UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

FROM COOPERATION TO COMPETITION:

HOW MICROBES AND INVERTEBRATES INTERACT IN A TROPICAL FOREST

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

Degree of

DOCTOR OF PHILOSOPHY

By

JANE MARGARET LUCAS
Norman, Oklahoma
2018

FROM COOPERATION TO COMPETITION:
HOW MICROBES AND INVERTEBRATES INTERACT IN A TROPICAL FOREST

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

BY

_____
Dr. Michael Kaspari, Chair

_____
Dr. Bradley Stevenson

_____
Dr. Lara Souza

_____
Dr. Michael Patten

_____
Dr. Michael Givel

I dedicate this dissertation to my parents, who instilled a love of the natural world in me, and to Dr. Adam Kay for letting me know I could make a career out of it.

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Michael Kaspari, for his support and guidance throughout my scientific career. His passion for ecology and constant curiosity towards how the world works has been an inspiration. From the early days on Barro Colorado Island, to our time together in Oklahoma, no other individual has shaped my identity as a scientist as much as you have, and for that I am eternally grateful. I would also like to extend my sincere appreciation towards my committee members: Dr. Bradley Stevenson, Dr. Lara Souza, Dr. Michael Patten and Dr. Michael Givel. Their guidance and positivity throughout my time at OU has strengthened my research and expanded my view of how to approach scientific questions. I extend a special level of thanks to Dr. Stevenson and the members of his lab. The guidance and advice I have gained by working with the team on GLCH floor 8 was essential to my success as a PhD student. Thank you to Brian Bill, Heather Nunn and Emily Junkins in particular for their constant support and willingness to teach and answer questions throughout my time at Oklahoma. I would also like to thank my collaborators both within and outside the University of Oklahoma. In particular, my external advisors, Dr. Alfonso Alonso and Dr. Tomas Roslin. The experiences I gained working with them has provided me with a much broader point of view and have helped shape my future as a scientist. Thank you to Dr. Laura Bartley for her guidance regarding plant-based sequencing protocols and the opportunity to teach her undergraduate course. Similarly, thank you to all of the OU Department of Biology faculty and staff. Your help in ordering supplies, dissertation preparation and general knowledge of how to navigate the University's requirements has been invaluable. Thanks to the University of

Oklahoma: Department of Biology, College of Arts and Science, Graduate College, and Graduate Student Senate, as well as the National Science Foundation and Graduate Assistance in Areas of National Need Fellowship for funding my dissertation research and its dissemination. Thank you to all my current and past lab mates: Karl Roeder, Jelena Bujan, Natalie Clay, Rebecca Prather, Ellen Welti and Michael Weiser. I want to extend a special thank you to Dr. Yanoviak and the students in his lab. Conversations spent with your lab have been foundational to me both as a scientist and as a person. To Max Adams, thank you for your friendship, advice and passion for entomology. Furthermore, thank you to everyone at the Smithsonian Tropical Research Institute for the incredible community and knowledge they have provided me. Dr. William Wcislo, Dr. Ben Turner, Dr. Joe Wright, Dr. Egbert Leigh, Dr. Scott Mangan and Dr. Meg Crofoot, thank you all for your invaluable insight and help in shaping my dissertation projects, as well as instilling a love of the tropics in me. Similarly, thank you to all of the students on BCI who have become my external network. The time I have spent with you all on the BCI balcony and comédor have been some of the most inspiring and enjoyable times in my graduate career. To my parents, thank you for encouraging me to follow my passions and teaching me to love the natural world. To my siblings, thank you both for your ability to remind me of who I am and what is important in this world. A profound thank you to Evan Gora for your love and unending inspiration. The countless hours of scientific talks with you have pushed me to become the scientist I am today. Lastly, I thank all of my family and friends for their love and support before and during my PhD.

Table of Contents

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Figures

Chapter 1

Chapter 2

Chapter 4

Abstract

Microbes and invertebrates are “the little things that run the world” (Wilson 1987, Moreau 2017), but the intricacies of how these organisms impact our environment remains underexplored. Here I investigate how microbes and invertebrates interact and how these interactions scale-up to impact communities and ecosystem-level processes. This work focuses on tropical brown food webs because they are dominated by a diversity of microbe-invertebrate relationships that span from obligate symbioses to fierce competition. Initially, I examine the symbiotic relationship between a dominant canopy ant, *Azteca trigona,* and their microbiota. Here I describe the diversity of microbial communities associated with these ants and demonstrate the role of invertebrate activity in microbial dispersal (Ch. 1). Furthermore, the microbial community within these canopy ants provides the basis for a facultative relationship between ants and their host plants, as the ant endosymbionts increase plant growth and facilitate nutrient exchange (Ch. 2). I then transition to explore how competition between microbes and invertebrates can shape the local community in the ephemeral environment of tropical leaf litter (Ch. 3). I demonstrate that antibiotic production by microbes—long considered a potent mechanism of competition between microbes—can also be effective against invertebrates. This cross-domain competition likely contributes to the diversity of detrital food webs (Ch. 4). Combined, the results of these studies demonstrate how invertebrate-microbe interactions drive ecosystem structure and function.

Chapter 1: The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest.

(Formatted for *Ecosphere*)

Citation: Lucas, J., B. Bill, B. Stevenson, and M. Kaspari. 2017. The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest. Ecosphere 8(2):e01639. 10.1002/ecs2.1639

Abstract

Microbial life is ubiquitous, yet we are just beginning to understand how microbial communities are assembled. We test whether relationships between ant microbiomes and their environments resemble patterns identified in the human home microbiome. We examine the microbial communities and chemical composition of ants, their waste, their nest and the surrounding soil. We predicted that the microbiome of the canopy ant, *Azteca trigona*, like that of humans, represents a distinct, relatively invariant, community compared to the soil community. Because *Azteca* build aboveground nests constructed from ant exudates mixed with chewed plant fibers, we predicted that nest-associated microorganisms should reflect their ants, not the surrounding environment. The ant microbiome was distinct from the soil, but contrary to initial predictions, ant microbiomes varied dramatically across colonies. This variation was largely driven by the relative abundance of *Lactobacillus*, a genus frequently associated with hymenopteran diets. Despite the origin of nests and their means of construction, nest-associated microorganisms were most similar to the surrounding soil. The microbiota of *Azteca* ants is thus distinct, but dimorphic across colonies, for reasons likely due to inter-colony differences in diet; microbiotas of the nests however mirror the surrounding soil community in similar to patterns of human home microbiota.

Introduction

Microbes are present in nearly every location on earth. Numerous studies are beginning to identify some of the rules by which microbial communities are assembled and vary geographically (such as the role of pH in microbial distribution (Fierer and Jackson 2005), or the high geographic endemism in fungal communities (Grantham et al. 2015, Barberan et al. 2015a)). Many of these studies have focused on the interactions humans and their microbiomes have with their "built environments" (hospitals, office buildings, and homes (Kembel et al. 2012, Hewitt et al. 2012, Barberan et al. 2015)). These have provided insight into how the geography of abiotic factors like climate and physical structure dictate which microbes colonize the home's exterior (Kembel et al. 2012, Matulich et al. 2015, Barberan et al. 2015a). Likewise, features of the home's occupants--their number, gender, and species--of the occupants, along with their associated microbiomes, can influence the home's internal microbial community (Taubel et al. 2009, Lax et al. 2014, Barberan et al. 2015a). Our study highlights another organism known for constructing elaborate dwellings: the ants. Like humans, ant colonies build structures to live in, produce waste, and interact in ways that produce distinct microbiomes (Wheeler 1910, Holldobler and Wilson 1990, 2009). We propose that like studies of the microbiome of the human home, ants and their built structures are intimately connected and capable of influencing one another's microbial assemblage.

The microbiota associated with social organisms are of particular interest as their colonial lifestyle provides a high risk of disease spread (Wilson 1975). To maintain colony health, many social organisms rely on associations with mutualistic

microbes (Currie et al. 1999, Currie et al. 2006, Koch and Schmid-Hempel 2011, Kellner et al. 2016). Microbiota can aid in nest mate recognition (Richard et al. 2007, Theis et al. 2013, Dosmann et al. 2016), or provide protection through production of antimicrobial compounds (Promnuan et al. 2009, Sen et al. 2009, Barke et al. 2010, Visser et al. 2012, Madden et al. 2013). Because of these relationships, the microbiota of social organisms and their built structures are being explored as potential sources for novel antibiotic compounds (Pelaez 2006, Bode 2009, Poulsen et al. 2011), though detailed investigations of these environments are lacking (Madden et al. 2013, Kellner et al. 2015).

The Neotropical ant *Azteca trigona*, forms high-density populations in Panama's seasonal forests (1-5 nests every 40 m) with colonies inhabited by >200 000 ants (Adams 1994, Clay et al. 2013). *A. trigona* societies build and maintain large papery carton nests (0.5 - 4 m in length) by chewing, regurgitating and gluing together plant fibers (Fig. 1). This process creates ample opportunity for the ant microbiome to inoculate the building material. These colonies may live up to 30 years (*M. Kaspari personal observation*), providing generous time for nests to develop distinctive microbiomes. Fueled on a diet of sugary honeydew and insects (Longino 2007), *A. trigona* are aggressive ants, with territories spanning multiple tree crowns and a consistent work force inhabiting, patrolling and defending the nest's exterior. Each colony produces up to 10 g of organic refuse a day, depositing it on the ground directly below the nest. This refuse mainly consists of ant waste, as well as occasional parts of carrion and nest material. The constant refuse input generates a constant, long-term interaction between canopy and forest floor microbial communities (Clay et al. 2013).

Our study uses *Azteca trigona* societies to pose similar questions pursued by studies of the microbiome of human societies: How do the microbiomes of individual colonies differ from the waste they produce, and to what extent do the bacterial communities shape the microbial communities of the nests they inhabit? We ask do the gut-origins of the exudates used in nest construction and maintenance make nest microbiotas an extension of the ant colony, or do they maintain microbiomes more similar to the surrounding environment? We further test the prediction, driven by assumption that core microbiota are maintained by ants (Hu et al. 2014), that inter-colony variation in the composition of the ant microbiome and refuse community will be smaller than, yet correlated with, the variation found in the nest and soil. Finally, because microbes are often metabolic and biogeochemical specialists, we explore how the chemical composition varies among the ants, their refuse, nest, and soil. Through these questions, we aim to shed light on how the microbiome of a species interacts with and is shaped by the surrounding environment.

Materials and Methods

All samples for this study were collected during July 2014 in the Barro Colorado National Monument (BCNM), Panama. BCNM consists of Barro Colorado Island (BCI) and the surround mainland Gigante peninsula. BCNM is a seasonally wet tropical forest that receives ca. 2600 mm of rain annually, with the majority of rain falling from mid-April to mid-December (Wieder and Wright 1995).

Field Samples

For this study, we located 10 nests along the Edwin Willis trail on the Gigante peninsula and 10 nests along the Thomas Barbour trail on BCI. Studied nest had no host tree specificity and ranged in size from 0.5 m to 3.5 m. We selected nests within 2 m from the ground to aid in sampling. Refuse collection buckets were placed below each nest to collect refuse before it could be inoculated with soil microbial communities, as described in Clay et al. (2013). Due to the close proximity to the forest floor, collection buckets capture >90% of the refuse fall. Each nest was given 5 days to allow for adequate refuse accumulation before sampling.

Microbial reference samples were taken from each colony's ants, refuse, nest and surrounding soil. Hydrogen peroxide and ethanol sterilized forceps were used to collect each sample. Roughly 20-30 ants (0.5 g total) were collected from the outside of the nest to ensure that workers from the same colony were being examined. Ants were surface sterilized with a 95% ethanol wash but not dissected, (Kautz et al. 2013). However, we acknowledge that a 95% ethanol wash may not be a fully sufficient way of eliminating surface bacteria (Moreau 2012), and therefore microbial ant samples represent entire ant microbiomes. Nest samples consisted of a 0.5 g piece of nest material taken from the external portion of the nest. Nest portions sampled were located at least 50 cm away from the bottom of the nest to avoid potential contamination with refuse material. For refuse samples, we collected 0.5 g of refuse from collection buckets (Clay et al. 2013). Finally, we took 0.5 g soil samples from locations 0.5 m away from directly below the center of nests. Due to collection buckets collecting the majority of refuse, and the distinct coloration difference between blackened refuse and red soils, we

are confident that samples taken 0.5 m away from nests were not contaminated by falling refuse.

Microbial Community Analysis

All samples were placed in sterile 1.5 ml tubes containing 750 ml of Zymo's Xpedition^{tm} Lysis/Stabilization solution and bashing beads. Within 2 hours of sampling, all samples were ground and homogenized by bead-beating tubes at a 1000 rpms for 10 mins using the Vortex-Genie$^{®}$ tube adaptor, after which, DNA was stabilized. Preserved field samples were stored at -40°C. Immediately prior to DNA extraction, samples were re-homogenized using a BioSpec© Mini-Beadbeater for 60 s. Total DNA was extracted according to the manufacturer's protocol (Zymo Soil/Fecal Xpedition^{tm} mini kit protocol).

Libraries of small-subunit (16S) rRNA gene fragments representative of bacterial phylotypes were generated from each DNA sample using the primers S-D-Arch-0519-a-S-15/S-D-Bact-0785-b-A-18 (Klindworth et al. 2013). The S-D-Arch-0519-a-S-15 primer was modified to include a 16 bp M13 sequence (GTAAAACGACGGCCAG) at the 5' end to allow for the attachment of a unique 12 bp "barcode" in a subsequent PCR reaction. The 50 µl PCR reaction containing 2 µl of 1:10 diluted template DNA, 0.2 µM each of forward and reverse primer and 1 µM of 5 Prime Master Mix (5 PRIME) were carried out in a Techne TC-512 Gradient Thermal Cycler (Techne Inc., Burlington, NJ, USA). Initial denaturation was held at 96 °C for 3 min, followed by 30 cycles, each consisting of 96 °C for 30 s, 52 °C for 45 s, and 72 °C for 45 s. The final extension was held for 10 min at 75 °C. Appropriate PCR products

were verified on 1% agarose gel. PCR products were purified using SPRIselect beads following the manufacturer's protocol (Beckman Coulter, Brea, CA, USA).

A unique 12 bp "barcode" was attached to each library using a subsequent 6 cycle PCR reaction. Unique barcode sequences are presented in Supplemental Table 1. The attached forward primers consisted of a unique barcode, two spacer nucleotides and the 16 bp adapter sequence (GTAAAACGACGGCCAG); the reverse primer was S-D-Bact-0785-b-A-18. This unique "barcode" labeling reaction was a total of 50 µl and contained 4 µl of the purified PCR product, 0.2 µM each of forward and reverse primer, and 1 µM of 5 Prime Master Mix (5 PRIME). Six cycles of PCR thermal cycling were carried out in a Techne TC-512 Gradient Thermal Cycler, as described above. The resulting products were cleaned using SPRIselect beads and quantified using the Qubit fluorometer and dsDNA HS assay kit (Life Technologies, Grand Island, NY, USA). Equimolar amounts of each uniquely barcoded PCR product were pooled and submitted for Illumina MiSeq using TruSeq 250 bp PE V2 chemistry.

Sequence Data Analysis

All 16s sequencing reads were analyzed and demultiplexed using QIIME (Caporaso et al. 2010). We removed sequencing reads that contained errors in the barcoded region, ambiguities, homopolymers (greater than 6 nucleotides in length), or an average quality score < 25. Primer sequences were trimmed, chimeric sequences were eliminated using USEARCH (version 6.1) and the "gold" reference database (Edgar 2010). Then sequences were clustered into *de novo* operational taxonomic units (OTUs) at 97% similarity. Microbial taxonomic classification was assigned via the SILVA reference database (Quast et al. 2013) using the pyNAST aligner. All raw data

is available in the NCBI BioSamples databank (Accession nos. SAMN04576300-SAMN04576371).

Chemistry Analysis

We analyzed how chemistry changes across environments by collecting additional samples (approx. 5 grams) from ants, refuse, nest and soil. Due to the partially destructive nature of nutrient sampling, all chemistry samples were taken after microbial samples were taken; however, we were only able to obtain large enough refuse samples from 11 of the 20 nests. Ant, nest, refuse and soil samples were air dried and then weighed to two grams. Samples analyzed for cations and P were extracted in Mehlich-3 solution (Mehlich 1984) with detection by ICP-OES on an Optima 2100 (PerkinElmer, Waltham, MA). Total C and N were measured in 0.5 M K_2SO_4 extracts and determined by automated colorimetry on a Lachat Quikchem 8500 (Hach Ltd). All samples were analyzed by the Soil Analysis Laboratory at the Smithsonian Tropical Research Institute (Panama City, Panama); detailed methods can be found in Turner and Romero (2009).

Statistical analysis

Rarefaction curves were constructed from the estimated number of OTUs in each sample using observed species richness in QIIME (Hu et al. 2014). Libraries were rarefied to 3000 reads (the size of the smallest sequence library) (Appendix S1: Fig. S1). Observed species richness and Chao richness were calculated in QIIME. Alpha diversity was compared among samples for each environment (i.e. ants, nest, refuse and soil) using a one-way ANOVA.

We compared microbial communities across environmental sites using PERMANOVA in QIIME (1000 permutations). We also ran pair-wise PERMANOVAs to identify differences among individual sample types and correct for multiplicity using a Bonferroni correction. Community similarity was calculated using weighted UniFrac distance (Luzopone and Knight 2005). We used a non-metric multidimensional scaling (NMDS) ordination to visualize relationships among microbial communities within ant workers, refuse, nest walls and soil. We used QIIME to generate NMDS coordinates and then fit environmental vectors on this ordination using the Vegan package in R v3.2.1 (Oksanen et al. 2011). Microbial community data were arcsine transformed to improve normality, and we confirmed normality both visually and with the Shapiro-Wilk test.

To examine which particular phyla were driving compositional differences, we determined differences among sample types using a Wilcoxon rank sum test and then effect size using soil as the control environment. The Wilcoxon test was performed in R (v3.2.1), and the effect size was calculated (Cohen's d (1988)) on all significant microbial phyla. Effect sizes allow a standardized comparison of strong differences in the units of standard deviations, and we treat effect sizes of >|1| as large.

Results

A total of 1 204 544 bacterial/archaeal 16 S rRNA gene sequences were retained and analyzed. Nest and soil samples averaged 58% more microbial OTUs than samples coming from ants and their refuse ($P < 0.001$, Fig. 2). Nest and refuse samples contained the highest percentage of unclassified at 5.8%, followed by soil at 4.0% and

ants at 3.6%. Our rarefaction analyses (at 97% identity threshold) indicated that the majority of our samples were adequately sampled.

Comparing microbial composition across the four sample types

The microbial community composition differed across all four sample types (full model: $F = 22$, $P = 0.001$, Fig. 3, Fig. 4; pairwise comparisons: $F > 8$, $P < 0.001$). Contrary to predictions, the microbiome of ants varied dramatically across colonies, and were more variable than refuse and nest samples ($F_{3,65} = 2.63$, $P = 0.049$, Fig. 3).

Ant microbiomes were unique in the dominance of one common order, Lactobacilliales (33% ± 23), that was bimodally distributed with >40% relative abundance in 13 of 18 colonies sampled, and <5% in the rest (Table 1). The four next most common orders were Oceanospirillales, Micrococcales, Corynebacteriales, Rhodospirillales, which made up 5 to 34% of the ant worker microbiome. These orders averaged >5% relative abundance in the other sample types.

The other three sample types were distinct from each other, but lacked a dominant order such as Lactobacilliales (Table 1). The five most common orders in refuse (Burkholderiales, Flavobacteriales ,Sphingobacteriales , Xanthomonadales , Chromatiales) were entirely distinct from those of ants. In nests, the top five dominant orders were Sphingobacteriales, Sphingomonadales, Xanthomonadales, Rhizobiales and Micrococcales; in the soil they were Xanthomonadales, Planctomycetales, Myxococcales, Rhizobiales, and Burkholderiales.

Variation in the Azteca microbiome and its products compared to the soil

The 20 ant colonies we sampled were at least ca. 50 m apart, with the furthest distance among any pair of colonies ca. 5 km. This likely represented a wide variety of

soil microbial communities (Barberan et al. 2015b). We used the soil community near each colonies as baseline against which to compare variation in the microbiomes of the *Azteca* ants, their nests, and refuse (Fig. 5). The abundance of some bacterial orders is highly correlated with a specific environment. The microbiota of ants consisted of >1 SD more OTUs of SR1 and BD1-5 (Firmicutes yielded a Cohen's d= 0.72, but with *Lactobacillus*, Cohen's d=1.7, driving the majority of separation). Compared to soil, ant microbiomes had fewer Armatimonadetes, Planctomycetes, Gemmatimonadetes, and Verrucomicrobia. As with ants workers, ant refuse had >1 SD more SR1, as well as Deinococcus-Thermus. Refuse had fewer members of the Armatimonadetes and Planctomycetes as well as Spirochaetae, and Acidobacteria. The microbiome of ant nests was most similar to the soil but contained higher levels of Actinobacteria (Cohen's d: 1.27); while hosting fewer Verrucomicrobia (Cohen's d: -1.69), Gemmatimonadetes (Cohen's d: -1.36) and Planctomycetes (Cohen's d: -1.07).

Chemistry composition correlates with microbial community structure

The biogeochemistry of the soil, ant workers, refuse, and nests were distinct, but the magnitude of these differences varied among nutrients (Table 2, Fig. 3). Nutrients that are correlated with microbial composition are displayed as vectors on the NMDS (Oksanen et al. 2013). Phosphorus had the strongest correlation with microbial community composition, while Mg had the weakest correlation (Appendix A1: Table S2). Ant workers were associated with the largest concentrations of P, N, Zn, and Na; refuse concentrated K, and C was relatively high in both, while soil was characterized by Fe, Mn, B, and Cu. Finally, the nests were relatively enriched in Mg, K and Ca.

Discussion

Distinct microbial communities exist across *A. trigona* and their refuse, and these communities are separate from the surrounding nest and soil communities (Fig. 3). The distinct community present within the ant samples compared to its surrounding environment is consistent with previous studies (Ishak et al. 2011, Kellner et al. 2015), and suggests that *A. trigona* microbial communities are not a result of accidental contamination (Kellner et al. 2015). This finding supports the hypothesis that ants are capable of shaping and maintaining their microbial symbionts (Fernandez-Marin et al. 2009, Kellner et al. 2015). Refuse, a product thought to mainly consist of ant frass, has a rapid and significant shift in its microbial composition upon introduction to the environment outside the nest. This is a pattern consistent with previous analysis of the refuse piles of leaf-cutter ant (Scott et al. 2010, Ishak et al. 2011) and this distinct shift from the ant microbiome suggests that refuse may be made up of a greater variety of materials than previous thought.

Microbiomes of ant nests

Despite the intimate nature in which ants build and inhabit their nests, the two are no more similar than the relationship seen between humans and the external microbiome of their homes (Barberan et al. 2015). The strong correlation between nest and soil samples suggests that the surrounding environment, rather than the occupants of the nest, is the main source for microbial colonization for external structures (Barberan et al. 2015). Furthermore, external portions of the nest are recycled frequently, allowing for constant resampling of the surrounding environmental community. Our results also support the hypothesis that microbial communities are

specialized to their environments and can experience rapid shifts once introduced to new environmental conditions. While additional sampling of internal portions of the nests is required to confirm whether colonization patterns are similar to those of the interiors of human homes, our results suggest that microbial assembly in ant built dwellings is comparable to those seen in human dwellings.

Nest communities had high levels (15% relative abundance) of the antimicrobial producing group Actinomycetes. Actinomycetes are commonly found in the nests of social organisms (e.g. paper wasps (Madden et al. 2013), termites (Visser et al. 2012), bees (Promnuan et al. 2009) and ants (Sen et al. 2009, Barke et al. 2010)). Social living brings an increased risk of disease spread, and many social organisms have developed relationships with antimicrobial producing organisms to help deter infections. Previous studies have emphasized the value in examining arthropod nest structures as a source of novel antibiotic-producing bacteria (Bode 2009, Poulsen et al. 2011, Madden et al. 2013). Further examination and isolation of the Actinomycete community occurring on *A. trigona* nests is required to assess its level of antimicrobial properties and potential role in nest hygiene.

Natural ant microbial community variability

The *A. trigona* microbiome was not highly conserved across individual colonies. This pattern is almost entirely driven by the relative abundance of the Firmicute *Lactobacillus.* The variability of *Lactobacillus* abundance is a pattern demonstrated in multiple ant species (Hu et al. 2013, Kellner et al. 2015), with diet likely driving the variability. *Lactobacillus* facilitates the breakdown of sugars into lactic acid and is known to increase dramatically in the presence of high sugar substrates (Shamala et al.

2000). Likewise, human microbiome studies found higher ratios of Firmicutes to Bacteroidetes in obese individuals compared with lean individuals; a ratio that was adjustable through the restriction of carbohydrate intake (Ley et al. 2006).

We suggest three working hypotheses for the bimodality in the relative abundance of Lactobacillus in *Azteca* microbiomes. First, *Azteca*, like most ants, are omnivorous, harvesting both sugars directly from plants and homopteran honeydew, as well as protein from both live and dead prey (Kaspari 2000). It is possible that this bimodality in microbiomes represents bimodality among colonies in feeding habits. We are currently manipulating food sources for colonies and extracting microbial communities from the ant gut and hind gut to determine diet is the main cause of variation across ant colonies. Secondly, Firmicutes, like *Lactobacillus*, are strongly associated with xylophagous insects. Because the nest building behavior of *A. trigona* includes consumption of woody material, this behavior is another possible source of *Lactobacillus* colonization (Colman et al. 2012). Finally, high and low Lactobacillus abundance may represent cryptic species differences in this currently poorly resolved genus (Longino 2007). We are currently exploring this possibility via DNA barcoding. We do not predict host tree identity to have a strong influence over ant microbiome, due to the large territory these ants inhabit and the variety of extra-floral nectaries they feed at.

Another feature of the *Azteca* microbiome is worth noting. The exclusive presence of the genus *Saccharibacter* (a bacterium isolated from pollen (Jojima et al. 2004)) in ant samples suggests that *A. trigona* are feeding on arboreal pollen. Ants from the arboreal genus *Cephalotes* often rely on pollen as an important source of protein and

may contain special internal structures for digesting pollen (Roche and Wheeler 1997). The presence of *Saccharibacter* in *A. trigona* suggests that pollen consumption by canopy ants may be more widespread than previously predicted, and that this genus may be a useful bacterial indicator for pollenophagy.

Ecological impacts of refuse deposition

Nutrient-rich refuse below *A. trigona* nests can accelerate decomposition and alter the composition of the invertebrate community in the soil (Clay et al. 2013). While previous studies of refuse dumps have emphasized an enrichment in nutrients and higher fine root density (Farji-Brener and Werenkraut 2015), our results suggest that the microbial community structure of refuse can also contribute to accelerated decomposition rates and provide a favorable environment for root growth. *A. trigona* refuse contains the bacterial fertilizer Bacillus *spp.* (Suslow et al. 1979) and plant-growth-promoting rhizobacteria (PGPR) such as Pseudomonas *spp.*, Rhizobiales *spp.* and Enterobacter *spp.* (Vessey 2003). Because refuse deposition is frequently on or close to the host tree's root system, this suggests a working hypothesis that trees hosting *A. trigona* benefit from the twin input of nutrients and beneficial bacteria. *A. trigona*, with stable, nutrient and microbe rich refuse piles, can provide long term "hot spots" for diversity and productivity, and may be an important driver of habitat heterogeneity.

Chemical composition and microbial community correlates

Each sample type in our study had a distinctive chemistry. Unsurprisingly, ant samples contained the highest levels of carbon and nitrogen, essential nutrients for animal life, but also high levels of metabolically active Zn. Nest samples were high in Ca and Mg, critical elements for cell wall structure and photosynthesis, respectively, in

plants (Shaul 2002, White and Broadly 2003). Refuse samples had elevated levels of K. Ants must regulate the amount of K consumed in order to maintain appropriate Na^+/K levels, a task made more difficult given the abundance of K, but not Na, in plant tissue (Kaspari et al. 2009). The 2-fold increase of K in refuse samples compared to ants emphasizes the constant effort ants must exert to maintain proper chemical balances. While the results of our chemical and microbial analysis are strictly correlative, they provide a foundation for future work to address the relationship between chemical availability and microbial community composition.

To conclude, the composition of local soils is a good predictor of the composition of the exterior of both *Azteca* nests and human homes. Similarly, we found that ants, like humans, show a distinct but variable microbiome. Whereas in humans, some of this variation can be due to diet, location, and genetics, (Shamala et al. 2000, Yatsunenko et al. 2012, Spor et al. 2011) the origins of Azteca's biomodal microbiome is still unresolved. It is intriguing, however, that the amount of sugar available to an ant colony, like a human, may be dramatically reflected in its microbiome. Quantification of diet preference and its relationship to internal microbial assemblage is thus important to discerning how microbial communities interact with and influence the surrounding environment.

Supplementary Material and Data Accessibility

Supplementary Information accompanies this paper on the Ecosphere website. All microbial data have been uploaded and are available at NCBI's BioSamples databank (Accession nos. SAMN04576300-SAMN04576371).

Acknowledgments

We thank Oris Acevedo, Belkys Jimenez and the STRI staff on BCI, Panama; ANAM for permits and the Turner soil lab. We thank the Stevenson lab at OU for their guidance in microbial processing techniques. We also thank EM Gora and the Yanoviak lab for providing useful comments on experimental design and earlier versions of the manuscript. Finally we thank the anonymous reviewers for their thoughtful comments. Jane Lucas conducted all field work, carried out molecular lab work, participated in data analysis, design of the study and drafted the manuscript; Brian Bill participated in molecular lab work and carried out the statistical analyses; Bradley Stevenson provided guidance on molecular lab work, supplied materials to conduct lab work, and aided in drafting the manuscript. Michael Kaspari participated in the design of study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication. This work was supported by the National Science Foundation (EF—1065844) to Michael Kaspari, the National Science Foundation Graduate Research Fellowship (2014170874) to Jane Lucas, Smithsonian Tropical Research Institute Short-Term Fellowship to Jane Lucas, and University of Oklahoma Biology Department Funds.

References

Adams, E. S. 1994. Territory defense by the ant Azteca trigona: Maintenance of an arboreal ant mosaic. Oecologia 97:202–208.

