Title: *Myrmecocystus* honeypot ants have species specific resident gut microbiome

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Abstract

Myrmecocystus honeypot ants rely on specialized workers, repletes, to store dissolved carbohydrates in their crops long term. The repletes store this liquid, which does not spoil in their crops, for many months at a time. When resources are scarce, repletes redistribute the stored nutrients to their colony members via trophallaxis. While we suspect that the gut microbiome of honeypot ants may aid in spoilage prevention, before we can investigate this, we must first characterize it. Here, we used 16S rRNA gene sequencing to determine the microbial community composition across six *Myrmecocystus* honeypot ant species, sampling multiple colonies, castes, and organs. We found that microbiome community composition was strongly shaped by species, with variation between colonies in *M. arenarius*, *M. depilis*, and *M. mexicanus*. Organ level differences were observed in the crop and midgut in *M. mexicanus*. Caste differences were observed in *M. flaviceps* and *M. mexicanus.* Replete crops of *M. mexicanus* and *M. depilis* were enriched in *Fructilactobacillus,* other lactic acid bacteria, and acetic acid bacteria, whereas halophiles were more prominent in the gut of species such as *M. flaviceps* and *M. wheeleri.* In this study we demonstrate that *Myrmecocystus* ants host species-specific gut microbiomes and identify an association between lactic acid bacteria, acetic acid bacteria, and halophiles within replete crops. While much work remains in understanding the roles of the microbes in the symbiosis with their host ants, the dominance of these particular taxonomic groups suggests an association with a high sugar environment and a potential microbial role in preventing spoilage of the crop contents.

Keywords

Replete, crop, lactic acid bacteria, halophiles

Introduction

Many animals, particularly those with specialized diets, rely on their microbiomes to supplement essential nutrients and provide beneficial services to the host (Engel & Moran, 2013). Compared to vertebrates, insects provide more tractable systems for studying host-microbiome relationships because they tend to have simpler gut morphology and gut microbiome communities. Host-associated microbiomes can contribute to a range of tasks, including nitrogen fixation and recycling (e.g., *Cephalotes* turtle ants), amino acid production (e.g.,

Onthophagus taurus dung beetles), toxin remediation (e.g., *Apis mellifera* honeybees), and the breakdown of complex plant polysaccharides the host cannot digest independently (e.g., *Nasuititermes* termites) (Brune, 2014; Auer et al., 2017; Hu et al., 2018; Flynn et al., 2021; Motta et al., 2022; Jones et al., 2025), However, not all insects require a resident gut microbiome. For example, despite their herbivorous diet, *Lepidoptera* caterpillars can meet their nutritional demands through host-encoded digestive enzymes and intrinsic mechanisms to degrade or tolerate plant toxins (Hammer et al., 2017).

Honeypot ants represent a compelling system for investigating animal-associated gut microbiomes. This convergently-evolved group comprises at least eight independently evolved genera spanning three Formicidae subfamilies (Sawh, 2023; Nogueira et al., 2026). In this study, we focus on the North American honeypot ants in the genus *Myrmecocystus*. This genus began diversifying approximately 14 million years ago, coinciding with the aridification of the southwestern United States and northern Mexico (Van Elst et al., 2021). The formation of new desert habitats created ecological niches that likely shaped the evolution of this group (Van Elst et al., 2021). To overcome irregular resource availability, honeypot ants have evolved specialized workers, known as repletes, that act as living food storage units to sustain the colony during dry, nutrient-limited seasons (Nogueira et al., 2026). Honeypot ant foragers collect extrafloral nectar, honeydew, and insect fluids to provide them to the repletes through trophallaxis, a regurgitation food-sharing process common to social insects (Meurville & LeBoeuf, 2021). As repletes accumulate the collected resources, the crops become enlarged and the intersegmental membranes of the gaster undergo extensive stretching (Fig. 1B, D-E) (Conway, 1977). While the basic structure of adult ant digestive tracts are conserved across all ants, honeypot ants are able to distend their crop far beyond the limits observed in non-replete ants, facilitating long term storage of liquid resources (Eisner & Brown, 1956). A 2023 study by Dong et al. found that the liquid stored in Australian honeypot ants, *Camponotus inflatus*, has antimicrobial properties. Based on whole ant sequencing, they concluded that the microbiome was dominated by the endosymbiotic bacterial genus *Candidatus Blochmannia* (99.75%) and the fungal genus *Neocelosporium* (92.77%) (Dong et al., 2023). *Blochmannia* is a well-known endosymbiont of *Camponotus* ants, residing within bacteriocytes associated with the midgut, and it contributes to host nutrition and development (Feldhaar et al., 2007). Since *Blochmannia* is restricted to midgut tissues, we suspect that the microbe responsible for the antimicrobial properties resides within the crop rather than the midgut (Feldhaar et al., 2007; Sauer et al., 2002). Therefore, we hypothesize that the gut microbiome of *Myrmecocystus* honeypot ants contributes to antimicrobial activity within their sugar-rich crop, thereby limiting the growth of pathogenic bacteria and fungi and preventing spoilage. However, before addressing the potential function, it is necessary to first characterize the gut microbiome of *Myrmecocystus* species.

In this study, we characterize the gut microbiome of six species of *Myrmecocystus* honeypot ants: *M. arenarius, M.depilis, M. flaviceps, M. mexicanus, M. mimicus* and *M, wheeleri*. We use community amplicon sequencing to determine whether a consistent resident gut microbiome exists. We compare the gut microbiome across scales: between different species within the

genus, between colonies of the same species, between castes within a colony, and between digestive organs within individual ants. If these ants possess a resident gut microbiome, we expect to observe the same community members in the same organs within a species, and potentially across the genus. Establishing whether *Myrmecocystus* hosts a resident gut microbiome is an important first step in understanding the relationship between these ants and their gut microbiome.

Materials and Methods

Field Sampling

Myrmecocystus ants were collected at five time points from 2022-2025 (Tab. S1), either from private property with permission or Bureau of Land Management (BLM) land, throughout the Southwestern United States (Tab. S1). Initial species identification was conducted onsite using worker morphological characteristics. We later confirmed ant identification through sequencing and analysis of the COI gene (Van Elst et al., 2021). Overall, we collected six *Myrmecocystus* species including *M. arenarius, M. depilis, M. flaviceps, M. mexicanus, M. mimicus*, and *M. wheeleri*. Our goal was to collect 10 repletes per colony, although this was not always achievable (Tab. S1). Alongside replete and worker samples, we also collected queens, alates, and brood however these collections were inconsistent and are saved for future examinations.

