone, requires endoglucanases and exoglucanases to cleave the rigid strands into cellobiose, which is then cleaved by β-glucosidases into glucose monomers (Lynd *et al.* 2002). Metazoans are typically unable to deconstruct recalcitrant plant polymers; herbivores use symbiotic microbes to aid them in these processes. Examples include, but are not limited to, bacteria in cow rumen (Hess *et al.* 2011), bacteria and protists in termite guts (Kudo 2009), and fungal and bacterial symbionts of *Sirex* spp. wood wasps (Talbot 1977; Adams *et al.* 2011).

While plants are high in energy, they tend to be low in certain nutrients, especially nitrogen, which can be a limiting factor for animals that consume plant biomass, including herbivores and detritivores. To compensate for this, some animals have developed symbioses with bacteria that can fix atmospheric nitrogen. Many examples of herbivore systems where bacterial mutualists provide fixed nitrogen to their hosts have been explored, including in bark beetles (Morales-Jiménez *et al.* 2009), ruminants (Granhall & Ciszuk 1971), and other systems (Harris 1993; Russell *et al.* 2009). The most deeply studied example of symbionts fixing nitrogen for their animal host is that of bacteria in the hind gut of termites (Benemann 1973; Potrikus & Breznak 1977; Noda *et al.* 1999; Warnecke *et al.* 2007). The diversity of microbial physiology creates other opportunities for aiding animals in nutrient acquisition. Examples include amino acid synthesis (Hansen & Moran 2011) and vitamin synthesis (LeBlanc *et al.* 2013). In general, microbes provide services to their hosts that allow them to consume substrates that do not contain sufficient nutrients for survival.

Defense compounds, and other secondary metabolites from plants, can be toxic to both the herbivore and the microorganisms that it associates with. Herbivores have developed many strategies in the arms race against plant defense compounds, including

behaviors, such as direct interference and avoidance and physiological strategies such as sequestration and metabolizing of plant defense compounds (Alba *et al.* 2011). Symbiotic microbes also appear to help herbivores through plant toxin mediation. For example, the mountain pine beetle uses bacteria and fungi to detoxify terpenes in the trees that it attacks (Wang *et al.* 2010; Adams *et al.* 2013; Boone *et al.* 2013). The microbes in these systems often provide metabolizing capabilities that animals alone lack (Dowd 1991).

While in some cases herbivores associate with a single microbial symbiont, many associate with communities of microbes, with different functions performed by various community members (Allison & Martiny 2008). By changing the relative abundance of various community members, the microbial community, as a whole, can respond to changes in substrate inputs, and allow for greater host flexibility. Some work has been done to examine changes in microbial community composition in symbiotic systems. Domingo et al. found that diet influenced the microbial community structure and biochemical activity in cricket hindguts (Santo Domingo *et al.* 1998). Zhou, et al. found that altering the diet of cows changed the microbial community composition in the rumen and affected methane production (2010). In both of these examples, it appears that changing the community composition is tied to biochemical processes that would affect the animal host. In addition to experimentation, it is also possible to gain insights into the function of a microbial community in a system, by comparing it to others. For example, Aylward *et al.* (2013), using comparative metagenomics, showed that host phylogeny, in addition to host substrate, are important for determining microbial community composition.

One symbiotic strategy that several insect groups employ is that of fungal agriculture. Fungus-growing termites, ants, and Ambrosia beetles, all grow fungal crops that they actively tend (Francke-Grosmann 1967; Aanen & Boomsma 2005; Schultz *et al.* 2005). In all three of

these cases, the fungus deconstructs plant biomass to various degrees, and the insects consume the fungus as their primary food source. Other insects are associated with fungi and their fitness is increased when the fungus is present, but they are not obligately reliant on it. Examples of insects that participate in this "pseudo fungal agriculture" include bark beetles (Harrington 2005) and bees (Brysch-Herzberg 2004). In both obligate and facultative fungal agriculture, the fungal crop is not grown in isolation. Bacteria are ubiquitous and insect fungus gardens contain a broad-scale characteristic bacterial community (Aylward *et al.* 2014).

1.2 Leaf-cutter ants

In this thesis, the microbial mediation of herbivory is studied in the context of the leaf-cutter ant system. Leaf-cutter ants are dominant herbivores in the Neotropics. Their dominance as herbivores can be viewed through many lenses: their diversity and abundance, their wide distribution, the diversity of substrates they consume, and through the impacts they have on the ecosystems in which they reside.

There are two genera of leaf-cutter ants, *Atta* and *Acromyrmex*, containing 15 and 24 described species, respectively (Cherrett *et al.* 1989). They range from the Southern United States to Uruguay (Cherrett & Peregrine 1976) and can be found in a broad diversity of ecosystems: tropical rainforests, dry forests, grasslands, disturbed habitats, intact habitats, wetlands and urban spaces. Some species of leaf-cutter ants have tremendously large ranges. *Atta cephalotes* can be found as far north as Mexico and as far south as Bolivia. *Atta sexdens* also has a wide range, from Colombia to Uruguay. It is thought that these species have such wide ranges, which are constantly expanding, because of their affinity for disturbed habitats. As humans encroach further into natural ecosystems, these cosmopolitan ants move with them (Cherrett & Peregrine 1976). In contrast to this, some species of leaf-cutters are

endemic to narrower niches and ranges. *Atta insularis* is restricted to the island of Cuba (Cherrett & Peregrine 1976) and *Atta robusta* is restricted to a thin strip of coastal *restingas* in Brazil (Teixeira *et al.* 2003). This latter species is especially sensitive to human activity and the encroachment of *Atta sexdens* colonies (Fowler 1995). Other species are restricted based on the substrates they consume. Grass-cutter ants (*Atta vollenweidiri, Atta capiguara* and *Atta bisphaerica*) are restricted to grasslands (Fowler *et al.* 1986).

Only a selection of species of leaf-cutter ants have been studied in detail in regards to the substrates that they incorporate into their gardens, but this work has demonstrated that leaf-cutter ants are generalist herbivores. The classic case study of a single colony of *Atta colombica* ants in Panama through one year is a prime example of the breadth of plant species leaf-cutter ant colonies can consume. Over the year, from just one of several foraging trails, the colony consumed leaves and flowers from 85 plants, belonging to 52 different species (Wirth *et al.* 1997; Wirth 2003). This diversity of plants is exceptional when compared to other insect herbivores. Bernays and Graham consider an insect a generalist herbivore if it consumes more than just three families of plants, and according to their estimates, less than 10% of insects fall into this category (1988). While there is interspecific variation in this regard for different species of leaf-cutters, the generalist nature of herbivory in this group is a definitive characteristic. Not only do leaf-cutter ants consume a large diversity of plants, they also consume massive quantities. A mature *Atta colombica* colony is capable of consuming 370 kg of plant material in one year. Leaf-cutter ants have been estimated to consume up to 17% of all the foliar biomass in some systems (Costa *et al.* 2008).

With their abundance, wide range, and voracious rates of plant consumption, leaf-cutter ants act as ecosystem engineers (Meyer *et al.* 2010; Corrêa *et al.* 2016). They affect canopy cover, soil mineral levels, and rates of carbon cycling, promoting the growth of

certain plants (Farji-Brener 2005; Sternberg *et al.* 2007; Sousa-Souto *et al.* 2012). They also tend to benefit exotic species and slow the regeneration of disturbed habitats (Farji Brener & Ghermandi 2008). They have the capacity to decimate both small-scale gardens (Belt 1874) and industrial agricultural plots (Fowler *et al.* 1986; Ferreira-Filho *et al.* 2015). Grass-cutter ants are estimated to cost ranchers 10-30% of their cattle production as they compete with them for forage (Fowler *et al.* 1986). While their overall effect on ecosystems is highly context-dependent (Farji Brener & Tadey 2016), it is undeniable that leaf-cutter ants affect biogeochemical cycles, their local ecology, and human agriculture.

Mediating the relationship between leaf-cutter ants and the plants that they utilize is an obligately mutualistic fungus, *Leucoagaricus gongylophorus*. The ants cultivate this fungus, feeding it fresh plant material, and in exchange, the fungus provides the ants with a usable source of energy and nutrients in specialized hyphal swellings called gongylidia (Holldobler & Wilson 2010). This fungal cultivar is vertically transmitted. When virgin queens depart from their mother's nest they take a wad of fungus garden with them, which they carry in their infrabuccal pocket. After mating, the queens lose their wings and dig a hole to establish their own colony. At first the queen will tend to her fungus garden by feeding it mostly fecal liquid, and she survives by consuming unfertilized eggs. Once the fungus garden has sufficiently stabilized, she will lay fertilized eggs that will result in workers, which will take over the duty of garden tending (Wirth 2003).

Garden workers engage in many activities to tend their crop (Wilson 1980). All surfaces, including fresh leaf material are licked, presumably to remove pathogens (Quinlan & Cherrett 1977; Bass & Cherrett 1994). Workers also spend time processing leaves before incorporation into fungus gardens to remove trichomes (Kitayama *et al.* 2010), waxes (Quinlan & Cherrett 1977) and plant endophytes (Van Bael *et al.* 2009). Next, most species

of leaf-cutter ants mulch the leaf material into smaller pieces (grass-cutter ants are an exception to this, leaving their grass pieces more intact) (Fowler *et al.* 1986). They then place a tuft of fungal hyphae from further down in the fungus garden on their newly incorporated piece to inoculate their cultivar on the new substrate (Bass & Cherrett 1994). They also defecate on the new material. This is because their feces contain biomass-degrading enzymes that originate in the gongylidia. The deposition of the enzyme-rich feces serves as a pre-treatment step in biomass degradation (De Fine Licht *et al.* 2013; Aylward *et al.* 2015). The ants are also important for keeping their gardens free from disease by "weeding" and "grooming" it (Currie & Stuart 2001). They remove pathogenic spores and diseased garden fragments, depositing both in the refuse dumps, which are either maintained in subterranean chambers below their fungus chambers, or externally in mounds away from their colony (Farji-Brener & Medina 2000).

1.3 Microbial mediation of herbivory in leaf-cutter ant fungus gardens

In the leaf-cutter ant system, the ants are responsible for the initial mechanical breakdown of plant material. Their complex and efficient foraging strategies ensure a steady supply of leaf material for their gardens (Werner 1973; Shepherd 1982; Roces 1993; Wirth *et al.* 1997; de Andrade *et al.* 2002; Roschard & Roces 2003). It is through the enzymatic capabilities of the fungus that the ants gain access to the energy and nutrients locked behind the recalcitrant polymers in the plants. Through previous work and the work in this thesis, we have learned that the fungus produces substrate-specific enzymes necessary for recalcitrant biomass degradation, especially of hemicellulose and cellulose (Nagamoto *et al.* 2011; Kooij *et al.* 2011; Suen *et al.* 2011; Grell *et al.* 2013; Aylward *et al.* 2013; Khadempour *et al.* 2016). While the fungus also produces laccases and has some potential to break down lignin, it does not seem to use it as an energy source, since the overall proportion of lignin

increases slightly as plant material passes through the fungus garden and other components are consumed (Suen *et al.* 2011). Furthermore, the fungus has lost one key lignin degradation domain that its closest saprophytic relatives possess (Nygaard *et al.* 2016).

Atta and *Acromyrmex* are the most derived genera in a tribe of fungus-growing ants, the Attini (Wetterer 1998). The tribe's phylogeny is congruent with that of their cultivar fungi. The most basal of fungus-growing ants are associated with fungi that are only facultative mutualists, rather than the obligate mutualists of the "higher attines". These basal fungi do not have specialized gongylidia (or similar) structures for the ants to consume (Schultz & Brady 2008). The more derived ants and their cultivars show some evidence for reciprocal genome evolution (Nygaard *et al.* 2016). While this co-evolutionary relationship has been demonstrated on the scale of the tribe, little has been explored in regards to the evolutionary relationships within one genus of ant. There are two recent studies that provide some evidence that particular species of ants grow separate lineages of fungi. One such study is restricted to *Acromyrmex* ants (Pereira *et al.* 2015), and the other was more broad (Mueller *et al.* 2017), but both suggest that there is a separation based on the ecology of the ants, specifically whether the ants cut grasses or dicotyledons.

It has become clear that the fungal cultivar in this system is responsible for accessing the energy from a diverse array of plant material. However, in order for the system to be able to consume plants, it must also contend with the low nutritive quality of plant material and with plant defense compounds. One study suggests that laccases produced by the fungus are responsible for degrading plant defense compounds (De Fine Licht *et al.* 2013), but beyond that, the fungus does not have the physiological capacity to completely mediate the relationship between the ants and the plants. It is thought that a bacterial community in the fungus gardens also plays an important role in the system.

Previous work by Aylward *et al.* (2012), Suen *et al.* (2010; 2011) and De Oliveira Barcoto *et al.* (2017) demonstrate that a bacterial community is consistently present in the fungus gardens. This community is dominated by Proteobacteria, particularly *Pantoea*, *Burkholderia*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*. On a broad scale, this community is conserved between different species of fungus-growing ants, and on an even broader scale, it is conserved between other fungus-growing insects (Aylward *et al.* 2014; De Oliveira Barcoto 2017). Along with the fungal cultivar, these bacteria are vertically transmitted. They have been found in the pellet that new queens carry in their infrabuccal pocket (Moreira-Soto *et al.* 2017). Besides the fact that these bacteria are consistently present, we know little about their roles in the garden. One study demonstrated that *Pantoea* and *Klebsiella* fix nitrogen in the system, which the ants incorporate into their bodies (Pinto-Tomás *et al.* 2009). Further work with metagenomics and metaproteomics suggests that bacteria are important for biomass degradation and vitamin production (Suen *et al.* 2010; Aylward *et al.* 2012), but without an experiment or comparative work, these studies have only been able to suggest the potential for these functions.

1.4 Introduction to thesis chapters

The aim of this thesis, overall, is to further explore the role of both the fungus and bacteria in the fungus gardens of leaf-cutter ants in mediating the relationship between ants and the plants that they consume.

In chapter 2, I conduct an experiment where sub-colonies of leaf-cutter ants are fed different substrates. Through metaproteomic analysis, I show that the fungal cultivar responds in a substrate-specific manner to different types of plant material that are incorporated into the fungus gardens. The fungus produces enzymes for the deconstruction of recalcitrant biomass, but only when a simpler carbon source is not available. This flexible,

substrate-specific response to plant substrates can help explain how the leaf-cutter ant system can function as an extreme generalist herbivore.

Chapter 3 focuses on the bacterial community in leaf-cutter ant fungus gardens. Here, I compared the bacterial community, both in terms of the relative abundance of particular groups and in terms of the functional potential, between grass- and dicot-cutter ant fungus gardens. While on the phylum level these fungus gardens are similar to other previously described bacterial communities associated with other fungus growing ants and insects, we can see that there is a clear difference on the genus and functional gene level between the two ant life histories. This difference is particularly pronounced in regards to nutrient supplementation genes, such as nitrogen fixation, amino acid metabolism and iron acquisition. All of these genes are more abundant in the grass-cutter ant fungus gardens, where the inputs are lower in these essential nutrients.

In Chapter 4, I compare the fungal genomes from grass- and dicot-cutter ants. This work is preliminary since only the first of eight fungal genomes have been sequenced for this project. Nevertheless, we see that *Leucoagaricus gongylophorus* contains a reduced number of genes compared to closely related fungi, with an expanded genome with large repeat regions. There is evidence for multiple different nuclei in each cell. Finally, in contrast to previous reports the there is no clear congruence between they phylogenies of *Atta* ants and the fungi they cultivate.

1.5 References

Aanen DK, Boomsma JJ (2005) Evolutionary dynamics of the mutualistic symbiosis between fungus-growing termites and *Termitomyces* fungi. In: *Insect-Fungal Associations* (eds Vega F, Blackwell M), pp. 191–210. Oxford University Press, New York, NY.

Adams AS, Aylward FO, Adams SM *et al.* (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology*, **79**, 3468–3475.

Adams AS, Jordan MS, Adams SM *et al.* (2011) Cellulose-degrading bacteria associated with the invasive woodwasp *Sirex noctilio*. *The ISME Journal*, **5**, 1323–1331.

Alba JM, Glas JJ, Schimmel BCJ, Kant MR (2011) Avoidance and suppression of plant defenses by herbivores and pathogens. *Journal of Plant Interactions*, **6**, 221–227.

Allison SD, Martiny JBH (2008) Colloquium paper: resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences*, **105 Suppl 1**, 11512–11519.

Aylward FO, Burnum KE, Scott JJ *et al.* (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. *The ISME Journal*, **6**, 1688–1701.

Aylward FO, Burnum-Johnson KE, Tringe SG *et al.* (2013) *Leucoagaricus gongylophorus* produces diverse enzymes for the degradation of recalcitrant plant polymers in leaf-cutter ant fungus gardens. *Applied and Environmental Microbiology*, **79**, 3770–3778.

Aylward FO, Khadempour L, Tremmel DM *et al.* (2015) Enrichment and broad representation of plant biomass-degrading enzymes in the specialized hyphal swellings of *Leucoagaricus gongylophorus*, the fungal symbiont of leaf-cutter ants. *PLoS ONE*, **10**, e0134752.

Aylward FO, Suen G, Biedermann PHW *et al.* (2014) Convergent bacterial microbiotas in the fungal agricultural systems of insects. *mBio*, **5**, e02077–14.

Barbosa P, Krischik VA, Jones CG (1991) *Microbial Mediation of Plant-Herbivore Interactions*. Wiley-Interscience, New York.

Bass M, Cherrett JM (1994) The role of leaf-cutting ant workers (Hymenoptera, Formicidae) in fungus garden maintenance. *Ecological Entomology*, **19**, 215–220.

Belt T (1874) *The naturalist in Nicaragua*. E.P. Dutton & Co., New York.

Benemann JR (1973) Nitrogen fixation in termites. *Science*, **181**, 164–165.

Bernays E, Graham M (1988) On the evolution of host specificity in phytophagous arthropods. *Ecology*, 886–892.

Boone CK, Keefover-Ring K, Mapes AC *et al.* (2013) Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. *Journal of Chemical Ecology*, **39**, 1003–1006.

Brysch-Herzberg M (2004) Ecology of yeasts in plant-bumblebee mutualism in Central Europe. *FEMS Microbiology Ecology*, **50**, 87–100.

Cherrett JM, Peregrine DJ (1976) A review of the status of leaf-cutting ants and their control. *Annals of Applied Biology*, **84**, 124–128.

Cherrett JM, Powell RJ, Stradling D (1989) The mutualism between leaf-cutting ants and their fungus. In: *Insect-fungus interactions* (eds Wilding N, Collins NM, Hammond PM, Webber J), pp. 93–120. Academic Press, London.

Corrêa MM, Silva PSD, Wirth R, Tabarelli M, Leal IR (2016) Foraging activity of leaf-cutting ants changes light availability and plant assemblage in Atlantic forest. *Ecological Entomology*, **41**, 442–450.

Costa AN, Vasconcelos HL, Vieira-Neto EHM, Bruna EM (2008) Do herbivores exert top-down effects in Neotropical savannas? Estimates of biomass consumption by leaf-cutter ants. *Journal of Vegetation Science*, **19**, 849–854.

Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. *Proceedings. Biological Sciences/ The Royal Society*, **268**, 1033–1039.

de Andrade A, Forti LC, Moreira AA *et al.* (2002) Behavior of *Atta sexdens rubropilosa* (Hymenoptera : Formicidae) workers during the preparation of the leaf substrate for symbiont fungus culture. *Sociobiology*, **40**, 293–306.

De Fine Licht HH, Schiøtt M, Rogowska-Wrzesinska A *et al.* (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences*, **110**, 583–587.

De Oliveira Barcoto M (2017) Fungus-growing insects host a convergent microbiome with functional similarities to other lignocellulose-feeding insects. Rio Claro, Brazil.

Dowd PF (1991) Symbiont-mediated detoxification in insect herbivores. In: *Microbial Mediation of Plant-Herbivore Interactions*, pp. 411–440. Wiley-Interscience, New York.

Farji Brener AG, Ghermandi L (2008) Leaf-cutting ant nests near roads increase fitness of exotic plant species in natural protected areas. *Proceedings. Biological Sciences/ The Royal Society*, **275**, 1431–1440.

Farji Brener AG, Tadey M (2016) Consequences of leaf-cutting ants on plant fitness: integrating negative effects of herbivory and positive effects from soil improvement. *Insectes sociaux*, **64**, 45–54.

Farji-Brener AG (2005) The effect of abandoned leaf-cutting ant nests on plant assemblage composition in a tropical rainforest of Costa Rica. *Ecoscience*, **12**, 554–560.

Farji-Brener AG, Medina CA (2000) The importance of where to dump the refuse: Seed banks and fine roots in nests of the leaf-cutting ants *Atta cephalotes* and *A. colombica*. *Biotropica*, **32**, 120–126.

Ferreira-Filho PJ, Wilcken CF, Neves DA *et al.* (2015) Does diatomaceous earth control leaf-cutter ants (Hymenoptera: Formicidae) in the *Eucalyptus* plantations? *Journal of economic entomology*, **108**, 1124–1128.

Fowler H (1995) The population status of the endangered Brazilian endemic leaf-cutting ant *Atta robusta* (hymenoptera, formicidae). *Biological Conservation*, **74**, 147–150.

Fowler H, Forti L, Pereira-da-Silva V, Saes N (1986) Economics of grass-cutting ants. In: *Fire ants and leaf-cutting ants* Fire Ants and Leaf-Cutting Ants. (eds Lofgren CS, Vander

Meer RK), pp. 18–35. Westview Pr, Boulder, CO.

Francke-Grosmann H (1967) Ectosymbiosis in wood-inhabiting insects. In: *Symbiosis: Associations of invertebrates, birds, ruminants, and other biota* (ed Henry SM), pp. 141–205. Academic Press, Inc., New York, NY.

Granhall U, Ciszuk P (1971) Nitrogen fixation in rumen contents indicated by the acetylene reduction test. *Journal of General Microbiology*, **65**, 91–93.

Grell MN, Linde T, Nygaard S *et al.* (2013) The fungal symbiont of *Acromyrmex* leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and other plant cell wall polysaccharides. *BMC Genomics*, **14**, 928.

Hansen AK, Moran NA (2011) Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *PNAS*, **108**, 2849–2854.

Harrington T (2005) Ecology and evolution of mycophagous bark beetles and their fungal partners. In: *Insect-Fungal Associations* (eds Vega F, Blackwell M), pp. 257–292. Oxford University Press, New York, NY.

Harris JM (1993) The presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis. *Microbial Ecology*, **25**, 195–231.

Hess M, Sczyrba A, Egan R *et al.* (2011) Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science*, **331**, 463–467.

Himmel ME, Ding S-Y, Johnson DK *et al.* (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science*, **315**, 804–807.

Holldobler B, Wilson EO (2010) *The Leafcutter Ants: Civilization by Instinct*. W.W. Norton and Company, New York, NY.

Hume ID, Warner ACI (1980) Evolution of microbial digestion in mammals. In: *Digestive physiology and metabolism in ruminants*, pp. 665–684. Springer Netherlands, Dordrecht.

Khadempour L, Burnum KE, Baker ES *et al.* (2016) The fungal cultivar of leaf-cutter ants produces specific enzymes in response to different plant substrates. *Molecular Ecology*, **25**, 5795–5805.

Kitayama K, Hay JD, Sousa-Souto L, Ottoni RJV, Uchoa de Aquino PDP (2010) Trichomes and *Atta sexdens* (Hymenoptera: Formicidae): a study of foraging behavior in the laboratory. *Sociobiology*, **55**, 107–116.

Kooij PW, Schiøtt M, Boomsma JJ, De Fine Licht HH (2011) Rapid shifts in *Atta cephalotes* fungus-garden enzyme activity after a change in fungal substrate (Attini, Formicidae). *Insectes sociaux*, **58**, 145–151.

Kudo T (2009) Termite-microbe symbiotic system and its efficient degradation of lignocellulose. *Bioscience, Biotechnology, and Biochemistry*, **73**, 2561–2567.

LeBlanc JG, Milani C, de Giori GS *et al.* (2013) Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Current opinion in biotechnology*, **24**, 160–168.

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*, **66**, 506–577.

Meyer ST, Leal IR, Tabarelli M, Wirth R (2010) Ecosystem engineering by leaf-cutting ants:

nests of *Atta cephalotes* drastically alter forest structure and microclimate. *Ecological Entomology*, **36**, 14–24.

Morales-Jiménez J, Zúñiga G, Villa-Tanaca L, Hernández-Rodríguez C (2009) Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microbial Ecology*, **58**, 879–891.

Moreira-Soto RD, Sanchez E, Currie CR, Pinto-Tomás AA (2017) Ultrastructural and microbial analyses of cellulose degradation in leaf-cutter ant colonies. *Journal of General Microbiology*, **163**, 1578–1589.

Mueller UG, Ishak HD, Bruschi SM *et al.* (2017) Biogeography of mutualistic fungi cultivated by leafcutter ants. *Molecular Ecology*, **26**, 6921–6937.

Nagamoto NS, Garcia MG, Forti LC *et al.* (2011) Microscopic evidence supports the hypothesis of high cellulose degradation capacity by the symbiotic fungus of leaf-cutting ants. *Journal of Biological Research-Thessaloniki*, **16**, 308–312.

Noda S, Ohkuma M, Usami R, Horikoshi K, Kudo T (1999) Culture-independent characterization of a gene responsible for nitrogen fixation in the symbiotic microbial community in the gut of the termite *Neotermes koshunensis*. *Applied and Environmental Microbiology*, **65**, 4935–4942.

Nygaard S, Hu H, Li C *et al.* (2016) Reciprocal genomic evolution in the ant-fungus agricultural symbiosis. *Nature communications*, **7**, 12233.

Pereira DIB, Botton S de A, Azevedo MI de *et al.* (2015) Isolation and molecular characterization of symbiotic fungus from *Acromyrmex ambiguus* and *Acromyrmex heyeri* ants of Rio Grande do Sul State, Brazil. *Ciência Rural*, **45**, 1256–1261.

Pinto-Tomás AA, Anderson MA, Suen G *et al.* (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science*, **326**, 1120–1123.

Potrikus CJ, Breznak JA (1977) Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. *Applied and Environmental Microbiology*, **33**, 392–399.

Quinlan RJ, Cherrett JM (1977) The role of substrate preparation in the symbiosis between the leaf-cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. *Ecological Entomology*, **2**, 161–170.

Ricklefs RE, Miller GL (2000) *Ecology*. W. H. Freeman and Company, New York, NY.

Roces F (1993) Both evaluation of resource quality and speed of recruited leaf-cutting ants (*Acromyrmex lundi*) depend on their motivational state. *Behavioral Ecology and Sociobiology*, **33**, 183–189.

Roschard J, Roces F (2003) Cutters, carriers and transport chains: Distance-dependent foraging strategies in the grass-cutting ant *Atta vollenweideri*. *Insectes sociaux*, **50**, 237–244.

Russell JA, Moreau CS, Goldman-Huertas B *et al.* (2009) Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proceedings of the National Academy of Sciences*, **106**, 21236–21241.

Santo Domingo JW, Kaufman MG, Klug MJ *et al.* (1998) Influence of diet on the structure and function of the bacterial hindgut community of crickets. *Molecular Ecology*, **7**, 761–767.

Schultz JC (1988) Many factors influence the evolution of herbivore diets, but plant chemistry is central. *Ecology*, **69**, 896–897.

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences*, **105**, 5435–5440.

Schultz TR, Mueller UG, Cameron R Currie, Rehner SA (2005) Reciprocal illumination: A comparison of agriculture in humans and fungus-growing ants. In: *Insect-Fungal Associations* (eds Vega F, Blackwell M), pp. 149–190. Oxford University Press, New York, NY.

Shepherd JD (1982) Trunk trails and the searching strategy of a leaf-cutter ant, *Atta colombica*. *Behavioral Ecology and Sociobiology*, **11**, 77–84.

Sousa-Souto L, de Jesus Santos DC, Ambrogi BG *et al.* (2012) Increased CO2 emission and organic matter decomposition by leaf-cutting ant nests in a coastal environment. *Soil Biology and Biochemistry*, **44**, 21–25.

Sternberg LDSL, Pinzon MC, Moreira MZ *et al.* (2007) Plants use macronutrients accumulated in leaf-cutting ant nests. *Proceedings. Biological Sciences/ The Royal Society*, **274**, 315–321.

Suen G, Scott JJ, Aylward FO, Currie CR (2011) The microbiome of leaf-cutter ant fungus gardens. In: *Handbook of Molecular Microbial Ecology, Volume III: Metagenomics in Different Habitats* Metagenomics in Different Habitats. (ed de Bruijn FJ), pp. 367–379. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Suen G, Scott JJ, Aylward FO *et al.* (2010) An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS genetics*, **6**, e1001129.

Talbot PHB (1977) The *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology*, **15**, 41–54.

Teixeira MC, Schoereder JH, Mayhé-Nunes AJ (2003) Geographic distribution of *Atta robusta* Borgmeier (Hymenoptera: Formicidae). *Neotropical Entomology*, **32**, 719–721.

Van Bael SA, Fernandez-Marin H, Valencia MC *et al.* (2009) Two fungal symbioses collide: endophytic fungi are not welcome in leaf-cutting ant gardens. *Proceedings. Biological Sciences/ The Royal Society*, **276**, 2419–2426.

Wang Y, DiGuistini S, Wang T-CT, Bohlmann J, Breuil C (2010) *Agrobacterium*-meditated gene disruption using split-marker in *Grosmannia clavigera*, a mountain pine beetle associated pathogen. *Current genetics*, **56**, 297–307.

Warnecke F, hl PLU, Ivanova N *et al.* (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature Biotechnology*, **450**, 560–565.

Werner F (1973) *Foraging activity of the leaf-cutter ant, Acromyrmex versicolor, in relation to season, weather and colony condition*. US International Biological Program, Desert Biome, Logan, UT.

Wetterer J (1998) Phylogeny of fungus-growing ants (tribe Attini) based on mtdna sequence and morphology. *Molecular Phylogenetics and Evolution*, **9**, 42–47.

Wilson EO (1980) Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). *Behavioral Ecology and Sociobiology*, **7**, 143–156.

Wirth R (2003) *Herbivory of leaf-cutting ants: a case study on Atta colombica in the tropical rainforest of Panama* (R Wirth, H Herz, RJ Ryel, W Beyschlag, B Hölldobler, Eds,). Springer-Verlag Berlin Heidelberg, Germany.

Wirth R, Beyschlag W, Ryel RJ, Holldobler B (1997) Annual foraging of the leaf-cutting ant *Atta colombica* in a semideciduous rain forest in Panama. *Journal of Tropical Ecology*, **13**, 1–741–75717.

Zhou M, Hernandez-Sanabria E, Guan LL (2010) Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. *Applied and Environmental Microbiology*, **76**, 3776–3786.

Chapter 2: The fungal cultivar of leaf-cutter ants produces specific enzymes in response to different plant substrates

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2.1 Abstract

Herbivores use symbiotic microbes to help derive energy and nutrients from plant material. Leaf-cutter ants are a paradigmatic example, cultivating their mutualistic fungus *Leucoagaricus gongylophorus* on plant biomass that workers forage from a diverse collection of plant species. Here, we investigate the metabolic flexibility of the ants' fungal cultivar for utilizing different plant biomass. Using feeding experiments and a novel approach in metaproteomics, we examine the enzymatic response of *L. gongylophorus* to leaves, flowers, oats, or a mixture of all three. Across all treatments, our analysis identified and quantified 1,766 different fungal proteins, including 161 putative biomass-degrading enzymes. We found significant differences in the protein profiles in the fungus gardens of sub-colonies fed different plant substrates. When provided with leaves or flowers, which contain the majority of their energy as recalcitrant plant polymers, the fungus gardens produced more proteins predicted to break down cellulose: endoglucanase, exoglucanase, and β-glucosidase. Further, the complete metaproteomes for the leaves and flowers treatments were very similar, while the mixed substrate treatment closely resembled the treatment with oats alone. This indicates that when provided a mixture of plant substrates, fungus gardens preferentially break down the simpler, more digestible substrates. This flexible, substrate-specific enzymatic response of the fungal cultivar allows leaf-cutter ants to derive energy from a wide range of substrates, which likely contributes to their ability to be dominant generalist herbivores.

2.2 Introduction

Herbivores are the most abundant and diverse animals on earth (Ricklefs & Miller 2000). Their success is shaped, at least in part, by different animal lineages evolving to specialize on different plant species and plant parts, each of which provide different barriers for herbivores to access stored carbon and other nutrients (Hansen & Moran 2013). Arguably, the most important strategy herbivores use to contend with these barriers to consumption is establishing symbiotic associations with microbes that broaden their physiological capacity (Dowd 1991).

The microbial mediation of herbivory has been studied at length in substrate-specialized herbivore systems. Microbial symbionts, which include bacteria, fungi and other microorganisms, mediate herbivory in three main ways: helping their hosts overcome recalcitrant plant material, supplementing nutrient-poor diets, and reducing the impact of plant defense compounds (Hansen & Moran 2013). For example, termites break down the highly recalcitrant biomass in wood through their association with both eukaryotic and bacterial symbionts (Tartar *et al.* 2009). The plant sap feeding aphids house intracellular *Buchnera aphidicola* that compensate for the absence of essential amino acids in their diet (Hansen & Moran 2011). Finally, when attacking trees the mountain pine beetle vectors fungi and bacteria, which break down terpenes that would otherwise be toxic to the developing larvae that specialize on tree phloem as a food source (Wang *et al.* 2012; Boone *et al.* 2013).

Unlike most herbivores, leaf-cutter ants are polyphagous, meaning that they occupy a generalist herbivore niche. These dominant herbivores belong to two genera, *Acromyrmex* and *Atta*, and forage on 2-17% of all the foliar biomass in some ecosystems in the Neotropics (Herz *et al.* 2007; Costa *et al.* 2008). Their success as herbivores can be attributed to their

obligate mutualism with a fungus, *Leucoagaricus gongylophorus*, which they cultivate for food: they provide the fungus with leaf material and, in turn, the fungus provides specialized hyphal swellings called gongylidia, which the ants feed on (Holldobler & Wilson 1990; Mayhé-Nunes & Jaffe 1998; Holldobler & Wilson 2008). The types of plant material that a colony consumes depends on the ant species, the location, and the season in which the colony is observed (De Vasconcelos 1990; Wirth 2003). In general, they tend toward young leaves with soft cuticles, less-toxic plant defense compounds, fewer trichomes, fewer endophytes and higher nutritional value (Howard 1987; 1988; Van Bael *et al.* 2011). Within these constraints, leaf-cutter ants incorporate many different types of plants into their fungus gardens and have been observed foraging at least 20 different species of plants over three days (Wirth *et al.* 1997). Ants also incorporate a variety of plant parts into their gardens such as leaves, flowers, seeds, and fruit parts in the wild, and oats and parboiled rice in laboratory settings (Wirth *et al.* 1997; Kooij *et al.* 2011).

Leaf-cutter ants tend to their mutualistic fungus in gardens, which can be viewed as an 'external gut'. These gardens contain both the fungus itself and a low diversity community of bacteria. Through enzymatic, metagenomic and metaproteomic analyses, the microbial communities in the fungus gardens of leaf-cutter ants *Atta sexdens* and *Atta cephalotes* have been explored. Many fungal amylases (Silva *et al.* 2006a), pectinases (Silva *et al.* 2006b), carbohydrate-active enzymes (CAZy), fungal oxidative lignin enzymes (FOLy), and secreted proteases have been identified (Aylward *et al.* 2012; 2013a), demonstrating that the fungus in this system is primarily responsible for the breakdown of plant biomass. The bacterial community in the fungus gardens was identified using isolation, metagenomics and 16S sequencing (Suen *et al.* 2010; Aylward *et al.* 2012). While the bacterial community has the

genetic capacity for biomass degradation (Suen *et al.* 2010), there is not yet evidence that this is actually occurring in the gardens.

In this study, we explore microbial mediation in a generalist herbivore by combining feeding experiments with metaproteomic analyses. Specifically, we fed sub-colonies of leaf-cutter ants leaves, flowers, oats or a mixture of all three. Using a novel multidimensional platform, coupling liquid chromatography, ion mobility spectrometry and mass spectrometry (LC-IMS-MS), we determined the metaproteomic response of fungus gardens on the different diets. Our working hypothesis is that the fungal cultivar *L. gongylophorus* responds to different plant substrates integrated into the garden by worker ants by producing specific proteins that have the capacity to break down the substrate provided.

2.3 Methods

2.3.1 Experimental design

Atta cephalotes fungus gardens were excised from colonies excavated in the secondary tropical moist forest surrounding the Smithsonian Tropical Research Institute (STRI) Gamboa research station in Panama between Dec. 27, 2012 and Jan 10, 2013. Five mature colonies were excavated. Since lab-reared sub-colonies without queens are unstable, five fungus chambers were excised from each colony to ensure that we would have sufficient numbers of replicates for proteomics. These fungus chambers were split into four sub-colonies each and were contained within a plastic container (10x10x8 cm) that was kept in a larger plastic container (14x19x9 cm). Care was taken to minimize disturbance to the fungus gardens and to ensure that a relatively even number of workers were distributed to each sub-colony.