Barberán, A., Dunn, R.R., Reich, B.J., Pacifici, K., Laber, E.B., Menninger, H.L., Morton, J.M., Henley, J.B., Leff, J.W., Miller, S.L., and N. Fierer. 2015. The ecology of microscopic life in household dust. Proc. R. Soc. B 282:1139.

Barberán, A., McGuire, K.L., Wolf, J.A., Jones, F.A., Wright, S.J., Turner, B.L., Essene, A., Hubbell, S.P., Faircloth, B.C., and N. Fierer. 2015. Relating belowground microbial composition to the taxonomic, phylogenetic, and functional trait distributions of trees in a tropical forest. Ecology Letters 18:1397-1405.

Barke, J., Seipke, R. F., Grüschow, S., Heavens, D., Drou, N., Bibb, M. J., Goss, R. J., Douglas, W. Y., and M. I. Hutchings. 2010. A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant Acromyrmex octospinosus. BMC Biology 8:109.

Bode, H. B. 2009. Insects: true pioneers in anti-infective therapy and what we can learn from them. Angew. Chem. Int. Ed. Engl. 48:6394-6396

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., and G. A. Huttley. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335-336.

Clay, N. A., Lucas, J. M., Kaspari, M., and A. D. Kay. 2013. Manna from heaven: Refuse from an arboreal ant links aboveground and belowground processes in a lowland tropical forest. Ecosphere 4:141.

Currie, C. R., Scott, J. A., Summerbell, R. C., and D. Malloch. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701-704.

Currie, C. R., Poulsen, M., Mendenhall, J., Boomsma, J. J., and J. Billen. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311:81 -83.

Cohen, J. 1988. Statistical Power Analysis for the Behavioral Sciences. L. Erlbaum Associates: Hillsdale, NJ.

Colman, D. R., Toolson E. C., and C. D. Takacs-Vesbach. 2012. Do diet and taxonomy influence insect gut bacterial communities? Molecular Ecology 21:5124–5137.

Dosmann, A., Bahet, N., and D. M. Gordon. 2016. Experimental modulation of external microbiome affects nestmate recognition in harvester ants (Pogonomyrmex barbatus). PeerJ 4:1566.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460-2461.

Farji-Brener, A. G., and V. Werenkraut. 2015. A meta-analysis of leaf-cutting ant nest effects on soil fertility and plant performance. Ecological Entomology 40:150-158.

Fernandez-Marin, H., Zimmerman, J. K., Nash, D. R., Boomsma, J. J., and W. T. Wcislo. 2009. Reduced Biological Control and Enhanced Chemical Pest Management in the Evolution of Fungus Farming in Ants. Proceedings of the Royal Society B: Biological Sciences 276.1665: 2263-269.

Grantham, N.S., Reich, B.J., Pacifici, K., Laber, E.B., Menninger, H.L., Henley, J.B., Barberán, A., Leff, J.W., Fierer, N., and R. R. Dunn. 2015. Fungi identify the geographic origin of dust samples. PloS ONE 10:0122605.

Hewitt, K. M., Gerba, C. P., Maxwell, S. L., and S. T. Kelley. 2012. Office space bacterial abundance and diversity in three metropolitan areas. PLoS ONE 7:37849.

Hölldobler, B., and E. O. Wilson. 1990. The Ants. Belknap of Harvard UP: Cambridge, MA.

Hölldobler, B., and E. O. Wilson. 2009. The Superorganism: The Beauty, Elegance, and Strangeness of Insect Societies. W.W. Norton: New York.

Hu, Y., Łukasik, P., Moreau, C. S., and J. A. Russell. 2014. Correlates of Gut Community Composition across an Ant Species (Cephalotes Varians) Elucidate Causes and Consequences of Symbiotic Variability. Molecular Ecology 23.6:1284-300.

Ishak, H. D., Plowes, R., Sen, R., Kellner, K., Meyer, E., Estrada, D. A., Dowd, S. E., and U. G. Mueller. 2011. Bacterial diversity in Solenopsis invicta and Solenopsis geminata ant colonies characterized by 16S amplicon 454 pyrosequencing. Microbial Ecology 61:821-831.

Jojima, Y. 2004. Saccharibacter Floricola Gen. Nov., Sp. Nov., a Novel Osmophilic Acetic Acid Bacterium Isolated from Pollen. International Journal of Systematic And Evolutionary Microbiology 54.6:2263-267.

Kaspari, M. 2000. A Primer of Ant Ecology. Measuring and Monitoring Biological Diversity: Standard Methods for Ground-living Ants. Washington, D.C.: Smithsonian Press: 9-24.

Kaspari, M., Yanoviak, S. P., Dudley, R., Yuan, M., and N. A. Clay. 2009. Sodium shortage as a constraint on the carbon cycle in an inland tropical rainforest. PNAS 106:19405-19409.

Kautz, S., Rubin, B. E. R., Russell, J. A., and C. S. Moreau. 2012. Surveying the microbiome of ants: comparing 454 pyrosequencing with traditional methods to uncover bacterial diversity. Applied and Environmental Microbiology 79.2:525-34.

Kellner, K., Ishak, H. D., Linksvayer, T. A., and U. G. Mueller. 2015. Bacterial Community Composition and Diversity in an Ancestral Ant Fungus Symbiosis. FEMS Microbiology Ecology 91.7.

Kembel, S. W., Jones, E., Kline, J., Northcutt, D., Stenson, J., Womack, A.M., Bohannan, B.J., Brown, G.Z., and J. L. Green. 2012. Architectural design influences the diversity and structure of the built environment microbiome. The ISME Journal 6:1469-1479.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and F. O. Glöckner. 2012. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 808.

Koch, H., and P. Schmid-Hempel. 2011. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proceedings of the National Academy of Sciences 108:19288-19292.

Lax, S., Smith, D. P., Hampton-Marcell, J., Owens, S. M., Handley, K. M., Scott, N. M. Gibbons, S. M., Larsen, P., Shogan, B. D., Weiss, S., and J. L. Metcalf. 2014. Longitudinal analysis of microbial interaction between humans and the indoor environment. Science 345:1048-1052.

Ley, R. E., Turnbaugh, P. J., Klein, S., and J. I. Gordon. 2006. Microbial ecology: human gut microbes associated with obesity. Nature 444:1022–1023.

Longino, J. 2007. A Taxonomic Review of the Genus Azteca (Hymenoptera: Formicidae) in Costa Rica and a Global Revision of the Aurita Group. Zootaxa 1491:1-63.

Lozupone, C., and R. Knight. 2005. UniFrac: A new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology 71.12:8228-235.

Madden, A. A., Grassetti, A., Soriano, J. A. N., and P. T. Starks. 2013. Actinomycetes with antimicrobial activity isolated from paper wasp (Hymenoptera: Vespidae: Polistinae) nests. Environmental Entomology 42:703-710.

Matulich, K.L., Weihe, C., Allison, S.D., Amend, A.S., Berlemont, R., Goulden, M.L., Kimball, S., Martiny, A.C., and J. B. Martiny. 2015. Temporal variation overshadows the response of leaf litter microbial communities to simulated global change. The ISME Journal.

Mehlich, A. 1984. Mehlich 3 soil test extractant: a modification of mehlich 2 extractant. Communications in Soil Science & Plant Analysis 15:1409–1416.

Moreau, C. S. 2012. Ant microbe protocols. Moreau Lab, Field Museum, Chicago, Illinois.

Simpson, G.L., Solymos, P., Stevens, M.H.H., and H. Wagner. 2011. Vegan: community ecology package. R package version 2.0-10.

Pelaez, F. 2006. The historical delivery of antibiotics from microbial natural products: can history repeat? Biochem. Pharmacol. 7:981-990.

Poulsen, M., Oh, D., Clardy, J., and C. R. Currie. 2011. Chemical analyses of wasp-associated Streptomyces bacteria reveal a prolific potential for natural products discovery. PLoS ONE 6:16763.

Promnuan, Y., Kudo, T., and P. Chantawannakul. 2009. Actinomycetes isolated from beehives in Thailand. World J. Microbiol. Biotechnol. 25:1685-1689.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and F.O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research, 41:590-596.

Richard, F. J., Poulsen, M., Hefetz, A., Errard, C., Nash, D. R., and J. J. Boomsma. 2007. The origin of the chemical profiles of fungal symbionts and their significance for nestmate recognition in Acromyrmex leaf-cutting ants. Behavioral Ecology and Sociobiology, 6:1637-1649.

Roche, R. K., and D. E. Wheeler. 1977. Morphological specializations of the digestive tract of *Zacryptocerus rohweri* (Hymenoptera: Formicidae). Journal of Morphology, 234:253–262.

Sen, R., Ishak, H. D., Estrada, D., Dowd, S. E., Hong, E., and U. G. Mueller. 2009. Generalized antifungal activity and 454-screening of Pseudonocardia and Amycolatopsis bacteria in nests of fungus-growing ants. Proceedings of the National Academy of Sciences, 106:17805-17810.

Scott, J. J., Budsberg, K. J., Suen, G., Wixon, D. L., Balser, T. C., and C. R. Currie. 2010. Microbial community structure of leaf-cutter ant fungus gardens and refuse dumps. PLoS One, 5:9922.

Shamala, T. R., Shri Jyothi, Y., and P. Saibaba. 2000. Stimulatory effect of honey on multiplication of lactic acid bacteria under in vitro and in vivo conditions. Letters in Applied Microbiology 30.6:453-55.

Shaul, O. 2002. Magnesium transport and function in plants: the tip of the iceberg. BioMetals 15:309-323.

Spor, A., Koren, O., and R. Ley. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. Nature Reviews Microbiology 9:279-290.

Suslow, T. V., Kloepper, J. W., Schroth, M. N., and T. Burr. 1979. Beneficial bacteria enhance plant growth. California Agriculture 33:15-17.

Täubel, M., Rintala, H., Pitkäranta, M., Paulin, L., Laitinen, S., Pekkanen, J., Hyvärinen, A., and A. Nevalainen. 2009. The occupant as a source of house dust bacteria. Journal of Allergy and Clinical Immunology 124:834-840.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E., and T. M. Schmidt. 2013. Symbiotic bacteria appear to mediate hyena social odors. Proceedings of the National Academy of Sciences 110:19832-19837.

Turner, B. L., and T. E. Romero. 2009. Short-term changes in extractable inorganic nutrients during storage of tropical rain forest soils. Soil Science Society of America Journal 73:1972.

Vessey, J. K. 2003. Plant growth promoting Rhizobacteria as biofertilizers. Plant and Soil 255.2:71-86.

Visser, A. A., Nobre, T., Currie, C. R., Aanen, D. K., and M. Poulsen. 2012. Exploring the potential for actinobacteria as defensive symbionts in fungus-growing termites. Microbial ecology, 63:975-985.

Wheeler, W. M. 1910. Ants: their Structure, development and behavior. Columbia UP: New York.

White, P.J., and M. R. Broadley. 2003. Calcium in plants. Annals of Botany 92:487-511.

Wieder, K., and J. S. Wright. 1995. Tropical forest litter dynamics and dry season irrigation on Barro Colorado Island, Panama. Ecology. 76:1971-1979

Wilson, E. O. 1975. Sociobiology: the new synthesis. Harvard University Press, Cambridge, Massachusetts.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., Magris, *et al*. 2012. Human gut microbiome viewed across age and geography. Nature 486:222-227.

Table 1: Core microbiota of ant, refuse, nest wall and surrounding soil. Values displayed are the percent relative abundance of bacterial genera in each sample type. Only genera present with more than 1% relative abundance are shown. (For a complete list of bacterial genera, see Data S1)

Sample Type	Bacteria genera	%	Sample Type	Bacteria genera	%
Ant	*Lactobacillus*	30	Refuse	*Rheinheimera*	6.2
	Marinobacterium	5.1		*Truepera*	5.1
	Acinetobacter	2.7		*Weeksella*	3.5
	Saccharibacter	2.3		*Acinetobacter*	3.1
	Gordonia	2.3		*Lampropedia*	2.5
	Weeksella	2.2		*Lactobacillus*	2.3
	Sulfrimonas	2.1		*Gordonia*	2.2
	Sandaracinaeceae Uncultured	1.4		*Azoarcus*	2.1
	Marinobacter	1.3		Saprospiraceae uncultured	1.9
	Corynebacterium	1.3		Comamonadaceae Other	1.8
	Chthoniobacteriales Uncultured	1.3		*Leucobacter*	1.8
	Truepera	1.2		*Olivibacter*	1.6
	Acidobacteria Uncultured	1.1		*Pseudofulvimonas*	1.5
	Proteobacteria Uncultured	1.1		*Muricauda*	1.4
	Myceligenerans	1.0		*Myceligenerans*	1.4
	Unassigned	3.3		*Achromobacter*	1.3
				Olivibacter	1.3
				Pseudomonas	1.3
				Myceligenerans	1.2
				Rhodobacteraceae Other	1.0
				Luteimonas	1.0
				Unassigned	5.8

Sample Type	Bacteria genera	%	Sample Type	Bacteria genera	%
Nest	*Sphingomonas*	3.4	Soil	Planctomycetaceae uncultured	2.5
	Nocardioides	3.1		*Opitutus*	2.1
	Pseudoxanthomonas	2.5		*Marinobacterium*	2.1
	Truepera	2.3		Xanthomonadales uncultured	1.9
	Olivibacter	2.0		*Planctomyces*	1.9
	Chryseobacterium	2.0		Comamonadaceae other	1.7
	Pedobacter	1.8		*Lactobacillus*	1.6
	Weeksella	1.7		*Blastocatella*	1.6
	Fructobacillus	1.6		*Dongia*	1.4
	Rhizobium	1.4		*Haliangium*	1.2
	Sphingomonadaceae Other	1.3		*Sorangium*	1.1
	Luteimonas	1.2		Xanthomonadaceae other	1.1
	Planctomyces	1.1		*Diaphorobacter*	1.1
	Brachybacterium	1.1		Myxococcales uncultured	1.1
	Cytophagia Other	1.0		Cytophagaceae uncultured	1.0
	Flavobacterium	1.0		Chitinophagaceae uncultured	1.0
	Unassigned	5.8		*Sphingomonas*	1.0
				Unassigned	3.5

Tables

Table 2: Average chemical concentration and the standard error in ants (17), nest (18), refuse (10) and soil (18).

Elements	Ants			Nest			Refuse			Soil		
Al	0.48	±	0.1	4.22	±	0.84	3.04	±	1.02	26.72	±	4.47
B	0	±	0	0.05	±	0	0.05	±	0	0.11	±	0.01
% C	49.61	±	0.75	40.19	±	0.56	43.28	±	0.39	30.76	±	2.86
Ca	2.4	±	0.29	10.3	±	0.68	9.05	±	1.14	9.07	±	1.15
Cu	0.02	±	0	0.04	±	0	0.06	±	0	0.08	±	0
Fe	0.53	±	0.09	4.14	±	0.95	3.1	±	0.92	34.19	±	6.5
K	16.84	±	1.46	35.42	±	2.76	35.67	±	3.8	7.34	±	1.72
Mg	1.45	±	0.05	3.21	±	0.22	3.48	±	0.16	2.98	±	0.4
Mn	0.15	±	0.02	0.29	±	0.07	0.29	±	0.08	1.16	±	0.23
% N	8.22	±	0.22	2.64	±	0.13	4.9	±	0.2	3.19	±	0.32
Na	1.93	±	0.13	0.91	±	0.23	0.59	±	0.07	0.27	±	0.04
P	7.71	±	0.21	2.98	±	0.2	5.24	±	0.4	1.9	±	0.37
Zn	0.19	±	0.01	0.06	±	0.01	0.09	±	0.01	0.08	±	0.01

Figure Legends

Figure 1: Photo of (A) *Azteca trigona* nest, (B) ant, (C) refuse in collection bucket and (D) environmental landscape. Photos A, C and D were taken by Jane Lucas. Photo B was taken by Shannon Hartman (www.antweb.org).

Figure 2: Alpha diversity for each sample type calculated from observed OTUs. Letters denote significant differences between sample types identified using ANOVA.

Figure 3: NMDS representation of bacterial communities of *A. trigona* ants, their refuse, nest wall and surrounding soil. Distances are based on dissimilarity matrices of sequence-based weighted UniFrac distances. Sample types differ significantly from each other (PERMANOVA: $P = 0.001$, $F = 22.27$). Chemical composition of all nutrients was correlated with compositional trends in ordination. The strength of each correlation is proportional to the vector length (P is the strongest ($r^2 = 0.60$).

Figure 4: Mean relative abundance of the bacterial phyla across sample types. Bacterial phyla present in > 0.01% relative abundance across samples are shown.

Figure 5: Bacterial phyla that differ significantly on each sample type compared to soil samples. Only phyla with large effect sizes (Cohen's $d > \pm 0.7$) are shown. Positive values represent an increase in sample type over soil; negative values represent higher abundance on soil samples.

Figure 1

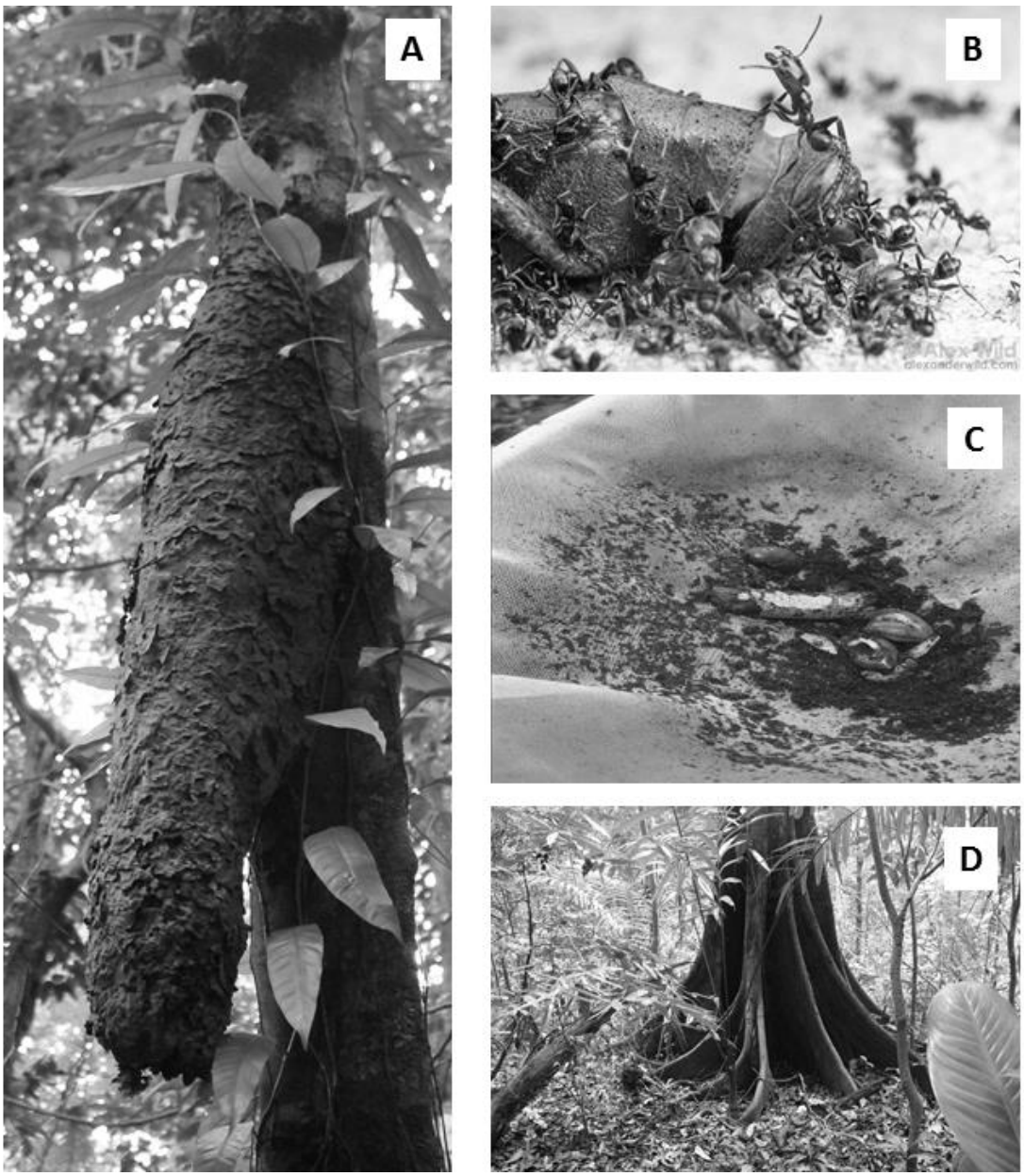


Figure 2

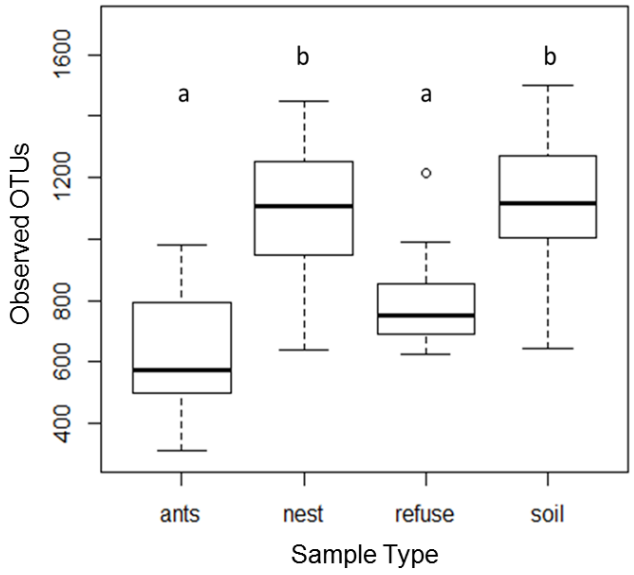

Observed OTUs
400
600
800
1200
1600
a
b
a
b
ants
nest
refuse
soil
Sample Type

Figure 3

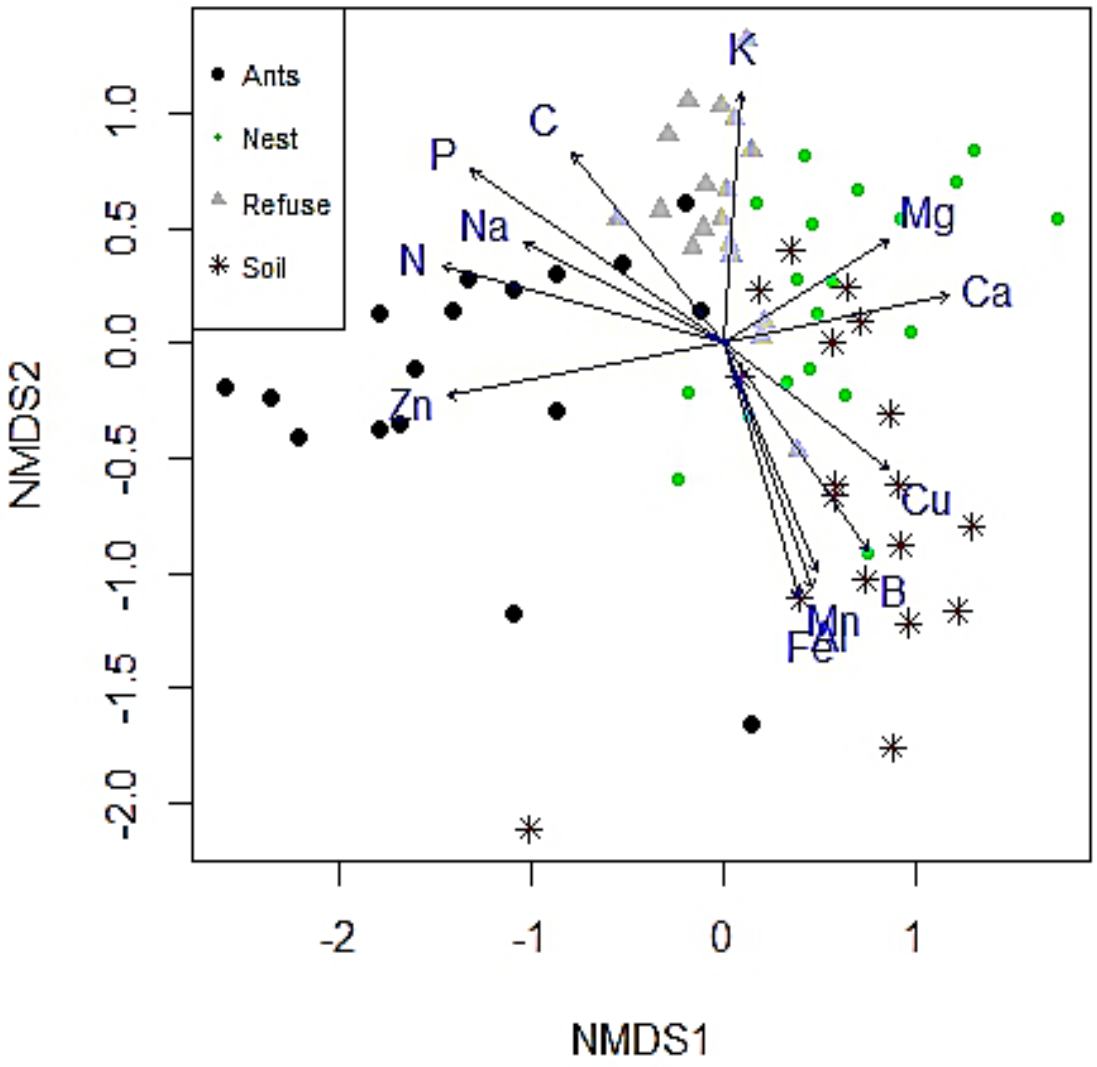

Ants
Nest
Refuse
Soil
K
C
P
Na
N
Mg
Ca
Zn
Cu
B
Mn
Fe
NMDS1
NMDS2
-2
-1
0
1
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0

Figure 4

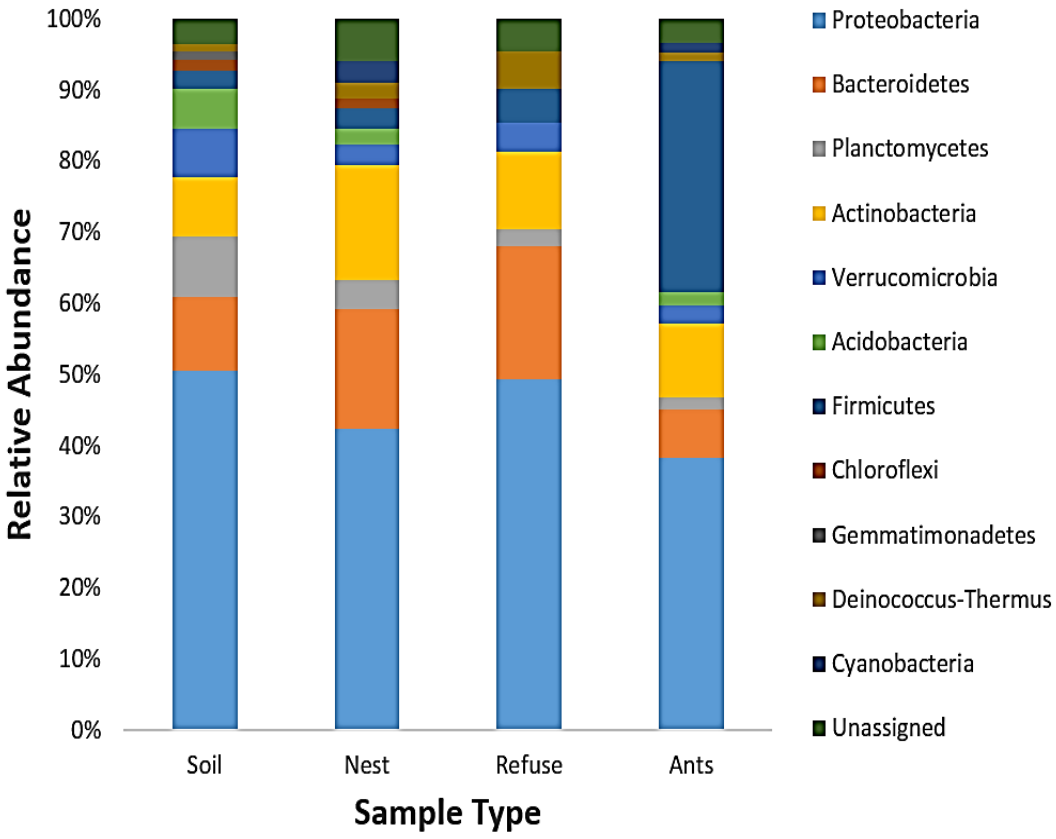
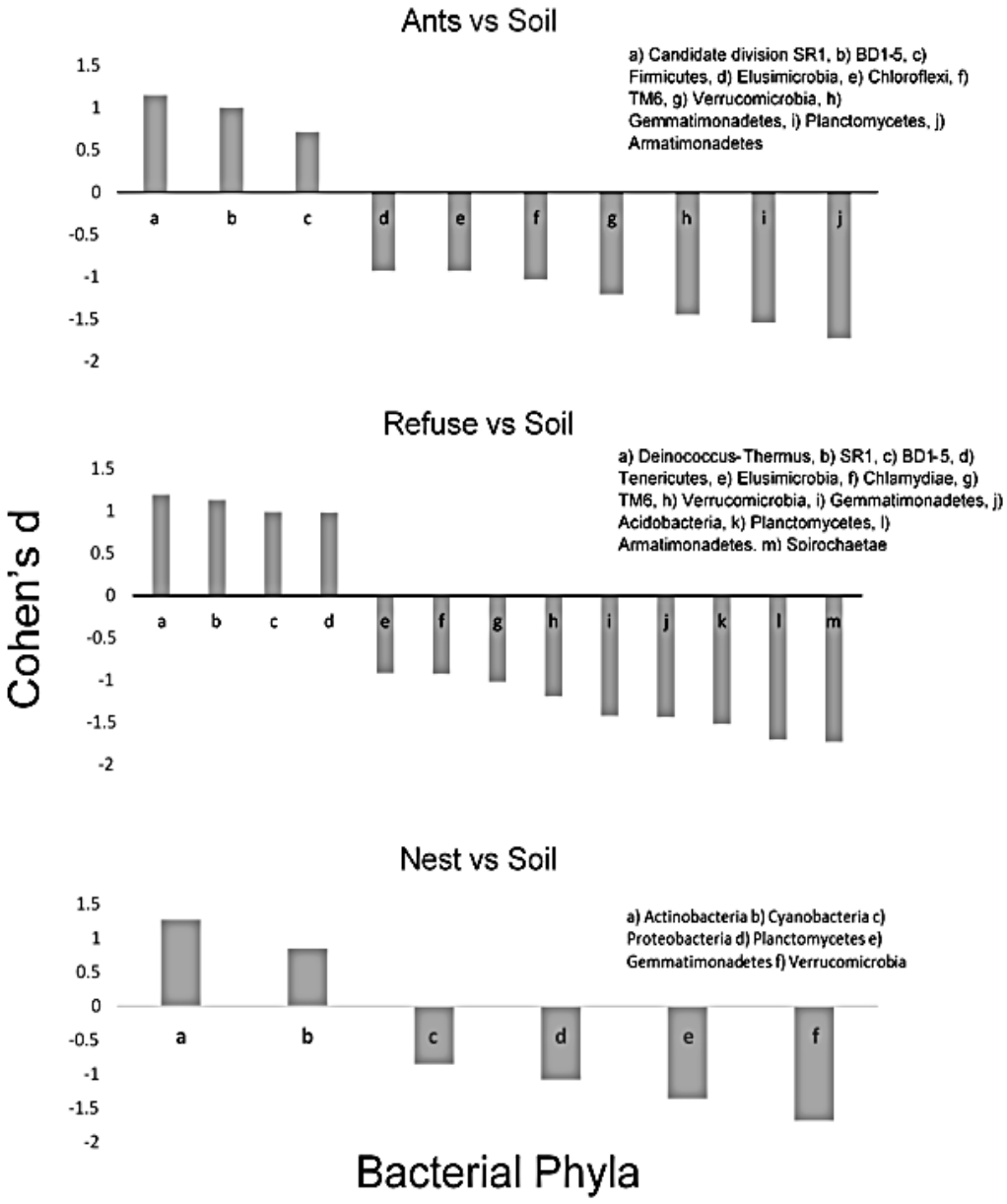

