First screening of bacterial communities of Microdon myrmicae and its ant host: do microbes facilitate the invasion of ant colonies by social parasites?

Giulia Scarparo , Paul Rugman-Jones , Marco Gebiola , Andrea Di Giulio , Quinn S. McFrederick

PII: S1439-1791(20)30124-9
DOI: https://doi.org/10.1016/j.baae.2020.11.008
Reference: BAAE 51299

To appear in: *Basic and Applied Ecology*

Received date: 24 February 2020
Accepted date: 28 November 2020

Please cite this article as: Giulia Scarparo , Paul Rugman-Jones , Marco Gebiola , Andrea Di Giulio , Quinn S. McFrederick , First screening of bacterial communities of Microdon myrmicae and its ant host: do microbes facilitate the invasion of ant colonies by social parasites?, *Basic and Applied Ecology* (2020), doi: https://doi.org/10.1016/j.baae.2020.11.008

First screening of bacterial communities of *Microdon myrmicae* and its ant host: do microbes facilitate the invasion of ant colonies by social parasites?

Giulia Scarparo[a,b], Paul Rugman-Jones[b], Marco Gebiola[b], Andrea Di Giulio[a]*, Quinn S. McFrederick[b]

[a]*Dipartimento di Scienze, Università Roma Tre, Viale Guglielmo Marconi 446, 00146, Roma, Italia;*

[b]*Department of Entomology, University of California, 165 Entomology Bldg. Citrus Drive, Riverside, CA 92521, USA*

Abstract

Many studies have highlighted how numerous bacteria provide their hosts essential nutrients or protection against pathogens, parasites and predators. Nevertheless, the role of symbiotic microorganisms in the interactions between social insects and their parasites is still poorly known. *Microdon* (Diptera, Syrphidae) is a peculiar fly genus whose larvae are able to successfully infiltrate ant colonies and feed upon the ant brood. Using high throughput 16S rRNA gene amplicon sequencing, we provide the first microbiome survey of *Mi. myrmicae* larvae and larvae and workers of its host, *Myrmica scabrinodis*, collected from two sites in England. We analyzed the microbiome of the external surface of the cuticle and the internal microbiome of the body separately. The results clearly show that the *Mi. myrmicae* microbiome significantly differs from that of its host, while no substantial dissimilarity was detected across the microbiome of ant workers and ant larvae. *Microdon myrmicae* microbiome varies across the two analyzed sites suggesting that bacteria communities of *Mi. myrmicae* are derived from the environment rather than by horizontal transmission between hosts and parasites. Families Streptococcaceae, Carnobacteriaceae and Rizhobiaceae are dominant in *My. scabrinodis*, and *Spiroplasma* is dominant in ant workers. Microbiome of *Mi. myrmicae* larvae is mainly characterized by the family Anaplasmataceae, with *Wolbachia* as predominant genus. Interestingly, we found *Serratia* within both *Mi. myrmicae* and *Myrmica* larvae. Bacteria of this genus are known to produce a family of pyrazines commonly involved in ant communication, which could play a role in *Microdon*/ant interaction.

Keywords: 16S rRNA; Syrphidae; Fly larvae; Ants; Myrmecophiles; Microbiome; *Myrmica scabrinodis.*

Introduction

Microbes are ubiquitous organisms that often establish symbioses of different degrees with a multitude of plants and animals. Interest in microbial symbionts and their ecological roles is receiving more and more attention from the scientific world and an increasing number of studies have investigated insect-bacteria associations (e.g., Douglas 2015; Meirelles et al. 2016; Kwong et al. 2017; McManus, Ravenscraft, & Moore 2018). Microbes influence the ecology and evolution of their insect hosts in a variety of ways. To ensure their spread throughout generations, bacteria provide a wide range of beneficial effects to their hosts so that they can in turn increase their own fitness. In fact, it has become clear that microbial symbionts can have profound effects on the lives of their insect hosts (Davis et al. 2013). For example, gut symbionts have been implicated in the mating preference of *Drosophila melanogaster* (Sharon et al. 2010). A pheromone promoting mating aggregation is produced by bacteria found in the gut of the desert locust, *Schistocerca gregaria* (Dillon, Vennard & Charnley 2000). In other insects, symbiotic bacteria have been shown to play

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

leading roles in defending their insect hosts against pathogens, parasitoids, and predators (Oliver et al. 2003; Kaltenpoth et al. 2005; Hedges et al. 2008; Brownlie & Johnson 2009), or in mediating thermal tolerance of their hosts (Dunbar et al. 2007).

To maintain symbiotic relationships with their hosts, bacteria have evolved two sophisticated strategies of infection: horizontal (i.e., the intra- or interspecific exchange of symbionts from one host to another) and vertical (i.e., inheritance of the symbiont from the mother or, more rarely, from both parents to the offspring). These strategies are not mutually exclusive (Bright & Bulgheresi 2010). Vertical transmission is characteristic of long-term obligate associations and is fundamental to guarantee symbiont survival through many generations of the same host species. In contrast, horizontal transfer promotes interspecific spread of symbionts via a shared environment. Intimate contact (e.g., parasitization) can create suitable conditions for the transfer of symbionts between species (Haapaniemi & Pamilo 2015).

Ants represent one of the most successful insect groups. They are able to exploit many environments and resources thanks to their complex social structure and their refined chemical communication, mainly based on pheromone production and sophisticated chemosensory receptors (Lenoir et al. 2001). Cuticular hydrocarbons (CHCs) play a key role for nestmate recognition, that is, discrimination between colony members and intruders (van Zweden and d' Ettorre 2010). However, it is generally acknowledged that microbial symbionts can also produce a wide variety of volatiles that are able to manipulate the behavior of individuals inside the colonies of many social insects (Davis et al 2013; de Bekker et al. 2018). Although the role of bacteria for the nestmate recognition in ants is still mysterious, for social termites it has been postulated that the presence of a colony-specific gut bacterial community is responsible for producing volatile digestive compounds, which are used by the termites to recognize nestmates (Matsuura 2001; Minkley et al. 2006).

Numerous arthropods have evolved complex associations with ants ranging from various degrees of mutualism and commensalism to predation, parasitism or parasitoidism (Hölldobler & Wilson 1990; Ivens et al. 2016). These organisms, known as “myrmecophiles”, belong to all the major extant lineages of arthropods, like arachnids, mites, myriapods, crustaceans and, most importantly in terms of the number of species involved, hexapods. These tight associations could potentially offer an optimal system in which endosymbiotic bacteria can spread horizontally between the associated species (Haapaniemi & Pamilo 2015). However, the role of symbiont microorganisms in fostering interactions between ants and their myrmecophilous hosts is still poorly known. One of the most striking examples of myrmecophiles are the larvae of *Microdon* hoverflies (Diptera; Syrphidae; Microdontinae), which feed undisturbed upon the ant brood, inside the ant colony, using a mixture of protective morphological features and chemical mimicry (Howard, Akre, & Garnett 1990; Howard, Stanley-Samuelson, & Akre 1990; Scarparo et al. 2017; Scarparo et al. 2019). Members of this genus are social parasites (here considered in its broad definition as any species that targets ant nests and inflicts a cost, or exploits the social structure of the colony for its own gain) associated with five ant subfamilies: Ponerinae, Dolichoderinae, Pseudomyrmecinae, Myrmicinae and Formicinae (Reemer 2013). Although *Microdon* is a species-rich genus of about 300 species mainly occurring in South America, it is still poorly known. In Europe only six species are known: *Mi. analis*, *Mi. major*, *Mi. devius*, *Mi. miki*, *Mi. mutabilis* and *Mi. myrmicae* (Speight, 2017). *Microdon myrmicae* is one of the best studied among *Microdon* species. It is mostly found in association with *Myrmica scabrinodis*, though many other *Myrmica* species are known to be suitable hosts (*e.g.*, *My. gallienii*, *My. rubra*, *My. vandeli*, and *My. sabuleti* (Bonelli et al. 2011). This social parasite is rare, infesting only 20%-26% of *Myrmica* colonies, but with a very low intracolonial density of the parasite larvae (an average of 2.5 larvae per colony) (Bonelli et al. 2011). Moreover, *Mi. myrmicae* is extremely localized, only occurring around wet grassland

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

usually dominated by *Sphagnum*, *Juncus*, and *Molinia coerulea* (Schönrogge et al. 2002). In contrast, its ant host, *Myrmica scabrinodis*, is one of the most common European *Myrmica* ants, and often occurs in wet and cool environments with nests being situated just above water level in moist grasslands, although it can be found even in drier patches associated with other *Myrmica* species. *Myrmica* colonies are usually small with about 200-500 workers and a variable number of queens (from 1 to many) (Radchenko & Elmes 2010), and host highly diverse myrmecophilous communities (Witek, Barbero & Markó 2014).

The study of these myrmecophiles is challenging because they are both rare and concealed within host ant nests, which themselves are found in extremely localized, increasingly degraded, and endangered patches of wetland. Many questions on their ecology and biology are still unanswered. As such, few studies have investigated the relationship of the microbiome in the ant-myrmecophile associations. In this work, we provide the first comparative screening of bacterial communities harbored by *Microdon myrmicae* and by its ant host, *Myrmica scabrinodis* (larvae and workers), aimed at addressing the following questions: (1) Is there similarity between host and parasite microbiome as a result of horizontal transmission (common in the ant colony through trophallaxis and allogrooming)? (2) Do bacterial communities change across geographical populations of hoverflies? (3) Does the *Mi. myrmicae* microbiome harbor bacteria that may be involved in the production of semiochemicals with a potential role in interspecific communication? (4) Does the ant microbiome change across developmental from larvae to workers?

Materials and Methods

Insect Collection

Larvae of *Microdon myrmicae*, and larvae and workers of its ant host, *Myrmica scabrinodis*, were collected from seven colonies at two sites in Southwestern England: Lower Prewley Moor (50.700239/ -4.070188) and Hollow Moor (50.788061/ -4.186153). Both sites were waterlogged, ungrazed, neutral grassland dominated by *Sphagnum* spp. and *Molinia caerulea*. All specimens were collected using flame-sterilized forceps, preserved in 100% ethanol, and stored in the freezer at -20 °C. We recognize that ethanol is not the best fixative to preserve the external microbiome, as it may have washed away some bacteria from the cuticle of insects. However, our samples were not vortexed during this phase and the connected microbes should not have been moved.

