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Los Angeles

Examining host-microbe interactions across multiple scales of organization

A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy in Biology

by

Denisse Alejandra Gamboa

2025

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2025

ABSTRACT OF THE DISSERTATION

Examining host-microbe interactions across multiple scales of organization

by

Denisse Alejandra Gamboa

Doctor of Philosophy in Biology

University of California, Los Angeles, 2025

Professor Noa Pinter-Wollman, Chair

Host-associated microbes impact individual host fitness, and it is therefore important to uncover the drivers of variation among hosts and their associated microbes. Animal microbiome composition and diversity is impacted by factors, including social interactions, phylogeny, and the environment. Although, all animals harbor microbes, delineating the intricate relationship of host-microbe interactions requires an expanded ecological perspective involving host behavior, evolutionary history, life stage, and the physical environment. To determine the relationship between eusocial colonies of harvester ants and their associated microbes we used a combination of observational, manipulative, and experimental approaches. We analyzed all harvester ant associated microbes using 16S rRNA gene sequencing. First, we ask whether reproductive role or behavioral task is more important in determining the composition and diversity of host-associated microbes in harvester ants (*Veromessor andrei).* We found that reproductive role was a better predictor of bacterial communities than behavioral task. Second, to

test if social interactions impact microbial communities, we experimentally manipulated the ability of ants to interact with one another. To examine the impact of the environment, we compared the microbiome of ants from a natural setting to that of captive lab ants. To examine the influence of evolutionary history, we compared the microbial communities of three harvester ant species, *Pogonomyrmex rugosus, P. californicus,* and *V. pergandei,* which differ in phylogeny. We found that the environment and phylogeny explained differences in microbial communities. However, social interactions did not impact microbial composition or diversity. Lastly, to determine how the physical environment and social organization affect the microbiome, we examined the bacterial communities of *V. andrei* nests. We collected samples across ants, brood, seeds, reproductives, and soil (from inside and outside the nest, and from different chamber types). We found that both the environment and social organization impact the bacterial communities of ant colonies. Collectively, this work 1) suggests that individual morphological differences are a better predictor for microbial community composition, 2) highlights the importance of examining multiple drivers of microbiome composition and diversity and 3) underscores the importance of considering environmental and social factors to understanding microbiome dynamics.

The dissertation of Denisse Alejandra Gamboa is approved.

Pamela Yeh

Nandita Garud

Elaine Hsiao

Noa Pinter-Wollman, Committee Chair

University of California, Los Angeles

2025

DEDICATION

In loving memory of my grandparents, Maria Concepcion and Martiniano. To my parents, Ana and Martin, and grandparents, Fernando and Coco, whose sacrifices allowed me to pursue this doctoral degree. A special thank you to my partner, Carina Lizarde, who has listened to every practice talk, stayed up with me through the night for this last stretch of writing, and has provided endless love and support. Thank you to my teacher and mentor, Natalie Gray, for your guidance and support throughout the last 20 years. Thank you to my family and friends, Anahi Gamboa, Andrew Gamboa, Martin Gamboa, Janin Escobedo, and Samuel Barajas for your unconditional love, friendship, and support.

Thank you to my dissertation committee, Dr. Pamela Yeh, Dr. Nandita Garud, and Dr. Elaine Hsiao, for your support and guidance. Thank you to my mentor, Dr. Noa Pinter-Wollman, for believing in me and for your unwavering support and guidance throughout this journey. Thank you to all the Pinter-Wollman lab members past and present. Thank you to my mentors Dr. Jenn Smith and Dr. Pedro Nava. Thank you to my AMEBA family, Robert, Gloria, Heidi, Berenice, Melissa, and Marissa.

This dissertation is dedicated to my younger self, whose resilience made it possible for me to be the first in my family to earn this doctoral degree.

Para mi gente.

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ACKNOWLEDGEMENTS

I affirm that the contents of this dissertation are my own work and all sources quoted, paraphrased, or otherwise are properly acknowledged in the references. I confirm that this dissertation has not been previously submitted for the awarding of a degree to this, or any other university. Any portions of this dissertation that resulted from the contributions of others are detailed below.

Chapters two to four were written as independent manuscripts for publication with co-authors (acknowledged below). For all chapters, I developed the questions, conducted analysis, wrote code, and drafted the manuscripts with advice and supervision by my advisor, Dr. Noa Pinter-Wollman. Each manuscript was prepared following the formatting of the respective journal or target journals. Therefore, there are minor formatting or structural differences between all chapters.

Chapter two is a version of the manuscript titled “Harvester ant microbiome is associated with reproductive role” that is in prep for submission to *Insectes Sociaux – International Journal for the Study of Social Arthropods* Special Issue: A glimpse into a little-known world: Organisms associated with social arthropods. This chapter follows the APA formatting. This manuscript was co-authored with Noa Pinter-Wollman.

Chapter three is a version of the manuscript titled “The effects of social interactions, physical environment, and evolutionary history on the microbiome of harvester ants” that is in prep for submission to the journal *Animal Behaviour.* The publication was co-authored with Noa Pinter-Wollman.

Chapter four was published in *Animal Microbiome* in 2025 (DOI 10.1186/s42523-025-00390-3) under the title “Social organization and physical environment shape the

microbiome of harvester ants". This chapter follows the *Animal Microbiome* formatting and is reproduced here with permission from Springer Nature. The publication was co-authored with Noa Pinter-Wollman, Peter J. Flynn, and Eva Sofia Horna-Lowell.

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VITA

Education

B.A. – Biology, Concentration in Ecology, Evolution, & Behavior – Mills College (2017)

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Gamboa, D.A., Flynn, P.J., Horna-Lowell, E.S. *et al.* Social organization and physical environment shape the microbiome of harvester ants. *anim microbiome* **7**, 29 (2025). https://doi.org/10.1186/s42523-025-00390-3

Smith, J. E., **Gamboa, D. A**., Spencer, J. M., Travenick, S. J., Ortiz, C. A., Hunter, R. D., & Sih, A. (2018). Split between two worlds: automated sensing reveals links between above- and belowground social networks in a free-living mammal. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 373(1753), 20170249. https://doi.org/10.1098/rstb.2017.0249

Presentations

Gamboa, DA (2025) Assessment of Prior Knowledge: Using reflective writing assignments to identify and address scientific misconceptions. *UCLA*. AMEBA x Biological Sciences Council Research Symposium. Los Angeles, CA (Poster)

Gamboa, DA (2025) Social insects and their associated microbes. *Victor Valley Museum*, Arthropolooza: The Ultimate Bugfest. Apple Valley, CA (Research talk)

Gamboa, DA (2025) Social insects and their associated microbes. *San Bernardino County Museum*, Arthropolooza: The Ultimate Bugfest. Redlands, CA (Research talk)

Gamboa, DA (2024) Social organization and the physical environment shape the microbiome of harvester ant nests. *Animal Behavior Society Annual Meeting.* London, Ontario, Canada (Research talk)

Gamboa, DA (2023) The microbiome and behavior: Examining host-microbe interactions in a superorganism. *Animal Behavior Society Annual Meeting.* Portland, OR (Research talk)

Gamboa, DA (2022) Beneficial microbes and social behavior in harvester ants. *International Union for the Study of Social Insects Meeting.* San Diego, CA (Research talk)

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Selected Research Grants

2024	Departmental Research Grant – UCLA EEB	$1,000
	Departmental Conference Grant – UCLA EEB	$950
2023	Departmental Research Grant – UCLA EEB	$1,200
	Departmental Conference Grant– UCLA EEB	$750
2022	Halde Conference Travel Grant – UCLA EEB	$750
2021	National Science Foundation – Graduate Research Fellowship Program	$138,000
	Departmental Research Grant – UCLA EEB	$2,000
	Department Fellowship – UCLA EEB	$7,000
2020	Department Fellowship – UCLA EEB	$7,000
2019	Department Fellowship – UCLA EEB	$21,000
	UCLA Alumni Fellowship	$10,000
	UCLA Competitive Edge: Summer Transition Program to the Doctorate	$6,000
2017 –	The Animal Behavior Society Charles H. Turner Award	$500

Teaching Positions

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Alaiza Guerrero (UCLA, 23-24), Angelica Soriano (UCLA, 2022), Monica Lu (UCLA, 2022), Martin Villa (Community College Field Biology Alliance, 2021), Cynthia Sanchez (Community College Field Biology Alliance, 2020)

Selected Service

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2022-2023 **Student Leadership Volunteer Organizer** – Department Seminar, UCLA EEB
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Chapter 1: Overview of the dissertation

Overview of the dissertation

Communities of associated microorganisms are integrated into macro-ecosystems, including eukaryotic hosts, and are crucial for their functioning and health. Host-associated microbes, collectively known as the microbiome, consist of microbial communities that inhabit specific environments and exhibit unique physiochemical properties (Berg et al., 2020; Whipps et al., 1988). These properties include the microbial structures, metabolites, and any interacting molecules produced by the host organisms, all of which are influenced by the surrounding environmental conditions (Berg et al., 2020; Whipps et al., 1988). The microbiome is a dynamic and interactive micro-ecosystem that changes over time and at various scales. Symbiotic microbial cells even outnumber the cells of the host (Archie & Theis, 2011; Lewin-Epstein et al., 2017; Parfrey et al., 2018; Theis et al., 2016). In these mutualisms, the host provides a habitat for microbial communities, which in turn have a beneficial impact on the host's health via nutrient acquisition, immune function, and even intraspecific communication (Archie & Theis, 2011; King et al., 2016; Mockler et al., 2018; Teseo et al., 2019). Host-associated microbes impact individual host fitness, thus making it crucial to study the drivers of variation among hosts and their associated microbes. Various factors, including social behavior, evolutionary history, and the environment, influence the composition and diversity of an animal’s microbiome.

The social environment mediates the exposure and susceptibility to microbes via social interactions (Archie & Theis, 2011; Braga et al., 2016; Collins et al., 2012). Social behavior determines patterns of physical contact and habitat overlap between individuals, thus playing a critical role in the transmission and acquisition of associated

microbes in social species. Socially mediated microbe transmission is observed in a variety of species from primates to eusocial insects, including termites, aphids, and bees (Bourguignon et al., 2018; Chen & Purcell, 1997; Engel et al., 2016; Goodfellow et al., 2019; Koch & Schmid-Hempel, 2011; Moeller et al., 2016b; Perofsky et al., 2017; Tung et al., 2015). For example, in bumblebees, beneficial microbes obtained via social interactions protect individuals from a deadly parasite (Koch & Schmid-Hempel, 2011). Social transmission of microbes between conspecifics may inoculate future generations, providing a collective benefit for the colony. Thus, social organization and behavior may promote the transmission of beneficial bacteria at the individual level via social interactions.

Host-microbe interactions often reflect the phylogeny and evolutionary history of the host, with closely related species tending to harbor more similar microbiomes. This pattern has been well documented in various mammalian species, including primates, deer mice, *Drosophila* flies, mosquitoes, and *Nasonia* wasps (Amato et al., 2017; Brooks et al., 2016; Moeller et al., 2016a; Ochman et al., 2010; Ross et al., 2018). In species-specific mutualisms between hosts and microbial communities, functional microbial communities play a crucial role in processes essential for host survival, including nutrient acquisition and immune function. For instance, fungus-farming ants have a well-known symbiotic relationship with their fungus symbiont, which provides food, and with their bacterial symbionts that protect against pathogens (Cafaro et al., 2010; de Souza et al., 2013; Little & Currie, 2007; Moreau, 2020; Nygaard et al., 2011; Ronque et al., 2020; Sapountzis et al., 2015). Similarly, the core gut microbes of herbivorous and predatory ants play a critical role in host nutrient acquisition (Funaro et

al., 2011; He et al., 2014; Łukasik et al., 2017; Russell et al., 2009). As a result, herbivorous and predatory ant species each form distinct groups with more similar microbiomes among themselves than with the other group. Ultimately, the intricate relationship between host phylogeny and evolutionary history makes it challenging to separate their respective effects on microbiome composition.

Animal hosts are constantly exposed to microbes from their physical environment (Gonzalez et al., 2011). All animals are colonized by microbes from their mother and their surroundings before, during, and after birth (Desbonnet et al., 2015; Schwarz et al., 2016; Vaishampayan et al., 2010). In humans, this process is said to take months, and bacterial communities present in the mother may or may not be the dominant communities in the infant. Consequently, the external biotic environment may play a larger role than direct transmission from conspecifics in individual host-microbial community composition. Additionally, free-living animals that live in rural environments harbor distinct microbiome communities compared to animals that live in urban (Dillard et al., 2022; Littleford-Colquhoun et al., 2019; Sugden et al., 2020) or captive environments (zoos and laboratories) (Alfano et al., 2015; Bornbusch et al., 2022; Bowerman et al., 2021; McKenzie et al., 2017; Rosshart et al., 2017). Consequently, the physical environment may play a larger role than direct transmission from conspecifics or phylogeny in individual host-microbial community composition.

Harvester ants offer an ideal system for studying host-microbe interactions, as they are a phylogenetically diverse, group-living species that inhabit various environments. Harvester ants are eusocial species, meaning that their colonies consist of overlapping generations, cooperative brood care, and reproductive division of labor

(sterile workers assist reproductive individuals) (Hölldobler & Wilson, 1990). The way in which individuals within a colony interact with one another results in collective behavioral phenotypes that influence the composition and success of the colony. Harvester ants are a functional group that includes any ant species that collects and stores seeds. This category is a broad classification that includes over 150 species across 18 genera and three subfamilies *(Myrmicinae, Ponerinae, Formicinae)* (Hölldobler & Wilson, 1990; MacMahon et al., 2000; Ward et al., 2015). Although prior studies have found evidence that the physical environment and life history shape the diversity of bacterial communities in *Pheidole* and *V. andrei* harvester ants, neither alone explains the bacterial diversity in this ecologically and evolutionarily diverse harvester ant group. To determine the relationship between eusocial colonies of harvester ants and their associated microbes, we used a combination of observational, manipulative, and experimental approaches. Using 16S ribosomal RNA gene sequencing of the V4 region, we analyzed the microbial communities associated with four species of harvester ants across two taxonomically diverse clades (*Pogonomyrmecini* and *Stenammini*): *Pogonomyrmex Californicus, Pogonomyrmex Rugosus, Veromessor pergandei,* and *Veromessor andrei.*

In **Chapter 2**, I investigate the relationship between bacterial community composition, behavioral tasks, and reproductive role by comparing the microbial communities of foragers, nest maintenance workers, and reproductives (winged female and male alates) from eight *V. andrei* colonies. These individual-level differences are crucial for explaining host-microbe interactions. We find that morphological differences are a reliable predictor of host-associated bacterial community differences in harvester

ants. Our findings align with previous work on the relationship between social roles and host-associated microbial communities in social insects.

In **Chapter 3**, I test for the impact of social interactions on microbial communities by experimentally manipulating the ability of ants to interact with one another. Second, I examined the impact of the environment by comparing the microbiome of ants from a natural setting to that of captive lab ants. Lastly, I examined the influence of evolutionary history by comparing the microbial communities of three taxonomically distinct harvester ant species, *Pogonomyrmex rugosus, P. californicus,* and *V. pergandei*. The physical environment and phylogeny were both important predictors of microbiome diversity and composition in the harvester ants that we studied. Despite ants being highly social animals, we did not find support for social interactions playing an important role in determining microbial diversity and composition across all species.

In **Chapter 4**, I investigate how social organization, and the physical environment influence the microbiome by sampling the bacterial communities surrounding and within the nests of harvester ant colonies (*V. andrei*). We collected samples from (i) ants, brood, seeds, reproductives, and soil, (ii) soil from inside and outside the nest, and (iii) soil from different chambers. We found that both the environment and social organization impact the bacterial communities of V. andrei colonies. This work was accepted and published in the journal, *Animal Microbiome.*

Overall, this work aims to understand the drivers of variation among hosts and their associated microbes across multiple scales of biological organization.

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Chapter 2: Harvester ant microbiome is associated with reproductive role

Harvest ant microbiome is associated with reproductive role

Abstract

Host-associated microbes can impact host fitness, and it is therefore important to uncover the drivers of variation among hosts in their associated microbes. In social insects, microbiome composition differs based on both reproductive role and behavioral task. Here, we asked whether reproductive role or behavioral task is more important in determining the diversity and composition of host-associated microbes in the harvester ant *Veromessor andrei*. Specifically, we compared the bacterial communities between workers and reproductives by sequencing the 16S rRNA gene region. We further compared the bacterial communities between workers that are foragers and those that perform nest maintenance, as well as between reproductives that are male alates and those that are female alates. We found that reproductive role was a better predictor of bacterial communities than behavioral task. Workers and reproductives differed in their bacterial communities. However, different types of workers (foragers and nest maintenance workers) and different types of reproductives (males and females) did not differ from one another. Our findings suggest that morphological differences are a better predictor for differences in microbial communities than behavioral differences.

Introduction

The relationship between insects and their associated microbial communities is vital to understand because of their ecological and evolutionary consequences. Gut bacteria, for example, facilitate nutrient acquisition in fruit flies (*Bactrocera dorsalis*) (Akami et al., 2019), honeybees (*Apis mellifera*) (Engel et al., 2012; Motta & Moran, 2024; Zheng et al., 2016), carpenter ants (*Camponotus floridanus*) (Feldhaar et al., 2007), and

herbivorous turtle ants (*Cephalotes spp.)* (Hu et al., 2018). In addition, host-associated microbes assist with pathogen defense in bumble bees (*Crithidia bombi*) (Koch & Schmid-Hempel, 2011; Mockler et al., 2018), honeybees (*A. mellifera*) (Engel et al., 2016; Jones et al., 2018), social wasps (family *Vespidae*) (Hoggard et al., 2011), and leaf-cutter ants (Genus: *Acromyrmex* and *Atta*) (de Souza et al., 2013; Heine et al., 2018). Thus, variation in host-associated microbes may have important implications for host health and physiology. Uncovering the drivers of variation within a population in host-associated microbes will provide important mechanistic insights into the role that microbes may play in shaping the structure and function of animal societies.

In social insects, there is a relationship between morphologically determined social roles and associated microbes (Liberti et al., 2024). Most social insect colonies are composed of morphologically distinct workers and reproductives and include a variety of life stages in the colony, such as brood and adults. This morphological diversity has been linked with microbiome diversity and composition. Larval developmental stages exhibit distinct microbial communities compared to those found in sterile workers and reproductive adults of the same species in parasitoid wasps (genus *Nasonia*) (Brucker & Bordenstein, 2012), hornets (Cini et al., 2020), arboreal trap jaw ants (*Daceton armigerum*) (Ramalho et al., 2020), and harvester ants (*Veromessor andrei*) (Gamboa et al., 2025). In fungus-farming termites, the gut microbiome of the queen and king is less diverse and differs from that of the morphologically distinct soldiers and workers (Otani et al., 2019). In honeybees, the gut microbiome composition of queens and males is less diverse than that of workers (foragers and nurses) (Kapheim et al., 2015). In carpenter ants, males harbor distinct microbial communities

compared to queens and workers (Koto et al., 2020). Finally, in the ant, *Diacamma. cf. indicum*, worker-specific bacteria are maintained and transmitted among workers but are not present in gamergates, which are morphologically different functional queens (Shimoji et al., 2021).

The behavioral tasks that workers perform have been linked to differences in host-associated microbial composition and diversity, primarily in honeybees and termites. Adult workers in a social insect colony perform a variety of tasks, including foraging, nest maintenance, and brood care. Workers who perform a certain task tend to interact more with one another due to spatial segregation between tasks (Mersch et al., 2013). Indeed, in honeybees, social interactions can lead to the homogenization of the microbiome (Liberti et al., 2022), and the social environment can determine microbial composition (Vernier et al., 2020). While Jones et al. (2018) found differences in the gut microbiome across honeybee workers that performed different tasks (foragers, nurses, and food processors), Kapheim et al. (2015) did not find such differences between honeybee foragers and nurses. More recent work using shotgun metagenomics (Baud et al., 2023) identified strain-level, but not species-level, differences in the gut bacterial communities of honeybee nurses and foragers. Thus, task-based differences in microbial communities in honeybees may be quite nuanced. Furthermore, soldiers and workers differ in gut microbiota in fungus-farming termites (Otani et al., 2019). However, there are morphological differences between soldiers and workers; therefore, the observed microbial differences are not strictly based on behavioral differences. Lab manipulations in which the microbiome is experimentally manipulated have shown a causative link between microbial communities and

behaviors, such as mating preferences in fruit flies (*Drosophila melanogaster*) (Sharon et al., 2010; Venu et al., 2014), food preference in mosquitoes (Verhulst et al., 2011), kin recognition in hyenas (Rojas et al., 2020; Theis et al., 2012, 2013), and other microbe-mediated behavioral changes via the host's neuroendocrine system in mice (Bravo et al., 2011). In social insects, such experimental manipulations of the microbiome have shown impacts on nestmate recognition in honeybees (Vernier et al., 2020). In leaf-cutter ants (Teseo et al., 2019) and harvester ants (*Pogonomyrmex barbatus*) (Dosmann et al., 2016), manipulating the microbiome has been shown to impact both nestmate recognition and aggressive behavior. These experimental manipulations demonstrate a relationship between behavior and the microbiome in various systems, including ants. Thus, while much is known about how tasks and reproductive roles impact the microbiomes of honeybees (Nguyen et al., 2024) and termites (Sinotte et al., 2020), it is surprising that there is little work on the differences in microbial communities among behavioral tasks in ants (Liberti et al., 2024).

Here, we investigate whether there is a relationship between social roles and host-associated microbes in the harvester ant *Veromessor andrei*. These ants are monogynous (one queen per colony), with monomorphic workers (similar sizes), and colonies can reach sizes of tens of thousands of individuals (Brown, 1999). Specifically, we examined the bacterial communities of the microbiome across individuals that perform different behavioral tasks (foraging and nest maintenance) and individuals that belong to different morphologically determined reproductive roles (female and male alates vs. sterile workers). Specifically, we test two non-mutually exclusive hypotheses that: (1) behavioral task (foraging and nest maintenance) relates to the composition and

diversity bacterial communities because individuals who perform similar tasks interact more often with one another and potentially share more microbes with one another than with individuals that perform other tasks; and (2) reproductive role relates to the composition and diversity of the bacterial communities due to physiological differences between reproductive (female and male alates) and sterile workers (foragers and nest maintenance workers). If behavioral task relates to microbial communities, we predict that bacterial communities extracted from workers of *V. andrei* will be more similar among workers who perform similar behavioral tasks (e.g., foragers) than among workers who perform different tasks (e.g., foragers vs. nest maintenance workers). If reproductive role (female and male alates vs. sterile workers) relates to microbial communities, we predict that *V. andrei* reproductives (female and male winged alates) will have different associated bacterial communities compared to those of sterile workers (foragers and nest maintenance).