To collect repletes and workers, we excavated colonies until we found chambers containing repletes. Compared to repletes, regular workers were easy to find and were located both in the replete chambers and throughout the rest of the colony chambers and tunnels. Typically, replete chambers would be found at least 1 m underground, but on rare occasions we would find them closer to the surface. Holes were carefully hand dug with small shovels, and were filled in once collections were completed. We removed repletes and workers with featherweight forceps or with fan paintbrushes into containers. Ants were not fed and kept alive until the dissection process. Colony fragments were maintained together prior to dissection, allowing possible trophallactic exchange to continue during this period. Ants were first surface sterilized with 70% ethanol followed by sterile phosphate buffered solution. Dissections were carried out under sterile conditions next to an open flame, using a dissecting scope, on a sterile petri dish. To minimize microbial contamination, all tools and equipment were surface sterilized repeatedly between each dissection using 70% ethanol and by passing them through the flame. The ants were surface sterilized and dissected by first removing the head, then the external membrane of the gaster was opened using sterile ultrafine forceps, and the digestive organs (crop and midgut) were transferred with contents intact and not leaking into individual sterile tubes. If any organ ruptured during dissection, the sample was discarded and not used for DNA extraction or subsequent analyses. Dissected midguts and crops were placed into tubes containing 100 µL of TES buffer (10mM pH 7.5 Tris, 10mM pH 8 EDTA, 0.5% SDS) for DNA extractions. For the repletes, dissected midguts were also placed into 100 µL of TES buffer. Because the replete crops contained a relatively large amount of fluid, we reserved some material for future proteomics, metabolomics and microbial isolation. However, this study is focused on characterizing the microbiome community composition, and so we describe only the methods for conducting community amplicon sequencing.

131 **DNA Extractions and Sequencing**
132 For individual ant crop and midgut samples stored in 100 µL of TES buffer (10mM pH 7.5 Tris,
133 10mM pH 8 EDTA, 0.5% SDS), our custom phenol/chloroform extraction protocol was followed.
134 Samples were incubated with 36 µL of lysozyme (100 mg/mL in 10mM Tris pH 7.5; Thermo
135 Scientific, Waltham, MA, USA) for 24 hours at 37℃. Then, 220 µL of TES and 5 µL proteinase K
136 (800 U/ml; New England Biolabs, Ipswich, MA, USA) were added to our samples. Samples were
137 incubated at 56°C for 20 minutes. Following incubation, 250 µL of Sigma Aldrich 3M sodium
138 acetate was added, and samples were frozen at -80°C for at least 15 minutes. Once the
139 samples had thawed, samples were spun at 14,000 rpm for 15 minutes at 4°C. The supernatant
140 is then transferred to a new tube, and a 1:1 volume of phenol:chloroform is added. Samples
141 were spun at 14,000 rpm for 15 minutes at 4℃. The supernatant was transferred to a new tube,
142 and a chloroform wash was performed. Before precipitating, 10% sodium acetate and 90% cold
143 isopropanol were added. Samples were spun at 14,000 rpm for 15 minutes at 4°C to pellet the
144 DNA. Then we performed two ethanol washes with 70% ethanol. The DNA was then
145 resuspended in 50 µL TE (10mM pH 7.5 Tris, 1mM EDTA). DNA concentrations were quantified
146 with Qubit HS dsDNA (Thermo Fisher Scientific, Waltham, MA, USA). Extracted DNA samples
147 were then shipped to the University of Connecticut's Microbial Analysis, Resources, and
148 Services (UCONN MARS) facility for 16S rRNA gene amplicon sequencing and ITS amplicon
149 sequencing.
150
151 According to the UCONN MARS facility protocol, the 16S rRNA V4 region was amplified using
152 515F and 806R with Illumina adapters and dual 8-basepair indices (Kozich et al., 2013). The
153 ITS2 region was amplified with ITS3 and ITS4 primers (White et al., 1990) using the same dual
154 indexing design as the V4. PCR reactions were performed with an initial denaturation of 95˚C
155 for 3.5 minutes, followed by 30 cycles of 30 seconds at 95.0°C, 30 seconds at 50.0°C, and 90
156 seconds at 72.0°C, with a final extension at 72.0°C for 10 minutes. PCR products were pooled
157 for quantification and visualization using the QIAxcel DNA Fast Analysis (Qiagen, Hilden,
158 Germany). PCR products were normalized based on the concentration of DNA from 250-400
159 bp, then pooled using the epMotion 3075 liquid handling robot. The pooled PCR products were
160 cleaned using Mag-Bind Beads (0.8x ratio; Omega Bio-Tek, Norcross, GA, USA) and
161 sequenced on an Illumina MiSeq platform (Illumina; San Diego, CA, USA) with a v2 2x250 base
162 pair kit. We only conducted ITS sequencing for the first set of collected samples. Because we
163 found almost no fungi in our samples, we chose to focus on bacteria and archaea only in
164 subsequent sequencing runs.
165
166
167 **Amplicon and Statistical Analysis**
168 We processed, aligned, and categorized sequence reads using DADA2 1.25.2 in R (Callahan et
169 al., 2016). The DADA2 pipeline was followed exactly, with the addition of the decontam package
170 to identify and remove potential contaminants (Davis et al., 2018). After filtering, trimming, and
171 merging reads, amplicon sequence variants (ASV) were taxonomically assigned using the
172 Greengenes v2 database. To construct the ASV-based phylogeny required for weighted
173 UniFrac analyses, we first made an alignment using MAFFT (Katoh et al., 2002). An ASV
174 phylogeny was inferred from these sequences using the GTR+G model in IQtree v2.4.0 (Minh et

al., 2020). The resulting tree was rooted in R using the phangorn package (Schliep, 2011). The rooted tree was incorporated into a phyloseq object (McMurdie & Holmes, 2013).

Weighted UniFrac distances were calculated using the phyloseq package, and ordinations were performed using non-metric multidimensional scaling (NMDS) with the vegan package (Oksanen et al., 2022). Microbiome composition was visualized using NMDS plots generated with the plot_ordinate function in phyloseq (McMurdie & Holmes, 2013).

Statistical significance between groups was evaluated using global permutational multivariate analysis of variance (PERMANOVA) and pairwise differences with ANOSIM in the vegan package (Oksanen et al., 2022). Analysis of compositions of microbiomes with bias correction (ANCOM-BC2) was used to identify differentially abundant taxa in each organ, in this case the log fold changes between the crop and midgut (Lin & Peddada, 2020). ANCOM-BC2 was mainly used for organ comparison because it allowed us to test whether the repletism phenotype is associated with differential abundance of specific taxa. Alpha diversity metrics, including observed richness, Shannon diversity, and inverse Simpson diversity, were calculated to measure the richness and evenness of microbial communities within the samples, using phyloseq (Shannon, 1948; Simpson, 1949; McMurdie & Holmes, 2013). Statistical differences in Shannon diversity index were first assessed using Kruskal-Wallis test, followed by pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg correction for multiple comparisons (Wilcoxon, 1945; Kruskal & Wallis, 1952; Benjamini & Hochberg, 1995). Bubble plots were generated in R using the ggplot2 and reshape2 package to visualize the presence and relative abundance of microbial taxa across species and across organs (Wickham, 2011, 2016).