A total of 20 parasites, 15 ant larvae and 13 ant workers were used in this work (Table 1).

DNA extraction, PCR amplification, and high-throughput sequencing

For all specimens, we attempted to extract the external and internal microbial DNA separately as follows. Whole specimens, either a pool of 2 ants (larvae or workers) or a single parasitic larva, were first placed in individual 1.5 mL microcentrifuge tubes containing 180 µl of washing/lysis buffer (Buffer A; 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton) and vortexed for 1 min to dislodge the external microbiome (Birer et al. 2017). The tubes were then transferred to a shaking bath for 15 min at 37 °C. The insect specimens were then removed from the washing buffer (now a microbial suspension) using flame-sterilized forceps and transferred to a 2.5% bleach solution for 1 minute to sterilize the outer surface from any remaining bacteria. The microbial suspension (i.e. external microbiome) was retained, immediately mixed with 25 µl of lysozyme (0.25 g/mL) and incubated at 37 °C for 1h. The surface-sterilized insect specimens (i.e. internal microbiome) were washed twice in 100% ethanol and then transferred to a clean 1.5 ml

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

microcentrifuge tube containing 180 µl of Buffer A. After collecting the external microbiome and sterilizing the surface of the samples with bleach, the ants and fly larvae were then homogenized using sterile and DNase/RNase free pestles, before adding 25 µl of lysozyme (0.25 g/mL) and being incubated at 37 °C, again for 1 hour. Due to the relatively large size of the parasitic *Microdon* larvae, they were cut in half longitudinally, and only one half was used in the extraction of the internal microbiome. After the incubation period, DNA extraction from internal and external microbiome followed the same protocol. DNA was precipitated and purified using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) using the manufacturer's protocol for gram-positive bacteria. One control blank sample was added to detect possible contaminants.

The bacterial V5-V6 domain of 16S rRNA was PCR amplified using 799F (CMGGGTATCTAATCCKGTT) and 1115R (AGGGTTGCGCTCGTTG) indexed primers. We used a dual barcoding approach with two primer sets to build the Illumina sequencing construct, as in Kembel et al. (2014) and McFrederick and Rehan (2016). To sequence these amplicons, we cleaned up the PCR reactions using the PureLink Pro PCR clean up kit (ThermoFisher Scientific, Waltham, MA), then normalized each sample to be equimolar with SequalPrep normalization plates (ThermoFisher Scientific, Waltham, MA). The bacterial library was sequenced on an Illumina MiSeq platform using 2 X 300 version 3 reagents. Raw sequence data are accessible as NCBI BioProject: PRJNA680377.

Statistical analyses

We used QIIME2 version 2019.1 (Bolyen et al. 2019) and the Past3 for data analyses. For quality control of the 16S rRNA gene data, we viewed quality scores of the DNA sequence and trimmed reads of low-quality regions. We used DADA2 (Callahan et al. 2016) to infer exact sequence variants (ESVs; bacteria that share the exact DNA sequence over the 16S rRNA gene region sequenced). To assign a taxonomy to each ESV, we trained the Silva database at 99% of similarity (v. 128 Quast et al. 2012) to our primer set in QIIME2, then assigned taxonomy using the *sklearn* classifier (Bokulich et al. 2018). For alpha and beta diversity analyses, we first aligned representative sequences with MAFFT (Katoh et al. 2002) and filtered out poorly aligned sections with QIIME2's alignment mask. Since the blank control was completely clean and free of contamination, we did not filter out any reads. After filtering and quality control we retained a total of: 38 specimens of parasites (12 for the first site [6 for the external microbiome, 6 for the internal microbiome] and 26 for the second site [13 for the external microbiome, 13 for the internal microbiome]); 24 specimens of ant larvae (9 for the first site [6 for the external microbiome, 3 for the internal microbiome] and 15 for the second site [9 for the external microbiome, 6 for the internal microbiome]); and a total of 26 specimens of ant workers (10 for the first site [5 for the external microbiome, 5 for the internal microbiome] and 16 for the second site [8 for the external microbiome, 8 for the internal microbiome]) (Table 1).

We built a phylogeny from the resulting alignment using FastTree (Price, Dehal & Arkin 2010) and conducted alpha and beta diversity analyses with QIIME2's core diversity metrics. Shannon and ESV richness were calculated to detect differences in the alpha diversity of the external and internal microbiome of parasites and hosts. Statistical significance was tested using Kruskall-Wallis test and p-values were adjusted by Benjamini-Hochberg correction. To detect differences in the microbiomes, we ran two different Permutational MANOVA (PERMANOVA) analyses with 9999 permutations and Bonferroni correction, all using Bray-Curtis distances calculated on the 16S rRNA gene feature table. First, we ran PERMANOVA to test differences among external and the internal microbiomes of the parasite (EP, IP) and the host (EW, IW, EL, IL) without taking in account the sites. Separately, we ran PERMANOVAs to determine

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

differences among sites in the external and internal microbiome of only *Microdon myrmicae*. We used Non-metric Multidimensional Scaling (NMDS) with Bray-Curtis dissimilarity to graphically display differences among and within groups. We perform NMDS using all the samples together to see differences among parasites and host. We ran then other NMDS separately per parasites and ants, to show dissimilarities among sites and external and internal microbiomes.

We tested for differential abundance of ESVs using an Ancom analysis in QIIME2. Additionally, the relative frequencies of the most abundant families (≥3%) were calculated and illustrated with a heatmap and two histograms of the external and internal microbiomes of *Mi. myrmicae and My. scabrinodis*, respectively.

Results

Data summary of the MiSeq analysis

A total of 2,100,632 rRNA V5-V6 reads were obtained from 96 samples. After quality control, filtering and merging of paired-end reads 1,055,197 sequences were retained, with a mean frequency of 10991.63 per sample. The DADA2 algorithm identified 5,086 unique ESVs with an average length of 332.49 bases. We rarefied to 3,600 reads per sample as rarefaction curves levelled off at that sequencing depth while simultaneously allowing us to retain 84% of 96 samples for downstream analyses.

Bacterial taxa associated with the parasitic larvae of Microdon myrmicae

A total of 29 bacterial phyla were detected. The main bacterial families on the external surface of *Mi. myrmicae* were Moraxellaceae (7.4%), Burkholderiaceae (7.2%), Nocardioidaceae (7%), Chitinophagaceae (6.9%). In contrast, the internal microbiome of the parasite larvae was constituted chiefly by Anaplasmataceae (40,9%), followed by other minor phyla (Fig. 1A, 2; see Appendix A). There were significant differences in the alpha diversity of bacterial communities between the external and internal larval microbiome (ESV richness/Kruskall-Wallis, p = 0.000005; Shannon diversity/Kruskall-Wallis, p = 0.000003) (Fig. 3). In fact, with an average of 312.8 ESVs observed, the external microbiome of the parasite was the richest in number of bacterial species across all samples. The internal microbiome of *Myrmica* larvae had the lowest species richness (Fig. 3). Significant differences were also detected with the analysis of the beta diversity across the external and internal larval microbiome (PERMANOVA, p =0.0015) (Fig. 4D). *Wolbachia* was by far the most abundant genus detected in *Microdon myrmicae*, accounting entirely for the family Anaplasmataceae (40.93%). As might be expected for an intracellular bacterium, *Wolbachia* was present almost exclusively inside the parasite body, with its occurrence outside the cuticle being close to 0 (0.33%). Interestingly, we detected *Serratia*, which is an opportunistic insect pathogen (Raymann et al. 2018) in the internal microbiome of *Mi. myrmicae* larvae.

Bacterial taxa associated with Myrmica scabrinodis workers and larvae

After rarefying to 3600 reads per sample, the bacterial phyla mainly represented across the external surface of *Myrmica* larvae were Streptococcaceae (17.1%) and Carnobacteriaceae (11.5%) (Fig. 1C, 2; see Appendix A). The internal microbiome was characterized by a simpler bacterial community composed mainly of only three families: Rhizobiaceae

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

(47.1%), Carnobacteriaceae (30%) and Enterobacteriaceae (5.7%) (Fig. 1C, 2; see Appendix A). The cuticular microbiome of ant workers was instead composed mainly of Streptococcaceae (14.3%), Vibrionaceae (7.4%), Weeksellaceae (7%), while the microbiome of internal body was dominated by Rhizobiaceae (51.7%), and Spiroplasmataceae (9.7%) (Fig. 1B, 2; see Appendix A). From the analysis of the alpha diversity, no significant differences were detected between the bacterial communities of workers and ant larvae both externally (ESV richness/Kruskall-Wallis, p = 0.89; Shannon diversity/Kruskall-Wallis, p= 0.98) and internally (ESV richness/Kruskall-Wallis, p = 0.08; Shannon diversity/Kruskall-Wallis, p= 0.46) (Fig. 3). This same ESV richness corresponds to extremely similar species communities, according to the beta diversity across the external (PERMANOVA, p = 1) and the internal (PERMANOVA, p = 0.39) microbiome, indicating no substantial differences among the microbiomes of two different developmental stages (Fig. 4B). *Spiroplasma* was the most abundant genus characterizing the internal microbiome of *Myrmica* workers but was almost completely absent in ant larvae. *Carnobacterium maltaromanticum* (Carnobacteriaceae) was instead the main species in both the external and internal microbiome of ant larvae. Although in low abundance, the presence of *Wolbachia* sp. on the external surface of ants (workers 3.6%; larvae 2.3%) was surprising, particularly since *Wolbachia* was nearly absent inside the body.