Methods

Study site and sample collection

To investigate the relationship between bacterial community composition, behavioral tasks, and reproductive role, we collected samples of foragers, nest maintenance workers, and reproductives (winged female and male alates) from eight *V. andrei* colonies in June 2023 (Figure 2.1). Ants were collected from colonies in a serpentine grassland at the Sedgwick Natural Reserve in southern California. We used gloves and soft tweezers, sterilized with 90% ethanol, to collect all samples. Foragers were identified as individuals traveling on foraging trails with seeds in their mandibles (Figure 2.1b). All foragers were collected from the foraging trail at least 1m from the nest

entrance. Nest maintenance workers were identified as individuals emerging from the nest entrance with dirt in their mandibles (Figure 2.1c). Reproductives (female and male alates) were identified by their distinct winged morphology and collected as they emerged from the nest entrance (Figure 2.1d). Female and male alates were distinguished by head size, with males having noticeably smaller heads than females. We collected at least 3-5 ants of each type from each colony. All collected ants were placed in a freezer at the field station and later transported in a cooler to the UCLA campus (approximately 2 hours away). All samples were stored at -20℃ until processing.

Sample processing

Sample preparation for DNA extraction: We washed all samples (ants) with 70% ethanol, 5% bleach, and sterile deionized water. Each washed sample was placed in a sterile 1.5 ml tube and crushed using a sterile pestle (Fisher Scientific). We followed the Qiagen DNEasy PowerSoil Pro kit protocol and added 800 µL of Solution CD1 (containing SDS for cell lysis) to the samples, then vortexed them. We incubated the samples overnight at 56℃ in a shaking incubator to maximize cell lysis.

DNA extractions: After 24 hours in the incubator, we extracted microbial DNA using the Qiagen DNEasy PowerSoil Pro kit, following the manufacturer's instructions. We sent 34 samples for 16S rRNA gene amplification and library sequencing at the UCLA Microbiome Center. These samples included eight forager samples (each consisting of five foragers crushed together), eight nest maintenance worker samples (each consisting of three workers crushed together), seven male alates sample (each consisting of three males crushed together), eight female alates sample (each

consisting of three female alates crushed together), and three control blank samples of 250 µl sterile distilled water, with no other material. Refer to the data in the GitHub repository: https://github.com/DAlejandraG/BT-microbes. Amplicon sequencing of the bacterial community targeted the V4 region of the 16S rRNA gene using primers 515F (59-GTGCCAGCMGCCGCGGTAA-39) and 806R (59-GGACTACHVGGGTWTCTAAT-39, following the Earth Microbiome Project (EMP) protocol (Caporaso et al., 2011; Jacobs et al., 2021).

Quantifying bacterial community diversity

To determine the bacterial community composition and diversity associated with each behavioral task (foraging and nest maintenance) and reproductive role (workers vs. winged alates), we processed the sequencing data using QIIME2 version 24.5 (Bolyen et al., 2019). We followed the demultiplexing and quality filtering steps described by Gamboa et al. (2025). The 16S rRNA gene amplicon sequencing raw reads are available from NCBI via BioProject record [PRJNA1311736]. To visualize bacterial taxonomic composition with relative abundance plots at the phylum and order levels, we exported the feature table and analyzed the data using the ‘phyloseq' package in R (McMurdie & Holmes, 2013). Following quality control, we assessed rarefaction, beta, and alpha diversity using the q2-diversity plugin, based on ASVs. We rarefied the dataset at a sampling depth of 4,853 and retained 150,443 features (1.23%) after refraction. All three control blank samples were removed from further analysis of alpha and beta diversity due to low read numbers. We show the blank control samples in Figure 2.3. The remainder of the analysis and figures include the 31 samples from the workers and reproductives but exclude blank controls.

To determine how behavioral tasks and reproductive roles influence host-associated bacterial communities, we examined the beta (between samples) and alpha (within samples) diversity of ASVs. Beta diversity reflects differences in bacterial community composition across multiple samples, grouping them based on similarities in sequence abundances or the presence and absence of sequences (Borcard et al., 2018). Alpha diversity describes the distribution of microbes within a sample. To compare the microbial diversity of workers and reproductives, we used six indices of alpha diversity: *Shannon's index* - a measure of how evenly species are distributed, independent of their richness (Kim et al., 2011; Lemos et al., 2011). *Faith's phylogenetic diversity (PD)* - a measure of species richness that accounts for phylogenetic diversity (Faith, 2007). *Pielou's evenness* - relative abundance of species (Pielou, 1966). *Simpson's index* - assesses the dominance of a few abundant species in a sample, with higher values indicating lower diversity (Kim et al., 2017). *Chao's richness estimator (Chao1)* - estimates the true number of species while considering rare species (Chao & Chiu, 2016). *Observed amplicon sequence variants (Observed ASVs)* - the number of observed unique sequences in a sample (Callahan et al., 2016).

Statistical analysis

Beta diversity: To determine if beta diversity differed among the different types of worker tasks (foraging and nest maintenance) and reproductive types (female and male alates), we applied a permutational multivariate analysis of variance (PERMANOVA) to a Bray-Curtis dissimilarity matrix, which assesses compositional dissimilarity among bacterial communities (Bray & Curtis, 1957). Principal Coordinate Analysis (PCoA) ordination was generated from this matrix using the Adonis function in the Vegan package

(Oksanen et al., 2001), with 999 permutations for the PERMANOVA. PCoA plots were visualized with the 'ggplot2' R package (Wickham, 2016).

Alpha diversity: To determine if alpha diversity differed between reproductive roles (workers vs. reproductives), we ran six linear mixed models (LMM) for the six alpha diversity measures: Shannon, Faith's phylogenetic distance, Pielou's evenness, Simpson, Chao1, and Observed ASVs. In each model, the dependent variable was one of the alpha diversity measures. We only included reproductive role (worker or reproductive) as the explanatory variable in the LLMs because the results from the beta diversity analysis did not identify differences among categories within each reproductive role (i.e., no difference between foraging and nest maintenance workers and no difference between female and male alates), only between reproductive roles (i.e., between workers and reproductives) (Table 2.1, Figure 2.2). We included 'colony' as a random effect in all LLMs to account for variation in microbial communities among colonies. We used the lmer() function in the 'lme4' R package for these models (Bates et al., 2015). We report the proportion variance explained by the random effects as the conditional R^2 minus the marginal R^2, obtained using the r2_nakagawa () function in the 'performance' R package (Lüdecke et al., 2021). Data and code are available on GitHub [https://github.com/DAlejandraG/BT-microbes].

Results

We found significant differences in microbial composition and diversity between reproductive roles (males and queens) and workers (foragers and nurses), but we did not detect differences within each reproductive role. Forager and nest maintenance workers did not differ in their microbial composition, and neither did male and female

alates. Bray-Curtis distances, visualized by a principal coordinate analysis (PCoA), demonstrated that ASVs clustered by reproductive role (workers vs reproductives) (Figure 2.2). Whether samples came from foragers, nest maintenance workers, female alates, or male alates explained approximately 28% of the total variation (PERMANOVA: $F_{DF=3} = 3.50$, $R^2 = 0.280$, p-value = 0.001). Beta diversity was significantly different between workers (foragers and nest maintenance) and reproductives (female and male alates) (PERMANOVA: $F_{DF=1} = 2.76$, $R^2 = 0.252$, p-value = 0.001) (Figure 2.2, Table 2.1). However, within each reproductive role, we did not detect a significant difference - foragers were not significantly different from nest maintenance workers, and male alates were not significantly different from female alates (Table 2.1).

The relative abundance of bacterial ASVs differed between workers and alates, but not within each reproductive role, especially at the level of 'order' but less at the level of the 'phylum' (Figure 2.3). Because we did not detect differences in beta diversity within workers and reproductives (Figure 2.2, Table 2.1), and because of our relatively small sample size, for the examination of alpha diversity, we lumped foragers and nest maintenance workers into one category (workers) and male and female alates into another category (reproductives) when comparing alpha diversity. We found that workers and reproductives had significantly different alpha diversity of their associated bacterial communities across all six measures of alpha diversity that we examined (Figure 2.4, Table 2.2).

Discussion

We found that reproductive role was a more important predictor of variation in host-associated microbes in *V. andrei* harvester ants than behavioral task (Figure 2.2). Workers and reproductives differed in the composition and diversity of their host-associated bacterial communities (Figures 2.3 and 2.4, Table 2.2). However, we did not find differences between forager and nest maintenance workers, nor did we detect a difference between male and female alates in their microbial communities (Table 2.1).

The bacterial phyla we found are similar to those identified in other ant species. Specifically, the *Firmicutes* bacterial phylum had the highest abundance in workers, while reproductives exhibited a more balanced distribution of *Firmicutes, Proteobacteria, and Actinobacteria* (Figure 2.3). Similar bacterial phyla were found to differ between workers and reproductives in the ant *D. cf. indicum* (Shimoji et al., 2021), which is consistent with our previous research on *V. andrei* (Gamboa et al., 2025). *Firmicutes, Proteobacteria,* and *Actinobacteria* are typical bacterial phyla in herbivorous and omnivorous ant species (Anderson et al., 2012; Russell et al., 2009). Interestingly, the *Firmicutes* bacterial phylum is frequently observed in predatory ant species such as army ants (*Eciton*) and bullet ants (*Paraponera clavata*) (Anderson et al., 2012; Valdivia et al., 2023), and the prevalence of *Firmicutes* found here in *V. andrei* is consistent with previous studies of the microbiome of the same ant population (Gamboa et al., 2025). There are slight differences in the bacterial orders identified here and in Gamboa et al., (2025), most likely because assignment of ASVs to taxonomic classifiers is less reliable at more detailed taxonomic levels. Additionally, the software used to classify the ASVs

taxonomically no longer supports the silva classifier (used Greengenes2 classifier), which was used to assign taxonomic groups in Gamboa et al. (2025).

Our findings are consistent with those of many other studies on social insects, which have found differences in microbial communities between reproductives and workers (Liberti et al., 2024; Sinotte et al., 2020). Reproductives of harvester ants (Gamboa et al., 2025) and *D. cf. indicum* (Shimoji et al., 2021) have higher alpha diversity of their bacterial communities compared to sterile workers. In contrast, the microbiomes of reproductives were less diverse than those of sterile workers in termites (Otani et al., 2019) and honeybees (Kapheim et al., 2015). It seems as though morphological differences and the various physiological pathways that are associated with polymorphism may mediate the observed differences in host-associated microbes. For example, queens receive a different diet during development (royal jelly), which may explain why their gut microbiome differs from that of workers (Nguyen et al., 2024). Perhaps in harvester ants, reproductives receive different diets during development compared to workers (Smith & Suarez, 2010). Interestingly, a recent study on the microbiome of *V. andrei* (Gamboa et al., 2025) found that the bacterial communities of reproductives and brood are very similar but distinct from those of workers. Perhaps if reproductives leave the nest soon after eclosion, their associated microbiota is more similar to that of their previous developmental stage (brood). Whereas outside workers have had more time elapse since their eclosion, exposing them to different microbes through their diet and/ or environment. Furthermore, (Gamboa et al., 2025)) found that the associated bacterial communities of brood and reproductives in *V. andrei* were more similar to those of seeds than to workers. Seeds are primarily fed to brood as a source

of protein, whereas workers rely on other food items for primarily carbohydrates (Csata & Dussutour, 2019). This difference in diet can further explain the differences we found here between workers and reproductives in their bacterial communities. Thus, the different nutritional needs of workers and reproductives might be an important driver of variation in host-associated microbial communities. Whether these differences in microbial communities are further determined by physiological mechanisms that drive morphological differences remains to be examined.

Our inability to detect differences in the host-associated bacterial communities of workers that perform different tasks is further consistent with prior work on social insects. Very few studies on the social insect microbiome have found differences in microbial communities based on behavioral tasks. This lack of relationship between the microbiome and behavior in social insects is surprising, given that work on other animals has found a relationship between the microbiome and behavior. For example, in fruit flies (*Drosophila melanogaster),* gut-specific microbes shape mating preferences (Sharon et al., 2010; Venu et al., 2014). The skin microbes of potential hosts influence food preference in mosquitoes (Verhulst et al., 2011). Furthermore, host microbiome mediates information transfer and social recognition, as seen in honeybees (Vernier et al., 2020), leaf-cutter ants (Teseo et al., 2019), and harvester ants (*P. barbatus)* (Dosmann et al., 2016). In the case of leaf-cutter and harvester ants, the eradication of the microbiome affected nest-mate recognition and led to increased aggression against the treated ants. While differences in microbial communities among termite workers have been observed (Hongoh et al., 2006), these workers varied in age and morphotype, suggesting that morphology still played an important role in determining

their host-specific microbial communities. In honeybees, no differences were found in the microbiome of nurses and foragers (Kapheim et al., 2015); however, a shotgun metagenomics approach identified strain-level differences between nurses and foragers of honeybees (Baud et al., 2023). Thus, task-related differences in host-associated microbial communities may be more nuanced and require a more fine-grained analysis of host-associated microbes to identify task-based differences in the microbiome. Finally, it is possible that because workers in harvester ants often switch among tasks (Gordon, 1996), there is enough mixing within the worker population, and diet might not be sufficiently different across tasks to drive differences in microbiome. Foragers and nest maintenance workers both spend substantial amounts of time outside the nest. Perhaps, if outside workers are compared to inside workers (such as nurses), more differences in host-associated microbial communities would be found. Indeed, outside work alters the CHC composition of harvester ants (Sturgis & Gordon, 2012). While Gamboa et al. (2025) sampled *V. andrei* workers from inside the nest, they did not distinguish between brood care workers and foragers that returned to the nest, or nest maintenance workers that are working inside, due to the destructive sampling of the nest itself. Thus, further work with more nuanced analysis of microbial communities and comparison of outside to inside workers might reveal differences in host-associated microbes based on tasks in ants.

Finally, we found that colony ID explained 42-62% of the variation in the diversity of the microbial communities. Other studies of ant-associated microbial communities have found a significant effect of colony identity on the diversity of bacterial communities. Interestingly, colonies with more diverse bacterial communities produce

more brood (Segers et al., 2019). The strong effect of colony ID that we observed could be driven by differences in the bacterial communities within the ant nests. The bacterial communities associated with the soil of *V. andrei* nests differ across nests and correspond to their geographical distribution. Nests that are more geographically distant harbor more distinct bacterial communities (Gamboa et al., 2025). Thus, the environment may play an important role in shaping the community of host-associated microbes in ants.

In conclusion, our work demonstrates that morphological differences are a reliable predictor of host-associated bacterial community differences in harvester ants. Our findings align with previous work on the relationship between social roles and host-associated microbial communities in social insects. We thus provide additional support for the potential role of diet, developmental trajectory, and environment in shaping the host-associated microbial communities of social insects.

Figures and Tables

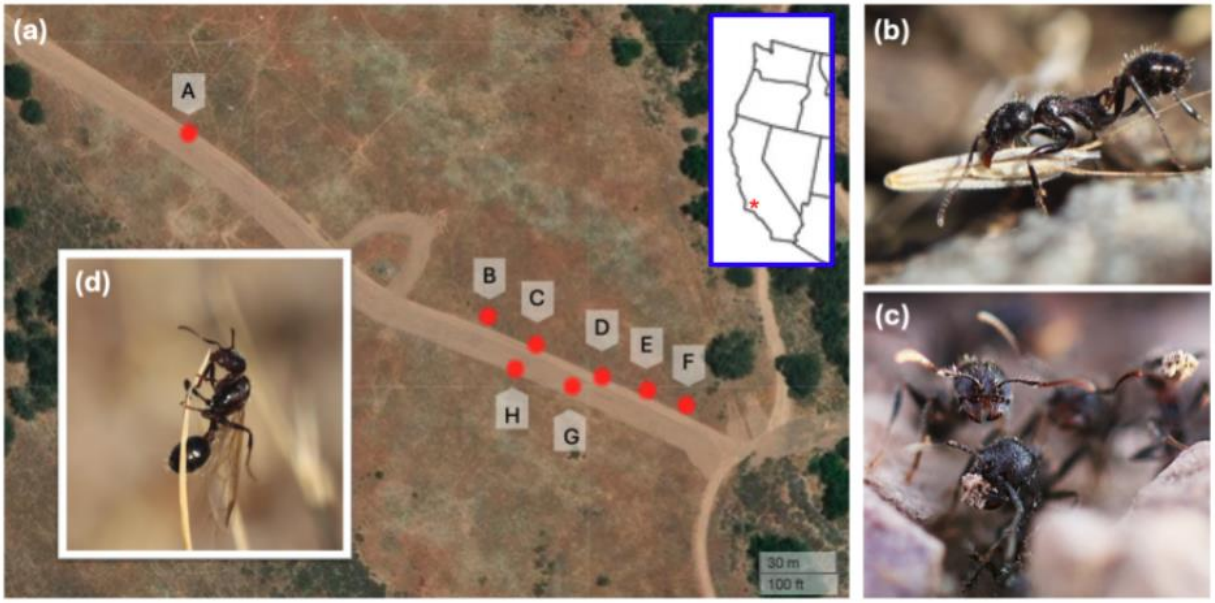

Figure 2.1: Sample collection.

(a) Map of the study site with the location of eight *V. andrei* colonies indicated with red dots and a corresponding letter ID for each colony. The inset shows the location of the field site on a map of the western United States, marked by a red asterisk. We collected at least (b) five foragers, (c) three nest maintenance workers, (d) three female, and three male alates (males not shown) from each of the eight colonies. All photo credits: Noa Pinter-Wollman.

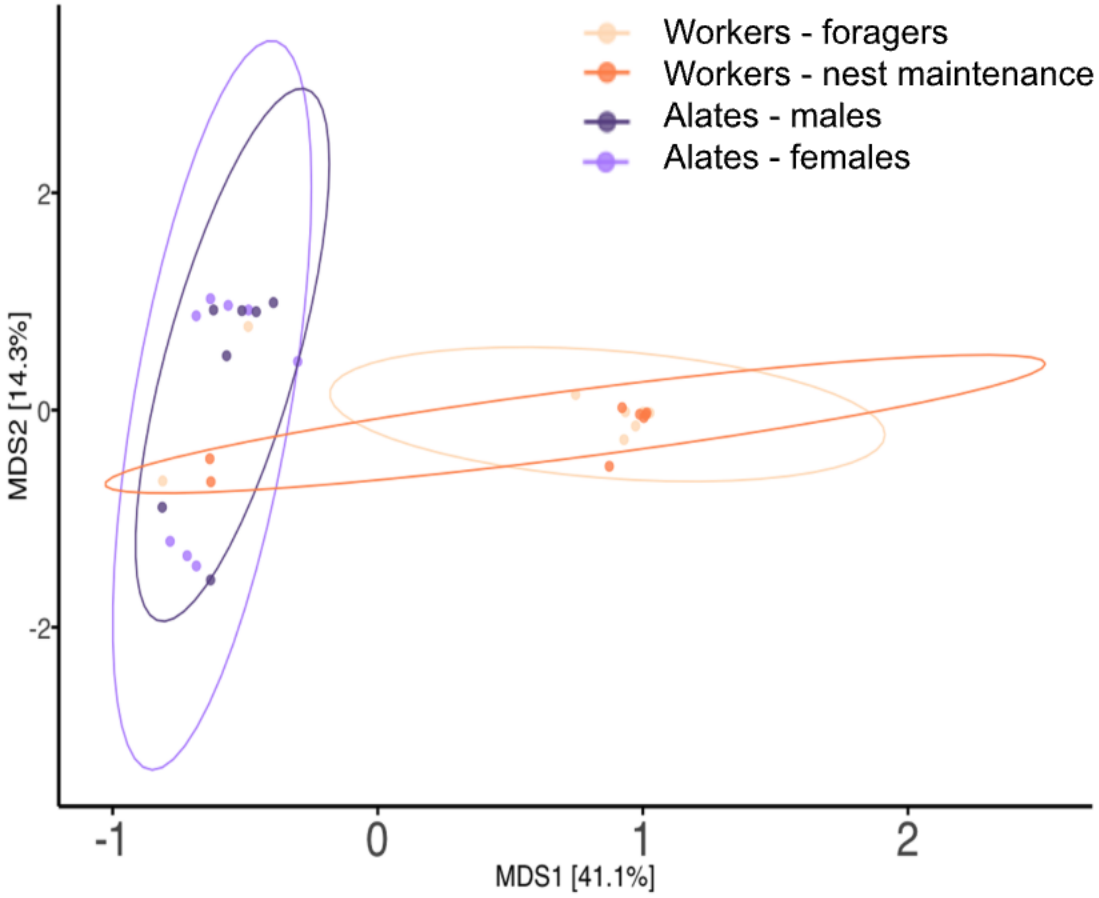

Figure 2.2: Beta diversity principal coordinate analysis (PCoA) from a Bray-Curtis dissimilarity matrix. Each point represents a single sample, and the closeness of the points indicates high similarity between bacterial communities. Workers are represented in light orange for foragers and dark orange for nest maintenance. Alates are in purple - dark for males and light for females.

Table 2.1: Pairwise PERMANOVA results comparing beta-diversity using Bray-Curtis distances between forager (FOR) and nest maintenance (NM) workers and female (FA) and male (MA) alates. Significant differences are in bold.

Pairs	DF	Sum of Squares	F Model	R^2	P value	P adjusted
FOR vs. NM	1	0.12	0.53	0.036	0.609	1
FOR vs. FA	**1**	**1.45**	**5.03**	**0.264**	**0.006**	**0.036**
FOR vs. MA	**1**	**1.33**	**4.44**	**0.255**	**0.008**	**0.048**
NM vs. FA	**1**	**1.60**	**5.57**	**0.285**	**0.007**	**0.042**
NM vs. MA	**1**	**1.45**	**4.90**	**0.274**	**0.005**	**0.030**
FA vs. MA	1	0.20	0.53	0.039	0.886	1

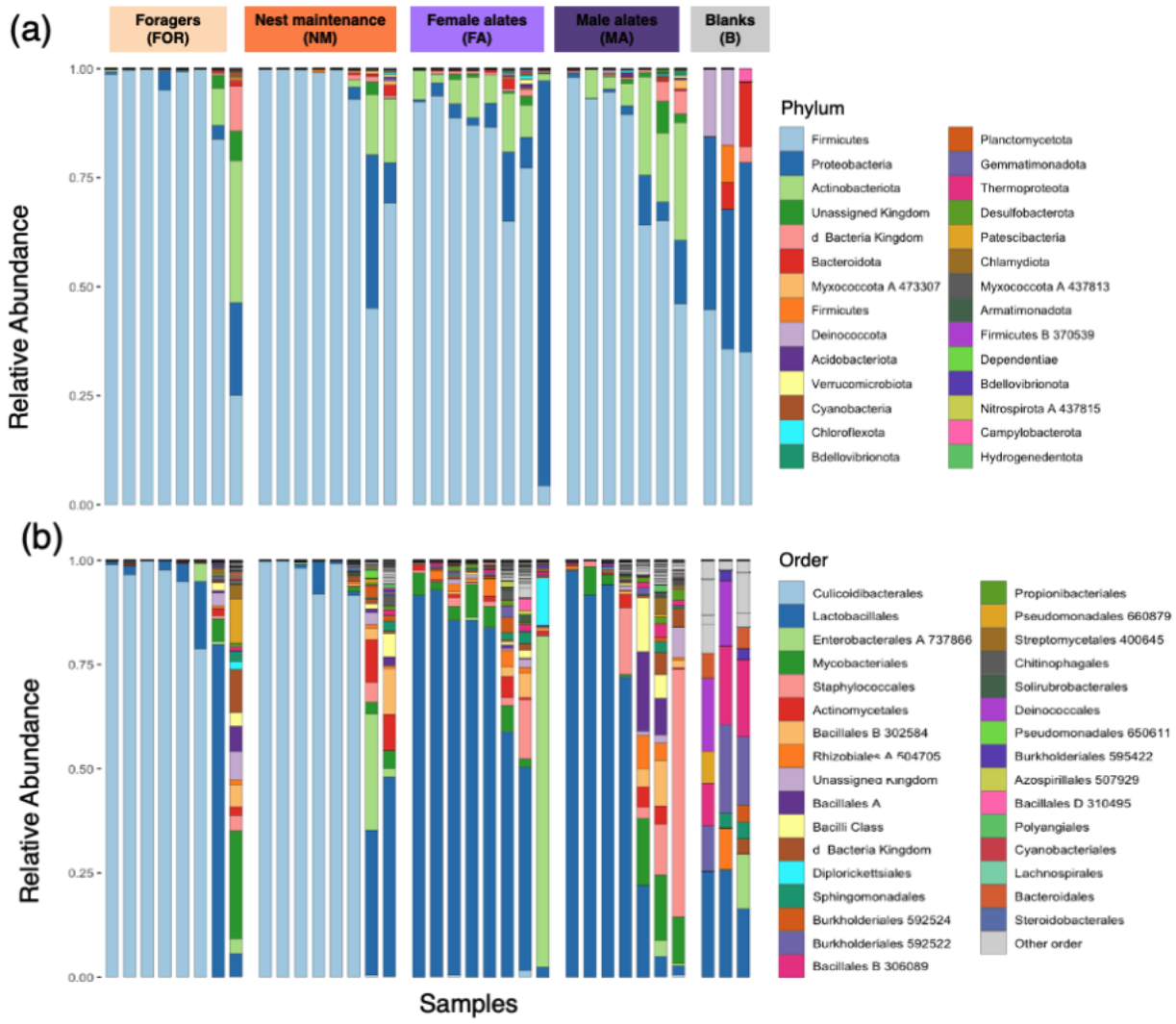

Figure 2.3: Relative abundance of bacterial taxa at the phylum (a) and order (b) levels. Samples are grouped by social role: forager workers (FOR), nest maintenance workers (NM), female alates (FA), and male alates (MA). The blank control samples are shown in the last three bars but are not included in the analysis of alpha and beta diversity. Each vertical bar represents one sample from a single colony, with color indicating the bacterial phylum or order based on ASV counts. The sampling depth was 4853 reads.