Each sub-colony was randomly assigned to one of four feeding treatments, and received different plant biomass to use as substrate for cultivating their fungal mutualist. The four feeding treatments were *Lagerstroemia speciosa* L. leaves, *Hibiscus rosa-sinensis* flowers, Quaker instant oatmeal, or a mixture of all three (Figure 2.1). The substrates that were selected were all readily available and were readily incorporated into the gardens by the ants, but they varied in terms of their energy availability. Leaves are the most recalcitrant substrate of the three. The flowers are similar to leaves in terms of cell wall structures but are more easily digestible (Amaglo *et al.* 2010). The oats are highly processed and have the most accessible energy in the form of sugars and starches (Cuddeford 1995; Welch 1995). The flowers and leaves were collected daily from plants in the immediate vicinity in Gamboa. The sub-colonies were fed *ad libitum*, typically every one or two days, depending on how quickly the ants would incorporate new substrate. The colonies were maintained at ambient temperature and humidity. After 15 days, the entire fungus garden from each sub-colony was frozen in PBS buffer at -20°C in a 50 mL conical tube, in preparation for further processing. One of the five colonies was excluded from metaproteomic analysis because it did not have surviving sub-colonies from all treatments but it was included it in the survivorship analysis. From the surviving sub-colonies we selected 16 samples for metaproteomics (four treatments and four colony replicates each). The sub-colonies that were selected for metaproteomics were all active and still incorporating new material into their gardens at the end of the 15 days of the experiment.

2.3.2 Mass spectrometry instrumentation

Analysis of the trypsin-digested peptide mixtures (Supplemental Methods) from the gardens was performed on both a *Thermo* Fisher Scientific *LTQ Orbitrap mass spectrometer (MS)* (San Jose, CA, USA) operated in tandem MS (MS/MS) mode and an in-house built ion-

mobility MS (IMS-MS) instrument that couples a 1-m ion mobility drift cell (Baker *et al.* 2007; 2010) with an Agilent 6224 time-of-flight (TOF) MS that was upgraded to have a 1.5 m flight tube for resolution around 25,000. The same fully automated in-house built 2-column HPLC system (Livesay *et al.* 2008) equipped with in-house packed capillary columns was used for both instruments with mobile phase A consisting of 0.1% formic acid in water and B comprised of 0.1% formic acid in acetonitrile. A 100 min LC separation was performed on the Velos MS (using 60-cm long columns having an o.d. of 360 μm, i.d. of 75 μm, and 3 μm C_{18} packing material) while only a 60 min gradient with shorter columns (30-cm long columns with the same dimensions and packing) that was used with the IMS-MS since the additional IMS separation helps address detector suppression and also faster LC analyses. Both gradients were linear with mobile phase B increasing from 0 to 60% until the final 2 min of the run when B was purged at 95%. 5 μL of each sample was injected for both analyses and the HPLC was operated under a constant flow rate of 0.4 μL/min for the 100 min gradient and 1 μL/min for the 60 min gradient. The Velos MS data was collected from 400-2000 m/z at a resolution of 60,000 (automatic gain control (AGC) target: $1x10^6$) followed by data dependent ion trap MS/MS spectra (AGC target: $1x10^4$) of the twelve most abundant ions using a collision energy setting of 35%. A dynamic exclusion time of 60 s was used to discriminate against previously analyzed ions. IMS-TOF MS data was collected from 100-3200 m/z.

2.3.3 Metaproteomic data processing and statistical analysis

Identification and quantification of the detected peptide peaks were performed using the accurate mass and time (AMT) tag approach (Zimmer *et al.* 2006; Burnum *et al.* 2012). Peptide database generation utilized Velos tandem MS/MS data (Kim *et al.* 2008; Piehowski *et al.* 2013) from pooled fractionated samples (Supplemental Methods). Due to the greater

sensitivity and dynamic range of measurements (Burnum *et al.* 2012) relative quantitation of the peptide peaks utilized the LC-IMS-MS data. Multiple in-house developed (Monroe *et al.* 2007; Jaitly *et al.* 2009) informatics tools were used to process the LC-IMS-MS data and correlate the resulting LC-IMS-MS features to the AMT tag database containing LC elution times, IMS drift times, and accurate mass information for each assigned peptide. Our in-house ion mobility mass spectrometry platform has previously provided novel insight into complex biological systems (Burnum *et al.* 2012; Baker *et al.* 2014; Cha *et al.* 2015; Baker *et al.* 2015; Kyle *et al.* 2016).

Data filtering was performed to remove peptides with inadequate data for statistics and samples that are extreme outliers (Webb-Robertson *et al.* 2010; Matzke *et al.* 2011). This resulted in 6,676 peptides and 1,766 proteins across the sixteen samples (four feeding treatments and four biological replicates for each treatment). Normalization approaches were evaluated using a statistical procedure for the analyses of peptide abundance normalization strategies (SPANS) and normalization factors were generated as the mean of the datasets that were observed consistently across technical replicates (Webb-Robertson *et al.* 2011). Peptide statistics were performed by comparing all treatment groups to one another using Analysis of Variance (ANOVA) with a post-hoc Tukey test to define peptide signatures. A BP-Quant quantification (Webb-Robertson *et al.* 2014) approach was used to estimate abundance at the protein level. Proteins were also evaluated with a Tukey test and deemed significant at a p-value<0.05. Only fungal proteins identified by ≥ 2 peptides are discussed (see Supplemental Table 2.1 for the full list of all detected proteins). Non-metric multidimensional scaling (NMDS) was conducted on these data with Bray-Curtis dissimilarity, using the vegan package in the R statistical programming environment (Oksanen *et al.* 2013; R Core Team 2013). To determine if the fungus gardens from different

treatments had significantly different protein profiles, function adonis was used to run a Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA).

2.4 Results

2.4.1 Fungal proteomics

With our metaproteomic analysis of the fungus gardens, we identified and quantified 1,766 different fungal proteins, including 161 putative biomass-degrading enzymes (Supplemental Table 2.1). NMDS analysis of the global proteome profiles across treatments and replicates revealed grouping according to treatment (Figure 2.2A). These differences according to treatment were significant (PERMANOVA $p<0.001$). Fungus garden proteomic profiles in both the leaves and flowers treatments showed low variability within-group and between-group, while the oats and mixed treatments had greater within-group variability and overlapped with each other. These groupings are evident when individual proteins are compared between treatments. To analyze the differential abundance of individual proteins, we conducted pair-wise comparisons of each protein in the four treatments. Numerous proteins with significantly different abundances were identified between the treatments (Supplemental Table 2.1). When individual protein differences are observed globally using heat maps, we can again see grouping according to treatment (Figures 2.3 and 2.4): the oats sub-colonies were most similar to the mixed sub-colonies, while the leaves sub-colonies were similar to the flowers. The significant changes for each protein pairwise comparison were identified by at least 2 peptides with: oats/mixed having 52 significantly changing proteins, leaves/flowers - 31, leaves/oats - 286, flowers/oats - 259, leaves/mixed - 135, and flowers/mixed - 125 (Supplemental Table 2.1).

All biomass-degrading enzymes observed to be significantly different ($p<0.05$) between treatments are listed in Table 2.1, where individual proteins are compared between the mixed and other treatments. We compared to the mixed treatment since it most closely resembles the ants' natural tendency to incorporate a mixture of substrates into their fungus gardens. In general, the leaves and flowers treatments had similar results with much higher abundances of CAZys, proteases and enzymes necessary for the breakdown of cellulose: endoglucanases (GH5 and GH6), exoglucanase (GH6), and β-glucosidases (GH3 and GH31), compared with the other two treatments. However, the oats treatment was very similar to the mixed treatment with a lower abundance of these proteins and proteases (Table 2.1, Figure 2.4).

2.4.2 Bacterial proteomics

We detected only 44 unique bacterial peptides and from these data we determined, through similar pairwise comparisons between treatments, that there were three bacterial proteins that differed significantly between treatments. Each of these proteins was identified with only a single peptide. These proteins were identified based on genomes of bacterial symbionts of leaf-cutter ants (*Enterobacter* strain FGI 35, *Serratia* strain FGI 94 (Aylward *et al.* 2013c), *Enterobacteriaceae* strain FGI 57 (Aylward *et al.* 2013b), *Pseudomonas* strain FGI 182, *Klebsiella variicola* strain AT-22 and *Pantoea* strain AT-9b (Aylward *et al.* 2014)). Malate dehydrogenase, which mapped equally to *Cronobacter, Pantoea, Serratia, Enterobacter,* and *Klebsiella* genomes, was more abundant in the leaf treatment. Periplasmic trehalase, which mapped to the *Enterobacter* genome, was more abundant in the flower treatments. ATP synthase subunit β, which mapped to all six bacterial genomes, was the least abundant in the leaf treatments. Overall, the global bacterial protein profiles did not differ between treatments (Supplemental Table 2.1, Figure 2.2B).

2.4.3 Sub-colony Survivorship

The fungus garden of some sub-colonies did not remain healthy throughout the experimental period, but instead dried out, were discarded by workers, or were overgrown by a pathogen. This was especially common for sub-colonies created from the gardens excised from the last two parent colonies. A sub-colony was considered failed when all the ants were dead or when the fungus garden was overtaken by a pathogen. Overall, sub-colonies fed exclusively on oats had significantly lower survivorship than the other colonies (Figure 2.5).

2.5 Discussion

The breakdown of plant biomass by *L. gongylophorus* is central to the success of leaf-cutter ant colonies and the function of this ant-fungus mutualism. Nevertheless, our understanding of the process of digesting leaves and other plant substrates within the fungus garden is limited. Specifically, the ability of *L. gongylophorus* to digest cellulose and other recalcitrant material has been debated. Some have argued that it does not effectively break down cellulose and instead relies on other plant components such as pectin for energy (De Siqueira *et al.* 1998; Silva *et al.* 2006b; Moller *et al.* 2011). In contrast to this, sugar composition analysis and microscopy shows a significant decrease in cellulose within fungus gardens and genomics and metaproteomics show a significant capacity of *L. gongylophorus* to degrade it (Suen *et al.* 2010; Nagamoto *et al.* 2011; Aylward *et al.* 2012; Grell *et al.* 2013; Aylward *et al.* 2013a). Our results here provide further support for the role of the fungus in recalcitrant biomass degradation. Specifically, our metaproteomic analysis detected 100 CAZys produced by *L. gongylophorus*, including 53 glycoside hydrolases (GH), 6 carbohydrate esterases (CE), 8 carbohydrate binding molecules (CBM), 4 polysaccharide lyases (PL), and 30 auxiliary activities enzymes (AA) (Figure 2.4, Supplementary Table 2.1). This suite of

enzymes includes all the components necessary for the breakdown of cellulose (endoglucanases GH5, GH12 and GH6, exoglucanase GH6 and β-glucosidase GH31).

Although our combination of proteomics and feeding experiments provide further evidence for the ability of *L. gongylophorus* to deconstruct cellulose, our findings indicate that this enzymatic response is context-dependent. Specifically, we found metabolic flexibility in the ants' fungal cultivar to preferentially digest various substrates; instead of consuming recalcitrant materials, the fungus digests the more readily accessible carbon sources when available. This is most clearly observed when comparing the mixed and oat treatment metaproteomes. In the mixed treatment the fungus does not produce an abundance of biomass-degrading enzymes, despite the presence of recalcitrant biomass. It instead has a metaproteome that is more similar to that of the oat treatment, suggesting that when given a mixture of substrates, the fungus derives its energy from the oats. The flexible, substrate-specific response of the fungus is important in a system where the ants cut a large diversity of substrates, which vary between seasons and environments. For example, in the dry season substrates that are rich in easily accessible nutrients may be more limited, such that the fungal cultivar needs to respond to and to derive energy from more recalcitrant sources. In contrast, in the wet season when substrates such as fruits and young leaves are more readily available, the fungal cultivar would benefit from reducing the energy expended on digesting recalcitrant material when easily accessible sugars are available.

Evidence supporting the substrate-specific response in the leaf-cutter ant fungus garden has been previously reported elsewhere. Kooij *et al.* (2011) manipulated the substrate for *A. cephalotes* fungus gardens and using Azurine-Crosslinked (AZCL) assays measured changes in specific enzymes of interest, observing an overall shift in enzyme activity between substrates. AZCL is a high throughput method used to detect enzyme activity, while

metaproteomics provides accurate detection and quantification of the specific proteins present. Thus, our approach represents a more thorough enzymatic response of the fungus garden, as follows. First, AZCL is conducted with a limited suite of substrates and only shows activity of enzymes to those substrates. This excludes any non-enzymatic proteins and any enzymes that did not have the appropriate substrate to respond to. Second, AZCL does not allow us to characterize specific proteins, whereas metaproteomics does..

Other systems where microbes are responsible for biomass breakdown also show substrate-specificity through fluctuations in the community structure of multiple microbes (Thoetkiattikul *et al.* 2013; Miyata *et al.* 2014). Here, a single vertically transmitted cultivar, with little variability between isolates (Silva-Pinhati *et al.* 2004) is responsible for the flexible, substrate-specific response of the system. The leaf-cutter ant system, which is optimized for the extraction of energy from plant material then fine-tunes the enzymatic response of the fungal cultivar. Previous work has shown that the lignocellulases and laccases from gongylidia are transferred by the ants from the middle of the garden and defecated on the top, serving as a pretreatment step for beginning rapid biomass degradation and detoxification (Cherrett *et al.* 1989; Moller *et al.* 2011; De Fine Licht *et al.* 2013; Aylward *et al.* 2015).

Recent work has identified the presence of an apparent consistent bacterial community in the fungus garden (Pinto-Tomás *et al.* 2009; Suen *et al.* 2010; Aylward *et al.* 2012). Although certain functional roles of the bacteria have been elucidated, such as nitrogen fixation (Pinto-Tomás *et al.* 2009) and the apparent capacity to provide vitamins (Aylward *et al.* 2012), our insights regarding the bacteria remain limited. Here, we did not observe a notable change in bacterial proteins, other than the three which are all part of central carbon metabolism and unlikely to play a direct role in substrate breakdown or

detoxification (Bergmeyer & Gawehn 1974; Boos *et al.* 1987). Only 1% of the unique peptides that were detected in these analyses were identified as bacterial. This is likely due to a considerable difference in the amount of fungal and bacterial biomass in the fungus gardens. It could also indicate that bacteria play a more limited role in the fungus gardens.

Interestingly, despite our finding that *L. gongylophorus* preferentially uses the simplest energy source (i.e., oats) when provided with a mixture of substrates, sub-colony survivorship dramatically decreased when this was the only substrate provided. This correlation between decreased health and feeding exclusively on a simple, energy rich diet has been observed in other animals. Cows that are fed a grain-rich diet gain weight quickly but suffer frequently from ruminal acidosis, which negatively impacts both production and animal welfare (Krause & Oetzel 2006). Ruminal acidosis results from different rates of fermentation in the standard grassy diet and has effects on the microbial community composition in the rumen (Steele *et al.* 2011; Hook *et al.* 2011). Humans also show a correlation between diet, the gut microbiome, and health (De Filippo *et al.* 2010; Martínez *et al.* 2013). While this experiment suggests that the fungus gardens of oat-fed sub-colonies are apparently less stable, colony health was not the focus of our study. However, we hypothesize that an exclusive diet of oats lacks required micronutrients that the ants, fungus or bacteria obtain from fresh plant material. While there have been thorough investigations into plant characteristics that are deterrents to leaf-cutter ant foraging and how this limits the diversity of plants they consume, no work has been done investigating whether a more diverse diet leads to higher fitness for leaf-cutter ants. Testing this hypothesis in future studies would help us to determine what minimum requirements exist for leaf-cutter ant forage and whether this is achieved more effectively with a diverse diet.

The mutualism between leaf-cutter ants and their fungal cultivar has been described as an "unholy alliance" (Cherrett *et al.* 1989), where the tasks of mechanical and enzymatic breakdown of plant material are partitioned to the ants and fungal cultivar, respectively. Through this alliance, leaf-cutter ants are capable of utilizing a wide diversity of plant material, unlike most other herbivores. Polyphagy in this system necessitates metabolic flexibility on the part of the fungus, and is a key factor in making leaf-cutter ants dominant herbivores. In this study, we dissect this unholy alliance at a previously unattainable depth, demonstrating that the cultivar does indeed have a flexible, specific response to different plant substrates. Our study provides an important step in building toward understanding the microbial mediation of a generalist herbivore system.

2.6 Acknowledgments

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2.7 Data accessibility

All of the metaproteomic data from this study is available in the Supplemental Materials (Supplemental tables 2.1 and 2.2).

Table 2.1 Fungal biomass-degrading enzymes that differ significantly from the mixed treatment

LAG	Protein Family	Annotation	Leaves	Flowers	Oats
CAZy					
1450	CE8	Pectin methylesterase			-
925	CBM57, CE15	Found attached to glycosidases			-
1065	GH31	α-glucosidase, and others			-
2832	GH6	Endoglucanase, exoglucanase, cellobiohydrolase		+	
1778	CBM32	Binding to galactose, lactose, polygalacturonic acid, LacNAc		+	-
4224	GH10	Xylan targeting	+		
3545	GH5	Endo-β-1,4-glucanase / cellulase and many others	+		
3581	CE5	Acetyl xylan esterase, cutinase	+	+	
3843	GH10	Xylan targeting	+	+	
830	GH105	Unsaturated rhamnogalacturonyl hydrolase; d-4,5-unsaturated β-glucuronyl hydrolase	+	+	
420	GH18	Lysozyme, chitinase, many others	+	+	
5098	GH3	β-glucosidase, and others	+	+	
811	GH3	β-glucosidase, and others	+	+	
1724	GH31	α-glucosidase, and others	+	+	
1811	GH92	Mannose targeting	-	-	
11012	AA5	Glyoxal oxidase			-
3543	AA5	Glyoxal oxidase			-
1590	AA3	Glucose oxidase	-	-	
3638	AA3	Alcohol oxidase 1	+	+	
2639	AA1	Laccase-1	+	+	
3464	AA1	Laccase-4	+	+	
5297	AA1	Laccase-2	+		
2404	AA1	Laccase-1	+		
5522	AA1	Laccase-2	+		
3730	AA2	Chloroperoxidase	+		
5105	AA2	Chloroperoxidase	-	-	
3594	AA3	Dihydrolipoyl dehydrogenase, mitochondrial		+	
Proteases					
3716	M36	Endopeptidase			-
971	C44	Self-processing precursor of Amidophosphoribosyltransferase		-	
2519	M67A	Isopeptidases that releases ubiquitin from ubiquitinated proteins		+	
3036	C01B	Endopeptidases or exopeptidases	+		
3725	M28E	Aminopeptidase	-		
439	A01A	Pepsin A	+	+	
100	M03A	Thimet oligopeptidase	+	+	
748	M13	Metalloendopeptidase	+	+	
1996	M41	ATP-dependent metalloendopeptidase	+	+	
15046	M67A	Isopeptidases that release ubiquitin from ubiquitinated proteins	-	-	
3735	S08A	Subtilisin Carlsberg	+	+	
2389	S08A	Subtilisin Carlsberg		+	
3512	S08A	Subtilisin Carlsberg		+	-
5096	S08A	Subtilisin Carlsberg		+	-
2939	S10	Carboxypeptidase Y	+	+	
4473	S10	Carboxypeptidase Y			-
2743	S10	Carboxypeptidase Y	-	-	-
924	S26B	Signalase 21 kDa component	+	+	+
2527	S53	Sedolisin	+	+	

A significant increase in abundance compared to the mixed treatment is indicated by + and a significant decrease is indicated by -.

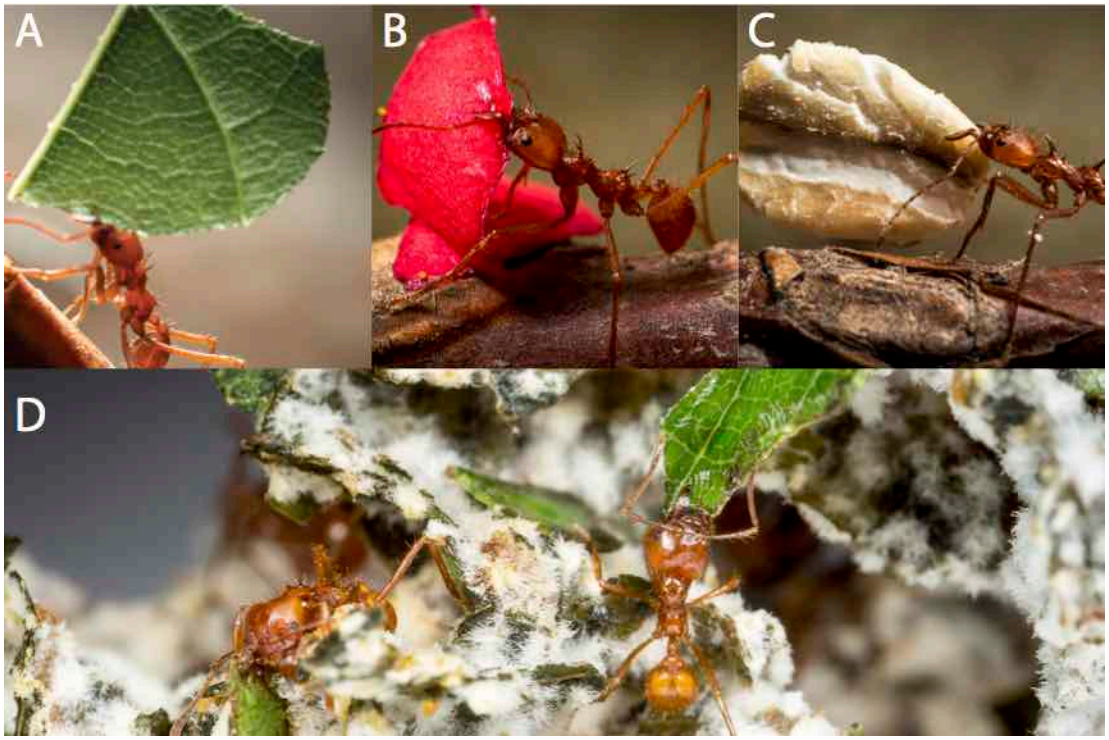

Figure 2.1 Leaf cutter ants carrying various substrates (A) a leaf, (B) a flower and (C) an oat. Ants tending to their fungus garden with newly incorporated leaf material (D) (photographs by Don Parsons).

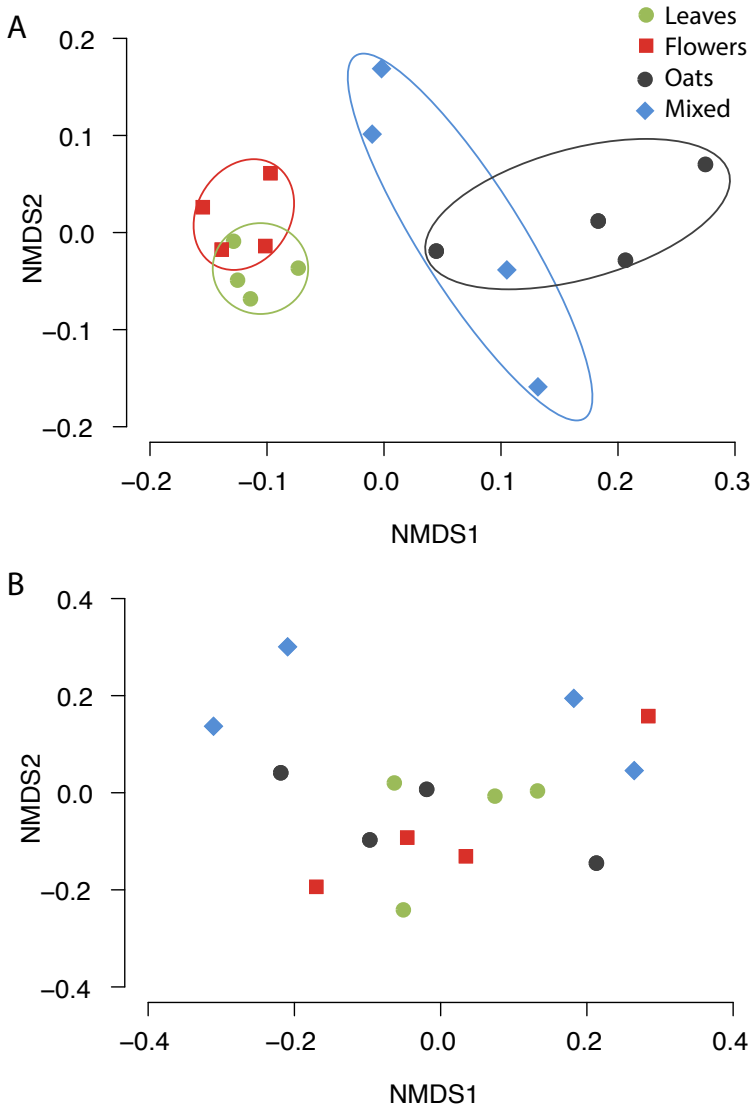

Figure 2.2 NMDS plot of (A) fungal and (B) bacterial whole-community metaproteomics. While the fungal results were significantly different between treatment groups, the bacterial metaproteomes were not possible to differentiate statistically.

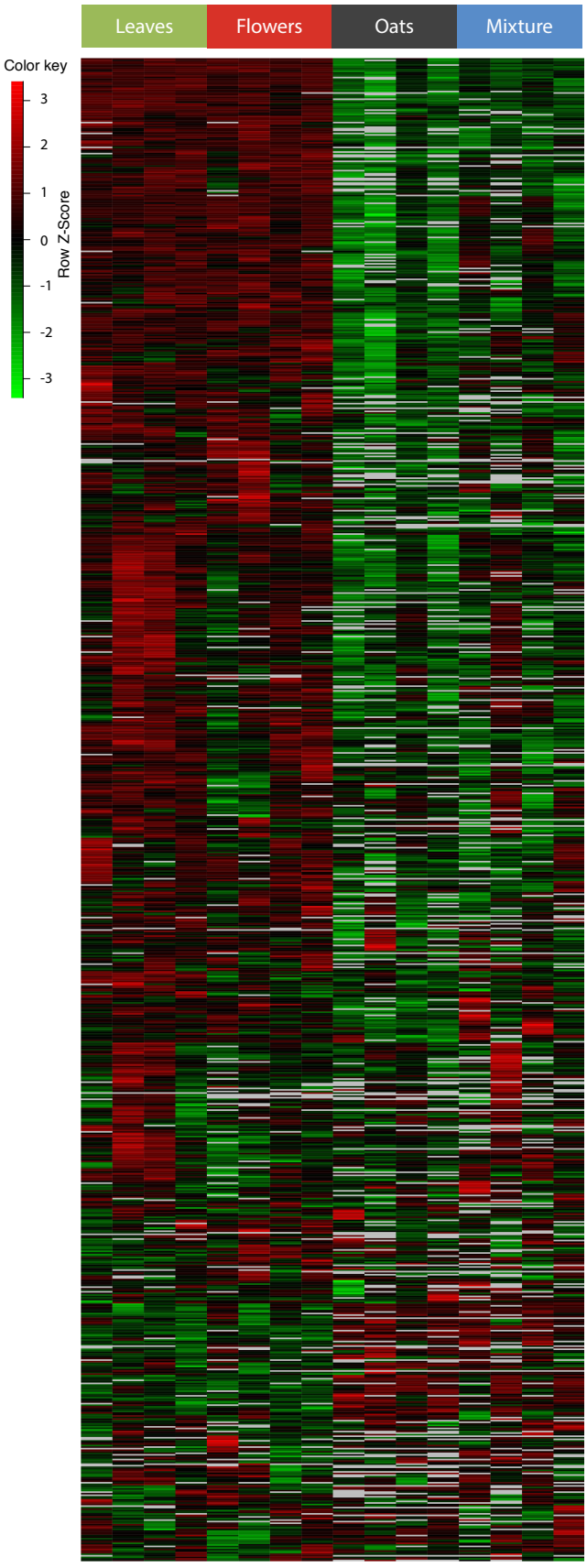

Figure 2.3 A heat map of the complete metaproteome. Columns represent each treatment and rows represent each protein. A clear division is visible between the two left columns (leaves and flowers) and the two right columns (oats and mixed).

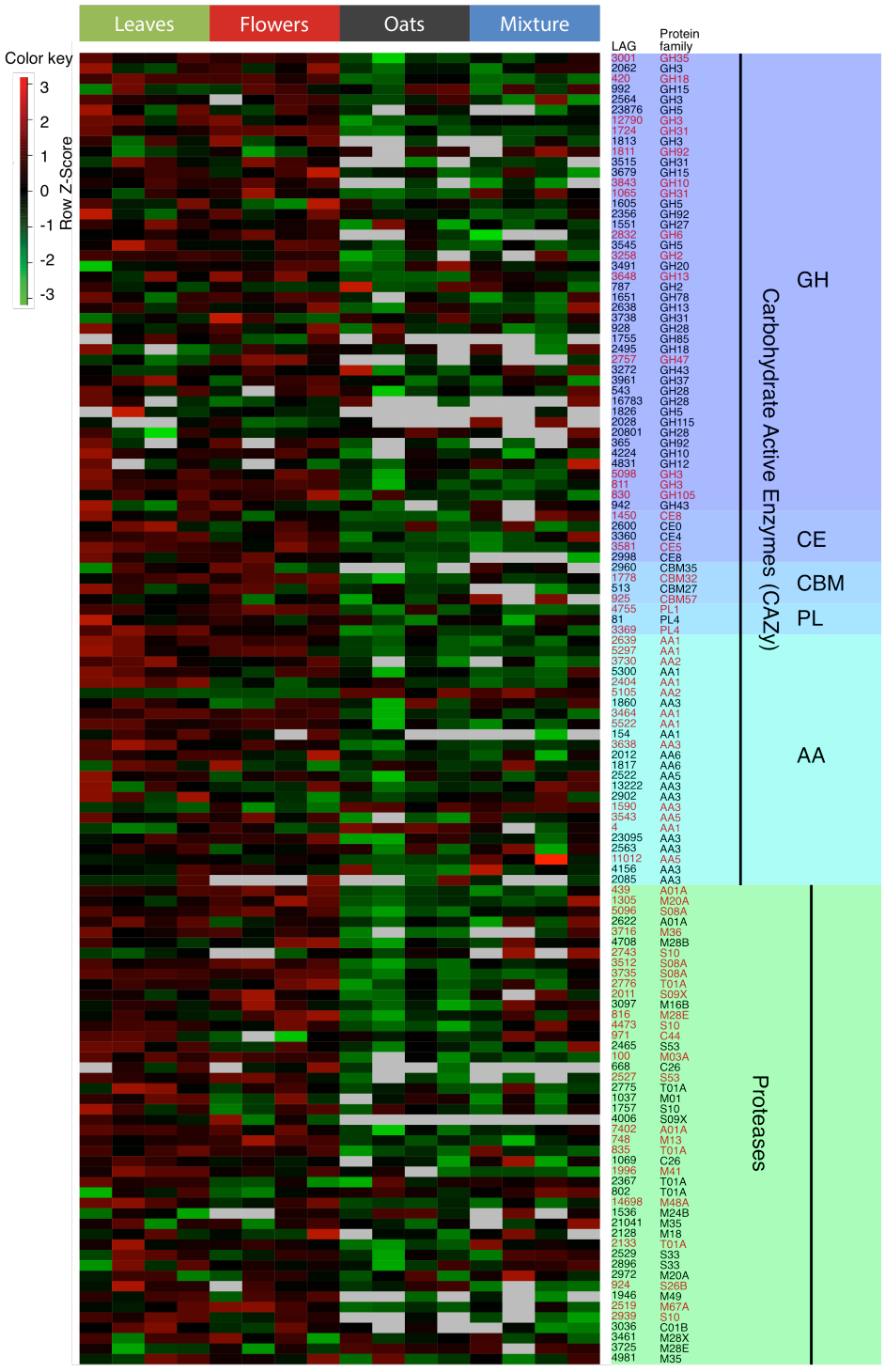

Figure 2.4 Heat map of higher or lower abundance of biomass degrading enzymes. A clear division can be seen between leaves and flowers on the left and oats and mixed on the right. GH – glycoside hydrolases, CE – carbohydrate esterases, CBM – carbohydrate binding molecules, PL – polysaccharide lyases, AA – auxiliary activities. Proteins in red text were significantly different between at least two treatments.

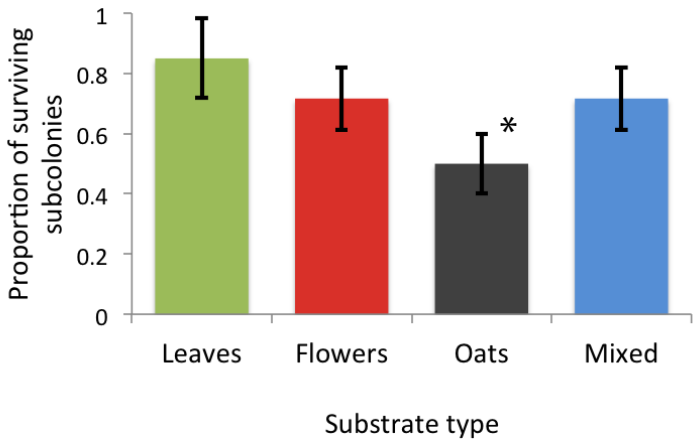

Figure 2.5 Sub-colony survival by treatment. Sub-colonies that were fed oats survived significantly (*) less than the other sub-colonies, over the course of the experiment (ANOVA $p<0.05$).

2.8 References

Amaglo NK, Bennett RN, Curto Lo RB *et al.* (2010) Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree Moringa oleifera L., grown in Ghana. *Food Chemistry*, **122**, 1047–1054.

Aylward FO, Burnum KE, Scott JJ *et al.* (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. *The ISME Journal*, **6**, 1688–1701.

Aylward FO, Burnum-Johnson KE, Tringe SG *et al.* (2013a) *Leucoagaricus gongylophorus* produces diverse enzymes for the degradation of recalcitrant plant polymers in leaf-cutter ant fungus gardens. *Applied and Environmental Microbiology*, **79**, 3770–3778.

Aylward FO, Khadempour L, Tremmel DM *et al.* (2015) Enrichment and Broad Representation of Plant Biomass-Degrading Enzymes in the Specialized Hyphal Swellings of Leucoagaricus gongylophorus, the Fungal Symbiont of Leaf-Cutter Ants. *PLoS ONE*, **10**, e0134752.

Aylward FO, Suen G, Biedermann PHW *et al.* (2014) Convergent Bacterial Microbiotas in the Fungal Agricultural Systems of Insects. *mBio*, **5**, e02077–14.

Aylward FO, Tremmel DM, Bruce DC *et al.* (2013b) Complete Genome of Enterobacteriaceae Bacterium Strain FGI 57, a Strain Associated with Leaf-Cutter Ant Fungus Gardens. *Genome Announcements*, **1**, e00238–12.

Aylward FO, Tremmel DM, Starrett GJ *et al.* (2013c) Complete genome of *Serratia* sp. strain FGI 94, a strain associated with leaf-cutter ant fungus gardens. *Genome Announcements*, **1**, e00239–12.

Baker ES, Burnum KE, Ibrahim YM *et al.* (2015) Enhancing bottom-up and top-down proteomic measurements with ion mobility separations. *Proteomics*, **15**, 2766–2776.

Baker ES, Burnum-Johnson KE, Jacobs JM *et al.* (2014) Advancing the High Throughput Identification of Liver Fibrosis Protein Signatures Using Multiplexed Ion Mobility Spectrometry. *Molecular & cellular proteomics*, **13**, 1119–1127.

Baker ES, Clowers BH, Li F *et al.* (2007) Ion mobility spectrometry-mass spectrometry performance using electrodynamic ion funnels and elevated drift gas pressures. *Journal of the American Society for Mass Spectrometry*, **18**, 1176–1187.

Baker ES, Livesay EA, Orton DJ *et al.* (2010) An LC-IMS-MS platform providing increased dynamic range for high-throughput proteomic studies. *Journal of proteome research*, **9**, 997–1006.

Bergmeyer H, Gawehn K (1974) Malate Dehydrogenase. In: *Methods of enzymatic analysis* (eds Bergmeyer H, Gawehn K), pp. 613–618. Academic Press, New York.

Boone CK, Keefover-Ring K, Mapes AC *et al.* (2013) Bacteria Associated with a Tree-Killing Insect Reduce Concentrations of Plant Defense Compounds. *Journal of Chemical Ecology*, **39**, 1003–1006.

Boos W, Ehmann U, Bremer E, Middendorf A, Postma P (1987) Trehalase of *Escherichia coli*. Mapping and cloning of its structural gene and identification of the enzyme as a periplasmic protein induced under high osmolarity growth conditions. *The Journal of*

biological chemistry, **262**, 13212–13218.

Burnum KE, Hirota Y, Baker ES *et al.* (2012) Uterine deletion of Trp53 compromises antioxidant responses in the mouse decidua. *Endocrinology*, **153**, 4568–4579.

Cha J, Burnum KE, Bartos A *et al.* (2015) Muscle Segment Homeobox Genes Direct Embryonic Diapause by Limiting Inflammation in the Uterus. *The Journal of biological chemistry*, **290**, 15337–15349.