Results

Species Differences

Microbiome composition differed strongly across *Myrmecocystus* species, with distinct taxa characterizing each species (Fig. 2, Fig. 3). In particular, *M. mexicanus* replete crops were dominated by *Fructilactobacillus*, with the top three ASV belonging to this genus (ASV1, ASV5, and ASV12) (Fig. 3, Fig S2). In contrast, *Fructilactobacillus* was largely absent from the replete crops of some species (e.g., *M. arenarius* and *M. wheeleri)* (Fig. 3, Fig S2). These differences were statistically significant across species and explained a large proportion of variation in community composition (PERMANOVA $p = 0.000999$; ANOSIM $p = 0.001$; Tab. S5). *Myrmecocystus* species accounted for approximately 61% of the variation in replete crop microbiome ($R^2 = 0.60726$) and 45% in the replete midgut ($R^2 = 0.45325$; Tab. S5). Shannon diversity differed significantly among species (Kruskal-Wallis chi-squared = 65.873, $p = 7.385e^{-13}$). *Myrmecocystus mexicanus* exhibited significantly lower Shannon diversity than all other species, whereas some species pairs (e.g., *M. depilis* and *M. mimicus*) did not differ significantly (Tab. S2).

Overall, species identity is a substantial factor of microbiome variation. Notably, *M. mexicanus* was characterized by a less diverse, *Fructilactobacillus*-dominated crop microbiome, whereas other species exhibited more compositionally diverse crop microbiomes (Fig. S2).

Colony Difference Within Species

Microbiome composition differed among some colonies within *Myrmecocystus* species, but this pattern was not universal (Tab. S5). Significant colony-level differences were observed in *M. arenarius, M. depilis,* and *M. mexicanus* (PERMANOVA, $p < 0.01$; Tab. S5), whereas *M. flaviceps, M. mimicus,* and *M. wheeleri* showed no detectable colony level differences. Within *M. mexicanus*, collection year (e.g., July 2022, August 2023, and July 2025) explained a relatively small proportion of variation in the crop microbiome (PERMANOVA $R^2 = 0.09736$; Tab. S6). Despite variation among colonies and across years, *Fructilactobacillus* remained a consistent and dominant genus in *M. mexicanus* crop, with ASV1 present in all samples (100% prevalence). However, the relative abundance of co-dominant taxa shifted across time points, including *Acetobacteraceae* G004843345 (ASV6), *Weizmannia* (ASV21), and lactic acid bacteria such as *Pediococcus* (ASV23) and *Lentilactobacillus* (ASV42). Similarly, *M. depilis* exhibited variation in microbiome composition within dominant genera between collection years, 2023 and 2025 (Tab. S6). The top ASV in *Myrmecocystus depilis* samples collected in 2023 included *Fructilactobacillus* (ASV1), *Acinetobacter* (ASV73), and *Vibrio* A 676397 (ASV7), while 2025 samples were characterized by increased abundance of *Saccharibacter* (ASV11) and other genera.

Here, we observe that colony differences were present in some but not all species, and that collection time can contribute to these patterns. Variations among colonies may partly reflect uneven sampling across species and time points.

Caste Differences (Replete vs. Worker)

Non-metric Multidimensional Scaling (NMDS) allows us to visualize overlap between replete and worker organ communities (Fig. 4). Overall, caste did not strongly structure microbiome composition, as no significant differences were detected between replete and worker samples in either the crop and midgut (PERMANOVA, $p > 0.05$; Tab. S5). However, caste differences were observed for certain species (Fig. 4; Tab. S5). In *M. depilis, M. flaviceps,* and *M. mexicanus,* worker and replete crop microbiomes differed significantly (PERMANOVA, $p < 0.05$; Tab. S5), whereas no caste differences were detected in other *Myrmecocystus* species (Tab. S5). These differences were driven by shifts in dominant taxa between castes. For example, in *M. depilis* replete crops contained *Fructilactobacillus* (ASV1: 0-99.28%), *Saccharibacter* (ASV11: 0-82.57%), and *Acinetobacter* (ASV73: 0-60.22%). While *M. depilis* worker crops were characterized by *Vibrio* (ASV7: 0-37.23%), *Pseudoalteromonas* (ASV22: 0-17.31%), and *Haloparvum* (ASV17). Similarly, in *M. flaviceps,* replete crops were dominated by *Haloparvum* (ASV17) and Bacili-associated taxa (ASV362 and ASV224), while worker crops showed higher relative abundance of *Weizmannia* (ASV21) and *Natronomonas* (ASV24). In *M. mexicanus*, both castes were dominated by *Fructilactobacillus*, but worker crops also differed in the relative abundance of the secondary taxon *Acetobacteraceae* G004843345 (ASV6 and ASV19). Shannon diversity also differed between castes in some species. Worker samples had a higher Shannon diversity than repletes in *M. flaviceps* (Wilcoxon test, adjusted $p = 0.0378$) and *M. mexicanus* (Wilcoxon test, adjusted $p = 0.0170$). However, no significant caste differences were observed for other species (Fig. S4).

Overall, caste did not strongly structure gut microbiomes across *Myrmecocystus* species. While crop communities differed between castes in some species (e.g., *M. depilis, M. flaviceps,* and *M. mexicanus),* the midgut communities remained similar between castes across all species in our analysis.

Organ Differences (Crop vs. Midgut)

Microbiome composition differed significantly between the crop and midgut across *Myremecocystus* species, independent of species identity (PERMANOVA $R^2 = 0.36251$, $p = 0.000999$; Tab. S5). Differences between the crop and midgut were detectable in *M. depilis*, *M. mexicanus*, *M. mimicus*, and *M. wheeleri* (PERMANOVA and ANOSIM, $p < 0.05$; Tab. S5). However, *M. arenarius* and *M. flaviceps* showed no detectable crop-midgut difference (Tab. S5). Shannon diversity was significantly higher in the midgut than the crop in *M. mexicanus* repletes (Wilcoxon rank-sum test, FDR-adjusted $p = 1.82e^{-13}$) (Fig. 5). While no significant differences in diversity were observed between organs in other species (all FDR-adjusted $p > 0.05$) (Fig. 5; Fig S3). ANCOM-BC2 identified several genera that are differentially enriched in either gut compartments in *M. depilis* and *M. mexicanus* (Fig. 6; Tab. S3-S4). In both species, *Fructilactobacillus* was significantly enriched in the crop (Fig. 6). In *M. mexicanus,* several additional lactic acid bacteria and Acetobacteraceae were also enriched in the crop, whereas taxa such as *Vibrio* and *Pseudoaltermonas* were enriched in the midgut (Fig. 6; Tab. S3-S4). No taxa were identified as differentially abundant between the organs in *M. mimicus, M. arenarius, M. flaviceps,* and *M. wheeleri.*

Overall, gut compartmentalization was observed in some *Myrmecocystus* species. Particularly in *M. mexicanus,* where the crop and midgut differed in both community composition and diversity.