Host/parasite comparison

The host microbiome appeared to differ substantially from that of the parasite in terms of beta diversity (PERMANOVA, $p < 0.05$). In Bray–Curtis/NMDS both external and internal microbiomes of *Microdon myrmicae* were clearly segregated from both host larvae and workers, which in turn were almost completely overlapping (Fig. 5). The microbiomes of the parasites also differed across two collection sites both internally (PERMANOVA, p = 0.006) and externally (PERMANOVA, p = 0.0006) (Fig. 4C). Nevertheless, apparently, the *Myrmica* microbiome did not change among sites (Fig. 4A), although we cannot be sure due to the low sample size after cleaning and filtering the reads.

Discussion

For the first time, we characterized and compared the external and internal microbiome of the social parasite *Microdon myrmicae* and its ant host *Myrmica scabrinodis*. Our research revealed that, (1) contrary to what we first hypothesized, beta diversity indicated strong differences between the bacterial communities of the microbiome of *Microdon myrmicae* compared to that of its host, *Myrmica scabrinodis* (both larvae and workers) (Fig. 5); (2) the external and the internal microbiomes of *Mi. myrmicae* significantly differed (Fig. 4D); (3) the *Mi. myrmicae* microbiome varied across the two analysed sites (Lower Prewley Moor and Hollow Moor) (Fig. 4C); (4) no substantial dissimilarity was detected across the external microbiomes of ant workers and ant larvae (Figs. 4B, 5).

Few works deal with the role of bacteria in social insects/social parasites associations (Di Salvo et al. 2019; Szenteczki et al. 2019; Kaczmarczyk-Ziemba et al. 2020). Recently, two studies compared the microbiome of the myrmecophilous butterfly *Maculinea alcon* with that of its ant hosts, *Myrmica scabrinodis* (Di Salvo et al. 2019) and *Myrmica schencki* (Szenteczki et al. 2019). *Maculinea* butterflies are thoroughly investigated myrmecophiles with a complex lifecycle which involves a first free-living stage, from the first to the third larval instar, feeding on one or few host plants, *Gentiana* spp. in the case of *Maculinea alcon.* The fourth instar larvae fall off the host plant and are adopted by *Myrmica* workers (Witek et al. 2008). Once inside the ant nest, *Maculinea* larvae employ a mixture of chemical and acoustical mimicry to undisturbedly feed on ant brood and induce regurgitation from host (Akino et al. 1999). Both

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

studies (Di Salvo et al. 2019; Szenteczki et al. 2019) argued that during larval development, the *Maculinea* microbiome undergoes marked modifications accordingly to the diet shift (from plant to insects), and once in the ant colony it significantly diverges from that of the ant host. Our results are in line with those two previous papers (Di Salvo et al. 2019; Szenteczki et al. 2019), showing that the microbiomes are different between *Microdon myrmicae* parasitic larvae and the ant host (both ant larvae and ant workers) (Fig. 5). This finding suggests that *Mi. myrmicae* larvae have a resistant microbiome and the bacteria acquired with the diet are not able to settle in the parasitic host. Moreover, bacterial communities in *Mi. myrmicae* sharply changed across the two geographic populations. Similar outcomes were also found for *Maculinea* caterpillars, where the analysis of the soil microbiome confirmed the environmental origin of the microbiome harbored by butterfly larvae (Di Salvo et al. 2019; Szenteczki et al. 2019). Although we did not analyze soil samples, we suspect that most bacteria outside and within hoverfly larvae were derived from the surrounding environment. In our initial analysis of alpha diversity (Fig. 3), we observed the highest number of ESVs in the external *Mi. myrmicae* microbiome, suggesting a weak selection of bacteria on the cuticle. Furthermore, we can exclude that the divergence of the microbiomes was due to the net genetic isolation of *Mi. myrmicae* populations. From a recent population genetics study on *Mi. myrmicae*, that involved the same two sites investigated in this paper, we found that *Microdon* adults can freely move across these sites and lay eggs in ant nests spaced across several kilometers, resulting in a panmictic population (Scarparo et al. 2020). Potentially *Mi. myrmicae* adults could vertically transmit their microbiome to the offspring, thus homogenizing the bacterial communities of the parasitic larvae among sites. But our results of a marked segregation among microbiomes in the two sampling sites suggests that the hoverfly mothers do not manipulate the microbiome of the offspring. Nevertheless, further investigations are needed to confirm this hypothesis. Such similarities among the microbiome variation in *Microdon* larvae and *Maculinea* caterpillars among sites and being distinct from the hosts suggest that these findings may be generalized to other social parasites. Below, we discuss the relevant taxa of bacteria found in both *Microdon myrmicae* and its host *Myrmica scabrinodis*, and present hypotheses about ways of infection and transmission, and their potential role in the symbiotic association.

Myrmica scabrinodis harbors a rich microbiome

We found that the *Myrmica* microbiome is rich in bacterial taxa, although the internal microbiome of both ant larvae and workers is mainly dominated by a few bacterial families. These data are interesting if contextualized in modern literature which deals with the characterization of the microbiome in different species of ants with different ecological habits. Previous works (Sanders et al., 2017; Russell, Sanders & Moreau 2017) have shown that many omnivorous ants have few bacteria in their guts and do not appear to require a microbiome (Hammer, Sanders, Fierer 2019). Whether the bacteria found here are simply passing through the gut as part of the ant's diet cannot be excluded by our analyses. That being said, our main question of whether the ants and parasites share microbes does not hinge on the abundance or host specificity of the ant's microbiome, and our data suggest that even if these are transient microbes, the ants and parasites acquire them from different sources and not one another. Some of the most abundant bacteria of the internal microbiome of *Myrmica scabrinodis* larvae and workers are those of the family Rhizobiaceae, which are plausibly obtained via the diet (Russell, Sanders & Moreau 2017). Bacteria of this family can form root- or stem-nodule symbioses with members of the plant family Leguminosae and are also generally found in phytophagous arthropods, which are able to assimilate nitrogen into amino acids and other essential organic compounds (Carareto Alves et al. 2014). Bacteria of the families Rhizobiaceae, Burkholderiaceae and Pseudomonadaceae, detected in *Myrmica* ants, are commonly found in herbivorous ants (Russell et al. 2009 a; Russell, Sanders & Moreau 2017; Hu et al. 2018; Ramalho

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

et al. 2020). Nevertheless, *Myrmica* species are common predators of a vast number of arthropods, including herbivores, and associations with aphids are widely documented. In this way, it is highly probable that *Myrmica* foragers could have absorbed part of these "herbivore-related" bacteria hosted in the gut of their prey or aphids and subsequentially transferred them to the larvae by trophallaxis. This same transfer from the host to the parasite seems partial or absent due to the low percentage of Rhizobiaceae observed within *Microdon* larvae. These nitrogen fixators could improve the host diet supplying or recycling essential amino acids and nitrogen (Russell et al. 2009 a; Hu et al 2018). It is also worth noting that the high abundance on the ant larval cuticle of *Carnobacterium maltaromanticum*, a species of Carnobacteriaceae isolated from *Sphagnum* ponds (Leisner et al. 2007). This moss is a typical element of the wet grassland areas where *My. scabrinodis* and *Mi. myrmicae* live (Schönrogge et al. 2002).

The microbiome of ants seems to be stable throughout development

We have found that the microbiome of ant larvae is very similar to the external microbiome of ant workers. This result is curious when compared with the many studies that deal with modifications of the internal microbiome between the stages of development of the ant (Ramalho, Bueno & Moreau 2017; Ramalho et al. 2020). For example, it has recently been discovered that bacterial communities hosted by the jaw trap ant, *Daceton armigerum*, undergo profound changes during the transition from larva to adult (Ramalho et al. 2020). The microbiome of *D. armigerum* larvae has a richer alpha diversity than that of workers. We did not find significant differences in the alpha and beta diversity of larvae and *Myrmica* workers. We believe that the repeated trophallaxis and the allogrooming that workers continuously perform towards the larvae may have contributed to unifying the microbiome across the two castes, although some bacteria may still be specific to a single stage of development. In fact, from the analysis of the relative abundances of bacterial families, we found some families present in higher abundances in ant workers rather than in ant larvae and vice versa (i.e. Carnobacteriaceae for ant larvae and Spiroplasmataceae for ants). Furthermore, we noticed intra-individual variability in the internal microbiome. In general, we found that the external microbiome of ant workers was very similar to the external microbiome of ant larvae, while the internal microbiome of the two castes showed a higher variability, although not significant. Nevertheless, we have to clarify that the study of microbiome variability throughout ant development was not the main aim of this work and the sampling of workers and larvae from many different colonies but with an insufficient number of replicates for each colony, may have biased the results.

Spiroplasma

Spiroplasma spp. are found in a wide variety of arthropods, including ants, although they are generally present at low levels compared with *Wolbachia* (Russell et al. 2012). Derived from the mollicutes (mycoplasma relatives), many *Spiroplasma* strains are pathogenic, male-killers, or protect their hosts (Jiggins et al. 2000; Oliver et al. 2003). *Spiroplasma* commonly live outside of cells, free in the hemolymph. We found *Spiroplasma* in the internal body of *My. scabrinodis* workers (9.7%). These bacteria are also found at high frequencies in many other *Myrmica* species (Ballinger, Moore & Perlman 2018), but it is still not clear what effects *Spiroplasma* might have on their *Myrmica* hosts. It has been hypothesized that *Spiroplasma* manipulates *Myrmica* reproduction, for example, by killing males, or protect its host against natural enemies (Ballinger, Moore & Perlman 2018). Although horizontal transfer of this bacterium is possible, it is claimed that vertical transmission of *Spiroplasma* seems to be more common due to the high infection prevalence in *Myrmica* larvae and pupae. Nevertheless, in our study case we can exclude maternal

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

transmission, since *Spiroplasma* was not detected in *My. scabrinodis* larvae. Additionally, *Spiroplasma* strains were not exchanged between the host and the social parasite.

