Animal Microbiome

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Gut microbiome mediates an evolutionarily conserved social behavior in eusocial insects

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Abstract

The gut microbiome is increasingly recognized as a key mediator of social behavior and division of labor in eusocial insects. In some species, trophallactic interactions facilitate nutrient exchange between larvae and adults, creating social interdependency. However, the role of gut bacterial symbionts in this process remains unclear. Here, we investigated the relative contributions of host identity, environment, and trophallactic interactions to gut microbiome assembly in two social wasp species, *Vespa orientalis* and *Vespula germanica*, using a cross-fostering, common garden experiment. Newly emerged workers and early-instar larvae were reciprocally exchanged and reared under controlled conditions. High-throughput 16S rRNA gene sequencing and functional inference revealed limited variation across treatments in worker gut communities, mainly shaped by shared environment, indicating stability and environmental dominance. Conversely, larval gut microbiomes were highly plastic, influenced by both larval species and the identity of their nursing workers, highlighting the impact of social interactions. Functional profiles reflected caste-specific roles: workers harbored microbiomes enriched for antimicrobial and detoxification pathways, while larvae microbiomes were enriched in metabolic functions for protein digestion and development. These findings demonstrate that metabolic division of labor in eusocial wasps is supported by life stage-specific microbial communities and functions and maintained by social interactions, positioning the gut microbiome as a key contributor to the maintenance of eusociality.

Keywords Sociality, Host/microbiome coevolution, Social insects, Division of labor, Eusocial insects, Metabolism, Wasp, Cross-fostering

Introduction

Host-associated microbiomes play a critical role in mediating social behaviors, such as mate selection [1], locomotion [2, 3], foraging decisions [4, 5] and interactions with conspecifics [6–9]. Among these behaviors are evolutionarily conserved functions such as division of labor in eusocial insects, supported by caste-specific microbiome profiles [10, 11], and nest-mate recognition that depends partially on shared microbial symbionts [12–14]. These colony-level interactions between nestmates, fundamental to their evolutionary success, make social insects a powerful model system for studying microbiome-behavior dynamics. Furthermore, in many eusocial

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insects, larvae depend on workers to feed, creating a critical interface between nutrients and social behavior where gut microbiome dynamics may play a significant role.

Despite the potential significance of the microbiome in maintaining social dynamics within a super-organism (e.g., colony), the relative contributions of the various factors shaping these microbial communities remain unclear. Several mechanisms of gut microbiota acquisition have been described for social insects, including vertical (mother-to-offspring) transmission [15, 16] and deposition of oral or anal secretions onto shared food substrate or comb material [17–19]. An additional source of microbiota transmission in eusocial species is through trophallaxis, the exchange of fluids between colony members, that can occur among siblings or between nursing workers and larvae. Proctodeal trophallaxis (hindgut-to-mouth) has been suggested to be the primary cause of maintenance of inter-generational host-specific microbial communities in termites [20, 21], but the dependence appears to be unidirectional (i.e., only larvae benefit). In some social insects, however, trophallaxis plays a bigger part in maintaining dependency between colony members; in two species of social wasps (family Vespidae), the Oriental hornet (*Vespa orientalis*) and the German wasp (*Vespula germanica*), workers feed larvae with high-protein food and, in turn, obtain most of their own nutritional requirements from the oral secretions produced by larvae, through trophallaxis [22]. However, it remains unclear whether gut bacterial symbionts are inoculated in either direction, or if the microbiota participates in the specialized larval digestion.

Therefore, to identify the drivers shaping the gut microbiome colonization in eusocial species, we executed a cross-fostering, common garden experiment between two species of social wasps, the Oriental hornet and the German wasp. This unique experimental design allowed contrasting the microbiome profiles of two distinct species, while leveraging innate nursing behaviors to disentangle the relative contributions of host species, the environment, and trophallactic interactions to gut microbiota establishment. We hypothesized that the significance of these factors will be reflected in differences in microbial community abundance and diversity. For example, if the environment has the greatest impact on the microbiome, most of the individuals will present with similar microbiome profiles regardless of their species or the identity of their nursing counterpart (i.e., worker or larva). Inversely, if host identity affects the microbiome the most, microbial communities from individuals from the same species will cluster. Alternatively, if trophallaxis acts as a tool for mediating bacterial symbionts, workers and larvae from the same treatment will be more similar than their respective counterparts in other treatments. Similarly, the control treatments (same-species worker-larvae combination) in this experimental design allow for identification of differences between life stages in *V. orientalis* and *V. germanica* as well as definition of a core microbiota.

Materials and methods

Colony care and sample collection

We collected wild colonies of two Vespid species, the Oriental hornet and the German wasp, at two locations (Oriental hornet – Kibbutz Beit Hashita, Israel, latitude: 32.550957, longitude: 35.438736, collection date: June 24th 2020; German wasp – the botanical garden at Tel Aviv University, Israel, latitude: 32.1141126, longitude: 34.8089881, collection date: August 10th 2020) using standard nest collection protocols [23]. The two sites are ~ 76.6 km apart, so while both are in Israel, they sample different foraging environments. Each species was represented by a single colony to minimize the impact of neutral diversity and standardize experimental conditions. Both colonies included the founding queen, workers, and additional offspring (eggs, larvae, pupae). We reared the colonies in laboratory conditions (28 °C, 70% relative humidity) in trapezoidal wooden boxes (14 L) with a Plexiglas front wall, supplied with *ad libitum* food (bumble bees (*Bombus terrestris*) from commercial rearing and raw chicken as protein sources and sugar solution (60% inverted sugar)) and tap water as well as cell building material (paper and soil). To reduce the influence of prior environmental exposure on the gut microbiomes, three weeks prior to the beginning of the experiment, we standardized the feeding regime of both colonies by supplying the same type of protein daily (bumble bees and raw organic chicken).

