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Azteca ants maintain unique microbiomes across functionally distinct nest chambers

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The microbiome of built structures has considerable influence over an inhabitant's well-being, yet the vast majority of research has focused on human-built structures. Ants are well-known architects, capable of constructing elaborate dwellings, the microbiome of which is underexplored. Here, we explore the bacterial and fungal microbiomes in functionally distinct chambers within and outside the nests of *Azteca alfari* ants in *Cecropia peltata* trees. We predicted that *A. alfari* colonies (1) maintain distinct microbiomes within their nests compared to the surrounding environment, (2) maintain distinct microbiomes among nest chambers used for different functions, and (3) limit both ant and plant pathogens inside their nests. In support of these predictions, we found that internal and external nest sampling locations had distinct microbial communities, and *A. alfari* maintained lower bacterial richness in their 'nurseries'. While putative animal pathogens were suppressed in chambers that ants actively inhabited, putative plant pathogens were not, which does not support our hypothesis that *A. alfari* defends its host trees against microbial antagonists. Our results show that ants influence microbial communities inside their nests similar to studies of human homes. Unlike humans, ants limit the bacteria in their nurseries and potentially prevent the build-up of insect-infecting pathogens. These results highlight the importance of documenting how indoor microbiomes differ among species, which might improve our understanding of how to promote indoor health in human dwellings.

1. Background

Shelters are distinct ecosystems that, though common, are scientifically underexplored [1]. Shelters can structure local biotic communities [2], with effects often persisting beyond the lifespan of their original occupants. For example, shelters exclude a subset of predators, parasites and diseases during and after their occupation [3]. However, much like human houses [4,5], shelters favour secondary inhabitants that can affect primary occupants, the most consequential of which are microbes.

A growing body of work considers microbial inhabitants that live inside human shelters (e.g. [5–7]). The microbiomes of built environments can affect human physical and mental health. For example, *Mycobacterium vaccae* increases cognitive function [7], and commensal bacteria in human homes help promote immunoregulation [8]. Microbiomes within human homes can reflect occupant identity [5], use (e.g. toilets tend to have more faecal microbes, pillows more mouth-associated microbes and kitchen more food microbes [6]) and sanitation

activities [9]. However, human-built microbiome research is still relatively new, and humans are just one of many tens of thousands of animal species that live in shelters. Even less explored than the microbiomes of human homes are those of other animals' shelters. The study of shelter microbiomes may give insight into the factors that structure microbial communities in human dwellings, as well as how we might better manage microbes in the future.

Ants are well-known architects that build or modify natural structures to create nests for their colonies. Some ants have nests that are large and subterranean, while others can fit entire colonies inside seeds [10]; others still are composed entirely of interconnected ant bodies (e.g. bivouacs [11]). Ant nests have the potential to be colonized by diverse microbes introduced via nest materials, food brought into the nests and/or the bodies of the ants themselves [12–14]. Just as human homes are divided into rooms that serve different functions (e.g. kitchen, bathroom, nursery, etc.), many ant species divide their nests into chambers for brood rearing, food storage or waste management. Different chamber types are likely to be colonized by distinct microbial communities depending on the materials present in the chamber and its location relative to the outside environment.

In the light of the many opportunities for microbial colonization, selection is likely to favour mechanisms by which ants maintain beneficial microbiota and minimize pathogens within their nests (e.g. [15–18]), as social insects are particularly susceptible to infection [19]. Ants exhibit behaviours that actively influence nest microbial communities. For example, certain ant species cultivate microbial symbionts within their nests as a food source or to aid in defense against pathogens [20,21]. In addition, many ants secrete potent antibiotic compounds and apply them to nest materials and colony members to maintain colony health [22–24].

Perhaps the nests in which ants have the greatest potential to control the composition of microbial co-inhabitants are those inside myrmecophytic plant species. Myrmecophytes are plants that have co-evolved relationships with ants, providing food or shelter, often in exchange for protection from herbivores and/or pathogens [25–28]. Myrmecophytic ant-nest microbiomes are unique in their potential to positively or negatively affect ants and their host plants. As a result, controlling microbes within the interior of a plant should be beneficial for both partners, though selection pressures for each may differ [29–32].

Here, we explore the microbial associates within the well-studied *Azteca–Cecropia* mutualism [25,26,33]. *Azteca* ants live within *Cecropia* trees that have internally segmented chambers (in essence, rooms) to which the ants assign different functional roles (e.g. food storage, brood chamber, waste chamber [34–36]). The chamber walls have large porous cavities that act as absorptive surfaces that allow plants to gain nutrients from ant waste [37,38]. Certain *Azteca* species further compartmentalize their homes by enlarging the domatium space [39] and through the production of carton, which is composed of macerated plant tissue combined with Chaetothyrialean fungi [40]. These beneficial fungal associates are vertically transmitted through newly colonizing queens [41,42]. We ask whether this compartmentalization and chamber specialization leads to similar patterns of microbial structuring as those seen in human-built environments [5,32,43], and whether ant colonies maintain distinct relationships with their microbial co-inhabitants. By investigating the microbiome of this widespread mutualism, we also set the stage for future studies on what behaviours promote sanitation for ants and therefore strategies humans might employ to limit pathogen exposure.