Wolbachia

Wolbachia is mostly a reproductive manipulator known to cause sex ratio distortion in its host via cytoplasmatic incompatibility, feminization, male-killing or parthenogenesis (Bourtzis & Miller 2006). Recent estimates indicate that *Wolbachia* is one of the most widespread parasites, infecting a significant proportion of insect species, with data ranging from 40%-52% (Weinert et al. 2015). However, its role in many arthropod associations is still unclear. Although *Wolbachia* is commonly maternally transmitted, horizontal transfer events are possible through an extracellular phase (Rasgon et al. 2006), and its widespread presence in a myriad of organisms supports the presence of horizontal transmission. It has been found in the hemolymph and in the fecal wastes of insects (Espino et al. 2009; Engel & Moran 2013). Furthermore, horizontal transfer between ants and their social parasite has been documented between the inquiline social parasite *Solenopsis daguerrei* and its hosts *S. invicta* and *S. richteri* (Dedeine et al. 2005). *Wolbachia* is the most proportionally abundant in *Mi. myrmicae* larvae, making up more than 40% of its internal microbiome. It is interesting to note the presence of *Wolbachia* also externally on the cuticle of *Myrmica* larvae (2.3%) and workers (3.6%), whereas it is surprisingly absent internally. Although *Wolbachia* and *Spiroplasma* can coexist in the same host, Szenteczki et al. (2019) in their analysis of the microbiomes of *Myrmica schencki* and *Maculinea alcon* showed a negative correlation between the abundance of these two endosymbionts. It is possible that the presence of *Spiroplasma* in *Myrmica scabrinodis* workers may have inhibited *Wolbachia* infection in the workers analyzed in this study. However, the possible mechanisms of competition between these two bacteria are still unknown.

Microbial volatile organic compounds (MVOCs): Serratia

The production of volatile compounds by bacteria and fungi is commonplace (Davis et al. 2013), and insects have evolved sophisticated chemoreception systems that are highly sensitive to volatile chemical signals, including microbial emissions (Ozaki et al. 2005; Davis et al. 2013). It has been recognized that microbial emissions have several ecological and/or physiological functions able to modulate insect behavior: attract or repel insects; stimulate oviposition; localize hosts and food resources; inhibit the growth of other microorganisms and many others (Davis et al. 2013 and references therein).

We detected the genus *Serratia* within both the parasite (1.7%) and the *Myrmica* larvae (0.7%). Recent evidence proved that a strain of *Serratia marcescens* produces a family of pyrazines commonly found to play a role in ant communication (Morgan 2009, Showalter et al. 2010, de Bekker et al. 2018). This includes various pyrazines, such as 3-Ethyl-2,5-dimethylpyrazine and 2,5-dimethylpyrazine, used as trail pheromones by many ant species, including several *Myrmica* spp. (Mander & Liu 2010; Silva-Junior et al. 2018). Furthermore, flies infected with *S. marcescens* produce a greater abundance of fly odors, including aggregation pheromones (Silva-Junior et al. 2018). It is possible that *M. myrmicae*, thanks to its symbiosis with *Serratia*, could exploit the ant's aggregation or trail pheromones to detect host brood, thereby guaranteeing availability of its food source. Furthermore, chemical mimicry due to biosynthesis of cuticular hydrocarbons (Howard, Akre, & Garnett 1990; Howard, Stanley-Samuelson, & Akre 1990; Scarparo et al. 2019), may allow the parasite to remain undetected within the ant colony. During lab observations we frequently saw

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

the ant workers bring their own larvae and eggs near or above *Microdon* dorsum (Scarparo et al. 2019), suggesting that the parasites were not easily detected by the ants. This bacterium was also detected in another social parasite of *Myrmica* ants, the lycaenid *Maculinea* caterpillars (Di Salvo et al. 2019).

Conclusions

Many studies have demonstrated that horizontal transfer mostly occurs among close relatives (Engelstädter & Hurst, 2006; Tinsley & Majerus 2007; Russell et al. 2009 b), although horizontal transfer across more distant taxa is also possible (Zug, Koehncke, & Hammerstein 2012). Nevertheless, our beta diversity results show significant dissimilarities in the microbiome composition of *Mi. myrmicae* and its ant host, *My. scabrinodis*, supporting the hypothesis that a complete horizontal transmission of the entire microbiome (or at least most of the microbiome) from the host to the parasite is unlikely, as shown for other host-parasite interactions, possibly due to the phylogenetic distance between hoverflies and ants, although a few groups of bacteria could still be exchanged. The external and internal microbiome of ant workers is similar to those of ant larvae, suggesting a horizontal bacterial transfer by trophallaxis and allogrooming across these two life stages. In contrast, although *Microdon* larvae are notably voracious predators of ant larvae, they do not assimilate the same internal microbiome of the host, suggesting that regardless of their diet, the parasite harbors a stable and species-specific bacterial community, as also observed for some ant species (Rubin et al. 2018; Ramalho et al. 2020). Even the constant contact with the ant brood and in some cases, the recently reported rubbing behavior (Scarparo et al. 2019), does not guarantee a transfer from the ants. These findings are consistent with recent characterizations of the microbiota of other ant social parasites (Di Salvo et al. 2019; Szenteczki et al. 2019). However, as one of the first contributions to the knowledge of the role of bacteria in social insects-social parasites associations, our work is purely descriptive and more specific studies are needed to directly test the horizontal bacterial transfer among ants and myrmecophiles.

In conclusion, the *Microdon* microbiome seems to be mainly related to environmental subterranean microbial communities. The considerable difference between host and parasite bacterial communities, both external and internal, found in this study suggests that the microbiome follows different mechanisms rather than horizontal transfer between host and parasite. We cannot exclude that single bacteria species could be essential for the success of the *Microdon* parasitic strategy, although more research on other host-parasite associations and environments is needed, possibly including experiments with selective antibiotic treatments.

Acknowledgments

We are deeply indebted to Prof. Richard Stouthamer for his support and help during the lab work. We are grateful to our friend Robert Wolton for the help during the samplings of *Microdon myrmicae*. We thank Marcus and Sally Vergette and Hazel Butt for kind permission to collect *Mi. myrmicae* on their farms in Devon and our colleagues Marco Molfini and Luigi Cao Pinna for their help during the field sampling. The Grant of Excellence Departments, MIUR-Italy (ARTICOLO 1, COMMI 314 – 337 LEGGE 232/2016) is gratefully acknowledged. This project was funded by USDA NIFA Hatch funds (CA-R-ENT-5109-H) to QSM. We kindly thank the three anonymous reviewers for their comments and suggestions which improved greatly our manuscript.

Appendix A. Supplementary data

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Supplementary data associated with this article can be found, in the online version, at XXXXX."

References

Akino, T., Knapp, J. J., Thomas, J. A., & Elmes, G. W. (1999). Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of *Myrmica* ant colonies. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *266*(1427), 1419-1426. https://doi.org/ 10.1098/rspb.1999.0796

Ballinger, M. J., Moore, L. D., & Perlman, S. J. (2018). Evolution and diversity of inherited *Spiroplasma* symbionts in *Myrmica* ants. *Applied and Environmental Microbiology*, *84*(4), e02299-17. https://doi.org/ 10.1128/AEM.02299-17

Birer, C., Tysklind, N., Zinger, L., & Duplais, C. (2017). Comparative analysis of DNA extraction methods to study the body surface microbiota of insects: a case study with ant cuticular bacteria. *Molecular ecology resources*, *17*(6), e34-e45. https://doi.org/ 10.1111/1755-0998.12688.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... & Bai, Y. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature biotechnology*, *37*(8), 852-857. https://doi.org/10.1038/s41587-019-0209-9.

Bonelli, S., Witek, M., Canterino, S., Sielezniew, M., Stankiewicz- Fiedurek, A., Tartally, A., Balletto, E., & Schönrogge, K. (2011). Distribution, host specificity, and the potential for cryptic speciation in hoverfly *Microdon myrmicae* (Diptera: Syrphidae), a social parasite of *Myrmica* ants. *Ecological Entomology*, *36*, 135-143.

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A., & Caporaso, J. G. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*(1), 90. https://doi.org/10.1186/s40168-018-0470.

Bourtzis, K., & Miller, T. A. (2006). *Insect Symbiosis, Volume 2*. CRC press.

Bright, M., & Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology*, *8*(3), 218. https://doi.org/ 10.1038/nrmicro2262.

Brownlie, J. C., & Johnson, K. N. (2009). Symbiont-mediated protection in insect hosts. *Trends in microbiology*, *17*(8), 348-354. https://doi.org/ 10.1016/j.tim.2009.05.005.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nature methods*, *13*(7), 581. https://doi.org/ 10.1038/nmeth.3869.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Carareto Alves, L. M., de Souza, J. A. M., Varani, A. M., & Lemos, E. G. M. (2014) The Family Rhizobiaceae. In Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.) *The Prokaryotes*. Springer, Berlin, Heidelberg

Davis, T. S., Crippen, T. L., Hofstetter, R. W., & Tomberlin, J. K. (2013). Microbial volatile emissions as insect semiochemicals. *Journal of chemical ecology*, *39*(7), 840-859. https://doi.org/ 10.1007/s10886-013-0306-z.

de Bekker, C., Will, I., Das, B., & Adams, R. M. (2018). The ants (Hymenoptera: Formicidae) and their parasites: effects of parasitic manipulations and host responses on ant behavioral ecology. *Myrmecological News*, *28*. https://doi.org/ 10.25849/myrmecol.news_028:001.

Dedeine, F., Ahrens, M., Calcaterra, L., & Shoemaker, D. D. (2005). Social parasitism in fire ants (*Solenopsis* spp.): a potential mechanism for interspecies transfer of *Wolbachia*. *Molecular Ecology*, *14*(5), 1543-1548. https://doi.org/ 10.1111/j.1365-294X.2005.02499.x.

Di Salvo, M., Calcagnile, M., Talà, A., Tredici, S. M., Maffei, M. E., Schönrogge, K., Barbero, F., & Alifano, P. (2019). The microbiome of the *Maculinea-Myrmica* host-parasite interaction. *Scientific reports*, *9*(1), 8048. https://doi.org/ 10.1038/s41598-019-44514-7.

Dillon, R. J., Vennard, C. T., & Charnley, A. K. (2000). Pheromones: exploitation of gut bacteria in the locust. *Nature*, *403*(6772), 851. https://doi.org/ 10.1038/35002669.

