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# Symbiont-Mediated Digestion of Plant Biomass in Fungus-Farming Insects

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## Abstract

Feeding on living or dead plant material is widespread in insects. Seminal work on termites and aphids has provided profound insights into the critical nutritional role that microbes play in plant-feeding insects. Some ants, beetles, and termites, among others, have evolved the ability to use microbes to gain indirect access to plant substrate through the farming of a fungus on which they feed. Recent genomic studies, including studies of insect hosts and fungal and bacterial symbionts, as well as metagenomics and proteomics, have provided important insights into plant biomass digestion across insect-fungal mutualisms. Advances in understanding of the divergent and complementary functions of complex symbionts not only reveal the mechanism of how these herbivorous insects catabolize plant biomass, but also represent a promising reservoir for carbohydrate-active enzyme discovery, which is of considerable biotechnological interest.



## INTRODUCTION

Agriculture only appeared within the past ten thousand years of human history, producing diverse domesticated crops with improved nutrition. It has been a dominant driving force in the expansion of human populations and the elaboration of human social systems. In parallel to human agriculture, millions of years ago, several distantly related insects, including ant, termite, beetle, and woodwasp lineages, evolved subsistence-scale cultivation of fungal crops for food, with some even transitioning to industrial-scale agriculture. In contrast to the internal digestion of other insects, fungus farmers have an external digestive system in the form of fungus gardens or galleries, which enables the degradation of recalcitrant plant biomass for nutrition, thereby allowing exploitation of novel ecological niches. By most measures, fungus-farming mutualisms represent the most successful of insect–microbe symbioses, unparalleled in ecological and economic impact, and the insects practicing these symbioses are among the most diverse and abundant lineages of animals on Earth (3, 12, 55, 95, 109). Mature colonies of some fungus-farming ants and termites comprise superorganisms of millions of workers and a single queen or pair of reproductives, making up a complex social entity with defined division of labor based on worker size and age. The key to colony success is not the insects alone, but rather elaborate and integrated interactions with symbionts that play key roles across colony functions (34). Genome-enabled approaches have begun to elucidate these interactions and functions. In this review, we provide an overview of recent omics-based research, including insect, fungal, and bacterial genomic and proteomic studies, which have led to a deeper understanding of plant biomass–degradation processes and uncovered the presence of only recently recognized bacterial symbionts. We review the four main insect groups that practice fungus farming and discuss the integrated role of fungal and bacterial symbionts in plant biomass–decomposition processes.

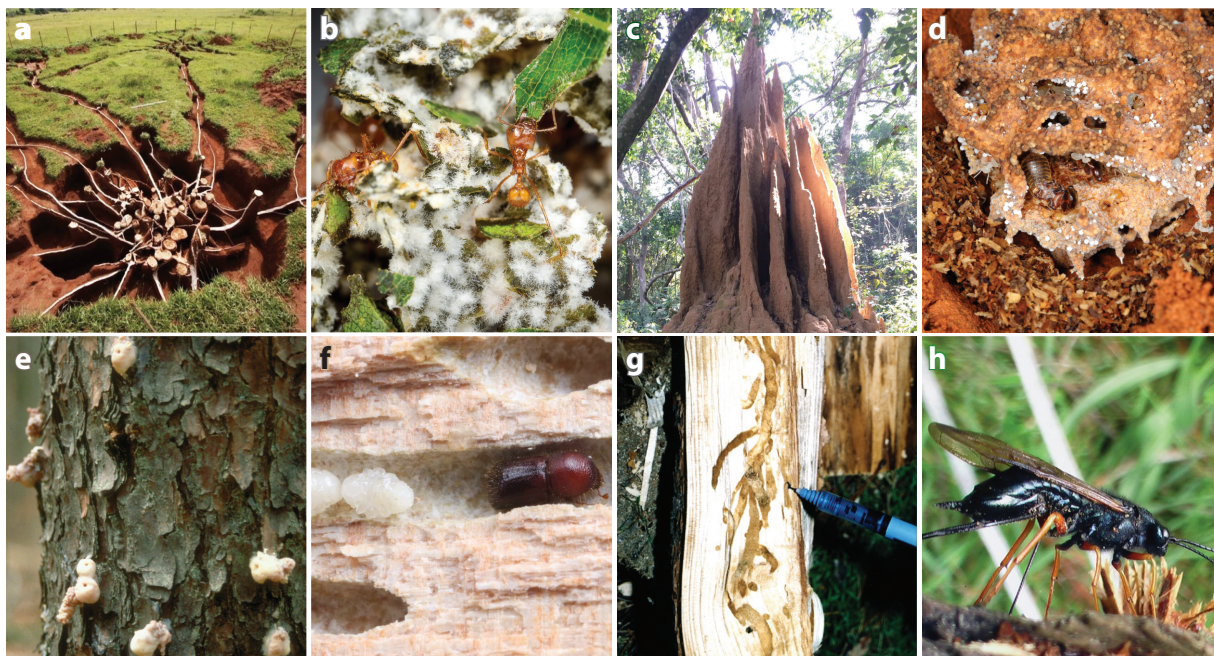
## INSECT FUNGICULTURE

### Insect Lineages Cultivating Fungus for Food

Insects from the orders Hymenoptera, Coleoptera, and Blattodea independently evolved fungus cultivation millions of years ago. The insects involved include a subtribe of ants, a subfamily of termites, and multiple lineages of beetles and woodwasps.

Fungiculture by ants has a single origin 55–65 million years ago (Mya) in South America (19, 78, 109). These exclusively New World ants range from southern Argentina to the northeastern United States and form the monophyletic subtribe *Attina* (subfamily *Myrmicinae*, tribe *Attini*), with 17 genera and approximately 250 described species. The *Attina* are composed of two major clades, the *Paleoattina* and *Neoattina*, that originated around 49 and 48 Mya, respectively (19, 78). *Attine* ants are obligate fungiculturists: Their cultivated fungi are the sole source of food for the larvae and an important source of food for adult ants (112, 132). Mature leaf-cutter colonies of the genus *Atta* are among the largest of any social insect, consisting of up to eight million workers and occupying an underground volume of more than 20 m<sup>3</sup>, making up a complex society with a highly refined division of labor based on worker size and age (48) (**Figure 1a,b**). Colonies of these superorganisms can harvest more than 240 kg dry weight of leaf material per year, making them dominant herbivores in Neotropical ecosystems (133).