We collected and identified bacteria and fungi from the nests of *Azteca alfari* colonies nesting inside *Cecropia peltata* trees as well as samples from the surrounding environment. We swabbed a variety of unique chambers within *C. peltata* trees housing workers, brood or nest carton material. We also sampled abandoned nest chambers and external locations that included tree stems, nest entrances, plant-produced food sources and soil at the base of each tree. Based on previous studies of human dwellings and research on the mechanisms by which microbial communities are structured in *Azteca* ant nests, we began with four *a. priori* predictions. First, we predicted that *A. alfari* colonies would maintain distinct microbial communities inside their nests from the surrounding environment and abandoned chambers. Second, we predicted that microbial communities within nests would differ among functionally distinct chambers (i.e. worker, brood and carton-containing chambers). Third, we predicted that ant- and plant-specific pathogens would be less common in ant-inhabited chambers compared with abandoned chambers or the surrounding environment. Finally, we predicted that microbial abundance and diversity should be lowest in brood chambers, as they are the chambers most likely to be cleaned [44].

2. Material and methods

(a) Study areas and sampling

We sampled nests and trees that are part of the model mutualism between *Azteca alfari–Cecropia peltata*. Additional information on the *Azteca–Cecropia* mutualism is provided in the electronic supplementary material, methods. We sampled one *C. peltata* tree in August 2013 and six more in December 2013 at the edges of yards in Gamboa, Panama (9°06′55.6″ N 79°41′59.2″ W). Only young, healthy-looking trees were selected for study (range in diameter at breast height 1.5–3.0 cm). Trees were cut with a machete dipped in 70% ethanol and flame sanitized, after which the trunk was split open longitudinally. Microbial (bacterial and fungal) samples were collected immediately following exposure by continuously rubbing the entire internal internode wall of one example of each target chamber type per tree for approximately 30 s with a sterile cotton swab. Only areas that made no direct contact with the machete were swabbed.

Outside of each chamber, we sampled the stem surface (hereafter outer stem surface), nest entrances and the area below the leaf axil on which the plant bears food propagules commonly harvested by its ant associates. We sampled (when present) chambers with only worker ants, nest carton material, empty (i.e. abandoned) chambers and chambers containing brood (figure 1). Each chamber may contain multiple components, but we found that even when chambers were shared, components were spatially separated. In the present study, we deliberately sampled chambers that fit into one of the discrete categories to prevent sampling of two adjacent regions within the same chamber. This sampling approach targets regions of the nest that are most representative of each category (e.g. for 'worker chambers', we sampled the internode with the most workers and no brood). We also swabbed the soil at the base of each tree.

(b) DNA extractions and sequencing

Total DNA was extracted from the swabs using the MoBio Power Soil DNA extraction kit (Qiagen, Germantown, MD, USA) as described previously [45]. Each swab tip was placed in PowerBead

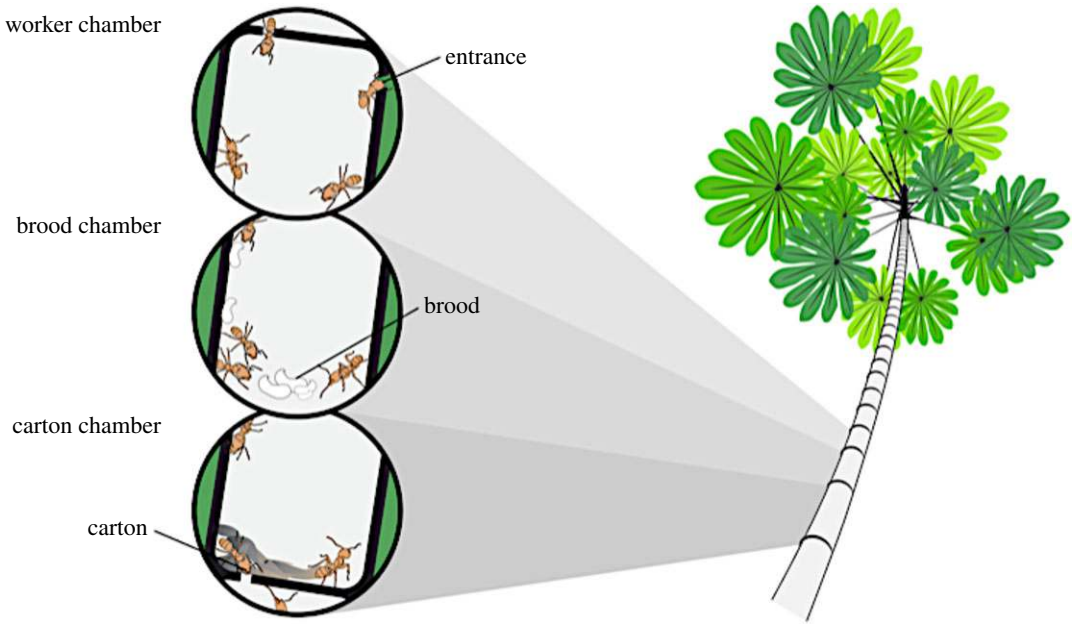

Figure 1. Diagram of *Azteca alfari* nest structure in *Cecropia peltata* trees. Worker chambers include only adults; brood chambers include larvae and pupae, with larvae often attached to nest walls using specialized body hairs; carton chambers included adult workers and nest carton constructed from chewed plant materials. Chambers are connected by small holes between internal partitions, and some chambers feature entrance holes that lead outside the nest. (Online version in colour.)