Douglas, A. E. (2015). Multiorganismal insects: diversity and function of resident microorganisms. *Annual review of entomology*, *60*, 17-34. https://doi.org/ 10.1146/annurev-ento-010814-020822.

Dunbar, H. E., Wilson, A. C., Ferguson, N. R., & Moran, N. A. (2007). Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS biology*, *5*(5), e96. https://doi.org/ 10.1371/journal.pbio.0050096.

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects–diversity in structure and function. *FEMS microbiology reviews*, *37*(5), 699-735. https://doi.org/ 10.1111/1574-6976.12025.

Engelstädter, J., & Hurst, G. D. (2006). The dynamics of parasite incidence across host species. *Evolutionary Ecology*, *20*(6), 603-616. https://doi.org/10.1186/1741-7007-6-27.

Espino, C. I., Gómez, T., González, G., do Santos, M. B., Solano, J., Sousa, O., Moreno, N., Windsor, D., Ying, A., Vilchez, S., & Osuna, A. (2009). Detection of *Wolbachia* bacteria in multiple organs and feces of the triatomine insect *Rhodnius pallescens* (Hemiptera, Reduviidae). *Applied and Environmental Microbiology*, *75*(2), 547-550. https://doi.org/ 10.1128/AEM.01665-08.

Haapaniemi, K., & Pamilo, P. (2015). Social parasitism and transfer of symbiotic bacteria in ants (Hymenoptera: Formicidae). *Myrmecological news*, *21*, 49-57.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Hammer, T. J., Sanders, J. G., & Fierer, N. (2019). Not all animals need a microbiome. *FEMS microbiology letters*, *366*(10), fnz117. https://doi.org/10.1093/femsle/fnz117.

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., & Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science*, *322*(5902), 702-702. https://doi.org/ 10.1126/science.1162418.

Hölldobler, B., & Wilson, E.O. (1990). *The Ants*. Springer, Berlin, 739 pp.

Howard, R. W., Akre, R. D., & Garnett, W. B. (1990). Chemical mimicry in an obligate predator of carpenter ants (Hymenoptera: Formicidae). *Annals of the Entomological Society of America*, *83*(3), 607-616. https://doi.org/ 10.1093/aesa/83.3.607.

Howard, R. W., Stanley-Samuelson, D. W., & Akre, R. D. (1990). Biosynthesis and chemical mimicry of cuticular hydrocarbons from the obligate predator, *Microdon albicomatus* Novak (Diptera: Syrphidae) and its ant prey, *Myrmica incompleta* Provancher (Hymenoptera: Formicidae). *Journal of the Kansas Entomological Society*, 437-443.

Hu, Y., Sanders, J. G., Łukasik, P., D'Amelio, C. L., Millar, J. S., Vann, D. R., ... & Pierce, N. E. (2018). Herbivorous turtle ants obtain essential nutrients from a conserved nitrogen-recycling gut microbiome. *Nature communications*, *9*(1), 1-14. https://doi.org/10.1038/s41467-018-03357-y.

Ivens, A. B., von Beeren, C., Blüthgen, N., & Kronauer, D. J. (2016). Studying the complex communities of ants and their symbionts using ecological network analysis. *Annual Review of Entomology*, *61*, 353-371. https://doi.org/10.1146/annurev-ento-010715-023719.

Jiggins, F. M., Hurst, G. D. D., Jiggins, C. D., vd Schulenburg, J. H. G., & Majerus, M. E. N. (2000). The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology*, *120*(5), https://doi.org/439-446.10.1017/s0031182099005867.

Kaczmarczyk-Ziemba, A., Zagaja, M., Wagner, G. K., Pietrykowska-Tudruj, E., & Staniec, B. (2020). First Insight into Microbiome Profiles of Myrmecophilous Beetles and Their Host, Red Wood Ant *Formica polyctena* (Hymenoptera: Formicidae)—A Case Study. *Insects*, *11*(2), 134. https://doi.org/10.3390/insects11020134.

Kaltenpoth, M., Göttler, W., Herzner, G., & Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology*, *15*(5), 475-479. https://doi.org/10.1016/j.cub.2004.12.084.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Katoh, K., Misawa, K., Kuma, K. I., & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic acids research*, *30*(14), 3059-3066. https://doi.org/10.1093/nar/gkf436.

Kembel, S. W., O'Connor, T. K., Arnold, H. K., Hubbell, S. P., Wright, S. J., & Green, J. L. (2014). Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *Proceedings of the National Academy of Sciences*, *111*(38), 13715-13720. https://doi.org/10.1073/pnas.1216057111.

Kwong, W. K., Medina, L. A., Koch, H., Sing, K. W., Soh, E. J. Y., Ascher, J. S., Jaffé, R., & Moran, N. A. (2017). Dynamic microbiome evolution in social bees. *Science Advances*, *3*(3), e1600513. https://doi.org/10.1126/sciadv.1600513.

Leisner, J. J., Laursen, B. G., Prévost, H., Drider, D., & Dalgaard, P. (2007). *Carnobacterium*: positive and negative effects in the environment and in foods. *FEMS microbiology reviews*, *31*(5), 592-613. https://doi.org/10.1111/j.1574-6976.2007.00080.x.

Lenoir, A., d'Ettorre, P., Errard, C., & Hefetz, A. (2001). Chemical ecology and social parasitism in ants. *Annual review of entomology*, *46*(1), 573-599. https://doi.org/10.1146/annurev.ento.46.1.573.

Mander, L., & Liu, H. W. (2010). *Comprehensive natural products II: Chemistry and Biology* (Vol. 1). Elsevier.

Matsuura, K. (2001). Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. *Oikos*, *92*(1), 20-26. https://doi.org/10.1034/j.1600-0706.2001.920103.x.

McFrederick, Q. S., & Rehan, S. M. (2016). Characterization of pollen and bacterial community composition in brood provisions of a small carpenter bee. *Molecular ecology*, *25*(10), 2302-2311. https://doi.org/10.1111/mec.13608.

McManus, R., Ravenscraft, A., & Moore, W. (2018). The bacterial associates of a gregarious riparian beetle with explosive defensive chemistry. *Frontiers in Microbiology*, *9*, 2361. https://doi.org/10.3389/fmicb.2018.02361.

Meirelles, L. A., McFrederick, Q. S., Rodrigues, A., Mantovani, J. D., de Melo Rodovalho, C., Ferreira, H., Bacci, M. Jr., & Mueller, U. G. (2016). Bacterial microbiomes from vertically transmitted fungal inocula of the leaf- cutting ant *Atta texana*. *Environmental microbiology reports*, *8*(5), 630-640. https://doi.org/10.1111/1758-2229.12415.

Minkley, N., Fujita, A., Brune, A., & Kirchner, W. H. (2006). Nest specificity of the bacterial community in termite guts (*Hodotermes mossambicus*). *Insectes sociaux*, *53*(3), 339-344. https://doi.org/10.1007/s00040-006-0878-5.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Morgan, E. D. (2009). Trail pheromones of ants. *Physiological entomology*, *34*(1), 1-17. https://doi.org/10.1111/j.1365-3032.2008.00658.x.

Oliver, K. M., Russell, J. A., Moran, N. A., & Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences*, *100*(4), 1803-1807. https://doi.org/10.1073/pnas.0335320100.

Ozaki, M., Wada-Katsumata, A., Fujikawa, K., Iwasaki, M., Yokohari, F., Satoji, Y., Nisimura, T., & Yamaoka, R. (2005). Ant nestmate and non-nestmate discrimination by a chemosensory sensillum. *Science*, *309*(5732), 311-314. https://doi.org/10.1126/science.1105244.

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS one*, *5*(3), e9490. https://doi.org/10.1371/journal.pone.0009490.

Provost, E., Blight, O., Tirard, A., & Renucci, M. (2008). Hydrocarbons and insects' social physiology. *Insect physiology: new research*, 19-72.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, *41*(D1), D590-D596. https://doi.org/10.1093/nar/gks1219.

Radchenko, A.G., & Elmes, G.W. (2010). Myrmica *Ants (Hymenoptera: Formicidae) of the Old World*. Natura Optima dux Foundation, Warszawa, Poland.

Ramalho, M. O., Bueno, O. C., & Moreau, C. S. (2017). Species-specific signatures of the microbiome from *Camponotus* and *Colobopsis* ants across developmental stages. *PloS one*, *12*(11), e0187461.

Ramalho, M. O., Duplais, C., Orivel, J., Dejean, A., Gibson, J. C., Suarez, A. V., & Moreau, C. S. (2020). Development but not diet alters microbial communities in the Neotropical arboreal trap jaw ant *Daceton armigerum*: an exploratory study. *Scientific reports*, *10*(1), 1-12. https://doi.org/10.1038/s41598-020-64393-7.

Raymann, K., Coon, K. L., Shaffer, Z., Salisbury, S., & Moran, N. A. (2018). Pathogenicity of *Serratia marcescens* Strains in Honey Bees. *mBio*, *9*(5), e01649-18. https://doi.org/10.1128/mBio.01649-18.

Reemer, M. (2013). Review and phylogenetic evaluation of associations between Microdontinae (Diptera: Syrphidae) and ants (Hymenoptera: Formicidae). *Psyche: A Journal of Entomology*, *2013*. https://doi.org/10.1155/2013/538316.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Rubin, B. E., Kautz, S., Wray, B. D. & Moreau, C. S. (2018). Dietary specialization in mutualistic acacia-ants affects relative abundance but not identity of host-associated bacteria. *Molecular Ecology, 28,* 900-916. https://doi.org/10.1111/mec.14834.

Russell, J. A., Funaro, C. F., Giraldo, Y. M., Goldman-Huertas, B., Suh, D., Kronauer, D. J., Moreau, C. S., & Pierce, N. E. (2012). A veritable menagerie of heritable bacteria from ants, butterflies, and beyond: broad molecular surveys and a systematic review. *PLoS One*, 7(12). https://doi.org/10.1371/journal.pone.0051027.