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Abundance
Soil
Nest
Refuse
Ants
Sample Type
Proteobacteria
Bacteroidetes
Planctomycetes
Actinobacteria
Verrucomicrobia
Acidobacteria
Firmicutes
Chloroflexi
Gemmatimonadetes
Deinococcus-Thermus
Cyanobacteria
Unassigned

Figure 5


Ants vs Soil
a) Candidate division SR1, b) BD1-5, c) Firmicutes, d) Elusimicrobia, e) Chloroflexi, f) TM6, g) Verrucomicrobia, h) Gemmatimonadetes, i) Planctomycetes, j) Armatimonadetes
1.5
1
0.5
0
-0.5
-1
-1.5
-2
a b c d e f g h i j
Refuse vs Soil
a) Deinococcus-Thermus, b) SR1, c) BD1-5, d) Tenericutes, e) Elusimicrobia, f) Chlamydiae, g) TM6, h) Verrucomicrobia, i) Gemmatimonadetes, j) Acidobacteria, k) Planctomycetes, l) Armatimonadetes. m) Spirochaetae
Cohen's d
1.5
1
0.5
0
-0.5
-1
-1.5
-2
a b c d e f g h i j k l m
Nest vs Soil
a) Actinobacteria b) Cyanobacteria c) Proteobacteria d) Planctomycetes e) Gemmatimonadetes f) Verrucomicrobia
1.5
1
0.5
0
-0.5
-1
-1.5
-2
a b c d e f
Bacterial Phyla

Appendix to:

Lucas, J., B. Bill, B. Stevenson, and M. Kaspari. 2017. The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest. Ecosphere 8(2):e01639. 10.1002/ecs2.1639

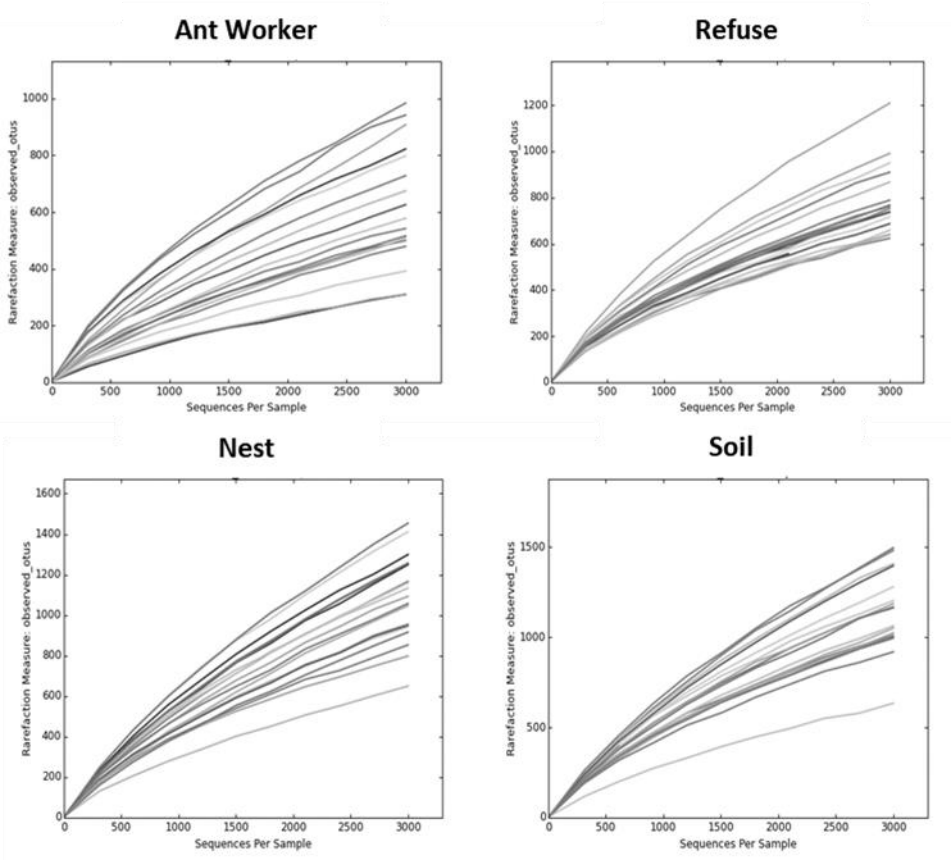


Fig. S1 Rarefaction curves of observed OTUS for *Azteca trigona* ant, refuse, nest wall and soil samples.

Table S1: List of Sample IDs the unique primer sequenced used.

SampleID	BC_number	BarcodeSequence	LinkerPrimerSequence
100008	Illumina_BC_112	AGCTGTCAAGCT	AGCTGTCAAGCTCCGTAAAACGACGGCCAG
100022	Illumina_BC_162	ACACGCGGTTTA	ACACGCGGTTTACCGTAAAACGACGGCCAG
100033	Illumina_BC_123	CATACCGTGAGT	CATACCGTGAGTCCGTAAAACGACGGCCAG
100039	Illumina_BC_181	GTGCAACCAATC	GTGCAACCAATCCCGTAAAACGACGGCCAG
100044	Illumina_BC_169	TTAAGACAGTCG	TTAAGACAGTCGCCGTAAAACGACGGCCAG
100046	Illumina_BC_265	TTCCTAGGCCAG	TTCCTAGGCCAGCCCGTAAAACGACGGCCAG
100058	Illumina_BC_252	CCTTGACCGATG	CCTTGACCGATGCCGTAAAACGACGGCCAG
100087	Illumina_BC_216	TTCTCCATCACA	TTCTCCATCACACCGTAAAACGACGGCCAG
100113	Illumina_BC_209	TTCGATGCCGCA	TTCGATGCCGCACCCGTAAAACGACGGCCAG
100122	Illumina_BC_253	CTATCATCCTCA	CTATCATCCTCACCCGTAAAACGACGGCCAG
100144	Illumina_BC_197	CGCTCACAGAAT	CGCTCACAGAATCGTAAAACGACGGCCAG
100159	Illumina_BC_154	GAAACATCCCAC	GAAACATCCCACCCGTAAAACGACGGCCAG
100170	Illumina_BC_116	TACCGAAGGTAT	TACCGAAGGTATCCGTAAAACGACGGCCAG
100179	Illumina_BC_150	ACGACTGCATAA	ACGACTGCATAACCGTAAAACGACGGCCAG
100208	Illumina_BC_242	GCCGTAAACTTG	GCCGTAAACTTGCCCCGTAAAACGACGGCCAG
100209	Illumina_BC_238	GGTTTAACACGC	GGTTTAACACGCCCGTAAAACGACGGCCAG
100217	Illumina_BC_260	GAACGGGACGTA	GAACGGGACGTACCCCGTAAAACGACGGCCAG
100013	Illumina_BC_126	TTCTCTCGACAT	TTCTCTCGACATCCGTAAAACGACGGCCAG
100014	Illumina_BC_182	GCTTGAGCTTGA	GCTTGAGCTTGACCGTAAAACGACGGCCAG
100020	Illumina_BC_121	AGTAGCGGAAGA	AGTAGCGGAAGACCGTAAAACGACGGCCAG
100035	Illumina_BC_155	CGTACTCTCGAG	CGTACTCTCGAGCCGTAAAACGACGGCCAG
100037	Illumina_BC_170	TCTGCACTGAGC	TCTGCACTGAGCCCGTAAAACGACGGCCAG
100062	Illumina_BC_250	CTCCCTTTGTGT	CTCCCTTTGTGTCCGTAAAACGACGGCCAG
100092	Illumina_BC_213	GTTCGGTGTCCA	GTTCGGTGTCCACCCGTAAAACGACGGCCAG
100106	Illumina_BC_113	GAGAGCAACAGA	GAGAGCAACAGACCGTAAAACGACGGCCAG
100115	Illumina_BC_255	CGATAGGCCTTA	CGATAGGCCTTACCCGTAAAACGACGGCCAG
100127	Illumina_BC_202	CGACTCTAAACG	CGACTCTAAACGCCGTAAAACGACGGCCAG
100180	Illumina_BC_195	GTGGTCATCGTA	GTGGTCATCGTACCCGTAAAACGACGGCCAG
100186	Illumina_BC_148	ACAACACTCCGA	ACAACACTCCGACCGTAAAACGACGGCCAG
100187	Illumina_BC_153	ACGGGTCATCAT	ACGGGTCATCATCCGTAAAACGACGGCCAG
100191	Illumina_BC_115	CGTGCTTAGGCT	CGTGCTTAGGCTCCGTAAAACGACGGCCAG
100204	Illumina_BC_257	CTTAGGCATGTG	CTTAGGCATGTGCGTAAAACGACGGCCAG
100222	Illumina_BC_241	ATTGTTCCTACC	ATTGTTCCTACCCCCGTAAAACGACGGCCAG
100239	Illumina_BC_237	CCTGTCCTATCT	CCTGTCCTATCTCCCGTAAAACGACGGCCAG
100244	Illumina_BC_233	CCGAGGTATAAT	CCGAGGTATAATCGTAAAACGACGGCCAG
JML046	Illumina_BC_263	ACTGACTTAAGG	ACTGACTTAAGGCGTAAAACGACGGCCAG
100003	Illumina_BC_117	CACTCATCATTC	CACTCATCATTCCCGTAAAACGACGGCCAG
100023	Illumina_BC_125	CCTGCGAAGTAT	CCTGCGAAGTATCCGTAAAACGACGGCCAG
100025	Illumina_BC_175	AGTTGTAGTCCG	AGTTGTAGTCCGCCGTAAAACGACGGCCAG

100030	Illumina_BC_194	GCATCAGAGTTA	GCATCAGAGTTACCCCGTAAAACGACGGCCAG
100036	Illumina_BC_156	TCAGTTCTCGTT	TCAGTTCTCGTTCCGTAAAACGACGGCCAG
100038	Illumina_BC_185	ACGATTCGAGTC	ACGATTCGAGTCCCGTAAAACGACGGCCAG
100097	Illumina_BC_251	AGCTGCACCTAA	AGCTGCACCTAACGTAAAACGACGGCCAG
100102	Illumina_BC_203	GTCTTCAGCAAG	GTCTTCAGCAAGCGTAAAACGACGGCCAG
100103	Illumina_BC_254	ACTCTAGCCGGT	ACTCTAGCCGGTCCCCGTAAAACGACGGCCAG
100147	Illumina_BC_198	ATTCGGTAGTGC	ATTCGGTAGTGCCCGTAAAACGACGGCCAG
100151	Illumina_BC_199	CGAGCTGTTACC	CGAGCTGTTACCCCCGTAAAACGACGGCCAG
100164	Illumina_BC_118	GTATTTCGGACG	GTATTTCGGACGCCGTAAAACGACGGCCAG
100183	Illumina_BC_152	AGCTATGTATGG	AGCTATGTATGGCCGTAAAACGACGGCCAG
100194	Illumina_BC_149	CGATGCTGTTGA	CGATGCTGTTGACCGTAAAACGACGGCCAG
100205	Illumina_BC_240	GCCACGACTTAC	GCCACGACTTACCCGTAAAACGACGGCCAG
100218	Illumina_BC_259	GAGAGTCCACTT	GAGAGTCCACTTCCCGTAAAACGACGGCCAG
100249	Illumina_BC_244	AGATGATCAGTC	AGATGATCAGTCCCGTAAAACGACGGCCAG
JML034	Illumina_BC_235	CTCGTGAATGAC	CTCGTGAATGACCCCGTAAAACGACGGCCAG
100004	Illumina_BC_183	CGCTGTGGATTA	CGCTGTGGATTACCGTAAAACGACGGCCAG
100005	Illumina_BC_124	ATGTGTGTAGAC	ATGTGTGTAGACCCGTAAAACGACGGCCAG
100021	Illumina_BC_160	GTAAATTCAGGC	GTAAATTCAGGCCCGTAAAACGACGGCCAG
100034	Illumina_BC_171	CGCAGATTAGTA	CGCAGATTAGTACCGTAAAACGACGGCCAG
100045	Illumina_BC_120	TTGCCAAGAGTC	TTGCCAAGAGTCCCGTAAAACGACGGCCAG
100047	Illumina_BC_193	GTCGAATTTGCG	GTCGAATTTGCGCCCGTAAAACGACGGCCAG
100059	Illumina_BC_249	ACCGTGCTCACA	ACCGTGCTCACACCCGTAAAACGACGGCCAG
100114	Illumina_BC_201	ATTCTCTCACGT	ATTCTCTCACGTCCCGTAAAACGACGGCCAG
100128	Illumina_BC_256	AATGACCTCGTG	AATGACCTCGTGCCGTAAAACGACGGCCAG
100141	Illumina_BC_114	TACTCGGGAACT	TACTCGGGAACTCCGTAAAACGACGGCCAG
100181	Illumina_BC_151	ACGCGAACTAAT	ACGCGAACTAATCCGTAAAACGACGGCCAG
100185	Illumina_BC_119	TATCTATCCTGC	TATCTATCCTGCCCGTAAAACGACGGCCAG
100196	Illumina_BC_147	AGAGTCTTGCCA	AGAGTCTTGCCACCGTAAAACGACGGCCAG
100227	Illumina_BC_243	GCAGATTTCCAG	GCAGATTTCCAGCCCGTAAAACGACGGCCAG
100231	Illumina_BC_239	AGACAGTAGGAG	AGACAGTAGGAGCGTAAAACGACGGCCAG
100236	Illumina_BC_258	CCAGATATAGCA	CCAGATATAGCACCGTAAAACGACGGCCAG
100241	Illumina_BC_200	CAACACATGCTG	CAACACATGCTGCCCCGTAAAACGACGGCCAG
JML036	Illumina_BC_230	ATCCCTACGGAA	ATCCCTACGGAACCCCGTAAAACGACGGCCAG

Table S2: Relationships between elemental and microbial community composition as determined by vector fitting.

Chemical	**r-squared**	***P*-value**
N	0.5909	0.001
C	0.3424	0.001
Al	0.3502	0.001
B	0.3587	0.001
Ca	0.3667	0.001
Cu	0.2673	0.001
Fe	0.3599	0.001
K	0.3142	0.001
Mg	0.245	0.001
Mn	0.3143	0.001
Na	0.3291	0.002
P	0.5985	0.001
Zn	0.5505	0.001

Table S3: NCBI BioSamples accession numbers for each sample

Accession No.	Sample Name	Tax ID
SAMN04576300	100008	1077528
SAMN04576301	100022	1077528
SAMN04576302	100033	1077528
SAMN04576303	100039	1077528
SAMN04576304	100044	1077528
SAMN04576305	100046	1077528
SAMN04576306	100058	1077528
SAMN04576307	100087	1077528
SAMN04576308	100113	1077528
SAMN04576309	100122	1077528
SAMN04576310	100144	1077528
SAMN04576311	100159	1077528
SAMN04576312	100170	1077528
SAMN04576313	100179	1077528
SAMN04576314	100208	1077528
SAMN04576315	100209	1077528
SAMN04576316	100217	1077528
SAMN04576317	100013	1077528
SAMN04576318	100014	1077528
SAMN04576319	100020	1077528
SAMN04576320	100035	1077528
SAMN04576321	100037	1077528
SAMN04576322	100062	1077528
SAMN04576323	100092	1077528
SAMN04576324	100106	1077528
SAMN04576325	100115	1077528
SAMN04576326	100127	1077528
SAMN04576327	100180	1077528
SAMN04576328	100186	1077528
SAMN04576329	100187	1077528
SAMN04576330	100191	1077528
SAMN04576331	100204	1077528
SAMN04576332	100222	1077528
SAMN04576333	100239	1077528
SAMN04576334	100244	1077528
SAMN04576335	JML046	1077528
SAMN04576336	100003	1077528
SAMN04576337	100023	1077528
SAMN04576338	100025	1077528
SAMN04576339	100030	1077528
SAMN04576340	100036	1077528
SAMN04576341	100038	1077528
SAMN04576342	100097	1077528
SAMN04576343	100102	1077528

SAMN04576344	100103	1077528
SAMN04576345	100147	1077528
SAMN04576346	100151	1077528
SAMN04576347	100164	1077528
SAMN04576348	100183	1077528
SAMN04576349	100194	1077528
SAMN04576350	100205	1077528
SAMN04576351	100218	1077528
SAMN04576352	100249	1077528
SAMN04576353	JML034	1077528
SAMN04576354	100004	1077528
SAMN04576355	100005	1077528
SAMN04576356	100021	1077528
SAMN04576357	100034	1077528
SAMN04576358	100045	1077528
SAMN04576359	100047	1077528
SAMN04576360	100059	1077528
SAMN04576361	100114	1077528
SAMN04576362	100128	1077528
SAMN04576363	100141	1077528
SAMN04576364	100181	1077528
SAMN04576365	100185	1077528
SAMN04576366	100196	1077528
SAMN04576367	100227	1077528
SAMN04576368	100231	1077528
SAMN04576369	100236	1077528
SAMN04576370	100241	1077528
SAMN04576371	JML036	1077528

Chapter 2: Nutrient transfer supports beneficial relationship between canopy ant, *A. trigona*, and host trees

(Formatted for *Ecological Entomology*)

Citation: Lucas, J.M., Clay, N.A., Kaspari, M. (2018) Nutrient transfer supports beneficial relationship between canopy ant, *A. trigona*, and host trees. In press *Ecological Entomology*.

Abstract

1. Energy fluxes between ants and plants has been a focal point for documenting mutualistic behavior. Plants can provide resources to ants through the production of extra-floral nectaries. In exchange, ants can fertilize plants through their nutrient and microbe-rich refuse.

2. Here, we provide a test of a potential facultative mutualism between the carton-nesting canopy ant, *Azteca trigona,* and their host trees. Through observational and experimental approaches, we document how nutrient transfer provides a basis for this beneficial ant-plant relationship.

3. In a greenhouse experiment, fertilization with refuse mineral nutrients alone increased seedling growth 3-fold and the microbial community of the refuse increased plant growth 11-fold.

4. Total root density was increased 3-fold in refuse piles compared to the surrounding area *in situ*. On average, refuse provides host trees with a > 800% increase of N, P and K relative to leaf litter.

5. *Azteca trigona* preferentially nests in trees with extra-floral nectaries and on large, longer lived tree species.

6. Given the nutrient-poor nature of Neotropics, host trees likely experience significant benefits from refuse fertilization. Conversely, *A. trigona* benefit from long-term stable structural support for nests, and access to nutrient-rich extra-floral nectaries. Without clear costs to either *A. trigona* or host trees, we propose that these positive interactions provide initial support for a facultative mutualism.

Introduction

Mutualisms, relationships where both species benefit from their interaction, are ubiquitous (Bronstein, 2001) and increase the diversity and stability of an ecosystem (Jander, 2015). To determine whether an interaction qualifies as a mutualism studies focus on documenting the benefits provided to each species involved. However, mutualistic actors may also incur costs, as long as the net benefit of their interaction is positive (Bronstein, 1994; 2001). Examples of this include the production of rewards, such as extra-floral nectaries by myrmecophytic plants (Fagundes *et al.*, 2017) or the trade of carbon for phosphorus between plants and mycorrhizae, respectively (Herre *et al*., 1999). Similarly, indirect benefits produced by interacting players can support mutualistic relationships with little cost. Fertilization from byproducts is one example of a low cost input that maintains mutualistic relationships (Sager *et al*., 2000).

Ant-plant interactions are among the most studied cases of mutualisms due the wide range of relationships that exist between them (Beattie, 1985; Heil & McKey, 2003). These interactions range from the obligate mutualisms that occurs between *Cecropia peltata* trees and their *Azteca* ants (Janzen, 1969) to loose facultative relationships seen between plants providing extra-floral nectaries to their protective ant communities (Bronstein *et al.*, 2006). These relationships highlight ants as "ecosystem engineers", with the potential to shape community structure (Sanders & van Veen, 2011). Furthermore, mutualistic ant plant relationships may be most pronounced in oligotrophic environments, where competition is high and nutrients are limited (Stachowicz, 2001, Pringle *et al.*, 2013). Current estimates suggest that in some forests, up to one third of all woody plants support ant associations (Schupp & Feener, 1991).

With such large estimates and their acknowledged ecological importance, studies on ant-plant interactions are still underexplored and require attention.

Energy fluxes between ants and plants has been a focal point for documenting the costs and benefits of mutualistic behavior. Myrmecotrophy—the transfer of nutrients from ants to plants—is a common phenomenon that can account for large portions of a plant's nutrient pool (Beattie, 1989, Dejean *et al.*, 2012). Studies of mymrecotrophy primarily focus on plants that provide internal chambers (domatia) that house colonies and their waste piles (Sager *et al.*, 2000, Mayer *et al.*, 2014). In these scenarios, the host plant is the sole benefactor of the nutritious waste piles created by ants. However, nutrient transfer from ants to plants can occur in a variety of nesting behaviors. External refuse dumps in *Atta colombica* demonstrate increased root foraging in refuse piles (Farji-Brener & Medina, 2000), while soil dwelling ant colonies increase nearby plants' access to nutrients (Wagner & Nicklen, 2010). Similarly, canopy ants can concentrate resources that provide essential nutrient supplies for epiphytes (Tresder *et al.*, 1995; Bluthgen *et al.*, 2001).

Extra-floral nectary (EFN) production is another important way nutrient transfer can support ant-plant mutualisms (Janzen, 1966). The primary hypothesis for EFN production by plants is to provide an enticing nutrient source for ant colonies in exchange for heightened protection against herbivores or competing plants (Heil & McKey, 2003; Chamberlain & Holland, 2009). However, work by Wagner and Nicklen (2010) proposes an additional benefit: extra floral nectaries encourage ant nesting behavior, which increases a plant's access to mineral nutrients. While their work focused primarily on soil dwelling ants, we suggest that this phenomena may occur

between the dominant carton-nesting canopy ant, *Azteca trigona*, and their host trees. *Azteca trigona* are territorially aggressive and forage throughout the canopy (> 40 m^2), concentrating resources in large carton nests that hang from host trees (Wheeler, 1942; Adams, 1994). The resulting waste products drop from the bottom of conical nests and accumulates in large piles near the host tree's trunk. In a previous study, we demonstrate that *A. trigona* waste piles are enhanced 7-fold in P, 23-fold in K and 3-fold in N, as well as in a variety of micronutrients, compared to the surrounding leaf litter (Clay *et al.*, 2013). Furthermore, *A. trigona* refuse contains a diverse microbial community with known plant growth promoting bacteria (*Pseudomonas* spp., *Rhizobiales* spp., and *Enterobacter* spp.; Lucas *et al.*, 2017). Moreover, refuse contains high levels of the antimicrobial-producing group, *Actinomycetes*, which may serve as a protection against pathogens to plants.

In this study, we test the nature of the relationship between *A. trigona* and their host trees. Due to the large refuse piles created by *A. trigona* and their use of extra-floral nectaries, we predict that nutrient transfer is an important component of this ant-plant interaction. Through an experimental greenhouse approach, we test whether the nutritious and microbe-rich refuse from *A. trigona* increases seedling growth and survival. Furthermore, we examine levels of root foraging in refuse piles to demonstrate the importance of this resource in natural environments. We also provide a test of the nutrient hypothesis proposed by Wagner and Nicklen (2010) by conducting a census of *A. trigona* nest density across the 50 ha forest dynamics plot on Barro Colorado Island (BCI). This census allows us to examine whether *A. trigona* preferentially nest on extra-floral nectary providing species, as well as examine the degree of specificity *A.*

trigona have with their host trees. Combined, our results demonstrate that a dominant canopy ant preferentially nests near food sources, and may benefit their host tree through long-term fertilization.

Material and Methods

Study site

This study was conducted throughout the Barro Colorado National Monument (BCNM) in Panama (BCI: 9'10'N, 79'51'W). The BCNM is a seasonally moist tropical forest that receives an average of 2,600 mm of rain year^{-1}. There is a pronounced dry season with < 10% of the rainfall occurring from mid-December to late April (Leigh, 1999).

Examining the influence of refuse fertilization on seedling growth

In 2015, ant refuse was collected from beneath 10 separate colony nests of *A. trigona* located along the Thomas Barbour trail on BCI. Buckets raised up on stilts and covered in fine mesh were placed below nests to catch the refuse before it could be colonized by soil microbial communities. More detailed descriptions can be found in Clay *et al.* (2013). Refuse was collected every three days. Soil located 10 m away from refuse buckets was collected at each point for soil addition treatments. Half of the refuse and soil collected was sterilized at 250°C for 1.5 hours, while the other portion was added as live microbial input on seedlings.

To test the prediction that refuse and associated microbes facilitate plant growth we setup four fertilization treatments: 1) refuse addition, 2) sterilized refuse addition, 3) soil addition, and 4) sterilized soil addition. For each fertilization treatment, we filled

twelve 24 cm tall, 10 cm wide 1.65 l tree pots with a 50:50 mixture of sterilized local soil and rock. Our focal species, *Ochroma pyramidale* (balsa wood), is a common pioneer tree on BCI whose seeds are wind dispersed and have physical dormancy. It has small seeds that require light gaps to establish and germinate (Croat, 1978). *Ochroma pyramidale* is a commonly used species in greenhouse experiments (Dalling *et al.*, 2013, Zalamea *et al.*, 2015), allowing us to compare our results to previous studies.

Seeds were collected from the soil seed bank below the crowns of three reproductive *O. pyramidale* adults. Seeds were then surface sterilized in a bath of 10 % sodium hypochlorite (bleach) solution for 10 mins. Immediately following surface sterilization, seeds were placed in 100°C water for 30s to break their physical dormancy (Zalamea *et al.*, 2015). They were then allowed to germinate in containers with fresh potting soil for two weeks (Fosforo Soil, Panama City, Panama). We transplanted into each pot three, two week old *O. pyramidale* seedlings, as described by Dalling *et al.* (2013). The initial dry mass of five randomly selected seedlings was determined at the time of transplant. Our pots were grown in full sun, in an open growing house with a clear plastic roof to regulate watering. Seedlings were watered every other day, and pot locations were randomized every 5 days. Seedlings received 5 g (roughly the average amount of refuse deposited each week by an *A. trigona* nest) of sterilized or unsterilized refuse or soil once a week and were grown for a total of 42 days.

At the time of harvest, seedlings were extracted and gently washed, then separated into root, leaf and stem fractions to examine above and below ground biomass. Wet leaf area was measured using an automated leaf area meter (LI-3000A, LI-COR, Lincoln, Nebraska). Final biomass was measured after drying for 72 h at 60^{o}C. Root mass ratio

(RMR; root mass per unit whole plant biomass) and specific leaf area (SLA; leaf area per unit leaf mass; cm^{-2} g^{-1}) were calculated from harvest data.

Quantifying root foraging in refuse piles

To assess whether trees were preferentially foraging in *A. trigona* refuse piles, we located 15 *A. trigona* nests along the Edwin Willis trail on the Gigante peninsula of BCNM. We measured the distance from the trunk of the tree to directly underneath the nest at each location. Soil cores were taken directly below nests (0 m), then in a randomized direction at 0.5 m, 1 m and 10 m away using a 5 cm diameter split-sleeve core sampler (AMS). Cores were taken to a depth of 10 cm. A 0.5 mm sieve was used to rinse roots and separate them from soil particles. Once cleaned, the roots were sorted into two categories: < 1 mm diameter and >1 mm as suggested by Cheng *et al.* (2009). The roots were dried to a constant mass at 60°C, and then weighed.

Azteca trigona nest survey: Testing for extra floral nectary preference and host tree specificity

To determine if *A. trigona* have host tree specificity and concentrate nests near extra floral nectary sources, we performed two surveys 5 years apart. In 2011, we surveyed the western most third of the 50 ha plot located on BCI (Hubbell & Foster, 1983). Using the 5 m post system of the 50 ha plot, we carried out a transect census of *A. trigona* colonies. The census area included the 5 m on either side of the post to ensure the entire plot area was examined. When a nest was located, we recorded its length, height from the ground and the tag of the tree it was located on. From this tree tag number we were able to determine tree species, diameter at breast height (hereafter dbh), and location within the plot. Nests within 10 m of each other were determined to

be the same colony. The plot area was re-censused in 2016, by the same individuals, using the same methodology as 2011. We were able to determine whether nests found in 2016 were the same as those in 2011 by comparing tree tag numbers as well as nest height and size records. Additionally we tested whether *A. trigona* non-randomly associated with trees with specific characteristics (i.e. extra-floral nectaries, large dbh).

To determine the relative size of host trees and potential to use refuse resources below nests, we extrapolated dbh of host trees to determine average crown area. From our survey we determined 30 cm as our most common host tree dbh. Using this value we calculated the average tree crown area using values provided by O'Brien *et al.* (1995). We also calculated daily refuse production values from calculations provided by Clay *et al.* (2013) and daily leaf litter fall values under a tree crown as provided by Sayer *et al.* (2012). Then, we used the leaf litter and refuse nutrient level data provided by Clay *et al.* (2013) to calculate the average amount of each element (by weight) a host tree has available to it refuse as compared to leaf litter inputs.