tubes containing solution C1 and swirled vigorously for approximately 10 s. For two methodological controls, we did the extractions using a clean swab or no swab. The extractions were performed as directed by the manufacturer, except that the final elution was performed in 50 µl of 70°C C6 elution buffer.

We used methods similar to those described previously by Barberan *et al*. [5] but modified them for sequencing with the Illumina platform using Illumina's two-step tailed amplicon approach (Illumina, Inc., San Diego, CA, USA). Briefly, amplicons were produced by PCR with universal bacterial/archaeal 515F [46] and 806R [47] primers and fungal ITS1F and ITS2 primers [48] to which adapters were added to allow for a second round of PCR. A no-template control PCR reaction was also performed. These initial PCR reactions were performed with 25 cycles in triplicates, the triplicates pooled and purified with UltraClean-htp 96 Well PCR Clean-Up Kit (Qiagen, Germantown, MD, USA). For the second PCR, 5 µl of each pooled and cleaned initial PCR reaction was used as the template using pairs of indexed primers that would allow the identification of each sample following sequencing. Each reaction was then cleaned again using the UltraClean-htp 96-well PCR Clean-up kit (Qiagen, Germantown, MD, USA) and quantified with a Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA). Equal masses of each sample were pooled into a single sample, concentrated by ethanol precipitation and sequenced on one run using the MiSeq platform (Illumina, Inc., San Diego, CA, USA) at North Carolina State University's Genomic Sciences Laboratory (Raleigh, NC, USA).

(c) Sequence processing

Demultiplexing was performed by the sequencing facility and resulted in 10 380 664 trimmed sequences of mixed ITS and 16S sequences. Primer and Illumina adapter sequences were trimmed from all sequences using cutadapt (v. 1.8.1 [49]). Trimmed sequences were processed following the UPARSE pipeline [50]. The 16S and ITS sequences were processed separately. For 16S analysis, reads were merged. For ITS, only Read 1 s were used due to the size variability of the ITS region. ITS reads were first filtered to exclude those shorter than 100 basepairs. Subsequent quality filtering of both merged 16S and single ITS reads removed sequences at a max *e* value of 1.0. This resulted in 5.5 million 16S reads and 6.6 million ITS reads. Singletons were removed from each dataset, and identical sequences were dereplicated. OTU groups were clustered at 97%. OTUs were then filtered to exclude sequences that matched less than 75% to those in the Greengenes (13.8) and the UNITE (12.11) databases for 16S and ITS reads, respectively.

The raw, trimmed sequences (merged for 16S and Read 1 s for ITS) were then mapped onto these representative sequence databases using a 97% similarity threshold. Approximately 4.1 million 16S sequences and 2.3 million ITS sequences mapped on to the database. Taxonomy was assigned to the sequences in these OTU tables in QIIME (1.9.1), using the RDP classifier (2.3) with a confidence threshold of 0.5. RDP was trained on the Greengenes (13.8) and UNITE (12.11) databases. This UNITE database was further curated manually to remove entries that were taxonomically unresolved at the phylum level. We normalized uneven sequence counts across all biological samples by rarefying each sample to a uniform sequencing depth; bacterial samples were rarefied to 1344 sequences per sample (including 31 of 32 samples) and 6130 sequences per sample (including 31 of the 32 samples) for fungi (electronic supplementary material, figure S1). Additional filtering methods are included in the electronic supplementary material, methods.

(d) Statistical analyses

Linear model analyses were performed in the R statistical environment [51]. We compared richness (observed OTUs) and the abundance of key microbial taxa across sampling locations using linear mixed-effect models that consisted of nested model reduction based on AIC values and *p*-values from likelihood ratio tests (*nlme* [52]). We performed pairwise comparisons using Tukey HSD post hoc tests, and we report associated *z*- and *p*-values. In all cases, we examined residuals to confirm appropriate model fits. We used the Bonferroni correction to correct for multiplicity and we report alpha when less than 0.05. For each model, we included a sampling location as a fixed effect and source tree as a random grouping factor. We did not include soil samples in our analyses as we did not have sufficient sample sizes.