Discussion

In this study, we characterize gut microbiomes across six *Myrmecocystus* species by analyzing the communities across species, colony, caste, and organ. Across our analyses, microbiome composition was primarily driven by ant species identity. Caste had minimal influence on microbiome structure, with repletes and workers generally retaining similar bacterial communities despite differences in morphology, varying tasks, and potential environmental exposures. While at first we were surprised that there were minimal differences between castes, in retrospect it makes sense for an ant species that engages in frequent trophallaxis, constantly sharing the contents of their digestive systems, especially the contents of their crops, also known as their social stomachs (LeBoeuf, 2017). Organ level differences between gut microbiomes were only statistically significant in the species *M. mexicanus*. While colony-level differences between gut microbiome composition were statistically significant for *M. mexicanus, M. arenarius, and M. depilis* they were not consistent across all species. Among all the species examined, *M. mexicanus* showed a specialized resident microbiome, with replete crops dominated almost entirely by *Fructilactobacillus.* This monodominance is striking when compared to other species within the *Myrmecocystus* genus, which generally retained more diverse crop microbial communities. Gut compartmentalization can play a role in shaping the microbial community. For example, in *Cephalotes* turtle ants, specific gut compartments support

specialized and conserved symbiont communities (Flynn et al., 2021). Here, we observe evidence of compartmentalization in the *M. mexicanus,* where the crop was dominated by *Fructilactobacillus*, while the midgut exhibited more microbial diversity.

Lactic acid and acetic acid bacteria

The complete dominance of *Fructilactobacillus* was only observed in *M. mexicanus* replete crops but it was also abundant in other species, just to a lesser degree (e.g., *M. depilis*). Where there was less *Fructilactobacillus,* there were often other genera of lactic acid or acetic acid bacteria present in high abundance (Fig 3). Lactic acid bacteria, particularly, are often found in the guts of sugar-feeding social insects. In aphid-tending *Formicine* ants and *Lasius* ants, their infrabuccal pockets and crops are abundant in lactic acid bacteria such as *Lactobacillus*, *Weissella*, and *Fructobacillus* that are capable of catabolizing sugars commonly found in hemipteran honeydew, including sucrose, trehalose, melezitose, and raffinose (Zheng et al., 2022). Beyond catabolizing sugar-rich foods, lactic acid bacteria can inhibit microbial competitors by producing lactic acid and other antimicrobial compounds, lowering pH, and generating acidic conditions that many bacteria and fungi cannot tolerate, as demonstrated in *Mycocepurus smithii* (Kellner et al., 2015). *Myrmecocystus* ants belong to the subfamily Formicinae, which are known to produce and consume formic acid from modified poison glands. Some ants in this subfamily have been shown to control their gut microbiomes by creating an acidic crop environment through formic acid consumption (Tragust et al., 2020). *Fructilactobacillus*, other lactic acid bacteria and acetic acid bacteria, which are acid tolerant, could be thriving due to these conditions in *Myrmecocystus* guts or they could be contributing to further acidification to control their environment in their favor. *Fructilactobacillus* is widely used in the food industry for the fermentation of dairy, meat, sourdough, and vegetables (Hammes & Vogel, 1995; Koga et al., 1998; Kellner et al., 2015; Byun et al., 2025). Acetic acid (vinegar) is also used in food production and preservation, both with live culture fermentation and with industrially derived vinegar (Sengun & Karabiyikli, 2011; De Roos & De Vuyst, 2018). Acetic acid bacteria inhibit the growth of foodborne pathogens (e.g., *Salmonella* spp.), as well as agents of food spoilage such as yeasts and molds (Levine & Fellers, 1940).

The acid-loving bacteria that dominate the digestive systems of *Myrmecocystus* ants could be commensal, not providing any benefit or harm to their host, but simply growing in an ideal niche. However, they could also be providing benefits to their hosts. First, they may facilitate the metabolism of sugar-rich diets by efficiently catabolizing carbohydrates (e.g., hemipteran honeydew and extrafloral nectar) stored in replete crops into sugars or other molecules that are more easily metabolized by the ants. Second, they may contribute to host fitness by acidifying the gut environment to inhibit the growth of pathogenic microbes. And third, their function may be analogous to lacto fermentation for human food preservation, where *Fructilactobacillus* prevents spoilage of nutrients in replete crops. In future work, we will examine the growth and metabolism of *Myrmecocystus* lactic acid and acetic acid bacteria, starting with *Fructilactobacillus* in *M. mexicanus* guts.

Halophiles

We also detected halophilic archaea and bacteria in multiple species collected in California, including *Haloparvum, Haloarcula, Halorubrum, Halobacterium* and *Natronomonas*. The high osmotic pressure generated by concentrated sugars in replete crops may favor the proliferation of halotolerant taxa, not because they are necessarily adapted to high salt environments, but because they are physiologically adapted to high osmotic pressures. Both high-salt and high-sugar environments reduce water activity and impose similar constraints on microbial growth (Kim et al., 2013). These halophiles could be adapted to the high sugar conditions within the crop. In *Myrmecocystus,* replete crops are concentrated in carbohydrate, composed primarily of fructose and glucose, with smaller amounts of maltose (Burgett & Young, 1974). Strains of *Tetragenococcus halophilus* typically associated with high-salt environments, have also been detected in sugar-rich beet juice (Justé et al., 2008; Lievens et al., 2015). Justé et al. (2008) found that some strains of *T. halophilus* were adapted to high salt, while others were adapted to high sugar environments, but not both. Halophilic archaea, including *Halobacterium* and *Haloarcula,* are capable of growing on sugars such as glucose, fructose, and sucrose (Johnsen et al., 2001). Together, these observations raise the possibility that halophilic bacteria and archaea detected in *Myrmecocystus* guts represent lineages adapted to high sugar conditions within the replete crop.

Where do the microbes come from?

The variation we see in microbiome composition across *Myrmecocystus spp.* is expected in social insects, where species in the same genus may differ in diet, habitat, foraging behavior, and exposure to environmental conditions (Hölldobler & Wilson, 1990). Even within *M. mexicanus*, variation among colonies and sampling periods suggest that beyond species identity, differences in vegetation, resource availability, and soil composition across habitats may influence microbial assembly across colonies. Similar patterns are observed in other social insects, where microbiome composition reflects environmental context. For example, *Oecophylla smaragdina* weaver ants gut microbiome composition differs between forest and urban habitats primarily due to variations in feeding habits and host diet (Chua et al., 2018).