Statistical Analyses

All analyses were performed in the statistical environment R (R Development Core Team 2013, version 0.99.903). All variables were tested for normality via the Wilks-Shapiro test (Sokal & Rohlf 1981). For plant growth analysis, we tested for treatment effects on plant growth by using linear mixed effect models using the *lme4* (Bates *et al.*, 2015). We tested the effect of the fertilization treatments as a fixed grouping variable on plant height, above and below-ground biomass, leaf number and leaf area, using pot location as a random grouping variable. We used the post-hoc Tukey HSD procedure to test for differences among levels of fertilization treatment on

plant height, above and below-ground biomass, leaf number and leaf area (Crawley, 2002). For root density analysis, we used a two-way ANOVA because we treated our four distances as a categorical variables. We followed this analysis with a Tukey HSD test to test the null hypothesis of no difference among distances in root density. We used the same methodology to test for differences in root density across nest locations. A G test with the Yates' correlation correction (McDonald, 2009) was used to determine if *A. trigona* nests were associated with common tree species, tree size classes and trees with extra-floral nectaries more than expected by chance.

Results

Plant growth is enhanced by refuse due to nutrient and microbial inputs

Trees supporting *A. trigona* nests receive a highly concentrated point-source fertilization of macro- and micronutrients. While refuse is only 6.7% the weight of leaf litter input per average tree crown (Sayer *et al.*, 2012), it accounts for 20% of the total available N, 41% of total P, and 136% of total K (Table S1) provided by leaf litter under a tree crown alone. More specifically, in the area where refuse accumulates under a tree (ca. 1 m^2), refuse input composes 833% of N, 1739% of P, and 5747% of K that is provided by leaf litter.

In our greenhouse experiment, refuse fertilization increased seedling growth and altered biomass allocation relative to soil fertilization. However, these effects were minimal without the microbial community of the refuse (Fig. 1). Unsterilized refuse (i.e. refuse with its unaltered microbial flora) caused an 11-fold increase in total biomass relative to soil treatments, whereas sterilized refuse only caused a 3-fold increase ($F_{3,44}$

= 101.35, $P < 0.0001$; Fig. 1). Fertilization also changed root mass ratios (RMR; $F_{3,44} = 3.11$, $P = 0.04$; Fig. 1). Unsterilized refuse-treated plants allocated 45% of their total biomass to roots, whereas sterilized refuse and both soil treatments averaged only 37% of its total biomass in their roots. Neither soil or refuse fertilization treatments had significant effects on specific leaf area ($F_{3,44} = 1.77$, $P = 0.17$).

Plants concentrate root foraging in Azteca trigona refuse

Dry mass of fine roots was 3-fold higher below nests and 0.5 meters away relative to locations at 1 and 10 meters away ($F_{3,39} = 9.44$, $P < 0.001$; Fig. 2). As predicted, coarse root mass did not differ between any locations ($F_{3,39} = 1.36$, $P = 0.27$; Fig. 2). There was no difference in root density across each nest, ruling out intercolonial differences ($F_{13,39}=6.84$, p=0.64).

Azteca trigona host tree demography

Our initial survey in 2011 found 97 colonies on 194 trees (223 total nests) in the 16.67 ha area examined. The same survey in 2016 found 123 colonies on 142 trees (164 total nests). Of the 194 trees hosting nests in 2011, 48 (34%) were hosting the same nests in 2016. Average colony density increased from 5.82 colonies/ha to 7.69 colonies/ha, though total nests decreased from 13.38 nests/ha to 9.84 nests/ha.

Azteca trigona nests were associated with trees with beneficial traits. Nests were aggregated on trees with extra-floral nectaries (2011: G = 57.91, d.f. = 1, $P < 0.001$; 2016: G = 34.14, d.f. = 1, $P < 0.001$; Muehleisen, 2013). Furthermore, nests were frequently found on larger trees, and their size class distribution is distinct from that of the trees on the 50 ha plot (Table 2, Table S1).

Azteca trigona were associated with specific host tree species. In 2011, 14.5% (44 of 303) of the tree and shrub species found on the plot had *A. trigona* nests in them. Similarly, in 2016, 13.9% (42 of 303) of the tree and shrub species found on the plot had *A. trigona* nests in them. *Azteca trigona* nests were most frequently found in the tree species *Trichilia tuberculate* (N=42 in 2011, N=19 in 2016) an over-canopy tree with an average density of 259 stems/ha (Belk *et al.,* 1989). The use of this species as a host tree was more often than would be expected based on its abundance within the plot (Table 1). Multiple additional species had a high association with nests and are detailed in Table 1. Host trees did not experience different rates of mortality compared to background rates (G= 0.654, d.f. = 1, $P = 0.42$).

Discussion

We provide initial evidence of a positive relationship between *A. trigona* and its host tree that could be the basis of a facultative mutualism. Specifically, we demonstrate that trees can benefit from the fertilization of nutrient and microbe-rich refuse deposited on its root system. Similarly, we show that *A. trigona* preferentially associate with trees that provide external nutrients (EFNs) and large, longer lived trees that could provide habitat for a colony throughout its lifespan. In the nutrient limited Neotropics, this unique relationship between *A. trigona* and its host tree may play an important role in shaping the local community through the exchange of key nutrients.

Seedlings fertilized with live microbial refuse saw an 11-fold increase in growth. While we acknowledge that in natural settings multiple trees may use refuse piles, we stress that host trees only need to exploit a portion of the nutrients to gain large

nutritional benefits. For example, if host trees only have access to 20% of the refuse pile, the nutritional benefit is equivalent to a substantial portion of the total P (8% of leaf litter) and K (27%) contained by all of the litterfall under its crown. This effect is particularly important because P and K were identified as nutrients limiting plant productivity at our study site (Kaspari *et al.*, 2008; Wright *et al.*, 2011).

Because *A. trigona* colonize trees long after the seedling stage (> 10 mm dbh) it is difficult to determine the extent to which *A. trigona* refuse benefits host trees during the duration of nest residency. However, post seedling stage fertilization can stimulate reproductive structures in plants (Willson & Price, 1980). If host trees benefit from increased access to limiting nutrients, then future studies should find increased investment in reproductive structures from host trees than conspecific trees without *A. trigona* nests. Furthermore, we find no evidence that host trees are hindered by the presence of *A. trigona*. Results from the 50 ha plot survey demonstrate that over a 5 year period trees hosting colonies do not experience increased mortality.

The potential beneficial effects of *A. trigona* refuse extends beyond its nutrient content. Our greenhouse experiment demonstrates that the refuse microbiome amplifies the effects of refuse on plant productivity (Fig. 1). Previous studies highlight the importance of beneficial microbial flora in supporting plant growth and defending against pathogens (Compant *et al.*, 2005; Van der Heijden *et al.*, 2007). *Azteca trigona* refuse provides multiple plant growth promoting bacterial taxa and an abundance of antimicrobial compound-producing *Actinomycetes* (Lucas *et al.*, 2017). Moreover, all seedlings fertilized with sterilized (non-living) microbial refuse had large amounts of visible detrimental fungal colonization (*pers. obs.*), whereas live refuse additions had no

visible fungal growth. This is anecdotal evidence that the rich microbial community found in refuse can provide protection against potential fungal pathogens.

Azteca trigona ants are major consumers of sugar and were non-randomly associated with tree species that produce extra-floral nectaries. In a similar study exploring the contribution of ant feces to plants, Pinkalski *et al.* (2015) demonstrated that ants given access to sugar produced more feces than those without access to sugar. Increased nutrient availability has been shown to increase the production of ant rewards (Folgarait & Davidson, 1994; Heil *et al.*, 2000), though the literature is conflicting (de Sibio & Rossi, 2016). Furthermore, ant aggression increases with increased levels of carbohydrate availability (Grover *et al.*, 2007; Gonzales-Teuber *et al.*, 2012). Thus, through a positive feedback loop, trees that provide extra-floral nectaries may benefit from increased nitrogen and macronutrient deposition, as well as increased ant-mediated defense (Pinkalski *et al.*, 2016; Gonzales-Teuber *et al.*, 2012). In return, ants may be rewarded with additional nutritional resources.

The results from our study provides initial support for a facultative mutualism between *A. trigona* and their host trees. To further explore the nature of this relationship, we identify three avenues that require additional attention. First is to examine whether *A. trigona* defend their host tree against herbivores, similar to the behavior of *Azteca* spp. mutualists with *Cecropia* spp. (Schupp, 1986). Although it is not resolved, early results indicate that *Azteca trigona* defend their tree from herbivores, and may be an important source of protection (*unpublished data*). Second is an exploration of where *A. trigona* colonies forage for nutrients, and whether this is strongly linked to host tree supplies. Finally, dendro-ecological measurements and

monitoring of reproductive structure production of host trees on the forest dynamics plot can determine whether *A. trigona* fertilization increases host tree growth and fitness.

Acknowledgments

We thank Carolyn Gigot, Kate Henderson, Annika Salzberg, and Andy Van Alst for their help in the field. We thank Carolina Sarmiento, Camilo Zalamea and Scott Mangan for their help in the design of our greenhouse study. We thank Jesse Czekanski-Moir and Evan Gora for help in analysis. We also thank Oris Acevedo and Belkys Jimenez and STRI staff on BCI, Panama, and ANAM for permits. We thank the Yanoviak lab for comments throughout the project design and manuscript writing process. This work was supported by funding from the Biology Department (OU), Graduate Student Senate (OU), NSF Graduate Fellowship Program ID: 2014170874 (awarded to J. Lucas), NSF DEB 0842038 (PI M. Kaspari, A. Kay), NSF Macrosystems EF-1065844 (PI M. Kaspari), and the Research Experience for Undergraduates program run by the Smithsonian Tropical Research Institute.

References

Adams, E.S. (1994) Territory defense by the ant *Azteca trigona*: maintenance of an arboreal ant mosaic. *Oecologia*, **97**, 202-208.

Addicott, J.F. (1986) Variation in the costs and benefits of mutualism: the interaction between yuccas and yucca moths. *Oecologia*, **70**, 486-494.

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R.H.B., Singmann, H. *et al.*, (2014) lme4: Linear Mixed-Effects Models Using Eigen and S4. *Journal of Statistical Software*, **67**, 1-48. doi:10.18637/jss.v067.i01.

Beattie, A. (1989) Myrmecotrophy: plants fed by ants. *Trends in Ecology & Evolution*, **4**, 172-176.

Beattie, A.J. (1985) *The evolutionary ecology of ant-plant mutualisms*. Cambridge University Press, Cambridge, U.K.

Belk, M.C., Black, H.L., Jorgensen, C.D., Hubbell, S.P. & Foster, R.B. (1989) Nest tree selectivity by the tropical ant, *Paraponera clavata*. *Biotropica*, **21**, 173-177.

Blüthgen, N., Schmit-Neuerburg, V., Engwald, S. & Barthlott, W. (2001) Ants as epiphyte gardeners: comparing the nutrient quality of ant and termite canopy substrates in a Venezuelan lowland rain forest. *Journal of Tropical Ecology*, **17**, 887-894.

Bronstein, J.L. (1994) Conditional outcomes in mutualistic interactions. *Trends in Ecology & Evolution*, **9**, 214-217.

Bronstein, J.L. (2001) The costs of mutualism. *American Zoologist*, **41**, 825-839.

Bronstein, J.L., Alarcón, R. & Geber, M. (2006) The evolution of plant–insect mutualisms. *New Phytologist*, **172**, 412-428.

Chamberlain, S.A. & Holland, J.N. (2009) Quantitative synthesis of context dependency in ant–plant protection mutualisms. *Ecology*, **90**, 2384-2392.

Cheng, X.Y., Chen, W.Y., Gu, B.H., Liu, X.C., Chen, F., Chen, Z.H. *et al.* (2009) Morphology, ecology, and contaminant removal efficiency of eight wetland plants with differing root systems. *Hydrobiologia*, **623**, 77-85.

Clay, N.A., Lucas, J., Kaspari, M., & Kay, A.D. (2013) Manna from heaven: refuse from an arboreal ant links aboveground and belowground processes in a lowland tropical forest. *Ecosphere*, **4**, 1-15.

Compant, S., Duffy, B., Nowak, J., Clément, C., & Barka, E.A. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology*, **71**, 4951-4959.

Crawley, M.J. (2002) *Statistical computing: An introduction to data analysis using s-plus*. John Wiley & Sons Publishing, Hoboken, NJ.

Croat, T.B. (1978) *Flora of Barro Colorado Island*. Stanford University Press, Palo Alto, CA.

Dalling, J.W., Heineman, K., Lopez, O.R., Wright, S.J. and Turner, B.L. (2016) Nutrient availability in tropical rain forests: the paradigm of phosphorus limitation. In G. Goldstein & L. S. Santiago (eds). *Tropical Tree*

Physiology, pp. 261-273. Springer International Publishing, Cham, Switzerland.

Dalling, J.W., Winter, K., Andersen, K.M., & Turner, B.L. (2013) Artefacts of the pot environment on soil nutrient availability: implications for the interpretation of ecological studies. *Plant Ecology*, **214**, 329-338.

de Sibio, P.R. & Rossi, M.N. (2016) Interaction Effect Between Herbivory and Plant Fertilization on Extrafloral Nectar Production and on Seed Traits: An Experimental Study With *Ricinus communis* (Euphorbiaceae). *Journal of Economic Entomology*, **109**, 1612-1618.

Dejean, A., Petitclerc, F., Roux, O., Orivel, J. & Leroy, C. (2012) Does exogenic food benefit both partners in an ant-plant mutualism? The case of Cecropia obtusa and its guest Azteca plant-ants. *Comptes Rendus Biologies*, **335**, 214-219.

Fagundes, R., DÁttilo, W., Ribeiro, S.P., Rico-Gray, V., Jordano, P. and Del-Claro, K. (2017) Differences among ant species in plant protection are related to production of extrafloral nectar and degree of leaf herbivory. *Biological Journal of the Linnean Society*, **20**, 1-13.

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

Folgarait, P.J. & Davidson, D.W. (1994) Antiherbivore defenses of myrmecophytic *Cecropia* under different light regimes. *Oikos*, **71**, 305-320.

González-Teuber, M., Silva-Bueno, J.C., Boland, W. & Heil, M. (2012) Increased host investment in extrafloral nectar (EFN) improves the efficiency of a mutualistic defensive service. *PLoS ONE*, **7**, e46598.

Grover, C.D., Kay, A.D., Monson, J.A., Marsh, T.C. & Holway, D.A. (2007) Linking nutrition and behavioural dominance: carbohydrate scarcity limits aggression and activity in Argentine ants. *Proceedings of the Royal Society B-Biological Sciences*, **274**, 2951-2957.

Heil, M. & McKey, D. (2003) Protective ant-plant interactions as model systems in ecological and evolutionary research. *Annual Review of Ecology, Evolution, and Systematics*, **34**, 425-553.

Heil, M., Hilpert, A., Kaiser, W., & Linsenmair, K.E. (2000) Reduced growth and seed set following chemical induction of pathogen defence: Does systemic acquired resistance (SAR) incur allocation costs? *Journal of Ecology,* **88,** 645-654.

Herre, E.A., Knowlton, N., Mueller, U.G. & Rehner, S.A. (1999) The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution*, **14**, 49-53.

Hubbell, S.P. and Foster, R.B. (1983) Diversity of canopy trees in a neotropical forest and implications for conservation. In T. C. Whitmore, A. C. Chadwick & A. C. Sutton (eds). *Tropical Rain Forest: Ecology and Management*, pp. 25-41. Blackwell Science Ltd, Hoboken, NJ.

Jander, K.C. (2015) Indirect mutualism: ants protect fig seeds and pollen dispersers from parasites. *Ecological Entomology*, **40,** 500-510.

Janzen, D.H. (1966) Coevolution of mutualism between ants and acacias in Central America. *Evolution*, **20**, 249-275.

Janzen, D.H. (1969) Allelopathy by myrmecophytes: the ant *Azteca* as an allelopathic agent of *Cecropia*. *Ecology*, **50**, 147-153.

Kaspari, M., Garcia, M.N., Harms, K.E., Santana, M., Wright, S.J., & Yavitt, J.B. (2008) Multiple nutrients limit litterfall and decomposition in a tropical forest. *Ecology Letters*, **11**, 35-43.

Leigh, E.G. (1999) *Tropical forest ecology: a view from Barro Colorado Island*. Oxford University Press, Oxford, U.K.

Lucas, J., Bill, B., Stevenson, B., & Kaspari, M. (2017) The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest. *Ecosphere*, **8,** e01639.

Mayer, V.E., Frederickson, M.E., McKey, D. & Blatrix, R. (2014) Current issues in the evolutionary ecology of ant–plant symbioses. *New Phytologist*, **202**, 749-764.

McDonald, J.H. (2009) *Handbook of biological statistics (Vol. 2)*. Sparky House Publishing, Baltimore, MD.

Muehleisen, A. (2013) Incidence of Extra-Floral Nectaries and their Effect on the Growth and Survival of Lowland Tropical Rain Forest Trees. PhD Thesis, The Ohio State University, Columbus, OH.

O'Brien, S.T., Hubbell, S.P., Spiro, P., Condit, R., & Foster, R.B. (1995) Diameter, height, crown, and age relationship in eight neotropical tree species. *Ecology*, **76**, 1926-1939.

Pinkalski, C., Damgaard, C., Jensen, K.M.V., Peng, R., & Offenberg, J. (2015) Quantification of ant manure deposition in a tropical agroecosystem: implications for host plant nitrogen acquisition. *Ecosystems*, **18**, 1373-1382.

Pinkalski, C., Damgaard, C., Jensen, K.M.V., Peng, R., & Offenberg, J. (2016) Macronutrient Exchange Between the Asian Weaver Ant *Oecophylla smaragdina* and Their Host Plant. *Ecosystems*, **19**, 1418-1428.

Pringle, E. G., Akçay, E., Raab, T. K., Dirzo, R., & Gordon, D. M. (2013) Water stress strengthens mutualism among ants, trees, and scale insects. *PLoS Biology*, **11**, e1001705.

R Development Core Team (2013) *R: A Language and Environment for Statistical Computing, Version 0.99.903*. R Foundation for Statistical Computing, Vienna, Austria [WWW document]. URL http://www.R-project.org.

Sagers, C.L., Ginger, S.M. & Evans, R.D. (2000) Carbon and nitrogen isotopes trace nutrient exchange in an ant-plant mutualism. *Oecologia*, **123**, 582-586.

Sayer, E.J., Wright, S.J., Tanner, E.V., Yavitt, J.B., Harms, K.E., Powers, J.S., *et al.* (2012) Variable responses of lowland tropical forest nutrient status to fertilization and litter manipulation. *Ecosystems*, **15**, 387-400.

Schupp, E.W. (1986) *Azteca* protection of *Cecropia*: ant occupation benefits juvenile trees. *Oecologia*, **70,** 379-385.

Schupp, E.W. & Feener, D.H., (1991) Phylogeny, lifeform, and habitat dependence of ant-defended plants in a Panamanian forest. In C. R. Huxley & D. F. Cutler (eds). *Ant-plant interactions*, pp. 175-97. Oxford University Press, Oxford, U.K.

Sokal, R.R. & Rohlf, F.J. (1981) *Biometry*, 2nd edn. W. H. Freeman and Company, New York, NY.

Stachowicz, J.J. (2001) Mutualism, facilitation, and the structure of ecological communities: positive interactions play a critical, but underappreciated, role in ecological communities by reducing physical or biotic stresses in existing habitats and by creating new habitats on which many species depend. *AIBS Bulletin*, **51**, 235-246.

Treseder, K.K., Davidson, D.W. & Ehleringer, J.R. (1995) Absorption of ant-provided carbon dioxide and nitrogen by a tropical epiphyte. *Nature*, **375**, 137-139.

Van Der Heijden, M.G., Bardgett, R.D., & Van Straalen, N.M. (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, **11**, 296-310.

Wagner, D. & Nicklen, E.F. (2010) Ant nest location, soil nutrients and nutrient uptake by ant-associated plants: does extrafloral nectar attract ant nests and thereby enhance plant nutrition? *Journal of Ecology*, **98**, 614-624.

Wheeler, W.M. (1942) Studies of neotropical ant-plants and their ants. *Bulletin of the Museum of Comparative Zoology at Harvard College*, **90,** 1-262.

Willson, M.F. & Price, P.W. (1980) Resource limitation of fruit and seed production in some *Asclepias* species. *Canadian Journal of Botany,* **58,** 2229-2233.

Wright, S.J., Yavitt, J.B., Wurzburger, N., Turner, B.L., Tanner, E.V., Sayer, E.J., *et al.* (2011) Potassium, phosphorus, or nitrogen limit root allocation, tree growth, or litter production in a lowland tropical forest. *Ecology*, **92**, 1616-1625.

Zalamea, P. C., Sarmiento, C., Arnold, A.E., Davis, A.S., & Dalling, J.W. (2014) Do soil microbes and abrasion by soil particles influence persistence and loss of physical dormancy in seeds of tropical pioneers? *Frontiers in Plant Science*, **5**, 799.

Tables

Table 1. Results of the *G*-tests with the Yates' correlation correction (McDonald, 2014) for tree species exhibiting high association with *Azteca trigona* nests. Trees with extrafloral nectaries (EFNs) are also noted.

Tree Species	Year	*G*-test	d.f.	*P*	EFNs
Alchornea costaricensis	2016	3.55164	1	0.059	YES
Alchornea costaricensis	2011	13.54191	1	>0.001	YES
Apeiba membranacea	2011	7.410328	1	0.006	NO
Astronium graveolens	2016	7.037124	1	0.008	NO
Chimarrhis parviflora	2016	3.922504	1	0.048	NO
Drypetes standleyi	2016	19.67892	1	>0.001	NO
Drypetes standleyi	2011	21.09855	1	>0.001	NO
Guarea Guidonia	2016	7.048582	1	0.008	YES
Gustavia superba	2016	10.55721	1	0.001	YES
Gustavia superba	2011	17.34683	1	>0.001	YES
Heisteria concinna	2016	9.775206	1	0.002	NO
Heisteria concinna	2011	12.2281	1	>0.001	NO
Hirtella triandra	2016	34.80945	1	>0.001	YES
Hirtella triandra	2011	33.30412	1	>0.001	YES
Inga marginata	2011	9.905782	1	0.002	YES
Pouteria reticulata	2016	15.8004	1	>0.001	NO
Sloanea terniflora	2011	3.658433	1	0.056	YES
Trichilia tuberculate	2016	7.738496	1	0.005	NO
Trichilia tuberculate	2011	33.16932	1	>0.001	NO

Table 2. Size class distributions of all the trees on the 50 hectare plot as well as the proportion of those trees hosting *A. trigona* colonies.

Size Class	Range (mm)	2011 Whole Plot (%)	2016 Whole Plot (%)	2011 *A. trigona* host trees (%)	2016 *A. trigona* host trees (%)
1	10 to 50	77.2	76.5	5.56	1.6
2	51 to 100	12.8	13.7	11.67	15.9
3	101 to 150	4.3	4.5	15.56	19.8
4	151 to 200	1.8	1.7	12.78	11.1
5	201 to 250	1.1	1	13.88	15.1
6	251 to 300	0.7	0.7	12.22	11.1
7	301 and up	2.0	1.9	28.33	25.4

Figure Legends:

Figure 1. Average growth of seedlings separated into above-ground, below-ground and total tissue biomass values across treatments after 6 weeks. Error bars represent standard deviations of the total biomass values and letters indicate significant differences among treatment type: live refuse (RL), sterile refuse (RS), live soil (SL) and sterile soil (SS). Live refuse treatments have significantly higher biomass.

Figure 2. Average biomass of coarse (> 1 mm) and fine (< 1 mm) roots at distances from underneath nests (in m). Error bars represent standard deviations for total root biomass values and letters indicate significant differences between distances. Root densities were highest in refuse piles and this effect was seen up to 0.5 m away.

Figure 1

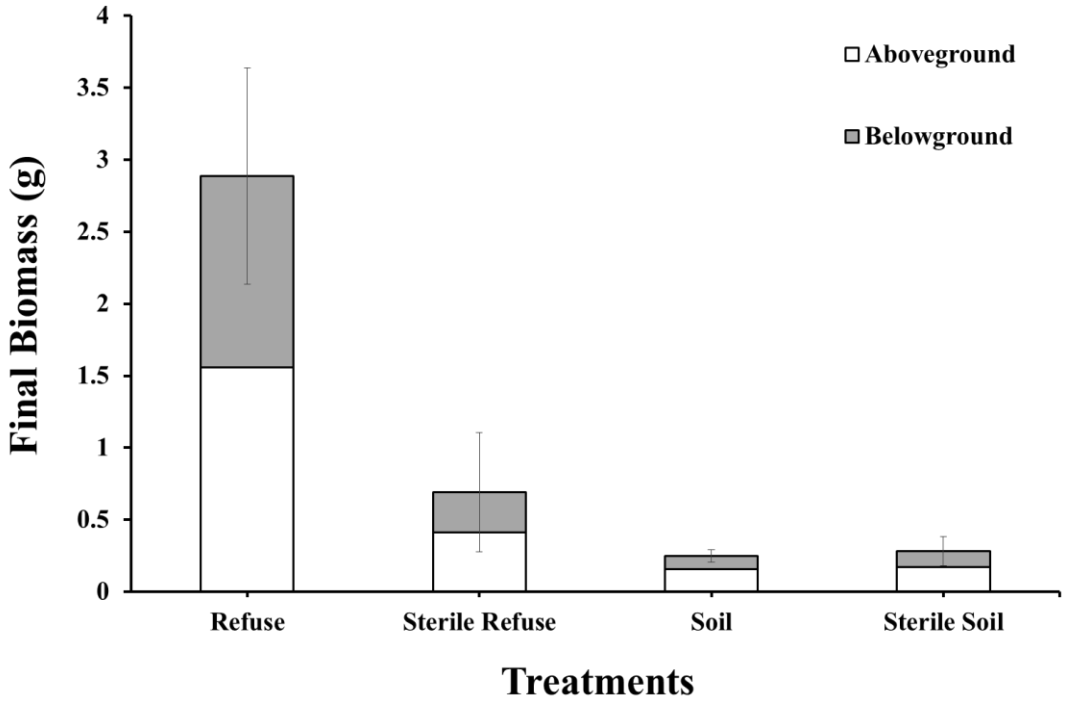
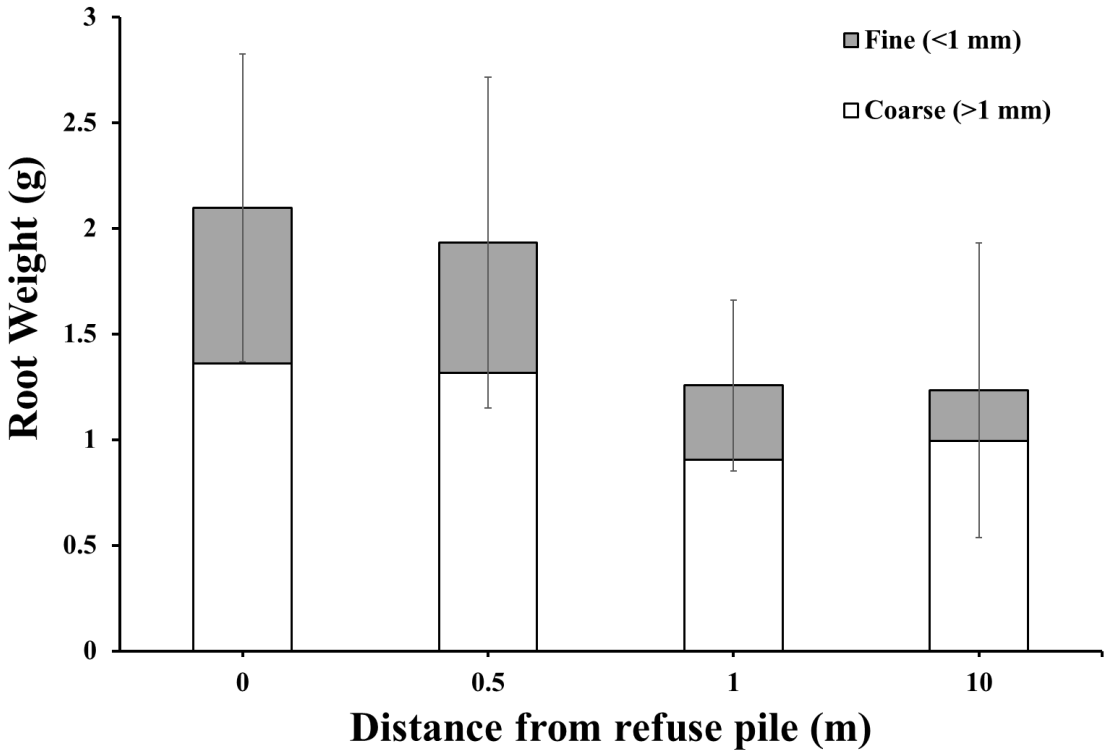

Final Biomass (g)
4
3.5
3
2.5
2
1.5
1
0.5
0
Aboveground
Belowground
Refuse
Sterile Refuse
Soil
Sterile Soil
Treatments

Figure 2


Fine (<1 mm)
Coarse (>1 mm)
Root Weight (g)
3
2.5
2
1.5
1
0.5
0
0
0.5
1
10
Distance from refuse pile (m)

Supplementary Appendix to:

Lucas, J.M., Clay, N.A., Kaspari, M. (2018) Nutrient transfer supports beneficial relationship between canopy ant, *A. trigona*, and host trees. In press *Ecological Entomology*.

Supplementary Methods

Calculations for nutrient availability

The largest proportion of *A. trigona* nests were found on trees in the 20 and 30 cm in diameter at breast height (dbh) size classes. Using the values supplied by O'Brien *et al.* (1995), we calculated the average tree crown area for trees in the 20 and 30 cm size classes. Daily leaf litter values were supplied by Sayer *et al.* (2012). Daily refuse production per nest and nutritional content of refuse and leaf litter was supplied by Clay *et al.* (2013).