We compared microbial community composition and beta diversity using Primer-E (v. 7.0.13). Microbial community data were square root transformed before calculating community

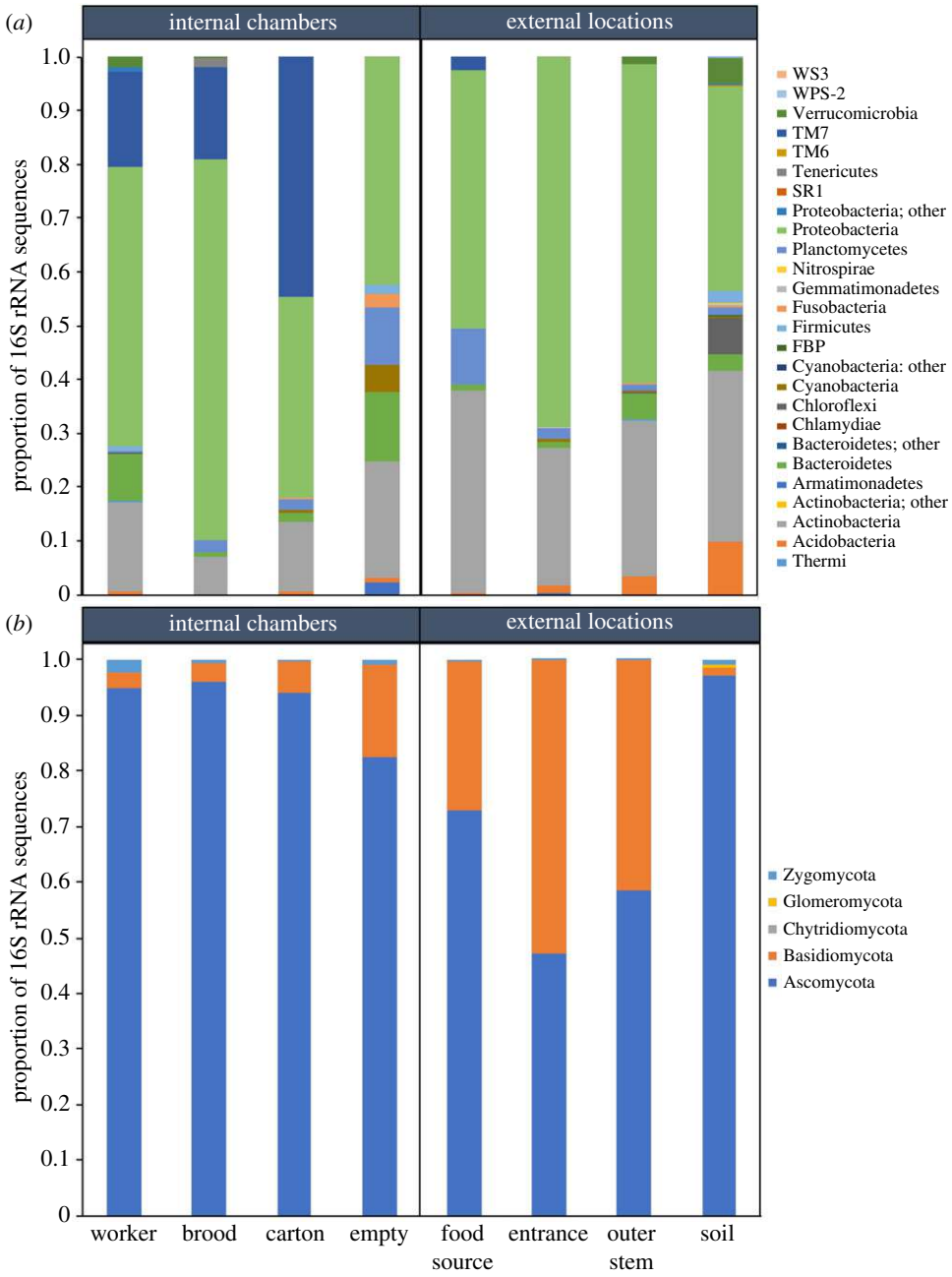

Figure 2. Taxonomic composition of (*a*) bacterial and (*b*) fungal phyla across internal and external nest sampling locations. Each bar represents the average proportion of sequence counts for all replicates in each sampling location.

dissimilarity between each swabbed location using Bray–Curtis dissimilarity. These distances were used to generate ordinations (non-metric multidimensional scaling, NMDS) for both bacteria and fungi. Next, we performed PERMANOVA with the community distance matrices to compare community compositions across sampling locations and individual trees using Primer-E (9999 permutations, v. 7.0.13 [53]). We also compared beta diversity across sampling locations using PERMDISP tests [53]. PERMDISP tests (i.e. an assessment of multivariate homoskedasticity across samples) calculate within sampling location dissimilarity in community composition and then compares the magnitude of dissimilarity across sampling locations (9999 permutations). To determine which sampling locations differed from each other, we conducted pairwise comparisons within the Primer-E environment and report pseudo-*t* scores and *p*-values from these post hoc analyses.

Identified fungal OTUs were classified to ecological function using the software FUNGuild [54]. We focused our functional analysis on fungal communities, as fungal infections are of particular concern for plants [55] and insects [56]. We used linear mixed effect models in R (as above) to compare the average relative OTU abundance of fungal putative functional groups across nest locations, again with source tree as a random grouping factor.

