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

Lily Khadempour[1,2,3], **Kristin E. Burnum-Johnson**[4], **Erin S. Baker**[4], **Carrie D. Nicora**[4], **Bobbie-Jo M. Webb-Robertson**[4], **Richard A. White III**[4], **Matthew E. Monroe**[4], **Eric L. Huang**[4], **Richard D. Smith**[4], and **Cameron R. Currie**[1,3,*]

[1]Department of Bacteriology, University of Wisconsin-Madison, Madison, WI, USA, 53706

[2]Department of Zoology, University of Wisconsin-Madison, Madison, WI, USA, 53706

[3]Department of Energy Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI, USA, 53706

[4]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA, 99352

Abstract

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

[*]**Corresponding author:** CR Currie, Department of Bacteriology, University of Wisconsin-Madison, 6155 Microbial Sciences Building, 1550 Linden Drive, Madison, WI 53706, USA Fax: (608) 262-9865 currie@bact.wisc.edu.

Data accessibility

All of the metaproteomic data from this study is available in the Supplemental Materials (Supplemental tables 1 and 2).

Introduction

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

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

Unlike most herbivores, leaf-cutter ants are polyphagous, meaning that they occupy a generalist herbivore niche. These dominant herbivores belong to two genera, *Acromyrmex* and *Atta*, and forage on 2–17% of all the foliar biomass in some ecosystems in the Neotropics (Herz *et al.* 2007; Costa *et al.* 2008). Their success as herbivores can be attributed to their obligate mutualism with a fungus, *Leucoagaricus gongylophorus*, which they cultivate for food: they provide the fungus with leaf material and, in turn, the fungus provides specialized hyphal swellings called gongylidia, which the ants feed on (Holldobler & Wilson 1990; Mayhé-Nunes & Jaffe 1998; Holldobler & Wilson 2008). The types of plant material that a colony consumes depends on the ant species, the location, and the season in which the colony is observed (De Vasconcelos 1990; Wirth 2003). In general, they tend toward young leaves with soft cuticles, less-toxic plant defense compounds, fewer trichomes, fewer endophytes and higher nutritional value (Howard 1987; 1988; Van Bael *et al.* 2011). Within these constraints, leaf-cutter ants incorporate many different types of plants into their fungus gardens and have been observed foraging at least 20 different species of plants over three days (Wirth *et al.* 1997). Ants also incorporate a variety of plant parts into their gardens such as leaves, flowers, seeds, and fruit parts in the wild, and oats and parboiled rice in laboratory settings (Wirth *et al.* 1997; Kooij *et al.* 2011).

Leaf-cutter ants tend to their mutualistic fungus in gardens, which can be viewed as an ‘external gut’. These gardens contain both the fungus itself and a low diversity community of bacteria. Through enzymatic, metagenomic and metaproteomic analyses, the microbial communities in the fungus gardens of leaf-cutter ants *Atta sexdens* and *Atta cephalotes* have been explored. Many fungal amylases (Silva *et al.* 2006b), pectinases (Silva *et al.* 2006a),

carbohydrate-active enzymes (CAZy), fungal oxidative lignin enzymes (FOLy), and secreted proteases have been identified (Aylward *et al.* 2012; 2013a), demonstrating that the fungus in this system is primarily responsible for the breakdown of plant biomass. The bacterial community in the fungus gardens was identified using isolation, metagenomics and 16S sequencing (Suen *et al.* 2010; Aylward *et al.* 2012). While the bacterial community has the genetic capacity for biomass degradation (Suen *et al.* 2010), there is not yet evidence that this is actually occurring in the gardens.

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

Methods

Experimental design

Atta cephalotes fungus gardens were excised from colonies excavated in the secondary tropical moist forest surrounding the Smithsonian Tropical Research Institute (STRI) Gamboa research station in Panama between Dec. 27, 2012 and Jan 10, 2013. Five mature colonies were excavated. Since lab-reared sub-colonies without queens are unstable, five fungus chambers were excised from each colony to ensure that we would have sufficient numbers of replicates for proteomics. These fungus chambers were split into four sub-colonies each and were contained within a plastic container (10×10×8 cm) that was kept in a larger plastic container (14×19×9 cm). Care was taken to minimize disturbance to the fungus gardens and to ensure that a relatively even number of workers were distributed to each sub-colony.

