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

Lily Khadempour¹²³, Huan Fan¹, Ken Keeover-Ring⁴, Camila Carlos¹³, Nilson S. Nagamoto⁵, Miranda A. Dam⁶, Monica T. Pupo⁷, Cameron R. Currie¹³

Affiliations:
¹ Department of Bacteriology, University of Wisconsin-Madison, Madison, WI, USA, 53706
² Department of Integrative Biology, University of Wisconsin-Madison, Madison, WI, USA, 53706
³ Department of Energy Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI, USA, 53706
⁴ Departments of Botany and Geography, University of Wisconsin-Madison, Madison, WI, USA, 53706
⁵ Department of Plant Protection, UNESP – São Paulo State University, Botucatu, SP, Brazil, CEP: 18603-970
⁶ Department of Nutritional Sciences, University of Wisconsin-Madison, Madison, WI, USA 53706
⁷ School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, CEP: 14040-903

Conceived of the project (L.K., C.R.C), performed computational and statistical analysis (L.K., H.F., C.C.), analyzed data (L.K.) designed sampling methodology (L.K.) performed lab work (L.K., M.A.D., K.K.), performed field work (L.K., N.S.N.) provided resources and facilities (M.T.P., C.R.C) and wrote the manuscript (L.K., H.F., K.K., C.R.C).
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 Pantoa, 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.
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, *Leucaagaricus gongylodes*, 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). Leucaagaricus 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.

Methods

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. bisphera 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.

**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 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).

**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.

**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.

**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.).

**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.).

**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).

Results

Metagenomic statistics
A summary of metagenome statistics is presented in (Table 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.

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%) (Supplemental Figure 1). Between fungus gardens, genus-level comparisons showed greater variability than phylum-level comparisons (Figure 2, Figure 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). 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, Empticia, Serratia and Klebsiella. DESeq2 analysis revealed that six bacterial genera (Entoplasma, Flavobacterium, Mesoplasma, Pantoaea, 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.

Bacterial functional analysis

Overall, we found significant differences in the predicted bacterial community functional profiles between grass- and dicot-cutter ant fungus gardens (Figure 4). All individual bacterial genes that were significantly different between grass- and dicot-cutter ant fungus gardens are listed in Supplemental Table 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 (Supplemental Table 2, Supplemental Figures 4-6). 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 5) and had a significantly higher abundance of a gene in the nitrogen fixation pathway, nitrogenase molybdenum-iron protein beta chain (Supplementary Table 2). Dicot-cutter ant fungus gardens were particularly enriched in membrane transport genes (Figure 5).

Plant taxonomy and consistency

The incorporated plant material was markedly different in consistency between the fungus gardens. Atta bipropria and A. atpignana gardens both contained material that was
clearly grass, which was not mulched (Figure 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 1). We detected 68 plant species based on the *MatK* gene query in the metagenomes, from 40 genera and 15 families (Table 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).

Iron content

The iron content of the fungus gardens is displayed in Figure 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.

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 \((1\rightarrow3),(1\rightarrow4)-\beta\)-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 implicated 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*) that was observed cutting considerable amounts of eucalyptus (Supplemental methods and Supplemental Figure 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, glutathione 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 1). We expected that since dicot-cutter ants incorporate a higher diversity of plants into their gardens (Table 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) we did not see a significant enrichment of plant defense compound degradation genes in these gardens (Figure 5, Supplemental Table 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), 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 (Supplemental Table 1). Other genes that are related to nutrient acquisition are also significantly more abundant in the grass-cutter ant fungus gardens (Figure 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 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 5, Supplemental Figure 2).

It is important to consider this system, not only from the perspective of the bacteria, but also that of the ants. One may ask: if grasses are so poor in nutrients, why do the ants use them as a substrate at all? 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 recaitrance 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) 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.

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Table 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           | 330013023        | 1-Feb-15        | S22°50'47.7"   | W48°26'9"    |
| *A. bisphaerica* 2     | Grass           | 330013025        | 3-Feb-15        | S22°50'48.4"   | W48°26'14"   |
| *A. bisphaerica* 3     | Grass           | 330013022        | 3-Feb-15        | S22°50'48.4"   | W48°26'23"   |
| *A. capiguara* 1      | Grass           | 330012994        | 2-Feb-15        | S22°50'32.1"   | W48°18'28.7" |
| *A. capiguara* 2      | Grass           | 330012996        | 3-Feb-15        | S22°50'47.2"   | W48°26'13"   |
| *A. capiguara* 3      | Grass           | 330012997        | 3-Feb-15        | S22°50'47.6"   | W48°26'12"   |
| *A. lariquita* 1      | Dicot           | 330013000        | 20-Jan-15       | S21°50'47.5"   | W48°19'31.3" |
| *A. lariquita* 2      | Dicot           | 330012995        | 17-Jan-15       | S21°50'33.5"   | W47°50'47.7" |
| *A. lariquita* 3      | Dicot           | 330012999        | 19-Jan-15       | S21°50'36.8"   | W47°50'50.7" |
| *A. sexdens* 1        | Dicot           | 330013001        | 30-Jan-15       | S21°50'53.4"   | W47°51'10.5" |
| *A. sexdens* 2        | Dicot           | 330013002        | 30-Jan-15       | S21°50'53.4"   | W47°51'10.5" |
| *A. sexdens* 3        | Dicot           | 330013001        | 31-Jan-15       | S21°50'53.4"   | W47°51'10.5" |