Experimental design

We conducted a cross-fostering, common garden experiment that included two groups in which the larvae of one species were nursed by workers of the second species and two control groups in which workers nursed their own larvae (Fig. 1). Accordingly, we carried out four treatment schemes: "control O" (*V. orientalis* workers nursing *V. orientalis* larvae), "control G" (*V. germanica* workers nursing *V. germanica* larvae), "cross O/G" (*V. orientalis* workers nursing *V. germanica* larvae), and "cross G/O" (*V. germanica* workers nursing *V. orientalis* larvae). The experiment was conducted in separate boxes, distinct from the original colony. Each treatment included four to six newly emerged workers, and a piece of comb cut off from the wild colony that contained between 20 and 30 larvae in early stages (up to 3rd instar, distinguished by body and head size and sclerotization [24]). We selected only early-stage larvae to minimize the effect of prior interactions with nursing workers outside of the timeframe

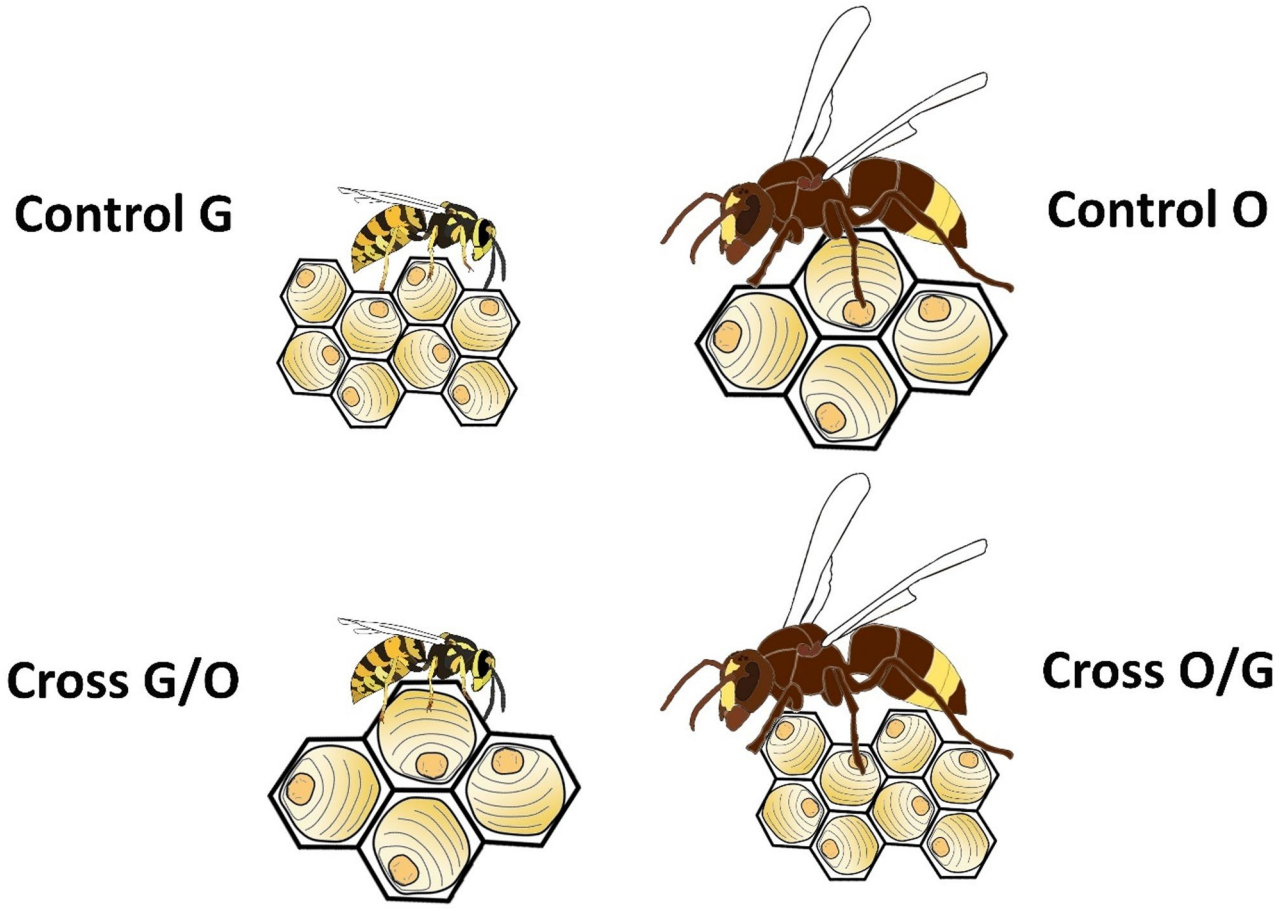

Fig. 1 Experimental design of the four treatments in this study. 'Control G' included German wasp (*Vespula germanica*) workers and larvae; 'Control O' included Oriental hornet (*Vespa orientalis*) workers and larvae; 'Cross G/O' included *V. germanica* workers and *V. orientalis* larvae; 'Cross O/G' included *V. orientalis* workers and *V. germanica* larvae. Analyzed sample size (n) is given for workers ('W') and larvae ('LV') separately as follows: Control G: n(W) = 22, n(LV) = 12; Control O: n(W) = 15, n(LV) = 11; Cross G/O: n(W) = 35, n(LV) = 10; Cross O/G: n(W) = 17, n(LV) = 8

of the experiment while ensuring that nursing behavior could still take place. Preliminary trials with eggs resulted in either abandonment by nursing workers or a complete lack of nursing behavior. Additionally, we used newly emerged workers as nursing workers. Because during metamorphosis, the larval gut content is expelled prior to pupation [25], newly emerged adults are thought to retain negligible gut microbiota [15]. The minimal microbiome at emergence likely reduces the potential influence of additional factors, such as life history traits or colony location, prior to microbiome establishment, although this has not been specifically demonstrated in the two species examined in this study.