Diet could contribute to the structuring of *Myrmecocystus* microbiome. *Myrmecocystus* foragers collect a wide range of resources, including gall secretions, aphid and coccid secretions, and extrafloral nectar (Burgett & Young, 1974). While all *Myrmecocystus* are considered scavengers, there are differences in dietary preferences and foraging habits among species (Snelling, 1976). Species that are primarily nocturnal, such as *M. mexicanus*, are thought to largely rely on liquid carbohydrate resources such as nectar and plant exudates (Snelling, 1976; Van Elst et al., 2021). Whereas more diurnal species, such as *M. wheeleri,* are described as more carnivorous, supplementing their diet with live or dead arthropods (Snelling, 1976; Van Elst et al., 2021). Comparative studies on Indo-Pacific ants, identified host dietary niche as the primary driver of microbial diversity and outweighing the contributions from the physical environment (Cunningham et al., 2025). In many cases, microorganisms do not just occupy the crop but actively define its physio-chemical state. In *Apis mellifera* honeybees, their diet, consisting of nectar and honey, introduces high levels of sucrose, glucose, and fructose in the crop, acting as a selective pressure that controls the assembly of local bacterial and fungal

389 communities (Callegari et al., 2021). Specialized microorganisms like acetic acid bacteria act as
390 oxygen scavengers to maintain suboxic conditions, while other fermentative bacteria produce
391 organic acids that acidify the gut environment (Callegari et al., 2021). We suspect that a similar
392 process is occurring in *Myrmecocystus,* where their diet favors microbes adapted to
393 carbohydrate metabolism, low pH tolerance, and osmotically stressful environments in the crop.
394 We expect that many members of the *Myrmecocystus* gut microbiome originate from their diets.
395 Many lactic acid bacteria that have been found in the crops of these ants have also been
396 isolated from nectar (Oliphant et al., 2023). It is also likely that halophilic archaea and bacteria
397 are also found in sources such as nectar with relatively high osmotic pressure. In the human
398 gut, it has been found that halophilic archaea have been associated with the dietary intake of
399 fermented and salted foods (Chang et al., 2008; Lee, 2013). We expect that diet could be a
400 source for microbiome community members, and that the crop conditions help to further shape
401 the community.
402
403 Another possible contributor to the origin of *Myrmecocystus* gut microbiome is through contact
404 of their immediate surroundings, such as soil-associated microbes. From our collected samples,
405 many of the colony entrances were located in close geographic proximity, which could increase
406 the likelihood of shared environmental exposure. In *Veromessor andrei* harvester ants, colonies
407 that are geographically closer harbor more similar microbial communities (Gamboa et al., 2025).
408 However, microbiome composition in this system is also shaped by host-associated factors
409 such as diet, life stage, and caste (Gamboa et al., 2025). If soil-associated microbes were a
410 dominant driver of microbiome assembly in *Myrmecocystus,* we would expect colonies that are
411 spatially close, regardless of species identity, to have similar microbial communities. Yet, our
412 results showed that colonies, such as *M. mexicanus* (X9 and X10) and *M. mimicus* (M1 and M2)
413 collected in July 2025, retained distinct species-specific microbiome compositions despite being
414 in close proximity (Fig. 3). Therefore, we do not expect that the immediate environment, namely
415 the soil, is the main contributor to the microbiome of these ants, however, more work will be
416 necessary to confirm this.
417
418 **Moving forward**
419 Future work will aim to disentangle the ecological and evolutionary drivers shaping these
420 microbial communities. In particular, we will be conducting field work to distinguish the
421 contributions of diet and physical environment (e.g., surrounding soil and food sources), as well
422 as to better understand how different transmission modes contribute to the shaping of the gut
423 community in *Myrmecocystus* honeypot ants. *Myrmecocystus* ants appear to have species-
424 specific resident gut microbiomes. Building on this finding the next steps are to examine the
425 relationship between the honeypot ants and their gut microbiome. We will investigate the
426 localization of these microorganisms throughout the digestive systems of the ants to better
427 understand if there are specialized structures for supporting these microorganisms. By
428 leveraging tools such as fluorescence in-situ hybridization and green fluorescent protein-tagged
429 microbes we will be able to explore the spatial distribution of lactic acid bacteria like
430 *Fructilactobacillus* or halophiles like *Haloparvum.* We will explore the nature of symbiosis
431 between the microbes and the ants (i.e., is the relationship commensal or mutualistic) through
432 various *in vivo* and *in vitro* physiological assays. We will couple these experiments with

metabolomics, transcriptomics, and proteomics to help identify the metabolic pathways, metabolites, and functional roles of gut microbiome community members. Beyond work within *Myrmecocystus*, comparative studies across convergently-evolved honeypot ant genera such as *Leptomyrmex*, *Camponotus*, and *Tapinolepis* could provide insight into the role of host physiology in structuring microbiome across independent evolutionary lineages.

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Data Availability

All raw sequencing data are available in the NCBI Sequence Read Archive (SRA) under the Bioproject PRJNA1449520: *Myrmecocystus* honeypot ants 16s rRNA.

Author Contributions

We followed CRediT's 14 Contributor Roles guidelines. **DVN**: Conceptualization; Data Curation; Formal Analysis; Investigation; Validation; Visualization; Writing (original draft); Writing (review and editing). **CF**: Conceptualization; Data Curation; Funding Acquisition; Investigation; Methodology; Visualization; Writing (original draft). **BN**: Investigation. **IS**: Investigation; Methodology. **ML**: Investigation; Methodology. **LK**: Conceptualization; Funding Acquisition; Project Administration; Supervision; Validation; Writing (review and editing)