Russell, J. A., Goldman- Huertas, B., Moreau, C. S., Baldo, L., Stahlhut, J. K., Werren, J. H., & Pierce, N. E. (2009 b). Specialization and geographic isolation among *Wolbachia* symbionts from ants and lycaenid butterflies. *Evolution: International Journal of Organic Evolution*, *63*(3), 624-640. https://doi.org/10.1111/j.1558-5646.2008.00579.x.

Russell, J. A., Moreau, C. S., Goldman-Huertas, B., Fujiwara, M., Lohman, D. J., & Pierce, N. E. (2009 a). Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proceedings of the National Academy of Sciences, 106(50),* 21236-21241. https://doi.org/10.1073/pnas.0907926106.

Russell, J. A., Sanders, J. G., & Moreau, C. S. (2017). Hotspots for symbiosis: function, evolution, and specificity of ant-microbe associations from trunk to tips of the ant phylogeny (Hymenoptera: Formicidae). *Myrmecological News*, *24*, 43-69.

Sanders, J. G., Łukasik, P., Frederickson, M. E., Russell, J. A., Koga, R., Knight, R., & Pierce, N. E. (2017). Dramatic differences in gut bacterial densities correlate with diet and habitat in rainforest ants. *Integrative and Comparative Biology*, *57*(4), 705-722. https://doi.org/10.1093/icb/icx088.

Scarparo, G., Cerretti, P., Mei, M., & Di Giulio, A. (2017). Detailed morphological descriptions of the immature stages of the ant parasite *Microdon mutabilis* (Diptera: Syrphidae: Microdontinae) and a discussion of its functional morphology, behaviour and host specificity. *European Journal of Entomology*, *114*, 565-586. https://doi.org/10.14411/eje.2017.071.

Scarparo, G., d'Ettorre, P., & Di Giulio, A. (2019). Chemical deception and structural adaptation in *Microdon* (Diptera, Syrphidae, Microdontinae), a genus of hoverflies parasitic on social insects. *Journal of Chemical Ecology*, 45(11-12), 959-971. https://doi.org/10.1007/s10886-019-01121-0.

Scarparo, G., Rugman- Jones, P., Gebiola, M., Di Giulio, A., & Purcell, J. (2020). Social parasite distancing: RADseq reveals high inbreeding in the social parasite *Microdon myrmicae* but low philopatry for host ant nest. *Ecological Entomology*, 1-11. https://doi.org/DOI: 10.1111/een.12944.

Schönrogge, K., Barr, B., Wardlaw, J. C., Napper, E., Gardner, M. G., Breen, J., Elmes, G.W., & Thomas, J. A. (2002). When rare species become endangered: cryptic speciation in myrmecophilous hoverflies. *Biological Journal of the linnean Society*, *75*(3), 291-300. https://doi.org/10.1046/j.1095-8312.2002.00019.x.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Sharon, G., Segal, D., Ringo, J. M., Hefetz, A., Zilber-Rosenberg, I., & Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, *107*(46), 20051-20056. https://doi.org/10.1073/pnas.1009906107.

Showalter, D. N., Troyer, E. J., Aklu, M., Jang, E. B., & Siderhurst, M. S. (2010). Alkylpyrazines: alarm pheromone components of the little fire ant, *Wasmannia auropunctata* (Roger) (Hymenoptera, Formicidae). *Insectes sociaux*, *57*(2), 223-232.

Silva-Junior, E. A., Ruzzini, A. C., Paludo, C. R., Nascimento, F. S., Currie, C. R., Clardy, J., & Pupo, M. T. (2018). Pyrazines from bacteria and ants: convergent chemistry within an ecological niche. *Scientific reports*, *8*(1), 2595. https://doi.org/10.1038/s41598-018-20953-6.

Speight, M.C.D. (2017) Species accounts of European Syrphidae(Diptera). *Syrph the Net, the Database of European Syrphidae* (eds by Speight M.C.D., Castella E., Sarthou J.-P. & Monteil C.),Vol.103, pp. 1–302. Syrph the Net Publications, Dublin.

Szenteczki, M. A., Pitteloud, C., Casacci, L. P., Kešnerová, L., Whitaker, M. R., Engel, P., Vila, R., & Alvarez, N. (2019). Bacterial communities within *Phengaris (Maculinea) alcon* caterpillars are shifted following transition from solitary living to social parasitism of *Myrmica* ant colonies. *Ecology and evolution*, *9*(8), 4452-4464. https://doi.org/10.1002/ece3.5010.

Tinsley, M. C., & Majerus, M. E. (2007). Small steps or giant leaps for male-killers? Phylogenetic constraints to male-killer host shifts. *BMC evolutionary biology*, *7*(1), 238. https://doi.org/10.1186/1471-2148-7-238.

van Zweden, J. S., & d'Ettorre, P. (2010). Nestmate recognition in social insects and the role of hydrocarbons. *Insect hydrocarbons: biology, biochemistry and chemical ecology*, 11, 222-243.

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150249. https://doi.org/10.1098/rspb.2015.0249.

Witek, M., Barbero, F., & Markó, B. (2014). *Myrmica* ants host highly diverse parasitic communities: from social parasites to microbes. *Insectes Sociaux*, *61*, 307-323.

Witek, M., Śliwińska, E., Skórka, P., Nowicki, P., Wantuch, M., Vrabec, V., Settele, J., & Woyciechowski, M. (2008). Host ant specificity of large blue butterflies *Phengaris* (*Maculinea*)(Lepidoptera: Lycaenidae) inhabiting humid grasslands in East-central Europe. *European Journal of Entomology*, *105*(5). https://doi.org/10.14411/eje.2008.115.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Zug, R., Koehncke, A., & Hammerstein, P. (2012). Epidemiology in evolutionary time: the case of *Wolbachia* horizontal transmission between arthropod host species. *Journal of Evolutionary Biology*, *25*(11), 2149-2160. https://doi.org/10.1111/j.1420-9101.2012.02601.x.

Table 1. Overview of analyzed samples. For ant workers and ant larvae, we pooled 2 individuals to obtain replicate. Each sample was used twice: first, to extract the external microbiome and then to extract the internal microbiome. The numbers between parenthesis refer to the sample effectively use to perform the analysis after the data filtering (external/internal). LPM – Lower Presley Moor (site 1); HM – Hollow Moor (site 2).

Nests	N° Parasites	N° 2 Ant Workers pools	N° 2 ant larvae pools
LPM-1	4 (3/3)	1 (1/1)	1 (1/1)
LPM-2	1 (1/1)	2 (2/2)	2 (3/1)
LPM-3	2 (2/2)	2 (2/2)	2 (2/1)
HM-1	4 (4/4)	1 (1/1)	1 (1/1)
HM-2	4 (4/4)	2 (2/2)	4 (4/3)
HM-3	1 (1/1)	2 (2/2)	2 (2/0)
HM-4	4 (4/4)	3 (3/3)	3 (2/2)
Total	20 (19/19)	13 (13/13)	15 (15/9)

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Table 2. PERMANOVA analysis showing differences among external and internal microbiome of parasites, ant workers and ant larvae. The analysis was performed with 9999 permutations and Bonferroni correction, using Bray-Curtis distances calculated on the 16S feature table. Significative p-values are in bold. EP- external microbiome of *Microdon myrmicae*; EW- external microbiome of *Myrmica scabrinodis* workers; EL- external microbiome of *Myrmica scabrinodis* larvae; IP- internal microbiome of *Microdon myrmicae*; IW- internal microbiome of *Myrmica scabrinodis* workers; IL- internal microbiome of *Myrmica scabrinodis* larvae.

Group 1	Group 2	Permutations	p-value	F-value
EP	EW	9999	**0.0015**	11.48
	EL	9999	**0.0015**	11.25
	IP	9999	**0.0015**	13.07
	IW	9999	**0.0015**	8.75
	IL	9999	**0.0015**	7.96
EW	EL	9999	1	1.45
	IP	9999	**0.0015**	18.71
	IW	9999	**0.0015**	4.55
	IL	9999	**0.0015**	5.92
EL	IP	9999	**0.0015**	16.56
	IW	9999	**0.003**	4.41
	IL	9999	**0.003**	4.61
IP	IW	9999	**0.0015**	14.03
	IL	9999	**0.0015**	10.47
IW	IL	9999	0.3885	2.32

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

Table 3. PERMANOVA anayisis showing the differences in microbiome composition of *Mi. myrmicae* larvae among the two sampling sites. The analysis was performed with 9999 permutations and Bonferroni correction, using Bray-Curtis distances calculated on the 16S feature table. Groups are divided according external or internal microbiome and the sampling site. Significative p-values are in bold. LPM-EP – external microbiome of *Microdon myrmicae* larvae collected from to Lower Prewley Moor site; HM-EP – external microbiome of *Microdon myrmicae* larvae collected from to Hollow Moor site; LPM-IP – internal microbiome of *Microdon myrmicae* larvae collected from to Lower Prewley Moor site; HM-IP – internal microbiome of *Microdon myrmicae* larvae collected from to Hollow Moor site.