Environmental and dietary conditions in all four treatments were identical. We conducted all four treatments in small wooden boxes (14.5 × 12 × 10 cm) with glass doors, with a minimum of four replications for each treatment (additional replications were conducted if an experiment ended without collected larvae, e.g., if all larvae died). The combs containing the larvae were glued to the ceiling of the wooden box. We supplied the workers and larvae with a protein source (bumble bees and organic chicken – to avoid the impact of antibiotics residues), a carbohydrate source (5 ml tube containing sterilized sugar solution (60% inverted sugar)), and tap water (in a 5 ml tube), all changed daily. We monitored larvae and workers daily and removed any individuals that died, storing them at – 20 °C for microbiome characterization. We terminated experiments when all or most larvae had died or reached the 5th instar (experiment length ranged between 5 and 39 days). Upon termination, we flash-froze all remaining workers and larvae and stored them at -20 °C until further analysis. We included workers even if no larvae had been collected from a particular treatment box, if nursing behavior had been observed. In two replicates, workers that had died were replaced by other newly emerged workers so that a minimum number of four nursing workers was maintained. These replacements are not expected to change the microbiome of the nursed larvae in these boxes because of the negligible microbiome of newly emerged workers.

Microbiome analysis

We dissected the digestive tracts (mid- to hindgut) of the frozen workers and larvae in a sterile environment under a Plan S 1.0x FWD 81 mm binocular (Zeiss, Discovery V12, Germany) and stored them at − 80 °C until further analysis. We extracted DNA from all samples (Table S1) using the PureLink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher; Waltham MA, USA) according to the manufacturer's instructions and following mechanical crushing using sterile L-shaped spreaders and a 2 min bead beating step. We then PCR-amplified the variable V4 region of the 16S rRNA gene using the 515 F-barcoded and 806R-non-barcoded primers [26]. Each PCR reaction consisted of 25µL PrimeSTAR Max PCR mix (Takara Kusatsu, Shiga, Japan), 2 µM of each primer, 11 µL of ultra-pure water, and 10 µL DNA template. Thermal cycler conditions were as follows: 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 20 s, followed by a final elongation at 72 °C for 1 min. We purified amplicons using Kapa Pure magnetic beads (Roche; Basel, Switzerland) and quantified them using the Picogreen dsDNA quantitation kit (Invitrogen, Thermo Fisher; Waltham, MA, USA). We then pooled equimolar amounts of DNA from individual samples and sequenced the pool using the Illumina MiSeq platform at the Genomic Center at the Azrieli Faculty of Medicine (Bar-Ilan University), Safed, Israel. Appropriate negative and positive controls were included.

We initially processed the 16S rRNA gene sequence data with QIIME2 version 2020.8 [27] using default parameters. We used DADA2 [28] to trim reads at the 5′ end by 13 bp and truncated them at 192 bp based on quality profiles. Default DADA2 parameters were used to correct sequencing errors, remove chimeric sequences, remove singletons, and dereplicate sequences into amplicon sequence variants (ASVs). We assigned taxonomy using classify-sklearn naïve bayes classifier against GreenGenes2 v2024.9 [29]. Following taxonomy assignment, we removed non-target features including chloroplasts, mitochondria, and eukaryotes. For phylogenetic analyses, we aligned ASV sequences using MAFFT, masked to remove hypervariable positions, and constructed a phylogenetic tree using FastTree as implemented in the QIIME2 align-to-tree-mafft-fasttree pipeline, using the rooted tree for downstream phylogeny-based analyses.

After the QIIME2 pipeline, we performed downstream analysis using phyloseq (version 1.34.0), an R/bioconductor package for handling and analysis of high-throughput phylogenetic sequence data [30]. First, we filtered out samples with a minimum sequence depth of less than 1,000. We normalized the data either by rarefaction or by scaling to relative abundances. Rarefaction was performed using the rarefy_even_depth function in phyloseq. Because different analyses were conducted on different subsets of samples, rarefaction was applied separately to each phyloseq object, using a threshold of 90% of the minimum library size within each dataset. This resulted in final rarefied depths of 3,972–12,342 reads per sample. Unless otherwise stated, diversity-based analyses (alpha diversity, beta diversity, PERMANOVA, and PCoA) were performed on rarefied datasets, whereas abundance-based analyses (MaAsLin2, taxa-specific tests, and heatmap visualizations) were conducted on non-rarefied data using relative abundances. Prior to downstream analyses, we assessed samples for potential outliers. We identified outliers based on their distance from the centroid in principal coordinate space (PCoA) and by multivariate Mahalanobis distance across the first five PCoA axes. Samples identified by both approaches were excluded from downstream analyses. Raw data and metadata are available online at https://www.ebi.ac.uk/ena/browser/view/PRJEB72672 [31].