Each sub-colony was randomly assigned to one of four feeding treatments, and received different plant biomass to use as substrate for cultivating their fungal mutualist. The four feeding treatments were *Lagerstroemia speciosa* L. leaves, *Hibiscus rosa-sinensis* flowers, Quaker instant oatmeal, or a mixture of all three (Figure 1). The substrates that were selected were all readily available and were readily incorporated into the gardens by the ants, but they varied in terms of their energy availability. Leaves are the most recalcitrant substrate of the three. The flowers are similar to leaves in terms of cell wall structures but are more easily digestible (Amaglo *et al.* 2010). The oats are highly processed and have the most accessible energy in the form of sugars and starches (Cuddeford 1995; Welch 1995). The flowers and leaves were collected daily from plants in the immediate vicinity in Gamboa. The sub-colonies were fed *ad libitum*, typically every one or two days, depending on how quickly the ants would incorporate new substrate. The colonies were maintained at ambient temperature and humidity. After 15 days, the entire fungus garden from each sub-colony was frozen in PBS buffer at −20°C in a 50 mL conical tube, in preparation for further processing. One of the five colonies was excluded from metaproteomic analysis because it did not have

surviving sub-colonies from all treatments but it was included it in the survivorship analysis. From the surviving sub-colonies we selected 16 samples for metaproteomics (four treatments and four colony replicates each). The sub-colonies that were selected for metaproteomics were all active and still incorporating new material into their gardens at the end of the 15 days of the experiment.

Mass spectrometry instrumentation

Analysis of the trypsin-digested peptide mixtures (Supplemental Methods) from the gardens was performed on both a Thermo Fisher Scientific LTQ Orbitrap mass spectrometer (MS) (San Jose, CA, USA) operated in tandem MS (MS/MS) mode and an in-house built ion-mobility MS (IMS-MS) instrument that couples a 1-m ion mobility drift cell (Baker *et al.* 2007; 2010) with an Agilent 6224 time-of-flight (TOF) MS that was upgraded to have a 1.5 m flight tube for resolution around 25,000. The same fully automated in-house built 2-column HPLC system (Livesay *et al.* 2008) equipped with in-house packed capillary columns was used for both instruments with mobile phase A consisting of 0.1% formic acid in water and B comprised of 0.1% formic acid in acetonitrile. A 100 min LC separation was performed on the Velos MS (using 60-cm long columns having an o.d. of 360 μm, i.d. of 75 μm, and 3 μm C_{18} packing material) while only a 60 min gradient with shorter columns (30-cm long columns with the same dimensions and packing) that was used with the IMS-MS since the additional IMS separation helps address detector suppression and also faster LC analyses. Both gradients were linear with mobile phase B increasing from 0 to 60% until the final 2 min of the run when B was purged at 95%. 5 μL of each sample was injected for both analyses and the HPLC was operated under a constant flow rate of 0.4 μL/min for the 100 min gradient and 1 μL/min for the 60 min gradient. The Velos MS data was collected from 400–2000 m/z at a resolution of 60,000 (automatic gain control (AGC) target: 1×10^6) followed by data dependent ion trap MS/MS spectra (AGC target: 1×10^4) of the twelve most abundant ions using a collision energy setting of 35%. A dynamic exclusion time of 60 s was used to discriminate against previously analyzed ions. IMS-TOF MS data was collected from 100–3200 m/z.

Metaproteomic data processing and statistical analysis

Identification and quantification of the detected peptide peaks were performed using the accurate mass and time (AMT) tag approach (Zimmer *et al.* 2006; Burnum *et al.* 2012). Peptide database generation utilized Velos tandem MS/MS data (Kim *et al.* 2008; Piehowski *et al.* 2013) from pooled fractionated samples (Supplemental Methods). Due to the greater sensitivity and dynamic range of measurements (Burnum *et al.* 2012) relative quantitation of the peptide peaks utilized the LC-IMS-MS data. Multiple in-house developed (Monroe *et al.* 2007; Jaitly *et al.* 2009) informatics tools were used to process the LC-IMS-MS data and correlate the resulting LC-IMS-MS features to the AMT tag database containing LC elution times, IMS drift times, and accurate mass information for each assigned peptide. Our in-house ion mobility mass spectrometry platform has previously provided novel insight into complex biological systems (Burnum *et al.* 2012; Baker *et al.* 2014; Cha *et al.* 2015; Baker *et al.* 2015; Kyle *et al.* 2016).