1. Calculations for daily leaf litter input (g under tree crown)

Average host tree crown area = 41.33 m^2

Average leaf litter fall = 2.75 g m^{-2}

Calculation for total leaf litter fall under a tree crown each day:

$$41.33\ \text{m}^2 \times 2.75\ \text{g m}^{-2}\ \text{day}^{-1} = 111.79\ \text{g day}^{-1}$$

2. Calculations for daily refuse input (g under tree crown)

Average daily leaf litter input under crown = 111.79 g day^{-1}

Average refuse input = 7.66 g day^{-1}

Calculation for percent of input refuse is compared to total leaf litter fall:

$$7.66\ \text{g day}^{-1} \div 113.79\ \text{g day}^{-1} = 6.7\ \%$$

3. Calculations for comparing nutrient content of refuse to leaf litter

Calculations to determine nutrient input in refuse compared to leaf litter:

1. Daily refuse production (g) $*$ Percent of focal nutrient
   $=$ g of nutrient produce by refuse daily

2. Daily leaf litter production (g) $*$ Percent of focal nutrient
   $=$ g of nutrient produce by leaf litter daily

Sample Calculation using N

1. 7.66 g of refuse day^{-1} * 5.032 % (Clay *et al.* (2013)) = 0.38 g of N from refuse day^{-1}

2. 113.57 g of leaf litter day^{-1}* 1.717 % (Clay *et al.* (2013)) = 1.95 g of N from leaf litter day^{-1}

Table S1. Average total values of nutrient input for each nest and host tree crown area.

Chemical	Refuse (g nest^{-1})	Leaf Litter (g nest^{-1})	Total Available	Percent from Refuse	Percent nutrients from refuse compared to leaf litter
Total N	0.38 ± 0.03	1.95 ± 0.27	2.33 ± 0.30	16.44	19.68 ± 10.94
Total C	3.45 ± 0.14	44.66 ± 6.54	48.10 ± 6.68	7.17	7.72 ± 2.17
P	0.43 ± 0.01	1.04 ± 0.01	1.47 ± 0.02	29.13	41.10 ± 54.19
K	0.20 ± 0.05	0.15 ± 0.07	0.34 ± 0.12	57.59	135.80 ± 64.84
Ca	0.64 ± 0.01	2.35 ± 0.61	3.00 ± 0.62	21.47	27.33 ± 0.93
Mg	0.23 ± 0.00	3.63 ± 0.14	3.86 ± 0.15	6.05	6.44 ± 2.34
Na	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	38.23	61.88 ± 50.18
S	0.03 ± 0.00	0.22 ± 0.03	0.24 ± 0.03	11.36	12.81 ± 13.38

Table S2 G test analysis of size class distributions on nests compared to whole plot values.

Size Class	Year	G value	d.f.	*P* value	Notes
1	2011	426.7811	1	8.16E-95	Less than expected
2	2011	ns	1	Ns	
3	2011	32.54788	1	1.16E-08	
4	2011	49.22732	1	2.28E-12	
5	2011	80.39137	1	3.07E-19	
6	2011	80.99441	1	2.26E-19	
7	2011	188.8781	1	5.59E-43	
1	2016	334.1906	1	1.18E-74	Less than expected
2	2016	ns	1	Ns	
3	2016	33.59228	1	6.8E-09	
4	2016	27.90309	1	1.28E-07	
5	2016	66.42309	1	3.64E-16	
6	2016	51.27871	1	8.01E-13	
7	2016	111.7515	1	4.05E-26	

Chapter 3: Antibiotics decrease primary consumers and disrupt microbial communities

(Formatted for *Ecology*)

Abstract

Bacteria and fungi secrete antibiotics to suppress and kill potential competitors and predators. Antibiotic compounds occur in most environments, potentially causing significant effects on the decomposer food web. Here, we examined the impact of antibiotics on terrestrial microbes and invertebrates in a diverse Neotropical soil and leaf litter community. We contrast two complementary hypotheses regarding how microbes use antibiotics. First, microbes produce antibiotics to compete with other free-living microbiota. Second, microbes produce antibiotics to deter invertebrate competitors, either through depletion of food resources or elimination of essential endosymbionts. The addition of antifungals and antibacterials to mesocosms caused microbial and invertebrate communities to shift in composition and become more dissimilar. Detritivorous invertebrate communities decreased in abundance by an average of 34% in antibacterial treatments and 18% in antifungal treatments. Relatively large soil invertebrates--millipedes, amphipods and isopods--decreased most when antibacterials were added, by an average of 39.1%, 59.1% and 75.3% respectively. Predatory invertebrate abundance did not vary with antibiotic treatment. In total, these experiments demonstrate that antibiotic compounds can shape community composition across domains and habitat heterogeneity in hyper-diverse and ephemeral forest floor environments.

Introduction

For forest detritivores, food exists in ephemeral patches across the forest floor (Burkepile et al. 2006, Treinens et al. 2010). These patchy resources are colonized and consumed by bacteria, fungi, and invertebrates. The resulting inter-taxon competition for resources, like leaf litter and carrion, can shape the diversity and abundance of the assemblage (Hibbing et al. 2010, Janzen 1977, Wardle and Yeates 1993, Rohlfs 2005, Sauvadet et al. 2016). Here we explore how one competitive tactic, antibiotics, shapes the composition and function of a Neotropical brown food web.

Foodfall resources –carrion, seeds, plant detritus— are rapidly colonized by microbial fauna, leading to intense local competition (Hibbing et al. 2010). One hypothesis suggests that bacteria and fungi use antibiotics to defend food patches from other free-living microbes (Stuttard and Vining 2014, Abrudan et al. 2015). Many microbial taxa, both common and rare, have evolved the antibiosis tactic (e.g., *Streptomyces, Peniclillium, Cephalosporium, Bacillus*; de Lima Procopio et al. 2012, Laidi 2014, Schlatter and Kinkel 2014, Becklund et al. 2016). Antibiotic producing bacteria are often themselves antibiotic resistant, as seen across the Actinobacteria phylum, suggesting an additional protective benefit of antibiotic production (Roughley et al. 1992, Huddleston et al. 1997, Cemark et al. 2008). Yet, how production of these poisons shapes microbial communities and the ecological processes they control remains unclear.

A complementary hypothesis states that microbes secrete antibiotics to deter animal competitors (Janzen 1977). A diversity of soil invertebrates—diplopods,

isopods, collembola, and oribatid mites—consume microbe-covered detritus (Cummins 1973, Hall and Meyer 1998). Some digest only the microbial turf; others contain a gut microbiome full of microbes and protists to help digest the detritus itself (Janzen 1977, Zimmer and Bartholme 2003). Therefore, invertebrates encountering an antibiotic-filled patch of litter may be harmed in at least two ways: through suppression of the microbial turf on which they feed (Hall and Meyer 1998), or by elimination of their mutualistic endosymbionts (Janson et al. 2012, Sommer and Backhed 2013, Engel and Moran 2013). If so, antibiotic compounds should especially target the vigor of detritivorous invertebrates with endosymbiotic gut assemblages (Boxall 2004, Baguer et al. 2000). Predators in brown food webs should, by contrast, be less impacted by antibiotics as they do not consume the poisonous compounds.

Studies of community effects of antibiosis remain uncommon (e.g., Williams and Vickers 1986, Abrudan et al. 2015), with most arising from simplified laboratory environments (e.g., Bizuye et al. 2013, de Lima Procopio et al. 2012). Here we attempt to close that gap using mesocosms constructed in the field from tropical soil and leaf litter microbial (bacteria, fungi and archaea) and invertebrate communities. We test the hypothesis that antibiotic compounds shift microbial communities in the soils of tropical forests, favoring Actinobacteria, given their production antibiotic compounds (Baltz 1998) and associated antibiotic resistance (Roughley et al. 1992, Huddleston et al. 1997, Cemark et al. 2008). We also test the prediction that antibiotics target and decrease the abundance of detritivorous soil invertebrates that compete with free living microbes for substrates. Finally, we test how antibiotic compounds impact decomposition rates, predicting that areas of high antibiotic activity will be slower to

decompose. In doing so, we explore the role antibiotic compounds play in shaping tropical brown food webs.

Materials and Methods

This study was conducted from May through August of 2014 in the Barro Colorado National Monument (BCNM), Panama. BCNM consists of Barro Colorado Island (BCI). BCNM is a seasonally wet tropical forest that receives ca. 2600 mm of rain annually, with the majority of rain falling from mid-April to mid-December (Wieder and Wright 1995).

We conducted a mesocosms study to examine how the amount of antibiotics present in a system influences brown food webs. Mesocosms were created with equal parts of soil, siftate and leaf litter from two sites on BCI; one from the nutrient poor area of the Shannon trail, the other from the nutrient rich region on the Schneirlar trail. We collected the top 2-3 cm of soil from each location, along with the course and fine leaf litter. Representative soil and siftate from each region was collected using Zymo Xpedition soil/fecal sampling kits^{tm} (Zymo Soil/Fecal Xpedition mini kit protocol, Zymo Research Corp., Irvine, California, USA) at time 0 in order to get an initial profile of the microbial communities. Leaf litter was placed in shaker sifters with 1-cm^2 metal mesh to separate the coarse leaf litter from the finer siftate. Each material type (soil, litter, siftate) was homogenized within locations to ensure equal initial mesocosm communities.

Mesocosms consisted of 1-litre containers with extra fine mesh on the top and bottom to aid in containment of organisms and drainage of stagnant water. Each

container consisted of 600 g of equal parts by volume of soil, siftate and coarse leaf litter. Treatments consisted of either a synthetic antifungal (Captan, Bionide Chemical, Oriskany, NY, USA) a natural, broad spectrum antibacterial (Streptomycin sulfate, Fischer Scientific, Grand Island, NY, USA) or controls (deionized H_20). We chose these specific antibiotics based on two criteria: their ability to reduce target populations, and their reported innocuousness towards non-target taxa (NCBI PubChem Compound Database, Colinas et al. 1994). Antibiotic treatment was dissolved in 15 ml of distilled water and evenly applied to mesocosm environments at levels of 0, .5, 1 or 2 times the product recommended dosages (Table 1). Furthermore, we used the same dosages as Baguer et al. (2000). We created seven mesocosms per treatment for each source location. Mesocosms were kept at natural temperatures on 12 hr light-dark cycles for a total of 21 days. Every 7 days they were replenished with distilled water representative of wet season rainfall rates. Each mesocosm was also given a litter bag containing pre-weighed 9-cm grade P8 filter paper (Fisher Scientific, Hampton, NH, USA) to monitor rates of decomposition.

After 21 days, mesocosms were tested for pH (Mettler Toledo, Columbus, OH, USA) and destructively harvested. We sampled microbial communities, ensuring an equal mixture of siftate and soil. One limitation in our study was our inability to determine whether DNA was from live cells at the time of sampling. To control for this, we ran our experiment for 21 days, which has been shown to be sufficient time for nonviable DNA to be eliminated from soil systems (Kell et al., 1998; Nocker and Camper, 2006). After microbial sampling, the untreated mesocosms were placed into a Berlese funnel for 48 hours to extract all living invertebrates. Invertebrate samples were

preserved in 95% ethanol. The invertebrates were identified at least to Class and represented the focal taxa that were most common in this study and others (Wardle 2002, Clay et al. 2013).

Microbial Community Analysis

All microbial samples were extracted and analyzed using the protocol outline in Lucas et al. (2017). Briefly, 0.5 g samples were disrupted and stabilized using Xpedition^{tm} Lysis/Stabilization solution and bashing beads (Zymo Research, Irvine, CA, USA). Total DNA was extracted according to the manufacturer's protocol (Zymo Soil/Fecal Xpedition^{tm} mini kit). A region of the 16S rRNA gene was amplified using the primers S-D-Arch-0519-a-S-15/S-D-Bact-0785-b-A-18 (Klindworth et al. 2013) that should amplify most bacteria and archaea with few biases against specific groups (Bates et al. 2010). The S-D-Arch-0519-a-S-15 primer was modified to include a 16 bp M13 sequence (GTAAAACGACGGCCAG) at the 5' end to allow for the attachment of a unique 12 bp "barcode" in a subsequent PCR reaction. After barcoded PCR products were cleaned, equimolar amounts of each uniquely barcoded PCR product were pooled and submitted for Illumina MiSeq using TruSeq 250 bp PE V2 chemistry.

Sequence processing

All 16S sequencing reads were analyzed and demultiplexed using QIIME (Caporaso et al. 2010). We removed sequencing reads that contained errors in the barcoded region, ambiguities, homopolymers (greater than six nucleotides in length), or an average quality score <25. Primer sequences were trimmed, and chimeric sequences were eliminated using USEARCH (version 6.1) and the "gold" reference database (Edgar 2010). Then sequences were clustered into *de novo* operational taxonomic units

(OTUs) at 97% similarity. Microbial taxonomic classification was assigned via the SILVA reference database release 119 (Quast et al. 2013) using the pyNAST aligner. All raw data are available in the Dryad digital repository (doi: XXXX)

Statistical Analysis

Microbial analysis was done in QIIME, unless otherwise stated. Rarefaction curves were constructed from the estimated number of OTUs in each sample using observed species richness. Libraries were rarefied to 3000 reads (the size of the smallest sequence library; Appendix Fig. S1). Observed species richness and Chao richness were calculated and used to compare alpha diversity across treatments and dosages using a series of two-sample nonparametric t-tests and Monte Carlo permutations to calculate p-values. We compared microbial communities across treatments, dosages and location using PERMANOVA (1000 permutations). Community similarity was calculated using weighted UniFrac distance (Lozupone and Knight 2005). We used a non-metric multidimensional scaling (NMDS) ordination to visualize relationships among microbial communities. To test for treatment effects on individual microbial taxa, we ran linear mixed effect models using the *lme4* package in R (R Development Core Team 2013, version 0.99.903; Bates et al. 2015). We used the effect size Cohen's d (Cohen 1988) to quantify the direction and magnitude of response for bacterial phyla when antibiotics were added as compared to controls. Cohen's *d* analysis divides the mean difference of each antibiotic treatment versus control by the pooled standard deviation (Kaspari et al. 2017). This analysis allows us to control for responses in taxa that vary in magnitude in species abundance. Values of Cohen's $d \geq |0.5|$ represent a 'medium'

effect size, while values larger than |0.8| are considered to be 'large'. Therefore, we only report phyla that responded with Cohen's *d* values ≥ |0.5|.

We compared invertebrate communities across treatments, and dosage using PERMANOVA in Primer-E version 7 (Clark and Gorley 2015). To examine if detritivores responded separately, we subsampled our invertebrate data and ran PERMANOVAs to examine the effect of treatment, dosage on the detritivore community. To visualize invertebrate communities by treatment type, we used NMDS ordination with coordinates generated in Primer-E. To test for treatment effects on individual invertebrate taxa, overall abundance and richness, decomposition rates and pH levels, we ran linear mixed effect models using *lme4* in R. Due to the fact that dosage did not have a significant effect in our community composition analysis (PERMANOVA microbial: $F_{96,7} = 1.37$, $P = 0.09$, invertebrate: $F_{96,7:}$ 1.48, $P = 0.08$), we treated dosage as a random grouping factor. We also treated litter source and mesocosm number as random grouping factors.

Results

In our litter mesocosms, antibiotics changed the composition of bacterial and invertebrate assemblages—but not pH nor cellulose decomposition--after 21 days. Bacterial community composition differed with antibiotic treatment (PERMANOVA: $F_{3,96}$: 1.84, $P = 0.02$; Fig. 1a) despite different starting communities (PERMANOVA Source Location: $F_{2,96}$: 3.32, $P = 0.01$). Antibiotics also changed invertebrate community composition (PERMANOVA: $F_{3,96:}$ 1.54, $P = 0.042$; Fig. 1b, Table 2), despite differences in starting compositions based on litter source (PERMANOVA

Source Location: $F_{3,96}$: 3.32, P = 0.01). In contrast, antibiotics had no effect on cellulose decomposition (dAIC = 3.66, X^2_2 = 0.33, P = 0.84, Table S1) or pH levels (dAIC = 2.1, X^2_2 = 1.90, P = 0.39, Table S1). Thus, against a background of litter patchiness, antibiotics shifted community composition but not a key ecosystem process of brown food webs.

Bacterial phyla responded similarly to both antibacterial and antifungal treatments (Fig. 2), but had differing responses at the family and genus level (Fig. S2). Contrary to our prediction, Actinobacteria relative abundance did not increase in either treatment (Cohen's *d*: antifungal -0.43, antibacterial 0.04), but we did see changes in multiple genera within the Actinobacteria phyla (Fig. S2). Changes in composition with antibiotic treatments were also accompanied by greater dispersion compared to controls (PERMDISP: $F_{3,90}$ = 151.68, P < 0.001). Composition changes however, were not accompanied by changes in alpha diversity (dAIC = 0.5, X^2_2 = 3.52, P = 0.17; Table S1).

Detritivore abundance decreased by an average of 34% in antibacterial treatments and 18% in antifungal treatments (Table 2). In antibacterial treatment, this response was primarily driven by the decrease of millipedes, amphipods and isopods by 39.1%, 59.1% and 71.68% respectively. Isopods and amphipods also responded strongly to antifungal treatments, decreasing by 75.3% and 60.2% respectively. While antibiotics suppressed detritivore abundance, the response was not uniform (Table 2). As predicted, we did not see an impact of antibiotic addition on any predatory taxa (e.g., Aranea, Gamasid, Pseudoscorpion; Table 2).

Discussion

Antibiotic compounds are well known by-products of the brown food web. Here we demonstrate that these compounds can shift the composition of this community across multiple taxonomic domains (Fig. 1). The significant suppression of key detritivorous invertebrates—and their failure to effect predatory taxa—is a significant field test of Janzen's hypothesis (1977) that animals that compete with free-living decomposers are targets of antibiosis. This resulted in a change in the composition of communities exposed to antibiotics, as well as greater dispersion within treatments. At the same time, we were surprised to find that antibiotic resistant Actinobacteria did not uniformly benefit from antibiotic application, nor did the application of these metabolic poisons have a net negative effect on the breakdown of a common carbon source in the litter: cellulose.

Bacterial communities are altered after antibiotic addition

Bacterial communities shifted in composition and became more dissimilar in the presence of antibiotic compounds. The response to antibacterial and antifungal treatments was similar at the phylum level for bacterial communities. This result is consistent with studies of similar dosages of Captan addition in southwestern Oregon soils (Colinas et al. 1994). The similarity in response could be due to the mechanism of action of our antibiotics. Both Captan and Streptomycin interfere with protein synthesis, although Captan targets thiols in fungi and should not directly impact bacteria (Gordon 2001). However, analysis at lower taxonomic levels demonstrates the variability in response to antibiotic treatment (Supplementary Material, Fig S2). For example, the relative abundance of Actinobacteria did not increase with antibiotic addition, but

multiple genera within the Actinobacteria phyla did (Fig S2). This is contrary to our prediction and previous work (Cermak et al. 2008).

The addition of antibiotics caused greater taxonomic dispersion across our mesocosms communities. This suggest that antibiotic compounds may contribute to the high levels of patchiness often observed in both microbial and invertebrate litter communities (Levings and Windsor 1984, Kaspari 1996, Hättenschwiler et al. 2005, Wang et al. 2008). Similarly, overall changes were seen regardless of dosage levels, a contrast to previous work (Ingrahm and Coleman 1984). This suggests that even trace amount of these active compounds can have large ramifying effects; a result that is of particular concern in the context of anthropogenic introductions of active antibiotics (Cytryn 2013).

Antibiotics suppress invertebrate detritivores but not predators

Our results build on previous work demonstrating that antibiotic compounds are capable of impacting fitness and abundance of invertebrate communities (Ingham and Coleman 1984, Colinas et al. 1994, Boxall 2004). These studies focus on aquatic ecosystems and the impact of anthropogenic introductions of antibiotic compounds (Capone et al. 1996, Kumar et al. 2005, Li et al. 2012). In contrast, we demonstrate that a naturally synthesizable antibiotic, Streptomycin, can shift terrestrial community composition as much as or more than synthetic compounds (Fig. 1, Table 2). Antibiotic production provides a potential mechanism for the heterogeneity of litter invertebrate communities (Coleman 2008, Donoso et al. 2013).

Millipede, amphipod and isopod communities decreased by 39.1%, 59.1% and 71.68% respectively, in environments with low antibacterial concentrations (1.5 mg g^{-}

[1]). These results contrast with Baguer et al. (2000), who found that common veterinary antibiotics did not have large impacts on soil invertebrates until high concentrations were introduced. However, that study was limited in focal taxa -- earthworms, collembola and enchytraeids – all of which demonstrated no response in our experiment. Millipede, amphipod and isopod assemblages are common in tropical environments, play important roles in the decomposition process and are in constant competition with microbial communities (Janzen 1977, Olson 1994, Bardgett and van der Putten 2014). Therefore, describing the mechanism by which antibiotics impact these taxa is fundamental to understanding ecosystem processes. Due to their reliance on endosymbiotic microbes, millipede and isopod raised in antibiotic laden environments likely have disrupted microbiomes responsible for decreased survival rates (Bouchon et al. 2016, Nardi et al. 2016).

We demonstrate that antibiotic compounds have the potential to disrupt non-target organisms spanning across domains. We suspect that antibiotic compounds in natural environments may contribute to the local heterogeneity of organisms in the decomposer food web. Further tests conducted in field settings are necessary to determine whether observed patterns sustain in the ephemeral landscape of hyper-diverse forest floors. Furthermore, our study provides insight on the potential ramifications anthropogenic introductions of antibiotic compounds can have on terrestrial ecosystems.

Acknowledgements

We thank Michael Weiser, Evan Gora, Brian Bill, Bradley Stevenson and Karl Roeder for their assistance in project design and comments on previous versions of the

manuscript. We thank the Stevenson Lab at OU for providing laboratory support and OMRF for their analysis of microbial samples. We also thank the staff of Barro Colorado Island, Panama for logistical support during the field portion of this project. This work was funded by the National Science Foundation's Graduate Research Fellowship Program and the Smithsonian Tropical Research Institute's Short-term Fellowship to JML.

References

Abrudan, M. I., F. Smakman, A. J. Grimbergen, S. Westhoff, E. L. Miller, G. P. van Wezel, and D. E. Rozen. 2015. Socially mediated induction and suppression of antibiosis during bacterial coexistence. *Proceedings of the National Academy of Sciences* **112**:11054-11059.

Baguer, A. J., J. Jensen, and P. H. Krogh. 2000. Effects of the antibiotics oxytetracycline and tylosin on soil fauna. *Chemosphere* **40**:751-757.

Baltz, R. H. 1998. Genetic manipulation of antibiotic producing Streptomyces. *Trends in Microbiology* **6:**76-83.

Bardgett, R. D., and W. H. van der Putten. 2014. Belowground biodiversity and ecosystem functioning. *Nature* **515**:505-511.

Bates, S.T., D. Berg-Lyons, J. G. Caporaso, W. A. Walters, R. Knight, and N. Fierer. Examining the global distribution of dominant archaeal populations in soil. *ISME J.* **2010**:908–917.

Becklund, K., J. Powers, and L. Kinkel. 2016. Tree species effects on pathogen-suppressive capacities of soil bacteria across two tropical dry forests in Costa Rica. *Oecologia* **182:**789-802.

Bizuye, A., F. Moges, and B. Andualem. 2013. Isolation and screening of antibiotic producing actinomycetes from soils in Gondar town, North West Ethiopia. *Asian Pacific Journal of Tropical Disease* **3**:375-381.

Bouchon, D., M. Zimmer, and J. Dittmer. 2016. The Terrestrial Isopod Microbiome: An All-in-One Toolbox for Animal-Microbe Interactions of Ecological Relevance. *Frontiers in Microbiology*. **23**:1472.

Boxall, A. B. 2004. The environmental side effects of medication. *EMBO Reports* **5**:1110-1116.

Burkepile, D. E., J. D. Parker, C. B. Woodson, H. J. Mills, J. Kubanek, P. A. Sobecky, and M. E. Hay. 2006. Chemically mediated competition between microbes and animals: microbes as consumers in food webs. *Ecology* **87:**2821-2831.

Capone, D. G., D. P. Weston, V. Miller, and C. Shoemaker. 1996. Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. *Aquaculture* **145**:55-75.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, and G. A. Huttley. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7:**335-336.

Čermák, L., J. Kopecký, J. Novotná, M. Omelka, N. Parkhomenko, K. Plháčková, and M. Ságová-Marečková. 2008. Bacterial communities of two contrasting soils reacted differently to lincomycin treatment. *Applied Soil Ecology* **40**:348-358.

Clarke, K. R. and R. N. Gorley. 2015. Primer v7. Primer-E Ltd.

Clay, N. A., J. Lucas, M. Kaspari, and A. D. Kay. 2013. Manna from heaven: refuse from an arboreal ant links aboveground and belowground processes in a lowland tropical forest. *Ecosphere* **4**:1-15.

Cohen, J. 1988. Statistical power analysis for the behavioral sciences. Second edition. L. Erlbaum, Hillsdale, New Jersey, USA

Coleman, D. C. 2008. From Peds to Paradoxes: linkages between soil biota and their influences on ecological processes. *Soil Biology and Biochemistry* **40**:271e289.

Colinas, C., E. Ingham, and R. Molina. 1994. Population responses of target and non-target forest soil organisms to selected biocides. *Soil Biology and Biochemistry* **26:**41-47.

Cummins, K. W. 1973. Trophic relations of aquatic insects. *Annual Review of Entomology* **18**:183-206.

Cytryn, E. 2013. The soil resistome: the anthropogenic, the native, and the unknown. *Soil Biology and Biochemistry* **63**:18-23.

de Lima Procópio, R. E., I. R. da Silva, M. K. Martins, J. L. de Azevedo, and J. M. de Araújo. 2012. Antibiotics produced by Streptomyces. *The Brazilian Journal of Infectious diseases* **16**:466-471.

Donoso, D. A., M. K. Johnston, N. A. Clay, and M. E. Kaspari. 2013. Trees as templates for trophic structure of tropical litter arthropod fauna. *Soil Biology and Biochemistry* **61**:45-51.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **67**:1-48.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:2460–2461.

Engel, P. and N. A. Moran. 2013. The gut microbiota of insects–diversity in structure and function. *FEMS Microbiology Reviews* **37**:699-735.

Gordon, E. B. 2001. Captan and folpet. *Handbook of Pesticide Toxicology* **2**:1711-1742.

Hall, R. O. and J. L. Meyer. 1998. The trophic significance of bacteria in a detritus-based stream food web. *Ecology* **79**:1995-2012.

Hättenschwiler, S., A. V. Tiunov, and S. Scheu. 2005. Biodiversity and litter decomposition in terrestrial ecosystems. *Annual Review of Ecology, Evolution and Systematics* **36**:191-218.

Hibbing, M. E., C. Fuqua, M. R. Parsek, and S. B. Peterson. 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews: Microbiology* **8**:15.

Huddleston, A. S., N. Cresswell, M. C. Neves, J. E. Beringer, S. Baumberg, D. I. Thomas, and E. M. Wellington. 1997. Molecular detection of streptomycin-producing streptomycetes in Brazilian soils. *Applied and Environmental Microbiology* **63**:1288-1297.

Ingham, E. R. and D. Coleman. 1984. Effects of streptomycin, cycloheximide, fungizone, captan, carbofuran, cygon, and PCNB on soil microorganisms. *Microbial Ecology* **10**:345-358.

Janzen, D. H. 1977. Why fruits rot, seeds mold, and meat spoils. *The American Naturalist* **111**:691-713.

Janson, B. and K. Thursky. 2012. Dosing of antibiotics in obesity. *Current Opinion in Infectious Diseases* **25**:634-649.

Kaspari, M. 1996. Litter ant patchiness at the 1-m2 scale: disturbance dynamics in three Neotropical forests. *Oecologia* **107**:265-273.

Kaspari, M., J. Bujan, M. D. Weiser, D. Ning, S. T. Michaletz, H. Zhili, B. J. Enquist, R. B. Waide, J. Zhou, B. L. Turner, and S. J. Wright. 2017. Biogeochemistry drives diversity in the prokaryotes, fungi, and invertebrates of a Panama forest. *Ecology* **98**:2019-2028.

Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer. 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Leeuwenhoek* **73**:169-187.

Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F. O. Glöckner. 2012. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* **41**:e1.

Kumar, K., S. C. Gupta, S. K. Baidoo, Y. Chander, and C. J. Rosen. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. *Journal of Environmental Quality* **34**:2082-2085.

Laidi, R. 2014. *Actinomycetes and Antibiotics Production: Isolation, Screening and Characterization of Novel Antibiotic Producing Streptomycetes Strains from Soil Origin*. Scholars' Press.

Levings, S. C. and D. M. Windsor. 1984. Litter moisture content as a determinant of litter arthropod distribution and abundance during the dry season on Barro Colorado Island, Panama. *Biotropica* **16**:125-131.

Li, W., Y. Shi, L. Gao, J. Liu, and Y. Cai. 2012. Occurrence of antibiotics in water, sediments, aquatic plants, and animals from Baiyangdian Lake in North China. *Chemosphere* **89**:1307-1315.

Lozupone, C. A., and R. Knight. 2007. Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences* **104**:11436.

Lucas, J., B. Bill, B. Stevenson, and M. Kaspari. 2017. The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest. *Ecosphere* **8**:e01639.

Nardi, J. B., C. M. Bee, and S. J. Taylor. 2016. Compartmentalization of microbial communities that inhabit the hindguts of millipedes. *Arthropod Structure & Development* **45**:462-474.

National Center for Biotechnology Information. PubChem Compound Database; CID=19649, https://pubchem.ncbi.nlm.nih.gov/compound/19649

Nocker, A. and A. K. Camper. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. *Applied and Environmental Microbiology* **72**:1997-2004.

Olson, D. M. 1994. The distribution of leaf litter invertebrates along a Neotropical altitudinal gradient. *Journal of Tropical Ecology* **10**:129-150.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**:D590-D596.

Rohlfs, M. 2005. Clash of kingdoms or why Drosophila larvae positively respond to fungal competitors. *Frontiers in Zoology* **2**:2.

Roughley, R. J., F. A. Wahab, and J. Sundram. 1992. Intrinsic resistance to streptomycin and spectinomycin among root-nodule bacteria from Malaysian soils. *Soil Biology and Biochemistry* **24**:715-716.

Sauvadet, M., M. Chauvat, D. Cluzeau, P. A. Maron, C. Villenave, and I. Bertrand. 2016. The dynamics of soil micro-food web structure and functions vary according to litter quality. *Soil Biology and Biochemistry* **95**:262-274.

