Bacteria contribute to plant secondary compound degradation in a generalist herbivore system

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Abstract

Insects and plants engage in a multitude of complex interactions. In antagonistic cases, such as herbivory, insects often specialize on a few closely related plant species to overcome physical and chemical defences. More rarely, herbivorous insects can feed on a range of plant species. Leaf-cutter ants are generalist herbivores that forage from a variety of plant species, which the ants bring to the fungus they farm, *Leucoagaricus* sp. While we show that anti-herbivory plant compounds can harm *Leucoagaricus* sp. *in vitro*, it is unknown how the ants' fungus gardens are able to incorporate a large diversity of plants with differing plant chemistry. Here, we investigate the fungus garden bacterial community's ability to degrade plant secondary compounds. We cultured fungus garden bacteria, sequenced the genomes of 42 isolates, and found genes involved in plant secondary compound degradation, including monoterpene epsilon-lactone hydrolase. Some of these genes show *in situ* expression in metatranscriptomes, such as limonene-1,2-monooxygenase. A majority of the bacterial isolates grew unhindered in the presence of plant secondary compounds and, using GC-MS, isolates from the genera *Pseudomonas, Klebsiella, Enterobacter,* and *Bacillus* could degrade either α-pinene, β-caryophyllene or linalool. Additionally, using a headspace sampler, sub-colonies of fungus gardens reduced α-pinene and linalool over a 36-hour period, while *Leucoagaricus* sp. strains alone only reduced linalool, not α-pinene. Our study provides evidence that *Leucoagaricus* sp. has a variable ability to tolerate and degrade plant secondary compounds, indicating that it may depend on bacteria to detoxify the diversity of plant chemistry the system encounters.

41

42 **Introduction**

43 The struggle between plants trying to avoid consumption and organisms trying to exploit plants
44 for energy define herbivorous interactions and provide an arena for antagonistic coevolution[1–4].
45 Herbivorous insects, one of the most well-studied and largest taxa of herbivores, are thought to
46 have had major impacts on shaping the diversity of the plant world[5,6] and vice versa[2,7]. Plants
47 have developed physical defences, such as trichomes, surface waxes, or leaf toughness, which
48 can hinder herbivory. In response, herbivorous insects have morphological and physiological
49 adaptations for consuming plant matter, such as claws for gripping trichomes[8], producing
50 adhesive material to attach to slippery wax layers[9], and sclerotized mandibles and enlarged head
51 musculature[10] for feeding on tough plant material.

52 Plants also produce an extraordinarily diverse set of plant secondary compounds (PSC), some of
53 which are used to deter herbivores[11]. Not only are the chemicals diverse in structure and toxicity,
54 the mechanisms behind the release of plant chemicals are complex, depending on the type of
55 damage, the taxon inflicting the damage, the species of plant, and environmental conditions (e.g.
56 hours of sunlight, temperature, moisture). Additionally, the synthesis, storage, and release of
57 these compounds are variable, which can affect the rapidity and severity of a plant's response to
58 herbivory. Some chemical responses are constitutively present, with toxins being slowly released
59 through open stomata, leaf cuticles, and gland walls. Other chemicals are sequestered in
60 specialized structures and released upon herbivore damage[12]. Many plant species produce toxins,
61 like hydrogen cyanide, where the enzyme and substrate involved are compartmentalized
62 separately. Upon wounding, the two components mix and hydrogen cyanide is released[13].
63 Finally, chemicals can be released as a direct response to being induced by herbivore or pathogen

attack[14]. Herbivorous insects engage in multiple strategies to overcome chemical plant defences, including harbouring gut microbiota that aid in the detoxification of PSC. Microbes associated with mountain pine beetles[15–17], red turpentine beetles[16], pine weevils[18], gypsy moths[19], apple maggot flies[20], termites[21], and coffee berry borers[22] have been found to play an important role in PSC degradation. The role of gut microbes in PSC detoxification indicates that microbes have the potential to determine the diet range of herbivorous insects[23].

Leaf-cutter ants are dominant herbivores in most of the Neotropical ecosystems and are able to forage from a diverse array of plants. In a long-term study, active colonies of two species of leaf-cutter ants, *Atta colombica* and *Atta cephalotes*, were observed cutting leaves from 67–77% of all plant species recorded in the foraging area[24]. In a year-long study by Wirth and colleagues[25], one colony of *A. colombica* foraged from the leaves or flowers of 126 species, representing 91 genera and 52 families. In mature colonies of *Atta* sp., the ants forage ravenously, forming massive foraging columns that create distinctive trails over time (Figure 1A). The leaf-cutter ants do not directly consume the plant substrate, but rather use it to feed *Leucoagaricus* sp. (Agaricales: Agaricaceae), the obligate fungal mutualist of the ants. In return, *Leucoagaricus* sp. degrades the leaf substrate and serves as food for leaf-cutter ants, providing usable energy and nutrients in the form of specialized hyphal swellings known as gongylidia[26,27]. This process occurs in structures known as fungus gardens (Figure 1B), which are maintained in underground chambers. Mature colonies of the genus *Atta* can be composed of hundreds of fungus garden chambers, supporting the nutrition of millions of larvae, pupae, and emerged workers.

The fungus garden, which functionally serves as the ants' external gut, includes the main fungal symbiont *Leucoagaricus* sp. and a diverse and abundant community of bacteria. In contrast, the internal gut of leaf-cutter ants has a reduced bacterial community, with adult worker guts

87 containing primarily *Wolbachia* or Mollicutes[28,29]. Culturing, scanning electron microscopy[30]
88 (Figure 1C), and metagenomics of fungus gardens demonstrate a consistent presence of garden
89 bacteria and have established a core bacterial community that mostly consists of Proteobacteria,
90 the majority being in the class Gammaproteobacteria[31–36]. While there is variation in the bacterial
91 diversity and abundance between colonies and geographic regions, certain bacterial genera are
92 consistently present and readily cultured, such as *Enterobacter*, *Pseudomonas, Pantoea,*
93 *Klebsiella,* and *Burkholderia*. With the exception of *Klebsiella* and *Pantoea*, which fix nitrogen
94 for the system[37], the role of the fungal garden's bacterial community remains a mystery.
95 However, metagenomic studies have yielded clues, suggesting several potential roles, including a
96 likely lead in PSC detoxification[36].

97 In this study, we examine the ability of garden bacteria from leaf-cutter ants to metabolize PSC.
98 Focusing on bacteria isolated from fungus gardens of fungus-growing ants collected in Brazil,
99 we investigate the potential of garden bacteria to tolerate and degrade PSC. First, we isolate
100 strains of *Leucoagaricus* sp. and determine susceptibility to eight PSC. Then, we sequence the
101 genomes of 42 isolates of garden bacteria and predict the presence of genes involved in PSC
102 degradation. Additionally, we analyse previously generated garden bacteria metagenomes,
103 *Leucoagaricus* sp. genomes, and metatranscriptomes to investigate the presence and expression
104 of genes involved in PSC degradation. We also expose garden bacteria to eight PSC and
105 determine susceptibility. Then, using Gas Chromatography-Mass Spectrometry (GC-MS), we
106 quantify the *in vitro* ability of 15 isolates of garden bacteria to degrade four PSC. Finally, we
107 measure reduction of two PSC by fungus gardens from our laboratory colonies of *Atta*
108 *cephalotes* using a headspace sampler coupled to a GC.

Results and Discussion

Sensitivity of *Leucoagaricus* strains to PSC. Fungi generally have extensive degradative capabilities[38] and *Leucoagaricus* sp. is known to produce laccases, which are used to treat incoming plant material and degrade plant phenolic compounds[39]. However, plant phenolics represent one class of plant chemicals so we tested if *Leucoagaricus* sp. could tolerate eight PSC: α-pinene, β-caryophyllene, eucalyptol, farnesol, limonene, linalool, *p*-cymene, and terpinolene. These compounds have been detected in fungus gardens collected in the field[36] and/or are known to be present in plants observed being foraged by leaf-cutter ants. We focused mostly on monoterpenes because terpenes often take a prominent place in the profile of blends released by damaged plants[40]. Five different strains of *Leucoagaricus* sp. from *Atta sexdens*, *Atta laevigata*, *Atta bisphaerica*, *Atta capiguara*, and *Paratrachymyrmex diversus* colonies were tested for their ability to grow in the presence of eight different PSC (Figure 2A/B). Of note, while *P. diversus* is not within the leaf-cutter ant lineage and largely collects substrate like seeds, insect frass, and dry plant debris for its garden, the species has been observed occasionally collecting fresh leaf and flower material as substrate for its fungus garden[41,42]. The *Leucoagaricus* sp. from *P. diversus* was the most generally inhibited, with complete growth inhibition from terpinolene, eucalyptol, linalool, and *p*-cymene, and high inhibition from α-pinene and limonene. The *Leucoagaricus* sp. from *P. diversus* and *A. laevigata* colonies were also the only *Leucoagaricus* sp. that were inhibited by β-caryophyllene, a sesquiterpene. The *Leucoagaricus* sp. from an *A. sexdens* colony also exhibited high sensitivity to PSC, with complete inhibition occurring from limonene, terpinolene, eucalyptol, and linalool. The *Leucoagaricus* sp. from an *A. capiguara* colony was the most resistant to the PSC tested, only exhibiting high inhibition in the presence of

linalool, while exhibiting low to no inhibition in the presence of the remaining seven compounds (Figure 2B).

Multiple studies have investigated the toxicity of various plant compounds to the fungal cultivars of leaf-cutter ants[43–46]. LaPointe *et al.*[45] isolated *Leucoagaricus* sp. from *Acromyrmex landolti* (a species in the other genus of leaf-cutter ants) and *A. laevigata* and found that growth of the isolates was reduced when grown on agar with aqueous plant extract or leaf homogenate compared to growth of the fungus on agar alone. In another study by Howard *et al.*[46], four terpenoids were tested for potential toxic effects against *Leucoagaricus* sp. from *A. cephalotes* and found that the four compounds had variable effects on fungal growth with certain compounds having little/no effect and others inhibiting the fungus greatly. Our results, combined with previous studies, suggest that the fungal cultivars of fungus-growing ants have varying levels of resistance to PSC, indicating that other microbes may be involved in detoxification.