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

Results

Fungal proteomics

With our metaproteomic analysis of the fungus gardens, we identified and quantified 1,766 different fungal proteins, including 161 putative biomass-degrading enzymes (Supplemental Table 1). NMDS analysis of the global proteome profiles across treatments and replicates revealed grouping according to treatment (Figure 2A). These differences according to treatment were significant (PERMANOVA p<0.001). Fungus garden proteomic profiles in both the leaves and flowers treatments showed low variability within-group and between-group, while the oats and mixed treatments had greater within-group variability and overlapped with each other. These groupings are evident when individual proteins are compared between treatments. To analyze the differential abundance of individual proteins, we conducted pair-wise comparisons of each protein in the four treatments. Numerous proteins with significantly different abundances were identified between the treatments (Supplemental Table 1). When individual protein differences are observed globally using heat maps, we can again see grouping according to treatment (Figures 3 and 4): the oats sub-colonies were most similar to the mixed sub-colonies, while the leaves sub-colonies were similar to the flowers. The significant changes for each protein pairwise comparison were identified by at least 2 peptides with: oats/mixed having 52 significantly changing proteins, leaves/flowers - 31, leaves/oats - 286, flowers/oats - 259, leaves/mixed - 135, and flowers/mixed - 125 (Supplemental Table 1).

All biomass-degrading enzymes observed to be significantly different (p<0.05) between treatments are listed in Table 1, where individual proteins are compared between the mixed and other treatments. We compared to the mixed treatment since it most closely resembles the ants' natural tendency to incorporate a mixture of substrates into their fungus gardens. In

general, the leaves and flowers treatments had similar results with much higher abundances of CAZys, proteases and enzymes necessary for the breakdown of cellulose: endoglucanases (GH5 and GH6), exoglucanase (GH6), and β-glucosidases (GH3 and GH31), compared with the other two treatments. However, the oats treatment was very similar to the mixed treatment with a lower abundance of these proteins and proteases (Table 1, Figure 4).

Bacterial proteomics

We detected only 44 unique bacterial peptides and from these data we determined, through similar pairwise comparisons between treatments, that there were three bacterial proteins that differed significantly between treatments. Each of these proteins was identified with only a single peptide. These proteins were identified based on genomes of bacterial symbionts of leaf-cutter ants (*Enterobacter* strain FGI 35, *Serratia* strain FGI 94 (Aylward *et al.* 2013c), *Enterobacteriaceae* strain FGI 57 (Aylward *et al.* 2013b), *Pseudomonas* strain FGI 182, *Klebsiella variicola* strain AT-22 and *Pantoea* strain AT-9b (Aylward *et al.* 2014)). Malate dehydrogenase, which mapped equally to *Cronobacter, Pantoea, Serratia, Enterobacter,* and *Klebsiella* genomes, was more abundant in the leaf treatment. Periplasmic trehalase, which mapped to the *Enterobacter* genome, was more abundant in the flower treatments. ATP synthase subunit β, which mapped to all six bacterial genomes, was the least abundant in the leaf treatments. Overall, the global bacterial protein profiles did not differ between treatments (Supplemental Table 2, Figure 2B).

Sub-colony Survivorship

The fungus garden of some sub-colonies did not remain healthy throughout the experimental period, but instead dried out, were discarded by workers, or were overgrown by a pathogen. This was especially common for sub-colonies created from the gardens excised from the last two parent colonies. A sub-colony was considered failed when all the ants were dead or when the fungus garden was overtaken by a pathogen. Overall, sub-colonies fed exclusively on oats had significantly lower survivorship than the other colonies (Figure 5).

Discussion

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

lyases (PL), and 30 auxiliary activities enzymes (AA) (Figure 4, Supplementary Table 1). This suite of enzymes includes all the components necessary for the breakdown of cellulose (endoglucanases GH5, GH12 and GH6, exoglucanase GH6 and β-glucosidase GH31).

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

Evidence supporting the substrate-specific response in the leaf-cutter ant fungus garden has been previously reported elsewhere. Kooij *et al.* (2011) manipulated the substrate for *A. cephalotes* fungus gardens and using Azurine-Crosslinked (AZCL) assays measured changes in specific enzymes of interest, observing an overall shift in enzyme activity between substrates. AZCL is a high throughput method used to detect enzyme activity, while metaproteomics provides accurate detection and quantification of the specific proteins present. Thus, our approach represents a more thorough enzymatic response of the fungus garden, as follows. First, AZCL is conducted with a limited suite of substrates and only shows activity of enzymes to those substrates. This excludes any non-enzymatic proteins and any enzymes that did not have the appropriate substrate to respond to. Second, AZCL does not allow us to characterize specific proteins, whereas metaproteomics does..