Schlatter, D. C. and L. L. Kinkel. 2014. Global biogeography of Streptomyces antibiotic inhibition, resistance, and resource use. *FEMS Microbiology Ecology* **88**:386-397.

Sommer, F. and F. Bäckhed. 2013. The gut microbiota--masters of host development and physiology. *Nature Reviews: Microbiology* **11**:227.

Stuttard, C. and L. Vining. 2014. *Genetics and Biochemistry of Antibiotic Production (Vol. 28).* Butterworth-Heinemann, Oxford, United Kingdom.

Trienens, M., N. P. Keller, and M. Rohlfs. 2010. Fruit, flies and filamentous fungi–experimental analysis of animal–microbe competition using Drosophila melanogaster and Aspergillus mould as a model system. *Oikos* **119**:1765-1775.

Wang, J., Y. Wu, H. Jiang, C. Li, H. Dong, Q. Wu, J. Soininen, and J. Shen. 2008. High beta diversity of bacteria in the shallow terrestrial subsurface. *Environmental Microbiology* **10**:2537-2549.

Wardle, D. A. 2002. *Communities and ecosystems: linking the aboveground and belowground components*. Princeton University Press, Princeton, New Jersey, USA.

Wardle, D. A. and G. W. Yeates. 1993. The dual importance of competition and predation as regulatory forces in terrestrial ecosystems: evidence from decomposer food-webs. *Oecologia* **93**:303-306.

Wieder, R. K. and S. J. Wright. 1995. Tropical forest litter dynamics and dry season irrigation on Barro Colorado Island, Panama. *Ecology* **76**:1971-1979.

Williams, S. T. and J. C. Vickers. 1986. The ecology of antibiotic production. *Microbial Ecology* **12**:43-52.

Zimmer, M. and S. Bartholmé. 2003. Bacterial endosymbionts in Asellus aquaticus (Isopoda) and Gammarus pulex (Amphipoda) and their contribution to digestion. *Limnology and Oceanography* **48**:2208-2213.

Tables

Table 1: Measurements of antibiotic dosages.

Antibiotic	Target Group	Source	Treatment Level	Amount Used
Streptomycin	Bacteria	Naturally Derived	0.5x	1.5 mg/g
			1x	3mg/g
			2x	6mg/g
Captan	Fungi	Synthetic	0.5x	12.5ug/g
			1x	25ug/g
			2x	50ug/g

Table 2: Average abundance levels of invertebrates from the mesocosm experiment.

DETRITIVORES	Captan	Control	Streptomycin	*P* value
Amphipod	**1.36 ± 2.01[a]**	3.42 ± 5.93[b]	**1.40 ± 2.23[a]**	0.06
Annelida	0.47 ± 0.92	1.14 ± 1.40	0.38 ± 0.58	ns
Blattaria	0.02 ± 0.15	0 ± 0	0 ± 0	ns
Diptera Larvae	5.71 ± 5.82	7.5 ± 5.08	4.57 ± 3.19	ns
Entomobryidae	8.5 ± 8.32	5.85 ± 3.71	7.02 ± 4.99	ns
Hypogastruridae	29.74 ± 31.61	26.35 ± 18.75	21.90 ± 25.19	ns
Isopoda	**0.97 ± 1.55[a]**	3.92 ± 6.73[b]	**1.11 ± 3.17[a]**	0.02
Isoptera	0.452 ± 1.52	0.28 ± 0.61	0.31 ± 1.13	ns
Millipede	2.45 ± 2.11[a]	3.71 ± 2.61[a]	**2.26 ± 2.52[b]**	0.04
Neelidae	4.69 ± 3.99	6.28 ± 6.94	7.71 ± 8.19	ns
Oribatid	120.52 ± 86.29[a]	152.85 ± 103.04[a]	**92.66± 71.00[b]**	0.07
Scolytidae	1 ± 1.68	0.57 ± 0.97	0.85 ± 1.42	ns
PREDATORS				
Ants	8.61 ± 13.48	6.78 ± 8.14	7.21 ± 10.94	ns
Aranea	1.78 ± 1.68	1.5 ± 1.51	1.81 ± 1.31	ns
Coleoptera	3.33 ± 3.68	2.92 ± 2.43	3.12 ± 2.15	ns
Dipluria	0.64 ± 1.83	0.78 ± 1.05	0.55 ± 1.25	ns
Diptera	20.23 ± 20.16	11.07 ± 8.16	13.74 ± 15.44	ns
Gamasid	19.30± 15.49	20.21 ± 10.86	14.17 ± 10.97	ns
Hemiptera	0.02 ± 0.15	0 ± 0	0 ± 0	ns
Ioxid	0.14 ± 0.35	0.14 ± 0.36	0.17 ± 0.58	ns
Neuroptera	0.04 ± 0.22	0.07 ± 0.26	0.07 ± 0.26	ns
Opilinoes	0.05 ± 0.31	0 ± 0	0 ± 0	ns
Pseudoscorpion	0.26 ± 0.49	0.64 ± 0.74	0.38 ± 0.58	ns
Staphylinidae	0.40 ± 0.76	0.57 ± 0.93	0.38 ± 0.85	ns

[1]**Bold** values represent taxa that were suppressed in abundance in antibiotic-laden environments.

Figure Legends

Fig. 1 NMDS representation of (a) bacterial and (b) invertebrate communities in mesocosms treated with antifungal (Captan, blue triangles), antibacterial (Streptomycin, pink circles) or control (deionized H_2O, black crosses). Microbial distances are based on dissimilarity matrices of sequence-based weighted UniFrac distances. Microbial sample types differ from each other (PERMANOVA: $F_{3,96} = 1.84$, $P = 0.02$). Invertebrate distances are based on dissimilarity matrices of Bray-Curtis distances. Invertebrate sample types differ from each other (PERMANOVA: $F_{3,96} = 1.54$, $P = 0.042$).

Fig. 2 Effect size (ES) expressed as Cohen's *d* of OTU abundance of bacterial and archaeal phyla in response to (a) antifungal (Captan) and (b) antibacterial (Streptomycin) additions. Values are in units of standard deviation above or below values recorded on control plots. Positive values represent increase with treatment, while negative values represent higher abundance in control environments.

Figure 1

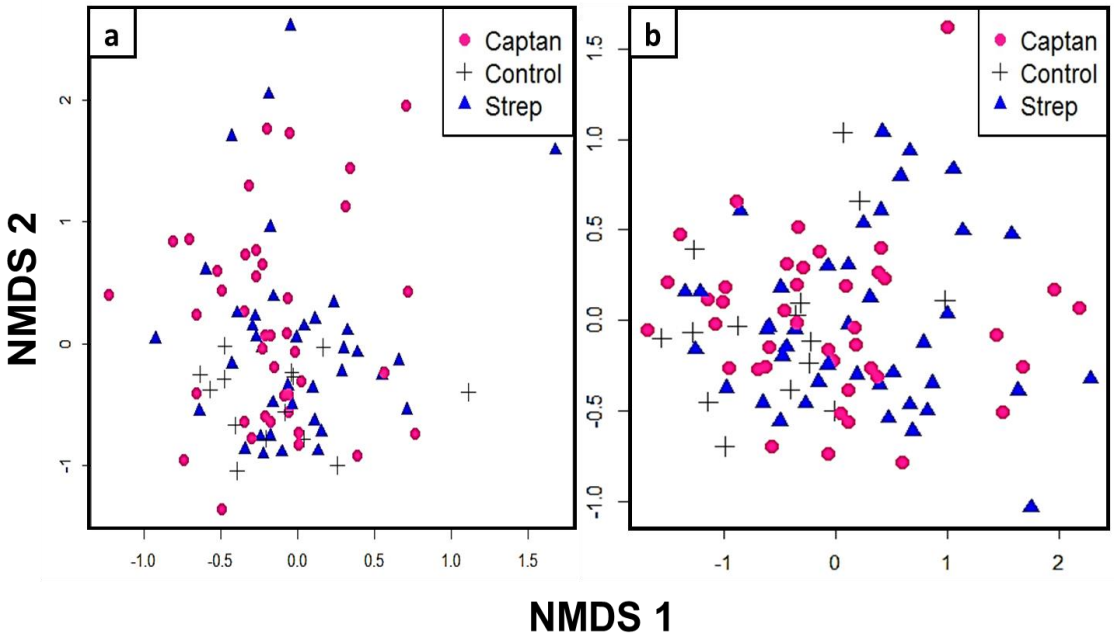
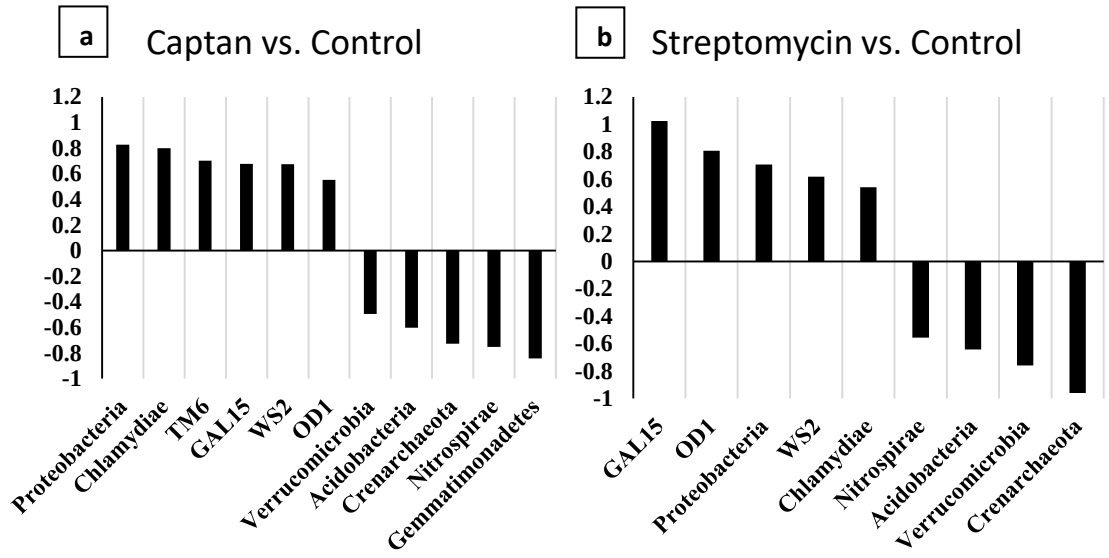


Figure 2


a
Captan vs. Control
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Proteobacteria
Chlamydiae
TM6
GAL15
WS2
OD1
Verrucomicrobia
Acidobacteria
Crenarchaeota
Nitrospirae
Gemmatimonadetes
b
Streptomycin vs. Control
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
GAL15
OD1
Proteobacteria
WS2
Chlamydiae
Nitrospirae
Acidobacteria
Verrucomicrobia
Crenarchaeota

Appendix to:

Lucas, J and M. Kaspari. Antibiotics decrease primary consumers and disrupt microbial communities.

Supp. Table S1. Average mass loss of cellulose (g), change in pH and alpha diversity (observed OTUs) in mesocosms by treatment.

Treatment	Ave. Mass Loss (± sd)	Ave. change in pH (± sd)	Ave. alpha diversity (± sd)
Captan	0.10891 ± 0.096	5.59 ± (0.28)	2647.38 ± 1038.19
Streptomycin	0.11957 ± 0.0495	5.52 ± (0.30)	3043.53 ± 1457.12
Control	0.11996 ± 0.0649	5.56 ± (0.32)	3295.71 ± 1399.06

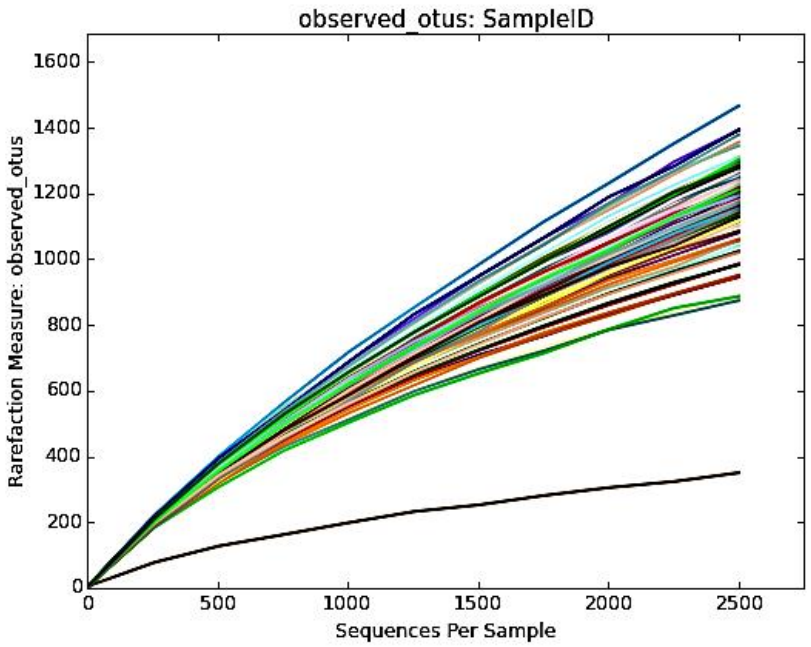


Fig. S1. Rarefaction curves were used to estimate richness in the observed OTUs. The vertical axis shows the bacterial and archaeal OTUs observed and the number of sequences per sample is shown on the horizontal axis.

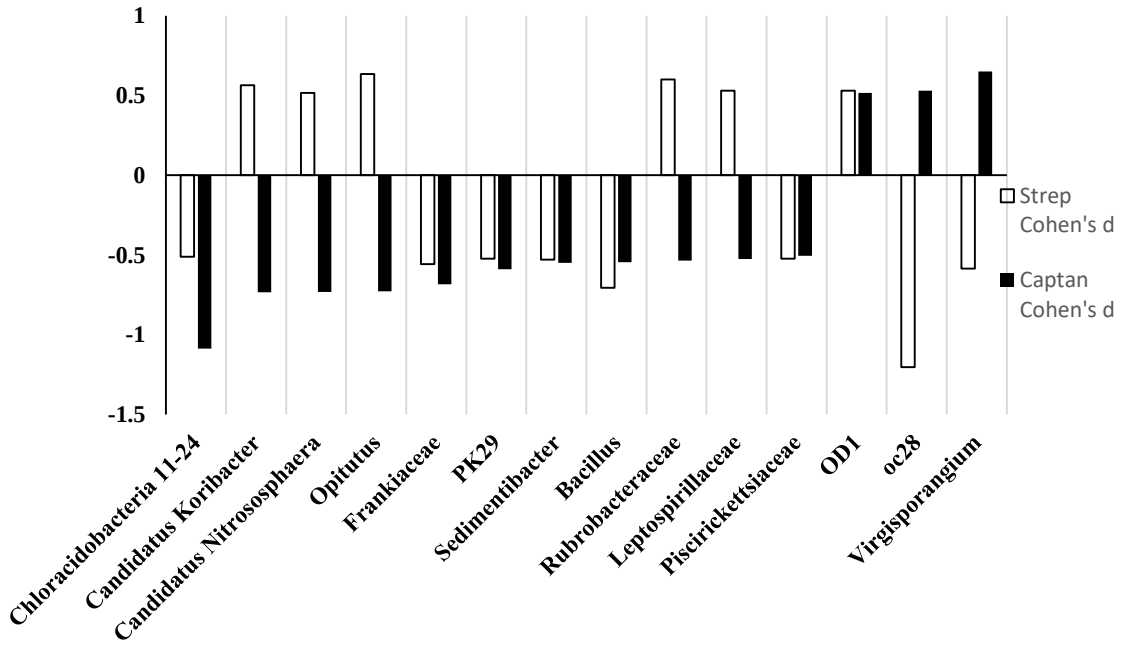


Fig. S2 Effect size (ES) as expressed by Cohen's *d* of OTU abundance of bacteria at family or genus level taxonomic assignment in response to antifungal (Captan) and antibacterial (Streptomycin) additions. Only bacteria that responded with ES > 0.5 on both treatments are displayed. Values are in units of standard deviation above or below values recorded on control plots. Positive values represent increase with treatment, while negative values represent higher abundance in control environments.

Chapter 4: Antimicrobials as chemical warfare against detritivorous invertebrates

(Formatted for *Ecology*)

Abstract:

Competition between microbes and animals is ubiquitous yet underexplored. Janzen (1977) hypothesized that microorganisms must render resources unpalatable to compete with animals, and that animals evolve avoidance or detoxification strategies in response. While microbial colonization of resources can deter animal competitors, the underlying mechanism of response remains unknown. Here we hypothesize that antibiotic production by microbes—long considered a potent mechanism of competition between microbes—can also be effective across phylogenetic domains. We test this by monitoring growth and survival of saprotrophic invertebrates (isopods, millipedes and termites) in antibiotic laden environments, and assess whether invertebrate survival is mediated by changes in their endosymbiont community. We also test whether these saprotrophic invertebrates have evolved avoidance behavior of antibiotic compounds. We find that antibiotic compounds had generally negative impacts on invertebrate survival, but little impact on the microbiome composition of these three saprotrophs. Moreover, we demonstrate that isopods and termites avoid areas of antibiotic activity, while millipedes do not. Combined, our results suggests that, consistent with Janzen (1977), antibiotic production is likely a key tactic that microorganisms use to deter animal competitors and, in turn, contribute to the diversity of the detrital food webs.

Introduction

Competition is expected to occur most frequently between closely related species (Darwin 1859, MacArthur 1958, Nottebrock et al. 2017). However, cross-domain competition between animals and microbes is a ubiquitous and underexplored interaction (Hochberg and Lawton 1990). The most prominent example of this phenomenon is food "spoilage" as a result of microbial warfare against animal competitors (Janzen 1977). In this context, animal avoidance of "spoiled" food is an evolved response driven by microbial competition. Consistent with Janzen's hypothesis (1977), microbial colonization can deter animal competitors (DeVault et al. 2003, Burkepile et al. 2006). Yet, such studies are few and rarely address the underlying mechanism driving this avoidance response.

For microbial fauna, the loss of food to animal competitors typically results in death by ingestion (Zimmer 2002, Nauseef 2007, Rozen et al. 2008). To combat this, fungi and bacteria secure resources for themselves by rendering food unpalatable (Janzen 1977). This includes changing the chemical composition of resources or producing toxic defensive compounds (Huis in't Veld 1996). Antimicrobial production is a wide-spread example of defensive compound production that is adopted by microbial organisms to harm microbial competitors (Abrudan et al. 2015). However, whether traditionally "antimicrobial" compounds (hereafter referred to as antibiotics) are more broadly effective against animal competitors remains unresolved. Animal consumption of antibiotics can increase the health of organisms by curing diseases or increasing weight (Bunyan et al. 1977, Walsh 2003). At the same time, antibiotic

compounds can increase susceptibility to disease (Gilchrist et al. 2007) and disrupt essential endosymbionts (Raymann et al. 2017).

Here we argue that this disruption of endosymbionts by antibiotic compounds is the most likely mechanism mediating microbial competition with invertebrates. Saprotrophic invertebrates are important competitors for microbial taxa due to their shared habitat and resources (Lussenhop 1992, Maraun et al. 2003). These saprotrophic invertebrates frequently host a microbe-rich endosymbiotic community necessary for their survival. In much the same way that antibiotics are used to experimentally disrupt microbiomes in the lab (Wilkinson 1998, Matsuura 2001, Hammer et al. 2017), we suggest that saprotrophic microbes use antibiotic compounds to disrupt invertebrate endosymbionts. This can decrease invertebrate viability and potentially result in invertebrate death. If microbes can readily kill invertebrate competitors, this challenges the traditional viewpoint that animals have an asymmetrical advantage in competition with microbes (Rozen et al. 2008). It also suggests that if maintenance of endosymbionts is essential for survival, animals must evolve protective behaviors such as avoidance or detoxification (Janzen 1977). Tropical leaf litter is a hot spot for invertebrate-microbe competition, and thus it is an ideal system for testing this mechanism (Maraun et al. 2003).

In this study, we examine the interactions between antibiotic compounds and three dominant, invertebrate detritivores: isopods (Philosciidae), millipedes (Spirostreptidae) and termites (Termitidae). These invertebrate taxa play essential roles in nutrient cycling and decomposition (Sugimoto et al. 2000, Crowther et al. 2015, Bouchon et al. 2016). While somewhat similar in their detritivorous nature, isopods,

termites and millipedes have important differences. Terrestrial isopods are agile organisms that occupy upper and middle layers of the leaf litter, inhibited by their inability to burrow (Zimmer 2002, Karagkouni et al. 2016). Millipedes by contrast are slower-feeding, burrowing organisms at the soil-litter interface that often produce and secrete defensive compounds (Kime and Golovatch 2000, Mans 2017). Both isopods and millipedes primarily consume detritus, though both can be omnivorous (Wooten and Crawford 1975, Bouchon et al. 2016). Termites are eusocial, creating huge colonies that can shape their environment (Jouquet et al. 2006). While some termites are wood consuming specialists, many species can persist on a variety of decomposing materials (Waidele et al. 2017). To aid in digestion of detritus, each of these invertebrates relies on a rich microbiome composed of diverse bacterial and fungal genera (Anderson and Bignell 1980, Bouchon et al. 2016, Nardi et al. 2016, Waidele et al. 2017) Termite microbiomes have also been demonstrated to play a role in kin recognition (Matsuura 2001), and therefore may be an important component of their eusociality. Thus ingesting substrate poisoned with antibiotics could have high impacts on invertebrate survival and fitness, and such invertebrate taxa should evolve sensory and behavioral mechanisms to avoid these compounds.

Here we hypothesize that antibiotic production is a viable tactic for bacteria and fungi to compete with invertebrates. We test whether antibacterial and antifungal compounds impact the survival and fitness of detritus feeding invertebrates. We quantify changes in invertebrate microbiomes to test whether microbiome disruption mediates antibiotic effects. Finally, we test whether invertebrates detect and avoid areas of antibiotic activity. Combined, our results provide some of the most complete support

of Janzen's (1977) hypothesis: that microbes and invertebrates compete for detritus, with antibiotics as one powerful weapon used by the microbes of the litter.

Materials and Methods

Study system, animal collection and antibiotic compounds

This study occurred during May-August of 2016 on Barro Colorado Island (BCI) in Panama. BCI is a seasonally wet tropical forest that receives ca. 2600 mm of rain annually, with the majority of rain falling from mid-May to mid-December (Wieder and Wright 1995). Study organisms and leaf litter were collected from the northern region of BCI. All millipedes (Spirostreptidae) were between 15 and 25 mm in length; isopods (Philosciidae) were between 5 and 10 mm in length. Termites (*Nasutitermes* spp.) were collected from one colony to control for potential inter-colonial differences in the microbiome.

In this study, we used three antibiotic compounds: streptomycin, sulfanilamide and Captan (Table 1, Fig. S1). Streptomycin is a naturally synthesized, broad-spectrum, bactericidal antibacterial derived from the soil bacteria, *Streptomyces griseus* (Kim et al. 2015). Streptomycin binds to the 30S ribosomal subunit, inhibiting protein synthesis. Sulfanilamide is a synthetic sulfonamide compound that has broad-spectrum antibacterial effects. Sulfanilamide competes with p-aminobenzoic acid (PABA), inhibiting bacterial synthesis of folic acid, which leads to cell death (Kim et al. 2015). Captan (ethanethiol) is a synthetic phthalimide antifungal that inhibits DNA and protein production by interacting with thiols. All antibiotic compounds were chosen based on

two criteria: their ability to reduce target populations, and their reported innocuousness towards non-target taxa (Colinas et al. 1994, Kim et al. 2015).

Invertebrate Growth and Survival

Survival arenas for isopods and millipedes were 80 mm diameter petri dishes, filled with 5 g of leaf litter-soil mixture collected from the same field location as the organisms (Fig. 1a). The environments were treated with antibiotic compounds prior to introduction of invertebrate organisms, and re-applied weekly. Antibiotic were dissolved in sterile, deionized water and added in concentrations specified by label instructions (Table 1), and used in previous studies (Bauger et al. 2000). Survival arenas were rehydrated with sterile H_2O (1.1 ml), every day to maintain the moisture content of leaf litter. A small cotton ball, soaked in sterilized H_2O and antibiotic treatment (at concentrations rates in Table 1) was added to each arena to maintain moisture levels and provide a water source.

Isopods or millipedes were assigned to one of four treatments: natural antibacterial streptomycin (n = 30), synthetic antibacterial sulfanilamide (n = 30), synthetic antifungal Captan (n = 30), or controls (sterile H_2O, n = 30). Isopods and millipedes were monitored daily for survival, which was determined visually by agitating the container and/or organism. If an organism was determined to be deceased, it was removed and placed in a sterilized container to confirm death. Due to the variability in response of millipedes and isopods, millipedes were kept in survival chambers for 6 weeks, while isopods were kept in survival chambers for 10 days.

An additional set of survival chambers (same conditions as described above) were created to monitor isopod and millipede growth. Organisms were assigned to one

of the four antibiotic treatments (n = 10 x treatment x organism). The weight of each individual was monitored ever 24-h (± 30 min), starting at the time of introduction, and ending after 7 days.

Due to the arboreal nesting behavior of termites, termite survival analysis consisted of 80 mm petri dishes filled with pre-weighed sterilized filter paper (cellulose). The filter paper was treated on the same schedule and in the same concentration as isopods and millipedes (n = 10 x treatment, Table 1). Environments were rehydrated every three days to maintain moisture. Due to the social nature of termites, we placed 30 individuals into each environment (10 soldiers and 20 workers). Survival was monitored daily over ten days. At the end of the survival trial, filter paper was gently washed, dried and weighed to determine mass loss. This provided a proxy measurement for consumption by termites in survival arenas.

Behavior Analysis

For behavior analysis, individual isopods and millipedes were placed in an 80 mm x 10 mm sterilized petri dishes. Each petri dish contained two cotton balls: one ball treated with streptomycin, sulfonamide, Captan, or sterile H_2O, and the other treated with sterile H_2O (Fig. 1b). We had 30 replicates per treatment per organism. Cotton was treated with the same dosage levels as survival experiments (Table 1). Cotton balls were placed on opposite sides of the arena, creating designated treatment and control sides (Fig. 1b). Preference trials began with placing millipedes or isopods in the center of the petri dish. Organisms were given 40 hours to acclimate to the environment. After the acclimation period, we recorded the location (treatment or control) of each organism at 8 hr intervals over a 32 hr period.

Termite preference arenas consisted of 20 cm x 10 cm x 4 cm containers with pre-weighed filter paper placed on each side (Fig. 1c). One side of the filter paper was treated with antibiotics or H_2O using the same dosages as the isopod and diplopod trials (Table 1). The other side of the arena was treated with H_2O. Each treatment had 10 replicates. Due to the social nature of termites, we placed 30 individuals into the center of each arena (10 workers and 20 soldiers). Termites were given 40 hours to acclimate. We then monitored which side of the environment each individual was on (treatment or control) every 8 hours for the subsequent 32 hours.

Survival, growth and behavioral statistical analysis

All survival, growth and behavior analyses were performed in R (R Core Team 2013). All variables were tested for normality via the Wilks-Shapiro test (Sokal and Rohlf 1981). To test for differences in survival across antibiotic treatments, isopods and millipedes were tested with the SurvDiff function, followed by a log-rank post-hoc test for pairwise differences, using the *survival* package (Therneau 2016). To control for random variation among arenas, termites were analyzed using Cox proportional hazard model (coxhp) in the *coxme* package (Therneau 2018). Because coxhp analysis does not allow for pair-wise comparisons, we tested for differences across treatments using the same log-rank post-hoc test described above. Kaplan-Meier survival curves were generated using the *survminer* package (Kassambara and Kosinski 2016). We used a one-way ANOVA and a post-hoc Tukey HSD, to test for differences in percent mass change in isopod and millipede growth in antibiotic environments. Differences in termite consumption of filter paper among antibiotic treatments were tested using a linear model and Tukey HSD. Behavioral analysis was performed using generalized

mixed-effect linear model and Tukey HSD, with dish and hour included as random effects and antibiotic treatment as a fixed effect, using the *lme4* package (Bates et al. 2014).

Microbiome Sampling

To examine the impact of antibiotic compounds on invertebrate microbiomes, we extracted the microbiome of the organisms used in our survival analysis. After 10 days, organisms raised in antibiotic laden environments were sampled (n = 3 x treatment x organism). All organisms were living at the time of sampling, to control for large shifts in microbial content post mortem (DeBruyn and Hauther 2017). Sampled organisms were surface sterilized by placing them in 95% ethanol for 2 mins, 5% bleach for 1 min and rinsed with sterile H_2O before DNA stabilization (Moreau 2014).

DNA extraction and stabilization was performed using the Xpedition Soil/Fecal DNA MiniPrep kit (Zymo Research, Irvine, CA, USA). The initial sampling step was modified to include ZR BashingBeads in sizes 0.1, 0.5 and 2 mm to ensure lysis of insect and microbial cells. All samples were ground and homogenized by bead-beating tubes at a 1000 rpms for 2 mins using a reciprocating saw with a tube adaptor.. Preserved field samples were stored at -40°C. Immediately prior to DNA extraction, samples were re-homogenized using a BioSpec© Mini-Beadbeater for 60 s. The remaining steps for DNA extraction followed the manufacturer's protocol (Zymo Research, Irvine, CA, USA).

Libraries of small-subunit (16S) rRNA gene fragments representative of bacterial phylotypes were generated from each DNA sample using the primers S-D-Arch-0519-a-S-15/S-D-Bact-0785-b-A-18 (Klindworth et al. 2013). The 50 µl PCR

reaction containing 2 µl of 1:10 diluted template DNA, 0.2 µM each of forward and reverse primer and 1 µM of 5 Prime Master Mix (5 PRIME) were carried out in a Techne TC-512 Gradient Thermal Cycler (Techne Inc., Burlington, NJ, USA). Initial denaturation was held at 96 °C for 3 min, followed by 30 cycles, each consisting of 96 °C for 30 s, 52 °C for 45 s, and 72 °C for 45 s. The final extension was held for 10 min at 75 °C. Appropriate PCR products were verified on 1% agarose gel. PCR products were purified using SPRIselect beads following the manufacturer's protocol (Beckman Coulter, Brea, CA, USA).