The *Macrotermitinae* is a monophyletic termite subfamily that includes approximately 330 described species in 11 genera that all obligately cultivate specialized fungi in the genus *Termitomyces* (Agaricales: *Lyophyllaceae*) (3, 35). Termite fungiculture originated in the sub-Saharan African rain forest approximately 30 Mya, after which the association dispersed to inhabit most of sub-Saharan Africa and spread to Southeast Asia (2). Fungus-farming termites



**Figure 1**

Fungus-farming insect symbioses. (a) The internal nest architecture of *Atta bisphaerica*. Panel adapted with permission from Reference 28. Image courtesy of L.C. Forti. (b) *Atta cephalotes* workers incorporating leaf fragments into their fungal garden. Image courtesy of D. Parsons. (c) A *Macrotermes bellicosus* mound in Côte d'Ivoire. Image courtesy of M. Poulsen. (d) A fungus garden of *Odontotermes formosanus*. Image courtesy of H. Li. (e) Pitch tubes produced by bark beetles attacking a pine tree. Image courtesy of Y. Li. (f) Bark beetle fungal gallery of *Ambrosiodmus minor*. Image courtesy of Y. Li. (g) *Sirex noctilio* ([https://commons.wikimedia.org/wiki/File:Sirex\\_noctilio\\_galleries.jpg](https://commons.wikimedia.org/wiki/File:Sirex_noctilio_galleries.jpg)). Image courtesy of P. Klasmer. (h) *S. noctilio* female on a pine tree ([https://commons.wikimedia.org/wiki/File:Sirex\\_noctilio\\_Pinus\\_radiata.jpg](https://commons.wikimedia.org/wiki/File:Sirex_noctilio_Pinus_radiata.jpg)). Image courtesy of Michaelbbecke. All photos used with permission.

remain diverse in African rainforests (35), but their contribution to plant biomass decomposition is greater in arid ecosystems, where they are dominant decomposers and account for between 20% and 90% of carbon mineralization (96, 134). Colonies of the genus *Macrotermes* construct some of the most remarkable nonhuman structures known, rising as conspicuous towers in dry savannah ecosystems (**Figure 1c,d**), and, along with colonies of the genus *Odontotermes*, display complex age-related division of labor.

The ambrosia beetles (Coleoptera: Curculionidae) are a polyphyletic assemblage of independently evolved clades within the weevils (12, 55). The most prominent groups are the Scolytinae and Platypodinae subfamilies, with 3,400 and 1,400 species, respectively. Ambrosia beetles transitioned to fungus farming approximately 97–110 Mya, achieving a near global distribution (12, 51) (**Figure 1e,f**). Ambrosia beetles usually do not colonize living trees, but instead burrow into diseased, dying, or dead trees, where they inoculate their fungal cultivar. Despite this, some species, particularly in the genus *Xylosandrus*, are important pests of tree nurseries (102). Adults and larvae feed on the cultivar fungus. Most ambrosia beetles live in small family units, with parental care; however, at least one species (*Austroplatypus incompertus*) is eusocial (12).

Fungus farming in the woodwasps (Hymenoptera: Siricidae) evolved independently three times in both the hosts and fungal symbionts approximately 150–200 Mya (12, 44) (**Figure 1g,h**). *Sirex* woodwasps infest tree species in the genus *Pinus* and are native to the coniferous forests of Eurasia and North Africa. There are 33 described species, including the European *Sirex noctilio*, a major

invasive pest in the Southern Hemisphere that has recently spread to North America and China (116). Alongside eggs, woodwasps inject spores of their fungal mutualist into live host trees. Subsequent fungal growth provides *Sirex* eggs and larvae with a suitable microenvironment for development by raising the carbon dioxide concentration and drying the wood substrate (86, 116). The larvae then feed on the fungus and partially digested wood (116).

### The Fungi Involved in Fungiculture

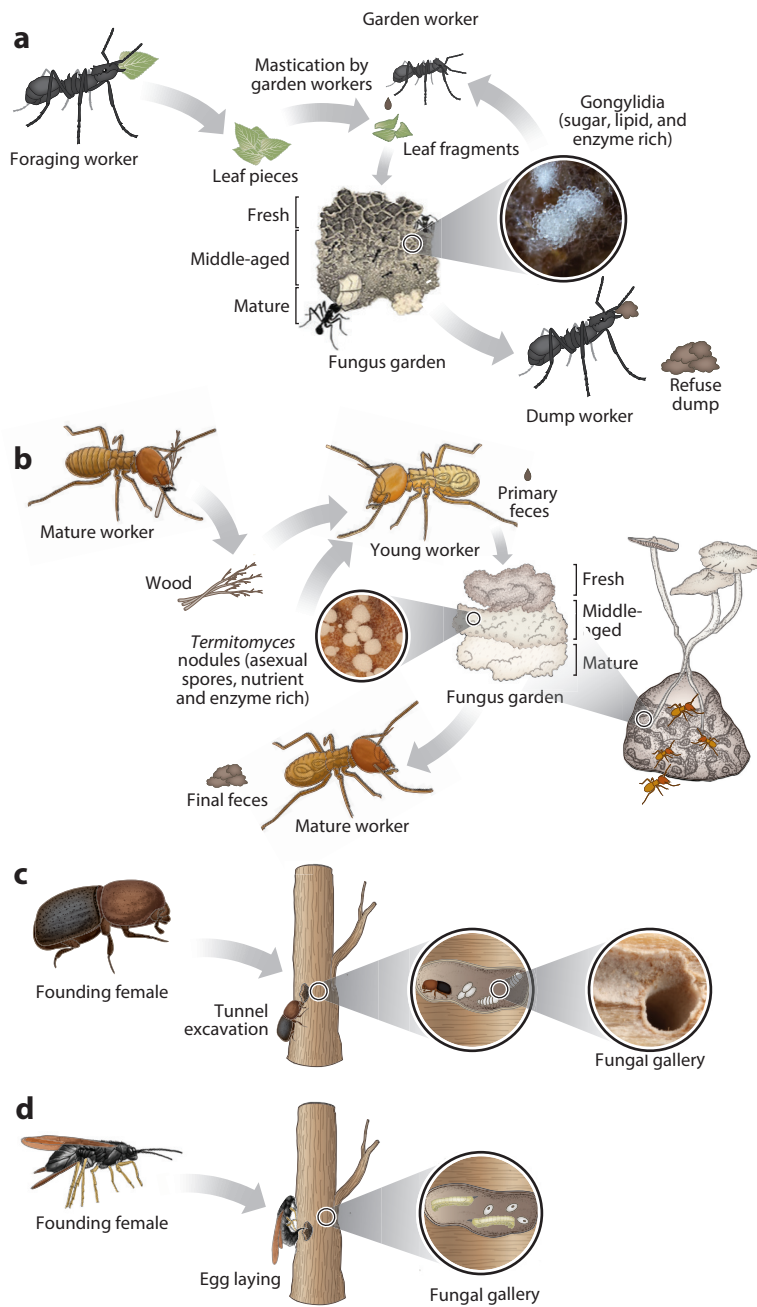
The success of these insect lineages, and their status as key ecosystem engineers, can be attributed to their mutualistic associations with fungi, which enable the use of otherwise inaccessible carbon. Fungus-farming ants, termites, and woodwasps associate with basidiomycetous fungi of the class Agaricomycetes. Ambrosia beetles associate with a diverse group of ascomycetous and basidiomycetous fungi, with the most well-known belonging to the order Ophiostomatales (Ascomycota). The sister taxa of these cultivated fungi consume substrates similar to their cultivated counterparts, suggesting that their ability to degrade dead and living plant material likely played an important role in the origin and success of these symbioses.