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

Recent work has identified the presence of an apparent consistent bacterial community in the fungus garden (Pinto-Tomás *et al.* 2009; Suen *et al.* 2010; Aylward *et al.* 2012). Although certain functional roles of the bacteria have been elucidated, such as nitrogen fixation (Pinto-Tomás *et al.* 2009) and the apparent capacity to provide vitamins (Aylward *et al.* 2012), our insights regarding the bacteria remain limited. Here, we did not observe a notable change in bacterial proteins, other than the three which are all part of central carbon metabolism and unlikely to play a direct role in substrate breakdown or detoxification (Bergmeyer & Gawehn 1974; Boos *et al.* 1987). Only 1% of the unique peptides that were detected in these analyses were identified as bacterial. This is likely due to a considerable difference in the amount of fungal and bacterial biomass in the fungus gardens. It could also indicate that bacteria play a more limited role in the fungus gardens.

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

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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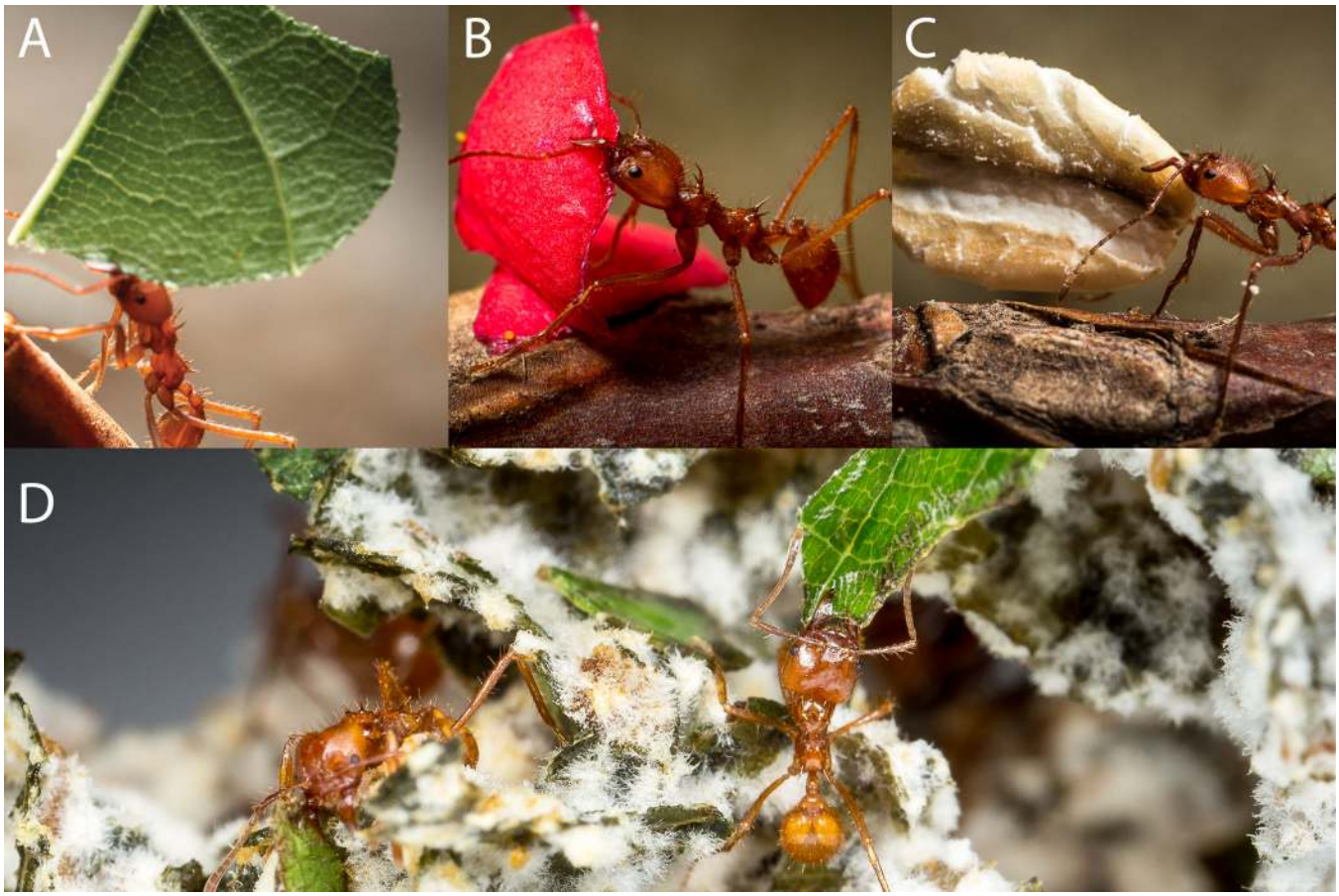

Figure 1.
Leaf cutter ants carrying various substrates (A) a leaf, (B) a flower and (C) an oat. Ants tending to their fungus garden with newly incorporated leaf material (D) (photographs by Don Parsons).