3. Results

(a) Bacterial and fungal community composition

The bacterial dataset contained 41 664 total reads across 1445 unique OTUs from the domatia and surrounding environmental samples. After subsampling (rarefaction), we identified 22 bacterial phyla across all of our sample locations out of the 30 bacterial phyla known globally. Yet just two phyla, Proteobacteria and Actinobacteria, accounted for 90% of all bacterial reads across all sampling locations (figure 2*a*). The fungal dataset contained 190 030 total reads across 973 unique OTUs. Five

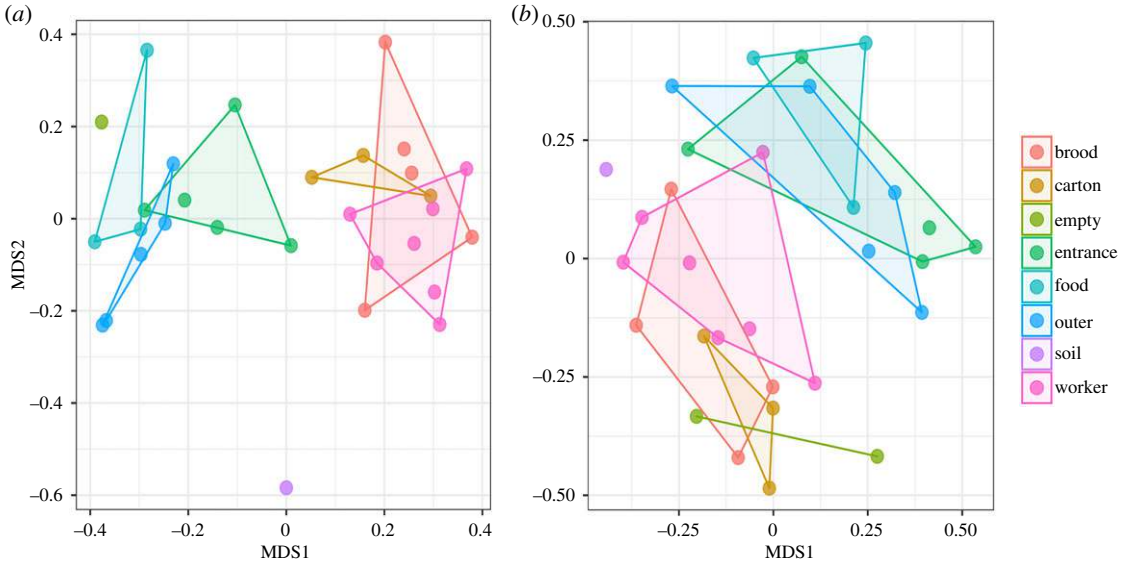

Figure 3. Non-metric multidimensional scaling for (*a*) bacterial and (*b*) fungal communities across sampling locations. Distances are based on dissimilarity matrices of sequence-based Bray–Curtis distances. Polygons connect the outermost points for each sampling location. Sample locations differ significantly from each other (PERMANOVA bacterial: pseudo-$F_{5,16} = 2.39$, $p < 0.001$, stress = 0.19; fungal: pseudo-$F_{6,15} = 2.46$, $p = 0.001$, stress = 0.22).

out of seven known fungal phyla were present in our samples, but the vast majority of reads were from Ascomycota (78.7%) or Basidiomycota (20.6%; figure 2*b*).

(b) Variation in community structure across nest locations

Our predictions that the diversity and composition of microbial communities would differ between internal and external locations as well as among nest locations as a function of chamber use were largely supported. Bacterial richness varied among sampling locations (dAIC = 4.75, $\chi^2 = 16.75$, d.f. = 6, $p = 0.01$; electronic supplementary material, figure S2), with worker chambers having the highest bacterial richness of all internal chambers (114.14 average OTUs per sample), and soil samples having the highest overall bacterial richness (498 average OTUs per sample). Particularly pronounced was the greater number of bacterial OTUs in worker chambers as compared to brood chambers (Tukey HSD; $z = 3.36$, $p = 0.012$; electronic supplementary material, figure S2). Low richness in brood chambers was primarily driven by the dominance of *Wolbachia* (30.58% average abundance). Unlike bacteria, overall fungal richness did not differ among sampling locations (dAIC = −2.58, $\chi^2 = 13.42$, d.f. = 8, $p = 0.09$; electronic supplementary material, figure S2). However, the abundance of some individual fungal orders did differ among sampling locations. Most notably, Chaetothyriales (Ascomycota), a diverse fungal order that contains many known ant mutualists and food sources, was in high relative abundance in internal chambers as compared to external sampling locations (dAIC = 13.87, $\chi^2 = 27.87$, d.f. = 7, $p < 0.001$; figure 4*c*).