Statistical analysis

We assessed alpha diversity using the Shannon diversity index [32] and beta diversity using weighted UniFrac distance [33] in the R package 'phyloseq' [30], both using the rarefied data set. To compare alpha diversity between sample groups, we employed Kruskal-Wallis non-parametric ANOVA, followed by Dunn's post hoc tests with Benjamini–Hochberg false discovery rate (FDR) correction for significant comparisons (adjusted $P < 0.05$). We analyzed beta-diversity patterns between treatments using permutational multivariate ANOVA (PERMANOVA; 9999 permutations) implemented with the function 'adonis2' in R package 'vegan' (version 2.6–10) [34]. Significance was assessed using permutation-based p-values (Pr(> F)). When the initial PERMANOVA indicated a significant overall effect, we assessed pairwise differences in microbial community composition among treatment groups using the 'pairwise.adonis' function in R package 'pairwiseAdonis' with weighted UniFrac distance matrix, 9,999 permutations, and Benjamini–Hochberg (FDR) correction for multiple comparisons [35]. We performed Principal Coordinate Analysis (PCoA) using the 'ordinate' and 'plot_ordination' functions in the R package 'phyloseq'. To identify drivers of microbiome composition, we implemented three additional PERMANOVA models. The full dataset model tested the fixed effects of species and caste. For the workers-only subset, we examined species and nursed larvae species identity ('counterpart identity', levels: same/other), while the larvae-only subset assessed species and nursing workers species identity ('counterpart identity', levels: same/other). Models included box number as a random factor to account for shared environmental conditions

(i.e., biological replicates), except when explicitly testing the trophallaxis hypothesis (i.e., that trophallaxis acts as a tool for mediating bacterial symbionts). For this specific analysis, we included 'box' as a nested fixed effect within counterpart identity to evaluate whether intra-box microbiome similarity (potentially mediated by trophallactic interactions) explains a substantial portion of the variance.

To assess gut microbiome differential abundance, we used MaAsLin2 (Multivariable Association Testing, version 1.18.0) [36], using the non-rarefied data set. We ran MaAsLin2 with a minimum prevalence of 0.1 and a minimum abundance of 0.001 and a single fixed variable representing information on both caste and treatment ('Caste_treatment'), with box number ('Box') as a random factor. Each feature was analyzed at its most specific available taxonomic assignment (e.g., species when available, otherwise genus, family, order, etc.). As a result, the set of differentially abundant features includes taxa resolved at different ranks, and counts reported reflect the number of significant features within each rank. We examined patterns of homogeneity of dispersion using the function 'betadisper' from the R package 'vegan' (version 2.6.10) [34].

We visualized differentially abundant taxa identified by MaAsLin2 using heatmaps (R package 'pheatmap', version 1.0.12 [37]) to highlight abundance patterns across samples. We then applied Kruskal-Wallis tests followed by Dunn's post-hoc comparisons to the differentially abundant taxa identified by MaAsLin2 ($P \leq 0.05$) to analyze taxa-specific differences in relative abundances across groups and visualized variation in individual taxa using the R package 'ggplot2' [38]. Finally, we identified core microbiota separately for workers and larvae in each species using the function 'core_members' from the R package 'microbiome' (version 1.26.0) [39] with an abundance detection threshold of 0.001 and minimal prevalence of 0.3. To minimize experimental design effects, we included only individuals from the control treatments in this analysis and used only rarefied data.

To infer the functional potential of the bacterial communities, we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States version 2 (PICRUSt2) [40] with the default parameters, generating predictions based on phylogenetic proximity to reference genomes for all taxa in the full dataset. The average NSTI (Nearest Sequenced Taxon Index) value and standard deviation for the full data set were 0.060 ± 0.100, indicating good coverage. We used the R package ggpicrust2 [41] to generate an error bar plot and a principal component analysis (PCA) plot of the predicted pathway abundances based on predicted Enzyme Commission (EC) number abundances, and then we performed a differential abundance analysis with the MaAsLin2 method [36] with a minimum prevalence of 0.4 and a minimum abundance of 0.001. The input data for KEGG pathway analysis with the ggpicrust2 R package was the unstratified predicted functional abundance table generated by PICRUSt2, with KEGG pathways as features.

Results

Experimental and sequencing success

We conducted 20 replicates overall of the four treatments, in which a total of 100 workers and 51 larvae were collected (Table S1). The treatment in which workers of the bigger species, *V. orientalis*, nursed larvae of the smaller species, *V. germanica* (Cross O/G), yielded the smallest number of larvae collected ($n = 9$). We posit that this occurred because the *V. germanica* comb cell size is much smaller than that of *V. orientalis*, which may have physically prevented the large Oriental hornets from properly reaching the early-stage, small German wasp larvae. In two out of the seven replicates of the cross treatment where German wasp workers nursed Oriental hornet larvae (Cross G/O), the workers destroyed the entire comb, leading to termination of the experimental replicate.

Microbial DNA from fourteen samples was not successfully extracted. Out of the 137 individual samples available for sequencing, six were omitted due to low sequencing depth and one was an outlier. In total, 89 workers and 41 larvae were available for analysis, representing all four treatments (Table S1, Figure S1).