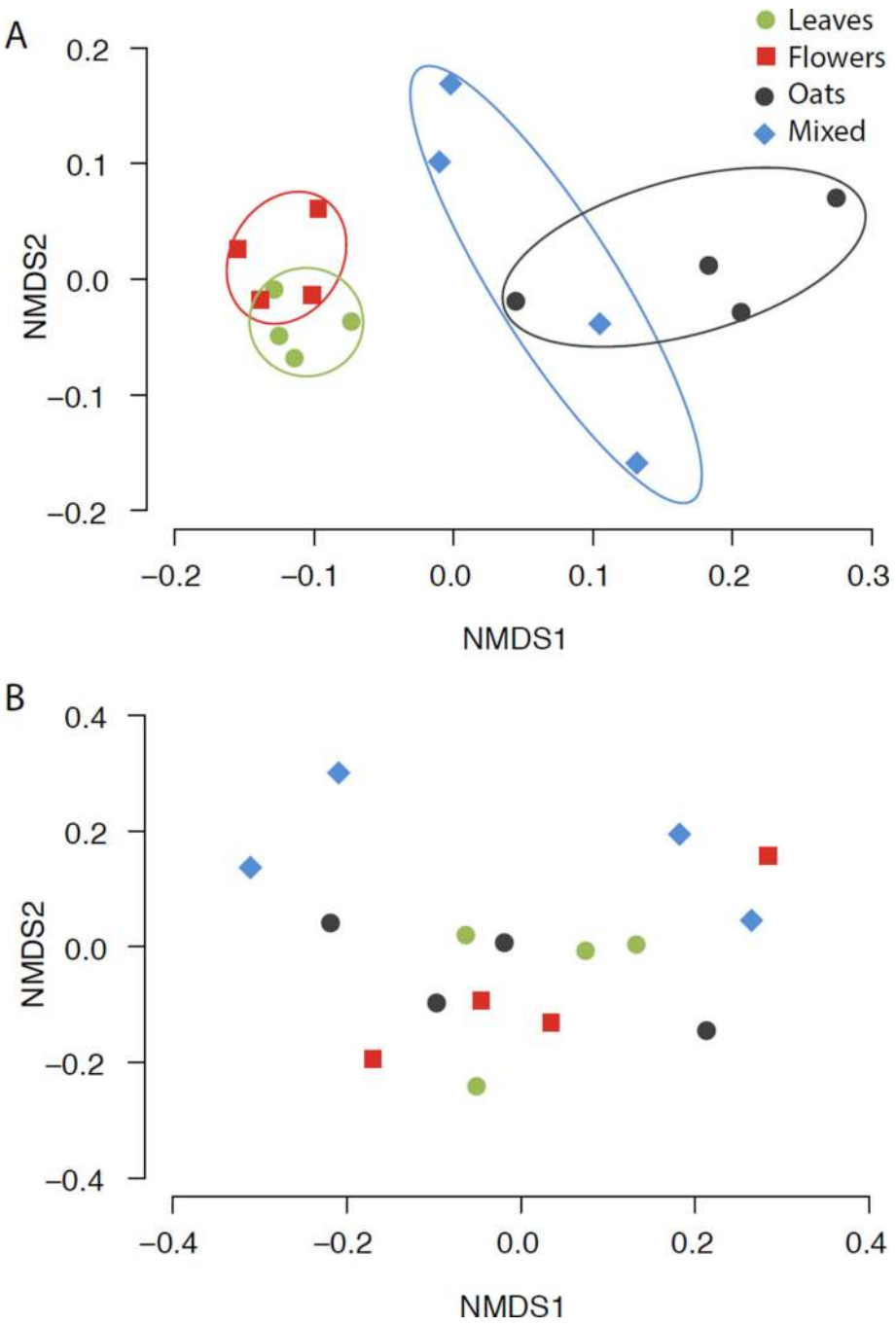

Figure 2.
NMDS plot of (A) fungal and (B) bacterial whole-community metaproteomics. While the fungal results were significantly different between treatment groups, the bacterial metaproteomes were not possible to differentiate statistically.