Gene content of bacterial isolates, garden bacteria metagenomes, garden metatranscriptomes, and *Leucoagaricus* sp. As the fungus garden is the external gut of leaf-cutter ants, the fungus gardens' microbiome likely fills roles similar to other gut microbiomes, including helping detoxify PSC. Taking advantage of the external and aerobic nature of the leaf-cutter ant fungal "gut", we cultured and sequenced bacteria from fungus gardens and tested the potential of isolates to degrade PSC. Garden bacteria were isolated from multiple fungus gardens of the two genera of leaf-cutter ants, *Atta* sp. and *Acromyrmex* sp., as well as three genera of other fungus-growing ants, *Paratrachymyrmex* sp., *Cyphomyrmex* sp., and *Apterostigma* sp., collected in Brazil (Table S1). After obtaining pure isolates, 42 bacterial genomes were sequenced (Table S2), including genera from both abundant and consistent genera (e.g., *Pseudomonas*, *Enterobacter*), as well as more sparse genera (e.g., *Chitinophaga, Bacillus*). To

assess the *in silico* potential of the 42 isolates for PSC degradation, we analysed the genomes (Figure 3A) for the presence of the diterpene degradation pathway[47], the cymene pathway (ko1220:module 00419), the saxA gene, the α-pinene/limonene degradation pathway (ko00903), the geraniol pathway (ko00281), the cumate pathway (ko01220: module 00539), the trans-cinnamate pathway (ko1220:module 00545), 20 cytochrome p450s known to be involved in the microbial transformation of PSC[48], and the squalene hopene cyclase gene (shc) (Dataset S1, Dataset S2). In addition to the 42 isolate genomes, we used the same genes and pathways to analyse 12 existing fungus garden bacteria metagenomes from Brazil (Dataset S1, Dataset S2), metatranscriptome data generated from the top portions of field-collected fungus gardens from two *Atta cephalotes* colonies and one *Atta colombica* colony (Table S3, Table S4), and two published genomes of *Leucoagaricus* sp. strains (Dataset S1, Dataset S2).

No genes belonging to the diterpene degradation cluster, the cymene pathway, or saxA were found in the 42 bacterial isolates analysed (Dataset S1, Dataset S2). However, the metagenome annotation did predict the presence of the cymene degradation pathway: one metagenome had the complete pathway, two metagenomes had ¾ of the pathway, five metagenomes had one gene from the pathway, and the remaining four had none of the genes (Dataset S2).

The α-pinene/limonene degradation pathway and geraniol pathway shared three genes between the two pathways: K01692 (enoyl-CoA hydratase), K01825 (fadB, 3-hydroxyacyl-CoA dehydrogenase), and K01782 (fadJ, 3-hydroxyacyl-CoA dehydrogenase). All isolates had at least one out of three shared genes, while one *Pantoea*, *Klebsiella*, *Enterobacter*, and *Acinetobacter* had all three. For the unique genes in the pathways (9 genes out of 12 for α-pinene/limonene and 12 genes out of 15 for geraniol), four of seven *Enterobacter* isolates had the highest proportion of unique α-pinene/limonene genes at 22%. All *Burkholderia* isolates, the *Acinetobacter* isolate,

four of seven *Enterobacter* isolates, one of four *Klebsiella* isolates, and one of 10 *Pantoea* isolates were predicted to contain the monoterpene epsilon-lactone hydrolase gene (K14731), which is involved in monocyclic monoterpene degradation[49]. Most isolates also contained an aldehyde dehydrogenase (K00128) involved in the α-pinene/limonene degradation pathway (Figure 3A). In the 12 metagenomes, the proportion of genes present from the α-pinene/limonene pathway ranged from 22% to 56% (Dataset S2). Additionally, we detected the expression of certain genes in the α-pinene/limonene pathway in the metatranscriptomic dataset (Table S4, Figure S2A). In the first *A. cephalotes* colony (FG1), we detected 33% of the α-pinene/limonene pathway in a range of 0.506-2831 transcripts per million (TPM), and all three shared genes in a range of 0.337-3.051 TPM. Of note, we detected limonene 1,2-monoxygenase (K14733) expression at 0.506 TPM, which is the first step of limonene transformation. In the second *A. cephalotes* colony (FG2), we detected 22% of the α-pinene/limonene pathway with 59.99 TPM and 3724 TPM, and all three shared genes in a range of 0.168-11.54 TPM. In the *A. colombica* colony (FG3), we detected 22% α-pinene/limonene pathway with 9.062 TPM and 2238 TPM, and one of the shared genes with 4.178 TPM. In all three metatranscriptomes monoterpene epsilon-lactone hydrolase (K14731) was expressed, reflecting its presence in the individual bacterial isolate genomes and confirming the gene's expression *in situ*.

For the unique genes in the geraniol pathway, *Acinetobacter* had the highest proportion of genes at 75%, while most other isolates were predicted to have between 8% and 17% of genes (Figure 3A). *Acinetobacter* contained all the genes except for the geraniol dehydrogenase (K19653, K17832) and one of the 3-hydroxyacyl-CoA dehydrogenases (K00022). The other isolates were predicted to have hydroxymethylglutaryl-CoA lyase and acetyl-CoA acyltransferase, in addition to the shared genes described above. In the metagenomic dataset, the proportion of completeness

for the geraniol degradation pathway was between 17% and 75% (Dataset S2). In the metatranscriptomic dataset (Table S4, Figure S2A), we found that FG1 expressed 50% of the geraniol pathway in a range of 0.115-25.47 TPM, including citronellol dehydrogenase (K13774) at 0.147 TPM, which is involved in the first step of geraniol and citronellol transformation. In FG2, we detected 42% of the geraniol pathway in a range of 0.033-27.18 TPM and in FG3, we detected 16% of the geraniol pathway with 1.334 TPM and 9.208 TPM.

Ten of the isolates contained one gene from the cumate pathway, with seven *Burkholderia* and two *Pseudomonas* isolates predicted to contain different components of the p-cumate 2,3-dioxygenase enzyme (K16303 or K16304) and one *Pantoea* isolate predicted to have 2,3-dihydroxy-p-cumate/2,3-dihydroxybenzoate 3,4-dioxygenase (K10621). On the other hand, more isolates had genes involved in the trans-cinnamate transformation pathway. Ten of twelve *Burkholderia*, all *Klebsiella*, and one of three *Pseudomonas* isolates had at least 50% of the genes necessary for cinnamate degradation (Figure 3A). Not all of the genes are necessary for a complete pathway, as there are two routes from trans-cinnamate to trans-2,3-dihydroxy-cinnamate. In the metagenomes, cumate and trans-cinnamate pathways had a range of 0%-100% and 50%-80% completeness, respectively (Dataset S2). We did not detect the expression of genes from these pathways in the metatranscriptomic dataset.

Twenty cytochrome p450s known to be involved in isoprenoid transformation were analyzed[48]. Most of the cytochrome p450s were not detected in the 42 bacterial isolates tested (Dataset S1). However, four cytochrome p450s were predicted to be present: CYP102A1, CYP106A2, CYP107H, and CYP108. CYP102A1 was detected in four out of 12 *Burkholderia* isolates, but none of the other genera (Figure 3A). CYP102A1 functions as a fatty acid hydroxylase and small mutations can result in the catalysis of oxidation of substances such as terpenes[50]. CYP106A2

was detected in *Bacillus* (ICBG1751) and is known to be a steroid hydroxylase, as well as a bacterial diterpene hydroxylase[51]. CYP107H and CYP108 were both detected in one *Pantoea* isolate (ICBG870). CYP107H is involved in a pathway that cleaves fatty acyl chains for biotin biosynthesis, while CYP108 is involved in α-terpineol hydroxylation[52]. We saw an increase in the amount of cytochrome p450s predicted to be present in the metagenomes, including CYP111, which catalyses the 8-methyl hydroxylation of linalool[53]. The other cytochrome p450s detected only in the metagenomes were CYP105A3, CYP101, CYP105A1 (Dataset S1), responsible for hydroxylation of compactin[54], oxidation of camphor[55], and hydroxylation of vitamin D3[56] and diterpene resin acids[7], respectively. We also detected the cytochrome p450s described above in the individual bacterial genomes: CYP102A1, CYP107H, CYP106A2, and CYP108 (Dataset S1). We did not detect the expression of these 20 cytochrome p450s in the metatranscriptomic dataset.

Squalene-hopene cyclase (shc) is responsible for synthesizing hopanoids, which integrate into biological membranes and increase structural order, resulting in a reduction of permeability and an increase in stability of baterial membranes[58]. The presence of this enzyme in garden bacteria isolates could explain the ability to tolerate stressful conditions, such as growth in the presence of PSC. All *Burkholderia* isolates and the *Asaia* isolate are predicted to have squalene-hopene cyclase, while none of the other isolates were predicted to contain this gene (Figure 3A). shc was detected in 11/12 metagenomes (Dataset S1).

In addition to the garden bacteria genomes, metagenomes, and metatranscriptomes, we analyzed two existing *Leucoagaricus* sp. for the same PSC degradation pathways. Overall, the two genomes, one from *Atta cephalotes* (*Leucoagaricus* sp. [Ac12]) and one from *Cyphomyrmex costatus* (*Leucoagaricus* sp. [SymC.cos]), lacked most of the gene sets analyzed (Dataset S1,

Dataset S2). This could be partially due to most of the genes used for annotation being of bacterial origin and so the annotation did not lend itself well to fungal genomes.

The presence of cytochrome p450s, α-pinene/limonene degradation genes, geraniol degradation genes, cumate degradation genes, trans-cinnamate degradation genes, *p*-cymene degradation genes, and squalene-hopene cyclase indicate that garden bacteria may be able to metabolize PSC, or at least survive in the presence of PSC. With the data available we were able to predict that both individual garden bacteria isolates and garden bacteria metagenomes contained the genes necessary to degrade or transform PSC that could harm the fungus gardens. Additionally, the higher completeness of pathways observed in the metagenomes suggests that while individual strains may not have the entire pathway for the degradation of a compound, as a community, garden bacteria have the capabilities to reduce PSC within the fungus garden. The metatranscriptomic dataset supports this hypothesis, as we found expression of genes involved in α-pinene/limonene and geraniol degradation. These metatranscriptomes came directly from field gardens, indicating that bacteria in fungus gardens in the natural environment are actively expressing genes involved in PSC/monoterpene degradation. In combination with the lack of genes predicted in *Leucoagaricus* sp. genomes within these pathways, we infer that fungus garden bacteria could be involved in detoxifying PSC based on their genomic content.

Bacterial tolerance of PSC. In addition to *in silico* analyses of the garden bacteria isolates, we assessed their tolerance by exposing garden bacteria isolates to the eight PSC described above (Figure 3B). Most bacterial isolates were able to grow uninhibited in the presence of a high concentration of the different compounds. Linalool was the most inhibitory against the bacterial isolates, causing some degree of inhibition against all isolates except *Pseudomonas* and three *Burkholderia* isolates. While none of the isolates were predicted to contain genes involved in the

degradation pathway, all isolates except for *Acinetobacter* and *Bacillus* were completely resistant to *p*-cymene, perhaps due to an alternative to degradation, such as efflux pumps. Farnesol, β-caryophyllene, and terpinolene did not inhibit the majority of bacterial isolates, causing small zones of inhibition in the *Bacillus* isolate as well as one to two other isolates. α-pinene and limonene caused slightly more inhibition, especially in *Bacillus* and two *Klebsiella* isolates, but most bacteria were resistant or only slightly susceptible to these two compounds. These trends were also seen in a larger set of samples (Figure S1). Like the tolerance of *Leucoagaricus* sp., bacterial isolates have varying susceptibilities to PSC.