As predicted, the composition of both bacterial and fungal communities differed between external and internal sampling locations as well as across nest chambers (PERMANOVA bacterial: pseudo-$F_{5,16} = 2.39$, $p < 0.001$; fungal: pseudo-$F_{9,21} = 2.46$, $p = 0.001$; figure 3). Differences in bacterial composition were driven by worker chambers differing from nest entrances (pseudo-$t = 1.82$, $p = 0.01$) and outer stem surfaces (pseudo-$t = 2.07$, $p = 0.005$), and brood chambers differing from stem surfaces (pseudo-$t = 1.69$, $p = 0.04$). For fungi, community composition differed between worker chambers and nest entrances (pseudo-$t = 1.92$, $p = 0.02$) and outer stem surfaces (pseudo-$t = 1.85$, $p = 0.02$). The identity of the individual tree in which a nest was located also influenced the composition of bacterial and fungal communities (PERMANOVA bacteria: pseudo-$F_{7,24} = 1.43$, $p = 0.01$; fungi: pseudo-$F_{7,24} = 2.58$, $p = 0.001$; electronic supplementary material, figure S3).

Beta diversity across sampling locations differed for fungal communities (PERMDISP Fungi: pseudo-$F_{5,21} = 5.56$, $p = 0.03$) and was marginally significant for bacterial communities (PERMDISP bacteria: pseudo-$F_{5,22} = 4.39$, $p = 0.07$). This was primarily driven by carton samples having lower fungal and bacterial beta diversity (i.e. were more homogeneous) as compared to worker (bacteria: pseudo-$t = 3.95$, $p = 0.02$, fungi: pseudo-$t = 3.89$, $p = 0.008$) and brood chambers (bacteria: pseudo-$t = 5.31$, $p = 0.02$, fungi: pseudo-$t = 3.91$, $p = 0.08$), as well as nest entrances (bacteria: pseudo-$t = 4.80$, $p = 0.03$, fungi: pseudo-$t = 6.34$, $p = 0.02$) and outer stem surfaces (bacteria: pseudo-$t = 5.01$, $p = 0.02$, fungi: pseudo-$t = 7.55$, $p = 0.02$). Contrary to predictions, worker and brood chambers did not have lower beta diversity than external sampling locations.

(c) Functional response of fungal communities across sampling locations

We were able to assign functional guilds to 59.71% of the filtered fungal OTUs in our dataset. Once unassigned OTUs were removed, putative plant pathogens were the most dominant functional guild (28% of the OTUs and 41% of reads). Contrary to initial predictions, putative plant pathogens were found in all locations, internal and external, and their average relative abundance did not differ across sampling locations (dAIC = 9.39, $\chi^2 = 6.58$, d.f. = 11, $p = 0.58$; figure 4*a*).

The second most diverse and common functional group was putative animal pathogens (15.5% of the OTUs and 26.6% of the sequences) and these were present in all sampling locations. However, as predicted, putative animal pathogens were in lower abundance in areas where ants were actively living and were abundant in empty chambers (dAIC = 19.06, $\chi^2 = 35.05$, d.f. = 11, $p < 0.001$; figure 4*b*). This effect was due