Most fungus-farming ants cultivate fungi in the genus *Leucoagaricus* (Agaricales: Agaricaceae), with the two leaf-cutter ant genera *Atta* and *Acromyrmex* farming a single fungal species, *Leucoagaricus gongylophorus* (21, 118). Foundress queens carry a small pellet of fungus from their natal nest in the infrabuccal pocket in their oral cavity, with which they start a new garden, resulting in the fungus being continually asexually propagated and leading to uniparental and clonal vertical inheritance (17) (**Figure 2a**). This contributes to the largely congruent phylogenies of the fungi and the ants, indicating long-term coevolution and codiversification (21, 22, 59). Fungus-farming ants and *Leucoagaricus* cultivars exhibit signs of coevolutionary change, with expansions of carbohydrate-active enzyme (CAZyme) genes and the formation of gongyliidia, specialized feeding structures, in the fungus and the loss of the arginine biosynthesis pathway in the higher attine ant hosts (95, 121). Notably, two groups of phylogenetically basal attine ants in the genus *Apterostigma* have secondarily switched to farming distantly related coral fungi in the family Pterulaceae (Agaricales) (92).

Termite-cultivated fungi all belong to the genus *Termitomyces* (3) (**Figure 2b**), with the closest known relatives being the wood-decaying genera *Tephrocye* and *Lyophyllum* (1). Approximately 40 *Termitomyces* species have been described, and cophylogenetic patterns indicate a single origin and host–symbiont codiversification (3). Most fungus-farming termites acquire their fungal mutualist horizontally. Mushrooms, the fruiting body of *Termitomyces*, grow from termite colonies and produce sexual basidiospores, which are collected by the first foraging workers of incipient colonies as starter inocula for a new fungus garden (113). Termites preferentially associate with specific cultivar species, possibly due to nutritional coadaptations (3, 26, 91), but host switching within relatively narrow host–symbiont clades exists (4, 94, 127). Vertical transmission has evolved in the genus *Macrotermes*, in which alate queens carry asexual spores within their guts, and in the species *Macrotermes bellicosus*, where the alate king is the sole carrier of spores (61, 70).

Across ambrosia beetles, fungal cultivars occur within at least 10 lineages spanning the Basidiomycota and Ascomycota (128) (**Figure 2c**). Most beetle genera associate with the genera *Raffaelea*, *Ambrosiella*, and *Fusarium* (Ascomycota: Sordariomycetes) (12, 55), while the two most phylogenetically diverse genera of ambrosia beetles (*Ambrosiodmus* and *Ambrosiophilus*) cultivate *Flavodon ambrosius* (Basidiomycota: Agaricomycetes) (81). Given the multiple origins of fungiculture in ambrosia beetles, the diversity of fungi that they cultivate compared to the ants, woodwasps,





**Figure 2**

Plant biomass integration and degradation in (a) leaf-cutter ants, (b) Macrotermitinae termites, (c) ambrosia beetles, and (d) woodwasps. Gongylidia images courtesy of D. Parsons, and ambrosia fungal gallery images courtesy of P. Biedermann. All images used with permission.

and termites is unsurprising (36, 128). Within each lineage, however, beetle–cultivar associations are species specific (71, 87, 115). Fungal dispersal occurs either through ingestion of fungal spores from their natal nest or, more commonly, via specialized structures called mycangia (51, 66, 87). During tree colonization, they use their mandibles and maxillae to excavate galleries, into which the fungi are inoculated (11).

The fungi cultivated by *Sirex* woodwasps have received comparatively little attention, with only three species identified, all in the genus *Amylostereum* (Russulales: Amylostereaceae) (125, 129); these species are closely related to *Amylostereum ferreum*, which causes wood rot in conifers (117) (Figure 2d). Mycangia are located in the ovipositor, where asexual fungal spores are collected by female woodwasps prior to dispersal, and they are then deposited along with phytotoxic venom into the tree during colonization (46). It was previously thought that *Amylostereum* symbionts were exclusively vertically transmitted, but associations between *Sirex* spp. and *Amylostereum* spp. are not strictly species specific and may be influenced by host tree species (37, 46), suggesting at least some degree of horizontal transmission.

### Bacterial Lineages Inhabiting Guts and Gardens

Recent studies across fungus-farming insects have revealed the presence of previously largely unknown bacterial symbionts in fungal gardens and galleries and that bacterial lineages are largely shared across species and genera within respective host insect clades. These nonrandom associations suggest that these bacterial symbionts likely play important and consistent roles in all four systems (10).