***in vitro* degradation of PSC by bacterial isolates.** Fifteen bacterial isolates that represent the diversity of garden bacteria across multiple fungus-farming ant lineages, as well as mostly being from genera that are consistently abundant between fungus gardens, were grown in liquid culture supplemented with one of four PSC (α-pinene, β-caryophyllene, eucalyptol or linalool) and degradation was assessed with GC-MS. To account for isolates unable to grow in the presence of certain PSC, we grew all bacterial isolates in two ways: (A) adding the compound during exponential phase (Figure 4) and (B) adding the compound after stationary phase (Figure S3). All *Enterobacter* isolates (ICBG810, $p=0.0004$; ICBG643, $p=0.0018$; ICBG832, $p=0.0062$) significantly reduced α-pinene (*t*-test, Bonferroni correction: $\alpha=.0033$) during exponential growth. Also, one of two *Klebsiella* isolates (ICBG873, $p=0.0026$) and one of two *Bacillus* isolates (ICBG1751, $p=0.0008$) significantly reduced α-pinene. Additionally, other bacterial isolates showed a varying range of reductions of α-pinene between vials, resulting in large variability and lack of significance with the Bonferroni corrected alpha-value. No isolates significantly reduced α-pinene within stationary growth. One of three *Pseudomonas* isolates (ICBG639, $p<0.0001$) significantly reduced β-caryophyllene in the exponential environment. The same *Pseudomonas*

292 isolate (ICBG639, p=0.0014) also significantly reduced β-caryophyllene at the stationary phase.
293 Linalool was reduced by two isolates, *Burkholderia* (ICBG637; exponential p=0.0036, stationary
294 p=0.0008) and *Pseudomonas* (ICBG967; exponential p=0.0020, stationary p=0.0024). Finally,
295 eucalyptol, the fourth compound tested, was not reduced by any of the isolates tested (Figure S4).
296 In all cases, no breakdown products were detected by GC-MS, which could be due to complete
297 degradation of the compounds into components of central metabolism[59,60].

298 Some genera isolated, such as *Pseudomonas* and *Burkholderia,* have been implicated in plant
299 compound degradation in other systems, such as in bark beetles and mountain pine beetles[16,61]. In
300 the current study, two isolates of *Pseudomonas* were found to significantly reduce either β-
301 caryophyllene or linalool *in vitro*. In addition to *Pseudomonas*, in the bark beetle system, *Serratia*
302 isolates were able to reduce 3-carene and (-)-β-pinene and *Rahnella* isolates were able to reduce
303 3-carene, (-)-α-pinene, (+)-α-pinene and (-)-β-pinene[16]. While *Serratia* and *Rahnella* were not
304 cultured in our study, metagenomic and culturing studies from fungus gardens of leaf-cutter ants
305 have detected *Serratia* (Figure S5) and *Rahnella*[32,33,36], suggesting that other isolates within fungus
306 garden bacterial communities could be involved in metabolizing PSC that simply were not tested
307 in this study.

308 **Fungus gardens exposed to α-pinene or linalool did not experience a shift in bacterial**
309 **community composition.** We exposed sub-colonies of *Atta cephalotes* to a low and high dose of
310 α-pinene or linalool for 48 hours and then extracted bacterial DNA for 16S rRNA amplicon
311 sequencing to determine if there was a shift in bacterial community, as previously observed for
312 mammalian herbivore gut microbiomes[62]. At the doses and exposure tested, we did not see a
313 change in abundance of certain expected community members known to degrade PSC (i.e.,
314 *Pseudomonas*), compared to the control sub-colonies (data not shown). However, the bacterial

315 genera observed are the same as genera observed from the fungus gardens of leaf-cutter ants
316 collected and immediately processed in the field[31–33,36] (Figure S5). The colonies used in the
317 experiments have been in lab for approximately 7 years (RM120223-02), 5 years (CR14) and 1
318 year (CF180406-01, CF180405-02, HH180403-03), which indicates that fungus garden bacterial
319 members – at the genus-level – are maintained even with a large change in environment: tropical
320 rainforest in Costa Rica to a controlled lab setting in Wisconsin. Specifically, we confirmed that
321 the genera we cultured directly in the field for our *in vitro* assays were found in our laboratory
322 colonies. All isolates tested in the GC-MS experiment that had been cultured from the Brazil
323 colonies were detected in the 16S data except for *Bacillus* (i.e. *Burkholderia, Pantoea,*
324 *Pseudomonas, Enterobacter,* and *Klebsiella* were present at varying abundances (Figure S5B).
325 For our subsequent experiments (described below), this means we can assume that our results are
326 relatable to environmental fungus gardens.

327 **Degradation of α-pinene and linalool by *Atta cephalotes* fungus gardens.** To examine if our
328 *in vitro* determination of PSC tolerance and reduction reflects an *in vivo* role in fungus garden
329 and the associated bacteria's behaviour in the environment, we use sub-colonies of fungus
330 gardens from *Atta cephalotes* colonies and measured headspace concentrations of two PSC, α-
331 pinene and linalool (i.e., the compounds that were degraded by garden bacteria and largely
332 inhibited *Leucoagaricus* growth). The headspace of the control vials was compared to the
333 headspace of vials containing fungus gardens and compound. When sub-colonies from three *A.*
334 *cephalotes* colonies were exposed to α-pinene (Figure 5A), there was significant reduction of α-
335 pinene in the fungus garden samples at 12 hours (only α-pinene vs fungus garden + α-pinene,
336 $p<0.0001$; cotton + α-pinene vs fungus garden + α-pinene, $p=0.1191$), 24 hours (only α-pinene
337 vs fungus garden + α-pinene, $p<0.0001$; cotton + α-pinene vs fungus garden + α-pinene,

338 $p<0.0001$) and 36 hours (only α-pinene vs fungus garden + α-pinene, $p<0.0001$; cotton + α-
339 pinene vs fungus garden + α-pinene, $p<0.0001$), compared to most control vials (mixed
340 regression model with time and treatment as fixed effects and ant colony as random effects, $\alpha =$
341 0.05). Additionally, the 36-hour sub-colonies had significantly reduced α-pinene compared to
342 the 12-hour sub-colonies ($p<0.0001$) and the 24-hour sub-colonies ($p<0.0001$), suggesting active
343 metabolizing by the fungus garden over this time period. *Leucoagaricus* sp. strains were also
344 tested in a similar fashion. Vials containing *Leucoagaricus* sp. were exposed to α-pinene and the
345 headspace was measured after 36 hours of exposure (Figure 5B). Compared to the control vials,
346 *Leucoagaricus* sp. strains did not reduce α-pinene significantly ($p=0.2786$, Welch two sample *t*-
347 test).

348 When sub-colonies from five *A. cephalotes* colonies were exposed to linalool (Figure S6A),
349 there was a significant reduction of linalool in the headspace between controls and 12-hour sub-
350 colonies (only linalool vs fungus garden + linalool, $p<0.0001$; cotton + linalool vs fungus garden
351 + linalool, $p=0.0728$), 24-hour sub-colonies (only linalool vs fungus garden + linalool, $p<0.0001$;
352 cotton + linalool vs fungus garden + linalool, $p=.0021$), and 36-hour sub-colonies (only linalool
353 vs fungus garden + linalool, $p<0.0001$; cotton + linalool vs fungus garden + linalool, $p=0.0049$).
354 However, we do not see any significant differences between the 12-hour, 24-hour, and 36-hour
355 linalool amounts, suggesting there may be a limit to the degradation possible with this compound
356 that was reached at 12 hours post-exposure. For the headspace sampling with *Leucoagaricus* sp.,
357 linalool was significantly reduced ($p=0.0036$, Welch two sample *t*-test), which was surprising
358 due to the high inhibition by this compound in the plate assay (Figure 2B, Figure S6B). This
359 could be due to a difference in dosage, which has been shown to have an effect on bacterial
360 tolerance and degradation of PSC[17]. The tolerance assays contained high amounts of compound,

whereas the headspace sampling had lower amounts of compound and *Leucoagaricus* sp. was allowed to obtain a higher biomass (grown for longer on PDA). Additionally, while we saw significant reduction of linalool by *Leucoagaricus* sp., strains from CF180405-02, CF180406-01, and AB1 had linalool amounts that were encompassed by the amount found in the control. Therefore, we conclude that *Leucoagaricus* sp. has variable abilities to tolerate and degrade common PSC at the strain level. Overall, the observed reductions of PSC indicate that the fungus gardens of leaf-cutter ants have the capability to reduce PSC that enter the system and *Leucoagaricus* sp. may depend on other microbes to aid in detoxifying these compounds.

Conclusions. In addition to the important morphological and physiological features of insects and plants, both organisms form symbioses with microorganisms to outcompete one another. In herbivorous insect systems, gut bacteria can be imperative in determining the plant range of its host. This study shows that bacteria associated with the fungus gardens of leaf-cutter ants can metabolize PSC, potentially enabling leaf-cutter ants to forage from a greater variety of plant sources. While earlier work with metagenomic sequencing in leaf-cutter ant fungus gardens showed the potential of the bacteria to degrade anti-herbivory compounds, our work combines *in silico, in vitro*, and *in vivo* evidence that indicates that garden bacteria can degrade PSC, some of which may otherwise inhibit the growth of *Leucoagaricus* sp. Like other herbivorous insect systems, the gut microbiome of leaf-cutter ants is demonstrably important for dictating palatable plant substrate, with the unique feature of the ants' gut being external in the form of fungus gardens. Overall, this study demonstrates the intricacy and nuance with which microbes serve as an interface between herbivores and the plants they consume.

Materials and Methods

Fungal tolerance assay. We selected compounds for testing based on leaf extracts from plant families that have been foraged by leaf-cutter ants[25], detection of terpenes in fungus gardens of *Atta laevigata*[36], and commercial availability: 98% (1R)-(+)-α-pinene (Acros Organics), >90% β-caryophyllene (TCI), 99% eucalyptol (Sigma-Aldrich), 95% farnesol(Sigma-Aldrich), 96%(S)-(-)- limonene (Sigma-Aldrich), 97% linalool (48.2% (R)-(-)-linalool/51.8% (S)-(+)-linalool) (Sigma-Aldrich), 99+% *p*-cymene (Acros Organics), and 85% terpinolene (Sigma-Aldrich). The eight PSC were tested against five strains of fungal cultivar from *Atta sexdens*, *Atta laevigata*, *Atta bisphaerica*, *Atta capiguara*, and *Paratrachymyrmex diversus* colonies (isolation information in Table S1). A 6 mm fungal plug of *Leucoagaricus* sp. was put onto a 60 mm Oxoid Malt Extract Agar (OMEA; per L: 30 g malt extract, 5 g mycological peptone, 15 g agar) plate and allowed to grow for two weeks. Then, for each PSC, the cultivar was exposed to a sterile disc with 15 µL of compound that had been allowed to dry in a biological safety hood for five min. The edges of fungal growth and the edge of the disc were 1 cm apart. Each compound was done in triplicate (three plates per plant defence compound per cultivar) and inhibition was monitored over the course of two weeks, with pictures being taken on day 2 and 14. Inhibition was determined by a qualitative scale where 0 =no inhibition, 1=no/slight inhibition at day 2 and resume normal growth by day 14(compared to control), 2=no/slight inhibition at day 2 and resume slow growth by day 14 (compared to control), 3= mostly inhibited by day 2, no additional growth by day 14, 4=complete inhibition at day 2 and day 14 (Figure 2A).