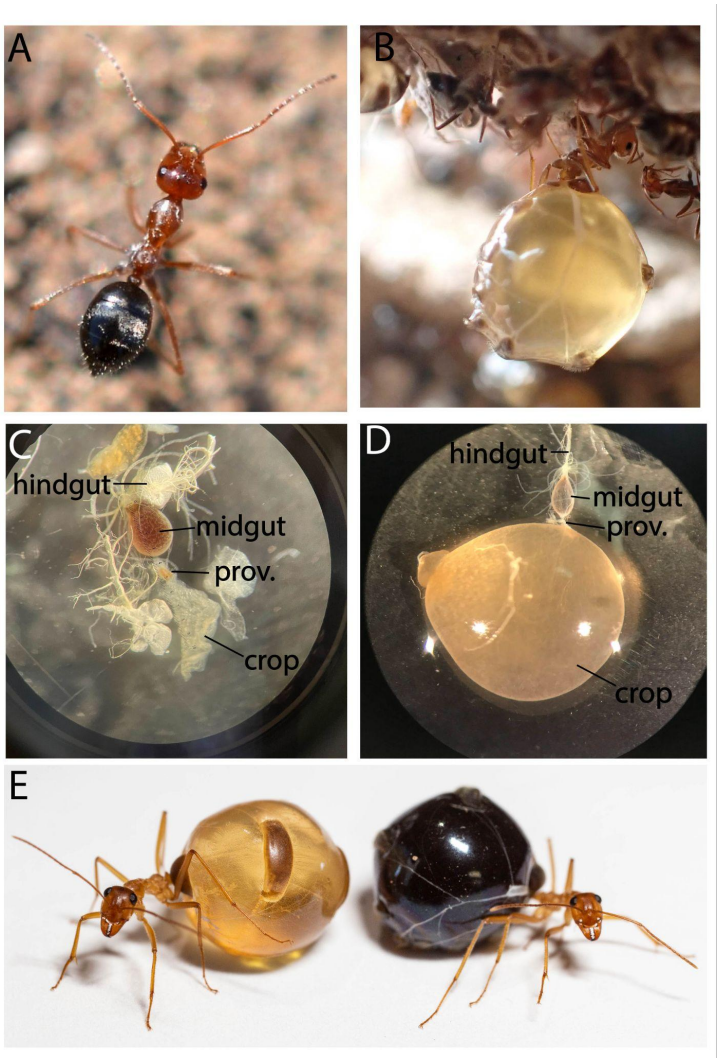

Figure 1. Honeypot ant colonies contain two castes, workers (A) and repletes (B). Compared to the crops of workers (C), the crops of repletes can expand to huge proportions as they are used to store fluids. (E) Depending on the resources collected, repletes within the same colony or chamber can have abdomens of different colors. Photo credits: (A) D. Caroll from buigguide.net, (B) M.P. Meurville, (C) I. Sawh, (D) C. Francoeur, (E) M. Lanan.

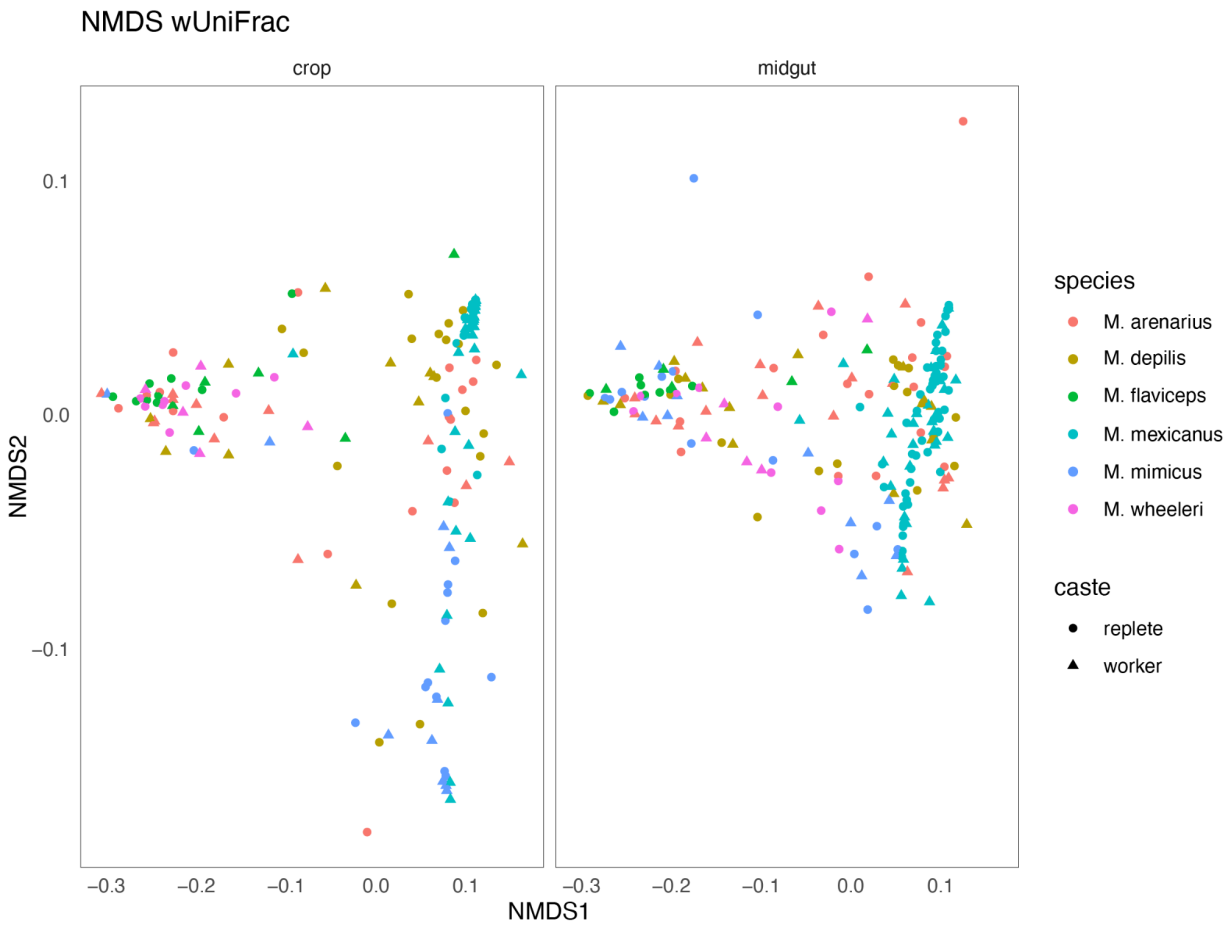

Figure 2. Non-metric Multidimensional Scaling (NMDS) ordination based on weighted UniFrac distances visualizes differences in microbial community composition across *Myrmecocystus* species, caste, and organ. Each point represents individual samples, species indicated by color, caste by shape, with panels corresponding to crop or midgut samples. Microbial communities show species associated clustering, with partial overlap among species. Crop and midgut occupy distinct regions of ordination space across species. Caste has a minimal effect on community composition, as no clear separation is observed.