Microbiome diversity of nursing workers

To test whether the microbiome of newly emerged nurses is shaped by identity (i.e., species), trophallactic interactions (i.e., identity of the nursed larvae) or the environment, we examined whether workers from different treatments are characterized by different gut microbial communities. We found that alpha diversity did not differ significantly between workers from different treatment groups, regardless of their identity or the identity of the larvae they nursed (Kruskal-Wallis, $P = 0.443$, Fig. 2A). Additionally, microbial beta diversity was not significantly different among workers ($F = 1.75$, $R^2 = 0.06$, $Pr(>F) = 0.115$, Fig. 2B). Consistent with this, relative abundance patterns in workers were comparable across species and treatments (Figure S2A).

To better understand how the microbial community changes in response to intrinsic and extrinsic conditions, we identified differential abundance of specific taxa as a function of treatment using MaAsLin2. Only one taxon (the species *Convivina intestine*) was found to be differentially abundant between workers of both species (reference: German wasp workers from the cross treatment, $P < 0.05$, Table S2). Visual examination of heatmaps,

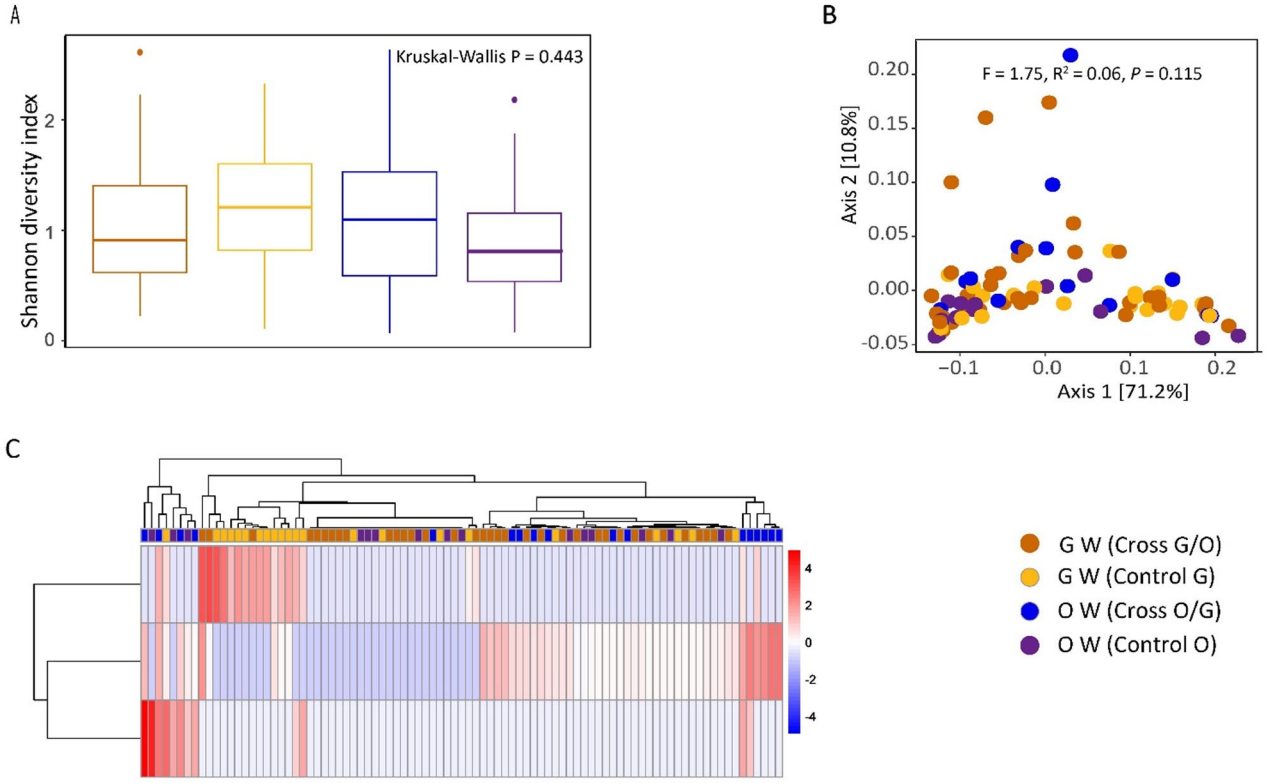

Fig. 2 Microbiome diversity and composition in nursing workers across the four treatments groups. (**A**) Shannon diversity index of nursing workers in each treatment. Differences between treatments, compared with a Kruskal-Wallis test, were not statistically significant ($P = 0.443$). (**B**) Principal Coordinate Analysis (PCoA) of bacterial community composition based on weighted UniFrac distances ($Pr(>F) = 0.115$). (**C**) Heatmaps of normalized, log_2-transformed abundances of bacterial taxa identified by MaAsLin2 analysis in workers. Taxa included show nominal significance (raw $P < 0.05$) in at least one comparison and are presented to illustrate relative abundance patterns across samples. Statistical significance was evaluated using Benjamini–Hochberg (FDR)–adjusted P values (see Table S2). Rows represent individual bacterial taxa; columns represent individual workers. Data were z-score scaled by row to emphasize relative differences across samples (blue = lower, red = higher abundance)

however, did not reveal treatment-specific patterns of differential abundance (Fig. 2C).