Cherrett JM, Powell RJ, Stradling D (1989) The mutualism between leaf-cutting ants and their fungus. In: *Insect-fungus interactions* (eds Wilding N, Collins NM, Hammond PM, Webber J), pp. 93–120. Academic Press, London.

Costa AN, Vasconcelos HL, Vieira-Neto EHM, Bruna EM (2008) Do herbivores exert top-down effects in Neotropical savannas? Estimates of biomass consumption by leaf-cutter ants. *Journal of Vegetation Science*, **19**, 849–854.

Cuddeford D (1995) Oats for animal feed. In: *The oat crop: production and utilization* (ed Welch RW), pp. 321–368. Chapman Hall, New York.

De Filippo C, Cavalieri D, Di Paola M *et al.* (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences*, **107**, 14691–14696.

De Fine Licht HH, Schiøtt M, Rogowska-Wrzesinska A *et al.* (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences*, **110**, 583–587.

De Siqueira CG, Bacci M Jr, Pagnocca FC, Bueno OC, Aparecida Hebling MJ (1998) Metabolism of plant polysaccharides by *Leucoagaricus gongylophorus*, the symbiotic fungus of the leaf-cutting ant *Atta sexdens* L. *Applied and Environmental Microbiology*.

De Vasconcelos HL (1990) Foraging activity of two species of leaf-cutting ants (Atta) in a primary forest of the central Amazon. *Insectes sociaux*, **37**, 131–145.

Dowd PF (1991) Symbiont-mediated detoxification in insect herbivores. In: *Microbial Mediation of Plant-Herbivore Interactions*, pp. 411–440. Wiley-Interscience, New York.

Grell MN, Linde T, Nygaard S *et al.* (2013) The fungal symbiont of *Acromyrmex* leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and other plant cell wall polysaccharides. *BMC Genomics*, **14**, 928.

Hansen AK, Moran NA (2011) Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *PNAS*, **108**, 2849–2854.

Hansen AK, Moran NA (2013) The impact of microbial symbionts on host plant utilization by herbivorous insects. *Molecular Ecology*, **23**, 1473–1496.

Herz H, Beyschlag W, Hölldobler B (2007) Assessing herbivory rates of leaf-cutting ant (*Atta colombica*) colonies through short-term refuse deposition counts. *Biotropica*, **39**, 476–481.

Holldobler B, Wilson EO (1990) *The Ants*. Belknap (Harvard University Press), Cambridge, MA.

Holldobler B, Wilson EO (2008) *The superorganism: the beauty, elegance, and strangeness of insect*

societies. W.W. Norton & Company, New York, NY.

Hook SE, Steele MA, Northwood KS *et al.* (2011) Impact of subacute ruminal acidosis (SARA) adaptation and recovery on the density and diversity of bacteria in the rumen of dairy cows. *FEMS Microbiology Ecology*, **78**, 275–284.

Howard JJ (1987) Leafcutting ant diet selection: the role of nutrients, water, and secondary chemistry. *Ecology*, **58**, 503–515.

Howard JJ (1988) Leafcutting and diet selection: relative influence of leaf chemistry and physical features. *Ecology*, **69**, 250–260.

Jaitly N, Mayampurath A, Littlefield K *et al.* (2009) Decon2LS: An open-source software package for automated processing and visualization of high resolution mass spectrometry data. *BMC bioinformatics*, **10**, 87.

Kim S, Gupta N, Pevzner PA (2008) Spectral probabilities and generating functions of tandem mass spectra: a strike against decoy databases. *Journal of proteome research*, **7**, 3354–3363.

Kooij PW, Schiøtt M, Boomsma JJ, De Fine Licht HH (2011) Rapid shifts in *Atta cephalotes* fungus-garden enzyme activity after a change in fungal substrate (Attini, Formicidae). *Insectes sociaux*, **58**, 145–151.

Krause KM, Oetzel GR (2006) Understanding and preventing subacute ruminal acidosis in dairy herds: A review. *Animal Feed Science and Technology*, **126**, 215–236.

Kyle JE, Zhang X, Weitz KK *et al.* (2016) Uncovering biologically significant lipid isomers with liquid chromatography, ion mobility spectrometry and mass spectrometry. *The Analyst*, **141**, 1649–1659.

Livesay EA, Tang K, Taylor BK *et al.* (2008) Fully automated four-column capillary LC-MS system for maximizing throughput in proteomic analyses. *Analytical chemistry*, **80**, 294–302.

Martínez I, Lattimer JM, Hubach KL *et al.* (2013) Gut microbiome composition is linked to whole grain-induced immunological improvements. *The ISME Journal*, **7**, 269–280.

Matzke MM, Waters KM, Metz TO *et al.* (2011) Improved quality control processing of peptide-centric LC-MS proteomics data. *Bioinformatics (Oxford, England)*, **27**, 2866–2872.

Mayhé-Nunes A, Jaffe K (1998) On the biogeography of Attini (Hymenoptera: Formicidae). *Ecotropicos*, **11**, 45–54.

Miyata R, Noda N, Tamaki H *et al.* (2014) Influence of feed components on symbiotic bacterial community structure in the gut of the wood-feeding higher termite *Nasutitermes takasagoensis*. *Bioscience, Biotechnology, and Biochemistry*, **71**, 1244–1251.

Moller IE, De Fine Licht HH, Harholt J, Willats WGT, Boomsma JJ (2011) The dynamics of plant cell-wall polysaccharide decomposition in leaf-cutting ant fungus gardens (J Chave, Ed,). *PLoS ONE*, **6**, e17506.

Monroe ME, Tolić N, Jaitly N *et al.* (2007) VIPER: an advanced software package to support high-throughput LC-MS peptide identification. *Bioinformatics (Oxford, England)*, **23**, 2021–2023.

Nagamoto NS, Garcia MG, Forti LC *et al.* (2011) Microscopic evidence supports the hypothesis of high cellulose degradation capacity by the symbiotic fungus of leaf-cutting ants. *Journal of Biological Research-Thessaloniki*, **16**, 308–312.

Oksanen J, Blanchet FG, Kindt R *et al.* (2013) vegan: Community ecology package. *CRAN.R-project.org*.

Piehowski PD, Petyuk VA, Sandoval JD *et al.* (2013) STEPS: a grid search methodology for optimized peptide identification filtering of MS/MS database search results. *Proteomics*, **13**, 766–770.

Pinto-Tomás AA, Anderson MA, Suen G *et al.* (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science*, **326**, 1120–1123.

R Core Team (2013) R: A language and environment for statistical computing. *R Foundation for Statistical Computing*.

Ricklefs RE, Miller GL (2000) *Ecology*. W. H. Freeman and Company, New York, NY.

Silva A, Bacci M Jr, Pagnocca FC, Bueno OC, Hebling MJA (2006a) Starch metabolism in Leucoagaricus gongylophorus, the symbiotic fungus of leaf-cutting ants. *Microbiological Research*, **161**, 299–303.

Silva A, Bacci M Jr, Pagnocca FC, Bueno OC, Hebling MJA (2006b) Production of polysaccharidases in different carbon sources by *Leucoagaricus gongylophorus* Möller (Singer), the symbiotic fungus of the leaf-cutting ant *Atta sexdens* Linnaeus. *Current microbiology*, **53**, 68–71.

Silva-Pinhati ACO, Bacci M Jr, Hinkle G *et al.* (2004) Low variation in ribosomal DNA and internal transcribed spacers of the symbiotic fungi of leaf-cutting ants (Attini: Formicidae). *Brazilian journal of medical and biological research*, **37**, 1463–1472.

Steele MA, Croom J, Kahler M *et al.* (2011) Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. *AJP: Regulatory, Integrative and Comparative Physiology*, **300**, R1515–R1523.

Suen G, Scott JJ, Aylward FO *et al.* (2010) An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS genetics*, **6**, e1001129.

Tartar A, Wheeler MM, Zhou X *et al.* (2009) Parallel metatranscriptome analyses of host and symbiont gene expression in the gut of the termite *Reticulitermes flavipes*. *Biotechnology for biofuels*, **2**, 25.

Thoetkiattikul H, Mhuantong W, Laothanachareon T *et al.* (2013) Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged 16S rRNA gene pyrosequencing. *Current microbiology*, **67**, 130–137.

Van Bael SA, Estrada C, Wcislo WT (2011) Fungal-fungal interactions in leaf-cutting ant agriculture. *Psyche: A Journal of Entomology*, **2011**, 1–9.

Wang Y, Lim L, DiGuistini S *et al.* (2012) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees. *New Phytologist*, **197**, 886–898.

Webb-Robertson B-JM, Matzke MM, Datta S *et al.* (2014) Bayesian proteoform modeling improves protein quantification of global proteomic measurements. *Molecular & cellular*

proteomics, **13**, 3639–3646.

Webb-Robertson B-JM, Matzke MM, Jacobs JM, Pounds JG, Waters KM (2011) A statistical selection strategy for normalization procedures in LC-MS proteomics experiments through dataset-dependent ranking of normalization scaling factors. *Proteomics*, **11**, 4736–4741.

Webb-Robertson B-JM, McCue LA, Waters KM *et al.* (2010) Combined statistical analyses of peptide intensities and peptide occurrences improves identification of significant peptides from MS-based proteomics data. *Journal of proteome research*, **9**, 5748–5756.

Welch RW (1995) The chemical composition of Oats. In: *The oat crop: production and utilization* (ed Welch RW), pp. 279–320. Chapman Hall, New York.

Wirth R (2003) *Herbivory of leaf-cutting ants: a case study on Atta colombica in the tropical rainforest of Panama* (R Wirth, H Herz, RJ Ryel, W Beyschlag, B Hölldobler, Eds,). Springer-Verlag Berlin Heidelberg, Germany.

Wirth R, Beyschlag W, Ryel RJ, Holldobler B (1997) Annual foraging of the leaf-cutting ant *Atta colombica* in a semideciduous rain forest in Panama. *Journal of Tropical Ecology*, **13**, 1–741–75717.

Zimmer J, Monroe ME, Qian WJ, Smith RD (2006) Advances in proteomics data analysis and display using an accurate mass and time tag approach. *Mass Spectrometry Reviews*, **25**, 450–482.

Chapter 3: Metagenomics reveals diet-specific specialization in fungus gardens of grass- and dicot-cutter ants

This chapter is in preparation for submission for publication, pending minor changes. Supplemental material for this chapter can be found in Appendix 2 of this dissertation.

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3.1 Abstract

Leaf-cutter ants are dominant herbivores in the Neotropics. While most leaf-cutter ant species cut dicots to incorporate into their fungus gardens, some species specialize on grasses. Here we examine the bacterial community associated with the fungus gardens of grass- and dicot-cutter ants to elucidate the potential role of bacteria in leaf-cutter ant substrate specialization. We sequenced the metagenomes of 12 *Atta* fungus gardens, across four species of ants, with a total of 5.316 Gbp of sequence data. We show that community composition was significantly different between dicot- and grass-cutter ants, with grass-cutter ant fungus gardens having significantly lower diversity and a significantly higher abundance of *Pantoea*, the most abundant genus overall. Reflecting this difference in community composition, the bacterial functional profiles between the fungus gardens are significantly different. Specifically, grass-cutter ant fungus garden metagenomes are particularly enriched for genes responsible for amino acid, siderophore, and terpenoid biosynthesis while dicot-cutter ant fungus gardens metagenomes are enriched in genes involved in membrane transport. Our results suggest that bacteria in leaf-cutter ant fungus gardens aid in nutrient supplementation, a function especially relevant for the fungus gardens of ants that forage grass, a plant source relatively lower in nutrient value.

3.2 Introduction

Understanding the role of microbial symbionts in aiding nutrient acquisition is fundamental to understanding the biology of herbivores. Most herbivores host microbial symbionts that serve as an interface between them and the plants that they consume. These microbes can compensate for the hosts' lack of physiological capacity to obtain energy and nutrients from plants (Hansen & Moran 2013). Herbivore microbial symbionts, often residing in the guts of animals, have been implicated in aiding plant biomass breakdown (Talbot 1977; Kudo 2009; Hess *et al.* 2011; Adams *et al.* 2011), plant defense compound remediation (Wang *et al.* 2010; Adams *et al.* 2013; Boone *et al.* 2013), and nutrient supplementation (Warnecke *et al.* 2007; Hansen & Moran 2011; LeBlanc *et al.* 2013). Microbial communities differ between hosts that specialize on different substrates (Muegge *et al.* 2011) and changes in these communities and their functional capacity are integral to their hosts' transition to utilizing novel substrates (Delsuc *et al.* 2013; Kohl *et al.* 2014; Li *et al.* 2014; Hammer & Bowers 2015; Kohl *et al.* 2016).

Leaf-cutter ants represent a paradigmatic example of the microbial mediation of herbivory. They are dominant herbivores in the Neotropics, consuming an estimated 17% of foliar biomass in the systems in which they live (Costa *et al.* 2008). These ants have a significant impact on their surrounding ecosystems, due to the volume of plant biomass they consume and soil that they excavate in building their underground colonies (Fowler *et al.* 1986; Moutinho *et al.* 2003; Gutiérrez & Jones 2006; Herz *et al.* 2007; Costa *et al.* 2008). Like other metazoans, leaf-cutter ants lack the capacity to break down recalcitrant plant material. Instead, they gain access to the nutrients in plant biomass by farming a fungus, *Leucoagaricus gongylophorus*, which serves as an external gut that enzymatically breaks down recalcitrant biomass in the leaf material that the ants forage (Nagamoto *et al.* 2011; Kooij *et al.* 2011;

Suen *et al.* 2011a; Grell *et al.* 2013; Aylward *et al.* 2013; Khadempour *et al.* 2016). *Leucoagaricus gongylophorus* produces gongylidia, specialized hyphal swellings that contain an abundance of sugars and lipids, that the ants consume and feed to larvae (Bass & Cherrett 1995; North *et al.* 1997).

Recent work has revealed that a community of bacteria reside within leaf-cutter ant fungus gardens (Suen *et al.* 2010; Aylward *et al.* 2012; Moreira-Soto *et al.* 2017). These communities were dominated by Gammaproteobacteria, and consistently contained strains of *Pseudomonas*, *Enterobacter* and either *Rahnella* or *Pantoea* and were consistent with communities of bacteria associated with other fungus-farming insects (Aylward *et al.* 2014). Some garden bacteria are vertically transmitted, as they are present in the fungus pellets that queens use to establish new fungus gardens (Moreira-Soto *et al.* 2017). The community consistency and their vertical transmission, suggest that the bacterial communities are important to the fitness of their hosts. One study, by Pinto-Tomás *et al.* (2009) showed that *Pantoea* and *Klebsiella* bacteria fix nitrogen that supplements the ant diet, which is important for a strict herbivorous system. Nevertheless, the functional role of most garden bacteria remains unknown.

While most leaf-cutter ants use dicots, at least three species of *Atta* are specialized on cutting grass, and another three species cut both grasses and dicots (Fowler *et al.* 1986). All previous studies on the microbial community in leaf-cutter ant fungus gardens have been focused on dicot-cutting ants, likely because dicot-cutters are more common and grass-cutter ants are notoriously difficult to maintain in the lab (Nagamoto *et al.* 2009). In this study, we compare the bacterial communities of fungus gardens from ants that cut grass and dicots. Given that grasses and dicots differ in terms of the cell wall composition (Ding & Himmel 2008; Popper & Tuohy 2010), plant defense compounds (Wetterer 1994; Mariaca *et al.* 1997)

and nutrient availability (Mattson 1980; Winkler & Herbst 2004), we hypothesize that the bacterial community in these fungus gardens will differ in terms of community composition and functional capacity, in response to the different composition of the substrates the ants incorporate into their gardens. To address this, we collected fungus gardens from grass- and dicot-cutter ants and obtained their metagenomes using Illumina sequencing. We analyzed the bacterial community in terms of its taxonomic composition and its functional capacity. We also conducted analyses on the fungus gardens to determine their plant composition, their nutritional composition and their plant defense compound contents.

3.3 Methods

3.3.1 Collection of fungus garden

Fungus gardens were collected on the campuses of the University of São Paulo (USP) in Ribeirão Preto, SP, Brazil and the State University of São Paulo (UNESP) in Botucatu, SP, Brazil. Collection dates and GPS coordinates are listed in Table 3.1. We collected fungus gardens from four species of *Atta* leaf-cutter ants: *A. bisphaerica* and *A. capiguara*, which both specialize on grass, *A. laevigata*, which cuts both grasses and dicots, and *A. sexdens*, a dicot-cutter ant (Fowler *et al.* 1986).

To collect the fungus gardens, we identified the ant species by worker morphology then followed the entrance tunnel by digging until we found a fungus garden. Care was taken to enter fungus garden chambers from the side, to avoid damaging the garden with digging tools and to avoid contamination with surrounding soil. Fungus gardens were transported to the laboratory and were aseptically transferred into 50 mL conical tubes. The majority of worker ants were removed from the fungus garden material before being transferred to the tubes. In order to further reduce the chance of soil contamination, only intact fungus garden

from the central region of the fungal mass was included in the tubes. Once filled, the tubes were frozen in liquid nitrogen and stored at -80°C. At least six 50 mL conical tubes were filled from each colony. For each colony, four tubes were used for metagenomics, one tube was used for gas chromatography, and one tube was used for iron content measurements.

3.3.2 DNA extraction

To target the bacteria in the fungus gardens, DNA was extracted by first using a differential centrifugation method (Aylward *et al.* 2012). PBS buffer with 1% tween 80 was added to the tubes and they were shaken for 30 min on a vortex. They were then kept at 4°C for 30 min so that large particles would settle. The liquid portion was decanted and passed through a 40 μm filter. The remaining leaf material from the fungus gardens was photographed after the differential centrifugation, to demonstrate the difference in leaf material consistency (Figure 3.1). The filtrate was centrifuged for 30 min at 4°C, after which a bacterial cell pellet was formed and the liquid was removed. This process was repeated with the original fungus garden tube. For each fungus garden, cell pellets from four tubes were combined and the DNA was extracted using the Qiagen Plant DNA Extraction Maxi Kit (Qiagen, Hilden, Germany).

3.3.3 DNA sequencing and assembly

All metagenomic sequencing was conducted at the Joint Genome Institute in Walnut Creek, CA. Since some of the DNA concentrations were too low for standard library prep, a low-input prep was completed for all of the samples. Sequencing was performed on an Illumina HiSeq-2500 platform (2 x 151 bp). BBDuk adapter trimming (Bushnell 2017) was used to remove known Illumina adapters. The reads were then processed using BBDuk filtering and trimming. Read ends were trimmed where quality values were less than 12.

Read pairs containing more than three ambiguous bases, or with quality scores (before trimming) averaging less than three over the read, or length under 51 bp after trimming, as well as reads matching Illumina artifact, spike-ins or phiX were discarded. Trimmed, screened, paired-end Illumina reads were assembled using the megahit assembler using with the "--k-list 23,43,63,83,103,123" option. Functional annotation and taxonomic classification were performed using the Integrated Microbial Genomes pipeline.

3.3.4 Plant genus richness

To determine the richness of plant substrate integrated in the fungus gardens of the ants, we used JGI's Integrated Microbial Genomes and Microbiomes (IMG) database "find gene" function to retrieve all genes annotated as *MatK* from the dataset. *MatK* is a widely used chloroplast plant DNA barcode (Hollingsworth *et al.* 2011). Retrieved *MatK* sequences for each metagenome were identified using BLAST. To ensure consistent and reliable certainty with the identified plants, we identified all sequences to the genus level. Because most of the plant biomass was removed from samples before DNA extraction only presence/absence of genera were considered, not abundance.

3.3.5 Bacterial taxonomic analysis

Abundance of bacterial groups (phyla and genera) were determined based on the IMG Phylogenetic Distribution tool, which is part of JGI's standard operating procedure (Huntemann *et al.* 2016). Briefly, IMG uses USEARCH (Edgar 2010) to compare metagenome gene sequences to all identified genomes in their database. One top USEARCH hit per gene is used to assign phylogenetic lineage. To determine relative abundance of bacterial taxonomic groups within each sample, we used the PhyloDist raw data from IMG and first removed all gene sequences that were identified as Eukaryote or Virus. We then

matched the PhyloDist data to the gene counts for each gene and normalized them to the total number of genes from Bacteria and Archaea. We used the relative abundances of each phylum and genus to run an non-metric multidimensional analysis (NMDS) using a Bray-Curtis dissimilarity index with the vegan package in the R statistical programming environment (Oksanen *et al.* 2013; R Core Team 2013). Also using the vegan package, we used ANOSIM and PERMANOVA to determine if groups (grass-cutters vs. dicot-cutters) were significantly different, and we used the Shannon diversity index to compare the diversity of each sample by bacterial genus. To test whether specific genera have significantly different relative abundances between grass- and dicot-cutter ant fungus gardens, we used DESeq2 in the R statistical programming environment (Love *et al.* 2014). Since DESeq2 requires inputs to be integers, we used number of gene copies per million genes in the metagenomes as our input (Alneberg *et al.* 2014).

3.3.6 Bacterial functional analysis

In order to make functional comparisons of the bacteria in grass- and dicot-cutter fungus gardens, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations of the metagenomes through IMG's KEGG Orthology (KO) pipeline, which is part of JGI's standard operating procedure (Huntemann *et al.* 2016). Briefly, genes were associated with KO terms (Kanehisa *et al.* 2014) based on USEARCH 6.0.294 results (Edgar 2010) and were filtered for minimum identity matches and gene sequence coverage. For an overall comparison of functional differences between the fungus gardens, we used the same ordination and statistical methods as for bacterial genus abundance. As with genus group differences, we used DESeq2 to determine what genes are significantly enriched between grass- and dicot-cutter ant fungus gardens, with number of gene copies per million genes in the metagenomes as our input (Alneberg *et al.* 2014).

3.3.7 Iron content

Separate 50 mL tubes of fungus garden material, from the same colonies as above, were used for determination of iron content. All ants were removed from fungus garden then the remaining material was analyzed at the UW Soil and Forage Lab in Marshfield, WI, using standard methods. Briefly, total iron content was determined by first digesting the fungus garden material in nitric acid/peroxide then analyzing by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Fassel & Kniseley 1974).

3.4 Results

3.4.1 Metagenomic statistics

A summary of metagenome statistics is presented in (Table 3.2). A total of 5.316 Gbp of assembled sequence data was produced in this study, with an average of 443 Mbp per metagenome. The smallest metagenome was from the grass-cutter colony *A. capiguara* 1 at 148.7 Mbp, and the largest metagenome was from the dicot-cutter colony *A. sexdens* 2 at 812.9 Mbp. Maximum scaffold lengths ranged from 61.96 Kbp to 701.42 Kbp, with an average maximum scaffold length of 266.6 Kbp. Between 91.63% and 99.31% of reads were aligned.

3.4.2 Bacterial taxonomic analysis

Proteobacteria (70-99%) were the most abundant bacterial phylum detected in the fungus gardens of *Atta* spp., followed by Actinobacteria (0.13-24%) and Firmicutes (0.096-2.4%) (Figure A2.1). Between fungus gardens, genus-level comparisons showed greater variability than phylum-level comparisons (Figure 3.2, Figure 3.3). Overall, *Pantoea* was the most abundant genus in all the fungus gardens (average 37%), followed by *Pseudomonas* (average 17%). The abundance of these two genera was especially pronounced in the grass-

cutter ant fungus gardens, where *Pantoea* and *Pseudomonas* averaged 45% and 28%, respectively. The high relative abundance of these two genera contributed to a lower overall diversity in the grass-cutter ant gardens (Shannon diversity index of 1.20-2.44) (Figure 3.3). While *Pantoea* and *Pseudomonas* were still abundant in fungus gardens of the dicot-cutter ants, *A. laevigata* and *A. sexdens*, it accounted for a lower proportion (28% and 6.5%, respectively) of the bacteria in these more diverse gardens (Shannon diversity index of 2.80-4.67). Other dominant bacterial genera included *Enterobacter, Burkholderia, Erwinia, Emticicia, Serratia* and *Klebsiella.* DESeq2 analysis revealed that six bacterial genera (*Entoplasma*, *Flavobacterium*, *Mesoplasma*, *Pantoea*, *Pseudomonas*, and *Spiroplasma*) were significantly different in relative abundance between the fungus gardens. They were all more abundant in the grass-cutter ant fungus gardens.

3.4.3 Bacterial functional analysis

Overall, we found significant differences in the predicted bacterial community functional profiles between grass- and dicot-cutter ant fungus gardens (Figure 3.4). All individual bacterial genes that were significantly different between grass- and dicot-cutter ant fungus gardens are listed in Table A2.1. In total, 514 predicted bacterial genes were significantly enriched in one group or another, with 313 and 201 genes significantly enriched in grass- and dicot-cutter ant gardens, respectively (Table A2.2, Figures A2.4-A2.6). Grass-cutter ant fungus gardens were enriched for amino acid biosynthesis genes for phenylalanine, tryptophan, tyrosine, histidine, arginine, lysine, cysteine, methionine, glycine, serine and threonine. They were also significantly enriched in terpenoid and siderophore biosynthesis genes (Figure 3.5) and had a significantly higher abundance of a gene in the nitrogen fixation pathway, nitrogenase molybdenum-iron protein beta chain (Table A2.2). Dicot-cutter ant fungus gardens were particularly enriched in membrane transport genes (Figure 3.5).

3.4.4 Plant taxonomy and consistency

The incorporated plant material was markedly different in consistency between the fungus gardens. *Atta bisphaerica* and *A. capiguara* gardens both contained material that was clearly grass, which was not mulched (Figure 3.1). In contrast, the leaf material in the fungus gardens of *A. laevigata* and *A. sexdens* was mulched to the point of being unrecognizable as plant material (Figure 3.1). We detected 68 plant species based on the *MatK* gene query in the metagenomes, from 40 genera and 15 families (Table 3.3). The fungus gardens of dicot-cutter ants had a significantly higher richness of plant genera than those of grass-cutter ants (ANOVA F=9.14, p=0.0128). As expected, the grass-cutter ant fungus gardens all contained grass (*Paspalum,* Poaceae). The dicot-cutter ant fungus gardens contained more genera and families of plants, which were mostly dicots, but three of these fungus gardens also contained some grass (Table 3.3).

3.4.5 Iron content

The iron content of the fungus gardens is displayed in Figure 3.6. The grass-cutter ant fungus gardens have lower amounts of iron than the dicot-cutter ant fungus, but this difference is not significant due to the high variability between *A. sexdens* gardens.

3.5 Discussion

Understanding how microbial symbiont communities change in relation to host substrate specialization can help inform on animal diet specialization and evolutionary transitions to utilizing novel substrates. *Atta* ants provide a relatively unique opportunity to examine a group of closely related herbivores that have transitioned from specialization on dicots to grasses. Dicots and grasses differ in terms of their cell wall composition, nutrient content and plant defense compounds. Here, using metagenomic sequencing, we examine this transition in the bacterial community in the fungus gardens of ants that are specialized

on these different substrates. The results of this study demonstrate that the bacterial community differs depending on type of substrate and likely facilitates the ants' ability to specialize on grasses, which represents a lower quality of substrate on which to grow their fungal crop.

If bacteria in fungus gardens are responsible for the breakdown of recalcitrant plant biomass, which is found in plant cell walls, we expect that the bacterial communities in the two ant groups examined here would be differentially enriched in the genes necessary for plant biomass breakdown. Grasses have a unique cell wall structure, containing (1→3),(1→4)-β-D-glucan chains and silica, neither of which are present in dicots (Popper & Tuohy 2010). In other systems specialized on grass biomass breakdown, the microbes responsible for this produce specialized enzymes (King *et al.* 2011) and have genomes that are adapted for this function (Wolfe *et al.* 2012). Since we do not observe any changes in abundance of these genes between these two systems, it is unlikely that the bacteria here are contributing to plant biomass breakdown. Indeed, recent work has implicate the fungal cultivar as the primary degrader of plant biomass in leaf-cutter ant fungus gardens (Nagamoto *et al.* 2011; Grell *et al.* 2013; Aylward *et al.* 2013; Khadempour *et al.* 2016).

Leaf-cutter ants, in general, cut an exceptionally broad diversity of plants (Mayhé-Nunes & Jaffe 1998; Solomon 2007) and thus, have the potential to encounter a myriad of plant defense compounds that are toxic to themselves and their fungal cultivar. The ants are not enriched in genes families for plant defense compound detoxification (Rane *et al.* 2016), so they must reduce the intake of these chemicals in other ways. Plant defense compound avoidance occurs in several steps. First, ants avoid cutting plants that contain plant defense compounds that are particularly toxic or abundant (Hubbell *et al.* 1984; Howard 1988; Wirth *et al.* 1997). Second, many plant defense compounds that the ants encounter are volatile

chemicals (Howard 1988; Howard *et al.* 1988), and in the time that the ants cut and carry the leaf material back to their colonies, some of the volatiles will have had time to dissipate. Finally, ants often leave leaf material in caches before they incorporate them into their fungus gardens (Hart & Ratnieks 2000; Roschard & Roces 2003), providing further opportunity for the defense compounds to evaporate. Nevertheless, some amount of volatiles can make their way into the gardens. In this study, using gas chromatography, we were able to detect eucalyptus-related compounds (eucalyptol, α-pinene, β-pinene, *p*-cymene and γ-terpinene) in the fungus garden of one ant colony (*A. laevigata* 1) that was observed cutting considerable amounts of eucalyptus (Supplemental methods and Supplemental Figure 3.2).

In order to mitigate the deleterious effects of plant defense compounds, we expect the fungal cultivar *L. gongylophorus* would produce enzymes to degrade them. Indeed, work by De Fine Licht *et al.* (2013) suggests that laccases from the fungal cultivar help detoxify plant defense compounds. Nevertheless, bacteria in the garden may also play a role in mediating plant defense compounds. The bacterial community contains the genes necessary for plant defense compound remediation, including many cytochrome P450s, gluthione S-transferases, and other genes involved in xenobiotic degradation, and aromatic compound degradation, but they are not consistently enriched in the dicot-cutter ant fungus gardens (Supplemental Table 3.1). We expected that since dicot-cutter ants incorporate a higher diversity of plants into their gardens (Table 3.3), that the diversity of bacteria would also be higher in these gardens, and that the bacteria would have a higher capacity for the degradation of these defense compounds. While we did observe a greater diversity of bacteria in the dicot-cutter ant fungus gardens (Figure 3.3) we did not see a significant enrichment of plant defense compound degradation genes in these gardens (Figure 3.5, Table A2.1). However, we still

cannot exclude the possibility that bacteria are taking part in this process. Since each dicot-cutter ant colony cuts a unique set of plants (Table 3.3), they potentially encounter a unique set of plant defense compounds. If the bacterial community were to respond in a substrate-specific manner to different plant defense compounds, our analysis in this study would not reveal that. To elucidate the role of bacteria in plant defense compound remediation, closely controlled experiments with particular defense compounds of interest applied to bacterial cultures and to fungus gardens would be necessary.

Pinto-Tomas *et al.* (2009) establishes that *Pantoea* and *Klebsiella* bacteria in Central American leaf-cutter ant fungus gardens are supplementing the ant diet through nitrogen fixation. Plant material, in general, is low in nitrogen, and many herbivores supplement their diets through bacterial nitrogen fixation (Douglas 2009; Hansen & Moran 2013). Grasses are especially low in nitrogen (Mattson 1980; Winkler & Herbst 2004), so we would hypothesize that grass-cutter ant fungus gardens would be enriched in nitrogen-fixing bacteria with a corresponding enrichment of nitrogen-fixing genes. Here we show that *Pantoea* are more abundant in the grass-cutter ant fungus gardens, and that a nitrogenase molybdenum-iron protein beta chain gene is significantly more abundant in grass-cutter ant fungus gardens (Table A2.1). Other genes that are related to nutrient acquisition are also significantly more abundant in the grass-cutter ant fungus gardens (Figure 3.5), such as genes in amino acid metabolism pathways. While it has been shown that nitrogen fixed by bacteria is incorporated into the bodies of ants (Pinto-Tomás *et al.* 2009), animals cannot simply absorb nitrogen as ammonium or nitrate, they require it to either be in the form of amino acids or other organic nitrogen-containing compounds (White 1993). The enrichment of arginine biosynthesis genes is of particular interest since the genome of *Atta* is deficient in genes in

this pathway (Suen *et al.* 2011b), and no evidence has been found that the fungus provides arginine (Aylward *et al.* 2013; Khadempour *et al.* 2016).

Other categories of genes enriched in the grass-cutter ant fungus garden bacteria are those involved in metabolism of terpenoids and secondary metabolites, especially their biosynthesis. Grass-cutter ant fungus gardens are significantly enriched in 67 of these genes. This list includes seven siderophores, which are responsible for iron acquisition (Crosa 1989; Winkelmann 2002). Siderophores are costly to produce so the enrichment of these genes suggests that iron acquisition is important in this system. The grass-cutter and fungus gardens examined in this study contained lower amounts of iron than the dicot-cutter ant fungus gardens (Figure 3.6). Terpenoids are the most abundant secondary metabolites found in plants, and serve diverse roles (Langenheim 1994; Gershenzon & Dudareva 2007). The majority of research into the connection between plant terpenoids and animal-microbe symbioses are in regards to the detoxification of terpenes that would be deleterious to the animal host (Wang *et al.* 2012; Cheng *et al.* 2013; Adams *et al.* 2013; Boone *et al.* 2013; Raffa 2013). However, not all terpenes are toxic to all organisms (Raffa 2013), and in at least one instance they have been shown to supplement a herbivore's diet after some modification by a gut bacterium (Berasategui *et al.* 2017). Dicots contain higher quantities of terpenoids (Wetterer 1994; Mariaca *et al.* 1997). One possibility is that the bacteria in these fungus gardens are producing terpenes as a nutritional additive, especially in the grass-cutter ant fungus gardens where there are lower terpene inputs and these genes are enriched (Figure 3.5, Figure A2.2).

Grass-cutter ants can be categorized as facultative specialists (Shipley *et al.* 2009), as they have the capacity to use grass, a difficult substrate, as their primary fungus garden input but they are also capable of consuming a wider range of substrates. The ants themselves are

adapted to cutting grass with shorter, wider mandibles than their dicot-cutter counterparts (Fowler *et al.* 1986; Silva *et al.* 2016). They also process leaves differently – they do not mulch the material, likely because the silica contained in grasses would dull their mandibles (Massey & Hartley 2009; Silva *et al.* 2016). The ants' adaptation to grass-cutting, combined with the community response of the bacteria in the gardens, allow grass-cutter ants to use grass as a substrate more efficiently than dicot-cutter ants can. This has allowed grass-cutter ants to exploit a novel niche, presumably reducing the amount of interspecific competition they experience. It should be noted, however, that grass-cutter ants, if given the opportunity to use palatable dicots, actually prefer those over grasses (Nagamoto *et al.* 2009), probably due to their lower recalcitrance and higher nutrient content. This indicates that grass-cutter ants' fundamental niche is broader than their realized one. Not only do grass-cutters cut dicots, when they are available to them, but in this study, we detected grasses in the fungus gardens of some dicot-cutters as well (Table 3.3) and both *A. laevigata* and *A. sexdens* workers were observed cutting grass (personal observation). Even though there is some grass in these fungus gardens, the majority of species that these ants cut are dicots. As well, *A. laevigata* and *A. sexdens* both process their leaves in the manner consistent with other dicot-cutter ants (Fowler *et al.* 1986)).

Grass-cutter ants forage on a substrate that is lower in nutritional quality than their dicot-cutter counterparts. The grasses they cut are lower in nitrogen and iron. Optimal foraging theory predicts that when the quality of forage is lower, the ants should be cutting a greater diversity of plants (Rockwood & Hubbell 1987). Instead, grass-cutter ants cut a significantly lower diversity of plants than their dicot-cutter counterparts. Grass-cutter ants to do not have access to the diversity of plants necessary to compensate for their low forage quality. Instead, the bacteria in their fungus gardens can provide the necessary nutrition that

a diverse diet provides in dicot-cutter ant fungus gardens, allowing grass-cutter ant species to exploit this novel niche. Not only does our work here provide further evidence of the importance of bacteria in the leaf-cutter ant system, it provides further support that microbial symbionts are important players in novel substrate utilization by animals.

3.6 Acknowledgements

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Table 3.1 Summary of collection details for leaf-cutter ant colonies used in this study

Leaf-cutter ant colony	Substrate niche	IMG genome number	Collection date	Latitude	Longitude
A. bisphaerica 1	Grass	3300013023	1-Feb-15	S22°50'47.7"	W48°26'.9"
A. bisphaerica 2	Grass	3300013025	3-Feb-15	S22°50'48.4"	W48°26'1.4"
A. bisphaerica 3	Grass	3300013022	3-Feb-15	S22°50'48.4"	W48°26'2.3"
A. capiguara 1	Grass	3300012994	2-Feb-15	S22°54'32.1"	W48°18'28.7"
A. capiguara 2	Grass	3300012996	3-Feb-15	S22°50'47.2"	W48°26'1.3"
A. capiguara 3	Grass	3300012997	3-Feb-15	S22°50'47.6"	W48°26'1.2"
A. laevigata 1	Dicot*	3300013000	20-Jan-15	S21°9'55.5"	W47°50'51.3"
A. laevigata 2	Dicot*	3300012995	17-Jan-15	S21°10'3"	W47°50'47"
A. laevigata 3	Dicot*	3300012998	19-Jan-15	S21°9'56.8"	W47°50'52.7"
A. sexdens 1	Dicot	3300012999	30-Jan-15	S21°9'50"	W47°51'26.9"
A. sexdens 2	Dicot	3300013002	30-Jan-15	S21°9'53.4"	W47°51'10.5"
A. sexdens 3	Dicot	3300013001	31-Jan-15	S21°10'2"	W47°51'5"

*While *A. laevigata* has been described as a grass/dicot-cutter ant (Fowler *et al.* 1986), due to its leaf-processing behavior and fungus garden plant composition observed in this study, we consider it a dicot-cutter.