A unique 12 bp "barcode" was attached to each library using a subsequent 6 cycle PCR reaction. The attached forward primers consisted of a unique barcode, two spacer nucleotides and the 16 bp adapter sequence (GTAAAACGACGGCCAG); the reverse primer was S-D-Bact-0785-b-A-18. This unique "barcode" labeling reaction was a total of 50 µl and contained 4 µl of the purified PCR product, 0.2 µM each of forward and reverse primer, and 1 µM of 5 Prime Master Mix (5 PRIME). Six cycles of PCR thermal cycling were carried out in a Techne TC-512 Gradient Thermal Cycler, as described above. The resulting products were cleaned and concentrated using SequelPrep Normalization Plates according to manufacturer's protocol (ThermoFischer Scientific, Waltham, MA, USA). Samples were then quantified using the Qubit fluorometer and dsDNA HS assay kit (Life Technologies, Grand Island, NY, USA). Equimolar amounts of each uniquely barcoded PCR product were pooled and submitted for Illumina MiSeq using TruSeq 250 bp PE V2 chemistry. Our Illumina 16S data is deposited on Dryad (accession no. pending).

Microbial bioinformatics and statistical analyses

All 16S sequencing reads were analyzed and demultiplexed using QIIME (Caporaso et al. 2010). We removed sequencing reads that contained errors in the barcoded region, ambiguities, homopolymers (greater than 6 nucleotides in length), or an average quality score < 25. Primer sequences were trimmed, chimeric sequences were eliminated using USEARCH (version 6.1) and the "gold" reference database (Edgar 2010). Sequences were clustered into *de novo* operational taxonomic units (OTUs) at 97% similarity. Microbial taxonomic classification was assigned via the SILVA reference database (Quast et al. 2012) using the pyNAST aligner. Sequences that failed to align were excluded from subsequent analyses. Rarefaction curves were constructed from the estimated number of OTUs in each sample using observed species richness in QIIME (Hu et al. 2014). Libraries were rarefied to 2080 reads (the size of the smallest sequence library; Fig. S2).

Alpha and Shannon diversity were compared using a linear model in the statistical environment R (team 2013). We compared microbial community composition and dispersion across organism types and antibiotic treatments using PERMANOVA and PERMDISP in QIIME (1000 permutations). We used Bray-Curtis distance to estimate community similarity. We used a non-metric multidimensional scaling (NMDS) ordination to visualize relationships among microbial communities across organism and antibiotic treatments. We used QIIME to generate NMDS coordinates and then fit vectors (using *envfit* and linear models in R) of significant bacterial taxa (greater than 1% relative abundance) on this ordination using the *vegan* package (Oksanen et al. 2013). We confirmed normality both visually and with the Shapiro-Wilk test.

Results

Antibiotics impact invertebrate growth and survival of three litter invertebrates

Growth and survival generally decreased with antibiotic treatments, but these effects varied among taxa. Isopods had lower survival rates with all three antibiotics relative to control groups (generalized linear model [GLM]: $\chi^2_3 = 428$, $P < 0.001$; Fig. 2). The fungicide had the weakest effect and the bactericides, especially the synthetic, were most lethal (log-rank post hoc test: $P < 0.001$; Fig. 2). However, isopods only lost body mass when exposed to synthetic antibacterial (sulfanilamide) environments (ANOVA: $F_{3,36} = 6.34$, $P < 0.001$; Fig. 3).

Millipede survival decreased with all three antibiotic treatments, but to a much small extent than the isopods (GLM: $\chi^2_3 = 10.5$, $P < 0.01$; Fig. 2). Differences across treatments were only marginally significant with a conservative post-hoc test (log-rank post hoc test: $P \geq 0.06$; Fig. 2) and millipede growth did not differ with antibiotic treatment (ANOVA: $F_{3,36} = 3.41$, $P > 0.05$; Fig. 3).

Termite survival decreased with the antifungal (Captan) and synthetic antibacterial (sulfanilamide) treatments (GLM: $\chi^2_4 = 627.26$, $P < 0.001$; Fig. 2), but the natural antibacterial had no effect on termite mortality. This response was consistent when we analyzed workers (GLM: $\chi^2_4 = 408.46$, $P < 0.001$; Fig. S3) and soldiers separately (GLM: $\chi^2_4 = 290.06$, $P < 0.001$; Fig. S3). Whereas we did not compare mass loss for termites (see Methods), termite consumption of cellulose increased with natural antibacterial treatment (LM: $F_{3,56} = 21.84$, $P = 0.003$; Fig. 4), but decreased with the synthetic antibacterial (LM: $F_{3,56} = 21.84$, $P < 0.001$; Fig. 4).

Antibiotics elicit avoidance in isopods, but not millipedes with termites in between

Isopods demonstrated unambiguous avoidance of areas with antibiotic activity (dAIC = 10.4, χ^2_3 = 15.61, P = 0.001; Fig. 5). Millipedes, by contrast, did not avoid antibiotics, regardless of the treatment (dAIC = 3.17, χ^2_3 = 2.83, P = 0.42; Fig. 5). Termites avoided areas treated with antifungals, but not antibacterials (dAIC = 20.6, χ^2_3 = 26.64, P < 0.001; Fig. 5). Avoidance of antifungal environments was consistent when we split the response between soldiers (dAIC = 17.8, χ^2_3 = 23.80, P < 0.001; Fig. S4) and workers (dAIC = 17.8, χ^2_3 = 23.80, P < 0.001; Fig. S4), although workers avoided the natural antibacterial and anti-fungal similarly (Fig. S4).

Millipedes, isopods, and termites have distinct microbiomes but none change with antibiotic treatment

Contrary to predictions, isopod, millipede and termite microbiome compositions generally were not altered by exposure to antibiotic treatments (PERMANOVA: $F_{4,11}$ = 0.94, P = 0.61, Fig. S6).The alpha diversity (observed OTUs) of isopods was 40% lower in sulfanilamide environments, as compared to controls ($F_{11,23}$ = 3.54, P = 0.04). Also, isopods raised in streptomycin environments had higher levels of Planctomycetacia compared to isopods raised in sulfanilamide environments ($F_{3,7}$ = 4.95, P < 0.042), though the relative abundance of Planctomycetacia was very low overall. All other classes and orders did not vary across treatments in isopod, millipede and termite microbiomes.

Of the three invertebrates, millipedes hosted the most diverse microbiomes. Alpha diversity (OTUs per individual host) varied (LM: $F_{2,32}$ = 7.09, P = 0.002), with millipedes hosting 31.6% more observed OTUs than termites (P = 0.01) and 15.2% more than isopods (P = 0.09). Millipedes also had higher Shannon diversity (combining

alpha diversity and evenness in relative abundance) than termites and isopods (LM: $F_{2,32} = 24.77$, $P < 0.001$; Tukey pair-wise $P < 0.001$). Beta diversity (within-group compositional dissimilarity) also varied among host taxa (PERMDISP: $F_{2,32} = 23.05$, $P < 0.001$, Fig. 6). Millipedes had the highest levels of beta diversity, followed by isopods, and then termites.

Each host had a unique, easily distinguished microbiome (PERMANOVA: $F_{3,35} = 20.87$, $P = 0.001$; Fig. 6) defined by specific classes of bacteria (Fig. S5). Termite microbiomes were unique in their high levels of Spirochaetea, whereas isopods were dominated by Proteobacteria (Fig. S5). *Wolbachia*, a common invertebrate endosymbiont, was not present in millipedes or isopods, and was only present in low levels in termites.

Discussion

In the detrital, or "brown" food web, falling fruits, leaves, and carcasses are quickly found, decomposed, and depleted. Microbial taxa that colonize these substrates can render them unpalatable to animals that also seek them for food (Janzen 1977, Burkepile et al. 2006). Here we develop and test a mechanism for this "spoilage": antibiotics that kill microbes in the litter also deter saprotrophs (that are themselves hosts to rich microbiomes). We show that some saprotrophic hosts suffer mortality when exposed to antibiotic-laced substrate. Moreover, as would be predicted by a frequent and potent inter-population interaction, antibiotics can elicit a strong avoidance response. At the same time, we document new and intriguing variation amongst three common saprotrophic taxa in patterns of mortality and avoidance.

Like the plant-based chemical defenses that are detected and avoided by herbivores (Bernays and Chapman 1977, Bernays and Chapman 1994, Rasmann et al. 2012, Rosenthal and Berenbaum 2012), a microbe's antibiotics serve best when they are detected by a hungry saprotroph and prevent it from eating the resource on which the microbe resides (Burkepile et al. 2006, Rozen et al. 2008). Moreover in plant-herbivore systems, the most susceptible herbivores are most likely to avoid plant toxins (Freeland and Janzen 1974, Molyneux and Ralphs 1992). Here we show a similar gradient of avoidance associated with susceptibility to antibiotics: avoidance of antibiotics by a taxon was roughly equivalent to its measured impact. Isopods that experienced the highest mortality rates also show the greatest avoidance; millipedes, the least effected of the three, did not. This has two implications. First, the upregulation of antibiotic production following microbial colonization may help generate the notoriously patchy nature of leaf litter invertebrate communities (Olson 1994, Shik and Kaspari 2010), as antibiotics "herd" invertebrates towards less defended patches. More generally, the similarity in function and effects of plant defenses like tannins and alkaloids, to microbial defenses like streptomycin, suggests a rich opportunity for collaboration and theoretical exchange between scholars of green and brown food webs.

Antibiotics and the life history of litter invertebrates

The contrasting responses of millipedes and isopods to antibiotics may reflect a larger life history gradient between these two dominant groups. Millipedes trend towards a K-selected life history (MacArthur and Wilson 1967), typically producing low numbers of brood that can take 1-2 years to mature (Hopkin and Read 1992). Comparatively, millipedes are slow moving organisms that rely on defensive

compounds rather than flight when confronted with competitors (Kime and Golovatch 2000, Billah et al. 2015, Stanković et al. 2016). Their lack of rapid mobility may restrict their diet to resources in the immediate vicinity, which can include toxic and difficult to digest materials. To combat this, millipedes are capable of digesting a vast array of compounds (Hopkin and Read 1992, Ashwini and Sridhar 2006). They have also evolved an ability to extract volatiles from plant material that they repurpose for their own defensive compounds (Meinwald et al. 1975, Clark et al. 2005, *but see* Shear 2015). Whether millipedes are capable of extracting and repurposing microbe-produced antibiotic compounds remains underexplored (Omura 2002). Their relatively sedentary nature combined with their advanced ability to digest and reuse complex compounds suggests that millipede may be evolved to neutralize rather than avoid antibiotic compounds.

Isopods by contrast were strongly impacted by antibiotic compounds and avoided all antibiotic-laden environments. Isopods produce larger brood sizes and typically have high infant mortality rates (Kight 2009), reflecting an r-selected life history (MacArthur and Wilson 1967). Furthermore, isopods are agile invertebrates that flee or hide when confronted with competitors (Hegarty and Kight 2014). They can move long distances to find high quality resources (Paris and Pitelka 1962) with the help of advanced chemoreceptors (Hassall and Rushton 1984, Zimmer 2002, Loureiro et al. 2005). The avoidance demonstrated by isopods in our study suggests that chemoreceptors used to identify food resources are also useful for escaping lethal compounds.

Contrary to our predictions, the microbiome of each invertebrate examined was resilient to the introduction of antibiotics. This suggests that disruption of endosymbionts may not be the mechanism used by antibiotics to impact animal competitors. Zimmer (1999) demonstrated that isopod microbiomes were relatively stable after a course of antibiotics, while Reyes and Tiejde (1976) used antibiotics to reduce microbiome bacteria. Eutick et al. (1978) found that termite microbiomes had mixed responses to difference antibiotic treatments. Alternatively, we may have failed to capture fatal changes in microbiomes. We only sampled invertebrate microbiomes from organisms that were living in order to control for microbiome turnover post-mortem (DeBruyn and Hauther 2017). Therefore, the microbiomes reported here may only represent those of particularly resilient individuals. Similarly, we did not measure levels of bacterial abundance. The antibiotic compounds tested were broad spectrum and therefore may have reduced overall microbial abundance without targeting specific taxa. Additional studies that capture microbiomes at the immediate time of death and account for true abundance levels are required to clarify the impact of antibiotics on endosymbionts.

Invertebrates were harmed by and avoided all three tested antibiotic compounds. This result is of particular concern when we consider that non-natural production and use of antibiotics are at historically high levels (Van Boeckel et al. 2015). Specifically, agricultural systems are the largest consumers of antibiotics, accounting for roughly 80% of antibiotic use in the USA (Sarmah et al. 2006, USDA 2012). When animal agriculture industries use antibiotics, active compounds are introduced into the environment through animal by-products, leading to increased levels of antibiotic

resistance (Kumar et al. 2005, Wepking et al. 2017). Our results suggest that the impact of these compounds may also lead to compositional shifts in native invertebrate communities. If native invertebrate communities are disrupted, we predict that the ecosystem processes they control (i.e. decomposition) will suffer as well (Vasconcelos and Laurance 2005).

In summary, we find that antibiotic compounds, traditionally considered as weapons for inter-microbial competition, also create a significant burden on invertebrates that live in and consume ephemeral resources. Survival rates of invertebrates were decreased in the presence of these compounds, leading isopods and termites to avoided areas of antibiotic activity. This response likely represents a long history of co-evolution between detritivores (and their microbiomes) and free-living microbes, one that may be profitably informed by comparisons to co-evolutionary races between plants and their herbivores. Our results add substantial support for Janzen's (1977) microbe-mediated animal deterrence hypothesis, and open up new opportunities for population interactions within the brown food web.

Acknowledgements

We thank our REUs Carolyn Gigot, Kate Henderson and Annika Salzberg for their work field and patience in rearing invertebrates. We also thank Evan Gora for his assistance in project design and comments on previous versions of the manuscript. We thank the Stevenson Lab at OU for providing laboratory support and OMRF for their analysis of microbial samples. In particular we thank Brian Bill, Emily Junkins and Heather Nunn for their advice throughout the microbial sequencing process. We also

thank the staff of Barro Colorado Island, Panama for logistical support during the field portion of this project. This work was funded by the National Science Foundation's Graduate Research Fellowship Program and the Smithsonian Tropical Research Institute's Short-term Fellowship to JML.

References

Abrudan, M. I., F. Smakman, A. J. Grimbergen, S. Westhoff, E. L. Miller, G. P. van Wezel, and D. E. Rozen. 2015. Socially mediated induction and suppression of antibiosis during bacterial coexistence. Proceedings of the National Academy of Sciences 112:11054-11059.

Anderson, J., and D. E. Bignell. 1980. Bacteria in the food, gut contents and faeces of the litter-feeding millipede *Glomeris marginata* (Villers). Soil Biology and Biochemistry 12:251-254.

Ashwini, K., and K. Sridhar. 2006. Breakdown of plantation residues by pill millipedes (*Arthrosphaera magna*) and assessment of compost quality. Current Science 954-959.

Bates, D., M. Maechler, B. Bolker, S. Walker, R. H. B. Christensen, H. Singmann, B. Dai, and G. Grothendieck. 2014. Package 'lme4'. R foundation for statistical computing, Vienna 12.

Bernays, E., and R. Chapman. 1977. Deterrent chemicals as a basis of oligophagy in *Locusta migratoria* (L.). Ecological Entomology 2:1-18.

Bernays, E., and R. Chapman. 1994. Behavior: the process of host-plant selection. Host-plant selection by phytophagous insects 95-165.

Billah, M. K., D. Kwang, C. Adofo, M. A. Olu-Taiwo, and G. A. Pesewu. 2015. Antibacterial activities of millipede extracts against selected bacterial pathogens. Journal of Microbiology and Antimicrobial Agents.

Bouchon, D., M. Zimmer, and J. Dittmer. 2016. The terrestrial isopod microbiome: An all-in-one toolbox for animal–microbe interactions of ecological relevance. Frontiers in microbiology 7:1472.

Bunyan, J., L. Jeffries, J. R. Sayers, A. Gulliver, and K. Coleman. 1977. Antimicrobial substances and chick growth promotion: The growth-promoting activities of antimicrobial substances, including fifty-two used either in therapy or as dietary additives. British Poultry Science 18:283-294.

Burkepile, D. E., J. D. Parker, C. B. Woodson, H. J. Mills, J. Kubanek, P. A. Sobecky, and M. E. Hay. 2006. Chemically mediated competition between microbes and animals: microbes as consumers in food webs. Ecology 87:2821-2831.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, and J. I. Gordon. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature methods 7:335.

Clark, V. C., C. J. Raxworthy, V. Rakotomalala, P. Sierwald, and B. L. Fisher. 2005. Convergent evolution of chemical defense in poison frogs and arthropod prey between Madagascar and the Neotropics. Proceedings of the National Academy of Sciences of the United States of America 102:11617-11622.

Colinas, C., E. Ingham, and R. Molina. 1994. Population responses of target and non-target forest soil organisms to selected biocides. Soil Biology and Biochemistry 26:41-47.

Crowther, T. W., S. M. Thomas, D. S. Maynard, P. Baldrian, K. Covey, S. D. Frey, L. T. van Diepen, and M. A. Bradford. 2015. Biotic interactions mediate soil

microbial feedbacks to climate change. Proceedings of the National Academy of Sciences 112:7033-7038.

Darwin, C. 1859. On the origin of the species by natural selection. London: J. Murray.

DeBruyn, J. M., and K. A. Hauther. 2017. Postmortem succession of gut microbial communities in deceased human subjects. PeerJ 5:e3437.

DeVault, T. L., O. E. Rhodes Jr, and J. A. Shivik. 2003. Scavenging by vertebrates: behavioral, ecological, and evolutionary perspectives on an important energy transfer pathway in terrestrial ecosystems. Oikos 102:225-234.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460-2461.

Eutick, M., P. Veivers, R. O'brien, and M. Slaytor. 1978. Dependence of the higher termite, *Nasutitermes exitiosus* and the lower termite, *Coptotermes lacteus* on their gut flora. Journal of Insect Physiology 24:363-368.

Freeland, W. J., and D. H. Janzen. 1974. Strategies in herbivory by mammals: the role of plant secondary compounds. The American Naturalist 108:269-289.

Gilchrist, M. J., C. Greko, D. B. Wallinga, G. W. Beran, D. G. Riley, and P. S. Thorne. 2007. The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. Environmental health perspectives 115:313.

Hammer, T. J., D. H. Janzen, W. Hallwachs, S. P. Jaffe, and N. Fierer. 2017. Caterpillars lack a resident gut microbiome. Proceedings of the National Academy of Sciences 201707186.

Hassall, M., and S. Rushton. 1984. Feeding behaviour of terrestrial isopods in relation to plant defences and microbial activity. Pages 487-505 *in* Symposia of the Zoological Society of London. Cambridge University Press. Cambridge, UK.

Hegarty, K. G., and S. L. Kight. 2014. Do predator cues influence turn alternation behavior in terrestrial isopods *Porcellio laevis* Latreille and *Armadillidium vulgare* Latreille? Behavioural processes 106:168-171.

Hochberg, M. E., and J. H. Lawton. 1990. Competition between kingdoms. Trends in ecology & evolution 5:367-371.

Hopkin, S. P., and H. J. Read. 1992. The biology of millipedes. Oxford University Press. Okford, UK.

Hu, Y., P. Łukasik, C. S. Moreau, and J. A. Russell. 2014. Correlates of gut community composition across an ant species (*Cephalotes varians*) elucidate causes and consequences of symbiotic variability. Molecular Ecology 23:1284-1300.

Huis in't Veld, J. H. 1996. Microbial and biochemical spoilage of foods: an overview. International Journal of Food Microbiology 33:1-18.

Janzen, D. H. 1977. Why fruits rot, seeds mold, and meat spoils. The American Naturalist 111:691-713.

Jouquet, P., J. Dauber, J. Lagerlöf, P. Lavelle, and M. Lepage. 2006. Soil invertebrates as ecosystem engineers: intended and accidental effects on soil and feedback loops. Applied Soil Ecology 32:153-164.

Karagkouni, M., S. Sfenthourakis, A. Feldman, and S. Meiri. 2016. Biogeography of body size in terrestrial isopods (Crustacea: Oniscidea). Journal of Zoological Systematics and Evolutionary Research 54:182-188.

Kassambara, A., and M. Kosinski. 2016. Survminer: drawing survival curves using 'ggplot2'. 2016. R package version 0.3 1.

Kight, S. L. 2009. Reproductive ecology of terrestrial isopods (Crustacea: Oniscidea). Terrestrial Arthropod Reviews 1:95-110.

Kim, S., P. A. Thiessen, E. E. Bolton, J. Chen, G. Fu, A. Gindulyte, L. Han, J. He, S. He, and B. A. Shoemaker. 2015. PubChem substance and compound databases. Nucleic acids research 44:D1202-D1213.

Kime, R. D., and S. I. Golovatch. 2000. Trends in the ecological strategies and evolution of millipedes (Diplopoda). Biological Journal of the Linnean Society 69:333-349.

Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F. O. Glöckner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research 41.

Kumar, K., S. Gupta, S. Baidoo, Y. Chander, and C. Rosen. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. Journal of environmental quality 34:2082-2085.

Loureiro, S., A. M. Soares, and A. J. Nogueira. 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environmental pollution 138:121-131.

Lussenhop, J. 1992. Mechanisms of microarthropod-microbial interactions in soil. Advances in ecological research 1-33.

MacArthur, R. H. 1958. Population ecology of some warblers of northeastern coniferous forests. Ecology 39:599-619.

MacArthur, R. H., and E. O. Wilson. 1967. The theory of island biography. Princeton University Press. Princeton, New Jersey, USA.

Mans, D. R. 2017. Exploring the global animal biodiversity in the search for new drugs–Spiders, scorpions, horseshoe crabs, sea spiders, centipedes, and millipedes. Journal of Translational Science 3.

Maraun, M., H. Martens, S. Migge, A. Theenhaus, and S. Scheu. 2003. Adding to 'the enigma of soil animal diversity': fungal feeders and saprophagous soil invertebrates prefer similar food substrates. European Journal of Soil Biology 39:85-95.

Matsuura, K. 2001. Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos 92:20-26.

Meinwald, J., J. Smolanoff, A. McPhail, R. Miller, T. Eisner, and K. Hicks. 1975. Nitropolyzonamine: a spirocyclic nitro compound from the defensive glands of a millipede (*Polyzonium rosalbum*). Tetrahedron Letters 16:2367-2370.

Molyneux, R. J., and M. H. Ralphs. 1992. Plant toxins and palatability to herbivores. Journal of Range Management 13-18.

Moreau, C. S. 2014. A practical guide to DNA extraction, PCR, and gene-based DNA sequencing in insects. Halteres 5:32-42.

Nardi, J. B., C. M. Bee, and S. J. Taylor. 2016. Compartmentalization of microbial communities that inhabit the hindguts of millipedes. Arthropod structure & development 45:462-474.

Nauseef, W. M. 2007. How human neutrophils kill and degrade microbes: an integrated view. Immunological reviews 219:88-102.

Nottebrock, H., B. Schmid, M. Treurnicht, J. Pagel, K. J. Esler, K. Böhning-Gaese, M. Schleuning, and F. M. Schurr. 2017. Coexistence of plant species in a biodiversity hotspot is stabilized by competition but not by seed predation. Oikos 126.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. O'hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2013. Package 'vegan'. Community ecology package, version 2.

Olson, D. M. 1994. The distribution of leaf litter invertebrates along a Neotropical altitudinal gradient. Journal of tropical Ecology 10:129-150.

Omura, S. 2002. Macrolide antibiotics: chemistry, biology, and practice.

Paris, O. H., and F. A. Pitelka. 1962. Population characteristics of the terrestrial isopod Armadillidium vulgare in California grassland. Ecology 43:229-248.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research 41:D590-D596.

R Core Team. 2013. R: A language and environment for statistical computing. R A Language Environment for Statistical Computing 55:275-286.

Rasmann, S., M. De Vos, C. L. Casteel, D. Tian, R. Halitschke, J. Y. Sun, A. A. Agrawal, G. W. Felton, and G. Jander. 2012. Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiology 158:854-863.

Raymann, K., L. M. Bobay, and N. A. Moran. 2017. Antibiotics reduce genetic diversity of core species in the honeybee gut microbiome. Molecular Ecology.

Reyes, V. G., and J. M. Tiedje. 1976. Ecology of the gut microbiota of *Tracheoniscus rathkei* (Crustacea, Isopoda). Pedobiologia.

Rosenthal, G. A., and M. R. Berenbaum. 2012. Herbivores: their interactions with secondary plant metabolites: ecological and evolutionary processes. Academic Press.

Rozen, D., D. Engelmoer, and P. Smiseth. 2008. Antimicrobial strategies in burying beetles breeding on carrion. Proceedings of the National Academy of Sciences 105:17890-17895.

Sarmah, A. K., M. T. Meyer, and A. B. Boxall. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65:725-759.

Shear, W. A. 2015. The chemical defenses of millipedes (Diplopoda): biochemistry, physiology and ecology. Biochemical Systematics and Ecology 61:78-117.

Shik, J. Z., and M. Kaspari. 2010. More food, less habitat: how necromass and leaf litter decomposition combine to regulate a litter ant community. Ecological Entomology 35:158-165.

Sokal, R., and F. Rohlf. 1981. 198 1. Biometry. Freeman San Francisco.

Stanković, S., I. Dimkić, L. Vujisić, S. Pavković-Lučić, Z. Jovanović, T. Stević, I. Sofrenić, B. Mitić, and V. Tomić. 2016. Chemical defence in a millipede: evaluation and characterization of antimicrobial activity of the defensive

secretion from *Pachyiulus hungaricus* (Karsch, 1881)(Diplopoda, Julida, Julidae). PloS one 11:e0167249.

Sugimoto, A., D. E. Bignell, and J. A. MacDonald. 2000. Global impact of termites on the carbon cycle and atmospheric trace gases. Pages 409-435 Termites: evolution, sociality, symbioses, ecology. Springer.

Therneau, T. 2016. A Package for Survival Analysis in S. version 2.38. 2015. Reference Source.

Therneau, T. 2018. Coxme and the Laplace Approximation.

USDA. 2012. Estimates of antibacterial drug sales in human medicine.

Van Boeckel, T. P., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences 112:5649-5654.

Vasconcelos, H. L., and W. F. Laurance. 2005. Influence of habitat, litter type, and soil invertebrates on leaf-litter decomposition in a fragmented Amazonian landscape. Oecologia 144:456-462.

Waidele, L., J. Korb, C. R. Voolstra, S. Künzel, F. Dedeine, and F. Staubach. 2017. Differential ecological specificity of protist and bacterial microbiomes across a set of termite species. Frontiers in microbiology 8.

Walsh, C. 2003. Antibiotics: actions, origins, resistance. American Society for Microbiology (ASM).

Wepking, C., B. Avera, B. Badgley, J. E. Barrett, J. Franklin, K. F. Knowlton, P. P. Ray, C. Smitherman, and M. S. Strickland. 2017. Exposure to dairy manure leads to greater antibiotic resistance and increased mass-specific respiration in

soil microbial communities. Page 20162233 *in* Proceeding of the Royal Society B.

Wieder, R. K., and S. J. Wright. 1995. Tropical forest litter dynamics and dry season irrigation on Barro Colorado Island, Panama. Ecology 76:1971-1979.

Wilkinson, T. 1998. The elimination of intracellular microorganisms from insects: an analysis of antibiotic-treatment in the pea aphid (*Acyrthosiphon pisum*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 119:871-881.

Wooten, R., and C. Crawford. 1975. Food, ingestion rates, and assimilation in the desert millipede *Orthoporus ornatus* (Girard)(Diplopoda). Oecologia 20:231-236.

Zimmer, M. 1999. The fate and effects of ingested hydrolyzable tannins in *Porcellio scaber*. Journal of Chemical Ecology 25:611-628.

Zimmer, M. 2002. Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-ecological approach. Biological Reviews 77:455-493.

Tables

Table 1: Measurements of antibiotic dosages.

Antibiotic	Target Group	Source	Mechanism of Action	Dosage Level
Streptomycin	Bacteria	Naturally Derived	Protein synthesis inhibitor, broad spectrum, bactericidal	1mg/g
Sulfanilamide	Bacteria	Synthetic	Inhibits enzyme activity, broad spectrum, bacteriostatic	1mg/g
Captan	Fungi	Synthetic	Inhibits fungal respiration, broad spectrum	25ug/g

Figure Legends

Fig 1 Example of a survival chamber (a), a preference chamber for millipedes and isopods (b) and a preference chambers for termites (c).

Fig 2 Survival probabilities of isopods, millipede and termites over time after antibiotic exposure, shown as Kaplan—Meier survival curves. The y-axis is zoomed in for millipedes, and the x-axis is in weeks, due to the low level of impact antibiotics had on their survival.

Fig. 3 Change in mass of isopods (■) and millipedes (∇). Change in mass is depicted as the proportion of initial weight remaining after 1 week in antibiotic laden environments. Error bars represent standard deviations.

Fig. 4 Amount of cellulose consumed by termites in survival chambers after 10 days. Cellulose consumed is measured as the percent of initial cellulose mass lost over 10 days of feeding by termites. Letters denote significant differences among treatments; error bars represent standard deviations.

Fig 5 Preferences of invertebrates when given a choice of control versus antibiotic-laden environments over a 32 hour period. Preference was determined as proportion of measured individuals on control versus treated environments. Gray boxes represent a preference of antibiotic-laden arenas, while white boxes demonstrate a preference for control environments. Letters denote significant differences in preference between treatments.

Fig. 6 NMDS ordination of bacterial communities across organisms using Bray-Curtis distances. Sample types differ significantly from each other (PERMANOVA: $F_{3,35}$ = 20.87, P = 0.001, stress = 0.02). Vectors represent the significant bacterial phyla (P < 0.05) driving the separation between each of the three organism types.