Sampling and bacterial isolations. Fungus-farming ant colonies were collected in January 2017 in the following general locations: Anavilhanas, AM; Ducke Reserve, AM; Itatiaia, RJ; Botucatu, São Paulo State; Ribeirão Preto, São Paulo State. Details regarding the exact GPS

406 coordinates and environment of the samples can be found in Table S1. In lab, pure isolates were
407 obtained after several rounds of subculturing based on morphology. A total of 317 isolates were
408 obtained. We identified 117 isolates to genus-level by 16S rRNA sequencing as previously
409 described[63]. Briefly, colonies were lysed and PCR was performed with 16S rRNA primers 27F
410 (5'- GAG AGT TTG ATC CTG GCT CAG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT
411 T-3'). Samples were sequenced using Sanger sequencing at the University of Wisconsin –
412 Madison Biotech Center (Madison, WI) and analysed using 4Peaks and CLC Sequence Viewer
413 7. The 16S rRNA gene sequence were matched using BLAST[64] and the SILVA database
414 (https://www.arb-silva.de) for the nearest genus-level identification.

415 **DNA Extraction, Assembly, and Annotation.** DNA from 42 bacterial isolates was extracted
416 using the Promega Wizard Genomic DNA Purification Kit using the Gram-Negative and Gram-
417 Positive Bacteria Protocol. Quality control was done using Qubit BR dsDNA kit (Invitrogen,
418 USA). Genomic DNA libraries for Illumina MiSeq 2x300bp paired-end sequencing were
419 prepared by the University of Wisconsin- Madison Biotechnology Center. Reads were corrected
420 with MUSKETv1.1, paired-ends were merged with FLASH v1.2.7, and assembled with SPAdes
421 3.11.0. Genome statistics can be found in Table S2. Species-level identification was determined
422 by uploading the genomes to JSpeciesSW, performing a Tetra Correlation Search, and taking the
423 first result. If there were conflicts in the top 5 results (i.e. different genera), the top 5 genomes
424 were pulled and ANI with pyani (github.com/widdowquinn/pyani) using the ANIm analysis was
425 performed. Then, the genome with the highest percent similarity was selected as our isolate's
426 taxonomic status. Predicted proteins putatively involved in PSC degradation were identified from
427 each genome in one of two ways: 1) Using the Kyoto Encyclopedia of Genes and Genomes
428 (KEGG) Automatic Annotation Server (KAAS). Specifically, genes encoding enzymes

429 putatively involved in monoterpene degradation or aromatic compound degradation, as defined
430 in the KEGG limonene and α-pinene degradation pathway (ko00903), geraniol degradation
431 (ko00281), degradation of aromatic compounds (ko01220, modules 00419,00539, 00545) were
432 investigated after annotation. 2) DIAMOND v0.9.21.122[65] BLASTP against the Uniprot Swiss-
433 Prot and TrEMBL databases (www.uniprot.org/downloads), downloaded on July 18, 2019. We
434 only kept the top 5% of hits (--top 5) that had an e-value below 1e-05 for each query sequence.
435 Then, using the grep command, we looked for the following Uniprot accession numbers that
436 corresponded to cytochrome p450s: P00183, P14779, Q2L6S8, P18326, Q59079, Q59831,
437 Q06069, P53554, P33006, U5U1Z3, A9F9S4, Q59723, Q59990, Q9K498, Q53W59, Q8VQF6,
438 A9FZ85, Q88LH7, Q88LH5, Q88LI2, Q65A64; the 20 genes in the diterpene degradation
439 cluster: Q9X4W9, Q9X4W8, Q9X4X8, Q9X4X7, Q9X4X6, Q9X4X5, Q9X4X4, Q9X4X2,
440 Q9X4X1, Q9X4X0, Q9X4W7, Q9X4W6, Q7BRJ3, Q7BRJ4, Q7BRJ5, Q7BRJ6, Q7BRJ7,
441 Q7BRJ8, Q7BRJ9, Q9X4X; saxA: A0A0N7FW12; squalene-hopene cyclase: P33990, P54924,
442 P33247. We also performed DIAMOND BLASTP using a custom database with solely these
443 gene sequences with a query coverage cut-off of 75% (--query-cover 75) and e-value cut-off of
444 1E-05. If there was alignment in both the Uniprot analysis and the custom analysis, then genes
445 were predicted to be present. The annotation methods were used on individual bacterial
446 genomes, two publicly available *Leucoagaricus* sp. genomes (BioProject: PRJNA179280 and
447 PRJNA295288), and the publicly available leaf-cutter ant garden bacteria metagenomes from
448 Brazil (Gold Analysis Project ID: Ga0157357 – Ga0157368). For the metagenomes, we used the
449 metagenomes KAAS option instead of the complete/draft genome KAAS option.

450 **Metatranscriptomic sequencing of fungus gardens.** Samples were collected directly from the
451 field into RNAlater buffer. Samples were taken from the top sections of three different colonies:

two *Atta cephalotes* colonies from La Selva and one *Atta colombica* colony from Golfito (Table S3). Total RNA extraction was identical to a method previously described[66]. cDNA library construction and Illumina HiSeq2000 sequencing were performed at the University of Wisconsin Biotechnology Center (Madison, WI). Metatranscriptomes were uploaded to MG-RAST and processed with their SOP[67]. We downloaded the reads post-processing (quality reads) and then metatranscriptomes were analysed using prodigal V2.6.2, DIAMOND v0.9.21.122, and kallisto v. 0.43.1[68]. First, we ran prodigal on the assembled fna files of the garden bacteria metagenomes (downloaded from JGI) with the metagenomic flag (-p meta). Then, a kallisto index was created with all of the combined prodigal garden bacteria metagenome output. The kallisto quant command was used to pseudo-align the garden bacteria index against the metatranscriptome reads. This gave a transcripts per million (TPM) value of bacterial transcripts in the metatranscriptome. Then, we used DIAMOND to blastp the metagenome coding regions against the Uniprot and KEGG databases described above. Using grep, we found the genes of interest (same as in the bacteria isolate annotation) and connected the gene of interest, the metagenomic transcript it mapped to, and the TPM in the metatranscriptome. For genes with multiple transcripts and different TPMs, we recorded the unique values (Figure S2) and summed the TPMs for total expression (Table S4). We also did the same workflow for four housekeeping genes (*gyrB*: K02470, *rpoB*: K03043, *rpoD/sigA*: K02086, *rpsL*: K02950).

***in vitro* garden bacterial tolerance.** We tested the effect of the eight PSC on 46 bacterial isolates using Whatman 6mm discs. Bacterial isolates were grown overnight (16-24 hours) until turbid (OD_{600} ~1-2). 100µL of overnight culture was spread and plated on Yeast Malt Extract Agar (YMEA; per L: 4 g yeast extract, 10 g malt extract, 4 g dextrose, 15 g agar). A disc with 15µL of PSC was deposited in the center of the bacterial lawn. Each PSC was done in triplicate

(3 plates per plate secondary compound per bacterial isolate). After 48 hours, pictures were taken of the plates using an Epson scanner and then uploaded into Fiji[69]. Fiji Version 1.0 was used to measure the zones of inhibition caused by each PSC (in centimetres). The average of the three zones of inhibition was calculated. The zones of inhibition were all scaled in reference to the largest zone observed so that 0 indicates inhibition (zone of inhibition = 3cm) and 1 indicates no inhibition (100% growth; zone of inhibition = 0cm)

Gas Chromatography-Mass Spectrometry (GC-MS) of bacterial isolates incubated with PSC. Bacterial isolates were prepared two ways for GC-MS: addition of compound during exponential growth or stationary growth. For both methods, bacterial isolates were grown overnight (16-24 hours) in 10% tryptic soy broth (TSB). All shaking was done at 300 rpm at room temperature. All experiments included an extra vial to read the OD_{600} to ensure bacterial growth in the presence of compound, which also served to confirm earlier patterns of compound tolerance by bacterial isolates (data not shown). *(A) Exponential phase* The OD_{600} was taken to calculate how much overnight culture would be needed to reach an OD_{600} of 0.08 (i.e. C_1V_1= (0.08)(1 mL)). The appropriate amount of overnight culture was inoculated into vials containing 10% TSB and 2.5 µL/mL of one of four PSC, α-pinene, β-caryophyllene, eucalyptol, linalool, that were added using a glass manual GC syringe (10 µL, Thermo Scientific). The bacterial cells and plant compound were left shaking for another two days at room temperature. This was done with 15 bacterial isolates representing six genera, as well as negative controls (no bacteria), in triplicate (16 x 4 compounds x 3 replicates). *(B) Stationary phase* 10 µL of overnight culture was pipetted into 987.5 µL of 10% TSB in vials. After two days of incubation at room temperature while shaking at 300 rpm, 2.5 µL/mL of one of four PSC (α-pinene, β-caryophyllene, eucalyptol, linalool) were added directly to the vials using a glass manual GC

498 syringe (10 µL, Thermo Scientific). Then, the bacterial cells and plant compound were left
499 shaking for another two days at room temperature. This was done with the same number of
500 samples listed above.

501 For both method A and B, PSC were extracted by pipetting 1mL of hexane into each vial and
502 shaking the vials overnight. After the phase were allowed to separate, 500 µL of the hexane-PSC
503 phase was removed and put into new vials containing 500 µL hexane and 5 µL/mL toluene (as
504 the internal standard). We then analysed the abundance of each PSC using GC-MS. Specifically,
505 the GC system consisted of a Thermo Fisher Trace 1310 Gas Chromatograph coupled with
506 Thermo ISQ LT Single Quadrupole Spectrometer. We injected 1 µL of each mono-/sesqui-
507 terpene sample directly, with a split flow ratio of 30:1. We used an oven profile of 40°C,
508 followed by a ramp of 3°C min^{-1} to 115°C (monoterpenes) or 130°C (sesquiterpenes) and then
509 30°C min^{-1} to 250°C with a 2 min hold. Peaks were integrated and analysed using the
510 Chromeoleon Chromatography Data System Software.