Table 3.2 Metagenome sequencing statistics for leaf-cutter ant fungus gardens

Leaf-cutter ant colony	Scaffold total	Scaffold sequence total (Mbp)	Main genome scaffold N/L50	Main genome scaffold N/L90	Max scaffold length (Kbp)	Scaffolds > 50 Kbp	Aligned reads	Protein coding genes
A. bisphaerica 1	628724	390.8	122506/ 740	467228/ 298	249.52	93 (2.07%)	163185122 (98.76%)	607 042 (99.39%)
A. bisphaerica 2	939707	630.9	148370/ 926	680177/ 292	253.54	69 (0.88%)	148406252 (96.49%)	910 609 (99.61%)
A. bisphaerica 3	285649	186.0	29401/ 972	244722/ 247	187.50	49 (1.90%)	167169016 (98.76%)	358 547 (98.19%)
A. capiguara 1	205334	148.7	16608/ 1403	178745/ 247	273.83	37 (2.49%)	199009346 (99.31%)	272 096 (98.99%)
A. capiguara 2	345332	261.2	35330/ 1420	303130/ 247	180.27	34 (1.06%)	204772230 (98.31%)	456 916 (98.70%)
A. capiguara 3	573737	359.5	83958/ 790	508567/ 247	135.51	13 (0.29%)	203079026 (98.77%)	644 865 (98.79%)
A. laevigata 1	853367	517.5	178678/ 686	645038/ 301	274.98	87 (1.53%)	178461364 (96.65%)	871 330 (99.42%)
A. laevigata 2	295365	189.2	32897/ 990	266928/ 247	252.44	92 (4.23%)	189659024 (96.85%)	332 737 (96.95%)
A. laevigata 3	601593	546.3	74824/ 1744	398992/ 340	241.71	17 (0.30%)	209535750 (96.06%)	722 718 (99.01%)
A. sexdens 1	674609	708.4	48220/ 3118	412923/ 341	701.43	167 (2.40%)	156148622 (97.92%)	822 403 (99.46%)
A. sexdens 2	857038	812.9	65552/ 2346	548662/ 328	61.96	17 (0.11%)	150809208 (95.32%)	1 088 719 (99.51%)
A. sexdens 3	1006806	564.7	221493/ 614	772141/ 277	386.55	68 (1.17%)	186976430 (91.63%)	1 029 784 (99.20%)

Table 3.3 Plant genera detected in each fungus garden sample using *MatK* gene

Sample	Family	Genus	Species *MatK* match %
A. bisphaerica 1	Fabaceae	*Chamaecrista*	99.3
	Poaceae	*Paspalum*	99.6
	Polygalaceae	*Polygala*	99.3
A. bisphaerica 2	Poaceae	*Paspalum*	99.4
A. bisphaerica 3	Fabaceae	*Chamaecrista*	99.3
	Fabaceae	*Zornia*	100
	Poaceae	*Paspalum*	99.5
	Polygalaceae	*Polygala*	99.0
A. capiguara 1	Poaceae	*Paspalum*	99.7
A. capiguara 2	Poaceae	*Paspalum*	99.6
A. capiguara 3	Fabaceae	*Chamaecrista*	99.3
	Poaceae	*Paspalum*	99.6
A. laevigata 1	Fabaceae	*Pterogyne*	99.4
	Myrtaceae	*Eucalyptus*	99.9
	Poaceae	*Paspalum*	99.5
	Poaceae	*Urochloa*	100
A. laevigata 2	Asteraceae	*Rensonia*	99.6
	Fabaceae	*Centrolobium*	98.2
	Fabaceae	*Schizolobium*	100
A. laevigata 3	Anacardiaceae	*Pachycormus*	98.6
	Asteraceae	*Kingianthus*	95.4
	Asteraceae	*Podanthus*	99.5
	Fabaceae	*Desmodium*	99.8
	Fabaceae	*Leucaena*	100
	Myrtaceae	*Eucalyptus*	99.8
	Poaceae	*Paspalum*	99.9
A. sexdens 1	Anacardiaceae	*Loxopterygium*	98.4
	Asteraceae	*Cymophora*	98.5
	Bignoniaceae	*Tabebuia*	98.1
	Fabaceae	*Andira*	98.6
	Fabaceae	*Batesia*	98.8
	Fabaceae	*Bussea*	100
	Fabaceae	*Libidibia*	99.8
	Fabaceae	*Pterogyne*	100
	Fabaceae	*Tipuana*	99.9
	Malvaceae	*Pachira*	100
	Myrtaceae	*Eucalyptus*	99.6
	Myrtaceae	*Eugenia*	99.8
	Poaceae	*Scutachne*	98.4
	Rubiaceae	*Genipa*	99.2
	Solanaceae	*Lycianthes*	100
A. sexdens 2	Bignoniaceae	*Tabebuia*	98.3
	Combretaceae	*Lumnitzera*	93.4
	Fabaceae	*Centrolobium*	98.2
	Fabaceae	*Pterogyne*	99.3
	Fabaceae	*Tipuana*	100
	Lecythidaceae	*Careya*	94.0
	Santalaceae	*Phoradendron*	99.6
A. sexdens 3	Asteraceae	*Echinacea*	99.3
	Asteraceae	*Eclipta*	100
	Asteraceae	*Perymeniopsis*	99.8
	Asteraceae	*Synedrella*	100
	Commelinaceae	*Commelina*	100
	Commelinaceae	*Murdannia*	92.0
	Fabaceae	*Desmodium*	99.8
	Fabaceae	*Leucaena*	100
	Malvaceae	*Sida*	99.7
	Myrtaceae	*Eucalyptus*	98.6
	Phyllanthaceae	*Phyllanthus*	100
	Rubiaceae	*Genipa*	100
	Solanaceae	*Acnistus*	99.4

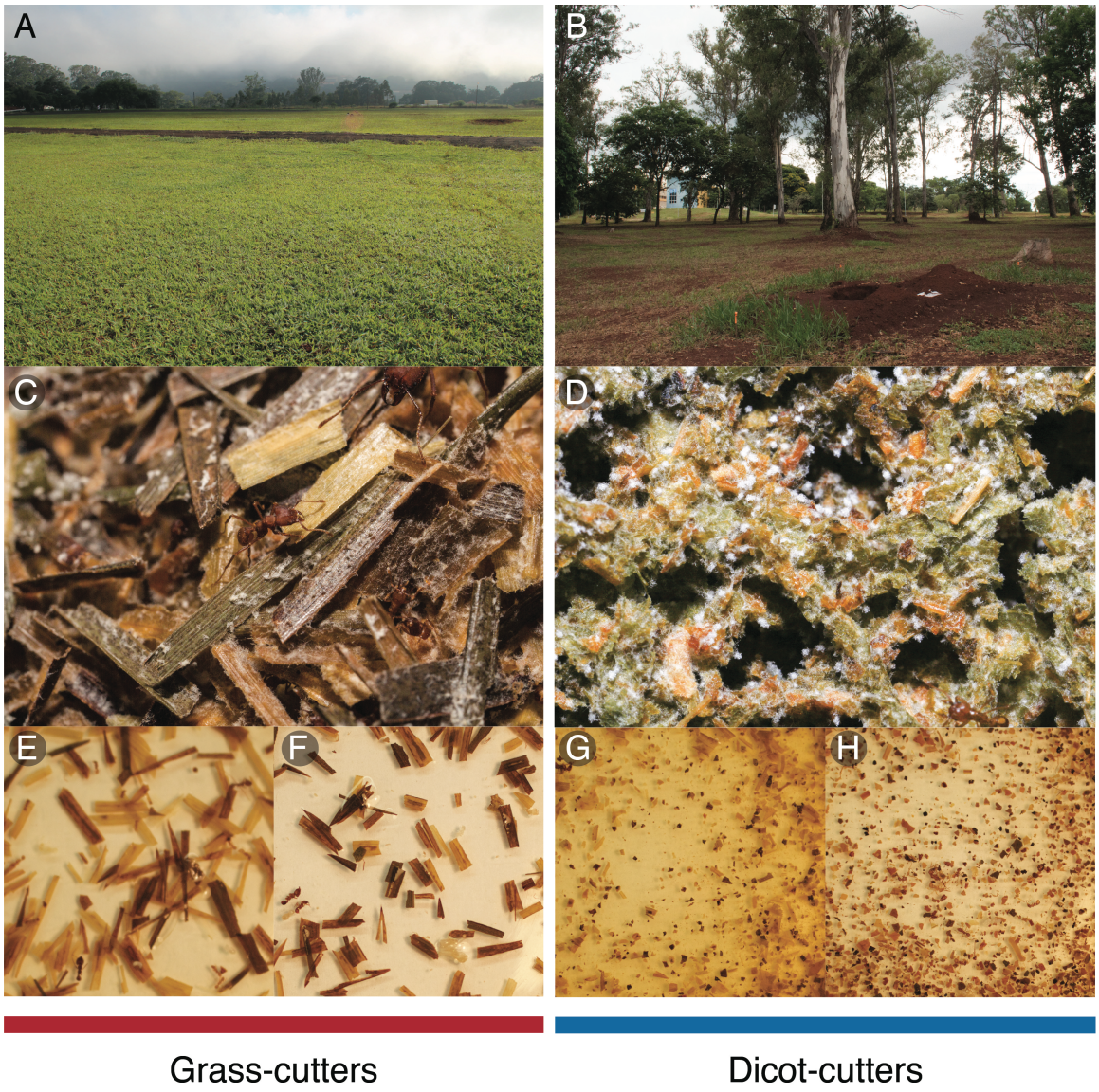

Figure 3.1 Grass- and dicot-cutter ants differ in the niches that they occupy, and the way that they cut and process leaf material. Field sites in (A) Botucatu, SP and (B) Ribeirão Preto, SP, Brazil. Fungus gardens of (C) grass- and (D) dicot-cutter ants. C. Visual inspection of leaf material from leaf-cutter ant fungus gardens demonstrates the degree of mulching that the different ants complete, with grass-cutters leaving the leaf material more intact (E – *A. bisphaerica* and F – *A. capiguara*), while dicot-cutters mulch to the point of unrecognizable leaf fragments (G – *A. laevigata* and H – *A. sexdens*).

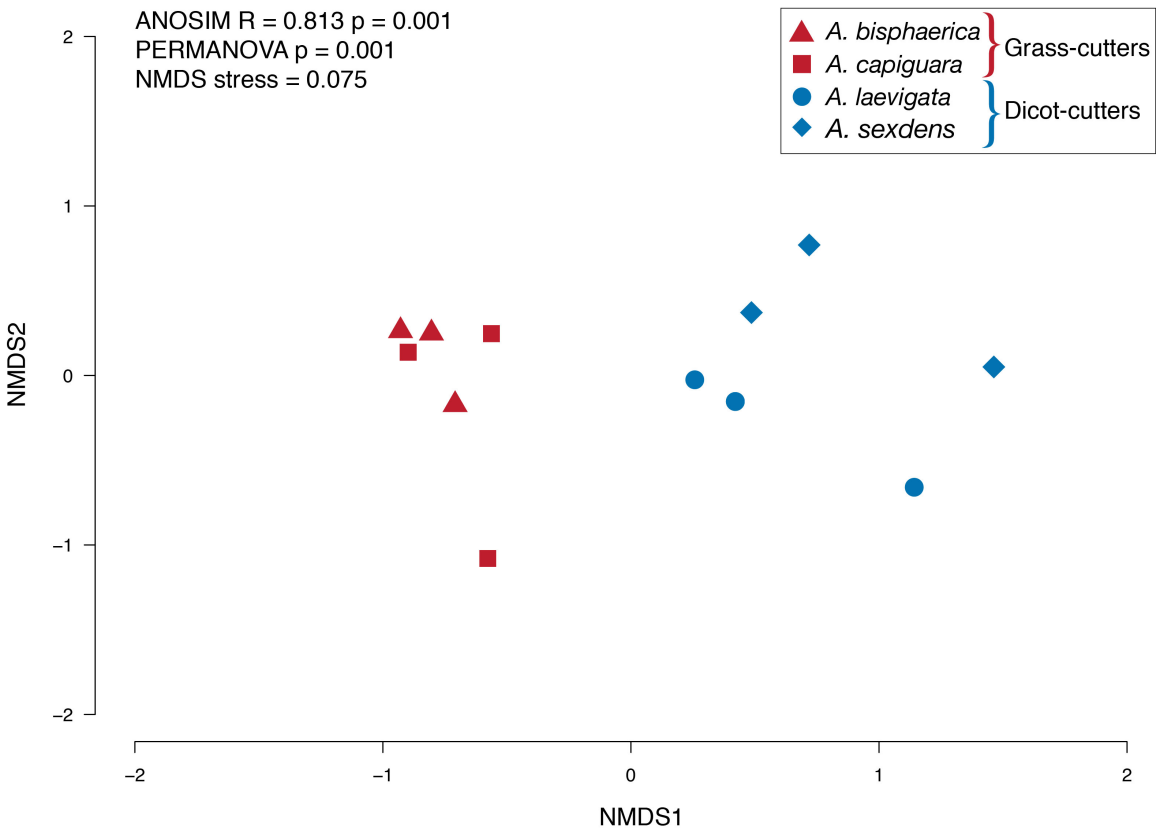

Figure 3.2 NMDS plot of the relative abundance of bacterial genera in fungus gardens of grass- and dicot-cutter ants. Grass- and dicot-cutter fungus garden bacterial communities are significantly different.

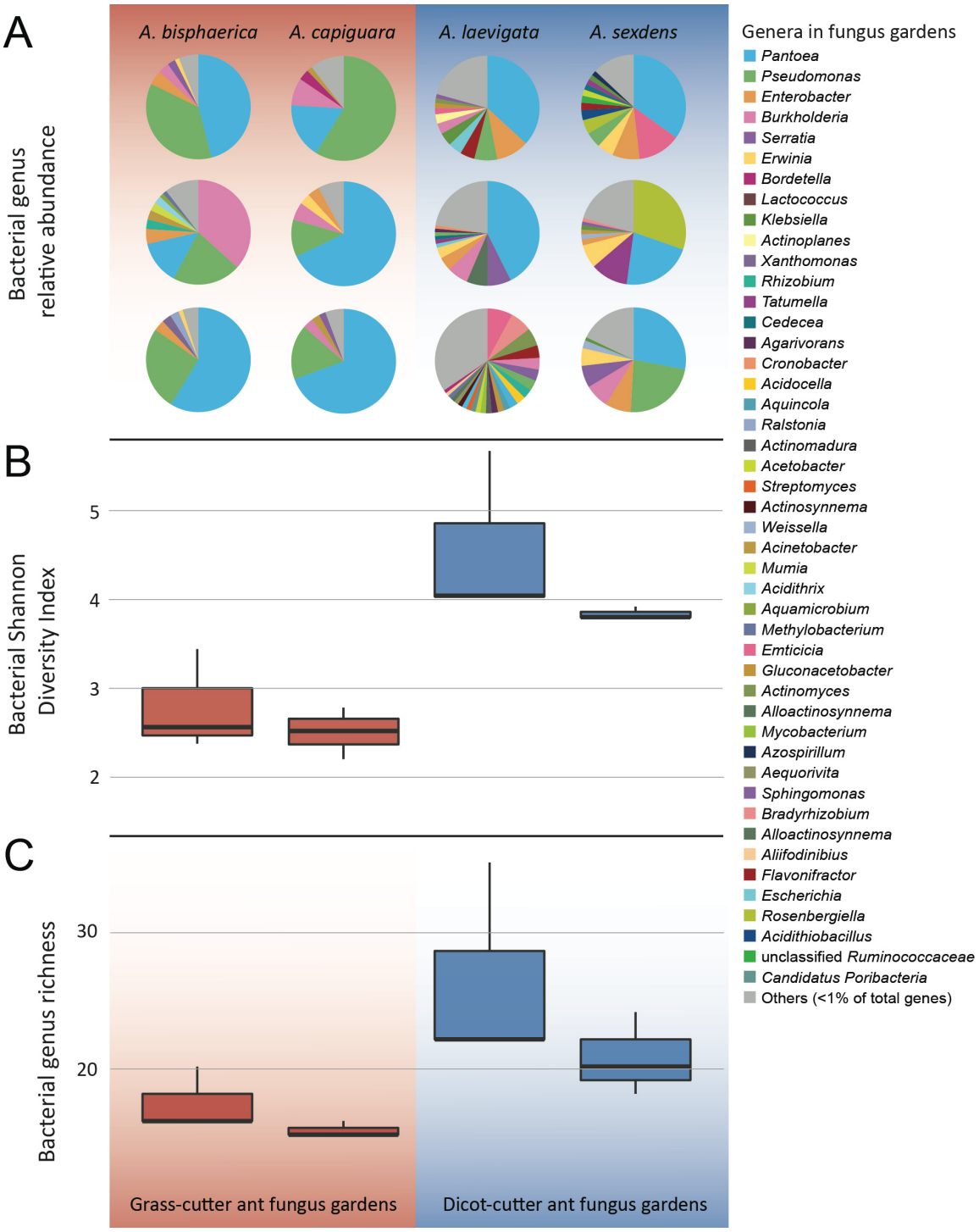

Figure 3.3 Genus-level bacterial community analysis of leaf-cutter ant fungus gardens from grass- and dicot-cutter ants, demonstrating that dicot-cutter ant fungus gardens have a higher diversity of bacteria. A. Pie charts showing proportions of different bacterial genera in the fungus gardens. B. Shannon diversity index of bacterial genera. C. Bacterial genus richness (for genera that consist of more than 1% of the total normalize gene count).

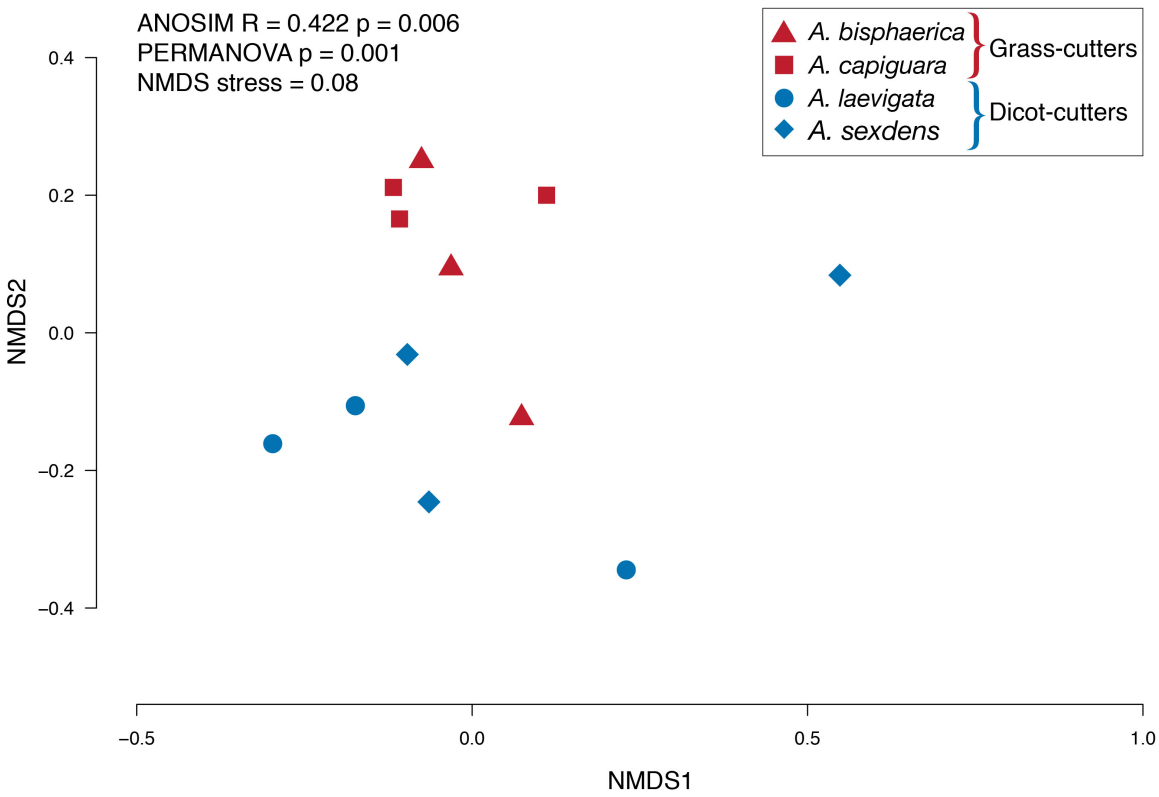

Figure 3.4 NMDS plot of KO functional genes from grass- and dicot-cutter ant fungus gardens. The KO profiles are significantly different between the fungus gardens of ants cutting the different substrates.

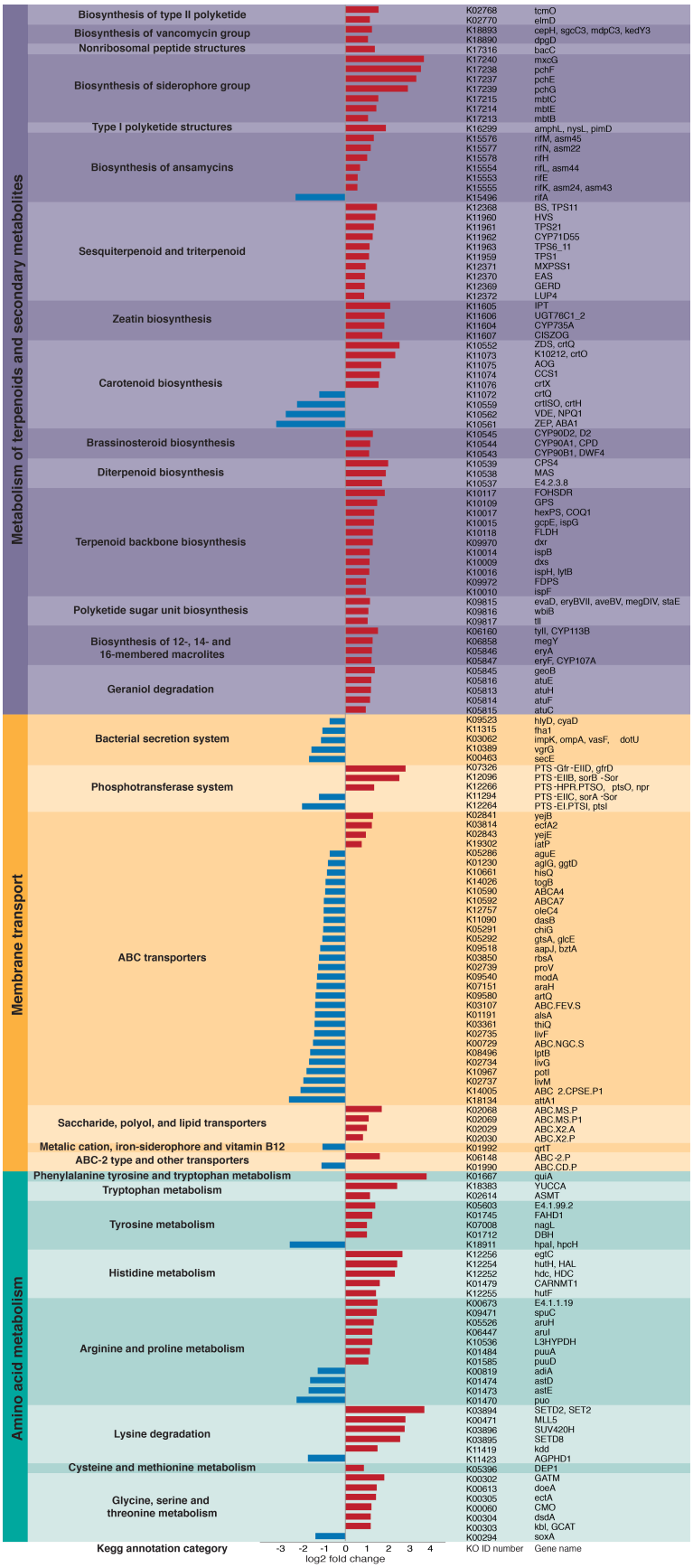

Figure 3.3 Particular groups of genes are enriched in either the grass- or dicot-cutter ant fungus gardens. Grass-cutter ant fungus gardens are enriched for genes involved in metabolism of terpenoids and secondary metabolites, as well as genes involved in amino acid metabolism. In contrast, dicot-cutter ant fungus gardens are enriched for genes involved in membrane transport. Bars extending to the left (blue) represent genes that are significantly more abundant in dicot-cutter ant fungus gardens and bars extending to the right (red) represent genes that are significantly more abundant in grass-cutter ant fungus gardens.

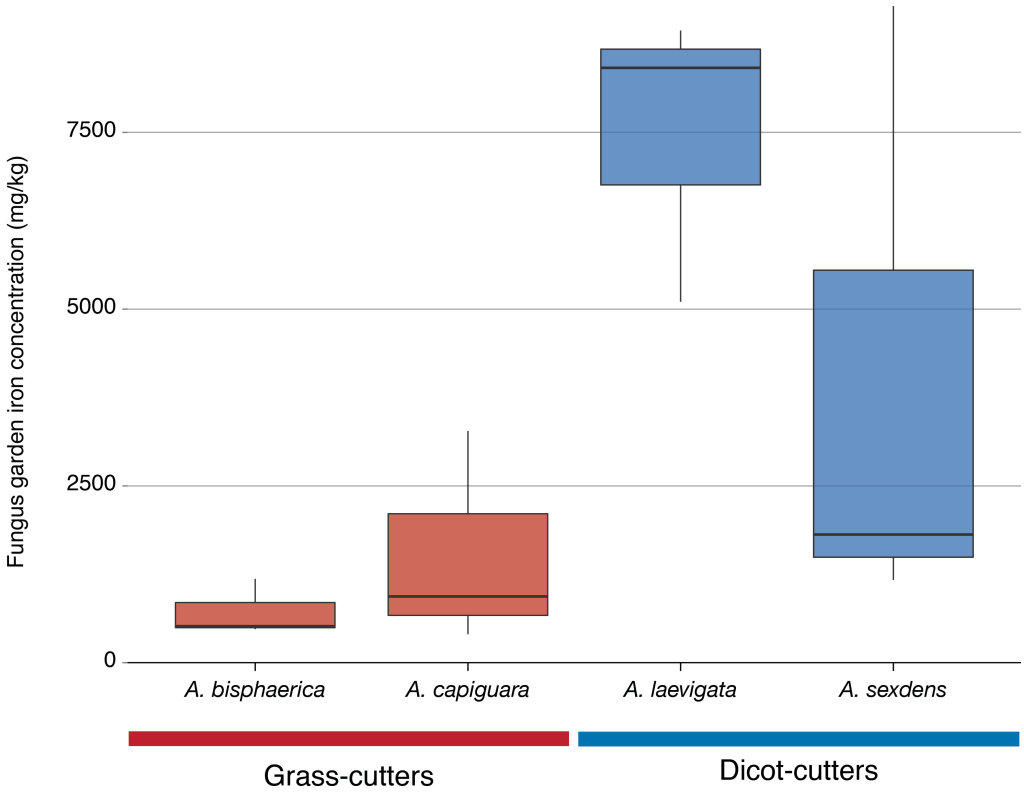

Figure 3.6 Iron content of fungus gardens from this study as measured by inductively coupled plasma optical emission spectroscopy. The iron content in the grass-cutter ant fungus gardens was lower than in the dicot-cutter ant fungus gardens. This difference is not statistically significant, however, since the *A. sexdens* fungus garden iron content is highly variable.

3.7 References

Adams AS, Aylward FO, Adams SM *et al.* (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology*, **79**, 3468–3475.

Adams AS, Jordan MS, Adams SM *et al.* (2011) Cellulose-degrading bacteria associated with the invasive woodwasp *Sirex noctilio*. *The ISME Journal*, **5**, 1323–1331.

Alneberg J, Bengtsson-Palme J, de Bruijn I *et al.* (2014) *Normalization of count data from the metagenomic data sets*. Stockholm, Sweden.

Aylward FO, Burnum KE, Scott JJ *et al.* (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. *The ISME Journal*, **6**, 1688–1701.

Aylward FO, Burnum-Johnson KE, Tringe SG *et al.* (2013) *Leucoagaricus gongylophorus* produces diverse enzymes for the degradation of recalcitrant plant polymers in leaf-cutter ant fungus gardens. *Applied and Environmental Microbiology*, **79**, 3770–3778.

Aylward FO, Suen G, Biedermann PHW *et al.* (2014) Convergent bacterial microbiotas in the fungal agricultural systems of insects. *mBio*, **5**, e02077–14.

Bass M, Cherrett JM (1995) Fungal hyphae as a source of nutrients for the leaf-cutting ant Atta sexdens. *Physiological Entomology*, **20**, 1–6.

Berasategui A, Salem H, Paetz C *et al.* (2017) Gut microbiota of the pine weevil degrades conifer diterpenes and increases insect fitness. *Molecular Ecology*, **26**, 4099–4110.

Boone CK, Keefover-Ring K, Mapes AC *et al.* (2013) Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. *Journal of Chemical Ecology*, **39**, 1003–1006.

Bushnell B (2017) BBMap.

Cheng X-Y, Tian X-L, Wang Y-S *et al.* (2013) Metagenomic analysis of the pinewood nematode microbiome reveals a symbiotic relationship critical for xenobiotics degradation. *Nature Publishing Group*, **3**, 237–10.

Costa AN, Vasconcelos HL, Vieira-Neto EHM, Bruna EM (2008) Do herbivores exert top-down effects in Neotropical savannas? Estimates of biomass consumption by leaf-cutter ants. *Journal of Vegetation Science*, **19**, 849–854.

Crosa JH (1989) Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiological reviews*, **53**, 517–530.

De Fine Licht HH, Schiøtt M, Rogowska-Wrzesinska A *et al.* (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences*, **110**, 583–587.

Delsuc F, Metcalf JL, Wegener Parfrey L *et al.* (2013) Convergence of gut microbiomes in myrmecophagous mammals. *Molecular Ecology*, **23**, 1301–1317.

Ding S-Y, Himmel ME (2008) Anatomy and ultrastructure of maize cell walls: An example of energy plants. In: *Biomass Recalcitrance: Deconstructing the Plant Cell Wall for Bioenergy* (ed

Himmel ME), pp. 38–60. Blackwell Publishing Ltd, Oxford, UK.

Douglas AE (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology*, **23**, 38–47.

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England)*, **26**, 2460–2461.

Fassel VA, Kniseley RN (1974) Inductively coupled plasma-optical emission spectroscopy. *Analytical Chemistry*, **46**, 1110A–1112A.

Fowler H, Forti L, Pereira-da-Silva V, Saes N (1986) Economics of grass-cutting ants. In: *Fire ants and leaf-cutting ants* (eds Lofgren CS, Vander Meer RK), pp. 18–35. Westview Pr, Boulder, CO.

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. *Nature chemical biology*, **3**, 408–414.

Grell MN, Linde T, Nygaard S *et al.* (2013) The fungal symbiont of *Acromyrmex* leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and other plant cell wall polysaccharides. *BMC Genomics*, **14**, 928.

Gutiérrez JL, Jones CG (2006) Physical ecosystem engineers as agents of biogeochemical heterogeneity. *BioScience*, **56**, 227–236.

Hammer TJ, Bowers MD (2015) Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia*, 1–14.

Hansen AK, Moran NA (2011) Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *PNAS*, **108**, 2849–2854.

Hansen AK, Moran NA (2013) The impact of microbial symbionts on host plant utilization by herbivorous insects. *Molecular Ecology*, **23**, 1473–1496.

Hart AG, Ratnieks FLW (2000) Leaf caching in Atta leafcutting ants: discrete cache formation through positive feedback. *Animal Behaviour*, **59**, 587–591.

Herz H, Beyschlag W, Hölldobler B (2007) Assessing herbivory rates of leaf-cutting ant (*Atta colombica*) colonies through short-term refuse deposition counts. *Biotropica*, **39**, 476–481.

Hess M, Sczyrba A, Egan R *et al.* (2011) Metagenomic Discovery of Biomass-Degrading Genes and Genomes from Cow Rumen. *Science*, **331**, 463–467.

Hollingsworth PM, Graham SW, Little DP (2011) Choosing and Using a Plant DNA Barcode (D Steinke, Ed,). *PLoS ONE*, **6**, e19254–13.

Howard JJ (1988) Leafcutting and diet selection: relative influence of leaf chemistry and physical features. *Ecology*, **69**, 250–260.

Howard JJ, Cazin J Jr., Wiemer DF (1988) Toxicity of terpenoid deterrents to the leafcutting ant *Atta cephalotes* and its mutualistic fungus. *Journal of Chemical Ecology*, **14**, 59–69.

Hubbell SP, Howard JJ, Wiemer DF (1984) Chemical leaf repellency to an attine ant: seasonal distribution among potential host plant species. *Ecology*, 1067–1076.

Huntemann M, Ivanova NN, Mavromatis K *et al.* (2016) The standard operating procedure of the DOE-JGI Metagenome Annotation Pipeline (MAP v.4). *Standards in Genomic*

Sciences, 1–5.

Kanehisa M, Goto S, Sato Y *et al.* (2014) Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Research*, **42**, D199–205.

Khadempour L, Burnum KE, Baker ES *et al.* (2016) The fungal cultivar of leaf-cutter ants produces specific enzymes in response to different plant substrates. *Molecular Ecology*, **25**, 5795–5805.

King BC, Waxman KD, Nenni NV *et al.* (2011) Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. *Biotechnology for biofuels*, **4**, 4–18.

Kohl KD, Connelly JW, Dearing MD, Forbey JS (2016) Microbial detoxification in the gut of a specialist avian herbivore, the Greater Sage-Grouse (A Endo, Ed,). *FEMS Microbiology Letters*, **363**, fnw144–6.

Kohl KD, Weiss RB, Cox J, Dale C, Denise Dearing M (2014) Gut microbes of mammalian herbivores facilitate intake of plant toxins (N van Dam, Ed,). *Ecology Letters*, **17**, 1238–1246.

Kooij PW, Schiøtt M, Boomsma JJ, De Fine Licht HH (2011) Rapid shifts in *Atta cephalotes* fungus-garden enzyme activity after a change in fungal substrate (Attini, Formicidae). *Insectes sociaux*, **58**, 145–151.

Kudo T (2009) Termite-microbe symbiotic system and its efficient degradation of lignocellulose. *Bioscience, Biotechnology, and Biochemistry*, **73**, 2561–2567.

Langenheim JH (1994) Higher plant terpenoids: A phytocentric overview of their ecological roles. *Journal of Chemical Ecology*, **20**, 1223–1280.

LeBlanc JG, Milani C, de Giori GS *et al.* (2013) Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Current opinion in biotechnology*, **24**, 160–168.

Li Z, Wright A-DG, Liu H *et al.* (2014) Bacterial community composition and fermentation patterns in the rumen of sika deer (*Cervus nippon*) fed three different diets. *Microbial Ecology*, **69**, 307–318.

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, **15**, 31–21.

Mariaca RG, Berger TFH, Gauch R *et al.* (1997) Occurrence of Volatile Mono- and Sesquiterpenoids in Highland and Lowland Plant Species as Possible Precursors for Flavor Compounds in Milk and Dairy Products. *Journal of Agricultural and Food Chemistry*, **45**, 4423–4434.

Massey FP, Hartley SE (2009) Physical defences wear you down: progressive and irreversible impacts of silica on insect herbivores. *Journal of Animal Ecology*, **78**, 281–291.

Mattson WJ (1980) Herbivory in relation to plant nitrogen content. *Annual Review Of Ecology And Systematics*, **11**, 119–161.

Mayhé-Nunes A, Jaffe K (1998) On the biogeography of Attini (Hymenoptera: Formicidae). *Ecotropicos*, **11**, 45–54.

Moreira-Soto RD, Sanchez E, Currie CR, Pinto-Tomás AA (2017) Ultrastructural and microbial analyses of cellulose degradation in leaf-cutter ant colonies. *Journal of General*

Microbiology, **163**, 1578–1589.

Moutinho P, Nepstad DC, Davidson EA (2003) Influence of leaf-cutting ant nests on secondary forest growth and soil properties in Amazonia. *Ecology*, **84**, 1265–1276.

Muegge BD, Kuczynski J, Knights D *et al.* (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, **332**, 970–974.

Nagamoto NS, Carlos AA, Moreira SM *et al.* (2009) Differentiation in Selection of Dicots and Grasses by the Leaf-Cutter Ants *Atta capiguara*, *Atta laevigata* and *Atta sexdens rubropilosa*. *Sociobiology*, **54**, 127–138.