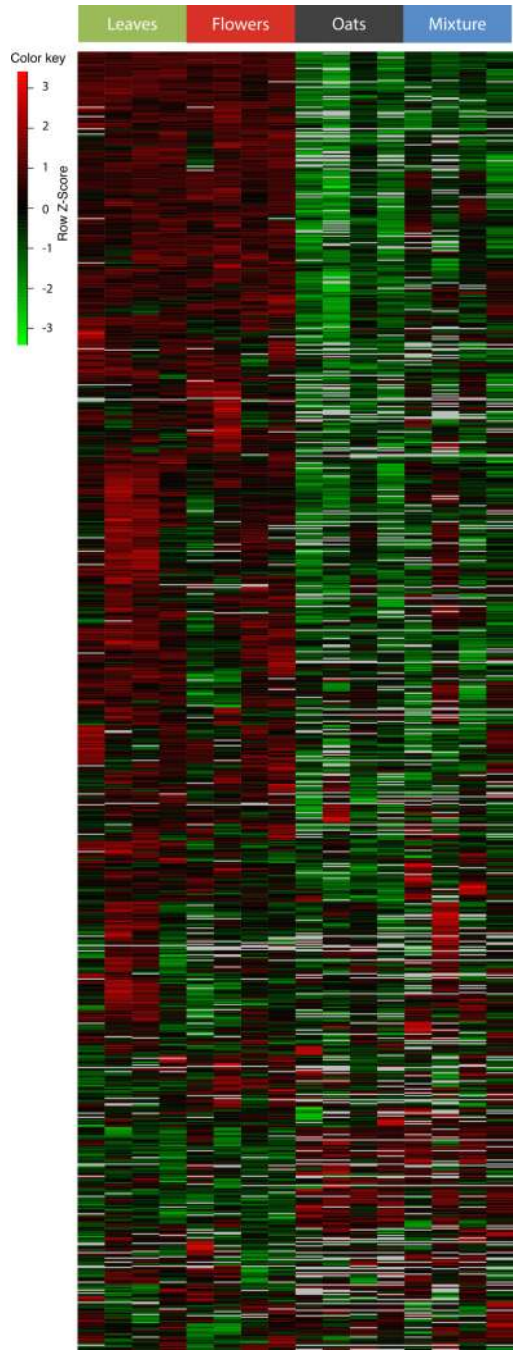

Figure 3.