All workers exhibited similar homogeneity of dispersion (Table S3). PERMANOVA models using weighted UniFrac distances ran with box number as strata did not converge. Therefore, we reran the models without this variable, and this revealed neither a significant effect of counterpart identity ($R^2 = 0.02$, $F = 1.52$, $Pr(>F) = 0.198$), nor of species ($R^2 = 0.02$, $F = 2.02$, $Pr(>F) = 0.125$) on worker community composition (beta diversity, weighted UniFrac distance), with the model explaining 4% of the total variation (Table 1). The broader model, including caste and species across all workers and larvae, explained 15% of the variation, with both caste ($R^2 = 0.12$, $F = 18.59$, $Pr(>F) < 0.001$) and species ($R^2 = 0.03$, $F = 5.13$, $Pr(>F) < 0.001$) emerging as significant effects.

Core microbiota analysis identified 20 shared taxa in workers from both species, 11 of which were also detected in larvae of both species (Table S4). Additionally, two taxa were only prevalent in *V. germanica* workers, while three taxa were uniquely prevalent in *V. orientalis* workers.

Microbiome diversity of larvae

Similarly, we aimed to assess whether larvae from different treatments shared similar gut microbial communities. Alpha diversity did not differ significantly between larvae from different treatment groups ($P = 0.106$, Fig. 3A). Inversely, beta diversity was significantly different among larvae from different treatments ($F = 6.41$, $R^2 = 0.34$, $Pr(>F) < 0.001$, Fig. 3B, Table S5), with relative abundance profiles differing visibly between treatments (Figure S2B).

Differences among larvae from different treatments were also observed when examining differential abundance of bacterial taxa after correcting for multiple comparisons. Four bacterial taxa (all at the species level) were found to be differentially abundant between larvae from the four different treatments (reference: German wasp larvae from the control treatment, $P < 0.05$; Table S6). These taxa belonged to three phyla: Bacillota I (*Leuconostoc fallax*, *Fructilactobacillus vespulae*), Actinomycetota (*Providencia huaxiensis*), and Pseudomonadota (*Aquabacter cavernae*). Heatmap visualization of taxa identified as differentially abundant at raw $P < 0.05$ showed

Table 1 Results of PERMANOVA tests evaluating the effects of caste/counterpart identity and species on microbiome community composition across different subsets of the data

Data set	Variable	Df	SumOfSqs	R^2	F	*P* value
Full (workers & larvae), 'Box' as random	Caste	1	0.37	0.12	18.59	0.000
	Species	1	0.10	0.03	5.13	0.000
	Residual	127	2.54	0.85		
	Total	129	3.01	1.00		
	Model R^2			0.15		
Workers (no 'Box' variable)	Counterpart identity	1	0.03	0.02	1.52	0.198
	Species	1	0.04	0.02	2.02	0.125
	Residual	86	1.50	0.96		
	Total	88	1.56	1.00		
	Model R^2			0.04		
Larvae (no 'Box' variable)	Counterpart identity	1	0.10	0.09	4.48	0.020
	Species	1	0.13	0.13	6.08	0.005
	Residual	38	0.84	0.79		
	Total	40	1.07	1.00		
	Model R^2			0.21		
Workers, 'Box' nested	Species	0	0.00	0.00	-Inf	NA
	Counterpart identity\Box	17	0.67	0.43	3.30	0.000
	Residual	69	0.83	0.53		
	Total	88	1.56	1.00		
	Model R^2			0.47		
Larvae, 'Box' nested	Species	0	0.00	0.00	Inf	NA
	Counterpart identity\Box	13	0.46	0.43	2.34	0.002
	Residual	25	0.38	0.36		
	Total	40	1.07	1.00		
	Model R^2			0.64		

The models include 'Box' either as a random factor or nested structure when applicable. Reported are degrees of freedom (Df), sum of squares (SumOfSqs), proportion of variance explained (R^2), F-statistics, and associated p-values (*Pr*(> F) - value). Model R^2 refers to the total variance explained by the fixed effects in each model

treatment-specific clustering patterns, corresponding to the four treatments (Fig. 3C).

Homogeneity of dispersion was similar across treatments in larvae (Table S3). PERMANOVA models using weighted UniFrac distances ran with box number as strata did not converge. Therefore, we reran the models without this variable, and this demonstrated that both counterpart identity (R^2 = 0.09, F = 4.48, Pr(> F) = 0.020) and species (R^2 = 0.13, F = 6.08, Pr(> F) = 0.005) significantly explained larva community composition (beta diversity). The model accounted for 21% of the total variation, a higher percentage than the broader model (Table 1).

Examining relative abundance patterns of specific taxa identified as significantly differentiated by MaAsLin2 showed that oriental hornet larvae shared a significantly elevated relative abundance of taxa from the orders Lactobacillales and Thermomicrobiales, the family Carnobacteriaceae, the genus *Corynebacterium*, and the species *Enterococcus G sulfureus* (Kruskal-Wallis, $P < 0.001$; post-hoc Dunn's test with Benjamini-Hochberg adjustment, Table S7; Fig. 4B-E). In addition, core microbiota analysis revealed that larvae from both species shared 19 taxa, 11 of which were also present in the cores of workers from both species (Figure S3, Table S4). Eleven taxa were only prevalent in the cores of *V. germanica* larvae, while ten taxa were only prevalent in the cores of *V. orientalis* larvae.

Microbiome transfer between workers and larvae

Stratified PERMANOVA models (box number as nested fixed effect) showed significant box-level effects, with this factor explaining the largest proportion of variance in both worker and larval microbiota (Table 1). The proportion of variation explained ('model R^2') was greater in models treating box number as a nested fixed effect than as a random variable, suggesting that box number accounts for a substantial proportion of microbiome differences across castes.