In fungus-farming ants, bacterial symbionts play an important role within fungus gardens. 16S rRNA amplicon sequencing, metagenomics, and bacterial genomics have revealed that fungus garden communities are dominated by the families Enterobacteriaceae and Pseudomonadaceae, the dominant genera being *Enterobacter*, *Pseudomonas*, *Klebsiella*, *Citrobacter*, *Escherichia*, and *Pantoea* (8, 10, 100, 120). Queens of several species of leaf-cutter ants consistently carry common garden bacteria (e.g., *Pseudomonas* and *Acinetobacter*) in the infrabuccal pocket, where the inoculum for their incipient fungus gardens is carried (88, 90), suggesting at least some vertical transmission between host generations. Garden bacteria in the genera *Klebsiella* and *Pantoea* fix atmospheric nitrogen that can be taken up by the fungus and ants, suggesting that they play an important role in adaptation to the nitrogen-limited plant diet (100). Garden bacteria also contribute to detoxification of plant defensive compounds (39) and have been implicated in defense against pathogens (104). Ant worker gut microbiomes are distinctly different from those of fungus gardens, being relatively simple (114). Amplicon sequencing of the 16S rRNA gene in fungus-farming ants from seven genera and 16 species showed that gut bacterial communities are dominated by Mollicutes but also include some Alpha- and Gammaproteobacteria and Actinobacteria (106). Work on *Acromyrmex* leaf-cutter ant guts has shown that bacterial community compositions are consistent across studies and colonies (106, 135). The community of bacteria present within ant fungus gardens belongs to genera that are common in mammalian digestive tract microbiota, while the gut microbiomes of individual worker ants are dominated by Mollicutes, which are not known as gut commensals. This suggests an evolutionary scenario involving a substantial functional reduction in biomass degradation by the gut microbiota of the ants themselves, in concert with the transition to the external digestive system in the form of fungus gardens.

Intestinal bacterial communities are far more diverse in fungus-farming termites, containing hundreds of bacterial lineages (33, 97–99). As in other higher termites, community structure in the fungus farmers reflects the phylogeny and diet of the host group (89). The most prominent bacterial lineages are from the phyla Synergistetes, Firmicutes (Clostridiales), Bacteroidetes (mostly

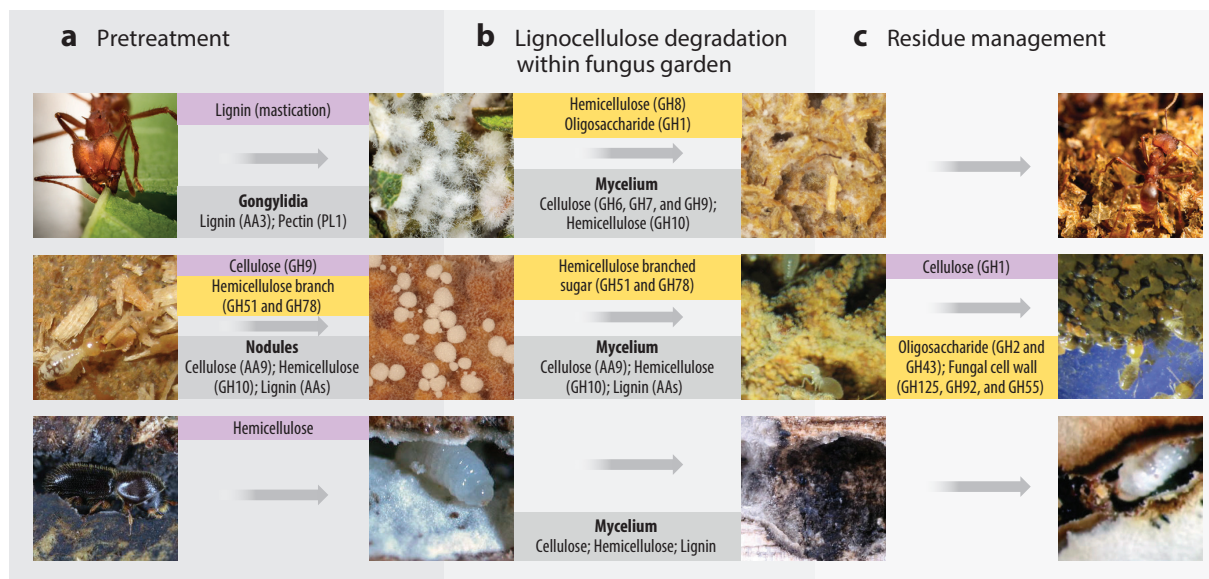
Bacteroidales), Proteobacteria (mostly Desulfovibrionales), and Spirochaetes (33, 76, 98, 99). Intriguingly, the termites appear to carry a core set of bacterial lineages that are either absent or rare in other higher termites but that resemble the bacterial lineages of the ancestral omnivorous cockroaches (33, 98). This is likely due to their altered role in plant biomass degradation and the adoption of the protein-rich diet of *Termitomyces* (50, 84, 99, 101). Putative gut bacterial roles include nitrogen fixation (105), defense through the production of antimicrobials (126, 130), and contribution of fungal- and plant biomass-degrading enzymes (see below). Gut-inhabiting lineages are prevalent in termite fungus gardens, but environmental contributions lead to garden communities that are distinct from those of guts (97). Gardens are dominated by Firmicutes (*Acetonea* and *Sporomusa*), Bacteroidetes (e.g., *Alistipes*), Proteobacteria, Actinobacteria, and Saccharibacteria (former candidate division TM7) (76, 97). In contrast to termite gut microbiomes, which are essentially identical between colonies of the same fungus-farming termite species across hundreds of kilometers (99), fungus gardens fluctuate in relative abundance of bacterial members over time (97), possibly associated with shifts in which plant substrates are used for fungiculture (82).