Nagamoto NS, Garcia MG, Forti LC *et al.* (2011) Microscopic evidence supports the hypothesis of high cellulose degradation capacity by the symbiotic fungus of leaf-cutting ants. *Journal of Biological Research-Thessaloniki*, **16**, 308–312.

North RD, Jackson CW, Howse PE (1997) Evolutionary aspects of ant-fungus interactions in leaf-cutting ants. *Trends in Ecology & Evolution*, **12**, 386–389.

Oksanen J, Blanchet FG, Kindt R *et al.* (2013) vegan: Community ecology package. *CRAN.R-project.org*.

Pinto-Tomás AA, Anderson MA, Suen G *et al.* (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science*, **326**, 1120–1123.

Popper ZA, Tuohy MG (2010) Beyond the Green: Understanding the Evolutionary Puzzle of Plant and Algal Cell Walls. *Plant physiology*, **153**, 373–383.

R Core Team (2013) R: A language and environment for statistical computing. *R Foundation for Statistical Computing*.

Raffa KF (2013) Terpenes tell different tales at different scales: glimpses into the chemical ecology of conifer - bark beetle - microbial interactions. *Journal of Chemical Ecology*, **40**, 1–20.

Rane RV, Walsh TK, Pearce SL *et al.* (2016) Are feeding preferences and insecticide resistance associated with the size of detoxifying enzyme families in insect herbivores? *Current Opinion in Insect Science*, **13**, 70–76.

Rockwood LL, Hubbell SP (1987) Host-plant selection, diet diversity, and optimal foraging in a tropical leafcutting ant. *Oecologia*, **74**, 55–61.

Roschard J, Roces F (2003) Cutters, carriers and transport chains: Distance-dependent foraging strategies in the grass-cutting ant *Atta vollenweideri*. *Insectes sociaux*, **50**, 237–244.

Shipley LA, Forbey JS, Moore BD (2009) Revisiting the dietary niche: When is a mammalian herbivore a specialist? *Integrative and Comparative Biology*, **49**, 274–290.

Silva LC, Camargo RS, Lopes JFS, Forti LC (2016) Mandibles of leaf-cutting ants: morphology related to food preference. *Sociobiology*, **63**, 881–8.

Solomon SE (2007) Biogeography and evolution of widespread leafcutting ants, *Atta* spp. (Formicidae, Attini). University of Texas at Austin.

Suen G, Scott JJ, Aylward FO, Currie CR (2011a) The microbiome of leaf-cutter ant fungus gardens. In: *Handbook of Molecular Microbial Ecology, Volume III: Metagenomics in Different*

Habitats Metagenomics in Different Habitats. (ed de Bruijn FJ), pp. 367–379. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Suen G, Scott JJ, Aylward FO *et al.* (2010) An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS genetics*, **6**, e1001129.

Suen G, Teiling C, Li L *et al.* (2011b) The genome sequence of the leaf-cutter ant Atta cephalotes reveals insights into its obligate symbiotic lifestyle. *PLoS genetics*, **7**, e1002007.

Talbot PHB (1977) The *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology*, **15**, 41–54.

Wang Y, DiGuistini S, Wang T-CT, Bohlmann J, Breuil C (2010) *Agrobacterium*-meditated gene disruption using split-marker in *Grosmannia clavigera*, a mountain pine beetle associated pathogen. *Current genetics*, **56**, 297–307.

Wang Y, Lim L, DiGuistini S *et al.* (2012) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees. *New Phytologist*, **197**, 886–898.

Warnecke F, hl PLU, Ivanova N *et al.* (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature Biotechnology*, **450**, 560–565.

Wetterer JK (1994) Nourishment and evolution in fungus-growing ants and their fungi. In: *Nourishment and Evolution in Insect Societies* (eds Hunt JH, Nalepa CA), pp. 309–328. Boulder.

White TCR (1993) *The Inadequate Environment*. Springer-Verlag, Berlin.

Winkelmann G (2002) Microbial siderophore-mediated transport. *Biochemical Society transactions*, **30**, 691–696.

Winkler JB, Herbst M (2004) Do plants of a semi-natural grassland community benefit from long-term CO2 enrichment? *Basic and Applied Ecology*, **5**, 131–143.

Wirth R, Beyschlag W, Ryel RJ, Holldobler B (1997) Annual foraging of the leaf-cutting ant *Atta colombica* in a semideciduous rain forest in Panama. *Journal of Tropical Ecology*, **13**, 1–741–75717.

Wolfe BE, Kuo M, Pringle A (2012) *Amanita thiersii* is a saprotrophic fungus expanding its range in the United States. *Mycologia*, **104**, 22–33.

Chapter 4: Genome sequencing of leaf-cutter ant fungal cultivar *Leucoagaricus gongylophorus* reveals between- and within-strain variation

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Conceived of the project (L.K., C.R.C), analyzed data (L.K.), designed sampling methodology (L.K.), performed lab work (L.K., E.W.-P.), performed field work (L.K., N.N.), provided resources and facilities (A.R., M.T.P., C.R.C), and wrote the manuscript (L.K., C.R.C.).

This manuscript contains preliminary data that will contribute to a larger fungal genome project, but will not be submitted for publication in this form.

4.1 Abstract

This work here is a preliminary investigation into the evolutionary transition from the deconstruction of dicots, an ancestral state in *Atta* leaf-cutter ants, to the deconstruction of grasses, a derived state. Specifically, I compare genomes of the fungal cultivars from ants that cut each of dicots and grasses. I compare the phylogeny of these fungi as well as their genetic potential for biomass degradation. Contrary to expectations, multiple unique sequences of EF1-α are present from individual fungus gardens, indicating either the presence of multiple strains of cultivar in each garden, or fungal cultivar strains containing multiple EF1-α gene sequences, likely in their multiple nuclei. In comparing the fungal cultivar genomes of *Atta cephalotes* (a dicot-cutter ant) to that of *A. bisphaerica* (a grass-cutter ant), I show that the dicot-cutter fungus has fewer genes encoding for biomass degrading enzymes likely due to this genome being more fragmented. The methods used in this chapter, as well as the preliminary data obtained, will help inform the analysis that will be completed once all genomes for this study are available.

4.2 Introduction

The relationship between leaf-cutter ants and their fungal cultivar has been studied for over a century (Belt 1874) and this conspicuous and fascinating example of non-human agriculture is a paradigmatic model of insect microbial symbiosis. Leaf-cutter ants belong to a tribe of fungus-growing ants called the Attini (Wetterer 1998). The relationship between attines and their fungal cultivars it thought to have originated approximately 60 mya (Schultz *et al.* 2015). Attine ants are separated into two groups, the "higher" and "lower" attines. The groupings are, in part, based on the types of fungi that they cultivate. Higher attine fungi are obligate mutualists, and have co-evolved with their ant hosts, with specialized structures that the ants consume as their main food source (Schultz & Brady 2008). The leaf-cutter ant genera (*Atta* and *Acromyrmex*) are the most derived lineage of the Attini, and both appear to be associated with one species of fungus, *Leucoagaricus gongylophorus* (Mueller *et al.* 2017).

There are 15 described species of *Atta,* which clade according to their biogeography and how they spread through South America and into Central America and the Southern United States (Bacci *et al.* 2009). When the substrates that these ants cut is superimposed on this previously-produced phylogeny, it also becomes apparent that there is a monophyletic group of *Atta* ants that have transitioned to cutting grass instead of dicots (Figure 4.1). It has been shown that grass-cutter ants are morphologically and behaviorally distinct from the other dicot-specialized *Atta*, likely for specialization on grasses. They have shorter and stronger mandibles than their dicot-cutting counterparts and they cut and process the leaves differently (Fowler *et al.* 1986) (Figure 4.1). These adaptations are thought to help the ant overcome the tougher cuticle of grasses as well as to make cutting the grass blades more efficient (Fowler *et al.* 1986).

Grasses differ from dicots in their cell wall structure, lignin content and sugar composition. Together these biochemical and structural differences represent a substantially different substrate for enzymatic hydrolysis (Ding & Himmel 2008). Thus, efficient deconstruction of grasses by the grass-cutter ant fungal cultivar is expected to require unique mechanisms that differ from those optimized for dicots. For example, cereal pathogens *Rhizoctonia cerealis, Fusarium culmorum* and *Pseudocercosporella herpotrichoides* all secrete similar cell wall degrading enzymes when grown on wheat. They secrete abundant amounts of arabanase, xylanase and laminarinase and low amounts of pectin enzymes, compared to dicot-pathogenic fungi (Cooper *et al.* 1988).

The work described in this chapter is the beginning of a study into the fungal mediation of an evolutionary transition in herbivory, from a dicot to grass diet. I am sequencing the genomes of two strains of *L. gongylophorus* from each of four species of ants: *A. bisphaerica* and *A. capiguara*, described as strict grass-cutters, *A. laevigata*, a transitional species, and *A. sexdens rubripilosa*, described as a strict dicot-cutter (Fowler *et al.* 1986). Here I present preliminary data since on the first of these eight genomes. I explore the phylogeny of the cultivar as well an overview of the genome and its biomass degrading potential.

4.3 Methods

4.3.1 Atta phylogeny annotation

The phylogeny of *Atta* was adapted from Bacci *et al.* (2009). Through searching the natural history literature on leaf-cutter ants, I determined if particular species of *Atta* are specialized to cut dicots, grasses, or both (Cherrett & Peregrine 1976; Fowler *et al.* 1986; Robinson & Fowler 2009). I mapped the substrate specialization onto the phylogeny (Figure 4.1). Grass-cutters are denoted with red, dicot-cutters with blue, ants that cut both with

purple and ants for which information could not be found are denoted with black (Figure 4.1).

4.3.2 Fungus collection and isolation

Fungal cultures were isolated from *Atta* fungus gardens in the state of São Paulo in Brazil between November 2014 and December 2015 (Table 4.1). Fungus gardens from four species of *Atta* leaf-cutter ants were collected: *A. bisphaerica* and *A. capiguara*, which both specialize on grass, *A. laevigata*, which cuts both grasses and dicots, and *A. sexdens*, a dicot-cutter ant.

Ant species were identified using worker morphology and by monitoring the substrates that the workers cut. Fungus gardens were then dug, taking care to enter chambers from the side, to avoid damaging the garden with digging tools and to avoid contamination with surrounding soil. Fungus gardens were transported to the laboratory where, under aseptic conditions, a small piece of mycelium was removed from fungus gardens and placed on potato dextrose agar (PDA) medium. Cultures were monitored and subcultured over the next month to ensure that pure cultures of fungal cultivar were isolated.

4.3.3 DNA and RNA extraction

To improve the quality of DNA and RNA extracted, and to avoid contamination by agar, fungal isolates were grown on 1% Oxoid malt extract agar plates overlaid with cellophane (gel dry grade, BioRad). Mycelium was scraped off the cellophane with a blunt scalpel and then frozen in aluminum foil at -80°C.

For DNA extraction, a modified version of the method by Möller *et al.* (1992) was used. Briefly, mycelium was crushed into a fine powder with liquid nitrogen and mortar and pestle then placed in tris-EDTA-SDS (TES) buffer with proteinase K. Impurities and RNA

were removed with CTAB and RNase A, respectively. This was followed by several rounds of purification with phenol and chloroform and further proteinase K and RNase A treatment. DNA was precipitated in a 10:1 mixture of isopropanol and ammonium acetate, cleaned twice with 70% ethanol, and suspended in tris-EDTA (TE) buffer. RNA was extracted using the phenol/chloroform extraction protocol from Book *et al.* (2014).

4.3.4 Genome and transcriptome sequencing and annotation

The *L. gongylophorus* AB2 genome was sequenced at the Joint Genome Institute (JGI) with Pacific Biosciences (PacBio) sequencing technology, assembled with Falcon (Gordon *et al.* 2016), and annotated using the JGI Annotation pipeline (https://genome.jgi.doe.gov/programs/fungi/FungalGenomeAnnotationSOP.pdf). Genome statistics of this genome and two closely related fungi that were previously sequenced at the JGI are presented in Table 4.2. Before gene prediction, assembly scaffolds were masked using RepeatMasker (Smit *et al.* 2012) and a genome-specific library of repeats was composed of the standard RepBase library (Jurka *et al.* 2005). Most frequent (>150times) repeats were recognized by RepeatScout (Price *et al.* 2005), and manually curated libraries of transposons (Figure 4.2). The Vista track provides access to the results of the comparative analysis of *L. gongylophorus* AB2 v1.0, *M. fuliginosa* MF-IS2 v1.0, and *L. gongylophorus* AC assemblies by The Berkeley Genome Pipeline (Couronne *et al.* 2003; Brudno *et al.* 2003). This approach combines local and global sequence alignment techniques to find conserved regions between two related genomes. Gateway at LBNL provides access to VISTA browser and all data related to this alignment (Figure 4.2).

4.3.5 Phylogeny construction

A phylogeny of EF1-α gene sequences was produced from previously sequenced amplicons obtained from (Mueller *et al.* 2017), from a blast search of EF1-α sequences against attine ant fungus gardens on the JGI Integrated Microbial Genomes and Microbiomes (IMG) dataset, and from BLAST searches against sequenced cultivar genomes (*L. gongylophorus* from *A. cepahalotes* and *A. bisphaerica*) as well as closely related *Lycoperdon perlatum* fungal genome. EF1-α sequences were aligned using MUSCLE (Edgar 2004) in the program MEGA7 (Kumar *et al.* 2016). Alignments were trimmed manually to ensure consistent length between sequences. The phylogeny was created using maximum likelihood based on the Tamura-Nei model (Tamura & Nei 1993).

4.3.6 CAZy analysis

CAZy annotations were completed using the methods from Book et al. (2016). Briefly, I utilized a locally created CAZy database (www.cazy.org) then used BLASTP to compare all protein-coding sequences from the three fungal strains *L. gongylophorus* AB2, *L. gongylophorus* AC and *M. fuliginosa* to the CAZy and pfam databases (ftp://ftp.ncbi.nih.gov/pub/mmdb/cdd/little_endian/Pfam_LE.tar.gz). These two annotations were compared and if a protein was annotated with both databases, it was included in the final CAZy annotation.

4.4. Results and Discussion

4.4.1 Genome statistics

At the time of writing, of the eight fungal genomes submitted for sequencing, the *L. gongylophorus* AB2 genome is the only one that is complete. Its host ant is *A. bisphaerica,* a grass-cutter ant species (Table 4.1). The remaining genomes are expected to be complete in

the spring of 2018. The AB2 genome assembly and annotation is of high quality. When compared to the previous *L. gongylophorus* fungal genome from an *A. cephalotes* colony, which was sequenced using 454 technology, the quality difference is apparent (Table 4.2). Genome coverage of the *L. gongylophorus* AB2 genome is at 222x, higher than that of the *L. gongylophorus* AC and *M. fuliginosa* genomes at 15x and 165x, respectively. This PacBio genome has the largest scaffolds of the three genomes and the highest proportion of scaffolds larger than 2 Kbp in length.

One important difference between the PacBio-sequenced *L. gongylophorus* AB2 genome and the other two comparison genomes is that it contains large swathes of repeat regions (Figure 4.2). These regions likely exist in the other two fungi as well, however, neither Illumina nor 454 technology are capable of successfully sequencing these regions. These repeat sequences may be useful in understanding the genome evolution of these fungi, and once the remaining genomes in this study have been sequenced, comparisons using these regions can occur.

4.4.2 Cultivar Phylogeny

The phylogeny of EF1-α genes from attine fungal cultivars is presented in Figure 4.3. There are two main clades of higher attine cultivars, with a sister group containing both free-living *Leucocoprinus* fungi, and lower attine cultivar strains. Clade A consists of several *Trachymyrmex* cultivar strains, distributed throughout the clade, as well as cultivar strains from *A. laevigata*, *A. sexdens*, *A. vollenweideri*, and *Acromyrmex* sp. Clade B contains a *Trachymymex arizonensis* cultivar strain, but it is primarily dominated by leaf-cutter ant cultivar strains, from *A. sexdens, A. bisphaerica, A. capiguara, A. cephalotes,* and *Acromyrmex* sp. *Atta sexdens* is the only species of *Atta* that has a cultivar with EF1-α sequences that fall into both clades. In fact, these sequences originate from the same fungus garden metagenome. There are other

instances of multiple, variable EF1-α sequences from the same metagenome, but those fall into the same major clade. This phylogeny is incongruent with that of *Atta* ants (Figure 4.3). Grass-cutters do not occur in a monophyletic clade, with clade A including fungi from the grass-cutters *A. vollenweiderii* and *A. laevigata*, and clade B including those from the grass-cutters *A. bisphaerica* and *A. capiguara.*

In some regards, this phylogeny is similar to that which is presented in the study by Mueller *et al.* (2017), where they present two cultivar clades. However, the results here dispute their conclusion that one clade contains all grass-cutter ant cultivars and that the other contains all dicot-cutter cultivars. It is important to note that in their work, they only consider *A. laevigata* and *A. vollenweiderii* as grass-cutter species, ignoring both *A. capiguara* and *A. bisphaerica*, which cultivate strains that contradict their conclusions.

One reason EF1-α is widely used as phylogenetic marker for fungi is that it is a conserved single copy gene (Stielow *et al.* 2015). There are three possible explanations for the observation of multiple, variable copies of this gene in the metagenomes used in this phylogeny. First, it is possible that there has been a duplication of EF1-α in the genomes of these fungi, as has occurred with bees (Danforth & Ji 1998). The second possibility is that there are multiple strains of cultivar in some fungus gardens, but there is no other evidence for this. The final, and most likely possibility is that the multiple nuclei in each cultivar strain contain different EF1-α sequences. This is the most likely explanation since Kooij *et al.* (2015) demonstrated that higher attine cultivar contain polykaryotic/heterokaryotic cells, with 7-17 nuclei per cell.

Once the remaining fungal genomes in this project have been sequenced, we will measure the variability in EF1-α and other traditionally single copy genes across the genomes using the original raw reads. While some mycorrhizal fungi are also polykaryotic, it has been

argued that they are homokaryotic (Tisserant *et al.* 2013). The multiple EF1-α sequences from the same cultivar fungus further support the conclusion of Kooij *et al.* (2015) that these fungi are heterokaryotic. The exploration of the polykaryotic nature of higher attine cultivars and whether there is a selective advantage to this phenomenon will be a valuable addition to our knowledge about fungal evolution. In addition to analyzing cultivar gene sequences in both genomes and metagenomes, I will also use microscopy to visualize the number of nuclei in each of these strains and will perform shotgun amplicon sequencing of single copy genes from these fungi to further measure any variability that exists.

4.4.3 CAZy comparison

To examine substrate specialization in the fungal genomes, I examined their biomass-degrading enzyme genes, through CAZy annotations (Figure 4.4). The *L. gongylophorus* AB2, *M. fuliginosa*, and *L. gongylophorus* genomes contained 7484, 7956 and 167 CAZy genes, respectively. This discrepancy in numbers of CAZy genes cannot be attributed to an evolutionary transition between the fungal strains, however, and is more likely an artifact of the highly fragmented nature of the *L. gongylophorus* AC genome (Table 4.2). Despite the differences in the total number of CAZy genes, the relative proportions of the groups are similar between the genomes. The largest group, the glycoside hydrolases comprise 44-53% of the total CAZy genes. This is followed by the auxiliary activities group at 16-25%, the glycosyltransferases at 9-14%, the carbohydrate-binding molecules at 7-13%, the carbohydrate esterases at 5-7%, and the polysaccharide lyases at 3-5%.

If particular strains of fungi are adapted to one substrate or another, we would expect there to be a difference in the presence/absence and abundance of particular types of CAZy genes. Substrate specialization has been demonstrated in other fungi, in both cases in the transition to grass-deconstruction (Cooper *et al.* 1988; Wolfe *et al.* 2012). Comparison of

CAZy annotations in the genomes of *L. gongylophorus* AB2, *L. gongylophorus* AC, and *M. fuliginosa*, present differences in the presence/absence of particular genes, but this is likely due to sequencing and annotation quality rather than biology. To demonstrate fungal substrate specialization through genomics, genomes of comparable quality are necessary.

4.4.4 Genome evolution and further work

While it appears that attine fungal cultivars have expanded genomes due to polykaryoty, the numbers of genes they encode are reduced compared to the most closely related free-living fungi that have had their genomes sequenced (Table 4.1). Previous work demonstrated a reduction in biomass degradation genes as fungi transitioned from free-living saprophytes to mycorrhizal mutualists (Kohler *et al.* 2015). In this case, the transition from free-living to mutualist is not associated with a loss of ability to break down plant biomass. Consequently, I do not expect that the cultivar will have a reduction in plant biomass degrading genes, but that there would be some specialization. Indeed, the CAZy profiles of *L. gongylophorus* AB2 and *M. fuliginosa* are similar. With the sequencing of additional strains I will be able to further investigate where the gene loss has occurred through the *L. gongylophorus* genome, and what differences exist between grass- and dicot-cutter ant fungal cultivars, if any. Further, I will be able to investigate the transition to grass-deconstruction in independent lineages of fungi, in comparing grass pathogen specialists (King *et al.* 2011) and grass saprophyte specialists (Wolfe *et al.* 2012), to see if there is genome convergence in fungi that consume grass.

4.6 Acknowledgements

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Table 4.1 *Leucoagaricus gongylophorus* isolate collection and sequencing information

Isolate	GPS coordinates	Date collected	Sequencing technology	Ant species
AB1	-22.84652, -48.43411	11/21/2015	Illumina	*A. bisphaerica*
AB2*	-22.84693, -48.43404	11/21/2015	PacBio	*A. bisphaerica*
AC1	-22.84642, -48.43427	11/21/2015	Illumina	*A. capiguara*
AC2	-22.9091, -48.30769	11/28/2015	Illumina	*A. capiguara*
AL1	-21.1675, -47.84638	12/05/2015	Illumina	*A. laevigata*
AL2	-22.84607, -48.43339	11/21/2015	PacBio	*A. laevigata*
AS1	-21.16337, -47.8615	11/27/2014	Illumina	*A. sexdens*
AS2	-21.16406, -47.8571	11/29/2014	PacBio	*A. sexdens*

* As of writing, AB2 is the only isolate from this project that has been sequenced, assembled, and annotated.

Table 4.2 Genome assembly statistics for three fungal genomes, *L. gongylophorus* isolated from *A. bisphaerica* and *A. capiguara* fungus gardens, as well as a closely-related free-living fungus *M. fuliginosa*.

Genome Assembly	*L. gongylophorus* AB2	*L. gongylophorus* AC	*M. fuliginosa*
Sequencing technology	PacBio	454	Illumina
Assembly date	2017	2012	2013
Genome Assembly size (Mbp)	72	101.58	46.4
Sequencing read coverage depth	222.02x	15.0x	165.4x
# of contigs	1765	92785	4852
# of scaffolds	1765	92785	3478
% of scaffolds >= 2Kbp	94.8%	13.0%	62.8%
Scaffold N50*	150	13933	199
Scaffold L50 (Mbp)	0.14	0	0.05
# of gaps	0	0	1374
% of scaffold length in gaps	0.00%	0.00%	1.60%
Three largest Scaffolds (Mbp)	0.86, 0.71, 0.70	0.10, 0.06, 0.03	0.63, 0.54, 0.45
# of gene models	8915	5420	15801

*JGI defines N50 as the number of scaffolds that constitute 50% of the assembly

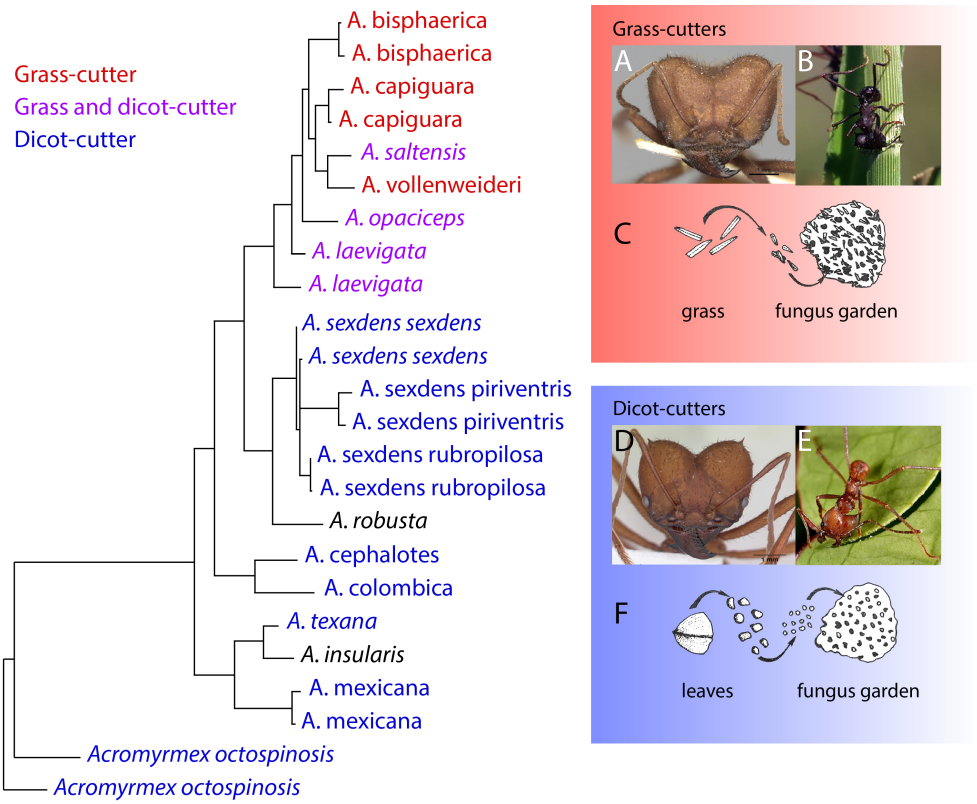

Figure 4.1 Maximum likelihood phylogeny of *Atta* species based on mtDNA and nDNA, adapted from Bacci *et al.* (2009). Colors of names of species indicate the type of substrate they forage. Black - unknown, red - dicots, blue - grasses, and purple - dicot/grasses. (A) *A. capiguara*, a grass-cutter has short, massive mandibles. (B) It orients downward and cuts in one direction. (C) Substrate preparation is simpler in grass-cutters – they do not form a pulp (D) *A. sexdens*, a dicot-cutter, has longer mandibles. (E) It pivots on its metathoracic legs and uses its mandibles like scissors. (F) Dicot-cutters pulp their leaves before incorporating them into the fungus garden. Figures modified from Fowler et al. 1986. Photographs from antweb.org: (A) April Nobile and (D) Shannon Hartman. (B) Dong Lin and (E) BBC.

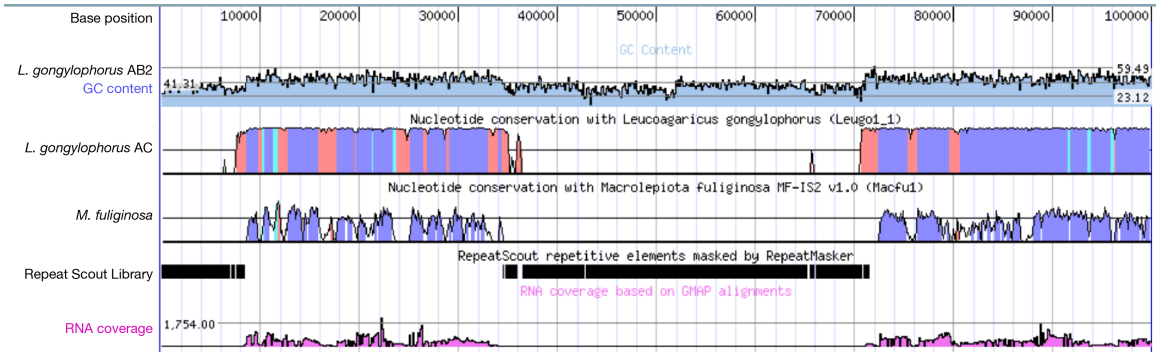

Figure 4.2 Nucleotide conservation between genomes of *L. gongylophorus* AB2, *L. gongylophorus* AC and *M. fuliginosa*, from the JGI's Mycobank genome browse tool. The top line is a graphical representation of the entire *L. gongylophorus* AB2 genome and its GC content, with the next two lines representing how other genomes are conserved in relation to it. This figure also depicts the Repeat Scout Library and RNA coverage based on GMAP. It is notable that the *L. gongylophorus* AB2 genome, sequenced with PacBio technology, shows non-coding repeat regions while the other two genomes displayed here do not.

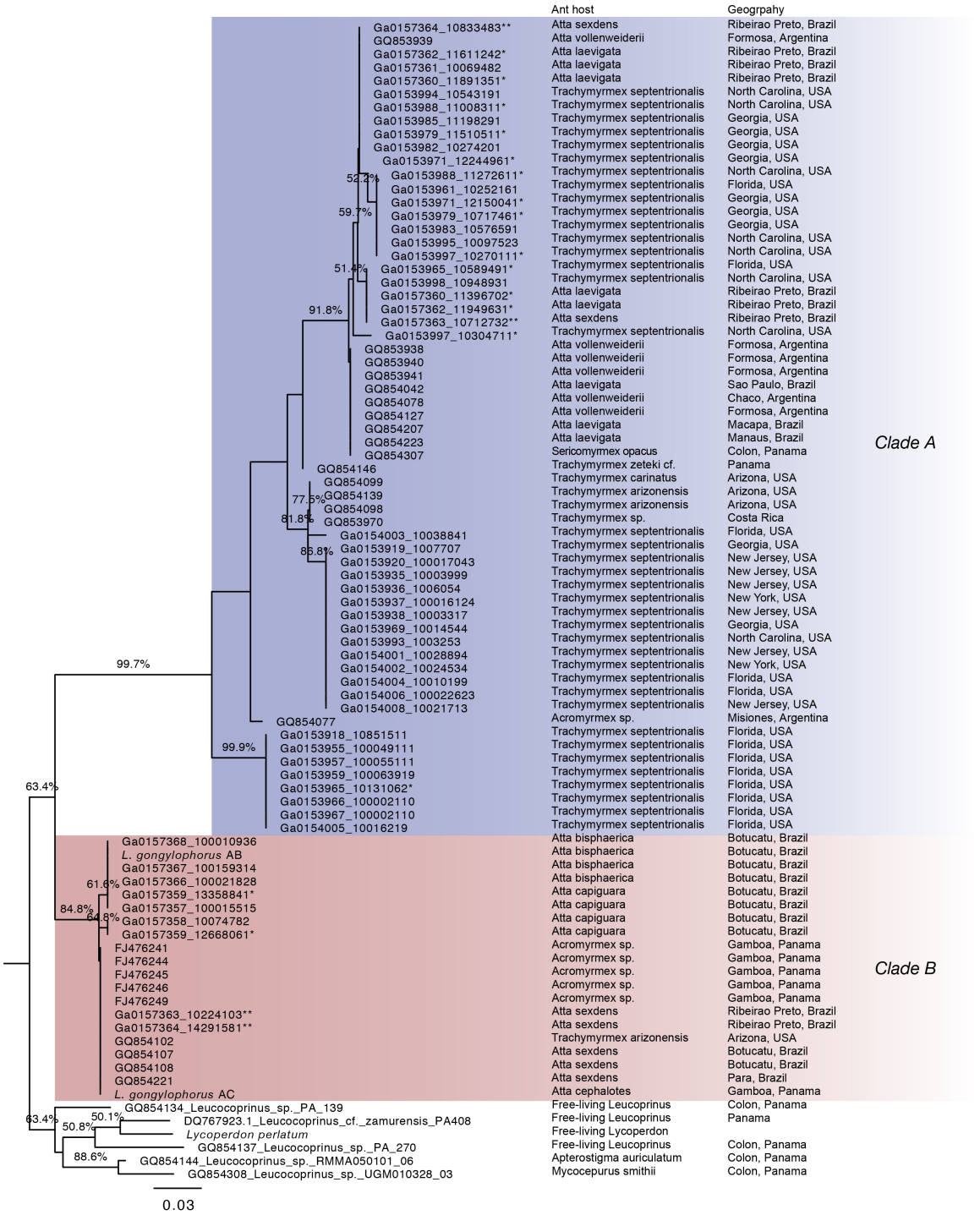

Figure 4.3 Maximum likelihood phylogeny constructed from the EF1-α gene. Bootstrap values above 50% are displayed on branches. Tree leaves with NCBI accession numbers starting with GQ or FJ were previously sequenced amplicons from (Mueller *et al.* 2017) and (Poulsen *et al.* 2009), respectively. NCBI accession numbers starting with Ga are extracted from attine fungus garden metagenomes stored on JGI's Integrated Microbial Genomes and Microbiomes online database. Sequences represented with fungal species names are EF1-α sequences from fungal genomes stored in the JGI Mycobank. A single asterisk (*) denotes different EF1-α sequences from the same metagenome that appear in the same large clade, and double asterisks (**) denote different EF1-α sequences from the same metagenome that appear in a different large clade.

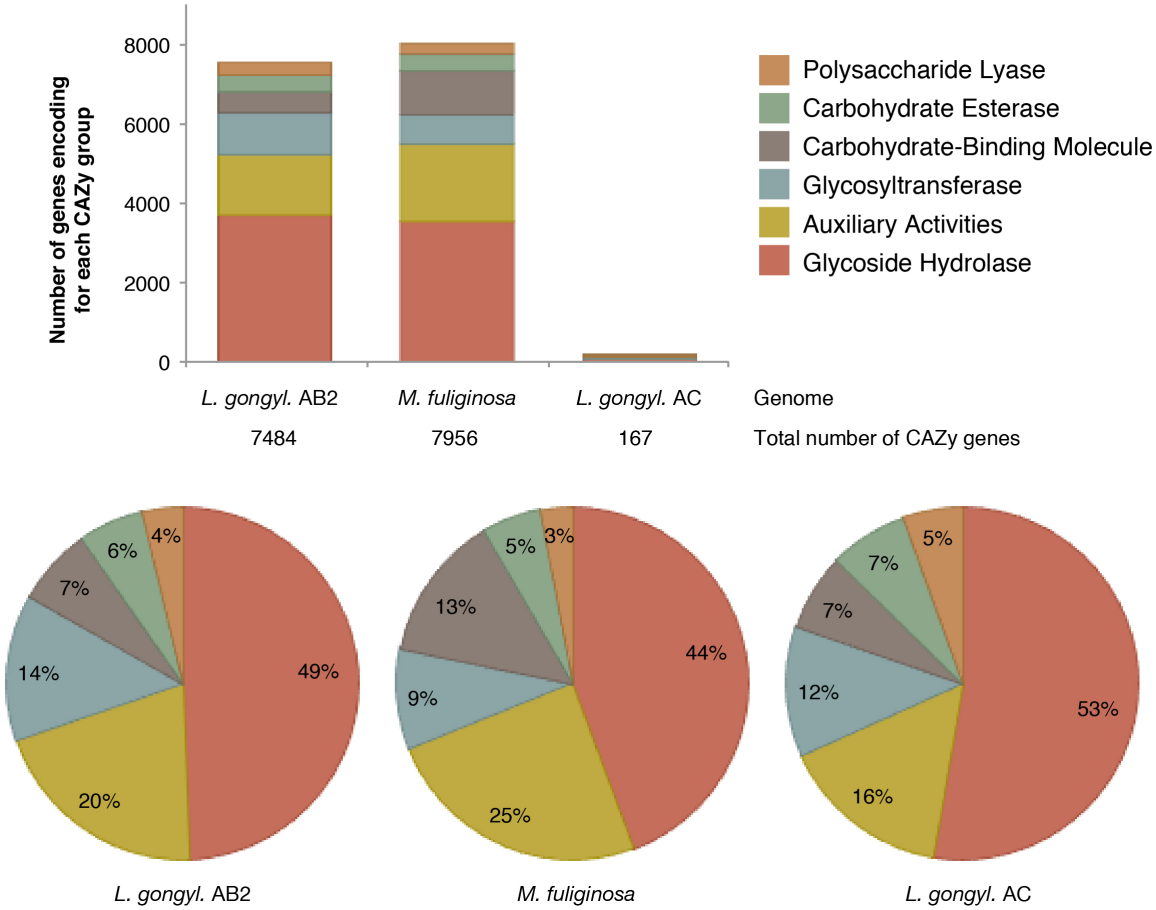

Figure 4.4 These graphs represent the number of CAZy genes found in each of the fungal genomes in this study, with bar graphs and pie charts showing absolute numbers and relative numbers of genes, respectively. While the *L. gongylophorus* AC genome contains far fewer total CAZy genes, likely due to quality of sequencing, assembly and annotation, the proportions of each group are similar to the two other fungal genomes.

4.7 References

Bacci M Jr, Solomon SE, Mueller UG *et al.* (2009) Phylogeny of leafcutter ants in the genus *Atta* Fabricius (Formicidae: Attini) based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, **51**, 427–437.

Belt T (1874) *The naturalist in Nicaragua*. E.P. Dutton & Co., New York.

Book AJ, Lewin GR, McDonald BR *et al.* (2014) Cellulolytic Streptomyces strains associated with herbivorous insects share a phylogenetically linked capacity to degrade lignocellulose. *Applied and Environmental Microbiology*, **80**, 4692–4701.