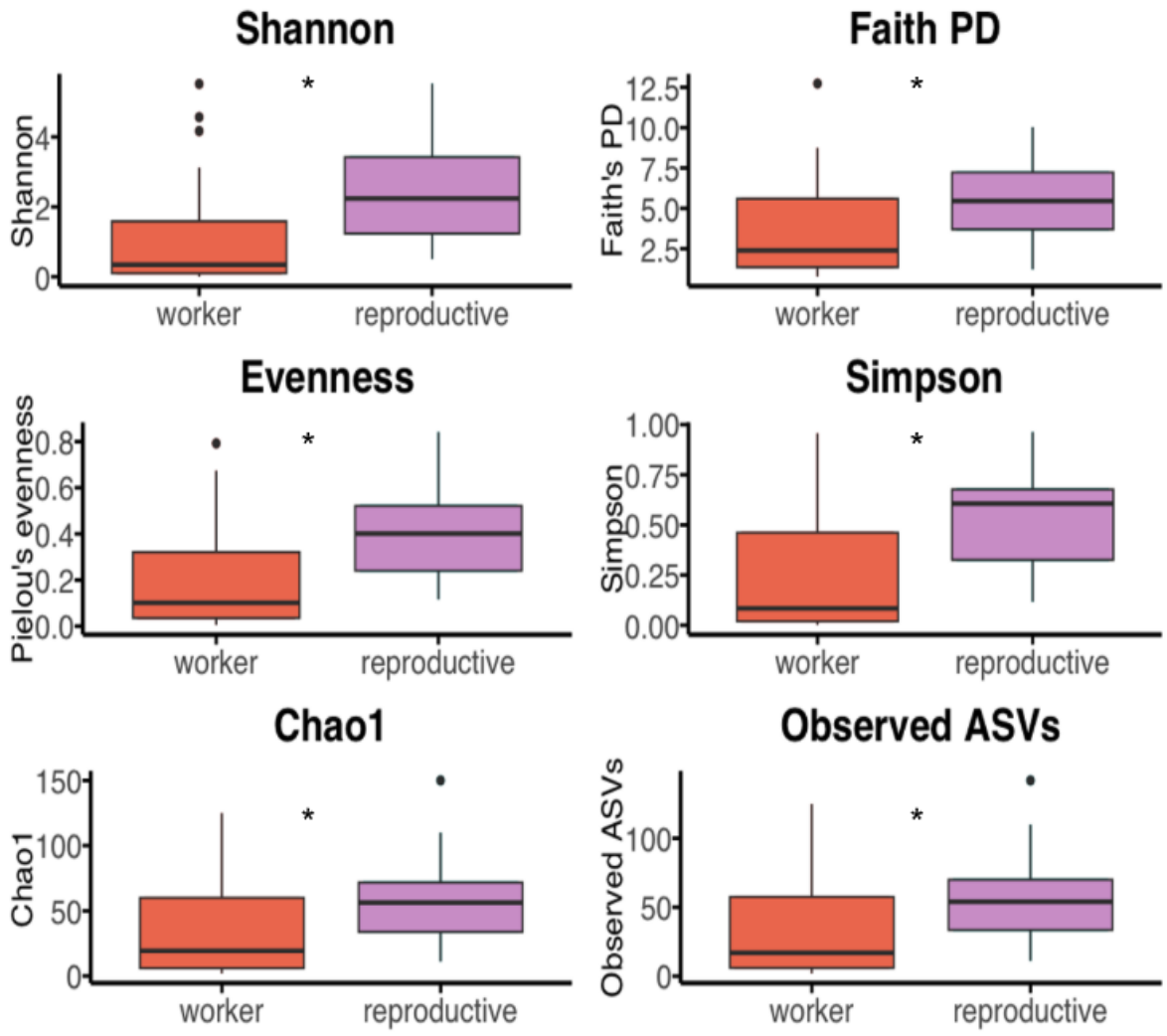

Figure 2.4: Alpha diversity measures (Shannon, Faith's phylogenetic distance (PD), Pielou's evenness, Simpson, Chao1, and Observed ASVs) of bacterial communities by reproductive role (worker vs. reproductives). The boxes indicate interquartile ranges, lines inside the boxes denote medians, whiskers extend to 1.5 times the interquartile range, and dots are outliers.

Table 2.2: Statistical output of the six linear mixed models that tested the effect of 'reproductive role' (workers vs. reproductives) on each of the six alpha diversity measures (Shannon, Faith's phylogenetic distance (PD), Pielou's evenness, Simpson, Chao1, and Observed ASVs). Number of samples in each statistical model: N=31. 'Reproductive role' was the only explanatory variable in each model. 'Colony' was included as a random effect in all models to account for variation in bacterial communities among colonies. The percentage of model variance explained by colony ID is shown as the difference between the conditional and marginal R-squared values.

Diversity measure	Estimate	SE	$\chi 2$	DF	p-value	% random effect 'colony ID'
Shannon	1.09	0.350	9.68	1	**< 0.001**	62%
Faith's PD	2.02	0.820	6.08	1	**0.01**	42.7%
Pielou's evenness	0.19	0.053	13.0	1	**< 0.0001**	55.6%
Simpson	0.273	0.070	5.13	1	**0.02**	48.9%
Chao1	22.49	9.94	14.9	1	**< 0.0001**	52.6%
Observed ASVs	22.0	9.71	5.13	1	**0.02**	48.3%

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Chapter 3: The effects of social interactions, physical environment, and evolutionary history on the microbiome of harvester ants

The effects of social interactions, physical environment, and evolutionary history on the microbiome of harvester ants

Abstract

The composition and diversity of animal microbiomes are impacted by a variety of factors, including the environment, behavior, and phylogeny. Uncovering which factors impact animal microbiomes is important for gaining a more in-depth understanding of the ways in which hosts interact with the microbial communities that they harbor. Here, we study three species of harvester ants to ask if social interactions, physical environment, and phylogeny relate to host microbial communities. To test if social interactions impact microbial communities, we experimentally manipulated the ability of ants to interact with one another. To examine the impact of the environment, we compared the microbiome of ants from a natural setting to the microbiome of ants that were kept in the lab. To examine the relationship between microbiome and evolutionary history, we compared the microbial communities of three harvester ant species, *Pogonomyrmex rugosus, P. californicus,* and *V. pergandei,* that have similar ecological roles but differ in phylogeny. We found that the environment (field vs. lab), as well as phylogeny, explained differences in microbiome composition and diversity among ants. However, despite ants being highly social animals, social interactions did not seem to impact microbial composition or diversity. The microbiome of ants that were kept in isolation did not significantly differ from the microbiome of ants that were allowed to interact with nestmates. Our work highlights the importance of examining multiple drivers of microbiome composition and diversity and provides a fertile foundation for future work on the mechanisms and function of microbiomes in harvester ants.

Introduction

Studying the bacterial communities that live on and within animals provides new insights into animal behavior. The internal and/or external microbiome of social individuals can have implications for intraspecific recognition of group members and kin, mate choice, and evolutionary costs and benefits of social relationships (Archie & Theis, 2011; Desbonnet et al., 2014; Lu et al., 2018; Vuong et al., 2017). Several new studies demonstrate the potential for microbial ecology to advance our understanding of animal behavior, particularly in relation to social recognition and the consequences of sociality (Archie & Theis, 2011; Redford et al., 2012; Vuong et al., 2017). The application of animal microbiomes to broad ecological and behavioral questions suggests that there is a relationship between an individual's microbiome and its social interactions, physical environment, evolutionary history, physiology, and genotype (Archie & Theis, 2011; Grieneisen et al., 2021). Here, we examine these relationships by manipulating social interactions and the physical environment in three different species of harvester ants, which differ in evolutionary history.

Social relationships mediate the exposure and susceptibility to bacteria from conspecifics (Archie & Theis, 2011). While the transmission of pathogens is a well-known cost of sociality, as it is facilitated through both direct physical contact and indirect contact from the environment (i.e., food, water, air, and refuges) (Ezenwa et al., 2012), the study of how sociality impacts the transmission of beneficial, or natural, microbes is emerging. Previous studies in group-living primates suggest that social contact facilitates the transmission and composition of various types of microbes, including both harmful, beneficial, and neutral (Goodfellow et al., 2019; Moeller et al.,

2016; Perofsky et al., 2017; Tung et al., 2015). A study by Raulo et al. (2021) found that social association strength was the strongest predictor of microbiome similarity among individuals in wild mice (*Apodemus sylvaticus*), even when controlling for spatial proximity and kinship. In Welsh mountain ponies, microbiome composition varied between groups, but there was significant individual variation within a group in microbiome composition and diversity despite communal living (Antwis et al., 2018). Similarly, bumblebees (*Bombus terrestris*) obtain functionally beneficial bacteria directly via social interactions (Koch & Schmid-Hempel, 2011). In carpenter ants (*Camponotus fellah*), there is a correlation between social network position and microbiome composition (Kay et al., 2023). Thus, group-living in animals shapes the microbiome composition at the individual and group level. In addition, the microbiome itself may shape social interactions and animal behavior. The composition of the gut-microbiome defines social group membership and influences the rate of social interactions in honeybees (Liberti et al., 2022; Vernier et al., 2020). Additionally, microbiome composition influences cuticular odor, which impacts nest-mate recognition and changes to it led to increased aggression levels in leaf-cutter ants (*Acromyrmex echinatior*) (Teseo et al., 2019). Furthermore, microbiome composition is related to learning performance in honeybees, with *Lactobacillus* Firm-5 cluster bacteria in the microbiome related to increased learning abilities (Li et al., 2021). Further research is needed to determine how changes in the microbiome relate to behavioral interactions because of the ways in which the microbiome influences the ability to recognize group members and because of the impact that interactions may have on microbial composition. The social environment is often confounded by the physical environment;

therefore, manipulating the social environment in a set physical environment may help identify how sociality shapes microbiome composition.

Animal hosts are constantly exposed to microbes from their physical environment (Gonzalez et al., 2011). Animals that live in their natural environment harbor distinct microbiome communities compared to animals that live in urban cities (Dillard et al., 2022; Littleford-Colquhoun et al., 2019; Sugden et al., 2020) or in captive environments, including zoos (Alfano et al., 2015; Bornbusch et al., 2022; McKenzie et al., 2017), and laboratories (Bowerman et al., 2021; Rosshart et al., 2017). For example, coyotes (*Canis latrans*) living in urban environments have increased gut microbiome diversity, poor average body condition, and increased susceptibility to parasites compared to rural coyotes (Sugden et al., 2020). Similarly, living in an urban environment reduced microbiome diversity and negatively impacted animal health in house sparrows (*Passer domesticus*) (Teyssier et al., 2018, 2020), American white ibises (*Eudocimus albus*) (Murray et al., 2020), tree frogs (*Dendropsophus minutus*) (Becker et al., 2017), lizards (*Zootoca vivipara*) (Bestion et al., 2017), and red columbus monkeys (*Procobus gordonoorum*) (Barelli et al., 2015). The observed decrease in microbiome diversity in urban environments and concomitant negative effects on survival was due to the loss of functional microbial symbionts that assist with essential metabolic and immunological pathways, thus decreasing body size and increasing susceptibility to pathogen exposure. Similarly, studies in ground beetles (*Carabus convexus*) (Magura et al., 2024) and carpenter bees (*Ceratina calcarata*) (Nguyen & Rehan, 2022) showed a decrease in microbial diversity in urban settings but did not find a negative impact on survival. White-crowned sparrows (*Zonotrichia leucophrys*) (Phillips et al., 2018) in urban settings

showed an increase in microbial diversity but no difference in body condition. Relatedly, studies on captive animals show differences in microbiome composition across natural and captive environments. For example, Florkowski et al. (2023) observed changes in the microbiome diversity of songbirds (*Passer domesticus*) that were moved from a 'wild' environment to a 'captive' environment. The birds were kept in captivity for 5-10 days, and even this brief exposure to captivity resulted in a decrease in both alpha and beta diversity of their microbiome. Animals kept in captive laboratory settings, such as deer mice (*Peromyscus maniculatus*) and house mice (*Mus musculus*), have an increased microbiome diversity (Bowerman et al., 2021; Diaz et al., 2023). The influence of the laboratory environment on microbiome diversity varies in ant species. Carpenter ants (*Camponotus fragilis*) reared in lab settings had an increased microbial diversity compared to ants collected in the field (He et al., 2014). Whereas lab conditions resulted in decreased microbial diversity in *Temnothorax nylanderi* (Segers et al., 2019). In a controlled lab setting, animals may be exposed to novel bacterial strains from humans, changes in their diet, or their housing containers. Although the directionality of microbial diversity varies in relation to the environment, it is clear that differences in the physical environment (natural vs captive) in which an animal resides can influence the acquisition and diversity of host-associated microbial communities.

The relationship between ants and their associated microbes varies widely across species and is influenced by host natural history, ecology, and phylogeny. We often see species-specific conserved mutualisms with microbial symbionts in ant species that have specialized diets. One of the most well-known examples is fungus-farming ants that have a symbiotic relationship with the fungus that they cultivate to feed

on and with bacterial symbionts (*Pseudonocardia*) that protect the fungus from pathogens (Little & Currie, 2007; Moreau, 2020). Previous work across ant species shows that species with highly specialized diets tend to have a higher density of gut-associated bacteria (Moreau, 2020). For example, herbivorous ants such as carpenter ants (*Camponotini*) and turtle ants (*Cephalotes*) will have a more similar microbiome composition compared to predatory ants, such as army ants (*Eciton*) and bullet ants (*Paraponera clavata*) (Anderson et al., 2012; Russell et al., 2009; Valdivia et al., 2023). The core gut microbes of herbivorous and predatory ants are essential and assist the host with nutrient acquisition (Funaro et al., 2011; Hu et al., 2018; Łukasik et al., 2017; Russell et al., 2009). However, the gut microbiome of ants with generalized diets tends to have less diverse and fewer functional gut-associated microbial communities (Hammer et al., 2019; Moreau, 2020; Russell et al., 2017). There is very little work on the microbiome of granivorous seed-harvesting ants. A previous study on various species of *Pheidole* ants (clade *Myrmicini*) found that geographic location and food source may influence their microbial communities (Martins & Moreau, 2020). Similarly, in the harvester ant, *V. andrei* (clade *Stenammini)*, reproductives and larvae had distinct microbial communities compared to adult workers (Gamboa et al., 2025). Both *Pheidole* and *V. andrei* were characterized by the presence of *Actinobacteria*, *Proteobacteria*, and *Firmicutes,* which is typical of herbivorous ant species (Anderson et al., 2012; Gamboa et al., 2025; Martins & Moreau, 2020; Russell et al., 2009). However, *Firmicutes* dominated the microbial communities of adult *V. andrei* workers, which is more typical of predatory ant species (Anderson et al., 2012; Gamboa et al., 2025; Valdivia et al., 2023). Although there is some information available about the

microbiome of *Pheidole* ants and *V. andrei*, harvester ants are a functional group that includes any ant species that collects and stores seeds. This category is a broad classification that includes over 150 species across 18 genera and three subfamilies *(Myrmicinae, Ponerinae, Formicinae)* (Hölldobler & Wilson, 1990; MacMahon et al., 2000; Ward et al., 2015). Although prior studies found evidence that the physical environment and life history shape the diversity of the bacterial communities of *Pheidole* and *V. andrei* harvester ants, none of them alone explains the bacterial diversity in this ecologically and evolutionarily diverse harvester ant group.

Here, we asked if social interactions, physical environment, and evolutionary history (phylogeny) impact the relationship between microbial communities and their hosts by studying three species of harvester ants. *Pogonomyrmex californicus, P. rugosus,* and *V. pergandei* harvester ant species provide an excellent opportunity to examine the relationship between host-associated bacterial communities and social interactions, physical environment, and evolutionary history. All three species are harvester ants that recruit to collect and store seeds (Branstetter, 2012; MacMahon et al., 2000; Ward et al., 2015). *Pogonomyrmex rugosus and V. pergandei* are from arid environments, whereas *P. californicus* is from a Mediterranean climate (Branstetter, 2012; MacMahon et al., 2000; Ward et al., 2015). Additionally, they come from two genera (*Pogonomyrmex* and *Veromessor)*, each from distinct clades, *Pogonomyrmecini* (*P. californicus* and *P. rugosus)* and *Stenammini (V. pergandei)* (Ward et al., 2015). To test if social interactions impact microbial communities, we experimentally manipulated the social environment. We predicted that ants living with nest mates would have more homogenous microbial communities (i.e., lower beta diversity) than solitary ants. To

examine the impact of the environment on microbial communities, we compared the microbiome of ants from a natural setting to the microbiome of ants that were kept in the lab for one week. We predicted that ants from the field would have a more diverse microbiome (higher alpha and beta diversity) than ants that were kept in a lab setting. Finally, to examine the relationship between microbiome and evolutionary history, we compared the microbial communities of three harvester ant species that have similar natural history and ecological roles but differ in their phylogeny. We predicted that the microbiome composition of ants from closely related species (*P. californicus* and *P. rugosus*) would be more similar to one another than to the composition of a species from a different genus and clade (*V. pergandei*).

Methods

Study site and sample collection

We collected worker ants from three species of harvester ants, *P.californicus, P. rugosus,* and *V. pergandei*. Ants from each species were collected from a specific study site in southern California (Figure 3.1A), and all ants were collected outside of their nest, from foraging trails. For each species, we collected approximately 100 ants from three distinct colonies. We used gloves (sterilized with 90% ethanol) to collect all ants. To test the effect of the physical environment on ant microbiomes, a subset of collected ants was immediately placed in separate test-tubes and stored on ice in a cooler as 'control' samples. To test the effect of social interactions on the microbiome, approximately two-thirds of the collected ants were maintained alive in plastic containers with a damp paper towel until arrival at the lab. All samples were transported to the UCLA campus, and the control samples were immediately stored at -20℃ until processing.

Testing the effect of the social environment

To determine the relationship between social interactions and microbial communities, we manipulated the social environment of the ants in the lab by assigning the live ants to either a ‘group’ or an ‘individual’ treatment (Figure 3.1B). Ants assigned to the ‘group’ treatment were placed in a group of 25 ants in a large plastic container (0.7 L (24 oz) deli containers), and ants assigned to the ‘individual’ treatment were each placed individually in small plastic containers (0.05 L (2 oz) plastic cups). All containers were covered with lids that had small holes created with a pin, to allow for air flow and prevent ants from escaping. To reduce mortality, all ants were provided with Perky-Pet Red Hummingbird Nectar Concentrate diluted 1:5, which provides necessary sucrose and contains food preservatives that reduce the likelihood of bacterial and fungal growth on the food. In the ‘group’ treatment, the food was provided in small Eppendorf tubes plugged with cotton balls to allow the fluid food to wick and be available to the ants. In the ‘individual’ treatment, food was provided by dipping small cotton balls in the liquid food and placing them in Eppendorf tube lids. Ants in both treatments were kept in their containers for seven days (Figure 3.1B) and then frozen in separate Eppendorf tubes at -20℃ until processing. When freezing the ants, individuals from all treatments, including the control and group treatments, were each placed in a separate tube such that each test tube for processing and DNA isolation contained a single ant.

Sample processing

Each sample contained a single ant from one of the three treatments (control, group, or individual). We analyzed a total of 135 samples: five ants from each treatment for each colony (N=15 per colony), three colonies from each species (N=15*3 = 45 per species),

and three species (N=45*3 =135 total). Following the same sample preparation protocol used in Gamboa et al. (2025), we washed each ant with 70% ethanol, 5% bleach, and sterile deionized water. We placed each washed ant in a new, sterile 1.5 ml tube and crushed it with a sterile pestle (Fisher Scientific). We followed the Qiagen DNEasy PowerSoil Pro kit protocol and added 800 µL of cell lysis CD1 solution to all samples and then vortexed each sample for 5 seconds. We incubated the samples overnight at 56℃ in a shaking incubator for 24 hours to facilitate cell lysis. We then extracted microbial DNA using the Qiagen DNEasy PowerSoil Pro kit protocol and sent the samples for 16S rRNA gene amplification and library sequencing at the UCLA Microbiome Center. Amplicon sequencing of the bacterial community targeted the V4 region of the 16S rRNA gene using primers 515F (59-GTGCCAGCMGCCGCGGTAA-39) and 806R (59-GGACTACHVGGGTWTCTAAT-39, following the Earth Microbiome Project (EMP) protocol (Caporaso et al., 2011; Jacobs et al., 2021). Information about the samples can be found in the GitHub repository: https://github.com/DAlejandraG/social-microbes. We processed the sequencing data using QIIME2 (version 24.5) (Bolyen et al., 2019) following the demultiplexing and quality filtering protocols from Gamboa et al. (2025). The raw 16S rRNA gene amplicon sequencing reads, totaling 7,290,950, are available on NCBI BioProject Record [PRJNA1311785]. We rarefied the dataset to a sampling depth of 21,788 and retained 3,486,080 features (47.8%) after refraction. Ten samples were removed due to low read numbers and are not shown in any of the figures. We then assessed microbial diversity using the q2-diversity plugin, based on ASVs. To generate relative abundance plots at

the phylum and order levels, we used the ‘phyloseq’ package in R (McMurdie & Holmes, 2013).