The guts, galleries, and mycangia of ambrosia beetles house rich bacterial communities (54, 58). Fungus galleries of *Xyleborinus saxesenii* are dominated by the genera *Pseudomonas*, *Enterobacter*, and *Rabnella*, while the galleries of *Xylosandrus germanus* are dominated by the classes Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, and Bacteroidetes (10, 54). The galleries of *Xyleborus affinis* and *X. saxesenii* also contain *Streptomyces* spp., which appear to play a role mediating gallery hygiene by producing antimicrobials (45). Microbiomes within mycangia are also diverse but are distinct from those of galleries. In *Xyleborus*, mycangial microbiomes differ between beetle species but often include members of the orders Pseudomonadales and Burkholderiales and a *Mycoplasma* sp. (54). *Streptomyces* isolates have also been identified in the mycangia of *Euplatypus parallelus* (123). The head and abdomen microbiomes of three *Xyleborus* species varied between lab-reared and wild beetles, indicating the importance of environment in seeding bacterial symbionts. However, a core gut microbiome appeared to be present and was dominated by *Burkholderia*, *Sphingobacterium*, *Trabulsiella*, and *Stenotrophomonas*. This core is functionally enriched in genes involved in metabolism and nutrition uptake, suggesting a bacterial contribution to digestion (58).

Very little is known about the gut or gallery microbiomes of *Sirex* woodwasps. Madden (85) isolated strains of *Flavobacterium*, *Azotobacter*, and *Achromobacter* from guts and frass of *S. noctilio*. Strains of cellulolytic *Streptomyces* have been isolated from the gut of *S. noctilio*, suggesting that bacteria play a role in breaking down decaying wood; several Enterobacteriales (genera *Plesiomonas*, *Rabnella*, *Yersinia*, *Erwinia*, *Pantoea*, and *Enterobacter*) have also been found in the *S. noctilio* gut (6).

## PLANT BIOMASS DIGESTION IN INSECT-FUNGAL MUTUALISMS

Plant biomass is the largest source of organic carbon on the planet, stored as recalcitrant aromatic lignin, cellulose, and hemicellulose. Cellulose microfibrils are linear polymers consisting of  $\beta$ -1,4-glycosidic linkages, which are only accessible to cellulases from the chain ends or in the amorphous regions. Hemicellulose is a general term for matrix polysaccharides consisting of polysaccharide backbones with frequent substitutions of sidechains by a variety of saccharides, which are able to cross-link with microfibrils, leading to the formation of cellulose-hemicellulose complexes in woody biomass. Gaining access to this source of organic carbon requires at least partial delignification, followed by subsequent enzymatic or chemical cleavage of plant polysaccharides. Most animals—including insects—require symbiotic microbes to do this (34). Despite being the energetic backbone of insect fungiculture, these processes have, until recently, largely remained a black box—understood simply as the cultivated fungi growing on plant material and providing energy



**Figure 3**

Overview of the enzymes that are active during the three-stage process of symbiont-mediated plant biomass degradation in leaf-cutter ants, Macrotermitinae termites, and ambrosia beetles: (a) substrate pretreatment, (b) degradation within gardens or galleries, and (c) residue management. Enzymes originating from the host insect are in purple, those from fungal cultivars are in grey, and those of bacterial origin are in yellow. Ant images courtesy of D. Parsons, termite images courtesy of H. Li, and beetle images courtesy of P. Biedermann and G. Kunz. All images used with permission. Abbreviations: AA, auxiliary activity; GH, glycoside hydrolase; PL, polysaccharide lyase.

to their insect hosts. Recent studies have revealed that plant substrate deconstruction is completed through elaborate step-wise processes involving pretreatment of plant substrates (**Figure 3a**), substrate inoculation (**Figure 3b**), and substrate residue management (**Figure 3c**).

### Biomass Degradation in Ant Fungiculture

Fungus-farming ants provision their fungus with plant substrates, including dry plant material, flowers, fruits, fresh leaves, and even partially degraded leaf substrate in the form of caterpillar frass. The exact makeup of foraged material is affected by ant species and time of year, with fresher materials like flowers and fruits making up a larger proportion of foraged substrate during the wet season (73). Leaf-cutter ants predominately forage and provision their cultivars with fresh plant material. They are generalist herbivores, foraging on a wide variety of plants, including grasses (53), but also, like other fungus-farming ants, on forage fruits and flowers (30, 64).

Foraged substrate is pretreated by fungus-farming ants prior to integration into the fungus gardens. In both *Acromyrmex* and *Atta* spp., pretreatment includes worker ants licking the plant material with their hypopharynx, removing any plant-associated or environmental microbes present on the substrate (23). In addition, within the fungus garden chamber, but not in the garden itself, workers remove the wax layers and then masticate the leaves into fragments to expose the leaf edges (41) (**Figure 2a**). These fragments are moved to the top of the fungus garden, and enzyme-concentrated fecal droplets are applied before inoculation with *L. gongylophorus* hyphae (32). Fecal droplets contain *L. gongylophorus*-derived plant cell wall- and plant secondary compound-degrading enzymes, such as the pectinase XEG1; serine-, aspartic-, and metalloproteases; and laccases (9, 69, 103, 108).



Initial growth of *L. gongylophorus* occurs at the top of gardens or across outer regions, where leaf fragments are first integrated, and involves utilizing easier-to-digest plant components. After ant pretreatment, leaf fragments have exposed internal tissue, allowing more direct fungal access to the simple sugars within leaf cells. Scanning electron microscope studies show in situ hyphal penetration into the exposed regions of leaf tissue (93), and the younger regions of fungus gardens in the grass-cutter *Atta bisphaerica* mostly degrade starch and hemicellulose (119). Comparative metaproteomic and stable isotope analyses of intact fungus gardens indicate that the fungal cultivar preferentially breaks down the simpler and more digestible substrates, which are then quickly vectored to the ants through the gongylidia (64, 111).

Culture-based studies of *L. gongylophorus* initially suggested poor growth on cellulose (5), creating a view that the more recalcitrant plant polymers are not degraded at all within fungus gardens. However, it is now clear that *L. gongylophorus*-derived deconstruction of cellulose does occur. Plant sugar composition studies of intact gardens of *Atta colombica* estimate that at least 30% of the cellulose in the leaves is degraded (61). Furthermore, genomic analyses of *L. gongylophorus* have confirmed the presence of genes predicted to encode biomass-degrading enzymes for the complete cleavage pathways necessary for the digestion of cellulose, as well as xylan and other plant polymers (7, 64, 107). Extracellular cellulases of fungal origin, including a glycoside hydrolase (GH) family 9 endoglucanase, GH6 and GH7 cellobiohydrolases, and hemicellulases (e.g., GH15 glucoamylases and CE5 xylan esterase/cutinase), have also been detected within fungus gardens using metaproteomic approaches (7). Colonies fed on leaves showed a considerably higher abundance of proteins predicted to be cellulases compared to colonies fed on easily digestible substrates such as oats, suggesting the existence of a flexible, substrate-specific enzymatic response from *L. gongylophorus* (64). In addition to the lignocellulolytic contribution of *L. gongylophorus*, metagenomic and metaproteomic studies suggest a role for garden bacteria in degrading hemicellulose and oligosaccharides (8, 10, 119, 120).