Differences between life stages

To characterize the microbiome profiles of larvae and workers, we analyzed Oriental hornets and German wasps from control treatments separately to minimize variation stemming from the species-specific core microbiome. Beta diversity was significantly different between

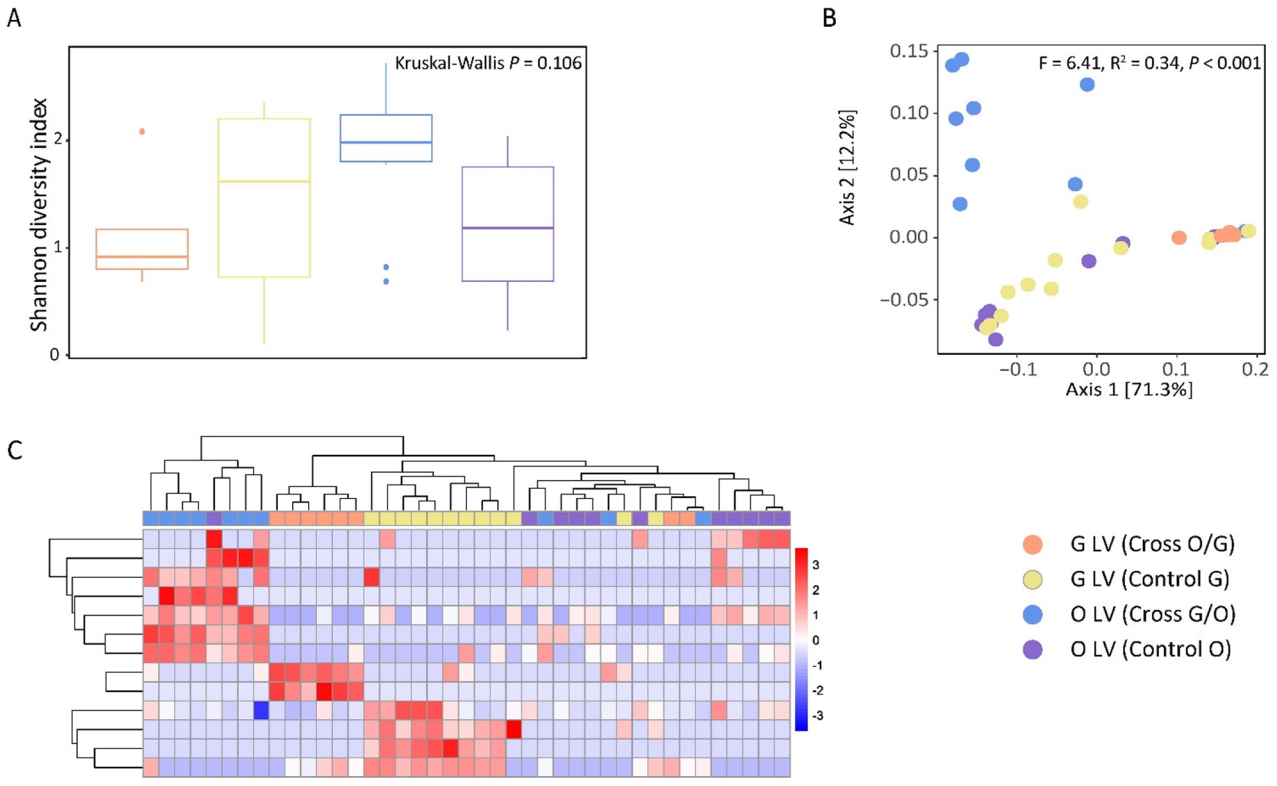

Fig. 3 Microbiome diversity and composition in larvae across four treatment groups. (**A**) Shannon diversity index of larvae in each treatment. Differences between treatments, compared via the Kruskal-Wallis test, were statistically insignificant ($P = 0.106$). (**B**) PCoA of bacterial community composition based on weighted UniFrac distances of larvae compared with PERMANOVA (Pr(> F) < 0.001). (**C**) Heatmaps of normalized, $\log_2$-transformed abundances of bacterial taxa identified by MaAsLin2 analysis in larvae. Taxa included show nominal significance (raw $P < 0.05$) in at least one comparison and are presented to illustrate relative abundance patterns across samples. Statistical significance was evaluated using Benjamini–Hochberg (FDR)–adjusted P values (see Table S6). Rows represent individual bacterial taxa; columns represent individual larvae. Data were z-score scaled by row to emphasize relative differences across samples (blue = lower, red = higher abundance)

larvae and workers, both in Oriental hornets ($F = 6.66$, $R^2 = 0.22$, $P = 0.001$, Fig. 5A) and in German wasps ($F = 5.75$, $R^2 = 0.15$, $P = 0.005$, Fig. 5B).

Differences between life stages were also evident in the differential abundance of specific microbial taxa. Based on MaAsLin2 results corrected for multiple comparisons (Benjamini–Hochberg (FDR), adjusted $P < 0.05$), 19 taxa were found to differ significantly between workers and larvae in *V. orientalis* (Fig. 5C, Table S8), and 23 taxa in *V. germanica* (Fig. 5D, Table S9). In Oriental hornets, most of the taxa that were differentially more abundant in larvae belonged to the phylum Bacillota. A very similar trend was observed in German wasps (Table S9). In addition, when analyzing relative abundance patterns of specific taxa across treatments, we found that the relative abundance of the genus *Lactococcus_A_346120* was significantly higher in workers of both *V. orientalis* and *V. germanica* compared with larvae (Kruskal-Wallis, $P < 0.001$; post-hoc Dunn's test with Benjamini-Hochberg adjustment, Table S7; Fig. 4F).