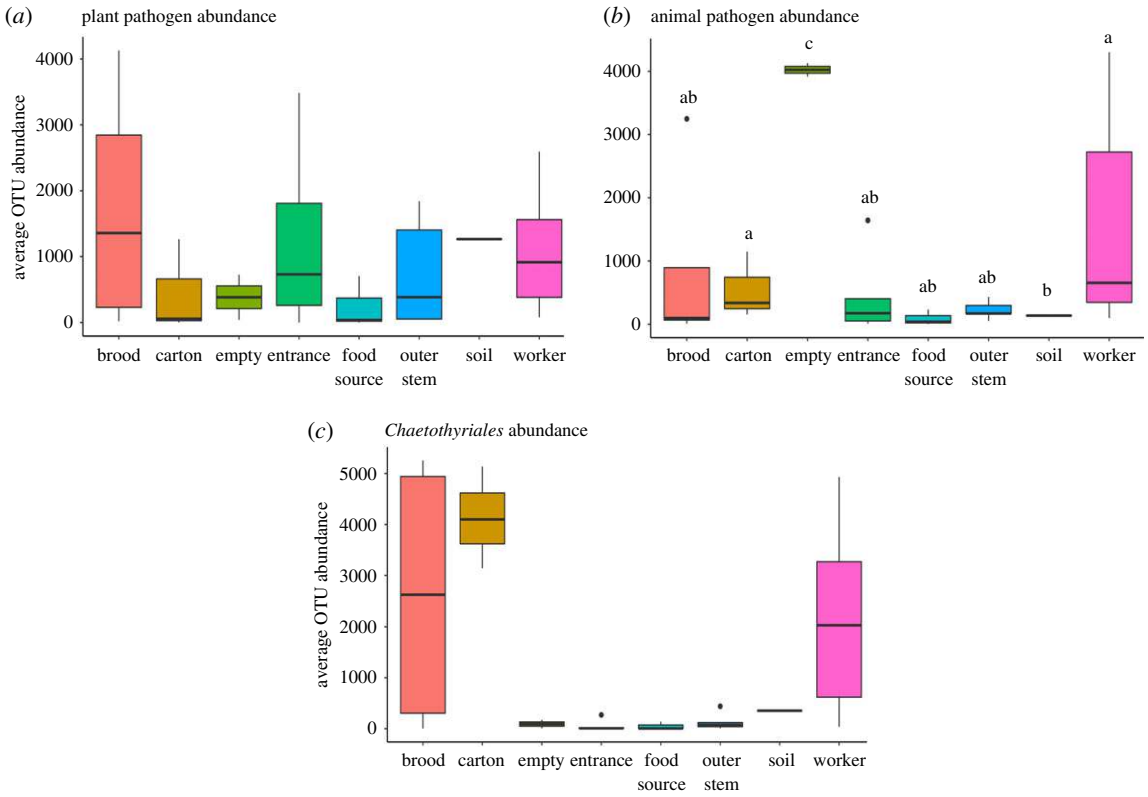

Figure 4. Boxplot showing the relative OTU abundance of (*a*) plant and (*b*) animal pathogens and (*c*) *Chaetothyriales* fungi across sampling locations. The boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively). Vertical lines inside the box define the median, while the whiskers represent the lowest and highest values within the 1.5 IQR. Samples exceeding those values (i.e. outliers) are represented as points outside of the box. Letters above boxes denote significant differences in OTU abundance across sampling locations. Note that plant pathogens did not differ across sampling locations, but animal pathogens did. (Online version in colour.)

in large part to the presence of the yeast *Cyberlindnera jadinii*, which accounted for an average of 41.7% of reads in empty chambers (electronic supplementary material, table S1). However, when we dropped *C. jadinii* from our analyses, we still found that empty chambers contained the highest levels of animal pathogens (dAIC = 5.56, $\chi^2 = 21.56$, d.f. = 11, $p = 0.005$, electronic supplementary material, figure S4).

4. Discussion

We investigated the microbiome of ant nests in a common, coevolved association between ants and plants. Consistent with our *a priori* predictions, the microbiome of ant nests inside trees differed markedly from the surrounding environment, and the nest microbiomes reflected chambers' functional roles. Also consistent with predictions, ants limited the prevalence of animal pathogens inside their *Cecropia* tree nests. However, contrary to our predictions, plant pathogens were prevalent within internal chambers, suggesting that *Azteca alfari* ants may not actively limit plant pathogens within their nests.

The distribution of microbes within *A. alfari* nests is parallel to that of human homes. Ants maintained distinct microbiomes depending on the chamber's function, similar to the unique microbiomes observed across rooms in human homes. One of the most interesting similarities between human homes and ant nests is the extent to which ants exclude external environmental microbes. Compared to the external nest entrances and outside stems, worker and brood chambers housed distinct microbiomes, and the carton within ant nests had lower beta diversity than the external sampling locations. The same is true of human homes, in which the phylogenetic diversity indoors is distinct and far lower within entire homes than it would be in even a pinch of nearby soils [6]. For humans, this loss of diversity is now largely viewed as negative, with corresponding negative health consequences [57]. For ants, circumstances in which this loss is negative or positive remain to be studied. Interestingly, the nests of soil-nesting ant species tend to have equal or higher microbial diversity compared with surrounding soil [58–62]. The positive or negative impacts of microbial diversity on ant colonies may therefore differ among ant species, and in particular, between soil- and arboreal-nesting species.

While patterns in microbial communities were similar between *A. alfari* nests and human homes, there were two key exceptions: (1) the setting in which developing ant brood live, and (2) the fate of abandoned rooms/chambers. Human young tend to spend large portions of time in daycares that are dominated by bodily microbiota, many of which can be pathogenic [63,64]. By contrast, ant 'nurseries' (brood chambers) demonstrated diminished levels of potentially pathogenic fungi and lower levels of bacterial richness overall. Brood are highly susceptible to infection due to their cuticles not being fully developed [65]. Consequently, many ant species exhibit intense sanitary care for brood via grooming or removal of brood from compromised environments [44]. Furthermore, larvae of *A. alfari* have specialized hairs that allow larvae to hang from the walls and ceiling of their nests which may limit their exposure to colonizing microbiota [66,67]. While this is a potentially useful comparison between the microbiomes ant and human young are exposed to, we acknowledge that there are some differences

between human nurseries and brood chambers that could explain why their microbiomes differ. In particular, human nurseries often concentrate individuals from various families, while ant chambers house highly related individuals. The related nature of ant brood is an important distinction and likely drives intense sanitary care in brood chambers. Ultimately, our study suggests that while *Azteca alfari* ants do not have complete control over the microbiomes of their nurseries, they are able to affect the composition of bacteria in the nursery in ways that both lower diversity and favour non-pathogenic species.