Statistical analysis

We examined the influence of social interactions, physical environment, and evolutionary history on the diversity of host-associated bacterial communities by analyzing beta diversity (between samples) and alpha diversity (within a sample). Beta diversity reflects the differences in bacterial community composition between samples, grouping them based on dissimilarities in sequence abundances or the presence/absence of sequences (Borcard et al., 2018). Alpha diversity describes the distribution of microbial taxa within a single sample. To determine if there are differences in microbial diversity based on ant species and treatment, we applied a permutational multivariate analysis of variance (PERMANOVA) on a Bray-Curtis dissimilarity matrix. Principal Coordinate Analysis (PCoA) ordination was generated from the dissimilarity matrix using the Adonis function in the Vegan package (Oksanen et al., 2001), with 999 permutations for the PERMANOVA. We used the ‘ggplot2’ R package (Wickham, 2016) to visualize PCoA plots.

To compare the microbial diversity between ant species and experimental treatments (control, individual, and group), we used four alpha diversity indices: (1) Shannon’s diversity index measures how evenly bacterial taxa are distributed, independent of their richness (Kim et al., 2011; Lemos et al., 2011). (2) Faith’s phylogenetic distance (PD) measures species richness, accounting for phylogenetic diversity (Faith, 2007). (3) Pielou’s evenness is a measure of the relative abundance of bacterial taxa (Pielou, 1966). (4) Observed amplicon sequence variants (ASVs)

measure the number of observed unique sequences in a sample (Callahan et al., 2016). To compare alpha diversity across species and treatments, we ran four linear mixed models (LMMs) for the four alpha diversity measures (Shannon, Faith's phylogenetic distance, Pielou's evenness, and Observed ASVs). In each model, the dependent variable was an alpha diversity measure. We included treatment (control, individual, and group) and species (*P. californicus, P. rugosus,* and *V. pergandei)* as the explanatory variables in the LLMs. We included 'colony' as a random effect in all LLMs to account for variation in microbial communities among colonies. We used the glmmTMB() function in the 'glmmTMB' R package for all models (Brooks et al., 2017; McGillycuddy et al., 2025). For post hoc tests, we used the R package 'emmeans' (Lenth et al., 2024). We report the proportion variance explained by the random effects as the conditional R^2 minus the marginal R^2, obtained using the r2_nakagawa() function in the 'performance' R package (Lüdecke et al., 2021). Data and code are available on GitHub:https://github.com/DAlejandraG/social-microbes

Results

Microbial composition was explained by species and by whether the ants were housed in the lab for a week before being frozen or whether they were frozen immediately upon being collected in the field (Figure 3.2). Analysis of the beta diversity of the samples showed clustering according to species and treatment (Figure 3.3). 'Species' explained 13.7% of the variation in beta diversity (PERMANOVA: $F_{DF=2} = 10.1$, $R^2 = 0.137$, p-value = 0.001). 'Treatment' explained approximately 5% of the total variation (PERMANOVA: $F_{DF=2} = 3.79$, $R^2 = 0.051$, p-value = 0.001). A post-hoc pairwise PERMANOVA showed that the microbial composition of *V. pergandei* differed significantly from the microbial composition of both *Pogonomyrmex* species, but that the

two *Pogonomyrmex* species did not differ significantly in their microbial composition from one another (Table 3.1). In addition, the post-hoc pairwise PERMANOVA analysis showed that the ‘control’ treatment was statistically significantly different from the ‘individual’ and ‘group’ treatments, but that the ‘individual’ and ‘group’ treatments did not significantly differ from one another (Table 3.1).

Furthermore, whether ants were frozen immediately after collection in the field or after being kept in the lab for 7 days significantly impacted microbial alpha diversity (Figure 3.4, Table 3.2). Control samples of ants that were frozen without spending time in the lab had higher alpha diversity compared to both the ‘group’ and the ‘individual’ treatments of ants that were kept in the lab, as measured by the Shannon and Faith’s phylogenetic distance diversity indices and Observed ASV (Figure 3.4A, B, D, Table 3.2). Alpha diversity according to Pielou’s evenness was significantly different between the ‘control’ and ‘group’ treatments, but the ‘individual’ treatment did not differ significantly from the other two treatment groups (Figure 3.4C, Table 3.2). Finally, there was a significant effect of ‘species’ in the statistical model examining Pielou’s evenness (Table 3.2). Still, the post-hoc pairwise Tuckey test did not reveal which species were different from one another (Figure 3.5), possibly because other effects in the statistical model masked any slight differences in Pielou’s evenness between species.

Discussion

We found that the physical environment had a large impact on the microbial diversity of harvester ants. Ants that were frozen immediately after being collected from the field differed substantially in their microbial communities compared to ants that were brought into the lab and kept there for a week (either isolated or in a group) before being frozen

(Figures 3.2, 3.3A, 3.4, and Tables 3.1, 3.2). Furthermore, ant species differed in their microbial diversity, with the two *Pogonomyrmex* species having similar microbial communities compared to *V. pergandei* (Figure 3.3B and Table 3.1). Interestingly, social interactions did not seem to impact the microbial composition of harvester ants. We did not detect differences in the microbial diversity between ants that were kept isolated in the lab and ants that were allowed to interact with nestmates for a week in the lab (Figures 3.3B, 3.4, and Table 3.1).

The bacterial communities we identified in the samples are typical of ant microbiomes. The most abundant phyla we detected are *Firmicutes*, *Proteobacteria,* and *Actinobacteria* (Figure 3.2). Other studies of ant microbiomes have also found these phyla to be the most abundant, in *V. andrei* (Gamboa et al., 2025) and other herbivorous and omnivorous ant species (Anderson et al., 2012; Russell et al., 2009). The presence of bacterial orders *Lactobacillates* and *Enterobacterales* was consistent with findings in adult *V. andrei* workers but not in reproductives (Gamboa et al., 2025). Additionally, the presence of the bacterial order *Rhizobiales,* which have been associated with nitrogen recycling and fixing, is consistent with bacterial communities found across various ant species, including leaf cutter ants, herbivorous turtle ants, arboreal ants, and predatory ants (Anderson et al., 2012; Eilmus & Heil, 2009; Hu et al., 2018; Nepel et al., 2022; Neuvonen et al., 2016; Russell et al., 2009; Sapountzis et al., 2015; Stoll et al., 2007). Previous studies on the harvester ant species *V. andrei* and *Pheidole* also found *Rhizobiales* in microbiome analysis (Gamboa et al., 2025; Martins & Moreau, 2020). Thus, *Rhizobiales, Firmicutes*, *Proteobacteria,* and *Actinobacteria* are present across three different clades and three genera of harvester ants

(*Pogonomyrmecini: P. californicus, P. rugosus, Stenammini: V. Andrei, V. pergandei, Myrmicini: Pheidole spp*), which may suggest a strong association of these bacteria with harvester ants. The nature of this relationship (e.g., whether it is symbiotic) should be investigated in future work.

Interestingly, the physical environment seemed to have a very large effect on the microbiome of the ants in our study. Ants that did not spend time in the lab had different and more diverse microbial composition compared to ants that were kept in the lab for a week (isolated or in a group) (Figures 3.2, 3.3A, 3.4, and Tables 3.1, 3.2). Indeed, Segers et al. (2019) found a similar pattern - ants that were kept in the lab for two months had different microbial communities compared to ants sampled in the field, with time in the lab decreasing microbial diversity. Interestingly, He et al. (2014) found an overall increase in gut bacterial diversity for lab-reared carpenter ants compared to field-collected ants. One possible explanation for these observed differences between ants from the field and those that spent time in the lab is a change in diet. In our study, in the lab, ants were fed with a simple liquid sugar water diet, which might have altered their gut microbiome. Indeed, the food that animals (and humans) eat changes their gut microbiome (Diaz et al., 2023; Gonzalez et al., 2011; Sugden et al., 2020; Zhang, 2022), and specifically in mammals and birds, sugars in the diet decrease the functional diversity of the gut microbiome (Sugden et al., 2020; Teyssier et al., 2018, 2020). While we did not specifically examine the gut microbiome, as done in other studies of ant microbiome (Flynn et al., 2021), we did sterilize the cuticle while processing the ant samples to obtain internal microbes, which would include the gut microbiome. Further

work on how sugars impact the gut microbiome of ants might inform lab rearing practices.

The three species we examined differed in their gut microbiomes despite all species being harvester ants and potentially sharing a similar diet composed predominantly of seeds. Interestingly, we found that the two species from the genus *Pogonomyrmex* had more similar microbial communities to one another than to *V. pergandei* ants (Figures 3.2, 3.3A). Specifically, at the phylum level, both *Pogonomyrmex* species were characterized by a higher abundance of *Firmicutes* compared to *V. andrei,* which was characterized by higher *Proteobacteria* abundance (Figure 3.1A). All species had similar *Proteobacteria* abundance (Figure 3.1A). Although *Firmicutes, Actinobacteria,* and *Proteobacteria* are consistent with other herbivorous ant species, predator ant species tend to have higher levels of *Firmicutes* (Anderson et al., 2012; Russell et al., 2009; Valdivia et al., 2023). While the *Pogonomyrmex* and *Veromessor* genera are in the same subfamily, phylogenetic work shows that they belong to different non-sister clades (Ward et al., 2015). The genus *Pogonomyrmex* is in the *Pogonomyrmecini* clade, consisting of two genera (*Pogonomyrmex* and *Hylomyrma)* and characterized by granivory and preference for arid (dry, low moisture) (MacMahon et al., 2000; Ward et al., 2015) or mesic (moderate moisture levels) environment (MacMahon et al., 2000; Ward et al., 2015). Additionally, certain species in this clade are not always specialist seed-harvesters (Ward et al., 2015). The *Veromessor* genus belongs in the *Stenammini* clade, which is nonmonophyletic and consists of five other species-rich genera (Ward et al., 2015). *Veromessor* species are characterized by a granivorous diet and preference for arid environments (Branstetter,

2012). Ant species differ substantially in their microbiome across taxa (Moreau, 2020), just like the species in our study. Specifically, microbial community composition has been shown to be shaped by host phylogeny. For example, *Formica* ants are characterized by core microbes that vary based on host species (Jackson et al., 2023). While both *V. pergandei* and *P. rugosus* live in dry deserts, compared to *P. californicus,* which lives in a more Mediterranean climate, we did not find that their microbiomes were more similar to one another (Figure 3.3A). Although geographic location has been shown to be a good predictor of microbiome similarity (Birer et al., 2020; Gamboa et al., 2025), the site from which we collected *P. rugosus* is similarly distant from the two other sites (Figure 3.1A). Yet, the microbiome of *P. rugosus* is more similar to that of *P. californicus* than to that of *V. pergandei* (Figure 3.3A). Furthermore, colony ID did not explain differences in microbial composition (Figure S3.1, Table 3.2). Thus, it seems as though phylogenetic similarity is a better predictor of microbiome similarity than geographic distance, climatic conditions, and colony identity.

Finally, the social environment did not appear to impact the microbial communities of harvester ants. Ants that were isolated did not differ in their microbial composition or diversity compared to ants that were allowed to interact with nestmates (Figures 3.2, 3.3B, 3.4). This finding is surprising because social interactions shape microbial composition in primates, rodents, equines, and certain social insects, including carpenter ants and bumblebees (Antwis et al., 2018; Goodfellow et al., 2019; Kay et al., 2023; Koch & Schmid-Hempel, 2011; Moeller et al., 2016; Perofsky et al., 2017; Raulo et al., 2021; Tung et al., 2015). However, it is possible that because both treatment groups received the same food in the lab, the impact that the food had on their gut

microbiome masked differences that might have emerged from social interactions. In other social insects, trophallaxis is one way in which microbial communities are shared (Koch & Schmid-Hempel, 2011; Onchuru et al., 2018; Salem et al., 2015). However, harvester ants do not engage in much trophallaxis compared to other ant species. Social interactions may further facilitate the exchange of microbes among ants through brief antennal contacts. However, because we sterilized the ant samples, we were unable to examine extra-cuticular microbes, potentially reducing our ability to detect differences between ants that were kept in solitary vs group conditions. Future work is needed to uncover the mechanisms and types of interactions by which social behavior impacts either internal or external microbial communities in ants.

Conclusions

Physical environment (lab vs nature) and phylogenetic similarity were both important predictors of microbiome diversity and composition in the harvester ants that we studied. Despite ants being highly social animals, we did not find support for social interactions playing an important role in determining microbial diversity and composition. Thus, our work highlights that many factors can interact, sometimes in surprising ways, to impact the microbiome of animals. Further work on the mechanisms of microbiome acquisition and the functional characteristics of the microbiome is necessary to uncover the intricate relationships between animals and their associated microbial communities.

Figures and Tables

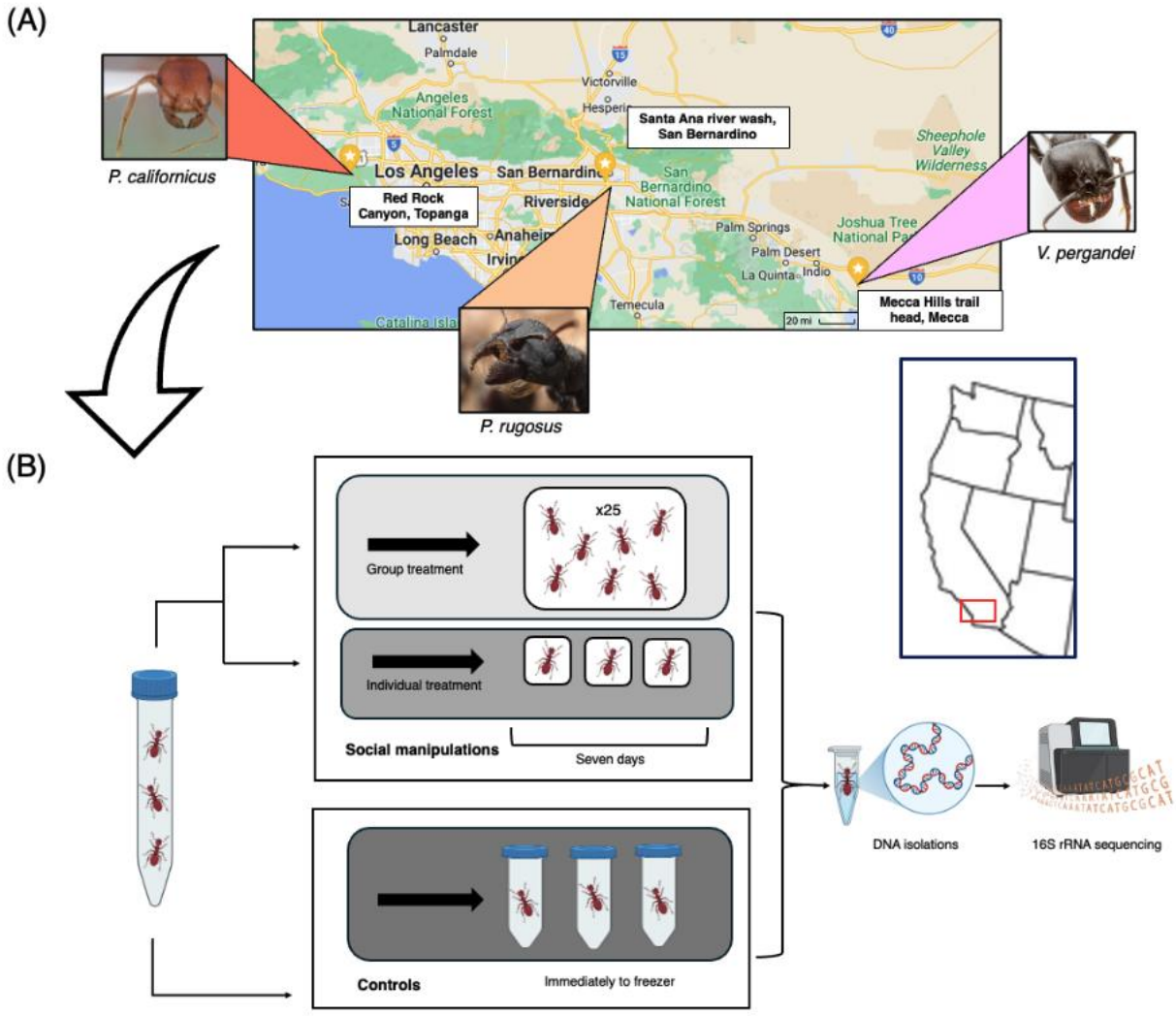

Figure 3.1. **Sample collection and processing.** (A) Map of the three study sites in southern California where we collected *P. californicus, P. rugosus,* and *V. pergandei* ants. At each site, we collected approximately 100 ants from each of three distinct colonies. At each site, we collected ants from a different harvester ant species - as shown on the map. The inset to the right shows in a red rectangle the location of the detailed map in the western United States. (B) Schematic representation of sample processing. After collecting ants in the field, they were separated into three different

treatments (control, individual, and group). Control ants were put on ice and stored in the freezer immediately after returning from the field. Ants in the ‘individual’ and ‘group’ treatments were brought to the lab and either kept together in groups of 25 ants (‘group’ treatment) or isolated in individual plastic cups (‘individual’ treatment). Ants were kept in these conditions for seven days and were then placed in the freezer and stored until processing. After DNA extractions, all samples were sent to the UCLA Microbiome Center for 16S rRNA sequencing.

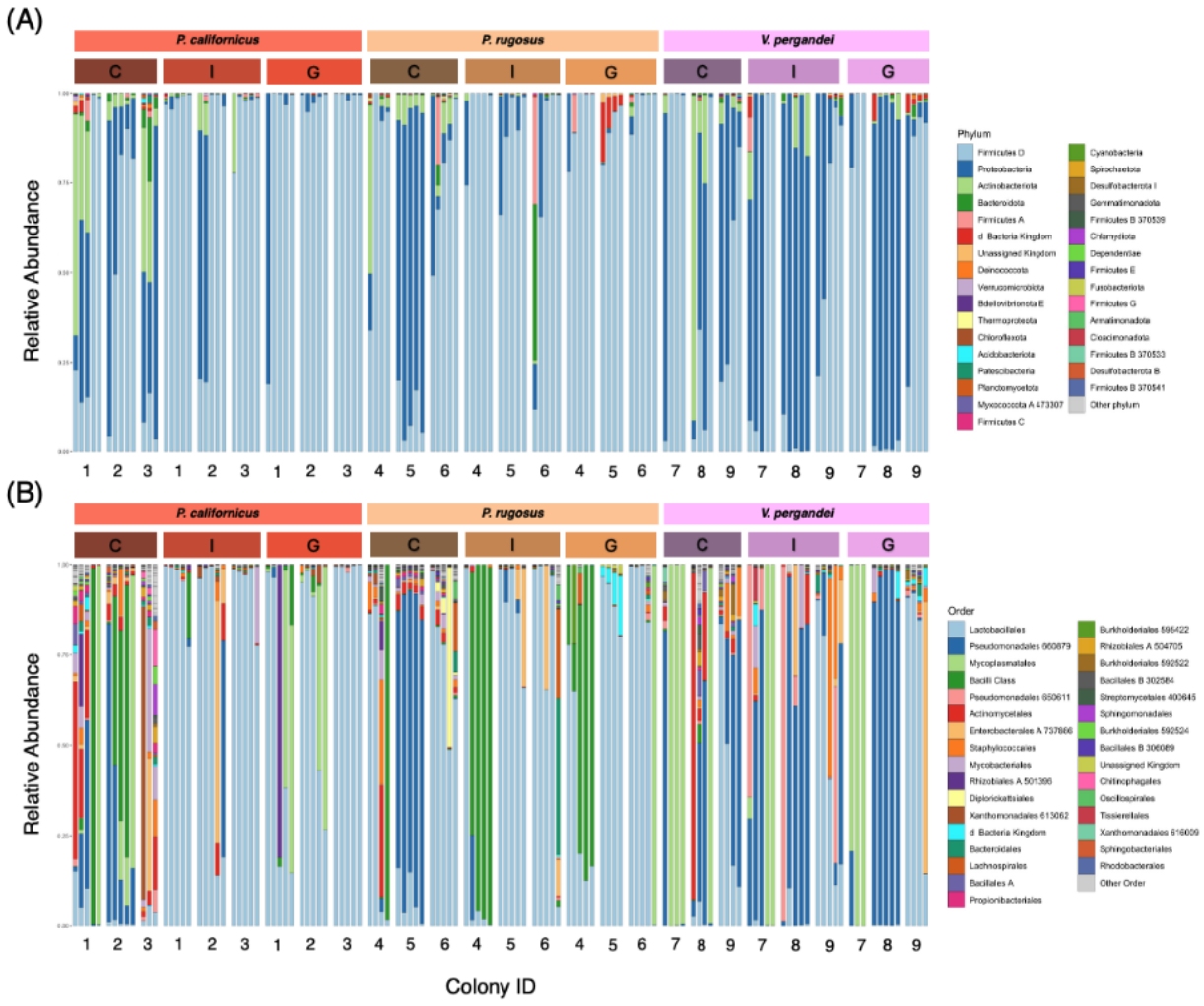

Figure 3.2: Relative abundance of microbial taxa at the level of (A) phylum and (B) order. In both panels, the samples are ordered by 1) ant species: *P. californicus, P. rugosus,* and *V. pergandei,* 2) treatment (C - control, I - isolated individuals, and G - groups), and 3) colony ID (1-9). Each vertical bar represents a single sample of a single ant as detailed in the text. The color of the bars represents the bacterial phylum or order, as determined by ASV counts, with a sampling depth of 21,788 reads.