Group 1	Group 2	Permutations	p-value	F-value
LPM-EP	HM-EP	9999	**0.0006**	4.09
	LPM-IP	9999	**0.0126**	5.4
	HM-IP	9999	**0.0012**	12.35
HM-EP	LPM-IP	9999	**0.0012**	9.74
	HM-IP	9999	**0.0006**	13.49
LPM-IP	HM-IP	9999	**0.006**	5.25

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

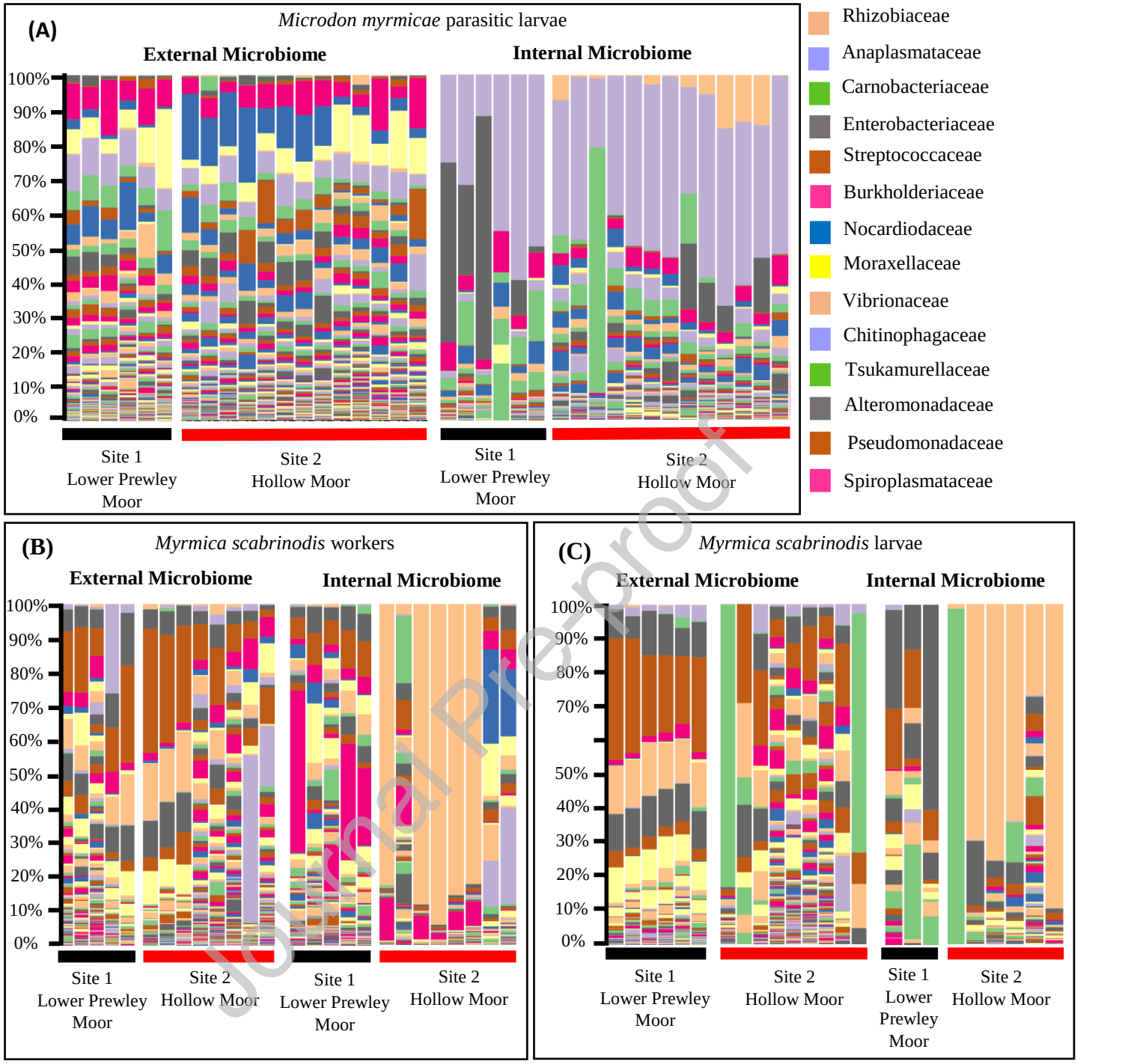

Fig. 1. Relative abundance for exact sequence variants (ESVs) coloured by bacterial families detected on the body surface and inside the parasites (A), and ant workers (B) and ant larvae. Each bar represents an individual pool from 2 ant workers and larvae or individual parasitic larvae.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

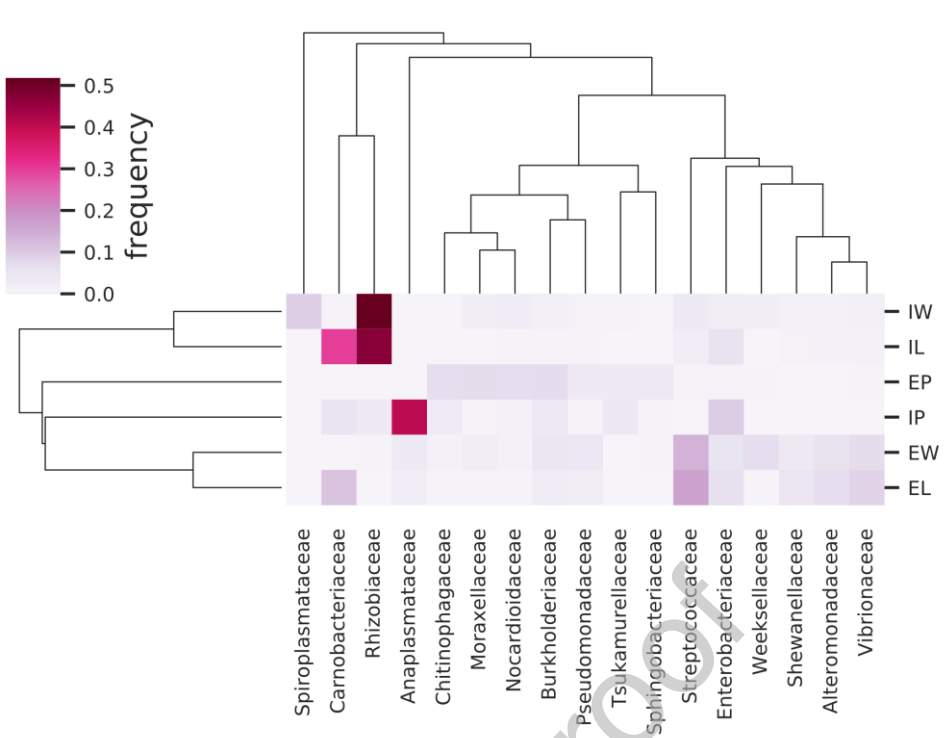

Fig. 2. Heatmap of the most abundant families, with Ward clustering of Bray-Curtis dissimilarities of sample type (groups collapsed by averaging family-level abundances) and bacterial families. IP- internal microbiome of *Microdon myrmicae*; EP- external microbiome of *Microdon myrmicae*; IW- internal microbiome of *Myrmica scabrinodis* workers; EW- external microbiome of *Myrmica scabrinodis* workers; IL- internal microbiome of *Myrmica scabrinodis* larvae; EL- external microbiome of *Myrmica scabrinodis* larvae.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

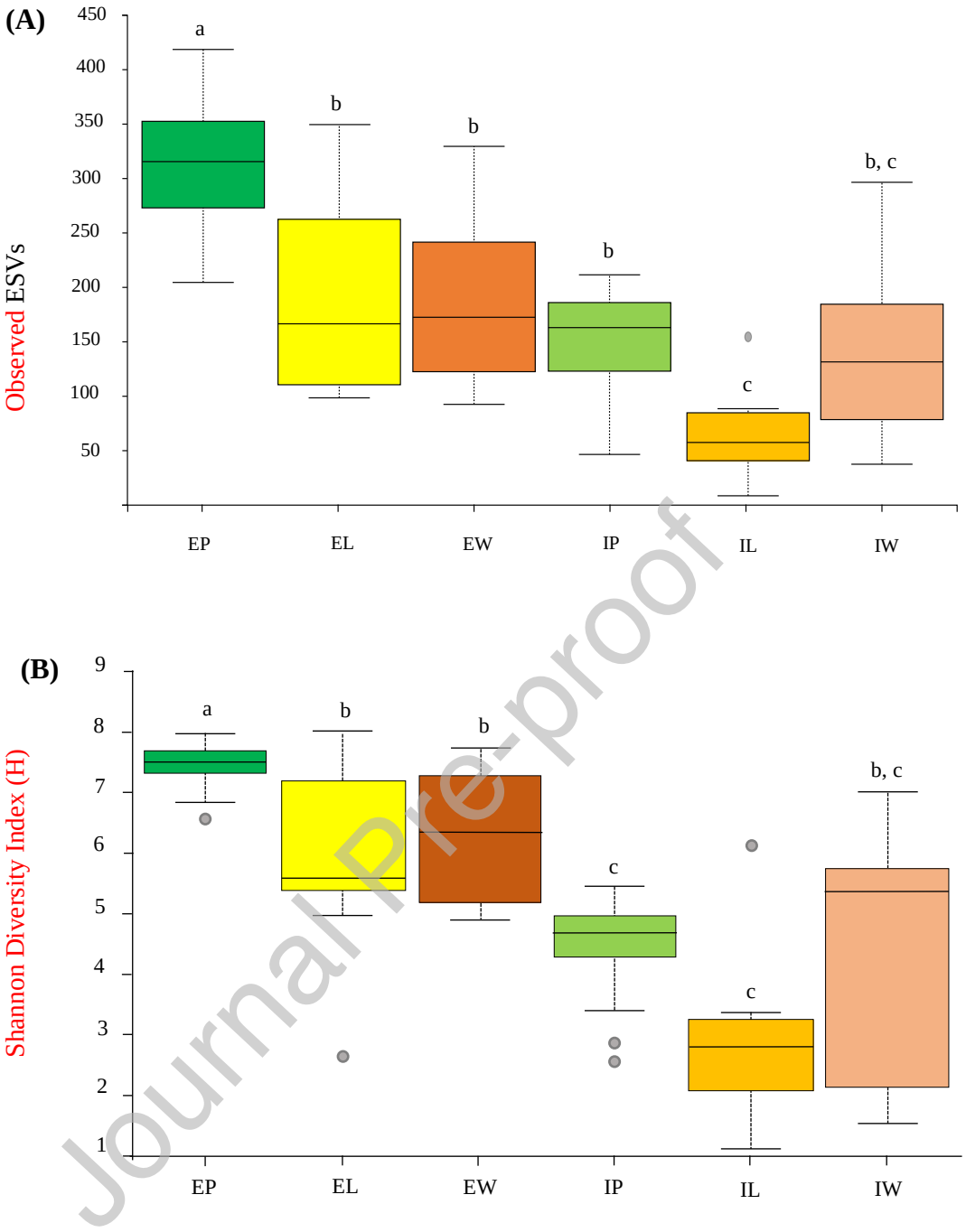

Fig. 3. Box plot of observed exact sequence variants (ESVs) (above) and Shannon diversity (below). A Benjamini-Hochberg post-hoc test revealed significance groups, represented by letters. The external microbiome of parasite cuticle is the most diverse, while the internal microbiome of ant larvae is the least diverse, although it does not differ significantly from internal microbiome of ant workers. EP- external microbiome of *Microdon* (green); EL- external microbiome of *Myrmica* larvae (yellow); EW- external microbiome of *Myrmica* workers (dark orange); IP- internal microbiome of *Microdon* (light green); IL- internal microbiome of *Myrmica* larvae (ocher); IW- internal microbiome of *Myrmica* workers (light orange).