Figure 1

Figure 2

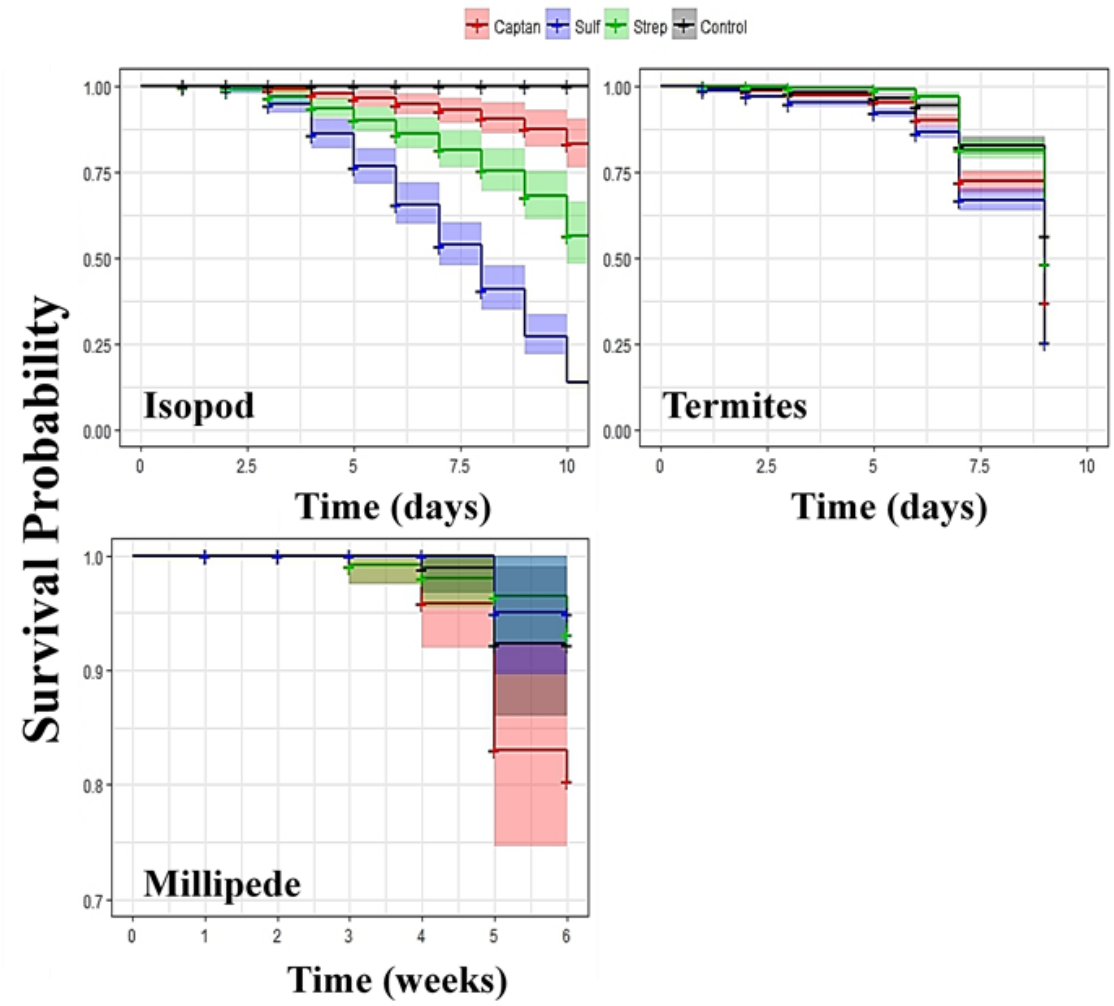

Captan
Sulf
Strep
Control
Survival Probability
Isopod
Termites
Millipede
Time (days)
Time (days)
Time (weeks)

Figure 3

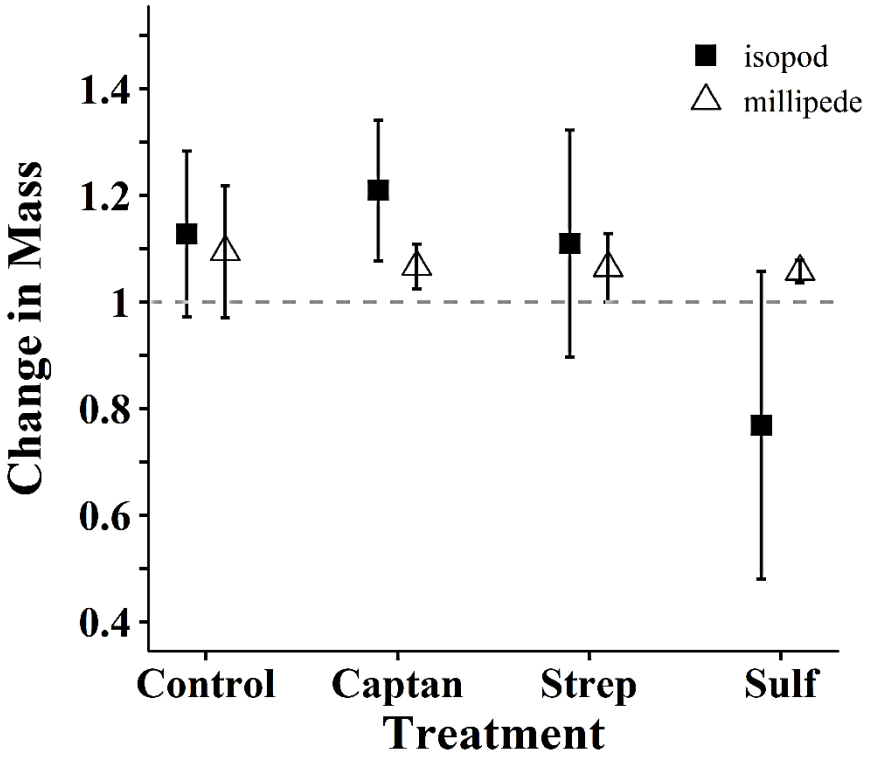

isopod
millipede
Change in Mass
1.4
1.2
1
0.8
0.6
0.4
Control
Captan
Strep
Sulf
Treatment

Figure 4

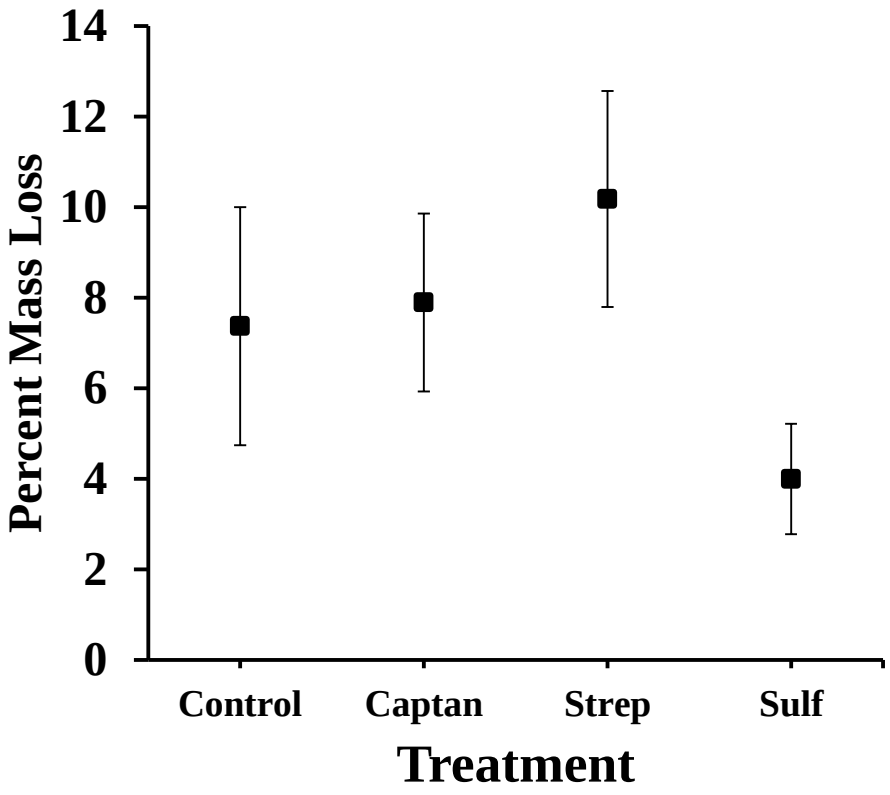

14
12
10
8
6
4
2
0
Percent Mass Loss
Control
Captan
Strep
Sulf
Treatment

Figure 5

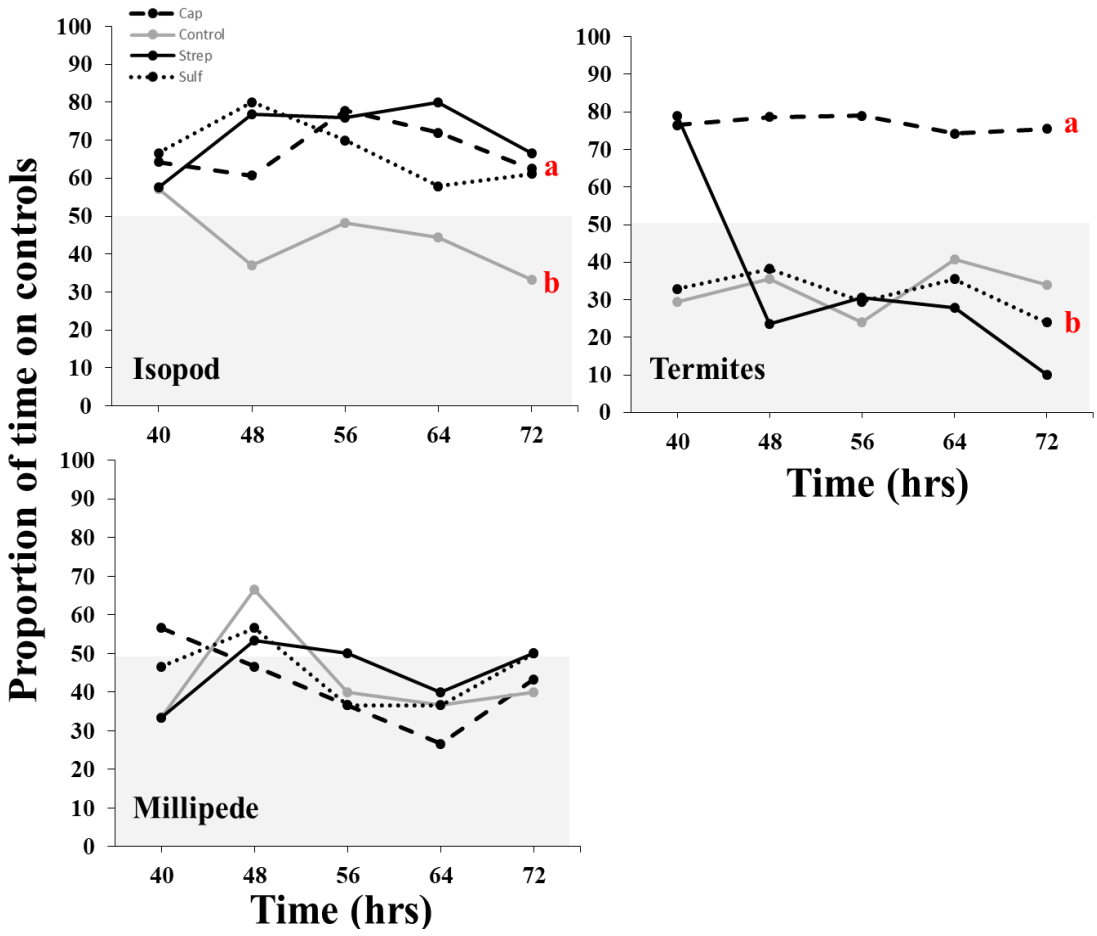
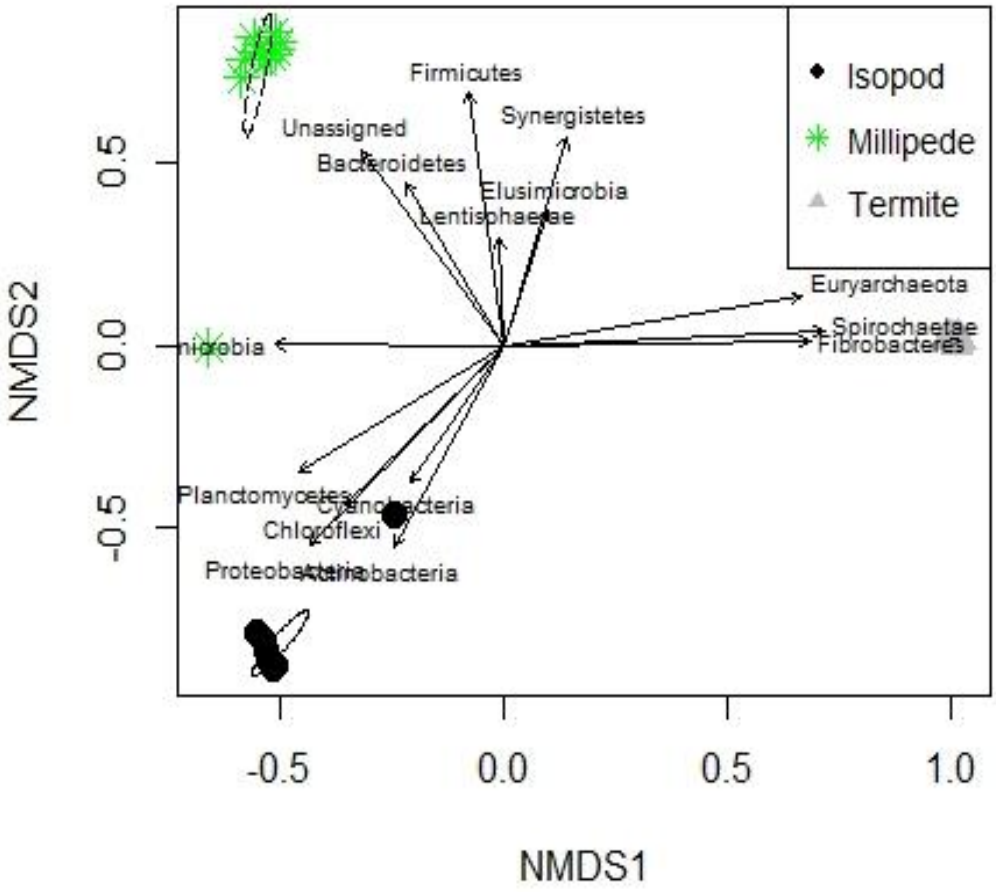

Cap
Control
Strep
Sulf
Proportion of time on controls
Isopod
Termites
Millipede
a
b
Time (hrs)
0
10
20
30
40
50
60
70
80
90
100
40
48
56
64
72

Figure 6


Isopod
Millipede
Termite
Firmicutes
Synergistetes
Unassigned
Bacteroidetes
Elusimicrobia
Lentisphaerae
Euryarchaeota
Spirochaetae
Fibrobacteres
Planctomycetes
Cyanobacteria
Chloroflexi
Proteobacteria
Actinobacteria
NMDS1
NMDS2
-0.5
0.0
0.5
1.0
-0.5
0.0
0.5

Appendix to:

Lucas, J and M. Kaspari. Antimicrobials as chemical warfare against detritivorous invertebrates.

Supplementary Figures

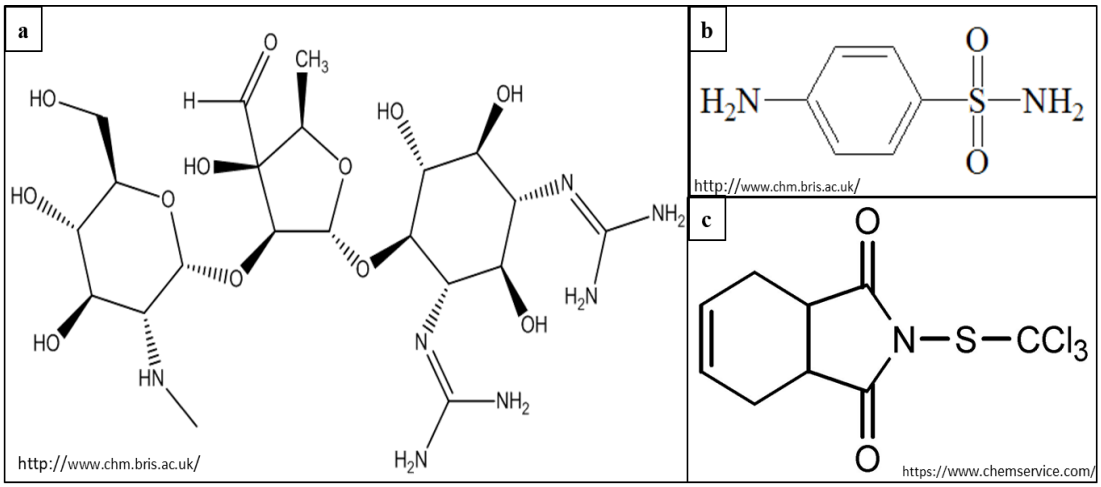


Fig. S1 Chemical structure of antibiotics streptomycin (a), sulfanilamide (b), and Captan (c).

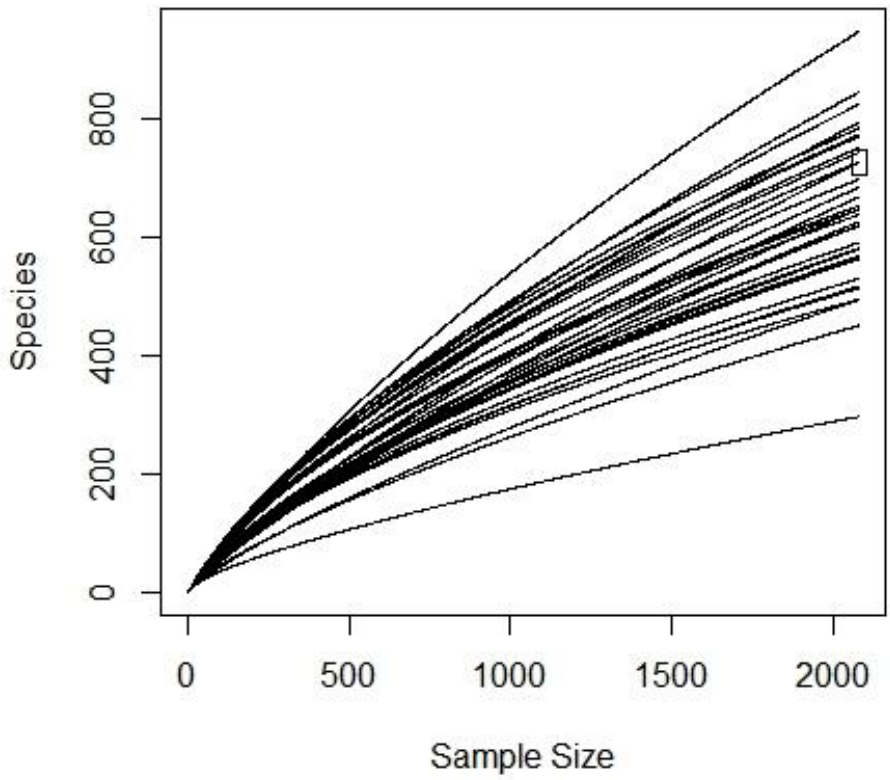


Fig. S2 Rarefaction curve demonstrating number of observed OTUs in each microbiome sample. The x-axis shows the bacterial OTUs observed and the number of sequences per sample is shown on the y-axis. Note that although sequencing covers thousands of Illumina reads, some samples have not reached the plateau.

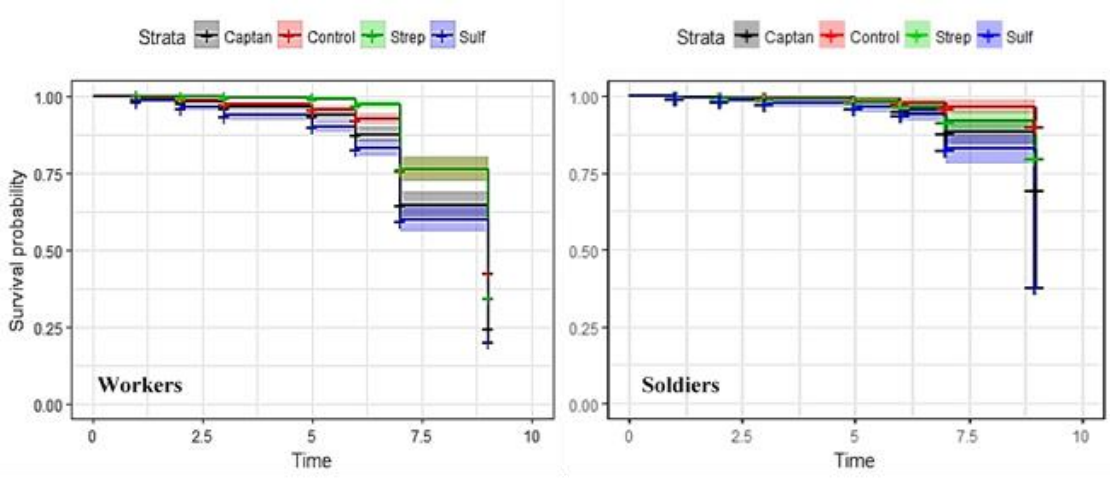


Fig. S3 Survival probabilities of termites split between workers and soldiers over time after antibiotic exposure. Survival probabilities are shown as Kaplan—Meier survival curves

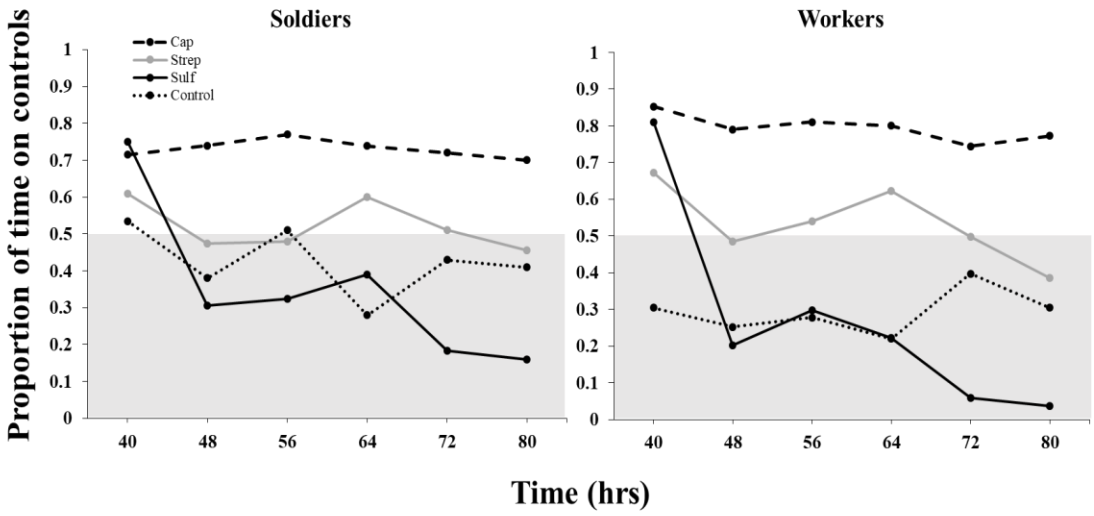


Fig. S4 Preferences of termite workers and soldiers when given a choice of control versus antibiotic-laden environments over a 32 hour period. Preference was determined as proportion of measured individuals on control versus treated environments. Gray boxes represent a preference of antibiotic-laden arenas, while white boxes demonstrate a preference for control environments.

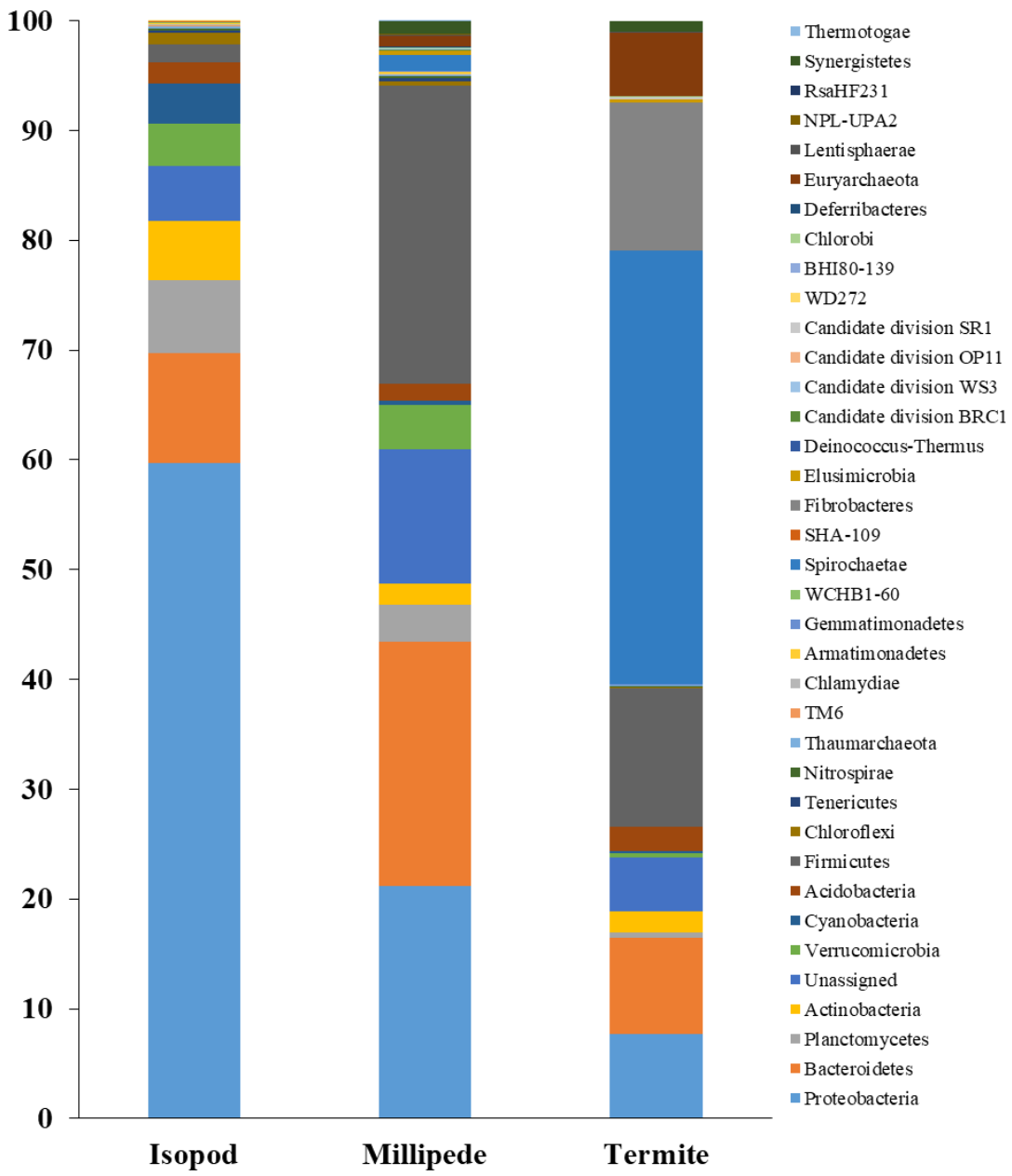


Fig S5 Average relative abundance of bacterial phyla found across organism microbiomes. Community composition differed between organisms (PERMANOVA: $F_{3,35} = 20.87$, $P = 0.001$).

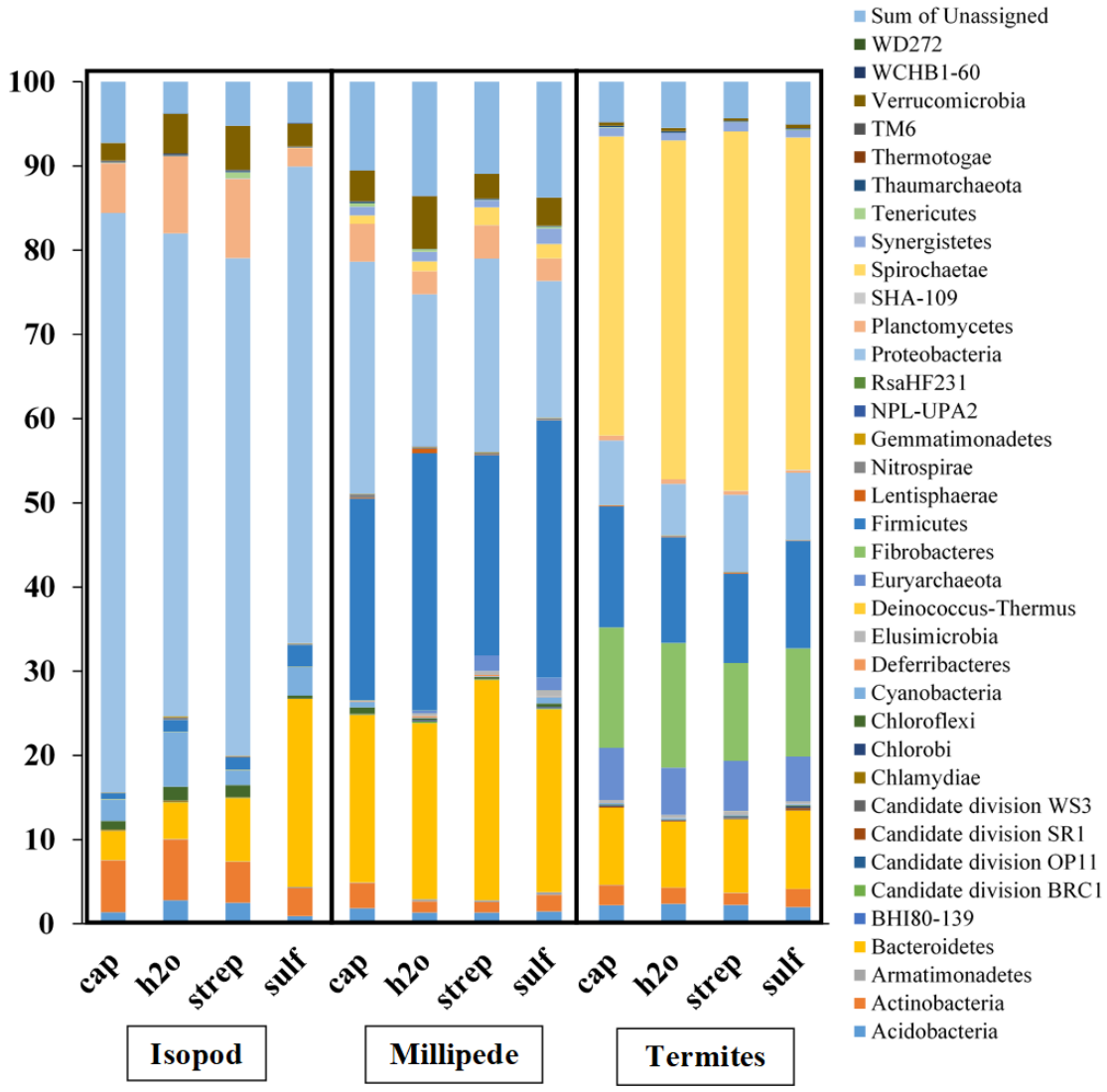


Fig S6 Relative abundance of bacterial taxa in each organism raised in antibiotic laden environments. Community composition within each organism across antibiotic treatments did not differ.