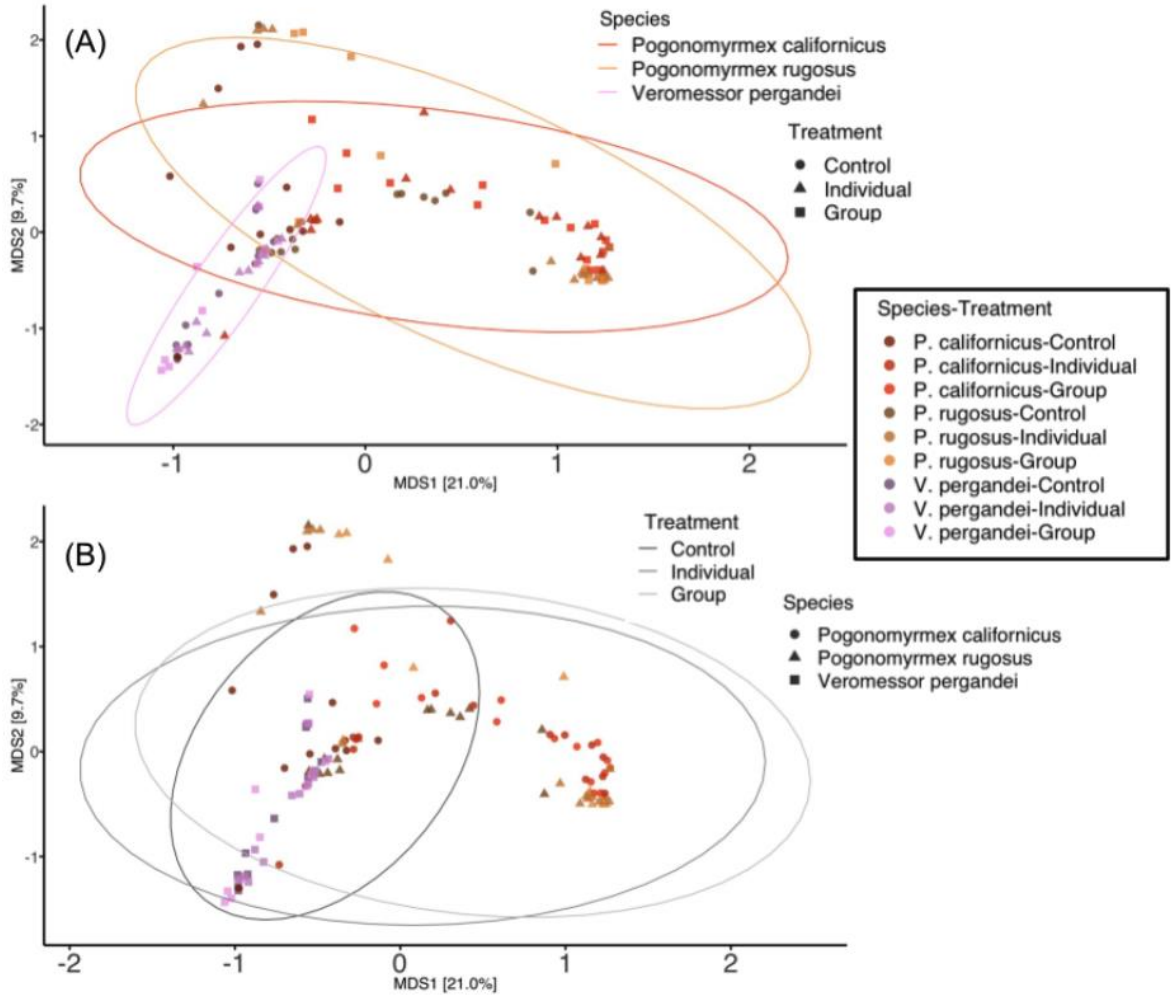

Figure 3.3: Microbial community beta diversity. Principal coordinate analysis (PCoA) plot of Bray-Curtis dissimilarity matrix of ant samples across treatments (control (darkest shades), individual (medium shades), and group (lightest shades)) and species (*P. californicus* (red), *P. rugosus* (orange), and *V. pergandei* (lavender)). In both plots, the colors of the points correspond to species-treatment combinations, as detailed in the legend that is in the box to the right. The data in both panels are the same, but we grouped them by (A) species as colored ellipses and shapes corresponding to treatments, and (B) treatment as ellipses and shapes as species. See the legend in each panel for details.

Table 3.1: Pairwise PERMANOVA results comparing beta-diversity using Bray-Curtis distances between treatments (control, individual, and group) and species. Statistically significant values are in bold.

Pairs	DF	Sum of Squares	F Model	R^2	P value	P adjusted
Control vs Group	**1**	**2.03**	**5.08**	**0.06**	**0.001**	**0.003**
Control vs Individual	**1**	**1.40**	**3.35**	**0.04**	**0.002**	**0.006**
Group vs Individual	1	0.62	1.58	0.02	0.093	0.279
P. californicus* vs *V. pergandei	**1**	**4.48**	**11.89**	**0.13**	**0.001**	**0.003**
P. californicus vs *P. rugosus*	1	1.02	2.84	0.03	0.008	0.024
V. pergandei* vs *P. rugosus	**1**	**5.25**	**14.28**	**0.15**	**0.001**	**0.003**

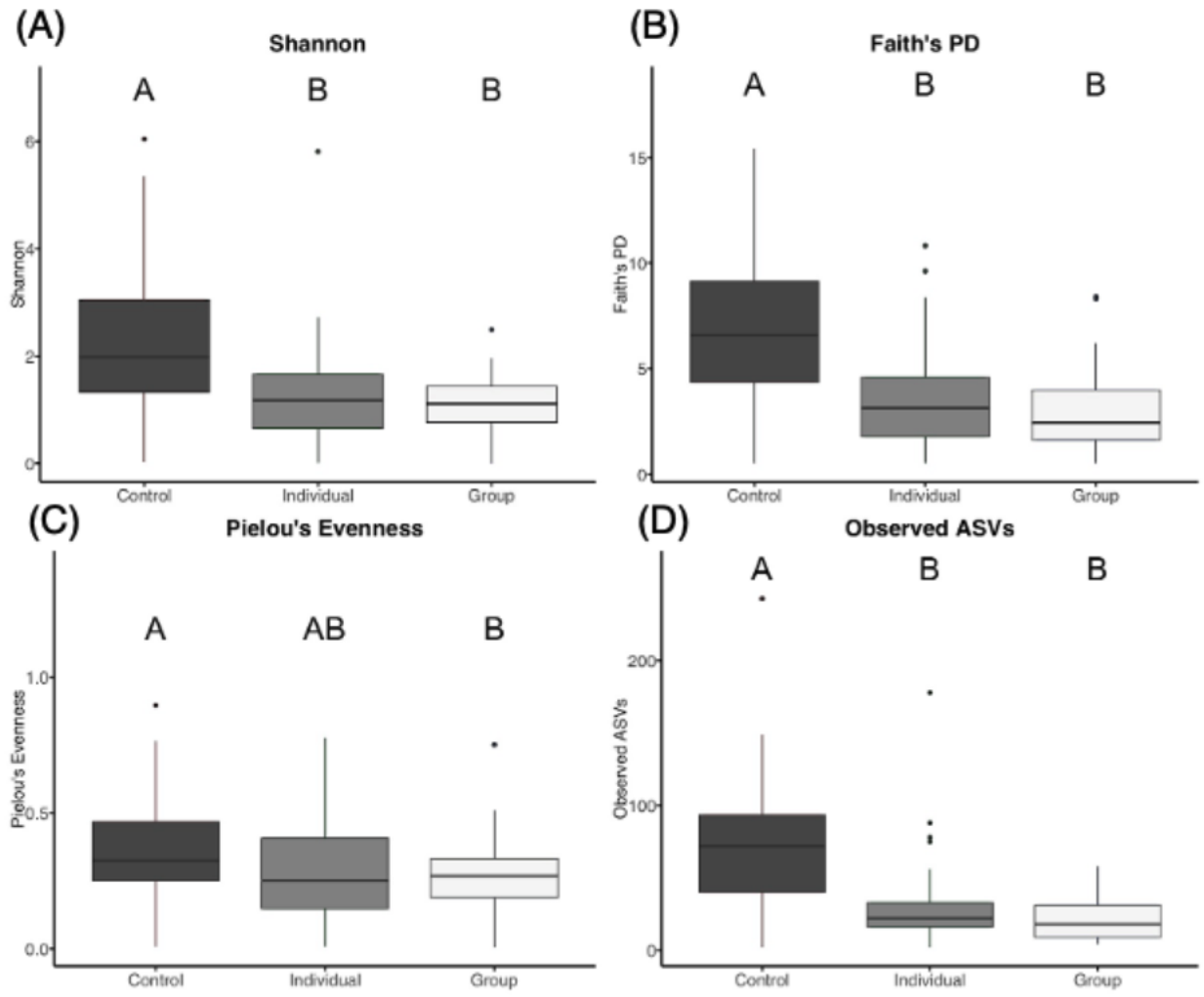

Figure 3.4: Microbial community alpha diversity across treatments (control, individual, and group) for all species. (A) Shannon diversity, (B) Faith's phylogenetic distance (PD), (C) Pielou's evenness, and (D) Observed ASVs. The boxes indicate interquartile ranges, horizontal lines inside the boxes denote medians, whiskers extend to 1.5 times the interquartile range, and dots are outliers. Boxes that do not share letters are statistically significantly different according to a post-hoc Tukey test (p-value < 0.05).

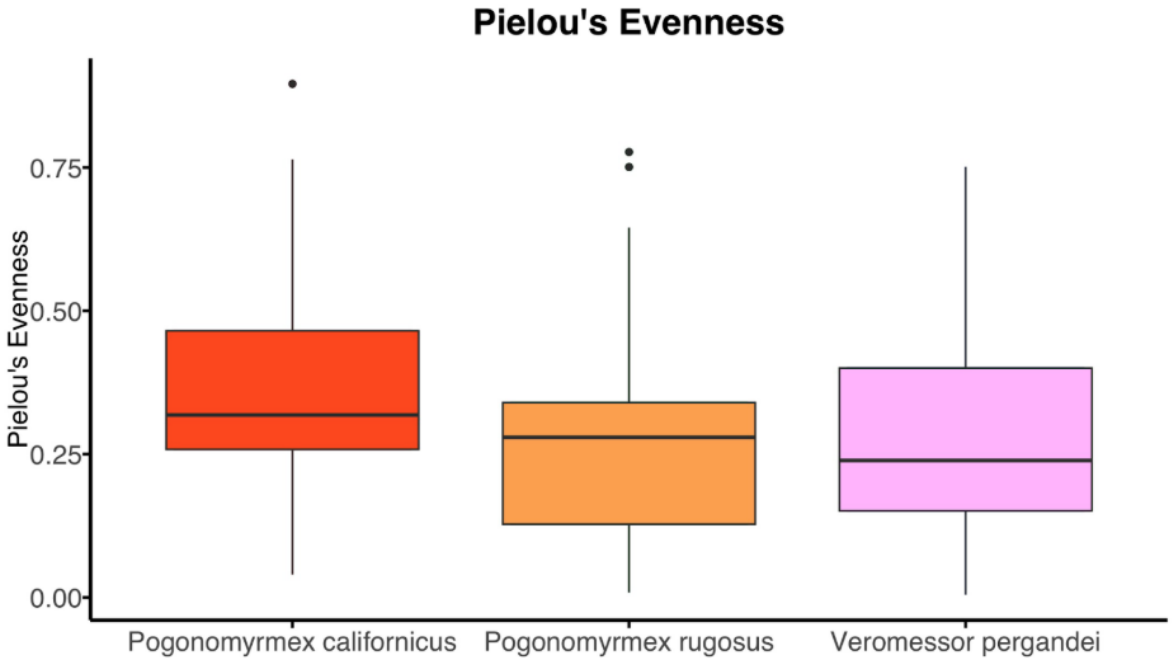

Figure 3.5: Microbial community alpha diversity, measured as Pielou's evenness, by ant species. While 'species' was a significant effect in the statistical model (Table 3.2), a post-hoc Tukey test did not reveal significant differences between the three species.

Table 3.2: Analysis of deviance for the statistical output of the four linear mixed models (LMMs) that tested the effect of 'treatment' (control, individual, and group) and 'species' (*P. californicus, P. rugosus,* and *V. pergandei*) on each of the four alpha diversity measures (Shannon, Faith's PD (phylogenetic distance), Pielou's evenness, and Observed ASVs). The number of samples in each statistical model: N = 125. 'Colony' was included as a random effect in all models to account for variation in bacterial communities among colonies.

Diversity measure	Effect	X^2	DF	p-value	% random effect 'Colony ID"
Shannon	**treatment**	**28.2**	**2**	**< 0.0001**	**0%**
	species	5.16	2	0.076	
Faith's PD (phylogenetic distance)	**treatment**	**50.8**	**2**	**< 0.0001**	**6.8%**
	species	0.23	2	0.893	
Pielou's evenness	**treatment**	**8.50**	**2**	**0.012**	**0%**
	species	**7.22**	**2**	**0.023**	
Observed ASVs	**treatment**	**54.9**	**2**	**< 0.0001**	**2.7%**
	species	1.29	2	0.527	

Supplementary Materials

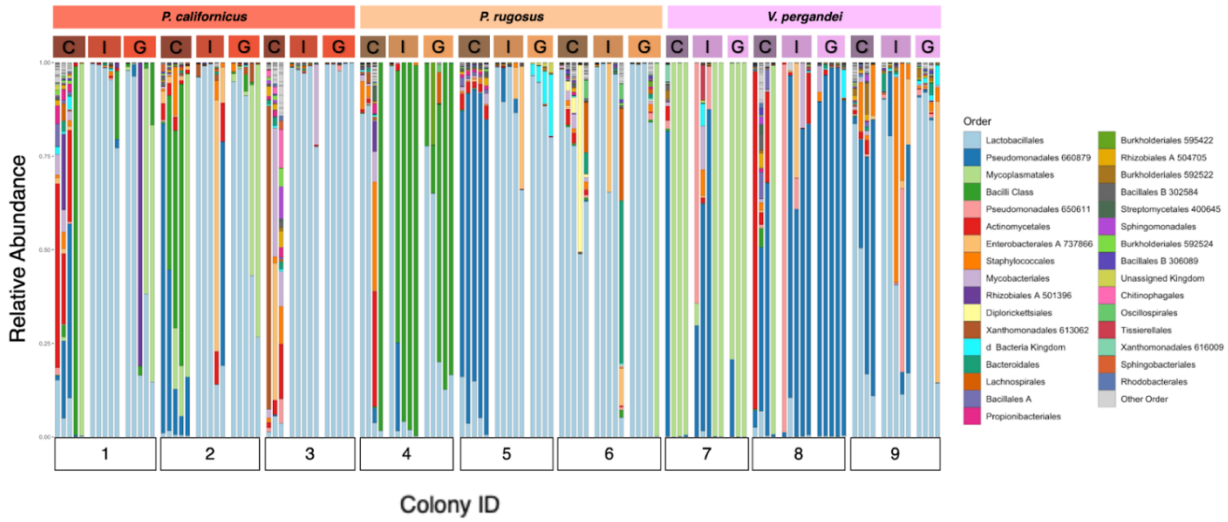

Figure S3.1: Relative abundance of microbial taxa at the order level. We use the same data as Figure 3.2, but the samples here are organized to emphasize colony ID on the x-axis. Each vertical bar represents a single sample of a single ant, as detailed in the text. The color of the bars represents the bacterial phylum or order, as determined by ASV counts, with a sampling depth of 21,788 reads.

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Chapter 4: Social organization and physical environment shape the microbiome of harvester ants

Social organization and physical environment shape the microbiome of harvester ants

Abstract

All animals harbor microbes, which are obtained from the surrounding environment and are impacted by host behavior and life stage. To determine how two non-mutually exclusive drivers - physical environment and social organization - affect an organism's microbiome, we examined the bacterial communities within and around nests of harvester ants (*Veromessor andrei*). We collected soil and nest content samples from five different ant nests. We used 16S rRNA gene sequencing and calculated alpha and beta diversity to compare bacterial diversity and community composition across samples. To test the hypotheses that physical environment and/or social organization impact ant colonies' community of microbes we compared our samples across (i) sample types (ants, brood, seeds and reproductives (winged alates), and soil), (ii) soil inside and outside the nest, and (iii) soil from different chamber types. Interestingly, we found that both the environment and social organization impact the bacterial communities of the microbiome of *V. andrei* colonies. Soil from the five nests differed from one another in a way that mapped onto their geographical distance. Furthermore, soil from inside the nests resembled the surrounding soil, supporting the physical environment hypothesis. However, the bacterial communities associated with the contents within the nest chambers, i.e., ants, brood, seeds, and reproductives, differed from one another and from the surrounding soil, supporting the social organization hypotheses. This study highlights the importance of considering environmental and social factors in understanding microbiome dynamics.

Introduction

Animals are associated with and are colonized by communities of microorganisms that are shaped by biotic and abiotic conditions, known collectively as the microbiome. In addition to vertical and horizontal acquisition, animals obtain microbes from the environment, and their behavior and life stage have a large impact on their microbiome. Throughout their lives, animals are colonized by microbes from their surroundings [1–3]. Because microbes are acquired in various ways, composition of microbial communities depends on how they are obtained or transmitted. Studies in humans and non-human primates suggest that the surrounding environment including habitat, diet, or social group can significantly influence microbiome composition [4–6]. The composition of the gut microbiome varies across captive, urban, and rural environments in many organisms such as Ring-tailed lemurs [7], Tasmanian devils [8], deer mice [9], water dragons [10], coyotes [11], beetles [12], carpenter bees [13], and honeybees [14]. In addition to the environment, host behaviors significantly impact host-microbiome dynamics. For example, communal nesting in four-toed salamanders (*Hemidactylium scutatum*) increases the transmission of beneficial, antifungal bacteria, enhancing hatchling survival compared to solitary nests [15,16]. Furthermore, coprophagy, a behavior involving the consumption of feces, regulates gut microbiota in vertebrates and invertebrates [17,18]. Thus, both the physical environment and the behavior of an individual impact its microbiome.

Host-associated microbiome studies often consider microbial communities inside or on the surface of the organism, however, many animals occupy stable burrows or construct nests [19]. Because of the large amount of time that animals spend in their

nests and burrows [20,21], the microbial communities of these built structures comprise much of the microbial communities that animals are exposed to through the physical environment. Indeed, the type of building materials used in nest construction can affect the health of the animals that build them, if materials such as resin and leaves with anti-bacterial properties are incorporated into the nest [22]. Despite this potentially large impact of nest microbial communities on its inhabitants, little is known about the relationship between the microbiome inside a nest and the microbiome of its inhabitants. Environmentally acquired microbes tend to be ephemeral and not host-specific due to the functional redundancy of bacterial species and the changing environmental conditions that both hosts and their microorganisms are exposed to (e.g., temperature, humidity, nutrients) [23]. Indeed, the microbiome of animals is often determined by the environment in which they live. For example, when the cuticular microbiomes of two arboreal ant species were compared, the physical location of their nest was a better predictor of their microbiome composition than the species of ant [24]. Similarly, when comparing the gut microbiome of weaver ants (*Oecophylla smaragdina*) from forest and urban environments, forest colonies had an increased abundance of *Acetobacteraceae* compared to urban colonies [25]. Therefore, the relative impact of the environment on the microbiome of an animal is important to consider, especially for animals whose environment is an integral component of their lives, such as soil-nesting ants. Microbial diversity and biomass in soils have been linked to a wide range of soil properties including factors that change with soil depth [26] such as soil pH, soil organic carbon, and oxygen [27,28]. Therefore, we expect that if the microbiome of animals that

live in soil is impacted by the environment, such subterranean animals will have microbiomes that mirror the soil's, including decreased microbial diversity with depth.

The behavior of an animal and the social organization of a population can impact the microbiome of an animal, and the microbiome can provide information specific to the host. For example, in spotted hyenas, microbiome varies with sex and age-class, and it is specific to individuals [29]. Further, the gut microbiome composition of individual chimpanzees from the same communities are similar due to their shared diets but long-term immigrants into the population show distinct gut microbiome composition, suggesting that immigrant individuals retain characteristics of their original community's gut microbes, despite moving to a new environment [30]. In social insect colonies individuals perform different behavioral tasks, which influence and structure the microbiome composition of individuals within colonies. For example, honeybee workers that perform different behavioral tasks, such as foraging, or nursing, show differences in gut bacterial community composition [31] and the gut microbiome can influence the onset of certain tasks, such as foraging [32]. Additionally, there are differences in the gut microbiome composition of reproductives and non-reproductive workers in termites [33,34], honeybees [35,36], and ants [37,38]. Thus, the behavioral role of individuals can have a strong impact on their microbiome.

Ants are highly social animals that shape the environment in which they live - their nest. Ants create nests, for example, by connecting leaves with silk [39], digging through wood [40], excavating soil [39] which alters soil distribution [41], among other means. The nest microbiome interacts with the ant microbiome. For example, the cuticular microbiome of two arboreal Amazon ant species overlaps with the bacterial

microbes found within their nests [24]. Most studies of ant microbiomes have focused on the gut microbiome, showing that ant species differ in the densities of bacterial communities in the gut according to diet type [42] and that ants can benefit from their microbiome via nutrient acquisition and defense against pathogens [42,43]. However, to our knowledge, the role of the nest in shaping the microbiome of ant colonies has only been explored in arboreal ants that occupy exiting tree cavities [44] and not in subterranean ants that construct and shape their own nest. In lab experiments, each nest region was found to have a different chemical signature that reflects the individuals that occupy that area [45]. Thus, it is likely that a similar relationship between the structure of subterranean nests and the materials inside each chamber shape the microbiome of a subterranean ant colony and its nest, as seen in arboreal ants [44]. Across ant species, behavioral tasks occur at specific locations within a nest, such that when not foraging, foragers are found near the entrance of the nest and brood nurses are found in the center, where the brood is located [45–48]. This spatial division of labor can structure the microbial composition of individuals within colonies of ants. The relationship between social organization and spatial position suggests that the physical environment and social organization combine to influence the microbiome of ant colonies. For example, nest chambers of ants might differ in their microbiome composition based on the behavioral tasks performed in them. Such potential differences in chamber microbiome can be driven by the chambers' content (e.g., the seeds or the brood) or by the ants that tend to the chamber material (Figure 4.1). Furthermore, the microbiome found inside ant nests might differ from the surrounding

soil, just as plant composition on nest mounds of ants differs from the surrounding environment [49].

Colonies of the harvester ants, *Veromessor andrei,* live in grasslands, where they turn and aerate the soil, redistributing nutrients and potentially creating favorable conditions for microorganisms within and around their subterranean nests [49–52]. Colonies of *V. andrei* are large, reaching sizes of tens of thousands of workers [53], and one queen, i.e., the colonies are monogynous [53,54]. They nest in grasslands habitats, where their primary diet is seeds, which workers gather by following long (up to tens of meters) foraging trails [54,55]. *Veromessor andrei* nests provide an excellent opportunity to examine the effects of the physical environment and social organization on the microbiome of the colony because of the strong effects that nest structure has on colony behavior. Nests of *V. andrei* are comprised of chambers connected with tunnels and the connectivity among nest chambers, and especially that of the entrance chamber, affects the speed of foraging recruitment [56]. The impact of nest structure on foraging behavior happens most likely through the impact of the nest structure on interactions among ants within the nest [57], which regulate foraging activity in other harvester ants [58–60]. Nest structure can potentially further segregate behavioral tasks such as brood care and food storage. However, it is not known how nest structure and social organization combine to impact the microbiome of harvester ant colonies.

To determine the roles of the physical environment and social organization in structuring an organism's microbiome we examined the bacterial communities of the microbiome within and around nests of *V. andrei*. Specifically, we test two non-mutually exclusive hypotheses: (1) the physical environment determines microbial community

structure; and (2) social organization determines microbial composition (Figure 4.1). We predict that if the physical environment influences microbial communities (1) the bacterial communities associated with the content of the nest (like ants, seeds, brood, etc.) will be similar to the bacterial communities in the surrounding soil; (2) the bacterial communities in the soil inside nest chambers will not differ from those in the surrounding soil; (3) bacterial diversity in nest chambers will decrease with nest depth, similarly to the relationship between depth and microbiome in soils [26]; and (4) nests in different locations will have different bacterial diversity because soils change their microbial composition and diversity spatially [27,28] (Figure 4.1a). We predict that if social organization influences microbial communities of ant nests (1) nest content (like ants, seeds, brood etc.) will differ in their bacterial composition according to their biological classification and will be different from the bacterial composition of the surrounding soil; (2) bacterial diversity of chamber soil will differ across chambers according to the content found in them, regardless of the bacterial communities in the surrounding soil; (3) bacterial diversity of chamber soil will differ from the bacterial diversity of the surrounding soil; and (4) bacterial composition of soil inside nest chambers will be conserved by the content of the chamber in a way that is consistent across different nests (Figure 4.1b).