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

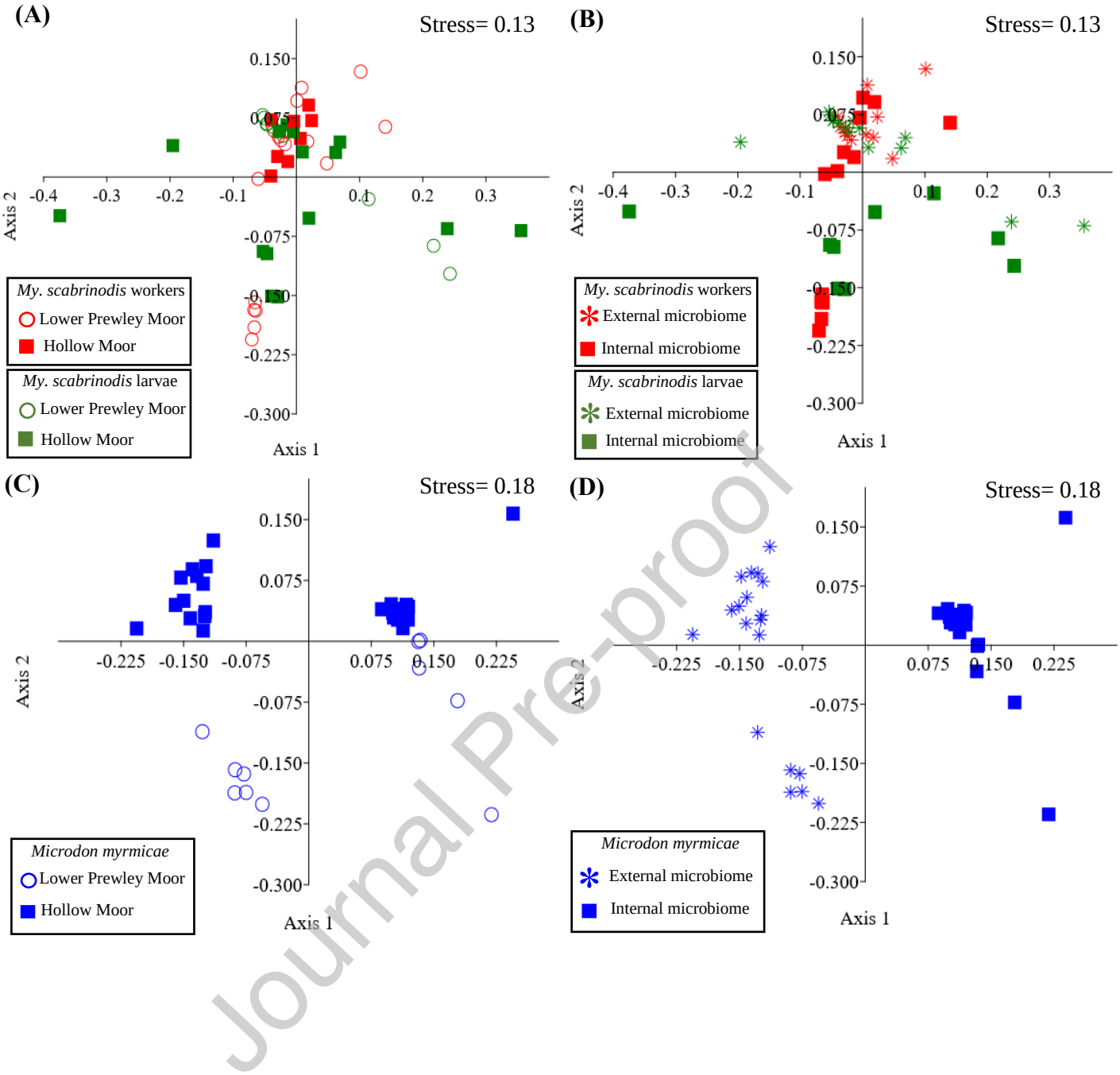

Fig. 4. Multivariate representations of bacterial community composition using nonmetric multidimensional scaling (NMDS) with Bray–Curtis distances showing *Myrmica* larvae and workers among two sampling sites (Lower Prewley Moor and Hollow Moor) (A) and between internal end external microbiomes (B); and *Microdon* larvae among the two sampling sites (C) and between external and internal microbiomes (D).

The microbiome composition of *Myrmica* larvae and workers seems to not change visibly in the two sampling sites (A), while the microbiome of *Microdon* larvae significantly diverge (PERMANOVA, $p < 0.05$) (C). Significant differences were detected between the external and internal microbiomes of both ants (B) and parasites (D), although no differences were detected among the two developmental stages (larvae and workers) on *Myrmica* (B). A-B – Ant workers are in red, ant larvae are in green: A- dots refer to Lower Prewley Moor site; squares refer to Hollow Moor site; B- stars refer to the external microbiome; squares refer to the internal microbiome; C-D *Microdon* larvae in blu: C- dots refer to Lower Prewley Moor site; squares refer to Hollow Moor site; D- stars refer to the external microbiome.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it

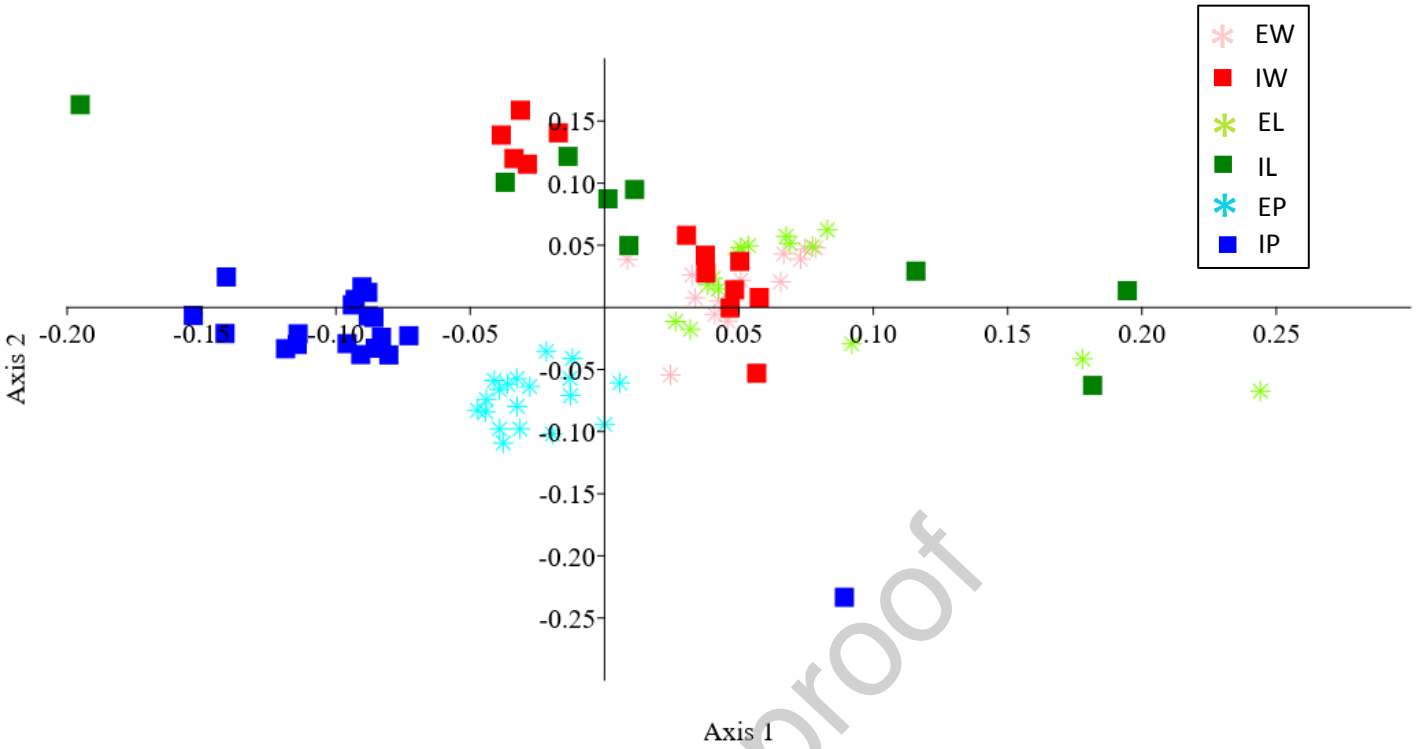

Fig. 5. Multivariate representation of bacterial community composition (beta diversity), using nonmetric multidimensional scaling (NMDS) of Bray–Curtis dissimilarity. The microbiome of *Microdon* larvae is significantly divergent from that of ant workers and larvae (PERMANOVA, $p < 0.05$). IP- *Microdon* internal microbiome (dark blue squares); Here differences among sites are not displayed. EP- *Microdon* external microbiome (light blue stars); IW- internal microbiome of *Myrmica* workers (red squares); EW- external microbiome of *Myrmica* workers (light red stars); IL- internal microbiome of *Myrmica* larvae (dark green squares); EL- external microbiome of *Myrmica* larvae (light green stars). Stress value: 0.18.

*Corresponding author. Tel.: +39 0657336323; fax: +39 57336321.
E-mail address: andrea.digiulio@uniroma3.it