The second difference between human environments and ant domatia was seen in abandoned chambers. When humans abandon homes, there is generally a decrease in human-associated microbes [43]. In *A. alfari* nests, abandoned chambers were almost entirely dominated by fungal guilds putatively assigned as animal pathogens. While the assignment of the animal pathogen is broad and does not necessarily suggest that all identified animal pathogens are harmful to ants, we did see significant suppression of these fungal groups when ants were present. If these pathogens are harmful to the colony, we can imagine two explanations for why animal pathogen abundance may be heightened in empty chambers. The first explanation is that eventually pathogens build up in chambers, and, as a result, the chambers are no longer healthy for the ants. The ants then abandon those chambers and seal them off. If this is the case, ants may have more sophisticated methods for monitoring the health of their chambers than we do for human homes. The other explanation is that once a chamber is abandoned, pathogenic fungi can grow more readily. Given that animal pathogens were common, though not dominant, where ants were present, we do not believe ants themselves were exerting strong suppression outside of the nursery. However, ants are known to produce a range of antimicrobial compounds that could be used to control pathogens inside the nest [24,68]. Additional studies that assess whether these putative animal pathogens affect *Azteca* fitness are needed to confirm whether their suppression of animal pathogens is evolutionarily advantageous or simply a by-product of ant activity.

Abandoned chambers were dominated by a single OTU, *Cyberlindnera jadinii*. This species of fungus is classified as an animal pathogen [54], but the pathogenic nature of *C. jadinii* for *A. alfari* requires further exploration. While some studies have documented harmful *C. jadinii* infections in immunocompromised humans [69], other studies have demonstrated that *C. jadinii* can be a potent attractant for some dipteran species [70]. Furthermore, many ant species have positive associations with a diversity of yeasts [71–73]. It is possible that *C. jadinii* yeast may be initially beneficial but may become pathogenic above some abundance threshold. Colonies of the acorn-nesting ant *Temnothorax curvispinosus* avoid regions of their nests that become overgrown with microbes, which suggest that even relatively benign microbes can have negative impacts on ants when microbes reach high abundances [67]. Whether the presence of *C. jadinii* is harmful for *A. alfari* is an important next step for understanding the microbial symbionts in this *Cecropia–Azteca* mutualism. Additionally, we acknowledge that relative sequence abundance may not directly infer viable absolute abundance for our observed OTUs. Therefore, further studies are needed to understand how these diversity patterns relate to levels of pathogen exposure.

In contrast with our observations of putative animal pathogens in nests, we found no evidence that ants control plant pathogens inside or outside their nests. Putative plant pathogens were the most dominant functional guild in our study, and they were present in all nest locations at similar levels regardless of whether ants were present of not. Our results are similar to those of Letourneau [74], who found that *Pheidole* ants colonizing *Piper* ant-plants did not decrease foliar fungal pathogens. One potential explanation is that there could be low selective pressure for ants to actively defend their host plants against pathogens if the pathogens are not deadly to the plant or if ant colonies are much shorter-lived than their host plant. Another potential explanation is that while these fungi are putative plant pathogens [54], they might not harm *Cecropia* trees, and therefore there is no need to limit their presence.

Outside of the general patterns of microbial communities and functional guilds inside *A. alfari* nests, the nests also contained a diverse set of beneficial ant-associated fungi. Specifically, the fungal order Chaetothyriales was in high abundance inside *A. alfari* nests (figure 4*c*). It has been proposed that founding queens carry Chaetothyriales fungal patches inside their infrabuccal pocket; however, whether these fungi are actively or passively transmitted is yet to be determined [42]. Chaetothyriales plays an important role in helping ants build their carton material and is a food source for the colony pupae and workers in some ant species [42,75]. Additionally, Chaetothyriales has been proposed to be part of a tripartite mutualism between ants and *L. africana* plants [76]. Our results support previous studies that have documented the prevalence of this fungus within ant dwellings [40,42,76]. Assessing whether this prevalent ant-nest associate benefits ants and/or host plants would be an interesting follow-up study.

One potential limitation to our study is that our sampling methodology may not have captured all microbial organisms inside and outside of nests. We therefore acknowledge that our results are an estimation of the diversity and abundance of microbial co-inhabitants. Similarly, our sampling represents one time point and does not determine whether patterns of nest microbiomes are influenced by tree or colony age. Follow-up work will determine the extent to which the nest microbiomes of *A. alfari* are consistent across time and space.

5. Conclusion

We show that the bacterial and fungal communities within the nests of *A. alfari* differ from the surrounding environment and that chamber function can shape microbial communities. This finding supports previous research showing that ants have an advanced ability to monitor, influence and culture microbiota within their nests. We also present preliminary evidence that *Azteca* ants are able to limit the build-up of fungal groups that could be detrimental to colony health. The results of this study highlight the complexity of microbiota associated with ants and their nests while providing insight into the commonalities that exist between human-built and animal-built dwellings. Understanding the mechanisms ants use to effectively control animal pathogens, especially in their nurseries, could help us gain insight into how we may be able to prevent pathogen build-up in human-built environments.

Data accessibility. All data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.f83t155 [77].

Authors' contributions. M.J.E., E.K.M., C.A.P. and R.R.D. conceived of the project. M.J.E. and P.R.M. collected samples. M.J.E., E.K.M., D.J.F. and C.A.P. carried out molecular analyses. J.M.L., E.K.M., J.L.S. and A.A.M. analysed data. All authors contributed substantially to drafts.

Competing interests. The authors declare no competing interests.

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