511 Signal peaks from the GC were integrated and standardized based on the toluene internal
512 standard (peak area/internal standard peak area) for each vial. Additionally, we used standard
513 curves of the four pure PSC to measure changes in concentration in the samples when compared
514 to controls. Standard curves were made to incorporate the possible ranges of concentrations (0
515 µL/mL to 3.5 µL/mL) within the experiment. Proportional change of bacterial treatments versus
516 the nonbacterial control was then calculated. Specifically, we took the average of the non-
517 bacterial control standardized peak areas and subtracted the control average from all the bacteria-
518 compound peak areas. Then, we divided the adjusted value by the non-bacterial control average
519 to obtain the percent change [(bacterial standardized peak area – average of control standardized
520 peak areas)/average of control standardized peak area]. We then analysed the standardized values

521 in JMP Pro 13 by performing one-sampled Student's *t*-tests for each compound with a null
522 hypothesis of μ=0, representing no change between compound abundance in bacteria-treated and
523 the non-bacterial control. Since we were performing 15 separate statistical tests for each
524 compound (between non-bacterial control and each of the 15 bacterial isolates), we used a
525 Bonferroni correction to avoid false positives (α=.05/15=.0033).

526 **Bacterial community changes when fungus gardens are exposed to PSC.** Two different doses
527 of compound were used in this experiment: 5 μL in 5.08 cm flame-sealed 50 μL capillaries and
528 25 μL in 5.08 cm flame-sealed 100 μL capillaries. For each dose there were three treatment
529 groups: (A) control (no compound) (B) α-pinene and (C) linalool. For each treatment,
530 approximately 5 g of the top-middle layer of fungus garden were taken from the same five *Atta*
531 *cephalotes* colonies used in the headspace experiment (5 colonies x 3 treatments x 2 doses = 30
532 sub-colonies). The fungus garden piece was placed in a 60 mm glass petri dish to ensure stability
533 and to make final collection easier. Then, the fungus garden piece was placed into a 12 oz glass
534 jar with metal lids (Nakpunar, New Jersey, USA). When all fungus garden pieces had been
535 randomly assigned a treatment for each dose, glass capillaries containing either (A) nothing (B)
536 α-pinene and (C) linalool were placed in a glass tube to hold the capillary upright, then put in the
537 appropriate jar containing fungus garden.

538 *DNA extraction + 16S rRNA sequencing* After 48 hours, fungus garden pieces were collected
539 into 50 mL conical tubes and weighed. Total DNA was extracted using a bacterial enrichment
540 method previously described [32]. Briefly, the fungus garden material was homogenized using a
541 sterile mortar and pestle. Then, the homogenized material was submerged in 1X PBS containing
542 0.1% Tween 80, followed by centrifugation for 15 min at 500-700rpm. This results in a layered
543 mixture containing leaf-material and fungal mass at the bottom, and bacteria in the middle/top.

544 This washing step was repeated until the bacterial layer became more transparent (about 3-5
545 times). Then the bacterial sample was spun down, the pellet was resuspended in 1X PBS + .1%
546 Tween 80 and passed through a 40 µm filter to remove any larger, non-bacterial debris. The filter
547 was flushed with an additional 5 mL 1XPBS + .1% Tween 80 then the entire sample was spun
548 down again. Total DNA was extracted using a Qiagen DNeasy Plant Mini Kit. DNA was
549 submitted to the Biotechnology Center for library preparation and sequencing. Specifically,
550 amplicon libraries spanning the V4 region of the 16S ribosomal gene were constructed and then
551 sequenced using Illumina MiSeq 2x300bp at the University of Wisconsin – Madison
552 Biotechnology Center.

553 Sequence reads were processed, aligned, and categorized using DADA2 1.12.1[70]. The DADA2
554 pipeline (https://benjjneb.github.io/dada2/tutorial.html) was followed almost exactly in July
555 2019. The only change was made in the filtering step using altered truncLen and trimLeft
556 parameters since we sequenced 2x300bp reads (truncLen=c(225,280); trimLeft=c(10,10)). The
557 statistical analysis was performed as described in the DADA2 pipeline provided above.
558 Additional microbiome analyses, including the violin plots in Figure 5C, were adapted from
559 https://bioconductor.org/help/course-
560 materials/2017/BioC2017/Day1/Workshops/Microbiome/MicrobiomeWorkflowII.html. The
561 adapted code can be found in github.com/cfrancoeur/PSC.

562 **Headspace sampling of fungus gardens with PSC.** *Atta cephalotes* colonies (Table S5) were
563 used in this experiment to create sub-colonies. 20 mL 18 mm Restek (Bellefonte, PA, USA) vials
564 (cat#23082) with magnetic screw-thread caps (cat#23090) were prepared three ways with α-
565 pinene or linalool: (1) Empty vials with a 20 µL Accu-Fill 90 micropet cut to 2.54 cm and flame-
566 sealed containing 1 µL of PSC (n=6). (2) Vials with approximately 0.3-0.4g of cotton with a 2.54

cm flame-sealed micropet containing 1 µL of PSC (n=3). (3) Vials with approximately 0.3g of fungus garden material with all ants manually removed. Vials contained a 2.54 cm flame-sealed micropet containing 1 µL of PSC (n=3 subsamples x 3-5 different *Atta cephalotes* colonies). Three colonies were used in the α-pinene experiment and five colonies were used in the linalool experiment. Three colonies with three subsamples was determined to have enough statistical power for the observed differences. We also prepared samples of vials with only fungus garden (i.e. no exposure to PSC) during certain runs to ensure that there were no detectable PSC innate to the system. This was done for three separate time points based on exposure to a PSC: 12 hours post-exposure, 24 hours post-exposure, and 36 hours post-exposure. At these given time points, each respective set of vials were destructively sampled and analysed by a Shimadzu HS20 Headspace Sampler coupled to a Shimadzu GC-2010 Plus with a flame ionization detector. Specifically, vials were loaded into the headspace sampler and injected into a column with a 50:1 split flow ratio. For the vials with α-pinene, the headspace sampler and oven were at 60°C, followed by a 20°C/min ramp up to 140°C. For the vials with linalool, which has a higher boiling point than α-pinene, the headspace sampler and oven were kept at 70°C, followed by a 25°C/min ramp up to 205°C. Then, compounds were identified using retention time (α-pinene=3.2 minutes, linalool=6.2 minutes) and areas under the curve were calculated in Shimadzu's LabSolutions software to determine the relative difference in α-pinene or linalool between vials. α-pinene and linalool are compounds that the garden bacteria can degrade and are inhibitory against *Leucoagaricus* sp.

Since we took subsamples (sub-colonies) from each *Atta cephalotes* colony (3 subsamples x 3 time points x 5 colonies), we employed a linear mixed-effects model to account for the correlation (non-independence) between subsamples. Specifically, to test if ant colony had an

590 effect on the observed value, we used the lmer package v. 3.1-0, holding time and treatment as
591 the fixed effects and ant colony as the random effect. Before the analysis, we divided the values
592 by 1,000,000 to rescale the response for the lmer optimization procedure. For the α-pinene
593 treatment, the colony variance is reported as 0, indicating that the variability with respect to ant
594 colony is much smaller than the variability with respect to the residual error. For the linalool
595 treatment, the colony variance was 0.000226, indicating that some of the variability observed
596 was due to the sampling from different colonies. We then used the estimated marginal means
597 (EMMs) with the emmeans package v. 1.3.5 for linear regression analysis of the data, using the
598 pairs() method. Marginal means were compared pairwise between exhaustive two-way level
599 combinations of treatment (control, cotton, fungus garden) and of time (12hr, 24hr, 36hr).
600 Assumptions of normality, linearity, and homoscedasticity for linear regression were examined
601 by plot diagnostics and were met for each analysis. All the code used in this analysis is available
602 at github.com/cfrancoeur/PSC.

603 **Headspace sampling of *Leucoagaricus* sp.** *Leucoagaricus* sp. strains were isolated by plating
604 small pieces of healthy fungus garden on Potato-Dextrose Agar (PDA). Laboratory fungus-
605 farming ant colonies are kept in a temperature-controlled 28°C room in separate large plastic
606 containers. Five *Atta cephalotes* colonies collected over the course of several years (2012-2018)
607 from Costa Rica were used for this experiment. Additionally, several isolates from Brazilian *Atta*
608 gardens used in the *Leucoagaricus* tolerance experiment (Figure 2) were also included.

609 2 mL of PDA was pipetted into 20 mL 18 mm Restek vials with magnetic screw-thread caps and
610 left to solidify on a slant. Then, 3x3 mm pieces of freshly growing *Leucoagaricus* sp. strains
611 were placed onto the slant and grown for one month at room temperature in the dark. Three vials
612 were prepared for each of the *Atta cephalotes* cultivars (n=5 strains x 3 vials x 2 compounds) and

613 one vial was prepared for three additional *Leucoagaricus* sp. strains: AB1, AL2, AS1 (n=3
614 strains x 1 vial x 2 compounds). After the month of growth, 20 µL Accu-Fill 90 micropet
615 (Becton, Dickinson and Company, N.J.) cut to 2.54 cm and flame-sealed were filled with (A)
616 nothing (B) 1 µL α-pinene or (C) 1 µL linalool and then added to the vials. After 36 hours of
617 exposure, the headspace of the vials was analyzed with the same methodology described for the
618 sub-colony headspace sampling. Signal peaks were statistically compared using a Welch two
619 sample *t*-test, comparing the peaks from the control vials to the vials containing *Leucoagaricus*
620 sp.