Book AJ, Lewin GR, McDonald BR *et al.* (2016) Evolution of high cellulolytic activity in symbiotic Streptomyces through selection of expanded gene content and coordinated gene expression. *PLoS Bology*, **14**, e1002475.

Brudno M, Do CB, Cooper GM *et al.* (2003) LAGAN and Multi-LAGAN: efficient tools for large-scale multiple alignment of genomic DNA. *Genome Research*, **13**, 721–731.

Cherrett JM, Peregrine DJ (1976) A review of the status of leaf-cutting ants and their control. *Annals of Applied Biology*, **84**, 124–128.

Cooper RM, Longman D, Campbell A, Henry M, Lees PE (1988) Enzymic adaptation of cereal pathogens to the monocotyledonous primary wall. *Physiological and Molecular Plant Pathology*, **32**, 33–47.

Couronne O, Poliakov A, Bray N *et al.* (2003) Strategies and Tools for Whole-Genome Alignments. *Genome Research*, **13**, 73–80.

Danforth BN, Ji S (1998) Elongation factor-1 alpha occurs as two copies in bees: implications for phylogenetic analysis of EF-1 alpha sequences in insects. *Molecular Biology and Evolution*, **15**, 225–235.

Ding S-Y, Himmel ME (2008) Anatomy and ultrastructure of maize cell walls: An example of energy plants. In: *Biomass Recalcitrance: Deconstructing the Plant Cell Wall for Bioenergy* (ed Himmel ME), pp. 38–60. Blackwell Publishing Ltd, Oxford, UK.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

Fowler H, Forti L, Pereira-da-Silva V, Saes N (1986) Economics of grass-cutting ants. In: *Fire ants and leaf-cutting ants* (eds Lofgren CS, Vander Meer RK), pp. 18–35. Westview Pr, Boulder, CO.

Gordon D, Huddleston J, Chaisson MJP *et al.* (2016) Long-read sequence assembly of the gorilla genome. *Science*, **352**, aae0344–aae0344.

Jurka J, Kapitonov VV, Pavlicek A *et al.* (2005) Repbase Update, a database of eukaryotic repetitive elements. *Cytogenetic and Genome Research*, **110**, 462–467.

King BC, Waxman KD, Nenni NV *et al.* (2011) Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. *Biotechnology for biofuels*, **4**, 4–18.

Kohler A, Kuo A, Nagy LG *et al.* (2015) Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nature Genetics*, **47**, 410–415.

Kooij PW, Aanen DK, Schiøtt M, Boomsma JJ (2015) Evolutionarily advanced ant farmers

rear polyploid fungal crops. *Journal of Evolutionary Biology*, **28**, 1911–1924.

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, **33**, 1870–1874.

Möller EM, Bahnweg G, Sandermann H, Geiger HH (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research*, **20**, 6115.

Mueller UG, Ishak HD, Bruschi SM *et al.* (2017) Biogeography of mutualistic fungi cultivated by leafcutter ants. *Molecular Ecology*, **26**, 6921–6937.

Poulsen M, Fernández-Marín H, Currie CR, Boomsma JJ (2009) Ephemeral windows of opportunity for horizontal transmission of fungal symbionts in leaf-cutting ants. *Evolution*, **63**, 2235–2247.

Price AL, Jones NC, Pevzner PA (2005) De novo identification of repeat families in large genomes. *Bioinformatics (Oxford, England)*, **21**, i351–i358.

Robinson SW, Fowler HG (2009) Foraging and pest potential of Paraguayan grass-cutting ants (*Atta* and *Acromyrmex*) to the cattle industry. *Zeitschrift für Angewandte Entomologie*, **93**, 42–54.

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences*, **105**, 5435–5440.

Schultz TR, Sosa-Calvo J, Brady SG *et al.* (2015) The most relictual fungus-farming ant species cultivates the most recently evolved and highly domesticated fungal symbiont species. *The American naturalist*, **185**, 693–703.

Smit AFA, Hubley R, Green P *RepeatMasker*.

Stielow JB, Levesque CA, Seifert KA *et al.* (2015) One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia - Molecular Phylogeny and Evolution of Fungi*, **35**, 242–263.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

Tisserant E, Malbreil M, Kuo A *et al.* (2013) Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *PNAS*, **110**, 20117–20122.

Wetterer J (1998) Phylogeny of fungus-growing ants (tribe Attini) based on mtDNA sequence and morphology. *Molecular Phylogenetics and Evolution*, **9**, 42–47.

Wolfe BE, Kuo M, Pringle A (2012) *Amanita thiersii* is a saprotrophic fungus expanding its range in the United States. *Mycologia*, **104**, 22–33.

Chapter 5: Summary and future directions

In this dissertation I explored the microbial mediation of herbivory in leaf-cutter ant fungus gardens. I built upon a field that has been studied in depth for many years but using novel methods with which I could answer new questions.

In chapter 1 I introduced the topic of the microbial mediation of herbivory and how it pertains to what we know about leaf-cutter ants. In chapter 2 I was able to begin answering how leaf-cutter ants are able to incorporate such a wide diversity of plants into their gardens, namely because their fungal cultivar responds in a flexible, substrate-specific manner. In chapter 3 I looked more closely at the bacterial community in the fungus gardens and provided further evidence for the mutualistic role of the bacteria, in this case, in helping the ants to transition to using grasses, a nutrient-poor substrate. In chapter 4 I present preliminary data comparing genomes of fungal cultivars from ants that are adapted to cutting grasses and dicots. In the appendix I present two more projects that I have contributed to that add to our knowledge about the functioning of leaf-cutter ant fungus gardens. Appendix 2 shows that fungal gongylidia contain an enrichment of biomass-degrading enzymes, which are deposited on fresh plant material through the ants' fecal droplets and serve as a pretreatment for biomass degradation in the gardens. In appendix 3 I show changes in the abundance of various lipids from leaf material through the fungus garden and into the gongylidia, where the ants can use them for energy and nutrients.

Of course, this thesis is only a step toward a greater understanding of how fungus and bacteria in leaf-cutter ant fungus gardens help the ants to use plants as a food source. We have not yet been able to obtain a thorough real-time picture of what the bacterial activity *in situ* since our attempts at targeting bacteria with metatranscriptomics and metaproteomics have not been successful. A more targeted approach, likely using Q-PCR,

combined with culture assays, would reveal how bacteria respond to various types of plant biomass, with different recalcitrance, different nutritive quality and different plant defense compounds. Using these methods we will be able to better understand the role of bacteria in the fungus gardens.

One clear future direction we will take is in the examination of the genomes of fungal cultivars. Already with the work presented in chapter 4, we can see that with the added genomes, we will be able to explore the unusual phenomenon of polykaryoty in these fungi. We will also be able to compare this group to other fungi that have transitioned from free-living to mutualist, and compare this group to others that have transitioned from degrading one type of substrate to another. All three of these will not only help us to better understand the biology of leaf-cutter ants and their fungal cultivar, but they will also expand our knowledge of fungal evolution and genomics more generally.

Appendix 1: Supplemental Materials for Chapter 2

A1.1 Methods

A1.1.1 Preparation of protein samples for mass spectrometry analyses

Frozen fungus was weighed to 1 g and placed into an ice-cold mortar and pestle and ground for 5 min with hydrated polyvinylpolypyrrolidone (PVPP). The ground sample was then placed in 10 mL of ice-cold phenol extraction buffer (0.7 M sucrose; 0.1 M KCl; 0.5 MTris-HCl, pH 7.5 and 50 mM EDTA (2% (vol/vol), β-mercaptoethanol, 1mM PMSF added directly before use)) and phenol extracted as described previously[1]. Briefly, 10 mL of phenol saturated with Tris-HCL, pH 7.5 was added to the sample and shaken at 4°C for 30 min and centrifuged at 5,000g for 30 min at 4°C. The upper phenolic phase was collected and again extracted using an equal volume of ice-cold phenol extraction buffer, repeated twice. The protein was then precipitated from the collected phenol phase with 5 volumes of cold 0.1 M ammonium acetate in methanol. The protein flakes were collected by centrifuging at 5,000g for 30 min at 4°C followed by two 100% methanol washes and allowed to dry. The protein pellet was then resuspended in 8 M urea, 100 mM NH_4HCO_3 pH 8 and assayed with Bicinchoninic acid (BCA) (Thermo Scientific, Rockford, IL) to determine the protein concentration. Proteins were reduced with 10 mM dithiothreitol (DTT) for 30 min at 60°C, with constant shaking at 800 rpm. Denatured and reduced samples were diluted 10-fold with 50 mM ammonium bicarbonate pH 7.8, and $CaCl_2$ was added to a final concentration of 1 mM prior to enzymatic digestion. Sequencing-grade modified trypsin was activated by incubating for 10 min at 37˚C. Activated trypsin was then added to the samples at 1:50 (w/w) trypsin-to-protein ratio, and samples were digested at 37˚C for 3 h with constant shaking at 800 rpm; reactions were quenched by rapid freezing in liquid nitrogen. Digested

samples were desalted using a 4-probe positive pressure Gilson GX-274 ASPEC™ system (Gilson Inc., Middleton, WI) with Discovery C18 100 mg/1 mL solid phase extraction tubes (Supelco, St.Louis, MO), using the following protocol: 3 mL of methanol was added for conditioning followed by 2 mL of 0.1% TFA in H_2O. The samples were then loaded onto each column followed by 4 mL of 95:5: H_2O:ACN, 0.1% TFA. Samples were eluted with 1mL 80:20 ACN:H_2O, 0.1% TFA. The samples were concentrated down to ~30 μL using a Speed Vac and a final BCA was performed to determine the peptide concentration. The samples were then individually vialed for mass spectrometry analysis.

A1.1.2 High pH RP C-18 Fractionation for accurate mass and time (AMT) tag database creation

To provide comprehensive coverage of the proteome, aliquots of individual sample protein digests were pooled and subjected to high pH reversed-phase liquid chromatography fractionation. All samples were equally pooled into one tube to a final mass of 300 μg and diluted to a volume of 900 μL with 10 mM ammonium formate buffer (pH 10.0), and resolved on a XBridge C18, 250x4.6 mm, 5 μM with 4.6x20 mm guard column (Waters, Milford, MA). Separations were performed at 0.5 mL/min using an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) with mobile phases (A) 10 mM Ammonium Formate, pH 10.0 and (B) 10 mM Ammonium Formate, pH 10.0/acetonitrile (10:90). The gradient was adjusted from 100% A to 95% A over the first 10 min, 95% A to 65% A over minutes 10 to 70, 65% A to 30% A over minutes 70 to 85, maintained at 30% A over minutes 85 to 95, re-equilibrated with 100% A over minutes 95 to 105, and held at 100% A until minute 120. Fractions were collected every 1.25 minutes (96 fractions over the entire gradient). The plate was partially dried in a speed vac and then every 24th fraction was combined for a total of 24 samples (each with n=4 fractions pooled) each with 50%

acetonitrile rinsing. The fractions were then completely dried down and 25 μL of 25 mM ammonium bicarbonate was added to each fraction for storage at -20°C until mass spectrometry analysis.

A1.2 Supplemental References

1. Isaacson, T., et al., *Sample extraction techniques for enhanced proteomic analysis of plant tissues.* Nat Protoc, 2006. **1**(2): p. 769-74.

Appendix 2: Supplemental Materials for Chapter 3

A2.1 Supplemental Figures

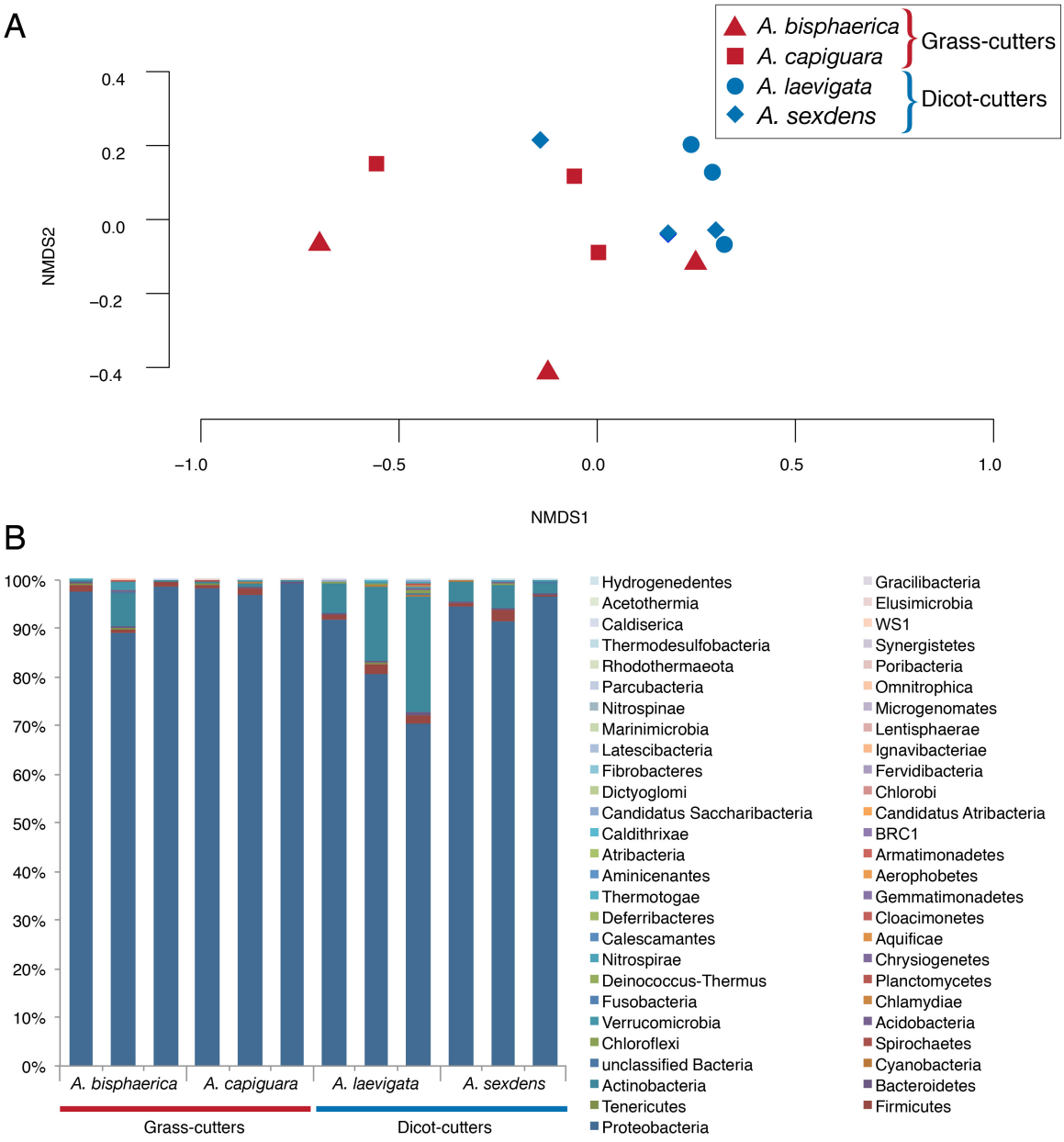

Figure A2.1 The bacterial communities do not differ significantly at the phylum level. A. NMDS plot of phylum-level differences in the bacterial communities in fungus gardens of grass- and dicot-cutter ants. B. Bar plots showing the relative abundance of different bacterial phyla, dominated by Proteobacteria.

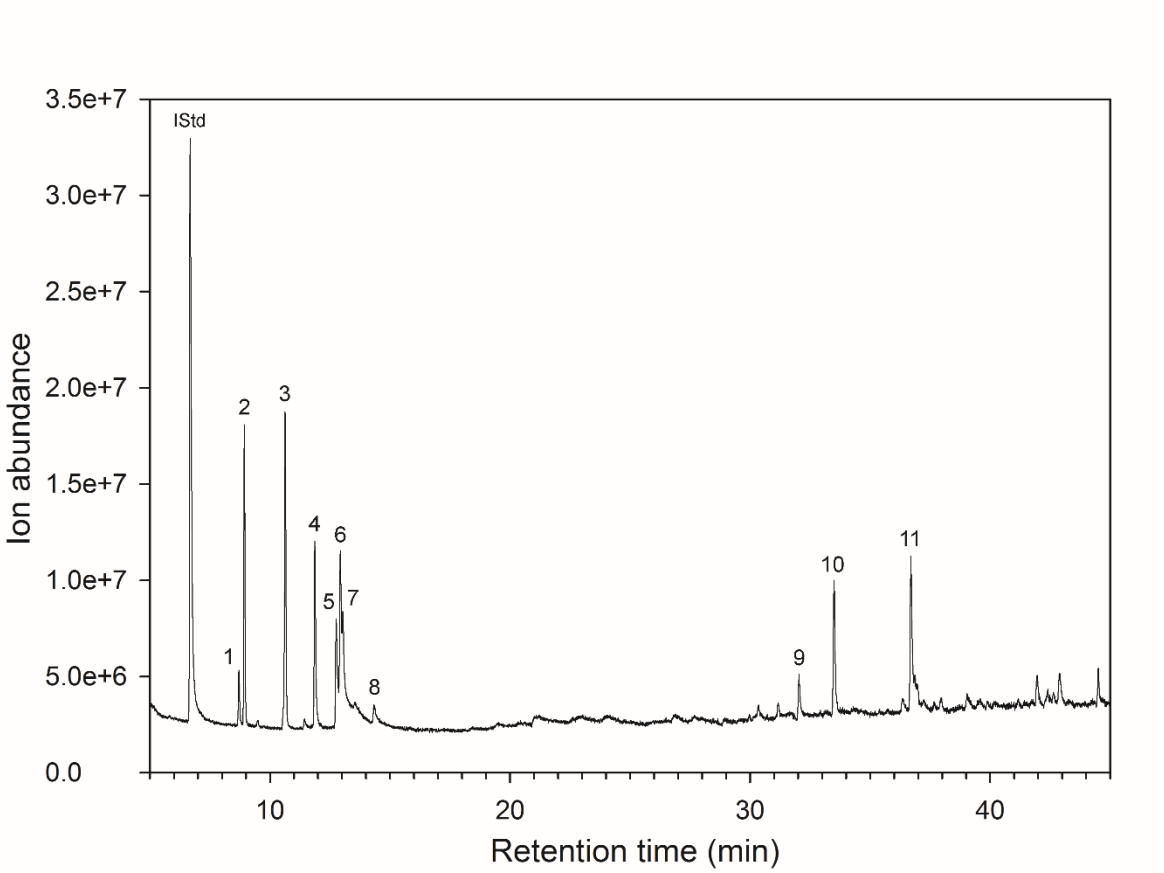

Figure A2.2 GC-MS total ion chromatogram of terpenes extracted from *Atta laevigata* (1) fungus garden material. IStd = internal standard (*m*-xylene), 1 = α-thujene, 2 = α-pinene, 3 = β-pinene, 4 = α-phellandrene, 5 = *p*-cymeme, 6 = limonene, 7 = eucalyptol (1,8-cineole), 8 = γ-terpinene, 9 = alloaromadendrene, 10 = unknown sesquiterpene, 11 = caryophyllene oxide. Terpenes were identified by both their mass spectra and retention indices calculated with an *n*-alkane series. No other samples presented identifiable peaks.

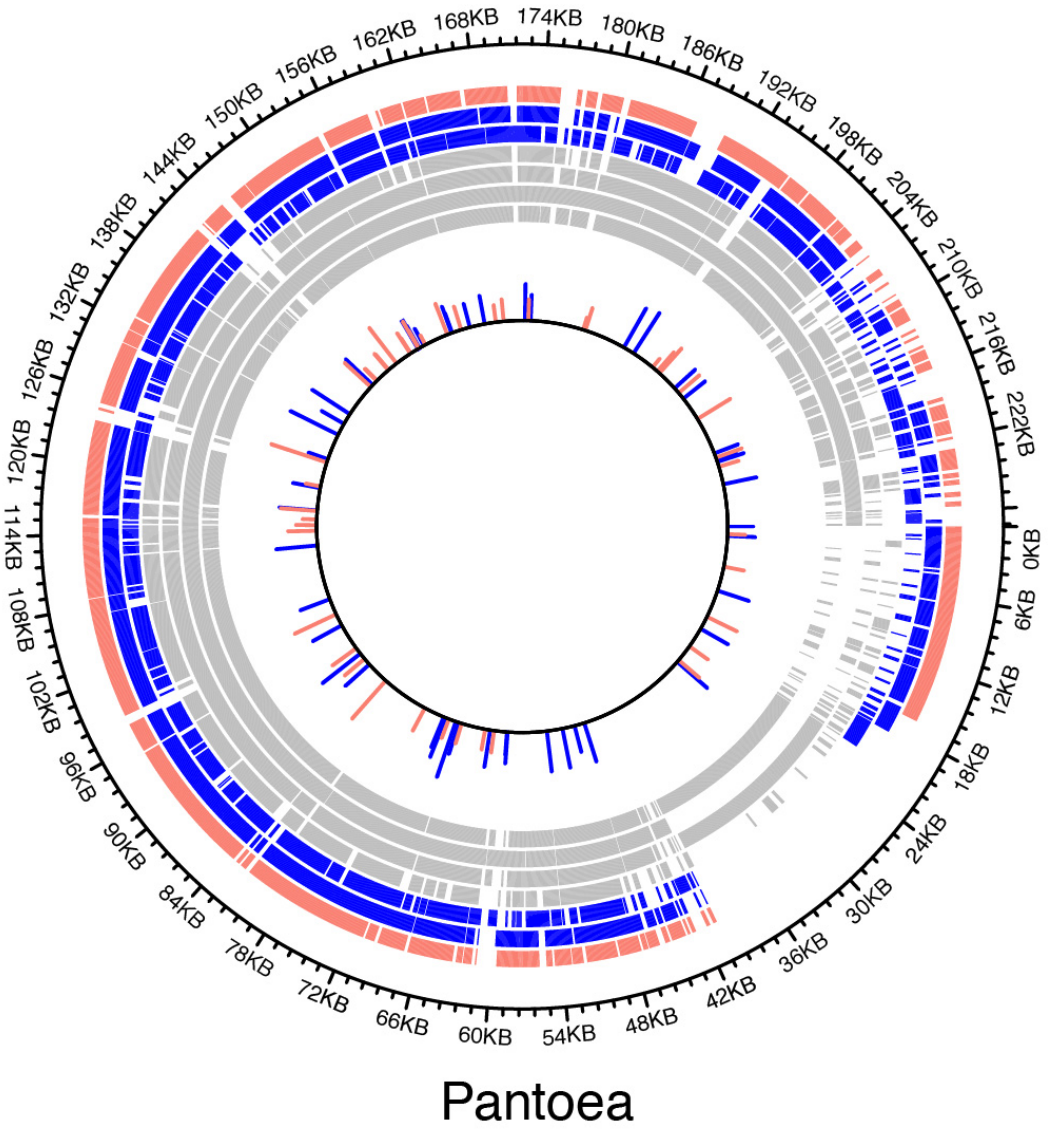

Figure A2.3 Pangenome of *Pantoea* composite genomes. The outermost band is a count of all kilobases represented in at least two out of seven *Pantoea* genomes, represented in the next severn rings. The red circle is from a grass-cutter ant fungus garden (*A. capigura* 2) the two blue genomes are from dicot-cutter ant fungus gardens (*A. laevigata* 2 and *A. sexdens* 1) and the next four grey genomes are from isolates of *Pantoea* (Accession numbers: SAMN08357569, SAMN08357571, SAMN08357570, and SAMN00203991). The inner ring represents genes that are significantly different between grass- and dicot-cutter ant fungus gardens, and are found in the *Pantoea* pangenome, but are not exclusive to it. Red and blue lines in the inner ring represent genes that are significantly more abundant in the grass- and dicot-cutter ant fungus gardens, respectively. Their lengths are proportional to their log2 fold abundance.

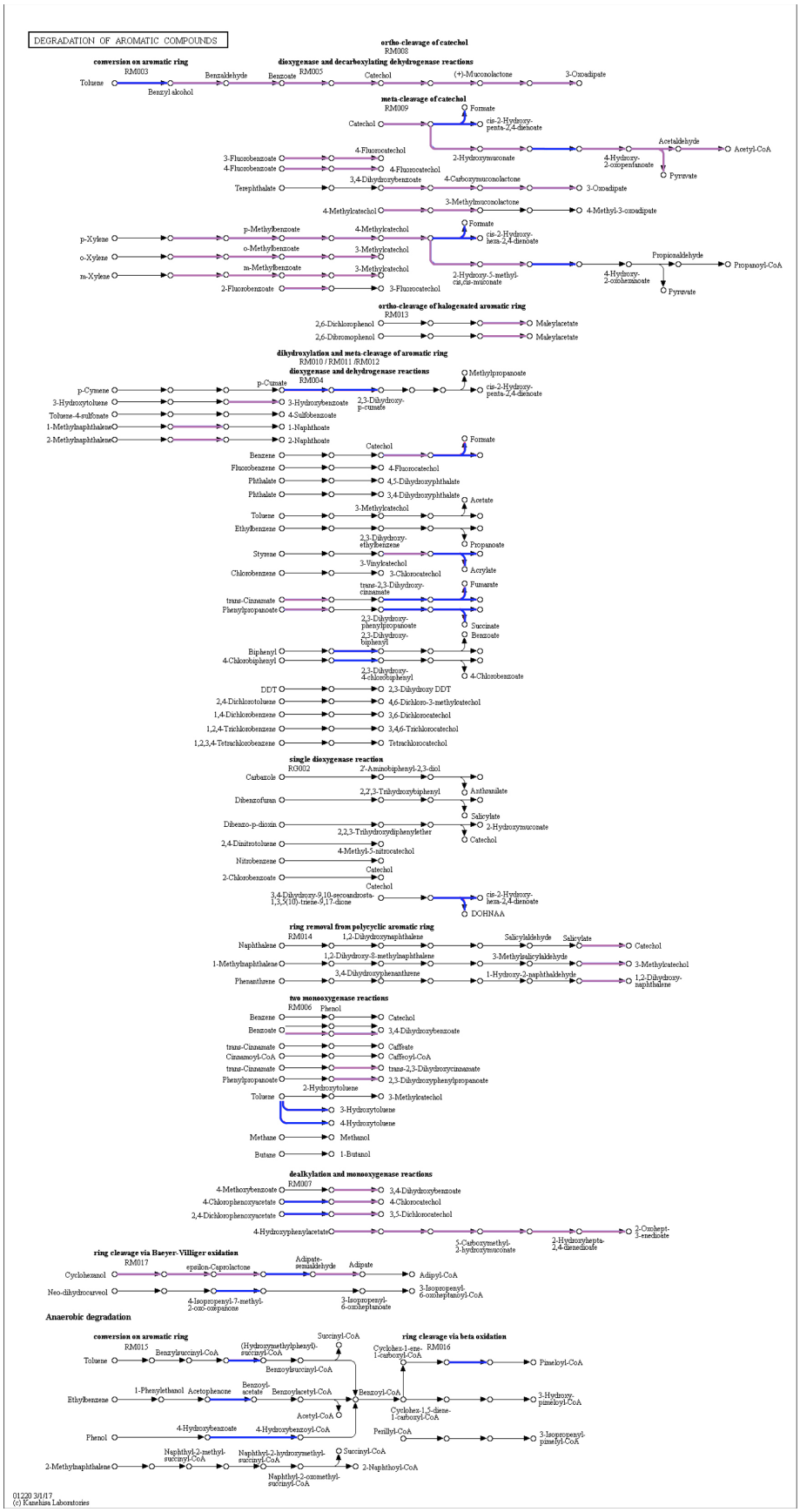

Figure A2.4 KEGG pathways involved in the degradation of aromatic compounds. Blue arrows indicate genes that are present in dicot-cutter ant fungus gardens, red arrows indicate genes that are present in grass-cutter ant fungus gardens and purple arrows indicate genes that are present in both.

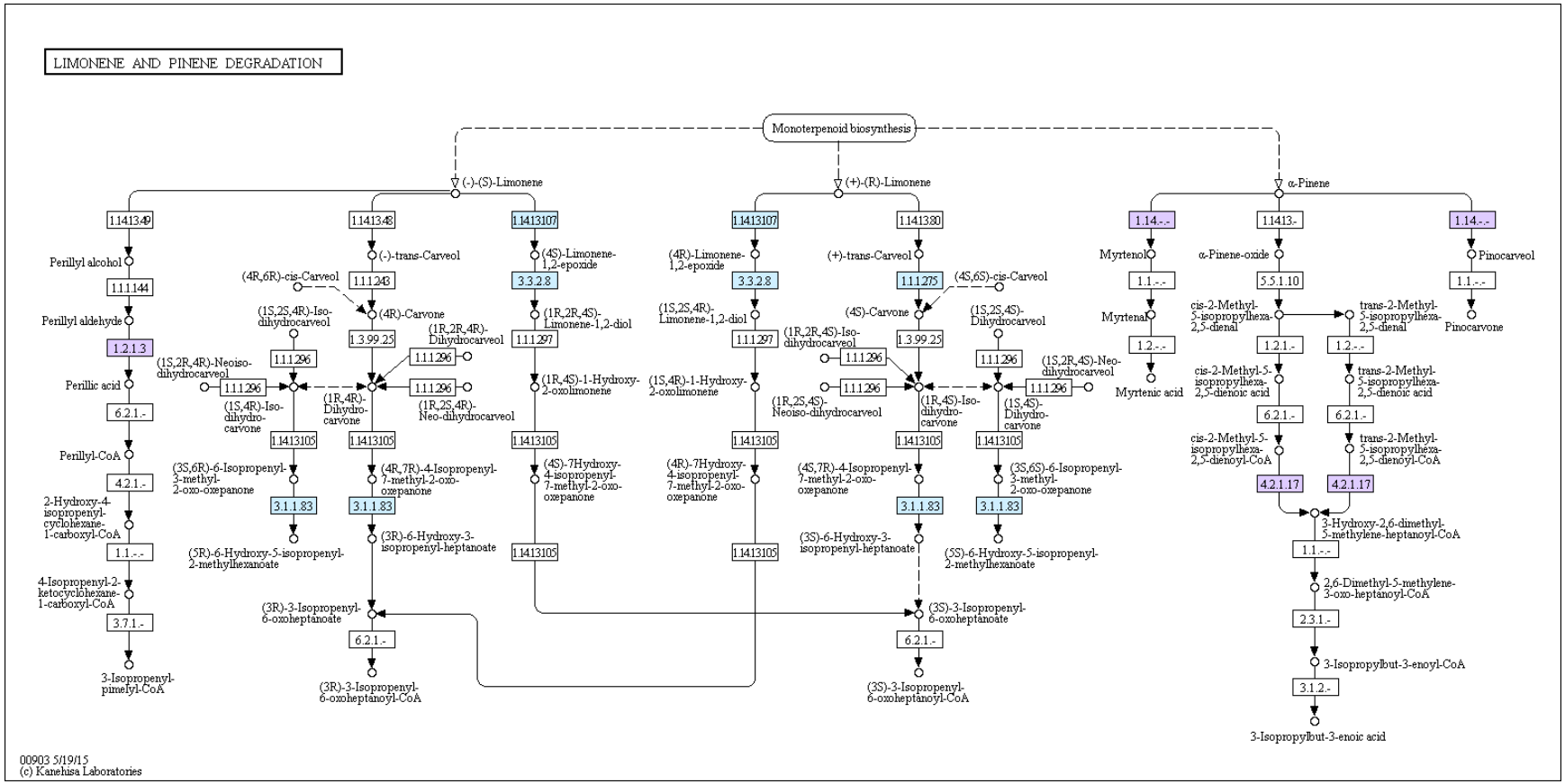

Figure A2.5 KEGG pathways involved limonene and pinene degradation. Blue boxes indicate genes that are present in dicot-cutter ant fungus gardens, red boxes indicate genes that are present in grass-cutter ant fungus gardens and purple boxes indicate genes that are present in both.


ABC TRANSPORTERS
Prokaryotic-type ABC transporters
Mineral and organic ion transporters
Sulfate CysP CysU CysW CysA
Tungstate TupA TupB TupC
Molybdate / Tungstate WtpA WtpB WtpC
Nitrate / Nitrite / Cyanate NrtA NrtB NrtC NrtD
Bicarbonate CmpA CmpB CmpC CmpD
Taurine TauA TauC TauB
Alkanesulfonate SsuA SsuC SsuB
HMP / FAMP ThiY ThiX ThiZ
Phthalate OphF OphG OphH
Molybdate ModA ModB ModC ModF
Iron (III) AfuA AfuB AfuC
Thiamin TbpA ThiP ThiQ
Spermidine / Putrescine PotD PotC PotB PotA
Putrescine PotF PotI PotH PotG
Mannopine AttC AttB AttA2 AttA1
2-Aminoethylphosphonate PhnS PhnV PhnU PhnT
Glycine betaine / Proline ProX ProW ProV
Osmoprotectant OpuBC OpuBB OpuBA
Oligosaccharide, polyol, and lipid transporters
Maltose / Maltodextrin MalE MalF MalG MalK
Galactose oligomer / Maltooligosaccharide GanQ GanP GanQ MsmX
Raffinose/ Stachyose/ Melibiose MsmE MsmF MsmG MsmK
Lactose /L-arabinose LacE LacF LacG LacK
Sorbitol / Mannitol SmoE SmoF SmoG SmoK
α-Glucoside AglE AglF AglG AglK
Oligogalacturonide TogB TogM TogN TogA
α-1,4-Digalacturonate AguE AguF AguG ?
Aldouronate LplA LplB LplC ?
Trehalose / Maltose ThuE ThuF ThuG ThuK
Trehalose TreS TreT TreU TreV
N-Acetylglucosamine NgcE NgcF NgcG ?
Cellobiose CebE CebF CebG MsiK
Chitobiose DasA DasB DasC MsiK
Chitobiose ChiE ChiF ChiG ?
Arabinooligosaccharide AraN AraF AraQ MsmX
Xylobiose BxlE BxlF BxlG ?
Multiple sugar? ClwE GguB GguA
Phospholipid MlaC MlaD MlaE MlaF MlaB
Phosphate and amino acid transporters
Phosphate PstS PstC PstA PstB
Phosphonate PhnD PhnE PhnC
Lysine / Arginine / Ornithine ArgT HisM HisQ HisP
Histidine HisJ HisM HisQ HisP
Glutamine GlnH GlnP GlnQ
Glutamine? GlnH GlnP GlnQ
Arginine ArtJ ArtI ArtM ArtQ ArtP
Glutamate / Aspartate GltI GltK GltJ GltL
Octopine / Nopaline OccT OccM OccQ OccP
General L-Amino acid AapJ AapQ AapM AapP
Glutamate GluB GluC GluD GluA
Cystine FliY YecS YecC
Cystine TcyJ TcyK TcyL TcyM TcyN
Arginine / Ornithine AotJ AotM AotQ AotP
Arginine / Lysine / Histidine / Glutamine BgtB BgtA
Lysine LysX LysX LysY
Branched-chain amino acid LivK LivH LivM LivG LivF
Neutral amino acid / Histidine NatB NatC NatD NatA NatE
Urea UrtA UrtB UrtC UrtD UrtE
D-Methionine MetQ MetI MetN
Amino acid YxeM YxeN YxeO
Peptide and nickel transporters
Oligopeptide OppA OppB OppC OppD OppF
Dipeptide / Heme / 5-Aminolevulinic acid DppA DppB DppC DppD DppF
Dipeptide DppE DppB DppC DppD
Cationic peptide SapA SapB SapC SapD SapF
Nickel NikA NikB NikC NikD NikE
Glutathione GsiB GsiC GsiD GsiA
Microcin C YejA YejB YejE YejF
Metallic cation, iron-siderophore and vitamin B12 transporters
Iron complex FhuD FhuB FhuC
Vitamin B12? BtuF BtuC BtuD
Manganese MntC MntB MntA
Zinc ZnuA ZnuB ZnuC
Iron (II, III) / Copper / Manganese / Zinc MtsA MtsC MtsB
Iron (II) / Manganese SitA SitC SitD SitB
Manganese / Zinc PsaA PsaC PsaB
Manganese / Zinc MntC MntB MntA
Zinc / Manganese / Iron (II) TroA TroC TroD TroB
Cobalt CbiN CbiM CbiQ CbiO
Nickel CbiK CbiN/L CbiM CbiQ CbiO
Biotin BioY BioN BioM
Biotin BioY EcfT EcfA1 EcfA2
ABC-2 and other transporters
Hemolysin CylB CylA
Capsular polysaccharide KpsE KpsM KpsT
Lipopolysaccharide RfbA RfbB
Teichoic acid TagG TagH
Lipo-oligosaccharide NodJ NodI
Na+ NatB NatA
Hemine HrtB HrtA
Oleandomycin OleC5 OleC4
Bacitracin BcrB BcrA
Bacitracin BceB BceA
Lantibiotics NukE NukG NukF
Lantibiotics NisE NisG NisF
Lipoprotein LolC LolE LolD
Heme CcmD CcmC CcmB CcmA
Lipopolysaccharide LptF LptG LptB
Fluoroquinolones Rv2686c Rv2687c Rv2688c
YydF peptide YydJ YydI
ABC-2 -type components without transporting function
Bacitracin BraE BraD Bacitracin resistance
Cationic antimicrobial peptide (CAMP) VraG VraF CAMP resistance
NosY NosF Cu2+-processing for N2O reductase
FtsX FtsE Cellar division involvment
YtrF YtrC/D YtrB YtrE Acetoin utilization
Monosaccharide transporters
Glucose / Arabinose GlcS GlcU GlcT GlcV
Glucose / Mannose GtsA GtsB GtsC MalK
Ribose / Autoinducer 2 / D-Xylose RbsB RbsC RbsD RbsA Auxiliary component
L-Arabinose AraF AraH AraG
Methyl-galactoside MglB MglC MglA
D-Xylose XylF XylH XylG
D-Allose AlsB AlsC AlsA
Fructose FrcB FrcC FrcA
Autoinducer 2 LsrB LsrC LsrD LsrA
Rhamnose RhaS RhaP RhaQ RhaT
Erythritol EryG EryF EryE
Xylitol XltC XltB XltA
myo-Inositol IbpA IatP IatA
myo-Inositol 1-phosphate InoE InoF InoG InoK
Glycerol GlpV GlpP GlpQ GlpS GlpT
sn-Glycerol 3-phosphate UgpB UgpA UgpE UgpC
02010 7/20/16
(c) Kanehisa Laboratories

Figure A2.6 KEGG pathways involved in ABC transporters. Blue boxes indicate genes that are present in dicot-cutter ant fungus gardens, red boxes indicate genes that are present in grass-cutter ant fungus gardens and purple boxes indicate genes that are present in both.