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

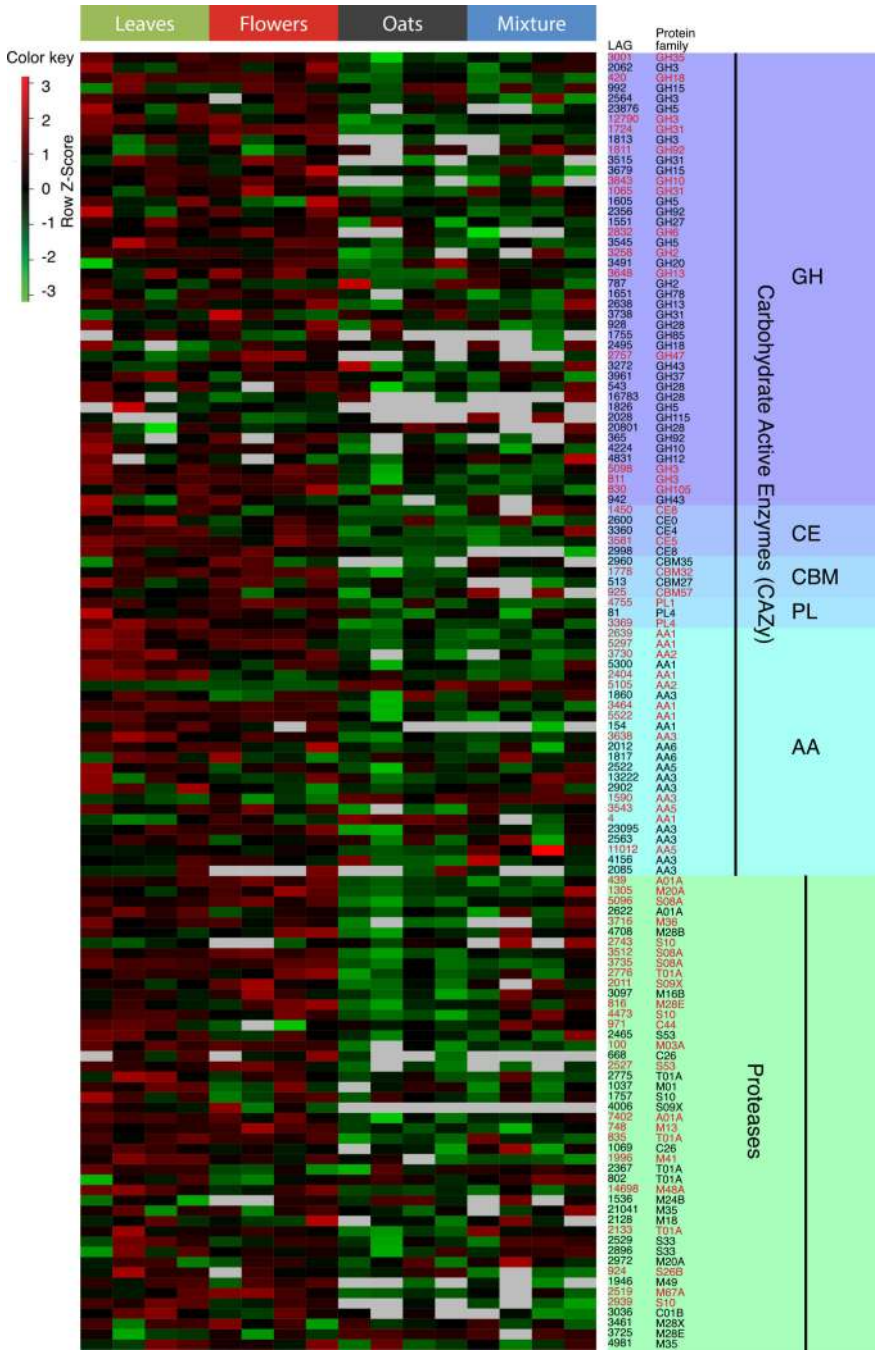

Figure 4.