*While *A. lariquita* 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 2 Metagenome sequencing statistics for leaf-cutter ant fungus gardens

| Leaf-cutter ant colony | Scaffold total (Mb) | Main genome scaffold N/1.50 | Main genome scaffold length (Kbp) | Max scaffold length (Kbp) | Scaffolds > 50 | Aligned reads Protein coding genes |
|------------------------|--------------------|-----------------------------|-----------------------------------|---------------------------|----------------|-------------------------------|
| *A. bisphaerica* 1     | 628724             | 122506/740                  | 467228/298                        | 249.52                    | 93             | 16318512 (97.42%)             |
| *A. bisphaerica* 2     | 939707             | 148570/926                  | 680177/292                        | 253.54                    | 69             | 14840692 (96.91%)             |
| *A. bisphaerica* 3     | 285649             | 29401/972                   | 244722/247                        | 187.50                    | 49             | 16716901 (98.76%)             |
| *A. capiguara* 1      | 205334             | 16608/1403                  | 178475/247                        | 273.83                    | 37             | 19900936 (98.99%)             |
| *A. capiguara* 2      | 345357             | 330130/1420                 | 308567/247                        | 180.27                    | 34             | 20477290 (98.70%)             |
| *A. capiguara* 3      | 573737             | 83938/790                   | 508567/247                        | 135.51                    | 13             | 20367906 (98.79%)             |
| *A. lariquita* 1      | 85367              | 178678/686                  | 645038/301                        | 274.98                    | 87             | 17816164 (96.86%)             |
| *A. lariquita* 2      | 295365             | 32897/990                   | 268628/247                        | 252.44                    | 92             | 18965904 (96.88%)             |
| *A. lariquita* 3      | 601593             | 74824/1744                  | 398992/340                        | 241.71                    | 17             | 20953750 (96.06%)             |
| *A. sexdens* 1        | 674609             | 48220/3118                  | 412923/341                        | 701.43                    | 167            | 15614862 (97.92%)             |
| *A. sexdens* 2        | 877038             | 65552/2346                  | 548662/328                        | 61.96                     | 17             | 15089208 (95.32%)             |
| *A. sexdens* 3        | 1006806            | 221493/614                  | 772141/277                        | 386.55                    | 68             | 18097643 (91.63%)             |

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Table 3 Plant genera detected in each fungus garden sample using *MatK* gene

| Sample         | Family      | Genus     | Species | Match % |
|----------------|-------------|-----------|---------|---------|
| *A. bisphaerica* 1 | Fabaceae    | Chamaecrista | 99.3    |         |
|                 | Poaceae     | Paspalum   | 99.6    |         |
|                 | Poaceae     | Paspalum   | 99.3    |         |
| *A. bisphaerica* 2 | Poaceae     | Paspalum   | 99.4    |         |
|                 | Fabaceae    | Chamaecrista | 99.3    |         |
| *A. bisphaerica* 3 | Fabaceae    | Zania      | 100     |         |
|                 | Poaceae     | Paspalum   | 99.5    |         |
|                 | Poaceae     | Paspalum   | 99.0    |         |
| *A. capiguara* 1  | Poaceae     | Paspalum   | 99.7    |         |
| *A. capiguara* 2  | Poaceae     | Paspalum   | 99.6    |         |
| *A. capiguara* 3  | Fabaceae    | Chamaecrista | 99.3    |         |
| *A. laevigata* 1  | Poaceae     | Paspalum   | 99.7    |         |
| *A. laevigata* 2  | Poaceae     | Paspalum   | 99.6    |         |
| *A. laevigata* 3  | Fabaceae    | Zania      | 100     |         |
| *A. sexdens* 1    | Poaceae     | Paspalum   | 99.7    |         |
| *A. sexdens* 2    | Poaceae     | Paspalum   | 99.6    |         |
| *A. sexdens* 3    | Poaceae     | Paspalum   | 99.7    |         |
Figure captions

Figure 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 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 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 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 5 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 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.
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ANOSIM $R = 0.813 \ p = 0.001$
PERMANOVA $p = 0.001$
NMDS stress $= 0.075$

Grass-cutters

- $A. \ bisphaerica$
- $A. \ capiguara$

Dicot-cutters

- $A. \ laevigata$
- $A. \ sexdens$
ANOSIM R = 0.422  p = 0.006
PERMANOVA p = 0.001
NMDS stress = 0.08
A. bisphaerica  |  A. capiguara  |  A. laevigata  |  A. sexdens

Fungus garden iron concentration (mg/kg)

Grass-cutters  |  Dicot-cutters