A2.2 Supplemental methods

A2.2.1 Composite genomes

Composite genomes were created using the MaxBin program with default settings (Wu *et al.*, 2014). Their quality, contamination and completeness were assessed using CheckM (Parks *et al.*, 2015). Only composite genomes with less than 10% contamination and more than 90% completeness were included in further analysis. CheckM reports the taxonomy level of each composite genome based on the most recent common ancestor of the annotation of the core genes identified, thus not all of them were identified to an informative level. To further elucidate the taxonomy of each composite genome, they were annotated separately using Prokka (Seemann, 2014). If a 16S sequence was recovered from a composite genome, then that sequence was blasted in NCBI. The only group that we had clear taxonomic identification, and which was present in both grass- and dicot-cutter ant fungus gardens, was *Pantoea*. Pangenomic analysis was carried out including three composite *Pantoea* genomes and four published *Pantoea* genomes using GET_HOMOLOGUES (Contreras-Moreira and Vinuesa, 2013). 2450 genes were shared at least by two out of those seven genomes. Circle plots showing the presence and absence of those 2450 genes in each genome were created using the circlize package (Gu *et al.*, 2014) in R (R Core Team, 2013). Genes that were found to be significantly different between grass- and dicot-cutter ant fungus gardens were marked on the *Pantoea* pangenome suggesting the fold change.

A2.2.2 KEGG orthology pathways

To map out the completeness of various KEGG pathways, first presence/absence was determined for each KEGG gene. A gene was considered present in either grass or dicot-cutter ant fungus gardens if it was present in at least 4 of the 6 metagenomes in that category. Presence and absence in grass-cutter ant fungus gardens, dicot-cutter ant fungus gardens or both were plotted on KO pathways through the Kegg Orothology Database website (http://www.genome.jp/kegg/ko.html). The pathways are represented in Supplemental Figures 3.4-3.6.

A2.2.3 Gas chromatography

Fungus samples were collected from each of the twelve leaf-cutter ant colonies, then dried for 24 hours at 105°C. Samples were then crushed by mortar and pestle with liquid nitrogen. 100 mg of crushed material from each sample was weighed out and placed into 2.0 mL self-standing graduated microcentrifuge tubes.

To extract the compounds from the samples, 750 µl of methanol was added to each of the 100 mg of frozen powdered material. Each sample was briefly vortex mixed, sonicated in a chilled water bath for ten minutes, vortex mixed again, then centrifuged at 20,000 rpm for ten minutes. For the analysis of the volatile compounds, 1 µL of methanol extract from each the samples was placed individually into a coupled Trace 1310 gas chromatograph and Thermo ISQ mass spectrometer with electron ionization at 70 eV (GC-MS) machine with the injector temperature set to 260°C. Compounds were identified using both of their mass spectra compared to the NIST 2014 MS library, and using their linear retention indices calculated from separate injections of a hydrocarbon series (C_8-C_{20}).

A2.3 Supplemental References

Contreras-Moreira B, Vinuesa P. (2013). GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis. *Applied and Environmental Microbiology* **79**: 7696–7701.

Gu Z, Gu L, Eils R, Schlesner M, Brors B. (2014). *circlize* Implements and enhances circular visualization in R. *Bioinformatics (Oxford, England)* **30**: 2811–2812.

Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. (2015). CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research* **25**: 1043–1055.

R Core Team. (2013). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. http://www.R-project.org/ (Accessed December 23, 2013).

Seemann T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics (Oxford, England)* **30**: 2068–2069.

Wu Y-W, Tang Y-H, Tringe SG, Simmons BA, Singer SW. (2014). MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. *Microbiome* **2**: 26.

Appendix 3: Enrichment and Broad Representation of Plant Biomass-degrading Enzymes in the Specialized Hyphal Swellings of *Leucoagaricus gongylophorus*, the Fungal Symbiont of Leaf-Cutter Ants

This chapter has previously been published in its entirety in *PLoS ONE*, 10(8): e0134752. The Supplemental Table and Dataset can be found at https://doi.org/10.1371/journal.pone.0134752.

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A3.1 Abstract

Leaf-cutter ants are prolific and conspicuous constituents of Neotropical ecosystems that derive energy from specialized fungus gardens they cultivate using prodigious amounts of foliar biomass. The basidiomycetous cultivar of the ants, *Leucoagaricus gongylophorus*, produces specialized hyphal swellings called gongylidia that serve as the primary food source of ant colonies. Gongylidia also contain plant biomass-degrading enzymes that become concentrated in ant digestive tracts and are deposited within fecal droplets onto fresh foliar material as ants incorporate it into the fungus garden. Although the enzymes concentrated by *L. gongylophorus* within gongylidia are thought to be critical to the initial degradation of plant biomass, only a few enzymes present in these hyphal swellings have been identified. Here we use proteomic methods to identify proteins present in the gongylidia of three *Atta cephalotes* colonies. Our results demonstrate that a diverse but consistent set of enzymes is present in gongylidia, including numerous plant biomass-degrading enzymes likely involved in the degradation of polysaccharides, plant toxins, and proteins. Overall, gongylidia contained over three-quarters of all biomass-degrading enzymes identified in the *L. gongylophorus* genome, demonstrating that the majority of the enzymes produced by this fungus for biomass breakdown are ingested by the ants. We also identify a set of 23 of these enzymes enriched in gongylidia compared to whole fungus garden samples, suggesting that certain enzymes may be particularly important in the initial degradation of foliar material. Our work sheds light on the complex interplay between leaf-cutter ants and their fungal symbiont that allows for the host insects to occupy an herbivorous niche by indirectly deriving energy from plant biomass.

A3.2 Introduction

Leaf-cutter ants of the genus *Atta* are prevalent consumers of foliar biomass in the New World tropics that play an important role in nutrient cycling and ecosystem engineering in many Neotropical habitats [1–3]. Rather than directly consuming the foliar material they collect, leaf-cutter ants use their forage to cultivate symbiotic gardens that they then consume for food [1,4]. Although the gardens cultivated by leaf-cutter ants contain a variety of microbes [5–10], the primary cultivar of these insects is the basidiomycetous fungus *Leucoagaricus gongylophorus*, which plays a major role in degrading the plant forage of the ants [1,11–15]. In addition to its biodegradative role, this fungus also produces the primary sustenance for leaf-cutter ants in the form of nutrient-rich hyphal swellings called gongylidia [1,16], thereby converting recalcitrant plant polymers into nutrients readily available to its host ants.

A central aspect of this insect-microbe symbiosis is the degradation of plant biomass, and a number of behaviors have evolved in these insects presumably to help them both exclude unwanted microbial pests from their gardens and enhance the biomass degrading capacity of *L. gongylophorus* [17–20]. Two examples of these behaviors, termed weeding and grooming, allow the ants to efficiently remove both patches of dead or contaminated fungus garden and selectively filter out spores of foreign fungi, respectively [17]. Moreover, entire fungus gardens are structured into different strata such that fresh foliar material is integrated only in top layers before being degraded in a step-wise process as it moved into lower layers [21,22]. When fresh foliar material is integrated into the top strata, the ants first macerate it into smaller pieces and deposit fecal droplets on top before inoculating fresh cultures of *L. gonylophorus* from older strata [1].

Previous work has shown that the fecal droplets of leaf-cutter ants contain plant biomass-degrading enzymes, which we define here to include Carbohydrate Active Enzymes (CAZymes),

Fungal Oxidative Lignin-degrading Enzymes (FOLymes), and proteases [23–25]. It has been postulated in previous studies that the deposition of these droplets onto fresh foliar material serves as a form of "pretreatment" [26,27] that initiates the degradation process before *L. gongylophorus* is inoculated. Many of the plant biomass-degrading enzymes in ant fecal droplets have been shown to be derived from *L. gongylophorus* [28,29], and gene expression analyses have shown that some of these enzymes appear to be highly expressed in gongylidia [30,31], suggesting that the enzymes concentrated by *L. gongylophorus* in gongylidia are still active after passing through the digestive tract of the ants. Furthermore, analysis of the *Atta cephalotes* genome has shown a reduction in the total number of encoded proteases compared to other insects [32], suggesting that the loss of these proteases may have been an adaptation allowing *L. gongylophorus* enzymes to pass through their digestive systems and become concentrated intact in fecal droplets.

Although the enzymes concentrated by *L. gongylophorus* in gongylidia have been shown to have broad hydrolytic activity against a variety of plant polymers [23–25,31,33] and are likely of great import to plant biomass degradation in this insect-fungal symbiosis, the full complement of enzymes present in gongylidia is unknown. Here we used proteomic methods to identify proteins present in the gongylidia of three laboratory-reared *Atta cephalotes* colonies. Our results provide insight into the full spectrum of enzymes used by leaf-cutter ants to pretreat foliar biomass and help clarify the extent of the co-evolutionary adaptations of leaf-cutter ants and their symbiotic fungus that underpin this ecologically-important symbiosis.

A3.3 Materials and Methods

A3.3.1 Sample collection

Samples of gongylidia were collected from three laboratory-reared colonies of *Atta cephalotes* fed a diet of maple *(Ace*r) and oak *(Quercus*) leaves three times weekly. Fungus garden material was taken from the middle strata of fungus gardens and gongylidia were collected using a sterile forceps and dissecting microscope set at 40X magnification. For each sample approximately 50 mg of gongylidia were placed into 50 uL of sterile water in a 1.5 uL microcentrifuge tube before the mixture was frozen at -80 °C prior to protein extraction and proteomic characterization.

A3.3.2 Preparation of gongylidia samples

To lyse cells, frozen gongylidia were placed in an ice cold mortar and pestle and ground under liquid nitrogen for several minutes before being transferred into centrifuge tubes with 0.1 mm zirconia beads and bead beat on a Bullet Blender (Next Advance, Averill Park, NY) for 3 minutes on speed 8. Samples were centrifuged and a methanol/chloroform extraction was performed to separate the protein, metabolites and lipids. Ice cold (-20 °C) cholorform:methanol mix (prepared 2:1 (v/v)) was added to the sample in a 5:1 ratio over sample volume and vigorously vortexed. The samples were then placed on ice for 5 minutes and then vortexed for 10 seconds followed by centrifugation at 10,000 x g for 10 minutes at 4 °C. The upper water soluble metabolite phase was collected into a glass vial, the lower lipid soluble phase was collected into a separate fresh glass vial, and both samples were dried in a SpeedVac before being stored at -80 °C until analysis. The remaining protein interlayer was placed in a fume hood to dry.

The protein pellets were resuspended in 100 mM NH_4HCO_3 and assayed with Bicinchoninic acid (BCA) (Thermo Scientific, Rockford, IL) to determine the protein concentration. 2,2,2-

Trifluoroethanol (TFE) (Sigma, St. Louis, MO) was added for a final concentration of 50% TFE. The samples were sonicated in an ice-water bath for 1 minute and incubated at 60 ºC for 2 hours with gentle shaking at 300 rpm. The samples were reduced with 2 mM Dithiothreitol (DTT) (Sigma, St. Louis, MO) with incubation at 37ºC for 1 hour with gentle shaking at 300 rpm. Samples were then diluted 5-fold with 50 mM NH_4HCO_3, 1 mM CaCl2 for preparation for digestion. Sequencing-grade modified porcine trypsin (Promega, Madison, WI) was added to the sample at a 1:50 (w/w) trypsin-to-protein ratio for 3 hours at 37 ˚C. The samples were concentrated in a SpeedVac (ThermoSavant, Holbrook, NY) to a volume of ~30 µl, acidified with trifluoroacetic acid to pH 4 and then centrifuged at 10,000 x g. The supernatant was cleaned with a strong cation exchange solid phase extraction procedure (SCX-SPE) on an OMIX tip (Agilent technologies, Santa Clara, CA) according to the manufacturer's instructions. The remaining pellet was re-digested, by resolubilization in 2M thiourea, 7 M urea and 1% CHAPS detergent and incubated at 60 ºC for 2 hours, reduced with 10mM Dithiothreitol (DTT) with incubation at 37 ºC for 1 hour, diluted 10-fold with 50 mM NH_4HCO_3, 1mM CaCl2. The sample was then re-digested with trypsin for 3 hours at 37 ˚C and cleaned with a strong cation exchange-solid phase extraction procedure (SCX-SPE). The supernatant and pellet samples were combined and concentrated down to ~30 µL using a SpeedVac and a final BCA assay was performed to determine the peptide concentration. The samples were then vialed for mass spectrometric analysis.

A3.3.3 Proteomic Analyses

Due to difficulties in collecting large quantities of gongylidia, on-line multidimensional fractions techniques were utilized in this study to increase measurement coverage given the small sample sizes available. Three biological replicates (5 µg injections) of each sample were analyzed utilizing an on-line 2-D liquid chromatography (LC) separation consisting of one strong cation exchange (SCX)

column and two reversed phase C18 columns and two solid phase extraction (SPE) columns for desalting. A total of 15 fractions were collected in this method, and while each fraction was collected, the previously collected fraction was analyzed by the analytical reversed phase separation with detection at the mass spectrometer. This system continuously acquired data for reversed phase separations through all 15 fractions. The 2D-LC system was custom built using two Agilent 1200 nanoflow pumps and one 1200 capillary pump (Agilent Technologies, Santa Clara, CA), various Valco valves (Valco Instruments Co., Houston, TX), and a PAL autosampler (Leap Technologies, Carrboro, NC). Full automation was made possible by custom software that allowed for parallel event coordination and therefore near 100% MS duty cycle through use of two trapping columns and two analytical columns. All columns were manufactured in-house by slurry packing media into fused silica (Polymicro Technologies Inc., Phoenix, AZ) using a 1-cm sol-gel frit for media retention (19). A detailed schematic of this setup with setting configurations and reagent details is provided in Figure A3.1.

Mass spectrometry analyses were performed using a Velos Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) outfitted with a custom electrospray ionization (ESI) interface. Electrospray emitters were custom made by chemically etching 150 um o.d. x 20 um i.d. fused silica [35]. The heated capillary temperature and spray voltage were 275 ºC and 2.2 kV, respectively. Data were acquired for 100 minutes after a 10 minute delay from when the gradient started. Orbitrap spectra (AGC $1x10^6$) were collected from 400-2000 m/z at a resolution of 100 k followed by data dependent ion trap CID MS/MS (collision energy 35%, isolation width 2.0, AGC $1x10^4$) of the ten most abundant ions. A dynamic exclusion time of 60 sec was used to discriminate against previously analyzed ions using a -0.6 to 1.6 Da mass window.

The peptide tandem mass spectra resulting from these procedures were mapped onto the *L. gongylophorus* genome [12] using MS-GF+ [36]. The resulting data were filtered by MS-GF < $1x10^{-10}$ and mass error < +/- 2.5 ppm which resulted in a spectral level FDR of 1.69% based on a decoy search (Dataset S1). For each of the samples, peptide spectral counts were summed across the 15 fractions.

A3.3.4 Enzyme annotations and statistical analyses

Carbohydrate Active Enzymes (CAZymes, [37]), Fungal Oxidative Lignin-degrading enzymes (FOLymes, [38]), and protease annotations (following the MEROPs classification system [39]) for the *L. gongylophorus* genome were predicted and annotated to the protein family level, as previously described [12]. Additionally, proteins of interest in the gongylidia samples to which a large number of spectra could be mapped were annotated using homology searches against the Swissprot database [40] (downloaded 5/12/2014) using LAST version 393 [41] (minimum 70% length coverage required, top hits retained). Analysis of the *L. gongylophorus* proteins was performed using the gene predictions previously generated using GeneMark-ES [12,42].

Spectral counts for the *L. gongylophorus* proteins were compared to those previously reported from proteomic characterization of *Atta cephalote*s whole fungus gardens [12]. Only those spectra previously identified as mapping to *L. gongylophorus* proteins were used in this comparison. Fisher's Exact Test ($P < 0.005$) was used to determine if the proportion of mass spectra mapping to plant biomass-degrading enzymes or their families was enriched in gongylidia compared to whole fungus gardens (top, middle, and bottom strata combined). Individual plant biomass-degrading enzymes were considered enriched if they were identified as statistically significant in at least two out of three gongylidia samples as compared to averaged whole fungus garden spectral counts. For ordination analyses raw spectral counts mapping to proteins identified in the gongylidia and fungus garden

samples were normalized using the arcsine square-root transform. Non-metric multidimensional scaling (NMDS) was conducted on these transformed normalized counts using the vegan package in the R statistical programming environment [43,44].

A3.4 Results and Discussion

The specialized manner in which leaf-cutter ants cultivate fungi for food has been of great interest to scientists for decades [4], and a central goal of many studies has been to characterize the process through which plant biomass is degraded in fungus gardens and converted into usable nutrients for the host ants [7,12,21,22,45,46]. Many earlier studies of leaf-cutter ants of the genus *Atta* as well as other related fungus-growing ant genera revealed the presence of proteases, glycoside hydrolases, and laccases in the fecal droplets of the ants [23–26,28,33], and many of these studies noted the probable fungal origin of these enzymes. More recently, studies of the leaf-cutter ant *Acromyrmex echinatior* have identified pectinases in the fecal droplets and gongylidia [45], and transcripts encoding for laccases, glycoside hydrolases, and proteases have been identified in the gongylidia of this species [30,31]. Moreover, a great deal of other research has investigated plant biomass degradation in leaf-cutter ants more generally without specific emphasis on gongylidia or fecal droplets [5,7,12,21,27,47–49]. Given the important role of gongylidia in supplying key biodegradative enzymes to the ants for subsequent deposition onto plant material, we sought to build on these previous studies by thoroughly identifying the CAZymes, FOLymes, and proteases present in the gongylidia of the leaf-cutter ant *Atta cephalotes* using novel proteomic methods.

We identified a total of 636 *L. gongylophorus* proteins using 27,313 total spectral identifications (17,660 unique spectral identifications after quality-filtering [50]) in the three gongylidia samples tested (275-498 proteins identified per sample) (Table A3.1 and Dataset S1). Of these proteins, 124 (19.5%) were predicted plant biomass-degrading enzymes including 50 Carbohydrate Active

Enzymes (CAZymes) likely involved in polysaccharide degradation, 31 Fungal Oxidative Lignin-Degrading Enzymes (FOLymes) likely involved in aromatic compound degradation, and 43 proteases likely involved in protein degradation. Of the 636 proteins identified, 185 (29%) were found in all three samples, while 338 (53%) were found in at least two samples (Figure A3.2). Many of the plant biomass-degrading enzymes identified were also shared between samples, with 21 CAZymes (42%), 17 FOLymes (55%), and 21 proteases (49%) identified in all three samples (Figure A3. 2).

Spectra mapping to CAZymes, FOLymes, and proteases were over-represented in the gongylidia samples (Table A3.2) compared to spectra mapping to the same enzyme sets in earlier proteomic experiments performed on whole fungus garden material [12] (Fisher's Exact Test, $p < 0.005$). Moreover, nonmetric multidimensional scaling (NMDS) analyses of overall protein profiles of the gongylidia and whole fungus garden samples confirmed distinct clustering between these two sample groups (Figure A3.3). Together, these results indicate that the overall protein content of gongylidia is distinct from that of whole fungus gardens, and that plant biomass-degrading enzymes in particular are enriched in these hyphal swellings. The enrichment of plant biomass-degrading enzymes in gongylidia relative to whole fungus gardens, the large portion of shared proteins in the gongylidia samples (Figure A3.2), and the overall similarity of gongylidia protein profiles relative to those of whole fungus garden samples (Figure A3.3) supports the hypothesis that a consistent and specialized set of enzymes is produced in these hyphal swellings.

Comparisons of the spectral abundance of individual proteins between gongylidia and whole fungus gardens revealed that 98 proteins were enriched at least one gongylidia sample, and 25 of these enriched in all three samples (Fisher's Exact Test, $p < 0.005$). The enzymes enriched in all three gongylidia samples include 6 CAZymes, 1 FOLyme, and 3 proteases, and another 12 CAZymes, 5 FOLymes, and 9 proteases were enriched in either one or two gongylidia samples (Figure A3.4). The

6 CAZymes enriched in all three gongylidia samples belong to enzyme families known to catalyze the degradation of pectin (family CE8) and glucosides such as starch (GH2, GH3, GH31). The three proteases enriched in all three gongylidia samples are metalloproteases (LAG_100, LAG_1037, and 1536), consistent with the identification of these enzymes in fecal droplets of *Atta texana* [23,28]. An LO1 laccase enriched in two gongylidia samples (LAG_2404) has previously been implicated in the detoxification of plant defense compounds potentially harmful to *L. gongylophorus* or the host ants [31]. The aryl-alcohol oxidase (LAG_4156) enriched in all three samples may play a role similar or auxiliary role to laccases, although in other basidiomycetous fungi enzymes of this class have been implicated in hydrogen peroxide generation and lignocellulose degradation [44,45], and so it may serve to target structural polymers in foliar biomass rather than plant defense compounds.

Our finding of a broad array of plant biomass-degrading enzymes in the gongylidia of *L. gongylophorus* has important methodological implications for assaying the presence and activity of enzymes in leaf-cutter ant fungus gardens. Although a number of recent studies have relied on transcriptomics or mRNA quantification as measures of enzyme occurrence in fungus gardens [30,31,48], the enrichment of CAZymes, FOLymes, and proteases in gongylidia and the likely presence of a broad array of these enzymes in the fecal droplets deposited by the ants on fresh foliar biomass [24,28,29,51] indicates that transcription and protein presence are decoupled in this environment through the specialized gardening behavior of the leaf-cutter ants. This is likely common in fungus gardens since enzymes can be stored in gongylidia and transported away from where they are produced to sites where they are active. The measurement of mRNA alone is thus likely to provide incomplete and even misleading assessments of the presence of enzymes or their activities in specific locations within fungus gardens. We propose that for the inference of biodegradative processes it is more appropriate to use other techniques that rely on the direct

measurement of proteins or their activities, such as the proteomic techniques used here and in other studies [12,45] or the direct measurements of enzymatic activity that have previously been used in fungus gardens [24,47,46].

In this work, our finding that plant biomass-degrading enzymes in general and 24 in particular were enriched in gongylidia supports the hypothesis that these enzymes play an important biodegradative role in leaf-cutter ant fungus gardens. Although only 10 CAZymes, FOLymes, and proteases were enriched in all three gongylidia samples, 36 plant biomass-degrading enzymes were enriched in gongylidia were enriched in at least one sample and 124 of these enzymes were identified in total. Recent analysis of the *L. gongylophorus* genome identified 145 putative plant biomass-degrading enzymes, and our finding of 119 of these enzymes (82% of the total, excluding the five glycoside hydrolases not reported previously) provides the surprising result that the majority of the biodegradative enzymes produced by the fungal cultivar are ingested by the ants in some quantity. These results strongly implicate the ingestion of plant biomass-degrading enzymes and subsequent deposition on fresh foliar material within fecal droplets as a critical aspect of the degradation of plant biomass in fungus gardens. The initial pretreatment of biomass with a diverse set of enzymes prior to inoculation may release free sugars or more labile substrates critical for the initial establishment and sustained growth of *L. gongylophorus* in fungus gardens. The apparent lack of cellulases and hemicellulases in gongylidia, indicates that these enzymes are not part of the pretreatment step, possibly because cellulose is used later in the degradation process. These results may partly explain why *L. gongylophorus* has been shown to grow slowly or not at all on numerous plant polymers in pure culture despite its apparent rapid growth in fungus gardens, where presumably the specialized gardening behaviors of the ants enhance biomass breakdown [1,11,14,52]. Together with these earlier results, our finding of widespread plant biomass-degrading enzymes in gongylidia therefore

underscores the importance of the specialized gardening behaviors of leaf-cutter ants to biomass degradation as well as the extent to which leaf-cutter ants have co-evolved with their symbiotic fungus to efficiently derive energy from foliar material.

A3.5 References

1. Weber NA. Fungus-Growing Ants. Science. 1966;153: 587–604. doi:10.1126/science.153.3736.587

2. Hölldobler B. The superorganism: the beauty, elegance, and strangeness of insect societies. 1st ed. New York: W.W. Norton; 2009.

3. Costa AN, Vasconcelos HL, Vieira-Neto EHM, Bruna EM. Do herbivores exert top-down effects in Neotropical savannas? Estimates of biomass consumption by leaf-cutter ants. J Veg Sci. 2008;19: 849–854. doi:10.3170/2008-8-18461

4. Belt T. The naturalist in Nicaragua [Internet]. London, J.M. Dent & Sons, Ltd.; New York, E.P. Dutton & Co; 1911. Available: http://archive.org/details/naturalistinnica01belt

5. Aylward FO, Burnum KE, Scott JJ, Suen G, Tringe SG, Adams SM, et al. Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. ISME J. 2012;6: 1688–1701. doi:10.1038/ismej.2012.10

6. Scott JJ, Budsberg KJ, Suen G, Wixon DL, Balser TC, Currie CR. Microbial Community Structure of Leaf-Cutter Ant Fungus Gardens and Refuse Dumps. PLoS ONE. 2010;5: e9922. doi:10.1371/journal.pone.0009922

7. Suen G, Scott JJ, Aylward FO, Adams SM, Tringe SG, Pinto-Tomás AA, et al. An Insect Herbivore Microbiome with High Plant Biomass-Degrading Capacity. PLoS Genet. 2010;6: e1001129. doi:10.1371/journal.pgen.1001129

8. Rodrigues A, Bacci M, Mueller UG, Ortiz A, Pagnocca FC. Microfungal "weeds" in the leafcutter ant symbiosis. Microb Ecol. 2008;56: 604–614. doi:10.1007/s00248-008-9380-0

9. Rodrigues A, Pagnocca FC, Bacci MJ, Hebling MJA, Bueno OC, Pfenning LH. Variability of non-mutualistic filamentous fungi associated with Atta sexdens rubropilosa nests. Folia Microbiol (Praha). 2005;50: 421–425.

10. Aylward FO, Suen G, Biedermann PHW, Adams AS, Scott JJ, Malfatti SA, et al. Convergent Bacterial Microbiotas in the Fungal Agricultural Systems of Insects. mBio. 2014;5: e02077–14. doi:10.1128/mBio.02077-14

11. Martin MM, Weber NA. The Cellulose-Utilizing Capability of the Fungus Cultured by the Attine Ant Atta colombica tonsipes. Ann Entomol Soc Am. 1969;62: 1386–1387.

12. Aylward FO, Burnum-Johnson KE, Tringe SG, Teiling C, Tremmel DM, Moeller JA, et al. Leucoagaricus gongylophorus Produces Diverse Enzymes for the Degradation of Recalcitrant Plant Polymers in Leaf-Cutter Ant Fungus Gardens. Appl Environ Microbiol. 2013;79: 3770–3778. doi:10.1128/AEM.03833-12

13. Chapela IH, Rehner SA, Schultz TR, Mueller UG. Evolutionary History of the Symbiosis Between Fungus-Growing Ants and Their Fungi. Science. 1994;266: 1691–1694. doi:10.1126/science.266.5191.1691

14. Siqueira CGD, Bacci M, Pagnocca FC, Bueno OC, Hebling MJ. Metabolism of Plant Polysaccharides byLeucoagaricus gongylophorus, the Symbiotic Fungus of the Leaf-Cutting Ant Atta

sexdens L. Appl Environ Microbiol. 1998;64: 4820–4822.

15. Jr MB, Anversa MM, Pagnocca FC. Cellulose degradation byLeucocoprinus gongylophorus, the fungus cultured by the leaf-cutting antAtta sexdens rubropilosa. Antonie Van Leeuwenhoek. 1995;67: 385–386. doi:10.1007/BF00872939

16. Bass M, Cherrett JM. Fungal hyphae as a source of nutrients for the leaf-cutting ant Atta sexdens. Physiol Entomol. 1995;20: 1–6. doi:10.1111/j.1365-3032.1995.tb00793.x

17. Currie CR, Stuart AE. Weeding and grooming of pathogens in agriculture by ants. Proc R Soc B Biol Sci. 2001;268: 1033–1039. doi:10.1098/rspb.2001.1605

18. Aylward FO, Currie CR, Suen G. The Evolutionary Innovation of Nutritional Symbioses in Leaf-Cutter Ants. Insects. 2012;3: 41–61. doi:10.3390/insects3010041

19. Fernández-Marín H, Zimmerman JK, Nash DR, Boomsma JJ, Wcislo WT. Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. Proc R Soc Lond B Biol Sci. 2009; rspb.2009.0184. doi:10.1098/rspb.2009.0184

20. Fernández-Marín H, Zimmerman JK, Wcislo WT. Fungus garden platforms improve hygiene during nest establishment in Acromyrmex ants (Hymenoptera, Formicidae, Attini). Insectes Sociaux. 2007;54: 64–69. doi:10.1007/s00040-007-0907-z

21. Kooij PW, Schiøtt M, Boomsma JJ, Licht HHDF. Rapid shifts in Atta cephalotes fungus-garden enzyme activity after a change in fungal substrate (Attini, Formicidae). Insectes Sociaux. 2011;58: 145–151. doi:10.1007/s00040-010-0127-9

22. Huang EL, Aylward FO, Kim Y-M, Webb-Robertson B-JM, Nicora CD, Hu Z, et al. The fungus gardens of leaf-cutter ants undergo a distinct physiological transition during biomass degradation. Environ Microbiol Rep. 2014;6: 389–395. doi:10.1111/1758-2229.12163

23. Boyd ND, Martin MM. Faecal proteinases of the fungus-growing ant, Atta texana: Properties, significance and possible origin. Insect Biochem. 1975;5: 619–635. doi:10.1016/0020-1790(75)90043-8

24. Martin MM, Boyd ND, Gieselmann MJ, Silver RG. Activity of faecal fluid of a leaf-cutting ant toward plant cell wall polysaccharides. J Insect Physiol. 1975;21: 1887–1892. doi:10.1016/0022-1910(75)90219-X

25. Martin MM, Gieselmann MJ, Martin JS. The presence of chitinase in the digestive fluids of ants. Comp Biochem Physiol A Physiol. 1976;53: 331–332. doi:10.1016/S0300-9629(76)80150-8

26. Cherrett, JM, Powell RJ, Stradling DJ. The Mutualism between leaf-cutter ants and their fungus. Insect Fungal Interactions. Academic Press; 1989. pp. 93–116.

27. Moller IE, De Fine Licht HH, Harholt J, Willats WGT, Boomsma JJ. The Dynamics of Plant Cell-Wall Polysaccharide Decomposition in Leaf-Cutting Ant Fungus Gardens. Chave J, editor. PLoS ONE. 2011;6: e17506. doi:10.1371/journal.pone.0017506

28. Boyd ND, Martin MM. Faecal proteinases of the fungus-growing ant, Atta texana: Their fungal origin and ecological significance. J Insect Physiol. 1975;21: 1815–1820. doi:10.1016/0022-1910(75)90247-4

29. Rønhede S, Boomsma JJ, Rosendahl S. Fungal enzymes transferred by leaf-cutting ants in

their fungus gardens. Mycol Res. 2004;108: 101–106.

30. De Fine Licht HH, Boomsma JJ, Tunlid A. Symbiotic adaptations in the fungal cultivar of leaf-cutting ants. Nat Commun. 2014;5: 5675. doi:10.1038/ncomms6675

31. Licht HHDF, Schiøtt M, Rogowska-Wrzesinska A, Nygaard S, Roepstorff P, Boomsma JJ. Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. Proc Natl Acad Sci. 2013;110: 583–587. doi:10.1073/pnas.1212709110

32. Suen G, Teiling C, Li L, Holt C, Abouheif E, Bornberg-Bauer E, et al. The Genome Sequence of the Leaf-Cutter Ant Atta cephalotes Reveals Insights into Its Obligate Symbiotic Lifestyle. PLoS Genet. 2011;7: e1002007. doi:10.1371/journal.pgen.1002007

33. Rønhede S, Boomsma JJ, Rosendahl S. Fungal enzymes transferred by leaf-cutting ants in their fungus gardens. Mycol Res. 2004;108: 101–106. doi:10.1017/S0953756203008931

34. Maiolica A, Borsotti D, Rappsilber J. Self-made frits for nanoscale columns in proteomics. Proteomics. 2005;5: 3847–3850. doi:10.1002/pmic.200402010

35. Kelly RT, Page JS, Luo Q, Moore RJ, Orton DJ, Tang K, et al. Chemically etched open tubular and monolithic emitters for nanoelectrospray ionization mass spectrometry. Anal Chem. 2006;78: 7796–7801. doi:10.1021/ac061133r

36. Kim S, Gupta N, Pevzner PA. Spectral probabilities and generating functions of tandem mass spectra: a strike against decoy databases. J Proteome Res. 2008;7: 3354–3363. doi:10.1021/pr8001244

37. Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 2014;42: D490–D495. doi:10.1093/nar/gkt1178

38. Levasseur A, Piumi F, Coutinho PM, Rancurel C, Asther M, Delattre M, et al. FOLy: an integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. Fungal Genet Biol FG B. 2008;45: 638–645. doi:10.1016/j.fgb.2008.01.004

39. Rawlings ND, Waller M, Barrett AJ, Bateman A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 2014;42: D503–D509. doi:10.1093/nar/gkt953

40. Consortium TU. Activities at the Universal Protein Resource (UniProt). Nucleic Acids Res. 2014;42: D191–D198. doi:10.1093/nar/gkt1140

41. Frith MC, Hamada M, Horton P. Parameters for accurate genome alignment. BMC Bioinformatics. 2010;11: 80. doi:10.1186/1471-2105-11-80

42. Borodovsky M, Mills R, Besemer J, Lomsadze A. Prokaryotic Gene Prediction Using GeneMark and GeneMark.hmm. Current Protocols in Bioinformatics. John Wiley & Sons, Inc.; 2002. Available: http://onlinelibrary.wiley.com/doi/10.1002/0471250953.bi0405s01/abstract

43. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria; 2014.

44. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. vegan:

Community Ecology Package [Internet]. 2013. Available: http://cran.r-project.org/web/packages/vegan/index.html

45. Schiøtt M, Rogowska-Wrzesinska A, Roepstorff P, Boomsma JJ. Leaf-cutting ant fungi produce cell wall degrading pectinase complexes reminiscent of phytopathogenic fungi. BMC Biol. 2010;8: 156. doi:10.1186/1741-7007-8-156

46. Semenova TA, Hughes DP, Boomsma JJ, Schiøtt M. Evolutionary patterns of proteinase activity in attine ant fungus gardens. BMC Microbiol. 2011;11: 15. doi:10.1186/1471-2180-11-15

47. De Fine Licht HH, Schiøtt M, Mueller UG, Boomsma JJ. Evolutionary Transitions in Enzyme Activity of Ant Fungus Gardens. Evolution. 2010;64: 2055–2069. doi:10.1111/j.1558-5646.2010.00948.x

48. Grell MN, Linde T, Nygaard S, Nielsen KL, Boomsma JJ, Lange L. The fungal symbiont of Acromyrmex leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and other plant cell wall polysaccharides. BMC Genomics. 2013;14: 928. doi:10.1186/1471-2164-14-928

49. Lange L, Grell MN. The prominent role of fungi and fungal enzymes in the ant–fungus biomass conversion symbiosis. Appl Microbiol Biotechnol. 2014;98: 4839–4851. doi:10.1007/s00253-014-5708-5

50. Piehowski PD, Petyuk VA, Sandoval JD, Burnum KE, Kiebel GR, Monroe ME, et al. STEPS: a grid search methodology for optimized peptide identification filtering of MS/MS database search results. Proteomics. 2013;13: 766–770. doi:10.1002/pmic.201200096

51. Boyd ND, Martin MM. Faecal proteinases of the fungus-growing ant, Atta texana: Properties, significance and possible origin. Insect Biochem. 1975;5: 619–635. doi:10.1016/0020-1790(75)90043-8

52. Abril AB, Bucher EH. Evidence that the fungus cultured by leaf-cutting ants does not metabolize cellulose. Ecol Lett. 2002;5: 325–328. doi:10.1046/j.1461-0248.2002.00327.x

A3.6 Acknowledgements

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Table A3.1. Summary of spectral counts and proteins identified.