Somera and colleagues (119) proposed a biphasic view of *Atta* spp. leaf-cutter gardens, involving fresh (outer) and mature (inner) regions. The fresh stage of *L. gongylophorus* growth involves utilization of the easily digested carbohydrates from fresh leaf substrate in the young regions of the fungus garden. This is then followed by the slower breakdown of more recalcitrant plant polymers, which are enriched in the older strata. In both *Atta* and *Acromyrmex* spp., the outer layer also has the highest expression and activity of enzymes involved in breaking down hemicellulose, cellulose, and lignin, further supporting the idea of a division of labor between fresh and mature fungus garden regions (43, 68, 69, 107).

Fungus-farming ants use their garden as an external digestive system that has evolved to efficiently convert plant substrate into fungal biomass, which then serves as the sole source of nutrients for larvae and nonforaging workers (111, 112). In many ant lineages, the fungus provides nutrients in the form of hyphal swellings called gongylidia, which are rich in lipids and carbohydrates (9, 95). Three polyols are secreted by *L. gongylophorus*, and these are consumed by the resident ants and microbes (31, 119). In *Atta* spp., garden bacteria provide supplemental nitrogen to workers (100), and metagenomics suggest that they may also play a role in ant nutrition through synthesis of B vitamins and amino acids (7).

After partial cellulose deconstruction in fungus gardens, older leaf-cutter worker ants move remaining cellulosic leaf material from the bottom strata of the fungus garden to refuse dumps (74). Similar to human-produced compost piles, metabolic activity is high within these dumps, primarily due to microbial degradation of cellulose. The concentration of cellulose continuously decreases from the top strata of the dump to the lower strata of the dump (110). Refuse dumps are enriched in nitrogen and phosphorus and play important roles in carbon and nitrogen cycling in neotropical forests (133).

### Biomass Degradation in Termite Fungiculture

Fungus-farming termites primarily provision their fungal cultivars with decaying plant material, such as wood, grass, leaf litter, and dung of herbivorous mammals (18, 24, 27). Some substrate preferences have been observed, but these are conceivably due to substrate availability, driven by seasonal variation and plant community composition (27). Flexibility in substrate use has been suggested to contribute to termites' abundance and dominant role as plant-biomass decomposers in African savannah ecosystems (24). In *Macrotermes* and *Odontotermes*, and possibly in other genera, older workers forage for plant material, which is brought back to the nest.

Efficient plant pretreatment is accomplished through a combination of mastication by workers and a first gut passage, where younger workers ingest the lignocellulosic material together with fungal nodules that contain asexual *Termitomyces* spores. These young workers excrete this mixture and use it to build fresh fungus gardens, and this continuous seeding allows for rapid growth of new mycelium and nodules containing asexual spores within fungus gardens (47, 78) (**Figure 2b**). Termite mastication is referred to as comminution and is carried out by the mandibles and the gizzards (20, 131). This step potentially reduces cellulose crystallinity of the plant substrate and breaks it into 20–80- $\mu\text{m}$  pieces (77, 80). As the first gut passage is fast, and plant decomposition is thought to be minimal, this passage mainly serves to mix the substrate with *Termitomyces* spores to facilitate rapid mycelial growth within the fungal gardens. A recent comprehensive study in *O. formosanus* with laboratory feeding experiments followed the passage of material using analytical approaches, including  $^1\text{H}$ - $^{13}\text{C}$  correlation nuclear magnetic resonance and thermochemolysis, to estimate the degree of pretreatment that takes place during this gut passage. The results showed extensive lignin depolymerization and degradation, leaving a residue almost completely devoid of the various C-C- and C-O-bonded lignin units that are normally considered to be the most recalcitrant (80). In addition, branched-chain sugars substituted along hemicellulosic backbones are depleted during this gut transition, suggesting that the host insect and gut microbiome likely make use of the more easily digestible sidechain sugars during this passage. However, work in other Macrotermitinae species has found that freshly deposited fungus garden contains high amounts of lignin, suggesting that the role of the first gut passage in lignin cleavage may differ between termite species (49, 76).

The pretreated plant material is embedded into the fungus garden, where *Termitomyces* fungi and garden bacteria are responsible for lignin, cellulose, and hemicellulose degradation (56, 57). Chemical analysis of fungus gardens from *O. formosanus* showed that significant polysaccharide cleavage occurs, leaving a substantial quantity of oligosaccharides and simple sugars in mature garden residues (80), consistent with the findings of work in *Macrotermes natalensis* and two other *Odontotermes* spp. (20). *Termitomyces* fungi associated with *Macrotermes* spp. have a broad range of extracellular plant polysaccharide-degrading enzymes, comparable to those of free-living saprophytic fungi, but do not grow well on glucose alone (60, 101); however, variation in the lignocellulose degradation capabilities of different *Termitomyces* spp. may exist (57). Transcriptomic analysis in *M. natalensis* and *Odontotermes* sp. has revealed that the highest expression of cellulases and cellobiohydrolases occurs in mature parts of the fungus garden (25). Collectively, it appears that *Termitomyces* preferentially breaks down the most complex polysaccharides, enriching the mature fungus substrate residue in oligosaccharides and glucose.