Methods

Study site and sample collection

To examine the microbiome of ant nests' soil and of the content of the nests, we collected samples from five colonies of *V. andrei* in May 24-29, 2021, from a serpentine grassland at the Sedgwick Natural Reserve in southern California, US. Sedgwick

Reserve is home to a thriving *V. andrei* population (>100 colonies), and we selected five colonies that could be easily accessed, were far from other ant nests, to reduce any negative impact of excavation on other colonies, and whose nests were off the road - so that nest excavation would not disrupt access (Figure 4.2a). To access the nest content, we first dug a trench approximately 1-1.5m away from the nest entrance, using a tractor fitted with a post hole digger, pickaxes, and shovels. Once the trench was established, we began digging towards the nest until we reached a chamber from its side and sampled its content, as detailed below. Once we completed sampling a chamber, we continued excavating in the direction of the tunnels leading out of the chamber, until we found another chamber and sampled it too. We proceeded to excavate and sample from all nest chambers until we could not find any more new chambers that were not sampled (Figure 4.2b). When we reached a chamber, we collected with gloves or soft tweezers (sterilized with ethanol) samples of its content, which included ants, brood, seeds, and reproductives (defined as male and unmated female winged alates and not the founding queen) (Figure 4.2e-h). Most chambers included ants, but not all chambers included brood, seeds, and reproductives. We placed each type of sample in a separate, labeled, 15ml tube. After sampling the chamber's content, we used a small disposable plastic spoon to collect soil from inside the excavated chamber (Figure 4.2d), referred to later as 'nest soil'. Nest chamber soil samples were classified into 'chamber types' according to the chamber content (e.g., if they had ants inside, they were considered 'ant' chamber type). If a chamber had more than one type of content (e.g., both brood and reproductives were found in the same chamber) the chamber was assigned a type based on all the material in it (e.g., brood + reproductives). We then

(using a new disposable plastic spoon) sampled soil from a location outside the nest, within approximately 5cm of the excavated chamber, and at the same depth as the chamber, which we referred to as 'near soil control' (Figures 4.2b, c). We recorded the depth of the chamber from the ground surface using a measuring tape. Once all chambers were excavated, we obtained the 'control far soil' samples by collecting soil from the side of the trench that was opposite the nest (Figure 4.2c). We used a measuring tape to sample soil from depths that matched those of the chambers we excavated. Thus, each chamber had several associated samples - three soil samples (from inside the chamber, near the chamber, and far (~1-2m) from the chamber - at the same depth) and samples of the content of the chamber (ranging from 1-3 additional samples - depending on the content we found). Each day we excavated one nest, with the first four nests (A, B, C, and D) excavated on consecutive days (May 24-27, 2021) and the fifth one (E) collected after a one-day break (on May 29th, 2021). Around noon and at the end of each day, around 5pm, we placed the samples we collected in a freezer at the field station. Nests differed in depth and number of chambers. All samples were transferred in a cooler to the UCLA campus (approximately a 2-hour drive from the field site), where they were stored in a -20 freezer until processing.

Sample processing

After collection, we stored and kept the samples at -20°C until extractions. To prepare the soil samples for extractions we weighed, using a microscale, up to 250 mg of soil and placed the soil into sterile 1.5ml tubes. To prepare the non-soil samples (ants, seeds, brood etc.) for extractions we first washed ant workers and alates three times: first in 70% ethanol, then in 5% bleach, and then with sterile deionized water. We

placed each washed sample in a sterile 1.5ml tube and used a sterile pestle (provided in the kit by Qiagen), that specifically fits in 1.5 ml tubes, to crush the ants. Seed and brood samples were crushed but not washed. Brood samples were not washed because the bleach and alcohol used in the wash protocol would have destroyed the samples. Seeds were not washed because we were interested in quantifying the microbial communities on their exterior. We added 800μl of Solution CD1 from the Qiagen DNEasy PowerSoil Pro kit, containing SDS for cell lysis to all samples (including soil) following manufacturer's instructions. To facilitate cell lysis, we vortexed and left the samples overnight in an incubator at 56°C. Microbial DNA was extracted using the same Qiagen DNEasy PowerSoil Pro kit following manufacturer's instructions. After DNA extraction, 285 samples were sent for sequencing at the UCLA Microbiome Center for 16S rRNA gene amplification and library sequencing. Amplicon sequencing of the bacterial community was performed using the V4 region of the 16S rRNA gene using the primers 515F (59-GTGCCAGCMGCCGCGGTAA-39) and 806R (59-GGACTACHVGGGTWTCTAAT-39) following the Earth Microbiome Project (EMP) protocol [61,62].

We collected and sequenced 285 samples (see data in the Github repository: https://github.com/DAlejandraG/nest-microbes). The 16S rRNA gene amplicon sequencing raw reads are available from NCBI via BioProject record PRJNA1147938. The raw dataset contained a total of 4,884,506 reads. We rarefied the dataset at a sampling depth of 3,618 and retained 955,152 features (25.73%) after refraction with a total of 264 samples (92.65%) after 22 samples were removed. We then removed 21 sample types that were obtained for only some nests or did not have a large enough

sample size to include in the analysis (e.g., entrance soil and soil from the mound). Lasty, we removed four samples due to errors in labeling during sample collection or during sample extraction. Code for this data cleaning is available on Github: https://github.com/DAlejandraG/nest-microbes.

Quantifying bacterial community diversity

To determine the bacterial community composition of each sample type (soil, seeds, brood, adult ants), bioinformatics were conducted using QIIME2 version 2024.2.4 [63]. Initial raw sequence data underwent demultiplexing and quality filtering with the q2-demux plugin, followed by denoising using DADA2 [64] through the q2-dada2 plugin. Amplicon Sequence Variants (ASVs) were aligned using MAFFT [65] via q2-alignment, and a phylogeny was constructed with FastTree2 [66] through q2-phylogeny. ASVs were assigned taxonomy using the q2-feature-classifier [67] classify-sklearn naive Bayes taxonomy classifier, referencing the Silva 13_8 99% OTU database [68]. ASVs are used as a proxy for bacterial species and are similar to OTUs (operational taxonomic units) but at a finer-scale resolution (100% similarity). Once the quality filtering steps were completed, we estimated refraction, alpha and beta diversity measures using q2 diversity based on ASVs. We created a summary feature table (see Github: https://github.com/DAlejandraG/nest-microbes) with information on how many sequences are associated with each sample. To create relative abundance plots at the phylum, order, family, and genus taxonomic levels, and assess species composition, we exported the feature table and used the '*phyloseq*' package in R [69]. To further examine the abundance and phylogenetic relationship among the most abundant ASVs in the ant and soil samples, we pruned the phylogeny using the drop.tip function from

the ape R package [70], retaining only the ASVs detected in each sample type. The ComplexHeatmap package in R [71] was used to generate heatmaps of the top 20 most abundant ASVs for ant and nest soil samples. Additionally, Similarity Percentage Analysis (SIMPER) was conducted to identify the specific contributions of ASVs to the composition of bacterial communities across ant and soil samples. The analysis was performed in R using the Vegan package [72] with 999 permutations.

To determine how the nest-associated bacterial communities are influenced by the physical and social environment, we examined diversity within (alpha diversity) and among (beta diversity) samples. The input for all diversity measures was ASVs. Alpha diversity indices provide information regarding the number of microbial taxa in a single sample. The alpha diversity indices we used include:

1. Shannon's index - describes how evenly species are distributed, independent of species richness [73,74]. A high Shannon index indicates more species diversity whereas a value of zero indicates that fewer species are present in the sample.
2. Faith's phylogenetic diversity - a weighted measure of richness that describes the amount of the phylogenetic tree that is covered by the communities, i.e. more evolutionary branches would result in greater diversity [75].
3. Pielou's evenness - provides information about the relative abundance of species in a sample, i.e., if some species are dominating others or if all species have similar abundances [76].
4. Observed amplicon sequence variants (Observed ASVs) - the number of observed unique sequences that are present in the sample [64].

Beta diversity provides information about the differences in bacterial community composition among multiple samples, classifying samples into groups according to similarities in their bacterial composition based on sequence abundances or the presence or absence of sequences [77]. Here we used the Bray-Curtis dissimilarity index as a beta diversity measure of compositional dissimilarity among bacterial communities [78]. We measured beta diversity differences between samples using a permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarity matrices. Principal Coordinate Analysis (PCoA) ordination was calculated based on these matrices using the Adonis [79] and Vegan package [72], with 999 permutations for the PERMANOVA. The resulting PCoA plots were visualized using ggplot2 [80]. We performed the PERMANOVA on Bray-Curtis distances calculated from the rarefied dataset to test for dissimilarities in bacterial community composition among samples based on the ASVs. This analysis tested for differences in beta diversity among all sample types, all soil types, and nest soil samples.

Statistical analysis

All analysis was conducted in R version 4.3.2 [81] and all of the best-fitting models met the required statistical assumptions – examined using the check_model() function in the 'performance' package [82].

All sample types: To determine if alpha diversity differed across all sample types, we ran four linear models (LM) - one for each of the four alpha diversity measures (Shannon, Faith's phylogenetic diversity, Pielou's evenness, and ASV richness) as the response variable. The explanatory variable was the type of sample (ants, seeds, reproductive, brood, or soil). We used the lm() function in R [81] for these models. For

post-hoc comparisons of bacterial diversity among sample types, we used a post hoc Tukey test by applying the Tukey HSD() function in R [81]. We further examined PCoA plots and used a PERMANOVA to examine beta diversity across sample types.

All soil samples: To determine if alpha diversity changed with soil depth and differed across locations, we ran linear models (LM) implemented as detailed above. In each model one of the four alpha diversity measures (Shannon index, Faith's phylogenetic diversity, Pielou's evenness, and ASV richness) was the response variable. The explanatory variables included: depth, nest ID, and soil type (chamber soil, control near, and control far). For post hoc tests we used the package 'emmeans' [83]. We used a model selection approach to determine which interaction terms to include in our final statistical model. We ran each model with either no interactions among soil type, depth, and nest ID; with the three-way interaction among the three variables; and three additional models with just one interaction each between a different pair of variables each time, totaling five statistical models per alpha diversity measure. We then compared the models using AIC [82] and selected the best fit model, i.e., the one with the lowest AIC score. The best fit models for all diversity measures included no interaction terms among the explanatory variables. For specific comparisons of bacterial diversity among soil types, we used a post hoc Tukey test [83]. We further examined PCoA plots and used a PERMANOVA to examine the beta diversity among soil samples and the five different nests. For specific comparisons of bacterial diversity among soil types, we used pairwise PERMANOVA tests by applying the pairwise.adonis( ) function in the package 'pairwiseAdonis' [79]. To assess differences in the amount of dispersion, we conducted a permutational analysis of multivariate

dispersions (PERMDISP) by applying the betadisperser( ) and permutest( ) functions in the package ‘Vegan [72].

Chamber soil samples: To determine if the bacterial composition in the soil inside nest chambers differed based on chamber type (i.e., the content found in the chamber: ants, seeds, reproductives, and brood), we ran four linear models (LM) and post hoc tests as detailed above. In each model, the response variable was one of the four diversity indices (Shannon index, Faith’s phylogenetic diversity, Pielou’s evenness, and ASV richness) and the explanatory variables included: nest ID, sample depth, and chamber type (based on the content listed above). We used the same model selection approach detailed above [82] and if the best fit model included an interaction term, but the collinearity was very high (VIF> 10), so we removed the interaction term. Due to high collinearity among terms in the models, we ended up keeping only models with no interaction terms. We further examined PCoA plots and used a PERMANOVA to examine beta diversity across chamber types.

Data and code are available in the Github repository: https://github.com/DAlejandraG/nest-microbes.

Results

All sample types

The alpha, and beta diversity of all samples differed significantly by sample type, supporting the social organization hypothesis. Ants, reproductives, brood, seeds, and soil had different ASV compositions, regardless of which taxonomic level we examined (Figures 4.3, S4.1-S4.4). The top three ASVs varied in relative abundance across sample type (Figure 4.4). Furthermore, ants, reproductives, brood, seeds, and soil

significantly differed in alpha diversity, calculated based on ASVs, regardless of which diversity measure we examined (Table 4.1, Figure 4.5). A post hoc Tukey test showed that ant samples had the lowest, and soil samples had the highest, alpha diversity compared to all other sample types, across all measures of alpha diversity. Brood and reproductives did not differ significantly in their alpha diversity across all diversity measures. Finally, seeds and reproductives showed significant differences in the Faith's Phylogenetic distance measure (Figure 4.5a).

The principal coordinate analysis (PCoA) demonstrated that the samples' ASVs clustered by 'sample type' (Figure 4.5b). 'Sample type' explained a significant amount of variation in the dataset, explaining approximately 22.42% of the total variation (PERMANOVA: $F_{DF = 4} = 16.91$, $R^2 = 0.22$, p-value = 0.001). Pairwise PERMANOVA results showed a significant difference between most pairwise comparisons (Adjusted $p < 0.01$, Table S4.1). Only samples of brood and reproductives were not significantly different. The comparisons with ants (ants vs. soil, ants vs. seeds, ants vs. brood, ants vs. reproductives) show high R^2 values, indicating that ants explain a substantial proportion of the variance in these comparisons (Table S4.1). Soil comparisons (soil vs. seeds, soil vs. brood, soil vs. reproductives) show lower R^2 values, suggesting less variance explained by soil (Table S4.1).

The similarity percentage (SIMPER) analysis, combined with heatmap visualizations, identified the top 20 bacterial ASVs contributing to the structural composition of bacterial communities within ant and nest soil samples (Figure 4.4a, b, Tables S4.2, S4.3). Among these, three ASVs (668c7, 94215, and 43e06), were classified within the phylum *Firmicutes* (class Bacilli) based on the bacterial ASV

phylogenetic analysis (Figures S4.5, S4.6). These top three ASVs exhibited markedly higher read abundances in ant samples compared to other sample types (Figure 4.4c, d, e), highlighting their distinct association with ants.

All soil samples

Bacterial alpha diversity of soil from inside and outside the nest differed only for one alpha diversity measure, providing stronger support for the 'physical environment' than the 'social organization' hypothesis (Table 4.2). Soil type (nest soil, control near, and control far) significantly impacted only the Pielou's Evenness index but none of the other alpha diversity measures (Table 4.2, Figure 4.7a). Soil depth did not have a statistically significant effect on any of the alpha diversity measures of the bacterial communities in the soil (Table 4.2). Finally, nest ID significantly impacted all alpha diversity measures except for Faith's phylogenetic distance (Table 4.2, Figure 4.6a).

Bacterial community beta diversity was explained both by soil type (nest soil, control far, control near) and nest ID (A, B, C, D, E). 'Soil type' explained 4% of the variation in beta diversity of all soil samples (PERMANOVA: $F_{DF = 2}$ = 12.76, R^2 = 0.04, p-value = 0.001), this variation was not due to differences in dispersion (PERMDISP: $F_{DF = 2}$ = 2.871, mean sq = 0.006, p-value = 0.061). 'Nest ID' explained 25% of the variation in beta diversity of all soil samples (PERMANOVA: $F_{DF = 4}$ = 12.62, R^2 = 0.25, p-value = 0.001). Thus, both Soil type and nest ID play an important role in determining the variance in the dataset. All pairwise comparisons among soil samples from the five different nests (A, B, C, D, E) were statistically significantly different except for the difference between nests D and E, with adjusted p-values ≤ 0.01 (Table S4.1). The R^2 values varied, with the highest being 0.228 and smallest being 0.051 (Table S4.1).

Comparisons of nest soil to control (near and far) were statistically significant but with lower R^2 values - ranging from 0.012 to 0.042 (Table S4.1) – and indeed grouping by soil type explains a smaller proportion of the variance (4%) compared to grouping by nest ID (25%). Pairwise PERMANOVA results indicate that control soils - near and far were not significantly different from one another (Table S4.1).

Nest soil samples

Our comparison across chamber types of bacterial diversity of soil inside the nest did not support either of our two hypotheses. We did not find a significant effect of chamber type or of sample depth on any of the alpha diversity measures (Table 4.3; Figure 4.8a). Similarly, the beta diversity of soil from inside the nest was not explained by chamber type (PERMANOVA: $F_{DF = 4} = 1.26$, $R^2 = 0.08$, p-value = 0.064). However, 'Nest ID' had a significant effect on the beta diversity of soil from inside the nest (PERMANOVA: $F_{DF = 4} = 5.55$, $R^2 = 0.35$, p-value = 0.001).

Discussion

Our study suggests that both social and environmental factors may shape the bacterial communities of V. *andrei* colonies. In support of the physical environment hypothesis (Figure 4.1) we found that the bacterial communities of nests in different locations varied significantly across alpha and beta diversity (Figure 4.6) and the relative abundance of the nest soil bacterial communities was similar to that of the control soil samples (Figure 4.3). In support of the social organization hypothesis, we found that the bacterial communities of the nest contents differed according to biological classification and was different from the bacterial communities of the surrounding soil (Figure 4.3, Figure 4.5). These differences in bacterial community composition were

consistent across the order, class, family, and genus taxonomic levels (Figures S4.1-S4.4). Furthermore, the beta diversity and evenness of the soil bacterial communities inside the nest was significantly different from the control soil samples (Figure 4.7, Table S4.1). However, the bacterial communities' diversity of the nest soil did not differ significantly across chambers according to the contents found in them (Figure 4.8).

Differences in the bacterial communities' composition within *V. andrei* nests (biotic and soil samples) provide partial support for the physical environment hypothesis. Overall, the bacterial communities of the biotic content of the nest (ants, reproductives, seeds, and brood) were significantly different from those in the soil inside the nest and the surrounding soil (Figures 4.3, 4.5, S4.1-S4.4). These findings align with previous research indicating that the microbiomes of *Formica exsecta* ants are different from those present in the nest and alpha and beta diversity were lower in ant samples compared to the nest material [84]. However, this work on *F. exsecta* did not differentiate between inside chambers and surrounding soils and only sampled nest material from the top layer of the soil (0-20cm). Further, our findings are consistent with other work on social insects, such as termites [34], honeybees [31], and ants [37,38,85,86] that show differences in the microbiome of brood, reproductives, and workers. In our study only brood samples were similar to soil samples, according to Faith's PD diversity measure and Observed ASVs, and reproductives were not significantly different from soil based on Pielou's evenness. The similarity between brood and soil can be explained by the fact that we did not wash the brood during processing because they would have disintegrated due to the lack of outer protection and contact with harsh chemicals [87]. In addition, we did not wash the seed samples

during processing, because we were interested in sequencing the microbes that were found on their exterior, yet we still found significant differences in alpha diversity between the seed and soil samples. Therefore, not washing the brood samples might not be the only explanation for not finding differences between brood and soil samples. The beta diversity of seeds, reproductives, and brood, were all similar (Figure 4.5b). This finding might be explained by the fact that brood are the primary consumers of protein [88,89], which comes from seeds. Protein is required for brood growth but is not required by worker ants – that do not grow in size after they eclose, and they rely mostly on carbohydrates for energy, and may metabolize lipids from seeds for water [90]. Furthermore, reproductives are most likely recently eclosed, being closer in developmental stage to brood than workers. Thus, it is possible that some bacterial species from seeds are present in the developmental stages that feed on them (brood) and the ants that recently fed on them (reproductives). These findings are consistent with other work on microbiome of honeybees [91] and ants [92], that have highlighted the role of developmental stage on microbiome composition, and with studies that found an impact of diet on microbiome composition of ants and honeybees [93–95].

In further support of the physical environment hypothesis, most alpha diversity measures of the soil bacterial communities inside the nest did not differ from the surrounding soil, either near, or far, from the nest. This result suggests that the bacterial species inside the nest come from the surrounding environment, as seen in nests of arboreal ant species [24,96]. Indeed, we also found that geographic location impacts the nest bacterial communities. As we predicted, nests in closer proximity had more similar bacterial communities than nests farther apart (Figures 4.2a, 4.6). This similarity can be

explained by the similar soil environments because nest bacterial communities' differences mirrored the physical location of the nests (Figure 4.2a), with colonies that were physically closer to each other exhibiting similar alpha and beta diversity (Figure 4.6). Such geographic clustering of microbial communities is seen in studies of soil microbiome [97,98] where microbial communities impact the soil's physical structure, chemical properties, and water content [97,99]. Future work might examine how geographical differences in soil microbial composition may affect the behavior of ant colonies and the structure of their nests.

In contrast to the physical environment hypothesis, the beta diversity and evenness of the soil bacterial communities inside the nest was significantly different from the control soil samples (Figure 4.7, Table S4.1). This finding suggests that there are differences in the identity of the taxa observed in soil samples collected from within the nest and soil samples collected approximately one meter away from the nest (Figure 4.7, Table S4.1). These findings align with previous research indicating that ant nests serve as unique microhabitats with distinct microbial activity and soil nutrient composition [51]. However, this previous work used core samples that cut through the nest, and do not distinguish between soil inside the nest and the soil immediately outside the nest chambers - as we did here. Furthermore, they only examined the very top layer of the soil (0-20cm), whereas, our study did not include samples from the surface of the soil, and most of our samples were from deeper than 20cm. Interestingly, in contrast with other studies of soil microbiome [27,100], we did not find a relationship between soil depth and bacterial diversity (Table 4.3, Figure S4.7). One possible explanation for this discrepancy could be the unique structure and activity within ant

nests, creating microenvironments that sustain higher microbial diversity even at greater depths. Indeed, the digging activity of ants moves soil materials vertically within the nest [41] possibly moving soil-associated microbes along with the moving soil. The unexpected lack of relationship between bacterial diversity and soil depth highlights the complexity of microbial dynamics within ant nests and suggests that additional factors, such as nest architecture and ant activity, may mitigate the typical depth-related decline in microbial diversity.

The social organization hypothesis was supported by the distinct bacterial communities' composition of each type of biotic nest content (ants, reproductives, seeds, brood). Each of these sample types had different bacterial composition and different alpha diversity (Figures 4.3, 4.5, S4.1- 4.4). The *Firmicutes* bacterial phylum dominated ant samples whereas *Actinobacteria*, *Proteobacteria*, and *Firmicutes* were more evenly distributed in brood and reproductives (Figure 4.3). The presence of *Actinobacteria*, *Proteobacteria*, and *Firmicutes* is typical of herbivorous and omnivorous ant species and larvae [93,101]. However, *Firmicutes* is dominant in *V. andrei* adult worker ants, similar to what has been observed in copious predatory ant species such as army ants (*Eciton*) and bullet ants (*Paraponera clavata*) [101,102]. Among the bacterial ASVs identified in our study, three *Firmicutes* (class *Bacilli*) ASVs specifically stood out due to their markedly higher read abundance across ant samples, irrespective of colony, compared to other sample types (Figure 4.4c, d, e). These three ASVs suggest a strong association with ants and may potentially play a role in symbiotic interactions within *V. andrei* ants. Considering past work found that in Azteca ants the microbiome inside chambers matches their content [44], that the chemical signature of

nest chambers is determined by their content [45], and that ants use certain nest chambers as latrines [103] it was surprising that we did not find a match between the content of a chamber and the bacterial communities of its soil (i.e., chamber type, Figure 4.8). Thus, we did not find support for the idea that spatial division of labor influences and structures the bacterial composition of the nest itself, only that of the biotic content within it. As discussed above, the difference in evenness and beta diversity between nest and control soils suggests that there is some influence of the ants on their nest soil microbiome, however, it does not relate directly to the content of the chambers.