Gongylidia Sample	Total Mapped Spectra	Total Proteins Identified	CAZymes Identified	FOLymes Identified	Proteases Identified
Sample 1	2,531	388	32	24	29
Sample 2	9,007	275	30	19	27
Sample 3	15,775	498	37	28	38
Total	27,313	636	49	31	43

Experimental Conditions						
Column Type	Ion Exchange				Trapping	Reversed Phase
Dimensions	150 µm ID X 15 cm				150 µm ID X 5 cm	75 µm ID X 40 cm
Packing Material	5 µm PolySULFOETHYLATM				5 µm Jupiter C18	3 µm Jupiter C18
Mobile Phase	A: 0.1 mM NaH_2PO_4 B: 3.0 mM NaH_2PO_4				A: 0.1% Formic Acid in H_20 B: 0.1% Formic Acid in Acetonitrile	
Flow Rate	1.5 µL/min Capillary Pump				0.4 µL/min (Nano Pump)	
Gradients	Semi-Continuous Gradient				Linear Gradient	
	Fraction	%B	Fraction	%B	Time (min)	%B
	1	0.0-0.0	9	8.0-9.0	0	0
	2	0.0-1.5	10	9.0-10.0	2	10
	3	1.5-3.0	11	10.0-11.0	12	20
	4	3.0-4.0	12	11.0-14.0	85	35
	5	4.0-5.0	13	14.0-17.0	95	95
	6	5.0-6.0	14	17.0-20.0	100	95
	7	6.0-7.0	15	20.0-70.0	103	0
	8	7.0-8.0				

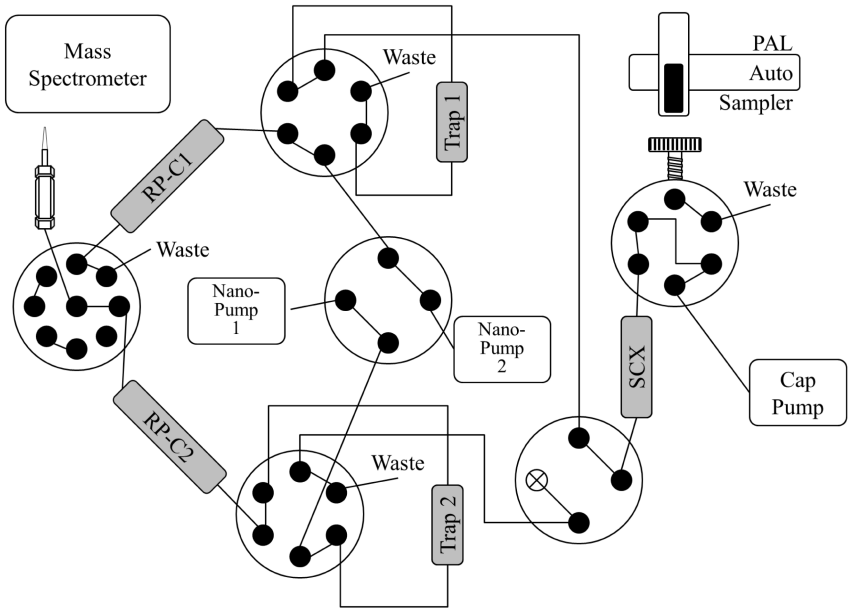

Figure A3.1. Schematic of the 2D Liquid Chromatography Mass Spectrometry (2D-LC-MS) setup used in this study. SCX: Strong cation exchange.

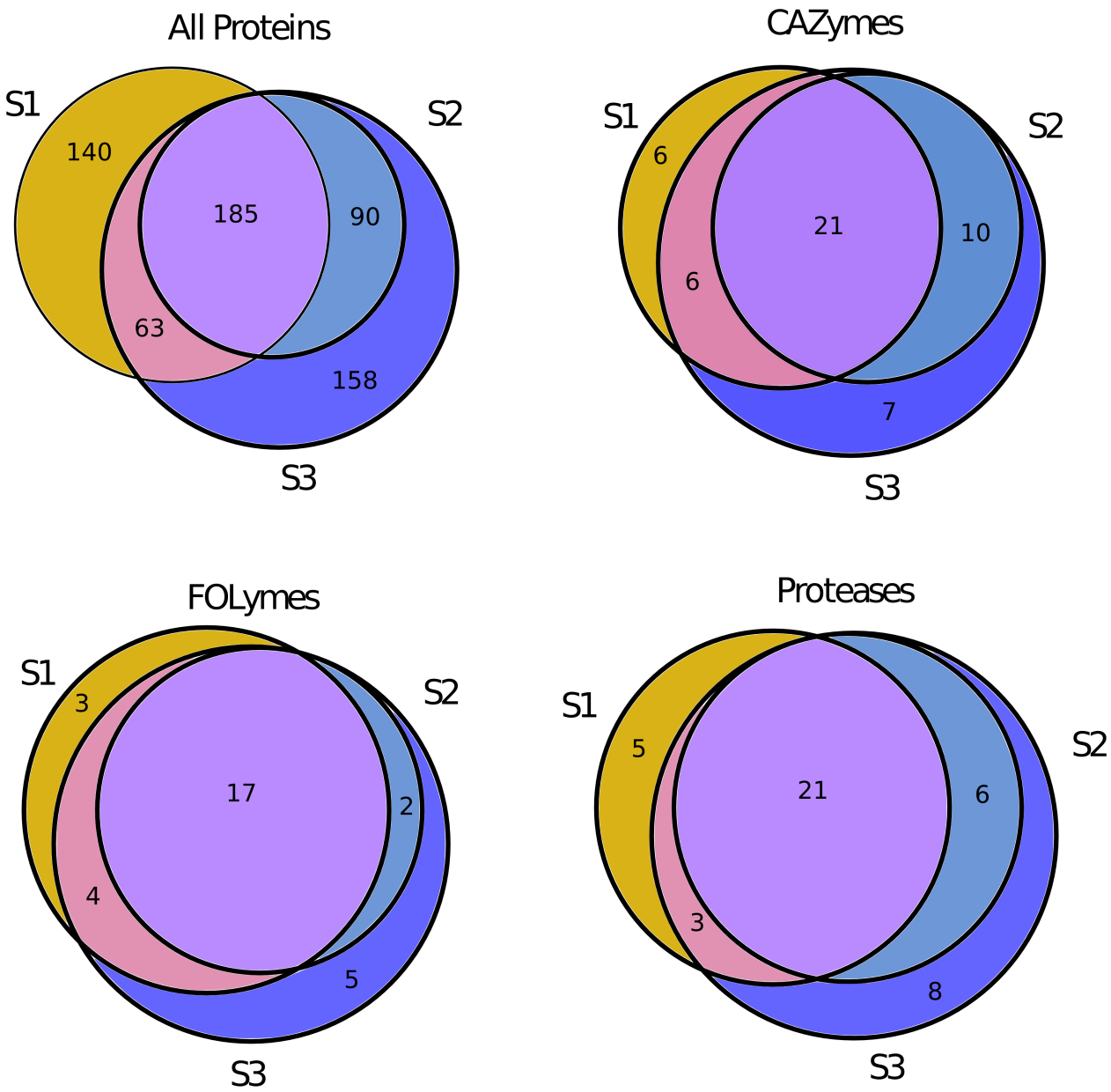

Figure A3.2. Venn diagram showing shared total proteins, CAZymes, FOLymes, and proteases identified in the three gongylidia samples (labeled S1-S3) analyzed in this study.

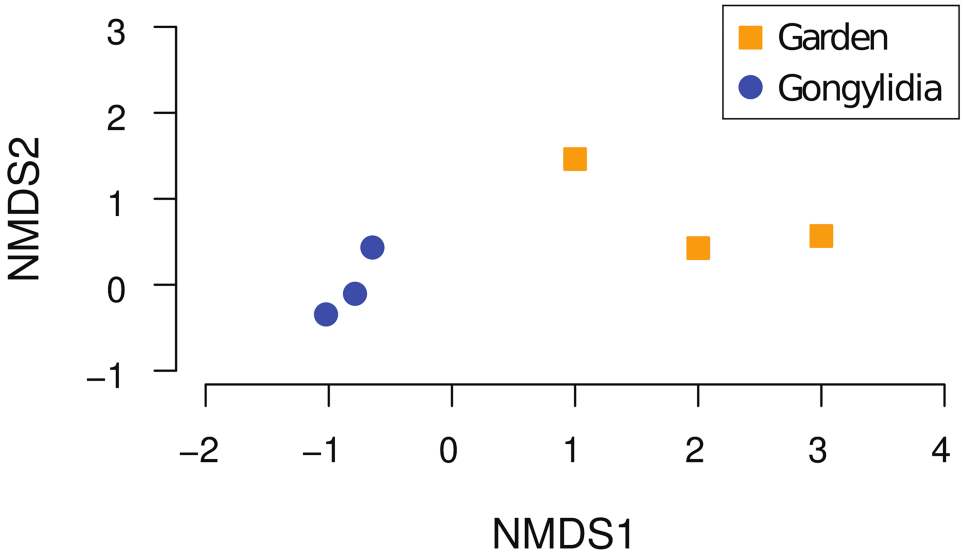

Figure A3.3. Non-metric multidimensional scaling (NMDS) ordination plot of fungus garden and gongylidia samples. Gongylidia samples are represented by blue circles, while orange squares represent fungus garden samples.

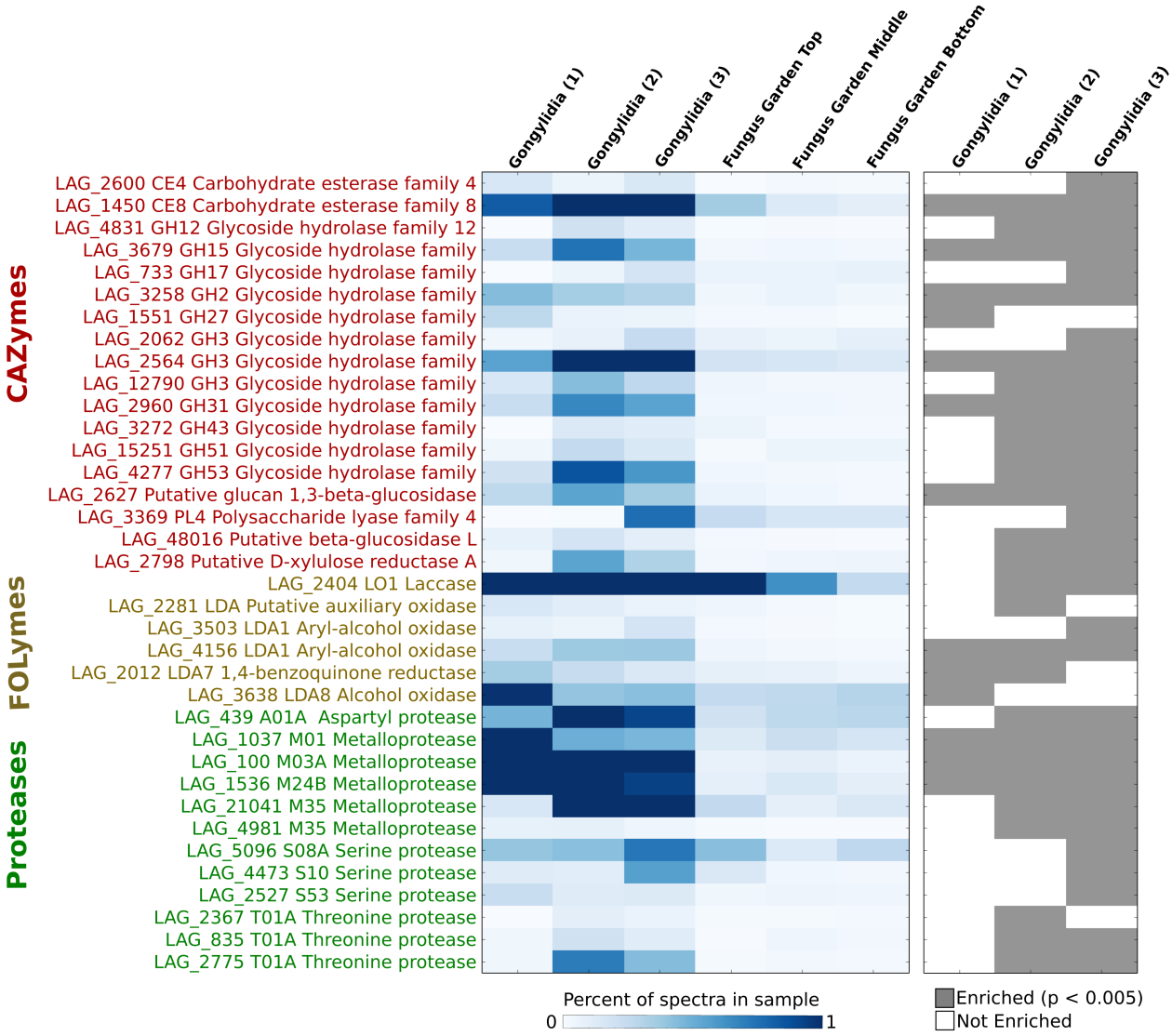

Figure A3.4. Heatmap are presented that show the relative percent of total spectra that could be mapped to specific CAZymes, FOLymes, and proteases (left) and those enzymes that were found to be enriched in at least one gongylidia sample (right; Fisher's Exact Test, $p < 0.005$). Only enzymes identified as enriched in at least one gongylidia samples compared to all fungus garden samples combined are shown.

Appendix 4: From plant to ant: lipids in the leaf-cutter ant fungus garden

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This appendix chapter is a work in progress and as such, it is not yet a cohesive unit.

A4.1 Introduction

The system of leaf-cutter ants and their microbial symbionts are pradigmatic in the field of insect microbial symbiosis. The ants, native to the Neotropics, cut foliar biomass from their environment and provide it as a growth substrate to a fungus, *Leucoagaricus gongylophorus* that they cultivate and consume (Holldobler & Wilson 2010). The ants masticate the fresh plant material and deposit it on the top of the fungus garden, transfer a tuft of fungal mycelium from an older part of the garden, defacate (the feces contain fungus-derived plant biomass-degrading enzymes) and allow the fungus to grow (De Fine Licht *et al.* 2013; Aylward *et al.* 2015). As the plant material is digested by the fungus, the material moves downward through the stratified layers of the garden toward the bottom. Once the material has been depleted of nutrients, it is removed from the bottom and deposited in a refuse dump, away from the fungus garden chamber (Farji-Brener & Medina 2000). Mostly in the middle region of the garden, the fungus produces gongylidia, which are hyphal swellings that worker ants consume and feed to larvae in the colony. While foragers also drink plant sap as they cut fresh leaf material, the gongylidia serves as the colony's main source of nutrition (Bass & Cherrett 1995; North *et al.* 1997).

Our previous work focused on elucidating the relationship between the ants and their fungal cultivar, mainly on the means by which the fungus is able to break down plant biomass (Aylward *et al.* 2013; Khadempour *et al.* 2016). One method that we used to study this was metaproteomics of the different layers of the garden and the gongylidia (Aylward *et al.* 2012; 2015). In examining the metaproteomic data, it became clear that there was an enrichment for lipid-associated proteins in the gongylidia (Supplemental figure 1). This result combined with previous work that demonstrated an enrichment of lipids and sugars in the gongylidia (Quinlan & Cherrett 1979), were the basis of this study. Our aim here is to examine the lipid content of leaf material and to track its progress through

the fungus garden and into the gongylidia, thereby learning more about how the fungus is capable of supporting the growth of an ant colony.

We used reverse phase LC-MS/MS analysis technology, which allowed us to keep total lipids intact. We examined the lipid contents of leaf material and the layers of the fungus garden as leaf material is broken down. We also directly compared the lipid contents of gongylidia with the middle layer of the fungus garden from which the gongylidia were harvested.

A4.2 Methods

A4.2.1 Material collection

Lab-maintained leaf-cutter ant colonies were provided exclusively maple leaves for the two weeks before sample collection. We collected leaf material as well as fungus garden from the top, middle, and bottom of leaf-cutter ant fungus gardens. These layers are differentiated based on color, texture and location in the garden.

Gongylidia are difficult to collect because of their small size and their sparseness in the garden. To collect gongylidia, we first collected fungus garden in deep petri dishes and removed the majority of workers. With fewer workers in the fungus garden, the gongylidia proliferate. After several days, the gongylidia were picked off by needlepoint under a dissecting microscope and they were placed in a tube with water.

The leaf material used in this experiment was collected in the summer and kept frozen at -20°C in a vacuum sealed bag so that the ant colonies can be fed during the winter when fresh leaves are not available.

A4.2.2 Total lipid extraction

The collected material was lyophilized for untargeted lipidomics analysis. Lipids were extracted using a modified Folch extraction (1957). To break up the biological material, approximately 25 mg of sample was bead beaded using a 3mm tungsten carbide bead in 750 µl of methanol for 2 min at a frequency of 30. The sample was then removed and transferred into a 20 mL clean EPS glass vial lined with a Teflon lined cap. Another 750 µl of methanol was added for a final volume of 1.5 ml methanol. 3 ml of chloroform and 200 µl of water were added to each sample. The samples were vortexed for 30 sec, sonicated for 30 min, vortexed again for 30 s, and then 0.925 µl of water was added to induce a phase separation. The samples were incubated at 4°C overnight and then the lower lipid layer was removed, dried down, and stored at -20°C at a concentration of 2 µg/µl in 2:1 chloroform/methanol until LC-MS/MS analysis.

A4.2.3 Reverse phase LC-MS/MS analysis

For the following, we used a modified method from Dautel *et al.* (2017). A Waters NanoAquity UPLC system interfaced with a Velos-ETD Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) was used for LC-ESI-MS/MS analyses. The total lipid extracts (TLE) were reconstituted in methanol for a final abundance of 0.4 µg TLE/µl. 7 µl of the TLE were injected onto Waters column (HSS T3 1.0 mm x 150 mm x 1.8 µm particle size). Lipids were separated over a 90 min gradient elution (mobile phase A: ACN/H_2O (40:60) containing 10 mM ammonium acetate; mobile phase B: ACN/IPA (10:90) containing 10 mM ammonium acetate) at a flow rate of 30 µl/min. Samples were analyzed in both positive and negative ionization using HCD (higher-energy collision dissociation) and CID (collision-induced dissociation) to obtain high coverage of the lipidome.

A4.2.4 Lipid targeted database and alignment

We used LIQUID software (Kyle *et al.* 2017) for lipid targeted database alignment. This is achieved by aligning all datasets (grouped by sample type and ionization mode) and matching unidentified features to their identified counterparts using MZmine2 (Pluskal *et al.* 2010). Aligned features are manually verified and peak apex intensity values are exported for statistical analysis.

A4.2.5 LC-IMS

The LC/IMS-MS analyses were performed in both positive and negative ion mode and collected from 100–3200 m/z at a MS resolution of 40,000. The LC/IMS-MS data were analyzed using in-house PNNL software for deisotoping and feature finding the multidimensional LC, IMS and MS data (Crowell *et al.* 2013). Features were then compared to the AMT tag database generated from LC/MS/MS for identification and quantitation (Zimmer *et al.* 2006).

A4.2.6 Statistical Analysis

Statistics were performed on the aligned lipid peak apex intensity values from each of the gardens and their respective sections from the bottom, middle and top of the gardens. Initial analysis evaluated if the samples from with a garden, irrespective of garden, are more highly correlated than across gardens. Data was compared using the Pearson correlation values for all samples to one another within gardens to the Pearson correlation values for all samples against samples in a different garden with a simple two-sample t-test. There is no obvious correlation structure by garden in Figure 1 and the t-test confirms this with p-values of 0.933 and 0.894, respectively, for the negative and positive d,atasets.

Outlier identification was conducted using the algorithm RMD-PAV (Matzke *et al.* 2011) none of which were found. As the data had very few missing values no lipid identifications were

removed. A standard Analysis of Variance (ANOVA) was used to evaluate each lipid for a statistical difference between the top, middle and bottom with a Tukey's post-hoc test to compare the individual levels to one another. For the negative set 43 of the 80 lipids were significant by ANOVA at a p-value of 0.05 and 104 of the 222 were significant at the same level for the positive set. Lastly, a Principal Component Analysis (PCA) scores plot was conducted using sequential projection pursuit to perform PCA without imputing missing values (Webb-Robertson et al. 2013).

A4.3 Results

The lipidome for the fungus gardens of leaf-cutter ants comprised of 188 intact lipids represented across 3 lipid categories (sphingolipids, glycerophospholipids, and glycerolipids) and 18 subclasses. Glycerophospholipids and glycerolipids were comprised of near equal of lipid species (89 glycerophospholipids and 91 glycerolipids) and presented 96% of the identified lipids. Sphingolipids were a minor component of the samples with a total of 8 identifications (or 4% of total identifications), 5 being ceramides and 3 hexosyl-ceramides (HexCer). Triacylglycerides (TGs) contained the greatest number of identified lipids (63 lipids or 33.5% of total) followed by diacylglycerophosphoethaolamine (PE) (26 identifications or 13.8% of total) and diacylglycerophosphoglycerol (PG) (19 lipids or 10.1% of total).

The leaves were dominated by PG as well as galactolipids (DGDG and SQDG). Minor amounts of PC, PE, and PIs were also identified however they were relatively less abundant when compared to the garden as were TGs. HexCer were the only sphingolipids identified in the leaves. Including leaf lipids in our analysis enabled us to track leaf lipids throughout the garden. For example, out of our 21 characterized PGs the 7 were below limits of confidence in the garden sample but in high abundance in the leaf sample.

Of the 188 lipids identified in the garden, 104 were found to be statistically significant almost exclusively between the top compared to the middle and bottom of the gardens. Comparing those lipids found to be statistically significant verse those that were not PE, PG, DG, and TG, significant lipids were more unsaturated (i.e., contained more double bonds in the fatty acid chains) with PG and TG also having longer fatty acid chains. SQDGs differed in that the significant lipids were less saturated (lower average number of double bonds). Examining the individual fatty acid composition of the intact lipids, 16:0, 18:2, and 18:3 dominated the fatty acids profile and composed the most abundant lipid per phospholipid and glycerolipid subclasses. Phospholipids were found to contain more odd chained fatty acids (approximately 12%) verse glycerolipids (approximately 6%) with C17 more common in the statistically significant phospholipids verses C15 in the glycolipids.

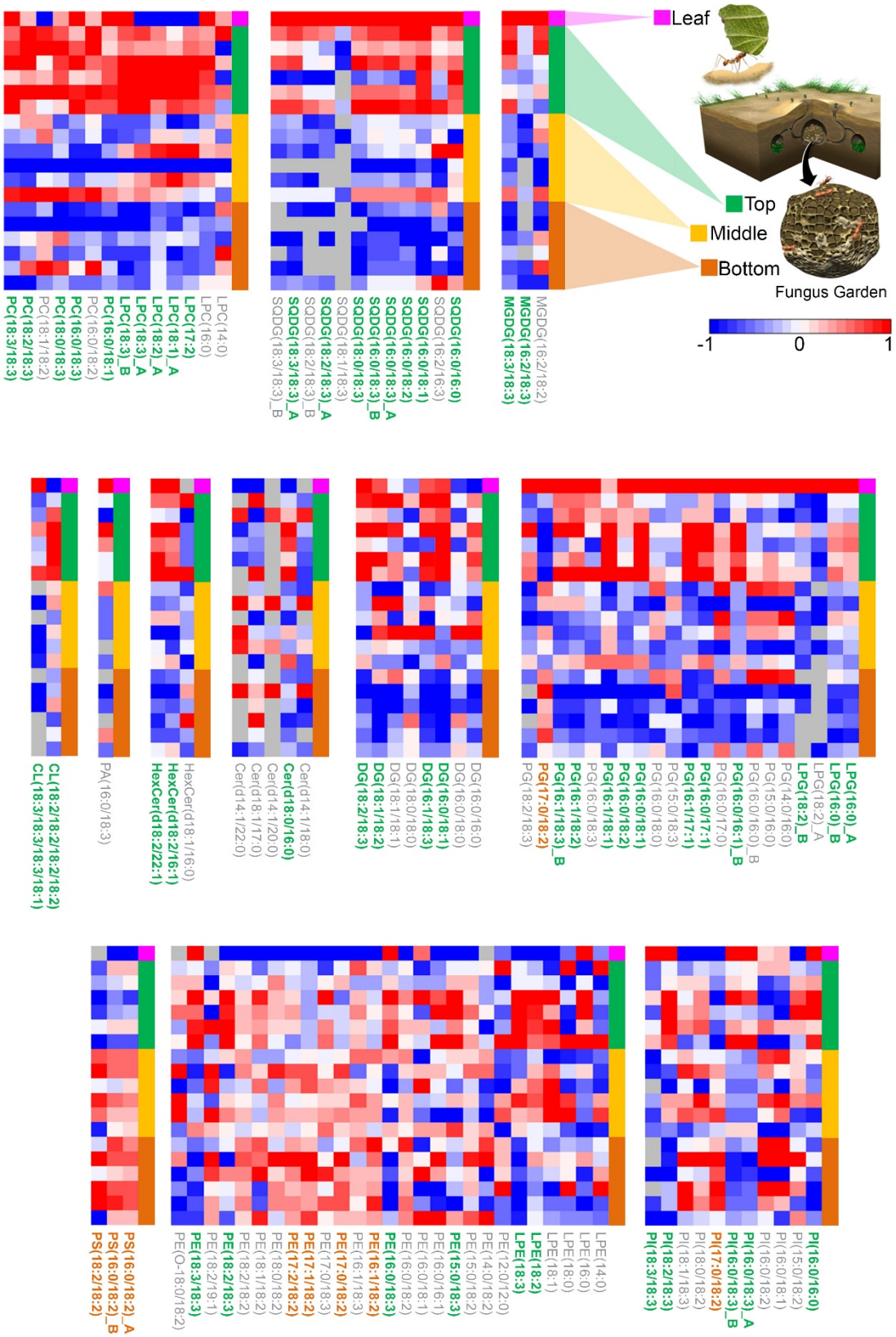
A majority of the lipids undergoing the greatest amount of log2 fold change per subclass from the top to the bottom strata of the six fungal gardens contained 18:3. For PCs, PEs, and PIs, the greatest decrease was seen with an 18:3 in both sn-1 and sn-2 fatty acid positions. This may also be true for DGs, however, that particular lipid was found to be co-eluting with DG(18:2/18:4). Both LPC(18:3) and LPE(18:3) were also undergoing the greatest decrease in their subclasses. Lipids containing a 16:0/18:3 underwent a considerable fold change with those attached to SQDG undergoing the greatest decrease and the second most for PCs, PEs, and PIs.

Strong trends in the degree of fold change were noted with chain length and degree of unsaturation for TGs (Fig. X). Almost no change in TGs with total fatty acids carbon lengths from C42-48 were detected. For those with C49 and C51, C52 and C54 as the number of total double bonds increased, the log2 fold change from top to bottom became more extensive. In other words, lipids with more polyunsaturated lipids (PUFA) decreased to a much larger extent from the top to

bottom verses the middle to bottom. Based on these results we hypothesized that 18:2 could be enriched in the Gongylidia.

Supplemental Figure A4.1 Heatmaps representing the relative log2 intensity change for lipids in the leaf food source and the top, middle, and bottom strata of six leaf-cutter ant fungus gardens. Lipids significantly increased in the top are denoted with green font; lipids significantly increasing in the bottom are denoted with orange font (p-values <0.05 were deemed significant); lipids not changing across the top to the bottom of the garden are denoted with grey font; _A and _B denote structural isomers. Diacylglycerophosphocholines (PC), monoacylglycerophosphocholines (LPC), sulfoquinovosyldiacylglycerol (SQDG), Monogalactosyldiacylglycerol (MGDG), Cardiolipin (CL), phosphatidic acid (PA), Hexosyl-Ceramide (HexCer), Ceramide (Cer), diacylglycerophosphoglycerol (PG), diacylglycerophosphoserine (PS), diacylglycerophosphoethanolamines (PE), monoacylglycerophosphoethanolamines (LPE), diacylglycerophosphoinositols (PI). Lipid abbreviations show the total number of acyl chain carbons: total number of double bonds.

Supplemental Figure A4.2 Heatmaps representing the relative log2 intensity change for lipids in the leaf food source and the Gongylidia and surrounding fungus garden of six leaf-cutter ant fungus gardens. Lipids significantly enriched in the Gongylidia compared to the surrounding fungus garden are denoted with purple font; lipids significantly enriched in the fungus garden are denoted with yellow font (p-values <0.05 were deemed significant); lipids not changing across the top to the bottom of the garden are denoted with grey font; _A and _B denote structural isomers. Diacylglycerophosphocholines (PC), monoacylglycerophosphocholines (LPC), sulfoquinovosyldiacylglycerol (SQDG), Monogalactosyldiacylglycerol (MGDG), Cardiolipin (CL), phosphatidic acid (PA), Hexosyl-Ceramide (HexCer), Ceramide (Cer), diacylglycerophosphoglycerol (PG), diacylglycerophosphoserine (PS), diacylglycerophosphoethanolamines (PE), monoacylglycerophosphoethanolamines (LPE), diacylglycerophosphoinositols (PI). Lipid abbreviations show the total number of acyl chain carbons: total number of double bonds.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Middle
Bottom
Fungus Garden
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MGDG(16:2/18:3)
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SQDG(16:0/18:1)
SQDG(16:0/18:2)
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SQDG(16:0/18:3)_B
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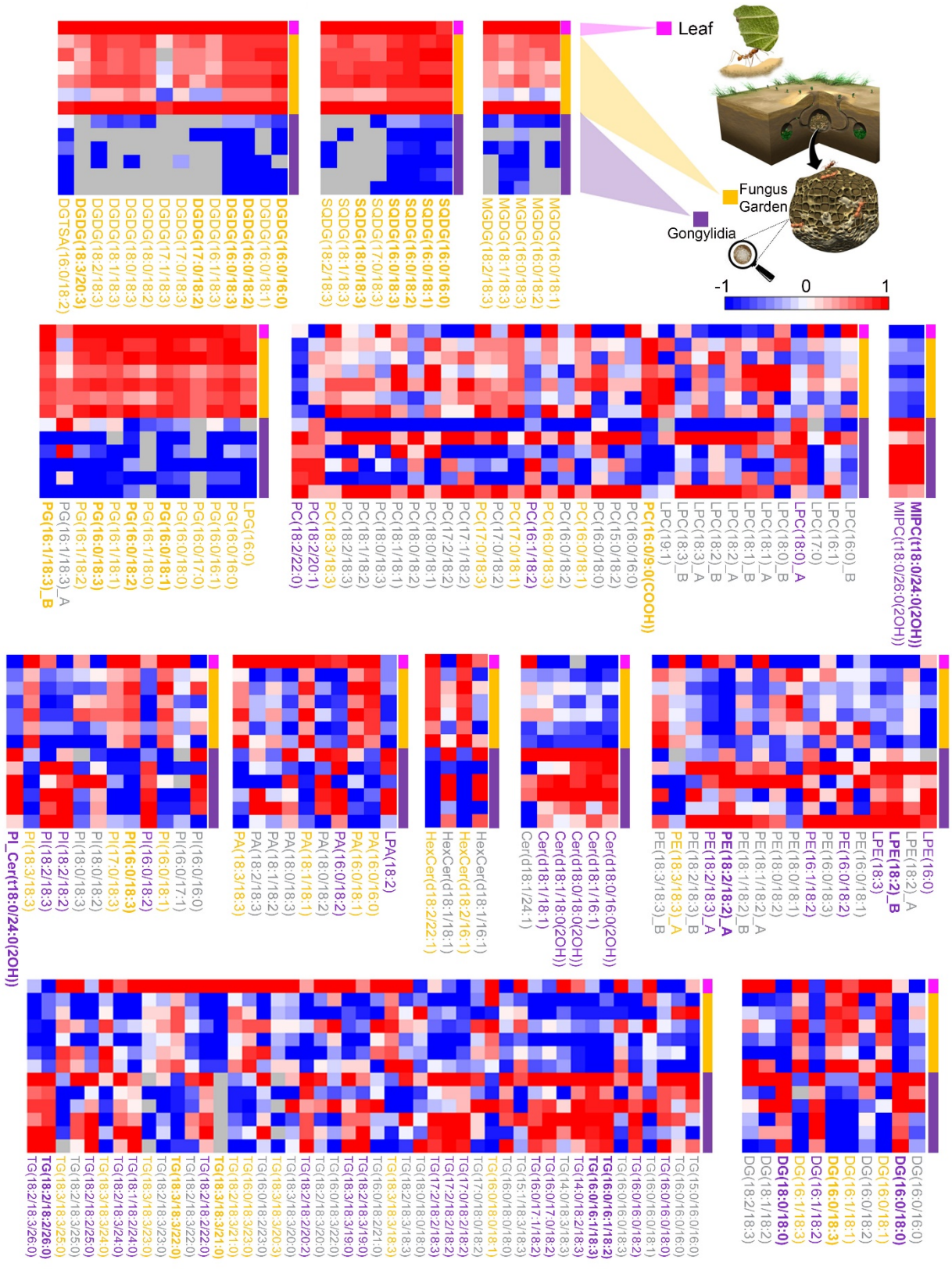
Leaf
Fungus Garden
Gongylidia
-1
0
1
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MIPC(t18:0/26:0(2OH))
LPC(16:0)_B
LPC(16:1)
LPC(17:0)
LPC(18:0)_A
LPC(18:0)_B
LPC(18:1)_A
LPC(18:1)_B
LPC(18:2)_A
LPC(18:2)_B
LPC(18:3)_A
LPC(18:3)_B
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TG(18:3/18:3/24:0)
TG(18:2/18:2/25:0)
TG(18:2/18:3/25:0)
TG(18:3/18:3/25:0)
TG(18:2/18:2/26:0)
TG(18:2/18:3/26:0)

A4.4 References

Aylward FO, Burnum KE, Scott JJ *et al.* (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. *The ISME Journal*, **6**, 1688–1701.

Aylward FO, Burnum-Johnson KE, Tringe SG *et al.* (2013) *Leucoagaricus gongylophorus* produces diverse enzymes for the degradation of recalcitrant plant polymers in leaf-cutter ant fungus gardens. *Applied and Environmental Microbiology*, **79**, 3770–3778.

Aylward FO, Khadempour L, Tremmel DM *et al.* (2015) Enrichment and broad representation of plant biomass-degrading enzymes in the specialized hyphal swellings of *Leucoagaricus gongylophorus*, the fungal symbiont of leaf-cutter ants. *PLoS ONE*, **10**, e0134752.

Bass M, Cherrett JM (1995) Fungal hyphae as a source of nutrients for the leaf-cutting ant Atta sexdens. *Physiological Entomology*, **20**, 1–6.

Crowell KL, Slysz GW, Baker ES *et al.* (2013) LC-IMS-MS Feature Finder: detecting multidimensional liquid chromatography, ion mobility and mass spectrometry features in complex datasets. *Bioinformatics (Oxford, England)*, **29**, 2804–2805.

Dautel SE, Kyle JE, Clair G *et al.* (2017) Lipidomics reveals dramatic lipid compositional changes in the maturing postnatal lung. *Nature Publishing Group*, **7**, 40555.

De Fine Licht HH, Schiøtt M, Rogowska-Wrzesinska A *et al.* (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences*, **110**, 583–587.

Farji-Brener AG, Medina CA (2000) The importance of where to dump the refuse: Seed banks and fine roots in nests of the leaf-cutting ants *Atta cephalotes* and *A. colombica*. *Biotropica*, **32**, 120–126.

Holldobler B, Wilson EO (2010) *The Leafcutter Ants: Civilization by Instinct*. W.W. Norton and Company, New York, NY.

Khadempour L, Burnum KE, Baker ES *et al.* (2016) The fungal cultivar of leaf-cutter ants produces specific enzymes in response to different plant substrates. *Molecular Ecology*, **25**, 5795–5805.

Kyle JE, Crowell KL, Casey CP *et al.* (2017) LIQUID: an-open source software for identifying lipids in LC-MS/MS-based lipidomics data. *Bioinformatics (Oxford, England)*, **33**, 1744–1746.

Matzke MM, Waters KM, Metz TO *et al.* (2011) Improved quality control processing of peptide-centric LC-MS proteomics data. *Bioinformatics (Oxford, England)*, **27**, 2866–2872.

North RD, Jackson CW, Howse PE (1997) Evolutionary aspects of ant-fungus interactions in leaf-cutting ants. *Trends in Ecology & Evolution*, **12**, 386–389.

Pluskal T, Castillo S, Villar-Briones A, Oresic M (2010) MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC bioinformatics*, **11**, 395.

Quinlan RJ, Cherrett JM (1979) The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). *Ecological Entomology*, **4**, 151–160.

Zimmer J, Monroe ME, Qian WJ, Smith RD (2006) Advances in proteomics data analysis and display using an accurate mass and time tag approach. *Mass Spectrometry Reviews*, **25**, 450–482.