The mature fungus garden is consumed by older workers through a second gut passage, in which a combination of insect- and gut community-derived enzymes secure oligosaccharide and fungal biomass digestion. Metagenomics studies on *Odontotermes* and *Macrotermes* species have elucidated bacterial contributions and found that worker gut microbiomes are enriched in enzymes such as GH92, GH43, and GH2, which are involved in the final digestion of

oligosaccharides, the ultimate step in plant material digestion (50, 84, 101). In addition, comparisons to termite species with plant-based diets indicate relatively more genes encoding mycolytic enzymes, such as chitinases,  $\beta$ -glucanases, and  $\alpha$ -mannanases, in bacteria dominating fungus-farming termite gut microbiomes, suggesting that they target fungal cell wall components (50, 84, 101). After the second gut passage, older workers produce final feces, which are placed within the nest but away from the fungal garden (47, 79). In sharp contrast to ant refuse dumps, these final feces are essentially devoid of organic material (96), leaving the combined effects of dual gut passage and external decomposition within fungal gardens effective at extracting carbon from the plant biomass.

### Biomass Degradation in Beetle Fungiculture

In contrast to fungus farming ants and termites, ambrosia beetles do not forage for plant substrate, but instead transport their fungus to host trees. Dispersing adult females identify a new tree trunk and excavate a tunnel into the living or freshly dead xylem and/or phloem to form the brood chamber for their eggs and fungal symbionts (12, 55) (**Figure 2c**). In the primitively eusocial ambrosia beetle *X. saxesenii*, larvae pretreat woody tissue by digging galleries and then smearing predigested feces containing small woody particles on the gallery walls (29), which serve as fungus beds. With frequent renewal of mandibles by molting, larvae engage in the mastication of tough woody substrates that, in a subsequent gut transit, are mixed with an endo- $\beta$ -1,4-xylanase targeting hemicellulose (12). Collectively, beetle larvae facilitate fungus cultivation and mechanical and enzymatic pretreatment of plant substrate.

To date, only an estimated 5% of the extraordinarily diverse ambrosia fungal symbionts have been described (55). Although current insights suggest that the fungal lineages differ in metabolic potential, these lineages generally appear to preferentially degrade hemicellulose and other simple sugars (29). The ambrosia fungi *Raffaelea brunnea* and *Raffaelea tritirachium* utilize various carbon compounds, such as xylose or cellobiose (9), while the fungal symbiont of *Xyleborus ferrugineus* metabolizes dextrose rather than cellulose (55). In *X. saxesenii*, the fungal cultivar degrades hemicelluloses, such as xylan, glucomannan, and callose (29). *Ambrosiophilus* beetles cultivate *F. ambrosius*, a basidiomycete fungus that is a more aggressive wood decomposer than known ascomycete ambrosia fungi. *F. ambrosius* causes significant weight loss and reduction in wood hardness and has high polyphenol oxidase activity in vitro, suggesting that this fungal cultivar likely produces cellulases and lignin-modifying enzymes (63). Scolytine beetles of the genus *Trypodendron* cultivate fungi of the genus *Phialophoropsis*, which shows strong and specific wood-degrading capabilities, as it is able to secrete xylanase and glucanase (29).

Although some ambrosia beetles may fit the traditional view of cultivation of fungi by converting cellulose and lignin from the plant woody tissue into lipids and sugars, the mechanisms of their plant biomass degradation still remain largely unknown (12, 55). The sparsity of studies on enzymatic profiles of ambrosia fungi, and the variation already documented, supports the hypothesis that the diverse lineages of ambrosia fungi will likely lead to the discovery of diverse enzymes and metabolic pathways. This may be even more true for bacterial contributions, which are likely to be as, if not more, diverse. *Pseudomonas* and *Enterobacter*, among others, likely play roles in nutrition and wood degradation, but little research on this has been done (10). Furthermore, plant biomass utilization may take place through the combined effort of endogenous cellulolytic enzymes in the insect midgut (131) and other ectosymbiotic fungi, yeasts, and bacteria that co-inhabit beetle galleries (12, 55).

Gallery hygiene maintenance, including waste-material management of sawdust, feces, and fungus, is critical for the turn-over of woody substrate. At least in *X. saxesenii*, dump management

includes balling of frass, which is mainly performed by larvae. These balls are then transported and removed by adult females (29). Interestingly, the gallery dump material has higher activity of cellulases, endoxylanases, and pectinases than does the material of the brood chamber and other gallery sites, likely due to the dominant presence of other secondary fungal symbionts (29).

Developing larvae feed on fungal biomass and, in some cases, on partially degraded plant tissue (29, 63). Ambrosia fungi form layers of conidiophores on the gallery walls and provide essential B vitamins and ergosterol, which beetles require for proper development but cannot synthesize themselves (52, 67). Comparisons of the transcriptomes of two ambrosia beetles, *Eurwallacea fornicates* and *Xyleborus glabratus*, with those of nonfarming beetles and other insects support the idea that genes involved in fructose metabolism, proteolysis, and controlling sugar concentration in hemolymph are under positive selection (13). Dietary specialization on their fungal symbiont is indicated by the high larval mortality observed when incidental fungi outcompete, and thus exclude, ambrosia fungus from the gallery (52, 55).

### Biomass Degradation in Woodwasp Fungiculture

*Sirex* woodwasps do not forage for substrate for their fungus, but rather provide access to plant biomass through the injection of their fungal cultivar directly into a host tree. This is typically concurrent with egg laying, when females using their ovipositor to drill through the bark into the cambium. Subsequent larval tunneling extends the access that the *Amylostereum* cultivar has within the sapwood, while the wood decay activity of the fungus facilitates larval tunneling. The best-studied woodwasp symbiosis is between *S. noctilio* and its obligate symbiotic fungus *Amylostereum areolatum*. During oviposition, *S. noctilio* adult females also engage in a pretreatment step, injecting phytotoxic mucus derived from their venom gland. The mucus increases laccase activity in *A. areolatum*, apparently contributing to lignin degradation during the early phase of tree attack (16, 75).

After establishing itself within trees, *A. areolatum* grows within the galleries, utilizing the sapwood as a growth substrate. Detailed understanding of the combined cellulolytic activities of the fungus, insect, and other associated microbiota remains limited. The genome of *A. areolatum* is rich in CAZyme genes, including the glycoside hydrolase family genes *GH3*, *GH5*, *GH6*, and *GH7* and auxiliary activity 3 (AA3), a family that may be involved in lignin degradation (40). It was also documented that *A. areolatum* produces cellulases and laccases in vitro, supporting the idea that it contributes to cellulose, to hemicellulose, and likely to lignin deconstruction (16, 75).