Conclusions

Our results contribute to a growing body of evidence that social insect nests are intricate ecosystems influenced by both intrinsic and extrinsic factors. Our study highlights the significant roles of both social organization and the physical environment in shaping the microbiome of *V. andrei* colonies. The influence of the surrounding soil bacterial communities on the nest bacterial communities especially underscores the intricate interplay between environmental and social factors in structuring nest microbiome. Thus, future work examining microbial ecology of animals should consider both the physical environment and social organization when studying the animal holobiont.

Figures and Tables

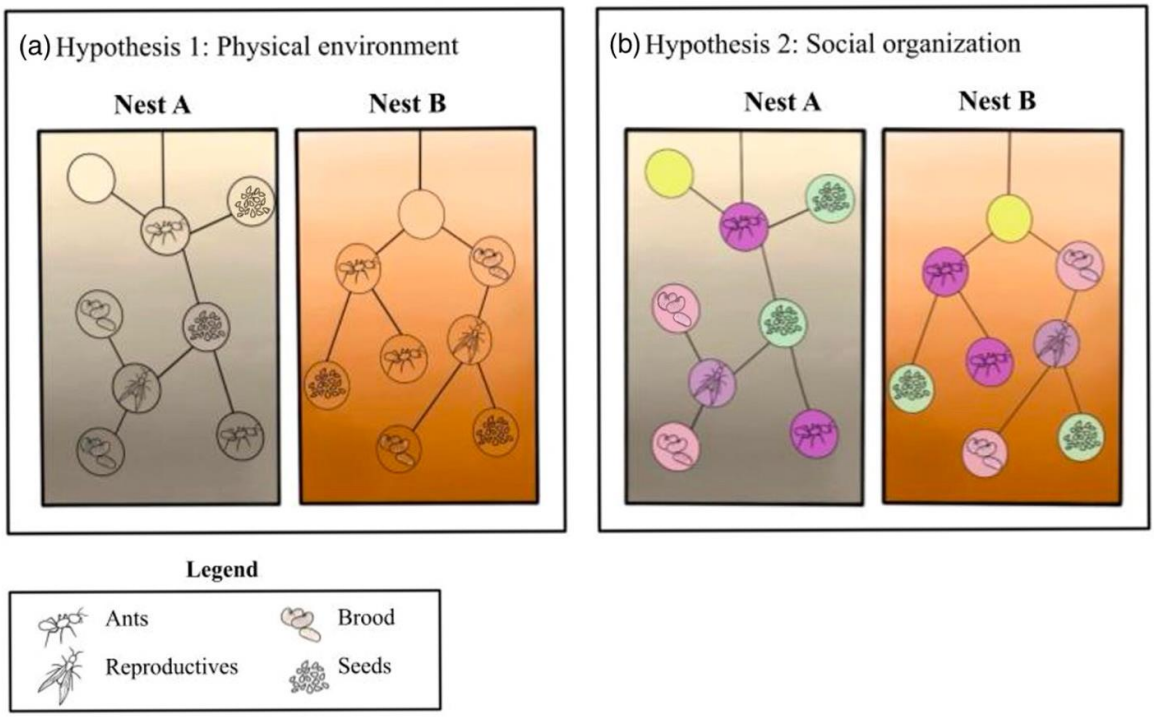

Figure 4.1: Visualization of hypotheses. (a) The physical environment determines microbial community structure. We expect the contents within the nest chambers (ants, reproductives, brood, and seeds) to have similar microbiome composition to the surrounding soil and that nests will differ from one another in their microbiome - based on differences in the surrounding soil. (b) Social organization determines microbial composition. We expect the microbiome composition of contents within the nests chambers to differ across chambers regardless of the microbial composition of the surrounding soil. The colors of the circles represent different microbiome compositions of the nest chambers. The background colors represent the microbiome composition of the surrounding soil.

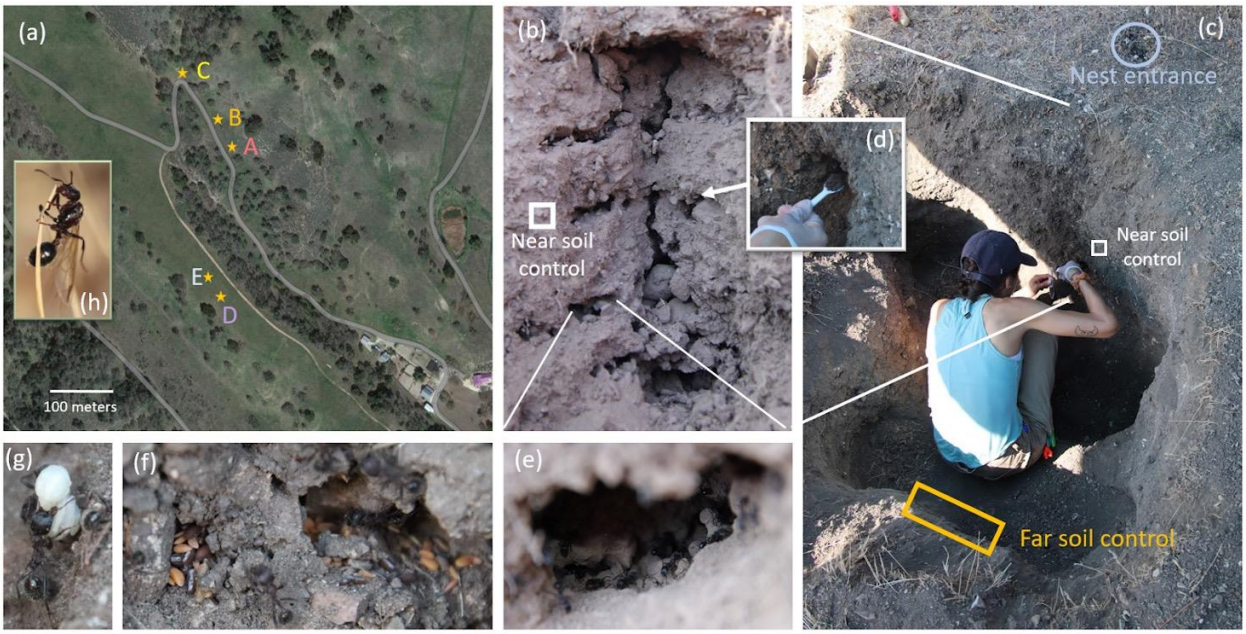

Figure 4.2: Sample collection. (a) Map of the study site with the locations of the five nests that we excavated indicated with orange stars and the letter ID of each colony, which is colored according to their representation in Figure 4.6. (b) the excavated nest of colony D; the white box indicates the approximate location where we sampled 'near soil control' for the chamber immediately to the right of the white box. (c) EHL crouching in the trench we dug to reach the nest chambers from the side, sampling soil from one of the chambers. A white box indicates the general area from which a 'near soil control' sample would be taken, and the yellow rectangle shows the approximate location where 'far control soil' samples were collected. The entrance of the nest is indicated at the top right in a gray circle. (d) sampling soil from inside a chamber with a plastic spoon. We collected each chamber's content, including (e) ants, (f) seeds, (g) brood, and (h) reproductives. All photo credits: Noa Pinter-Wollman.

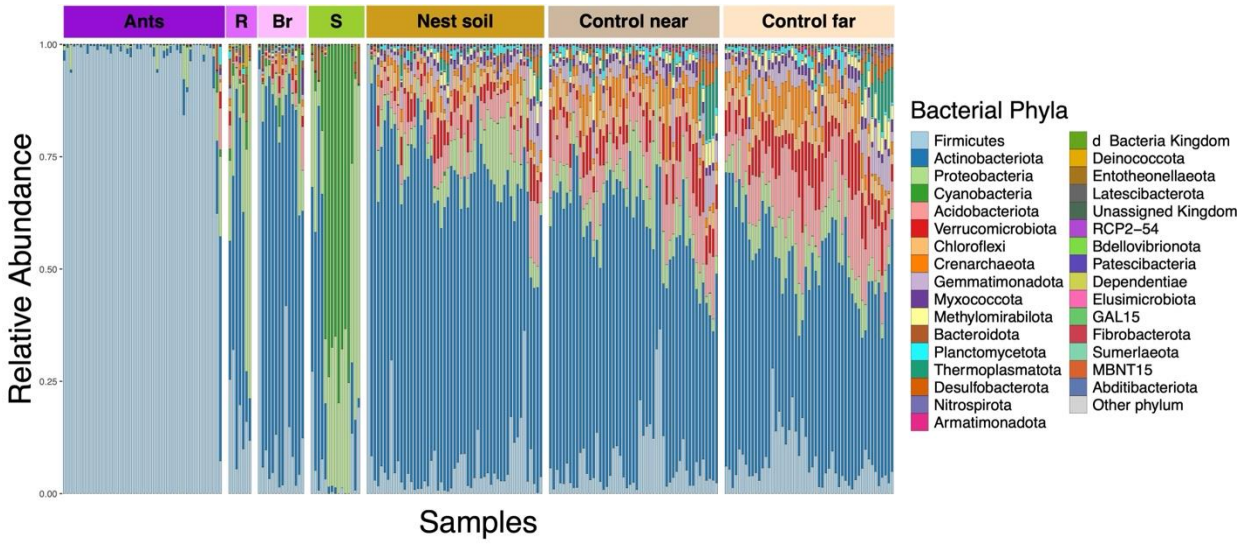

Figure 4.3: Relative abundance of bacterial phyla ordered by sample type: ants, reproductives (R), brood (Br), seeds (S), nest soil, control near soil, control far soil. Each vertical bar is an individual sample with color indicating the bacterial phyla according to ASV. The sampling depth was 3618 reads. For abundance plots by class, order, family, and genus see Figures S4.1, S4.2, S4.3, and S4.4 respectively.

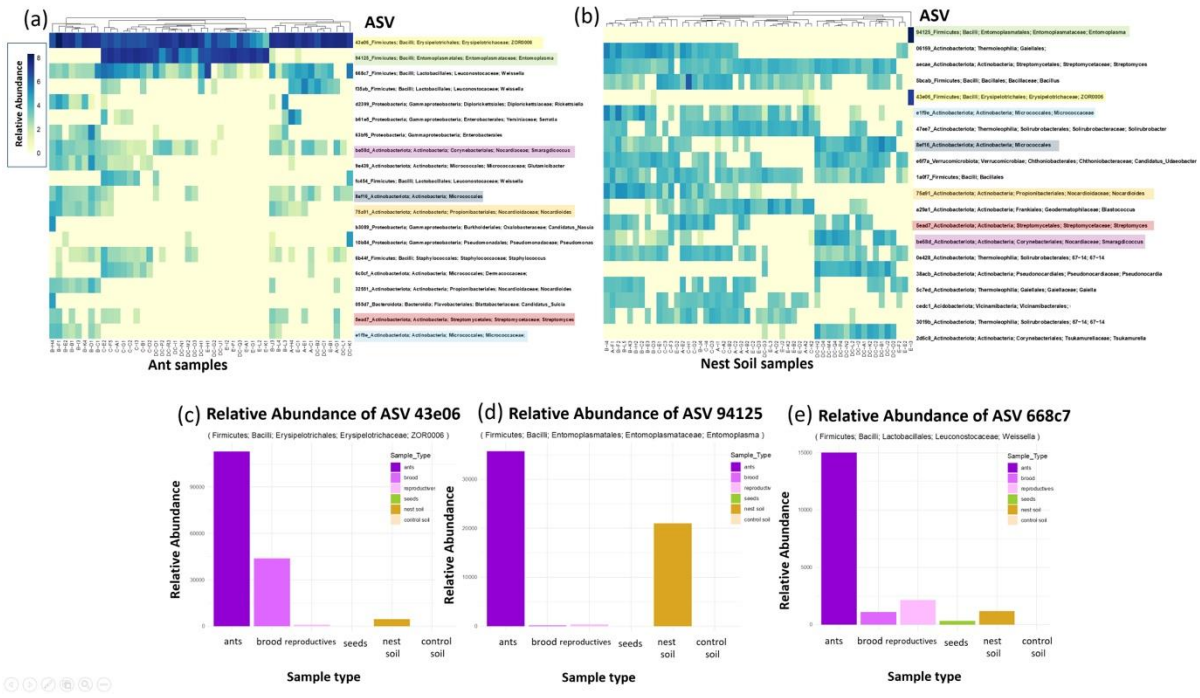

Figure 4.4: **Analysis of ASVs in soil and ants.** (a) Heatmap of the abundance of the top 20 ASVs for all ant samples and (b) top 20 ASVs for all nest soil samples. Names of ASVs which are the same between ant and nest soil samples are highlighted in the same color in (a) and (b). Color in the heatmaps indicates relative abundance – see color bar to the left. Heatmaps are arranged by overall abundance of ASVs – with the most abundant ASV at the top row of each heatmap and the lowest abundance ASV at the bottom row. (c) Relative abundance of the most abundant ASV (43e06) across all sample types. (d) Relative abundance of second most abundant ASV (94215) across all sample types. (e) Relative abundance of the third most abundant ASV (668c7) across all sample types.

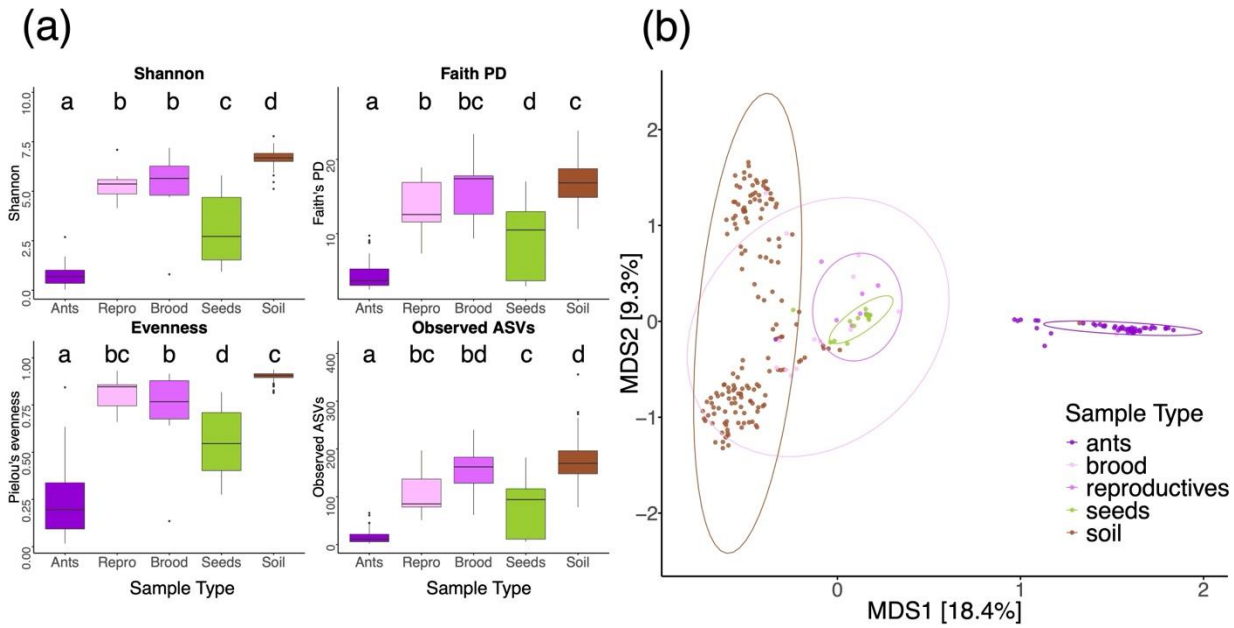

Figure 4.5: Alpha and beta diversity measures by sample type: ants, reproductives (repro), brood, seeds, and soil. (a) Box plots of microbiota alpha diversity measures (Shannon, Faith's Phylogenetic distance, and Observed ASVs) by sample types. Here, and in all following figures, boxes indicate interquartile ranges, lines inside the boxes denote medians, whiskers extend to 1.5 times the interquartile range, and dots are outliers. Boxes that do not share letters are statistically different according to a post hoc Tukey test (p-value < 0.05). (b) Beta diversity Principal coordinate analysis (PCoA) from Bray-Curtis dissimilarity matrix by sample types. Each point represents one sample and is color coded by sample type. The closeness of points indicates high community similarity.

Table 4.1: Statistical output of the four linear models that tested the effect of 'sample type' on each of four alpha diversity measures (Shannon, Faith, Pielou's Evenness, and Observed ASV's). Number of samples in each statistical model N = 239. 'Sample type' was the only explanatory variable in each model and results of the post-hoc test for models in which 'sample type' was a significant effect (p-value < 0.05) are shown in Figure 4.5.

Diversity measure	**Sum of squares**	**DF**	**F value**	**p-value**
Shannon	1363.2	4	691.59	**<0.0001**
Faith	6041.3	4	186.99	**<0.0001**
Pielou's Evenness	16.924	4	387.15	**<0.0001**
Observed ASVs	972137	4	161.61	**<0.0001**

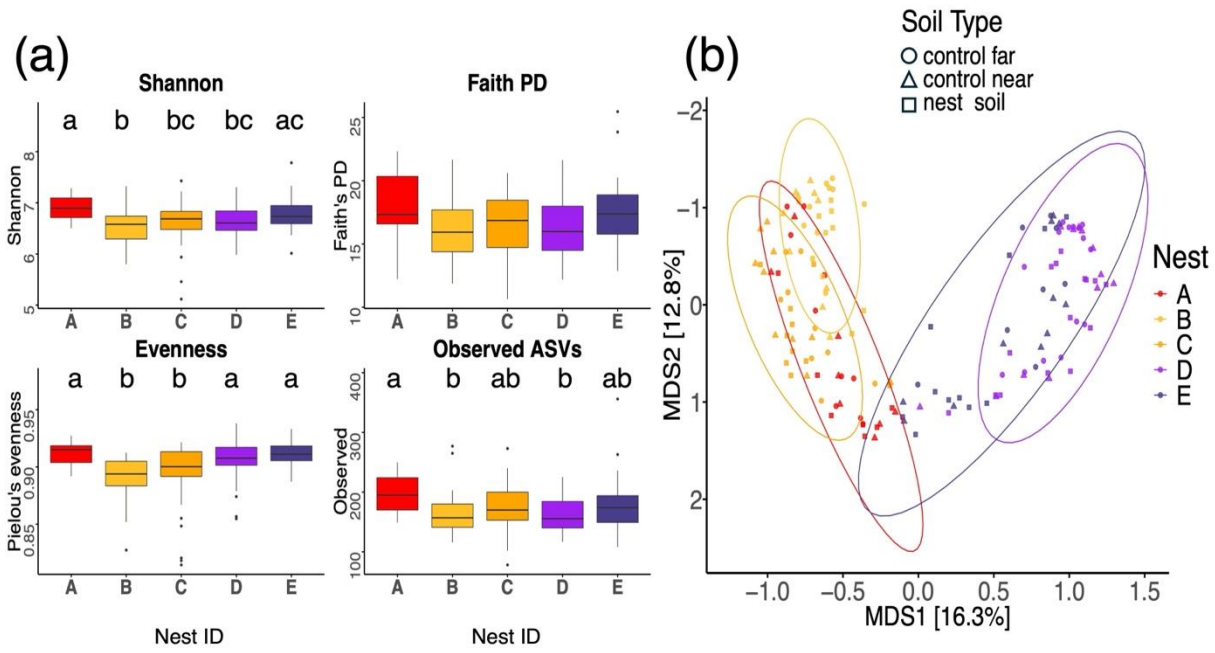

Figure 4.6: Alpha and beta diversity measures of all soil samples by nest. (a) Effect of nest ID (A, B, C, D, E) on alpha diversity measures (Shannon, Faith's phylogenetic distance, Pielou's Evenness, and Observed ASVs) of soil samples only. For measures in which nest ID was a significant effect, boxes that do not share a letter are statically significant according to a post hoc Tukey test. (b) PCoA plots from a Bray-Curtis dissimilarity distance matrix. Each point is a soil samples with colors corresponding to colony ID and point shape representing soil type (nest - circles, control near - triangles, and control far - squares).

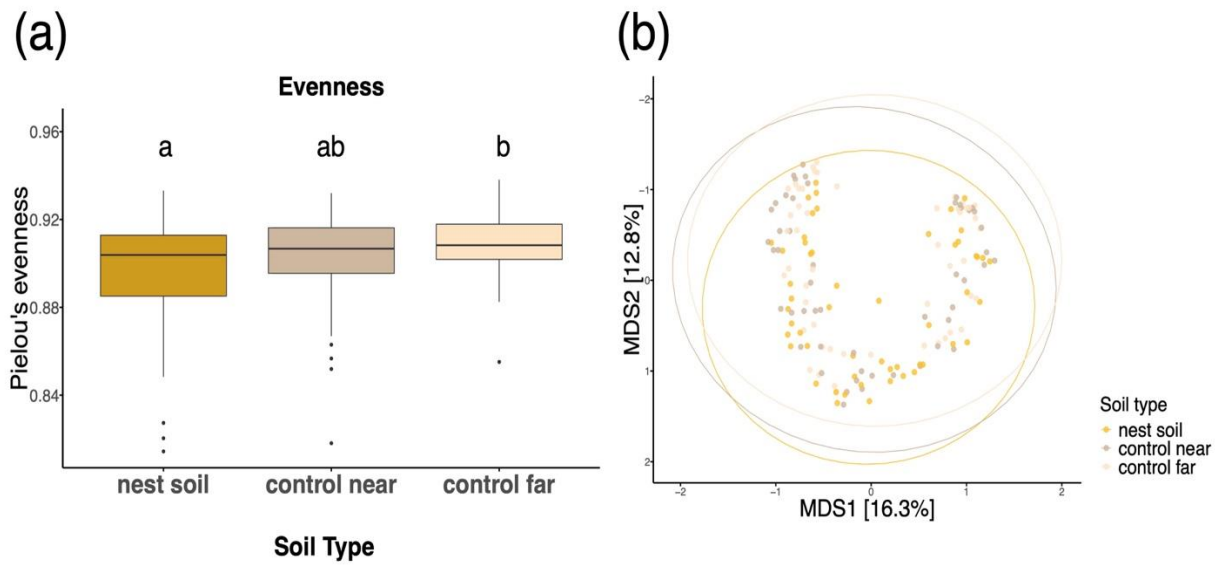

Figure 4.7: Alpha and beta diversity measures of all soil samples by soil type - same soil samples shown in Figure 4.6 with colors corresponding to soil type (nest soil, control near, and control far). Here we show a different view from Figure 4.6 to emphasize the effect of soil type. (a) Effect of soil type on the alpha diversity measure Pielou's evenness. Boxes that do not share a letter are statistically significant according to a post hoc Tukey test. (b) PCoA plot with Bray-Curtis dissimilarity distance matrix. Each point represents one soil sample, and colors correspond to soil type (nest, control near, and control far).