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

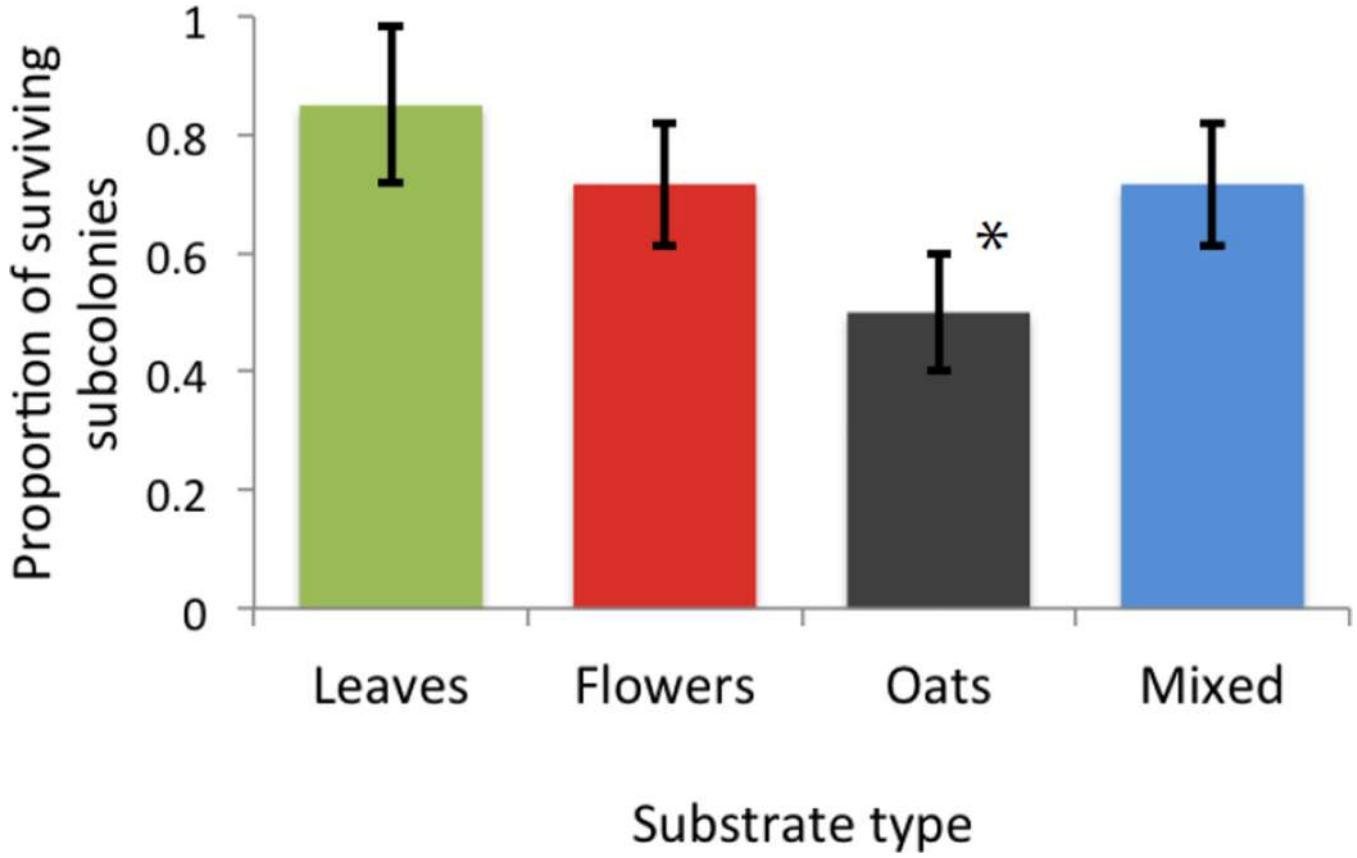

Figure 5.
Sub-colony survival by treatment. Sub-colonies that were fed oats survived significantly (*) less than the other sub-colonies, over the course of the experiment (ANOVA $p<0.05$).

Table 1

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

LAG	Protein Family	Annotation	Leaves	Flowers	Oats
CAZy					
1450	CE8	Pectin methylesterase			–
925	CBM57, CE15	Found attached to glycosidases			–
1065	GH31	α-glucosidase, and others			–
2832	GH6	Endoglucanase, exoglucanase, cellobiohydrolase		+	
1778	CBM32	Binding to galactose, lactose, polygalacturonic acid, LacNAc		+	–
4224	GH10	Xylan targeting	+		
3545	GH5	Endo-β-1,4-glucanase / cellulase and many others	+		
3581	CE5	Acetyl xylan esterase, cutinase	+	+	
3843	GH10	Xylan targeting	+	+	
830	GH105	Unsaturated rhamnogalacturonyl hydrolase; d-4,5-unsaturated β-glucuronyl hydrolase	+	+	
420	GH18	Lysozyme, chitinase, many others	+	+	
5098	GH3	β-glucosidase, and others	+	+	
811	GH3	β-glucosidase, and others	+	+	
1724	GH31	α-glucosidase, and others	+	+	
1811	GH92	Mannose targeting	–	–	
11012	AA5	Glyoxal oxidase			–
3543	AA5	Glyoxal oxidase			–
1590	AA3	Glucose oxidase	–	–	
3638	AA3	Alcohol oxidase 1	+	+	
2639	AA1	Laccase-1	+	+	
3464	AA1	Laccase-4	+	+	
5297	AA1	Laccase-2	+		
2404	AA1	Laccase-1	+		
5522	AA1	Laccase-2	+		
3730	AA2	Chloroperoxidase	+		

LAG	Protein Family	Annotation	Leaves	Flowers	Oats
5105	AA2	Chloroperoxidase	–	–	
3594	AA3	Dihydrolipoyl dehydrogenase, mitochondrial		+	
Proteases					
3716	M36	Endopeptidase			–
971	C44	Self-processing precursor of Amidophosphoribosyltransferase		–	
2519	M67A	Isopeptidases that releases ubiquitin from ubiquitinated proteins		+	
3036	C01B	Endopeptidases or exopeptidases	+		
3725	M28E	Aminopeptidase	–		
439	A01A	Pepsin A	+	+	
100	M03A	Thimet oligopeptidase	+	+	
748	M13	Metalloendopeptidase	+	+	
1996	M41	ATP-dependent metalloendopeptidase	+	+	
15046	M67A	Isopeptidases that release ubiquitin from ubiquitinated proteins	–	–	
3735	S08A	Subtilisin Carlsberg	+	+	
2389	S08A	Subtilisin Carlsberg		+	
3512	S08A	Subtilisin Carlsberg		+	–
5096	S08A	Subtilisin Carlsberg		+	–
2939	S10	Carboxypeptidase Y	+	+	
4473	S10	Carboxypeptidase Y			–
2743	S10	Carboxypeptidase Y	–	–	–
924	S26B	Signalase 21 kDa component	+	+	+
2527	S53	Sedolisin	+	+	

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