References

1. Agrawal, A. A. Phenotypic plasticity in the interactions and evolution of species. *Science (80-.).* **294**, 321–326 (2001).
2. Ehrlich, P. R. & Raven, P. H. Butterflies and Plants : A Study in Coevolution. *Evolution (N. Y).* **18**, 586–608 (1964).
3. Berenbaum, M. R. & Zangerl, A. R. Chemical phenotype matching between a plant and its insect herbivore. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13743–13748 (1998).
4. Cornell, H. V. & Hawkins, B. A. Herbivore Responses to PSC: A Test of Phytochemical Coevolution Theory. *Am. Nat.* **161**, 507–522 (2003).
5. Marquis, R. J. Herbivores Rule. *Science (80-.).* **305**, 619–621 (2004).
6. Fine, P. V. A., Mesones, I. & Coley, P. D. Herbivores promote habitat specialization by trees in Amazonian forests. *Science (80-.).* **305**, 663–665 (2004).
7. Interface, I., Farrell, B. D., Mitter, C. & Futuyma, D. J. Diversification at the Insights from phylogenetics. **42**, 34–42 (1964).
8. Kennedy, C. E. J. Attachment may be a basis for specialization in oak aphids. *Ecol. Entomol.* **11**, 291–300 (1986).
9. Gorb, E. V. & Gorb, S. N. Attachment ability of the beetle Chrysolina fastuosa on various plant surfaces. *Entomol. Exp. Appl.* **105**, 13–28 (2002).
10. Bernays, E. A. Diet-Induced Head Allometry among Foliage-Chewing Insects and its Importance for Graminivores. *Science (80-.).* **231**, 495–497 (1986).
11. Walling, L. L. The myriad plant responses to herbivores. *J. Plant Growth Regul.* **19**, 195–216 (2000).
12. Maffei, M. E. Sites of synthesis, biochemistry and functional role of plant volatiles. *South African J. Bot.* **76**, 612–631 (2010).
13. Gleadow, R. M. & Woodrow, I. E. Constraints on effectiveness of cyanogenic glycosides in herbivore defense. *J. Chem. Ecol.* **28**, 1301–1313 (2002).
14. Kessler, A. & Baldwin, I. T. Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature. *Plant Cell Physiol. Proc. Natl. Acad. Sci. U.S.A. Sci. J. Zurzycki K. Nozue al. Proc. Natl. Acad. Sci. U.S.A. Plant Cell* **41**, 415–50 (2000).
15. Adams, A. S., Boone, C. K., Bohlmann, J. & Raffa, K. F. Responses of Bark Beetle-Associated Bacteria to Host Monoterpenes and Their Relationship to Insect Life Histories. *J. Chem. Ecol.* **37**, 808–817 (2011).
16. Boone, C. K. *et al.* Bacteria Associated with a Tree-Killing Insect Reduce Concentrations of Plant Defense Compounds. *J. Chem. Ecol.* **39**, 1003–1006 (2013).
17. Howe, M., Keefover-Ring, K. & Raffa, K. F. Pine Engravers Carry Bacterial Communities Whose Members Reduce Concentrations of Host Monoterpenes With Variable Degrees of Redundancy, Specificity, and Capability. *Environ. Entomol.* **47**, 638–645 (2018).
18. Berasategui, A. *et al.* Gut microbiota of the pine weevil degrades conifer diterpenes and increases insect fitness. *Mol. Ecol.* **26**, 4099–4110 (2017).
19. Mason, C. J., Couture, J. J. & Raffa, K. F. Plant-associated bacteria degrade defense chemicals and reduce their adverse effects on an insect defoliator. *Oecologia* **175**, 901–910 (2014).
20. Lauzon, C. R., Potter, S. E. & Prokopy, R. J. Degradation and Detoxification of the Dihydrochalcone Phloridzin by Enterobacter agglomerans, a Bacterium Associated with the Apple Pest, Rhagoletis pomonella(Walsh) (Diptera: Tephritidae). *Environ. Entomol.*

32, 953–962 (2003).
21. Brune, A., Miambi, E. & Breznak, J. A. Roles of oxygen and the intestinal microflora in the metabolism of lignin- derived phenylpropanoids and other monoaromatic compounds by termites. *Appl. Environ. Microbiol.* **61**, 2688–2695 (1995).
22. Ceja-Navarro, J. A. *et al.* Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nat. Commun.* **6**, 1–9 (2015).
23. Douglas, A. E. Microbial Brokers of Insect-Plant Interactions Revisited. *J. Chem. Ecol.* **39**, 952–961 (2013).
24. Rockwood, L. L. Plant Selection and Foraging Patterns in Two Species of Leaf-Cutting Ants (Atta). *Ecology* **57**, 48–61 (1976).
25. Wirth, R., Herz, H., Ryel, R. J., Beyschlag, W. & Holldobler, B. *Herbivory of leaf-cutting ants : a case study on Atta colombica in the tropical rainforest of Panama*. (Berlin ; New York : Springer, [2003] ©2003, 2003).
26. Holldobler, B. & Wilson, E. O. *The Ants*. (Harvard University Press, 1990).
27. Holldobler, B. & Wilson, E. O. *The Superorganism: The Beauty, Elegance, and Strangeness of Insect Societies*. (W.W. Norton & Company, 2008).
28. Zhukova, M., Sapountzis, P., Schiøtt, M. & Boomsma, J. J. Diversity and transmission of gut bacteria in Atta and Acromyrmex leaf-cutting ants during development. *Front. Microbiol.* **8**, (2017).
29. Sapountzis, P. *et al.* Acromyrmex leaf-cutting ants have simple gut microbiota with nitrogen-fixing potential. *Appl. Environ. Microbiol.* **81**, 5527–5537 (2015).
30. Moreira-Soto, R. D., Sanchez, E., Currie, C. R. & Pinto-Tomás, A. A. Ultrastructural and microbial analyses of cellulose degradation in leaf-cutter ant colonies. *Microbiol. (United Kingdom)* **163**, 1578–1589 (2017).
31. Aylward, F. O. *et al.* Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. *ISME J.* **6**, 1688–1701 (2012).
32. Suen, G. *et al.* An insect herbivore microbiome with high plant Biomass-degrading capacity. *PLoS Genet.* **6**, (2010).
33. Aylward, F. O. *et al.* Convergent bacterial microbiotas in the fungal agricultural systems of insects. *MBio* **5**, 1–10 (2014).
34. Kopac, S. *et al.* High-Quality Draft Genome Sequences of Eight Bacteria Isolated from Fungus Gardens Grown by Trachymyrmex septentrionalis Ants. *Microbiol. Resour. Announc.* 1–3 (2018).
35. González, C. T., Saltonstall, K. & Fernández-Marín, H. Garden microbiomes of Apterostigma dentigerum and Apterostigma pilosum fungus-growing ants (Hymenoptera: Formicidae). *J. Microbiol.* (2019). doi:10.1007/s12275-019-8639-0
36. Khadempour, L. *et al.* Metagenomics reveals diet-specific specialization of bacterial communities in fungus gardens of grass- and dicot-cutter ants. *bioRxiv* 250993 (2019). doi:10.1101/250993
37. Pinto-Tomás, A. A. *et al.* Symbiotic Nitrogen Fixation in the Fungus Gardens of Leaf Cutter Ants. *Science (80-.).* **326**, 1120–1123 (2009).
38. Dowd, P. F. Insect fungal symbionts: A promising source of detoxifying enzymes. *J. Ind. Microbiol.* **9**, 149–161 (1992).
39. De Fine Licht, H. H. *et al.* Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 583–7 (2013).

40. Schoonhoven, L., van Loon, J. J. & Dicke, M. Insect-plant biology. in 49–86 (Oxford, 2005).
41. De Fine Licht, H. H. & Boomsma, J. J. Forage collection, substrate preparation, and diet composition in fungus-growing ants. *Ecol. Entomol.* **35**, 259–269 (2010).
42. Schultz, T. R. & Brady, S. G. Major evolutionary transitions in ant agriculture. *Proc. Natl. Acad. Sci.* **105**, 5435–5440 (2008).
43. Hubbell, S. P., Howard, J. J. & Wiemer, D. F. Chemical Leaf Repellency to an Attine Ant : Seasonal Distribution Among Potential Host Plant Species. *Ecology* **65**, 1067–1076 (1984).
44. Maria, F. *et al.* Activity of Ricinus communis (Euphorbiaceae) and ricinine against the leaf-cutting ant Atta sexdens rubropilosa (Hymenoptera : Formicidae) and the symbiotic fungus Leucoagaricus gongylophorus. **938**, 933–938 (2004).
45. Lapointe, S. L., Serrano, M. S. & Corrales, I. I. Resistance to leafcutter ants (Hymenoptera: Formicidae) and inhibition of their fungal symbiont by tropical forage grasses. *J. Econ. Entomol.* **89**, 757–765 (1996).
46. Howard, J. J., Cazin, J. J. & Wiemer, D. F. Toxicity of terpenoid deterrent to the leafcutting ant Atta cephalotes and its mutualistic fungus. *J. Chem. Ecol.* **14**, 59–69 (1988).
47. Martin, V. J. J., Yu, Z. & Mohn, W. W. Recent advances in understanding resin acid biodegradation: Microbial diversity and metabolism. *Arch. Microbiol.* **172**, 131–138 (1999).
48. Janocha, S., Schmitz, D. & Bernhardt, R. Terpene Hydroxylation with Microbial Cytochrome P450 Monooxygenases. in *Biotechnology of Isoprenoids* **148**, 215–250 (2015).
49. van der Vlugt-Bergmans, C. J. B. & van Der Werf, M. J. Genetic and Biochemical Characterization of a Novel Monoterpene ε-Lactone Hydrolase from. *Appl. Environ. Microbiol.* **67**, 733–741 (2001).
50. Whitehouse, C. J. C., Bell, S. G. & Wong, L.-L. P450 $_{BM3}$ (CYP102A1): connecting the dots. *Chem. Soc. Rev.* **41**, 1218–1260 (2012).
51. Schmitz, D., Janocha, S., Kiss, F. M. & Bernhardt, R. CYP106A2—A versatile biocatalyst with high potential for biotechnological production of selectively hydroxylated steroid and terpenoid compounds. *Biochim. Biophys. Acta - Proteins Proteomics* **1866**, 11–22 (2018).
52. Peterson, J. A. *et al.* Cytochrome P-450(terp). Isolation and purification of the protein and cloning and sequencing of its operon. *J. Biol. Chem.* **267**, 14193–14203 (1992).
53. Ullah, A. J. H., Murray, R. I., Bhattacharyya, P. K., Wagner, G. C. & Gunsalus, I. C. Protein Components of a Cytochrome P-450 Linalool 8-Methyl Hydroxylase. *J. Biol. Chem.* **265**, 1345–1351 (1990).
54. Matsuoka, T. *et al.* Purification and characterization of cytochrome P-450sca from Streptomyces carbophilus. *Eur. J. Biochem.* **184**, 707–713 (1989).
55. Unger, B. P., Gunsalus, I. C. & Sligar, S. G. Nucleotide sequence of the Pseudomonas putida cytochrome P-450(cam) gene and its expression in Escherichia coli. *J. Biol. Chem.* **261**, 1158–1163 (1986).
56. Sawada, N. *et al.* Conversion of vitamin D3 to 1α,25-dihydroxyvitamin D 3 by Streptomyces griseolus cytochrome P450SU-1. *Biochem. Biophys. Res. Commun.* **320**, 156–164 (2004).
57. Janocha, S. *et al.* Resin Acid Conversion with CYP105A1: An Enzyme with Potential for

the Production of Pharmaceutically Relevant Diterpenoids. *ChemBioChem* **14**, 467–473 (2013).