Table 4.2: Statistical output of the four linear models that tested for the effect of nest, depth, and soil type (chamber soil, control near, and control far) on each of four alpha diversity measures (Shannon, Faith, Pielou's Evenness, and Observed ASV's). Number of samples in each statistical model N = 155. Effects that are statistically significant (p-value <0.05) are in bold and results of the post-hoc analysis are shown in Figures 4.6 and 4.7.

Diversity measure	Effect	Sum of Squares	DF	F value	p-value
Shannon	**Nest**	2.675	4	5.524	**<0.001**
	Depth	0.037	1	0.306	0.581
	Soil type	0.482	2	1.993	0.140
Faith	Nest	49.53	4	1.885	0.116
	Depth	13.96	1	2.125	0.147
	Soil type	9.65	2	0.735	0.481
Pielou's Evenness	**Nest**	0.014	4	**9.368**	**<0.0001**
	Depth	< 0.001	1	0.080	0.777
	Soil type	0.005	2	**6.376**	**0.002**
Observed ASVs	**Nest**	19929	4	**3.340**	**0.012**
	Depth	135	1	0.093	0.764
	Soil type	665	2	0.223	0.801

Table 4.3: Statistical output of the four linear models that tested for the effect of nest, depth, and chamber type (based on the content found in the chamber) on each of four alpha diversity measures (Shannon, Faith, Pielou's Evenness, and Observed ASV's). Number of samples in each statistical model N = 53.

Diversity measure	Effect	Sum of Squares	DF	F value	p-value
Shannon	Nest	2.054	4	3.492	0.165
	Depth	0.252	1	1.710	0.199
	Chamber type	0.834	4	1.417	0.248
Faith	Nest	55.438	4	1.648	0.184
	Depth	5.455	1	0.649	0.426
	Chamber type	34.502	4	1.026	0.407
Pielou's Evenness	Nest	0.005	4	2.345	0.073
	Depth	0.0008984	1	1.591	0.215
	Chamber type	0.004	4	1.877	0.136
Observed ASVs	Nest	13147	4	2.393	0.069
	Depth	665	1	0.484	0.491
	Chamber type	6806	4	1.239	0.312

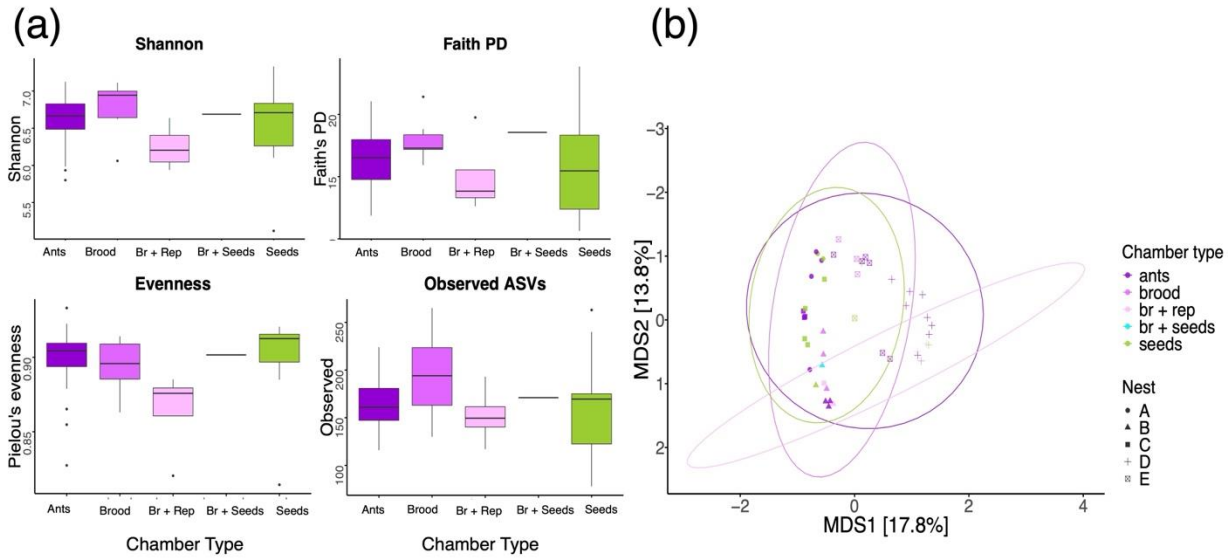

Figure 4.8: Alpha and beta diversity of soil samples only from inside the nest, by chamber type (ants, brood, brood + reproductive (Br + Rep), brood/seeds (Br + Seeds), and seeds). (a) Effect of chamber type on alpha diversity measures (Shannon, Faith's Phylogenetic distance, Pielou's Evenness, and Observed ASVs). Chamber type did not have a statistically significant effect on any of the alpha diversity measures. (b) PCoA plot with Bray-Curtis dissimilarity matrix for nest soil by chamber type and nest - colors represent chamber type (ants, brood, brood + seeds, brood + reproductives, and seeds) and point shape corresponds to nest ID.

Supplementary Materials

Supplementary Figures

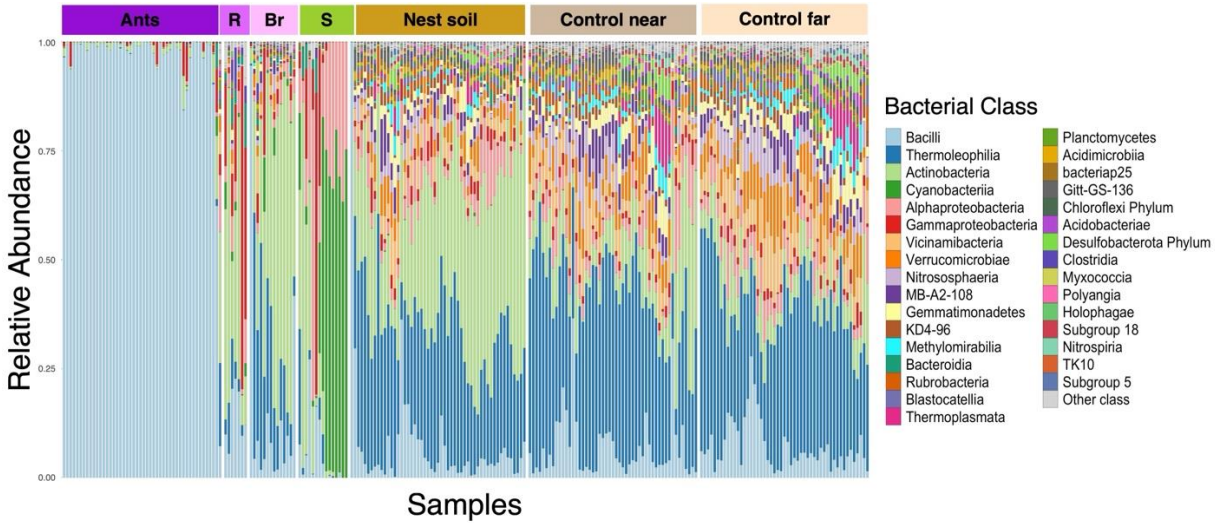

Figure S4.1: Relative abundance of bacterial class ordered by sample type: ants, reproductives (R), brood (Br), seeds (S), nest soil, control near soil, control far soil. Each vertical bar is an individual sample with color indicating the bacterial class according to ASV. The sampling depth was 3618 reads.

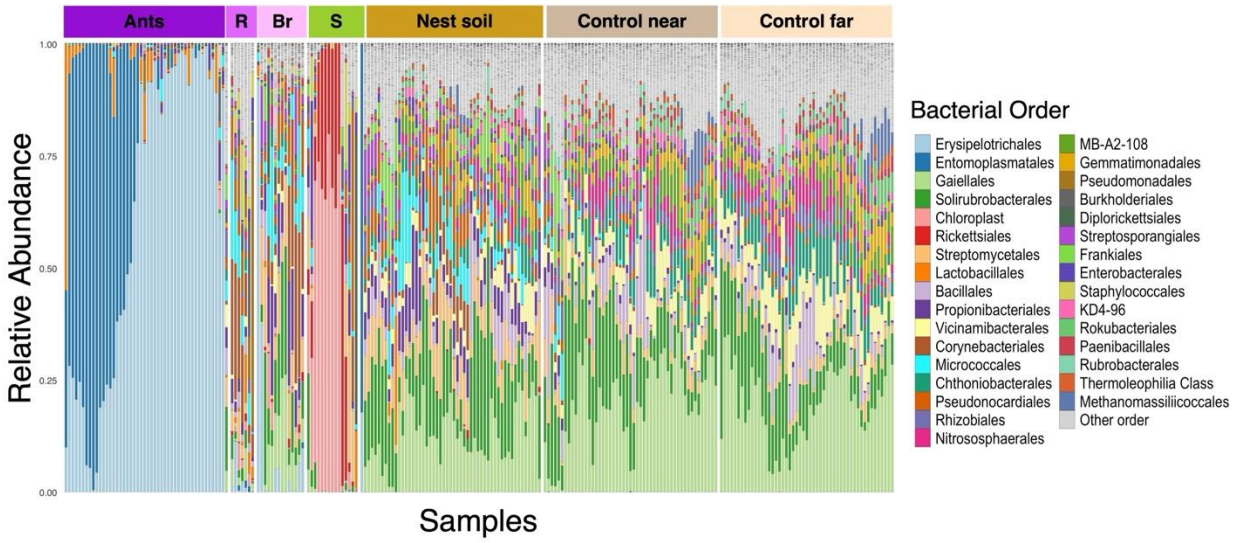

Figure S4.2: Relative abundance of bacterial order organized by sample type: ants, reproductives (R), brood (Br), seeds (S), nest soil, control near soil, control far soil. Each vertical bar is an individual sample with color indicating the bacterial order according to ASV. The sampling depth was 3618 reads

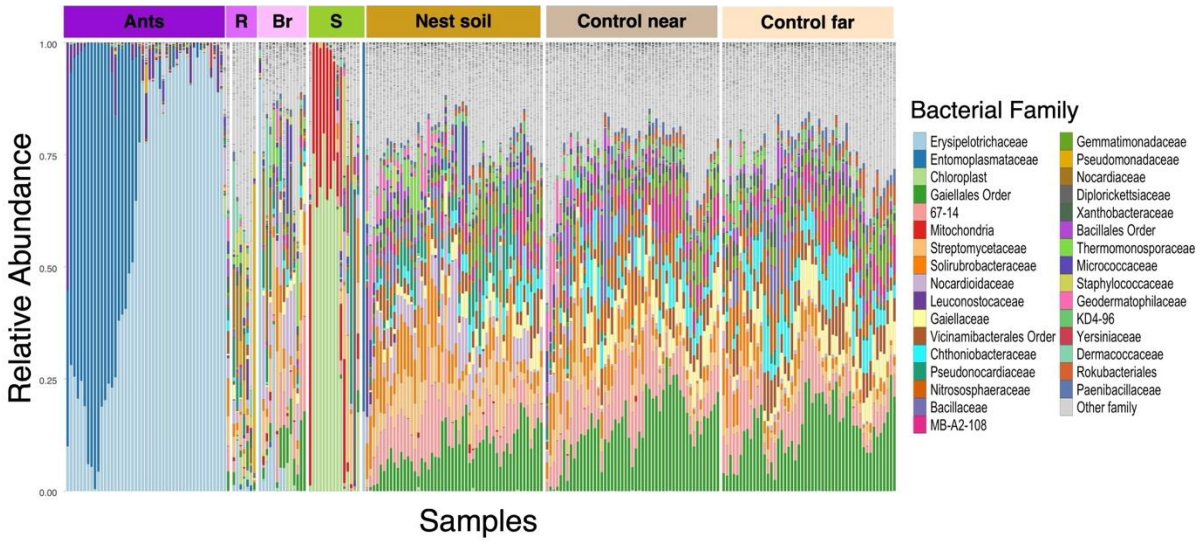

Figure S4.3: Relative abundance of bacterial family ordered by sample type: ants, reproductives (R), brood (Br), seeds (S), nest soil, control near soil, control far soil. Each vertical bar is an individual sample with color indicating the bacterial family according to ASV. The sampling depth was 3618 reads.

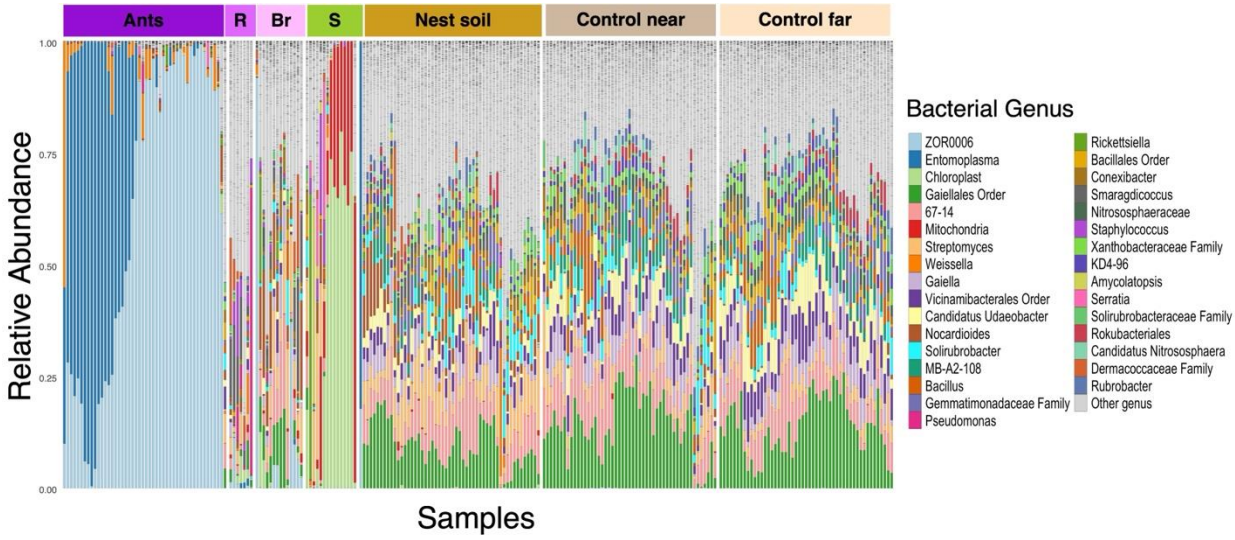

Figure S4.4: Relative abundance of bacterial genus ordered by sample type: ants, reproductives (R), brood (Br), seeds (S), nest soil, control near soil, control far soil. Each vertical bar is an individual sample with color indicating the bacterial genus according to ASV. The sampling depth was 3618 reads.

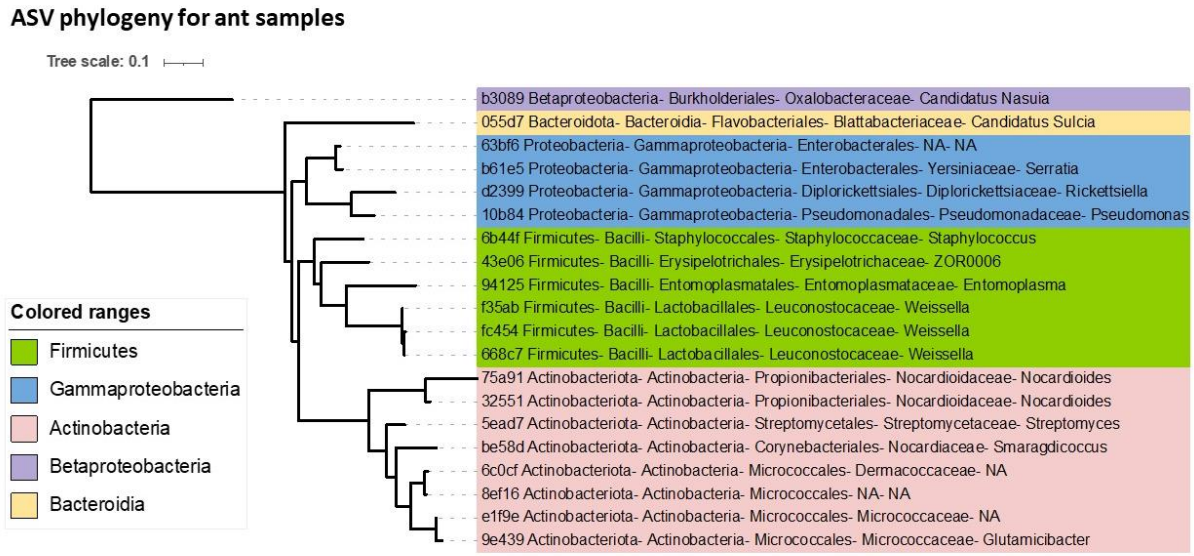

Figure S4.5: Phylogenetic tree of top 20 bacterial ASVs from ant samples. ASVs are colored by bacterial class.

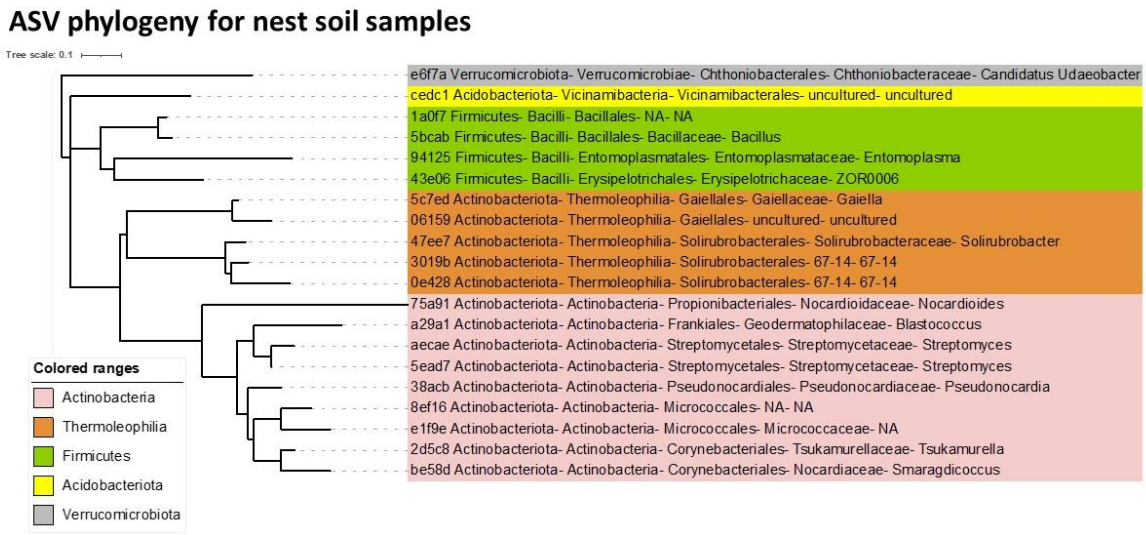

Figure S4.6: Phylogenetic tree of top 20 bacterial ASVs from nest soil samples. ASVs are colored by bacterial class.

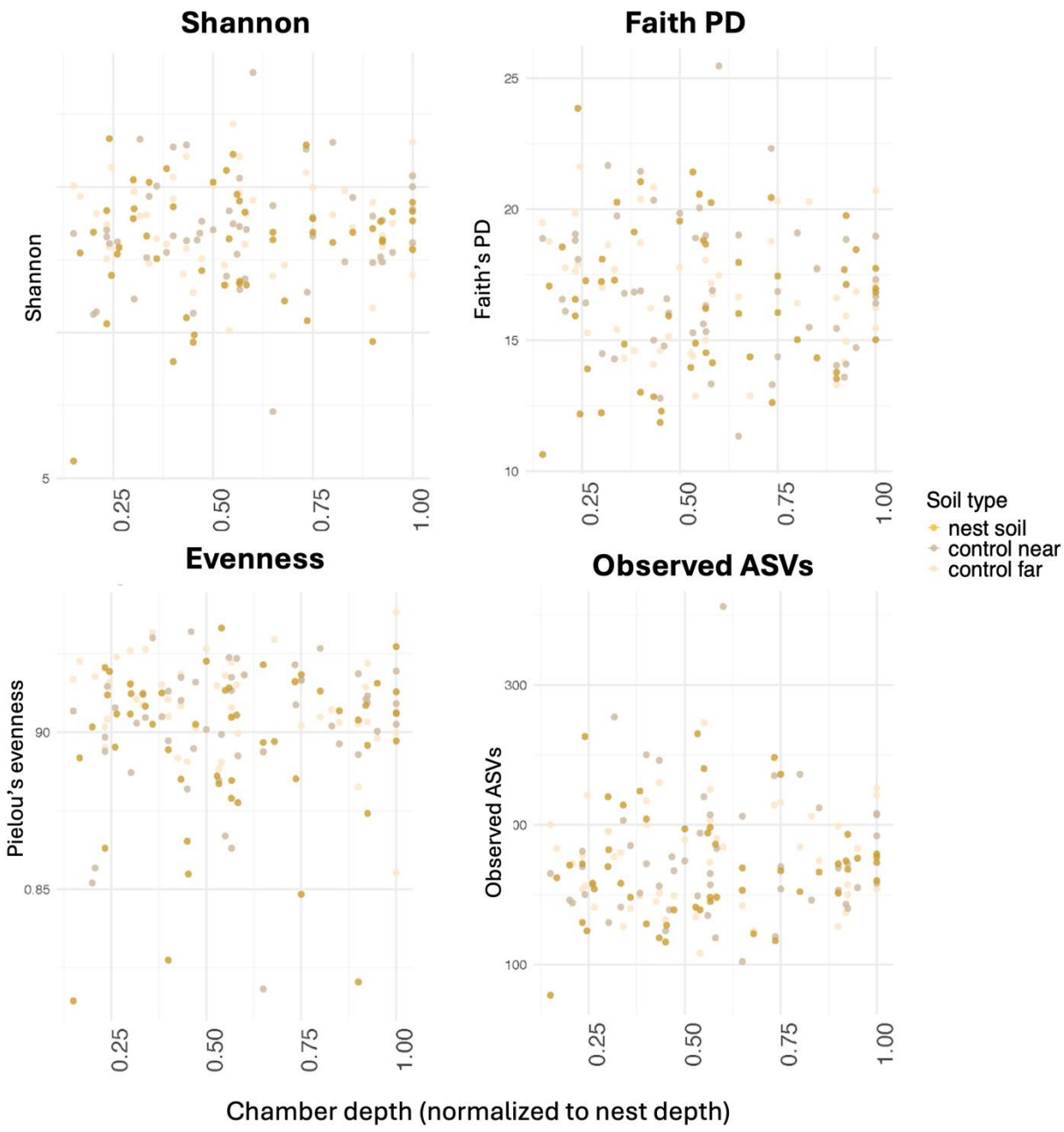

Figure S4.7: Alpha diversity was not related to chamber depth (normalized by nest depth). Each point represents a chamber in a nest, color indicates soil sample type (nest, control near, control far).

Supplementary Tables (in attached excel spreadsheets)

Table S4.1: Pairwise PERMANOVA results comparing beta-diversity using Bray-Curtis distances between 1) All sample types, 2) All soil types, and 3) Nest Soil.

Table S4.2: SIMPER analysis of ant samples. Includes the top 20 ASV taxa which contribute to the observed differences in community structure in the ant samples.

Table S4.3: SIMPER analysis of nest soil samples. Includes the top 20 ASV taxa which contribute to the observed differences in community structure in soil samples.

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