Research on the microbiome of *S. noctilio* woodwasps suggests that bacteria may contribute to plant biomass degradation: Cellulolytic strains of *Streptomyces* bacteria have been isolated from this species in eastern North America (14). Comparative studies revealed one of these strains to be among the most cellulolytic *Streptomyces* known (15), and biochemical characterization and targeted gene deletions showed that nonreducing-end exoglucanase (*GH6*) genes and the CebR transcriptional repressor play important roles in its cellulolytic capacity (14, 15). Given that it secretes these high-efficiency cellulolytic enzymes, it may contribute to plant cell wall degradation in woodwasp fungiculture. Nevertheless, given how little is known about the relative and absolute contributions of specific fungal and bacterial cellulolytic enzymes toward plant biomass degradation, woodwasp fungiculture is not included in **Figure 3**.

*Sirex* woodwasp larvae tunnel into the tree for one to two years, during which they feed on fungus biomass and/or fungus-altered wood. The first few larval instars of *Sirex* exclusively feed on *Amylostereum* (86), whereas later instars consume fungal biomass and partially digested sapwood (46, 116). The *Amylostereum* cultivar provides the larvae with sterols, such as ergosterol, and



woody matter-degrading enzymes, which are retained in their midgut and may assist in nutrient acquisition (72, 124).

## SYNTHESIS: INSECT FUNGICULTURE AND BIOENERGY APPLICATIONS

Recalcitrant plant cell wall components, such as cellulose and hemicellulose, make up the largest source of renewable organic carbon on the planet (42). Just as the development of agriculture enabled humans to produce food on a large scale, several groups of insects evolved the capacity to efficiently utilize the vast stored carbon in plant biomass to grow their own food. By cultivating fungus, these insects readily access plant polysaccharides, enabling them to enter new niches and become major and, in the case of ants and termites, dominant plant biomass decomposers in their respective ecosystems (95, 134). Using microbial symbionts to mediate plant biomass digestion is not unique to fungus-farming insects and appears to be shared across most, if not all, plant-feeding animals. The major innovation in fungus-farming insects is external plant substrate digestion, which allowed these insects to greatly expand the space available for digesting plant substrate by removing the limitation imposed by physical space restriction of guts. External digestion also allows for greater behavioral manipulation and management of substrate degradation, such as the movement of digestive enzymes in leaf-cutter ants and the Macrotermitinae.

Although all four fungus-farming symbioses evolved independently, they share several key similarities. Most notably, they all obligately rely on fungi to serve as the main food source for the insects and produce the majority of the plant biomass-degrading enzymes. This is perhaps not surprising, as many of the most cellulolytic organisms on the planet are fungi. Being modular organisms, with a filamentous growth form, also makes fungi better suited to engage in mutually beneficial symbioses that involve sacrificing modules to feed their insect host. Another similarity among these symbioses is that bacteria play important roles in host nutrition by aiding final digestion of partially digested plant biomass and supplementing nitrogen and possibly vitamins and amino acids. Lastly, effective host pretreatment of the plant substrate is critical in all four symbioses to initiate efficient plant biomass breakdown.

Unsurprisingly, given the independent origins and long evolutionary histories of the four fungus-farming insect mutualisms, there are appreciable differences in the specifics of plant biomass degradation among the symbioses. In leaf-cutter ant fungiculture, turnover of plant substrate is rapid, facilitating fast cultivar growth on the more easily digested plant polymers. In contrast, the fungus-farming termites rely more on worker gut bacteria through dual gut passages and thus achieve more complete digestion of the plant biomass. Research into the specifics of biomass degradation in woodwasps and ambrosia beetles, including comparative omics studies, are needed before conclusions can be drawn about differences in breakdown processes in these systems. A highly cellulolytic *Streptomyces* may contribute to wood degradation in *Sirex* woodwasps, which would be unique within insect fungiculture in terms of having a single dominant bacterial symbiont aiding in plant biomass degradation.

The parallels between fungus-farming insects and human agriculture have fascinated biologists for more than a century. More recently, human efforts to produce cellulosic biofuels, to shift to more carbon-neutral energy sources, have created another parallel, tapping into the stored carbon within plant cell walls. Humans have been producing cellulosic ethanol through microbial fermentation for more than a decade (65). However, scalable production of biofuels using ligno-cellulosic matter is still not economically viable except during the highest peaks of global oil prices, due largely to inefficiencies in lignocellulose deconstruction. Currently, industrial saccharification, the breakdown of complex polysaccharides into monosaccharides, employs strategies involving a



combination of chemical pretreatment and enzymatic hydrolysis to obtain simple sugars for subsequent fermentation (62). This parallels insect fungiculture, which also relies on pretreatment of plant substrates and microbially derived lignocellulolytic enzymes. The design of industrial cellulosic biofuels could thus benefit from insights derived from the saccharification strategies of fungus farmers, which are taken to industrial scales in the genera *Atta*, *Macrotermes*, and *Odonotermes*. The depolymerization of lignin is challenging and a major barrier in accessing inner polysaccharides, yet fungus-growing termites rapidly achieve significant lignin depletion during the first gut passage (80); improving our understanding of this incredibly efficient process could aid industrial biofuel design.

Fungus-farming insect symbioses represent a particularly promising source of novel cellulolytic enzymes, or enzyme cocktails, for industrial cellulosic biofuel production. The microbial enzymes associated with insect fungiculture have evolved over millions of years to become finely tuned to aid in the success of their associated insect hosts. Work on the fungal-farming symbioses continues to lead to discoveries of novel and diverse microbe-derived enzymes that sequentially and synergistically provide the necessary tools to effectively decompose plant substrates. Indeed, *Streptomyces* sp. SirexAA-E associated with *Sirex* woodwasps has emerged as a strain of high interest in biofuel research (14, 38, 83, 122). Nevertheless, the most extensive work on fungus-farming insects has been done on the most charismatic host lineages, leaf-cutter ants in the genus *Atta* and termite fungus farmers in the genus *Macrotermes*. The vast majority of the diversity of hosts and microbial symbionts remains unexplored, indicating that the vast majority of the enzymatic repertoire also remains untouched. An abundance of enzyme identities, functions, and physicochemical properties have yet to be discovered and elucidated—all with promising potential to inform plant biomass-decomposition strategies and for biotech applications.

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