58. Siedenburg, G. & Jendrossek, D. Squalene-hopene cyclases. *Appl. Environ. Microbiol.* **77**, 3905–3915 (2011).
59. Fuchs, G., Boll, M. & Heider, J. Microbial degradation of aromatic compounds- From one strategy to four. *Nat. Rev. Microbiol.* **9**, 803–816 (2011).
60. Marmulla, R. & Harder, J. Microbial monoterpene transformations-a review. *Front. Microbiol.* **5**, 1–14 (2014).
61. Adams, A. S. *et al.* Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Appl. Environ. Microbiol.* **79**, 3468–3475 (2013).
62. Kohl, K. D. & Dearing, M. D. Experience matters: Prior exposure to plant toxins enhances diversity of gut microbes in herbivores. *Ecol. Lett.* **15**, 1008–1015 (2012).
63. Lewin, G. R. *et al.* Cellulose-enriched microbial communities from leaf-cutter ant (Atta colombica) refuse dumps vary in taxonomic composition and degradation ability. *PLoS One* **11**, 1–22 (2016).
64. Altschup, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **215**, 403–410 (1990).
65. Buchfink, B., Xie, C. & Huson, D. H. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **12**, 59–60 (2014).
66. Book, A. J. *et al.* Cellulolytic Streptomyces strains associated with herbivorous insects share a phylogenetically linked capacity to degrade lignocellulose. *Appl. Environ. Microbiol.* **80**, 4692–4701 (2014).
67. Wilke, A. *et al. MG-RAST Manual for version 4, revision 3*. (2017).
68. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* **34**, 525–527 (2016).
69. Schindelin, J. *et al.* Fiji: An open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).
70. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).

Data Availability. All sequencing data has been uploaded to NCBI under the following BioProject numbers: PRJNA564151, PRNJNA429666, PRNJNA429667, PRNJNA429668, PRNJNA565936, PRJNA577467. Individual accession numbers for each dataset are included in Table S2, Table S3, and Table S5.

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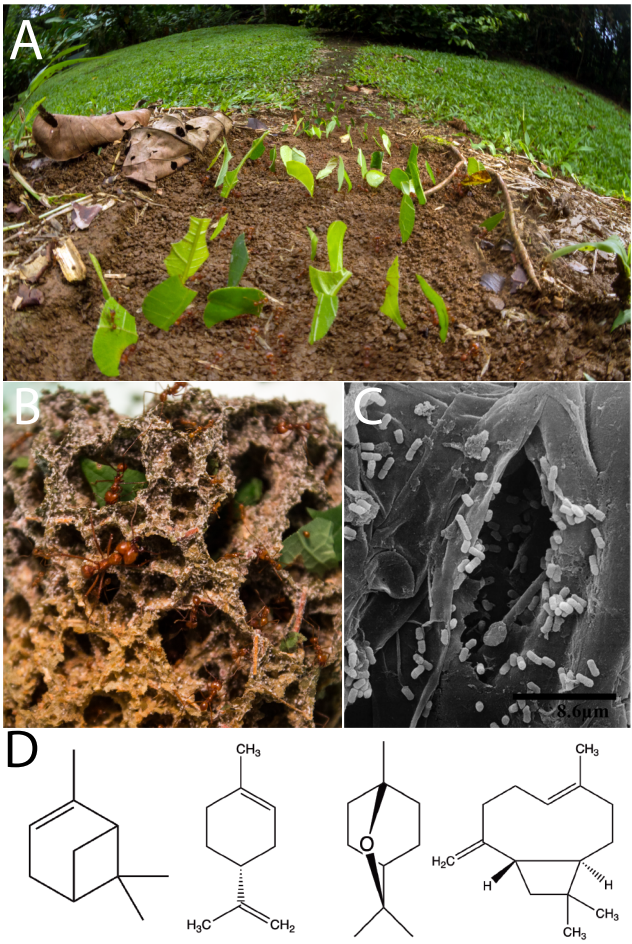

Figure 1. Leaf-cutter ants (*Atta cephalotes*) cut fresh leaf material at large scales, forming visible foraging trails (a) to bring the leaf material as a food source to their specialized fungus gardens (b). Bacteria are known to co-exist within the fungus garden, as can be seen from this SEM image (c). We can detect terpenes from the fresh leaf material in the fungus gardens using GC-MS. One *Atta laevigata* sample collected in an area with eucalyptus had detectable amounts of PSC, such as α-pinene, *p*-cymene, eucalyptol, and caryophyllene oxide (36) (d). Photo credits: panel A Alexander Wild; panel B Lily Khadempour; panel C Rolando Moreira-Soto, reprinted from reference (30).

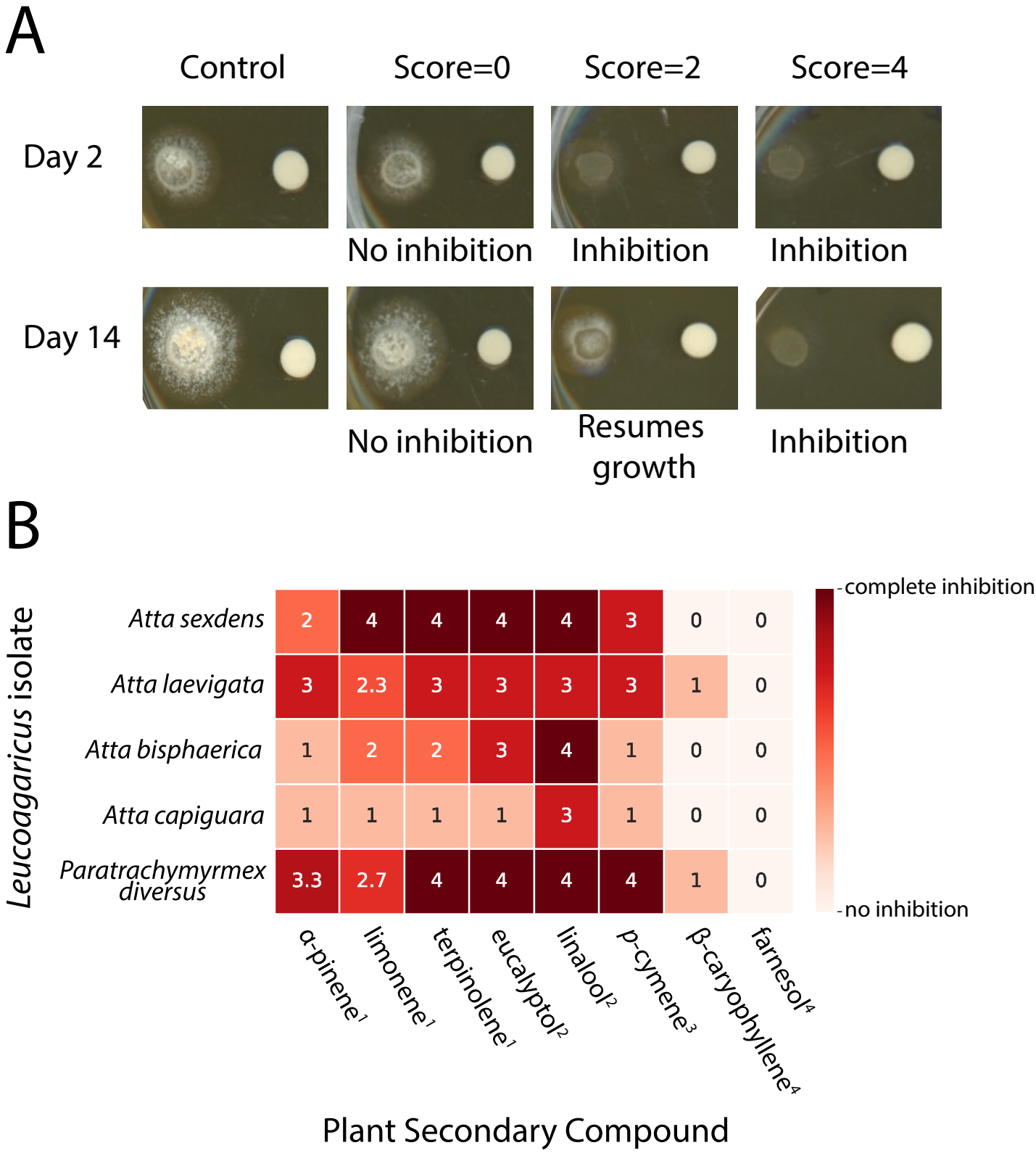

Figure 2. Tolerance of different *Leucoagaricus* sp. strains when exposed to eight PSC. The growth of *Leucoagaricus* sp. was qualitatively scaled (a). Each isolate was exposed to eight compounds using a disc assay, done in triplicate. The growth for each isolate was then scored and averaged across the 3 technical replicates (b). The terpene class is indicated by the superscript number. 1= monoterpene, 2=terpenoid, 3= alkylbenzene related to monoterpene, 4=sesquiterpene.