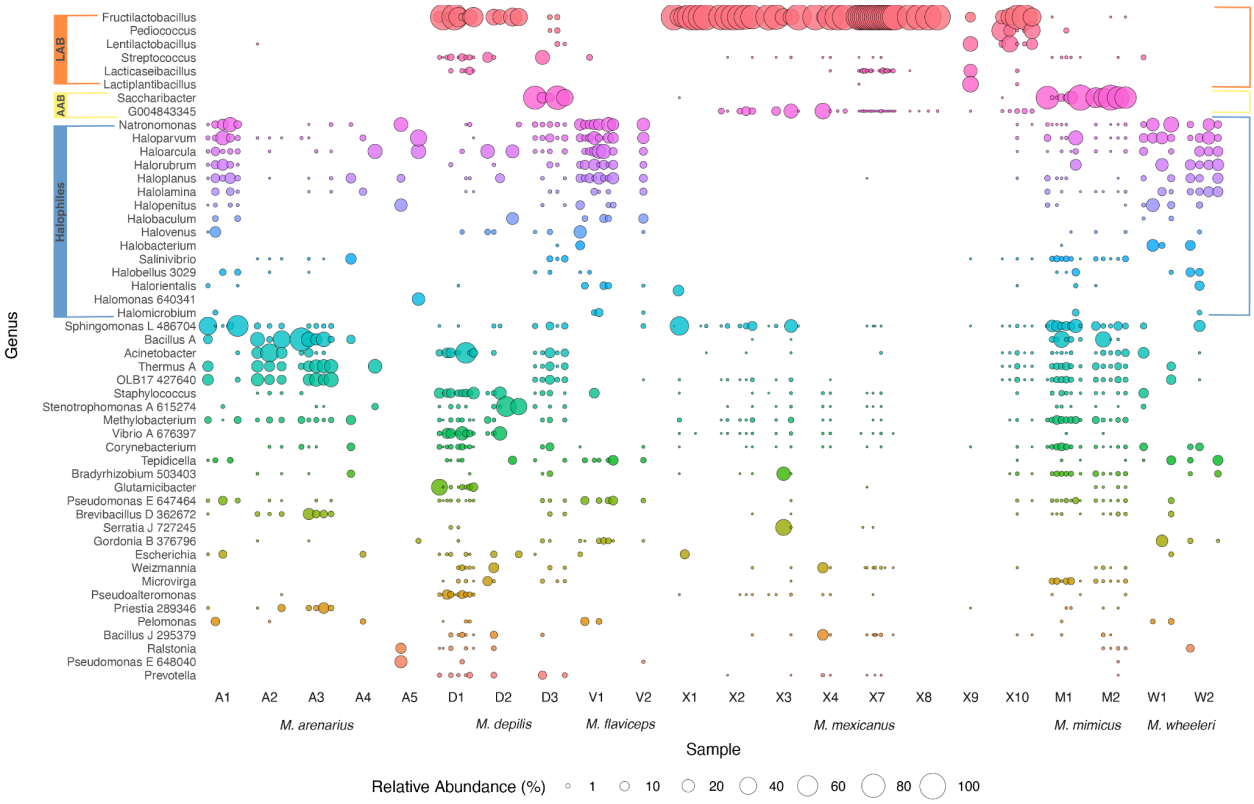

Figure 3. The bubble plot depicts the relative abundance (as percentage) of bacterial genera in replete crop samples across *Myrmecocystus* species. Each point represents a genus within a sample, with the bubble size indicating its relative abundance, and the color signifying genus identity. Genus names are grouped by lactic acid bacteria (LAB = orange), acetic acid bacteria (AAB = yellow), and halophilic archaea and bacteria (blue). The microbial communities in replete crops exhibit species-specific structuring. In particular *M. mexicanus* replete crops are strongly dominated by *Fructilactobacillus*, whereas other species show more variable community composition.

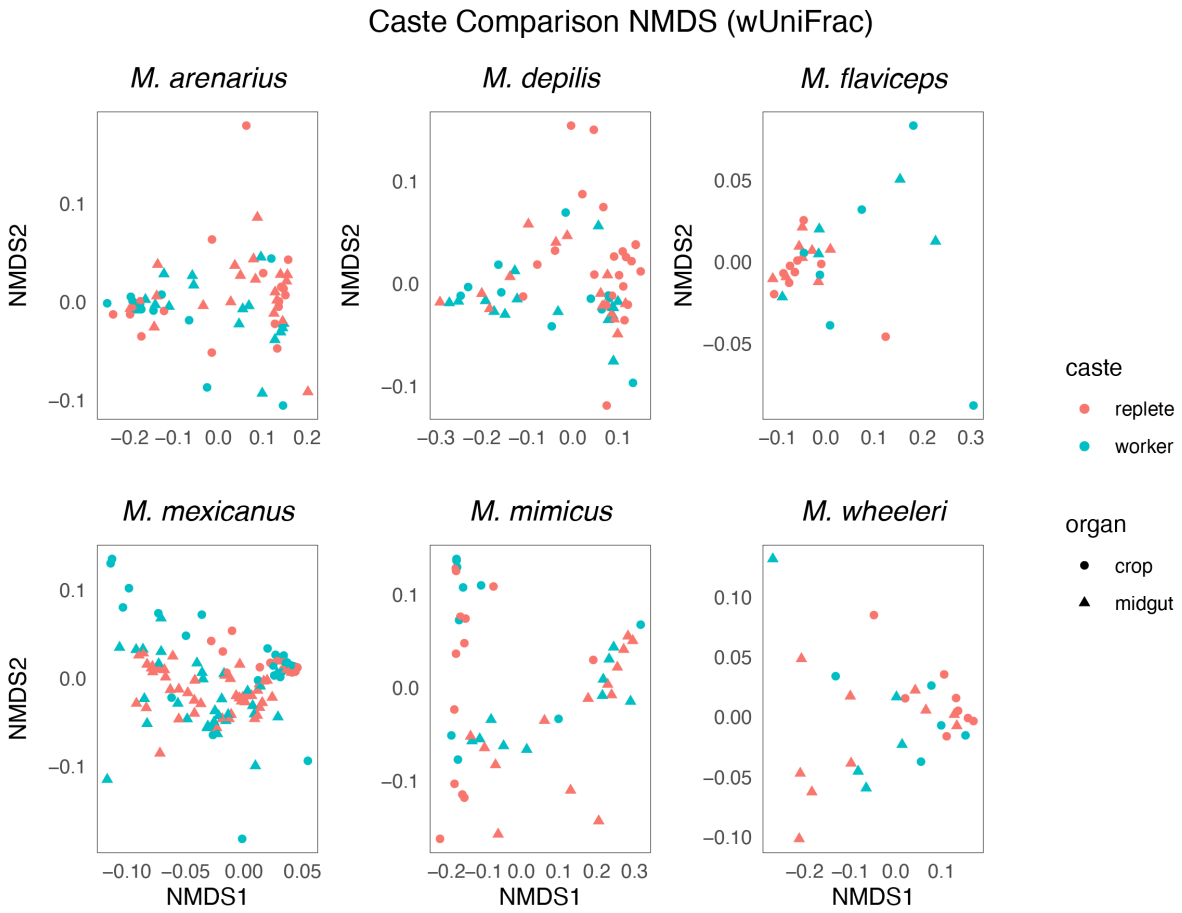

Figure 4. NMDS ordination with weighted UniFrac distances comparing the microbial community composition across caste replete (red) and worker (blue). Caste did not strongly structure gut microbiomes across *Myrmecocystus* species. However, within certain species, *M. depilis, M. flaviceps,* and *M. mexicanus,* replete and worker crops differed significantly in microbial composition. Whereas the midgut communities remained similar between castes across all species.