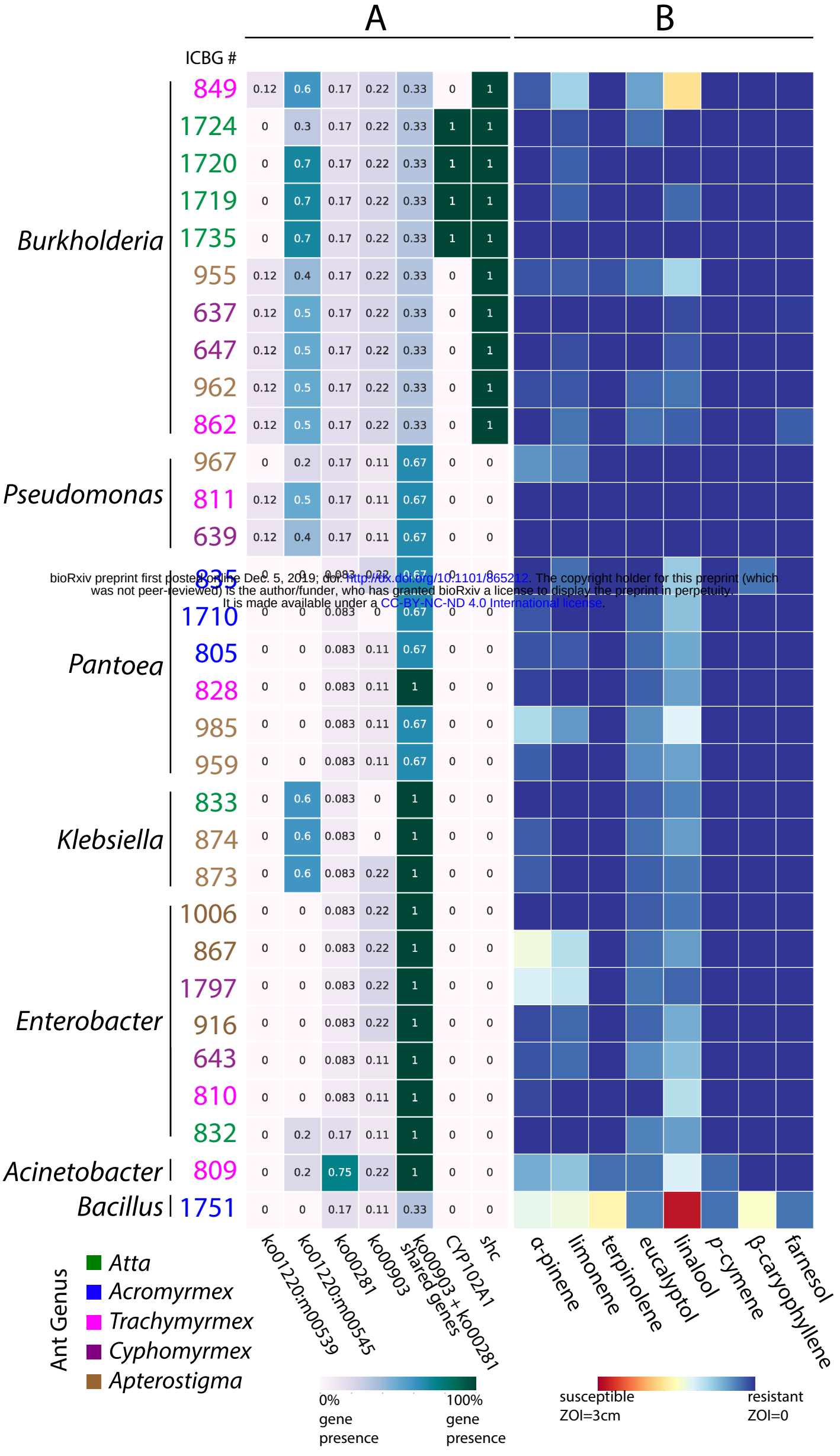

Figure 3. Bacteria isolated from different genera of fungus-farming ants (isolate numbers color-coded by colony of origin) are predicted to have genes involved in PSC transformation (a) and are able to survive in the presence of eight PSC (b). (a) ko01220:m00539 corresponds to the cumate degradation module in the degradation of aromatic compounds pathway. ko01220:m00545 corresponds to the trans-cinnamate degradation module in the degradation of aromatic compounds pathway. ko00281 corresponds to the geraniol degradation pathway. ko00903 corresponds to the α-pinene/limonene degradation pathway. The shared genes between the geraniol pathway and the α-pinene/limonene pathway are K01692, K01825, and K01782. All of the pathways are presented as proportions with 1 being 100% of the genes predicted to be present and 0 being 0% of the genes predicted to be present. CYP102A1 and shc are single genes and so 0 or 1 indicates the absence or presence of that sequence, respectively. 31/42 annotations are displayed here, the annotations for the remaining 11 genomes are included in supplementary, in addition to the metagenome and metatranscriptome annotations (Tables S3, S4, S7) (b) 46 isolates were tested but 31 isolates are displayed as these are isolates that have genomes and were tested for tolerance. Data from all 46 isolates are provided in Figure S1.

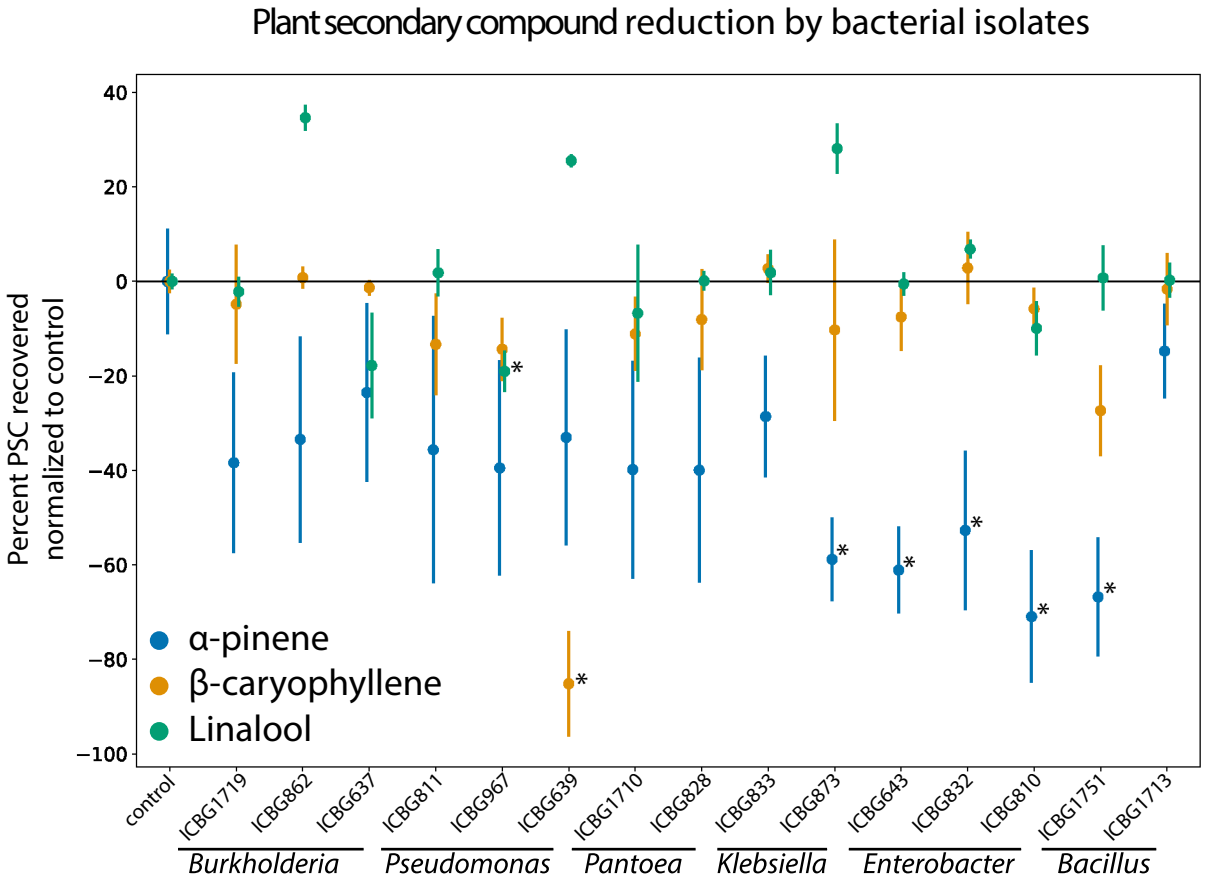

Figure 4. PSC reduction in vitro by 15 bacterial isolates. Bacterial isolates are grouped by their genus-level identification on the x-axis. The y-axis is the percent change of PSC recovered compared to a non-bacterial control vial (10% TSB + compound). Each point represents the average of three GC-MS measurements and the bars are the standard deviation of the observations.

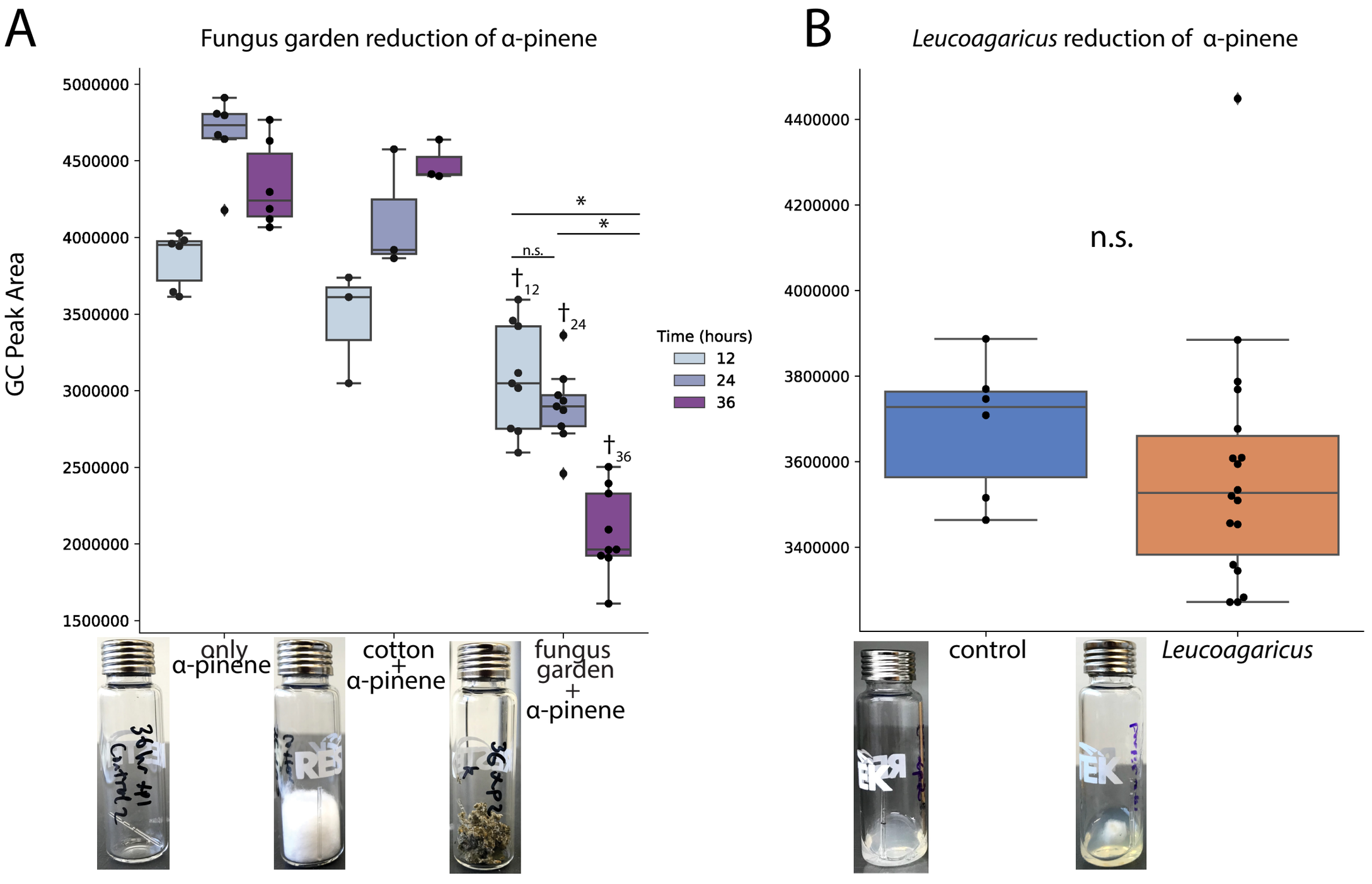

Figure 5. Three sub-colonies of three *Atta cephalotes* colonies were exposed to α-pinene for 12, 24, 36 hours and reduction of α-pinene was measured in the headspace (a). *Leucoagaricus* sp. from the lab colonies, as well as the *Atta* sp. strains tested in Figure 2, were grown on PDA and then exposed to α-pinene for 36 hours. Headspace measurements were collected and compared to a vial with only PDA (b). In (a) and (b) each point represents a single measurement and is superimposed on top of a boxplot. The y-axis indicates the area under the curve with a retention time of ~3.2 minutes, indicating the level of α-pinene in the headspace of each vial. The asterisks (*) indicate signifiance ($p < 0.05$) between the fungus garden samples at different time points. The cross symbol (†) indicates significance ($p < 0.05$) between the fungus garden sample and the control vials at the same time point. The pictures are examples of the experimental set up for each treatment.