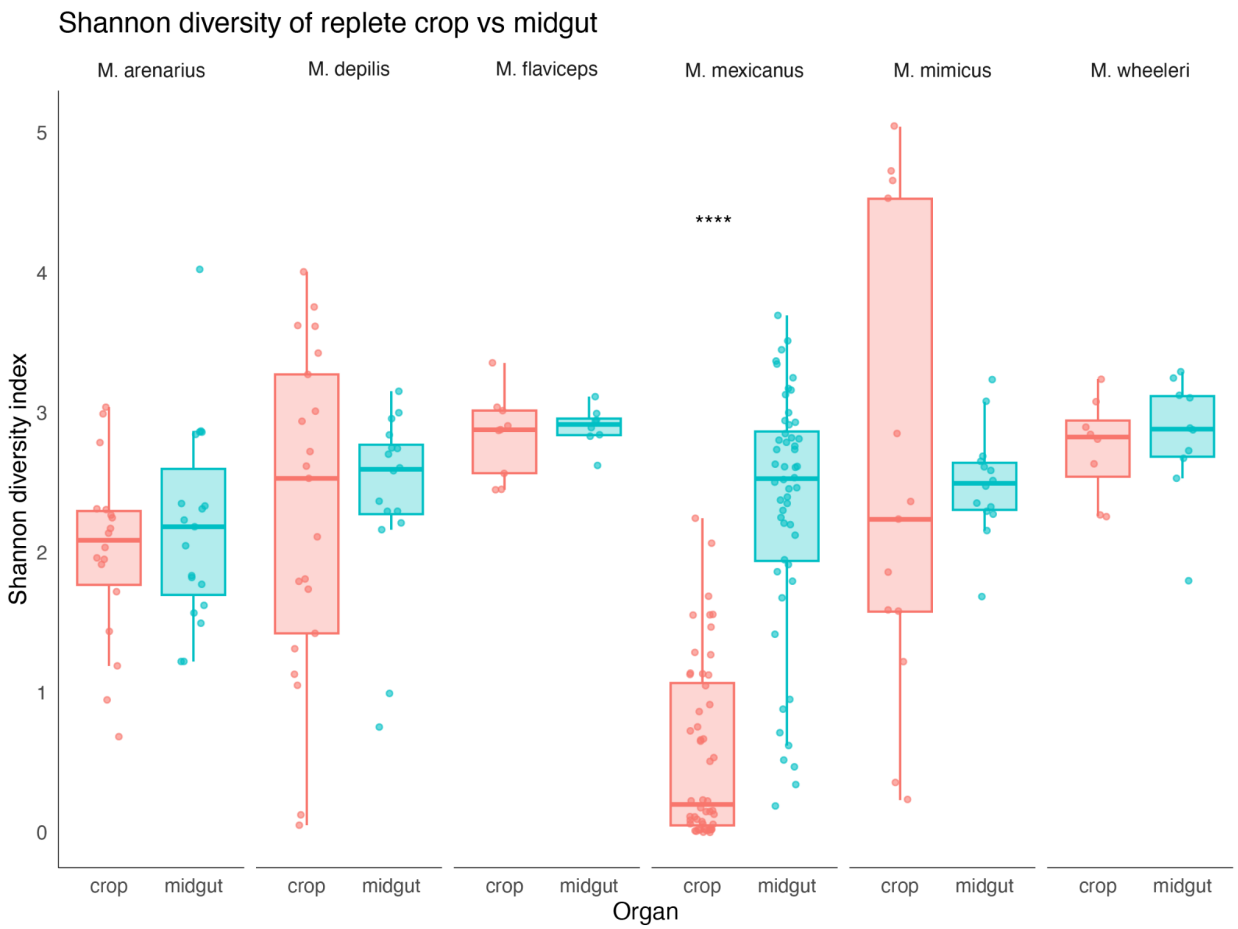

Figure 5. Shannon diversity index comparing the microbial diversity between replete crop and midgut samples across *Myrmecocystus* species. Each point represents individual samples, with boxplots summarizing the distribution of diversity for each organ within species. The midgut communities generally exhibit higher diversity than crop communities, however, this difference is only statistically significant in *M. mexicanus* (Wilcoxon rank-sum test, FDR-adjusted p = $1.82e^{-13}$). No significant differences in Shannon diversity were observed in other *Myrmecocystus* species (all FDR-adjusted p > 0.05).

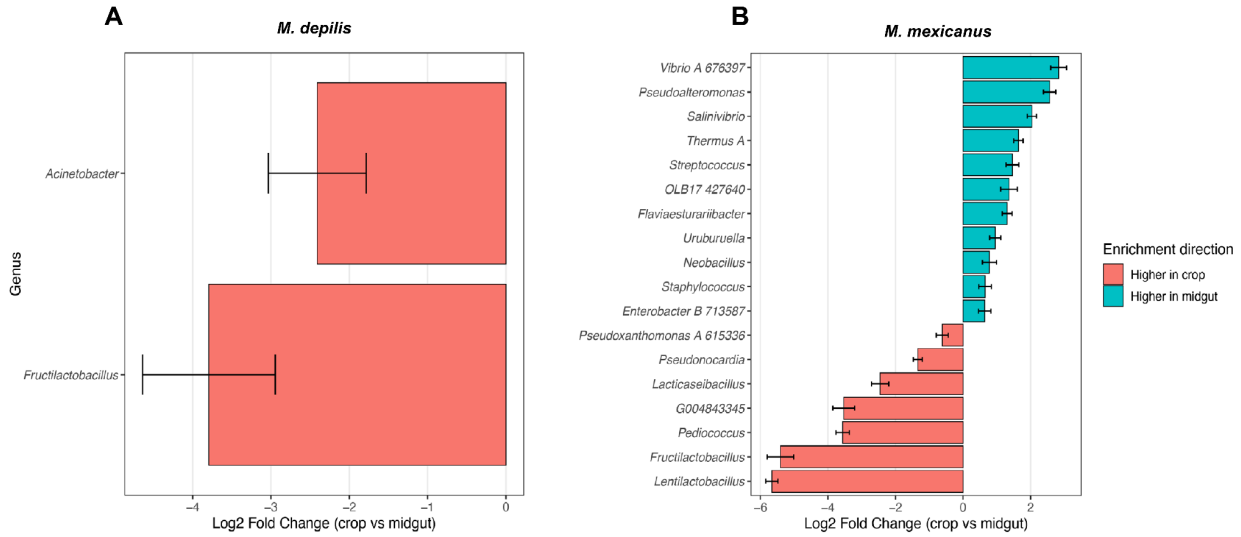

Figure 6. Differentially abundant bacterial genre between replete crop and midgut samples identified using ANCOM-BC2. Negative Log_2 fold changes (red) indicate genera differentially abundant in the crop, while positive log_2 fold change values (blue) indicate enrichment in the midgut. (A) *Fructilactobacillus* and *Acinetobacter* were found to be differentially abundant in the crop of *M. depilis*, while no genera was differentially abundant in the midgut. (B) Lactic acid bacteria such as *Fructilactobacillus*, *Lentilactobacillus*, *Lacticaseibacillus*, and *Pediococcus*, are differentially more abundant in the crop of *M. mexicanus* than the midgut.

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