We also tested differentially abundant functions and pathways between life stages in the full dataset, regardless of species. 108 predicted functional pathways (KEGG Orthology) were differentially abundant between workers and larvae (Table S10). Annotation of these taxa suggests that functions more abundant in larvae correspond largely to metabolism of lipids (fatty acids degradation; glycerolipid metabolism), processes that support growth and biosynthesis (citrate (TCA) cycle; oxidative phosphorylation; steroids biosynthesis), mechanisms for decreasing oxidative stress (flavone & flavonol biosynthesis; peroxisome; beta-alanine metabolism), and developmental capacities (carotenoid biosynthesis) (Figure S4A). Conversely, in workers, most of the differentially abundant features were related to carbohydrate metabolism, such as metabolism of starch, sucrose, fructose, mannose and galactose, insulin signaling pathway, and phosphotransferase system (PTS). Additional enriched functions included defense and detoxification (dioxin degradation; cyanoamino acid metabolism) and microbial antibiotics (biosynthesis of vancomycin group antibiotics; stilbenoid, diarylhepatonoid and gingerol biosynthesis; streptomycin, penicillin and cephalosporin biosynthesis; polyketide sugar unit biosynthesis).

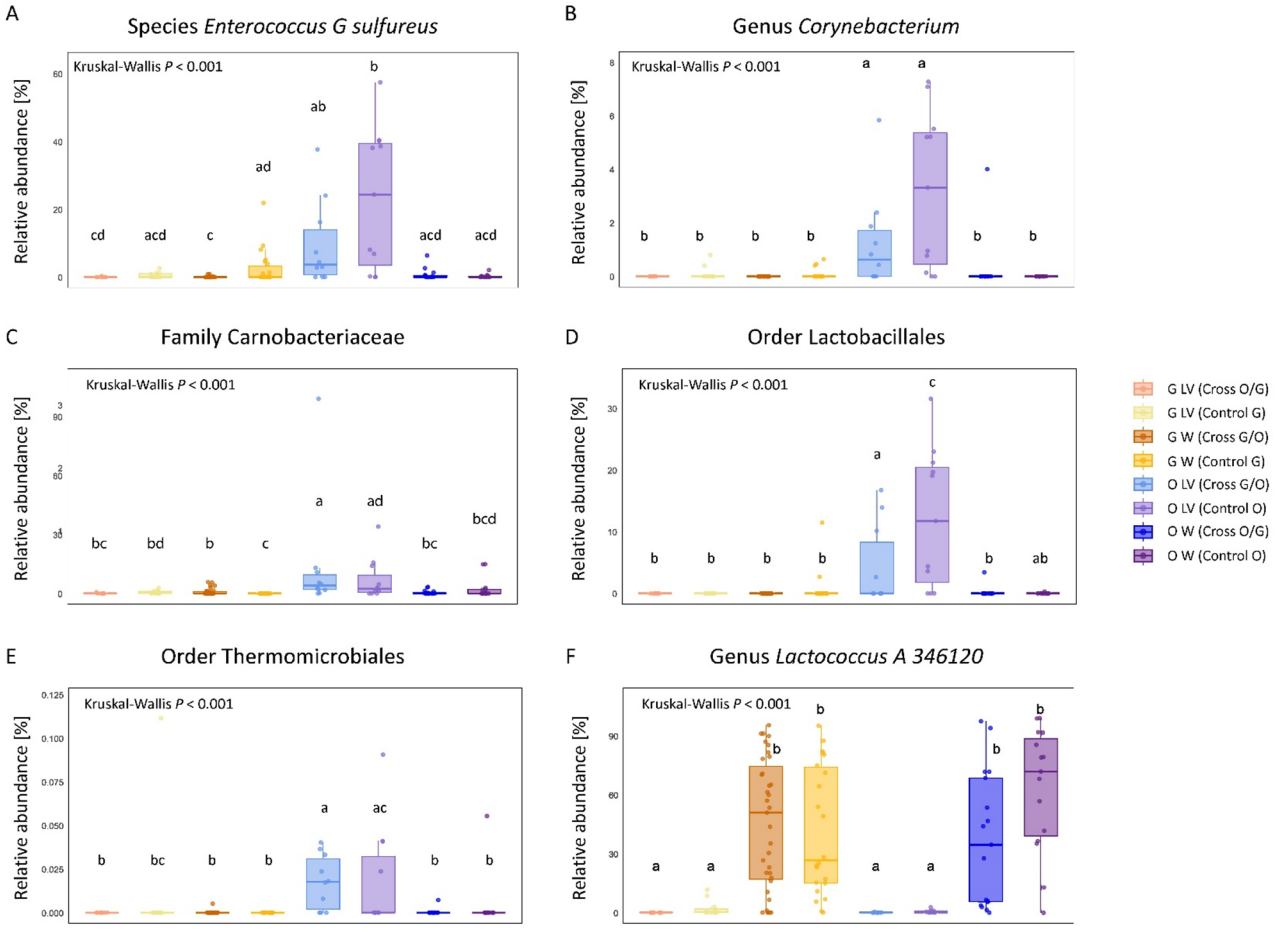

Fig. 4 Bacterial taxa with significantly higher relative abundance in larvae and workers. (**A**–**E**) Boxplots showing taxa with higher relative abundance in larvae ('LV') of Oriental hornets. (**F**) Taxa with differential abundance in workers ('W') across the four treatment groups. Each box represents the distribution of relative abundance across replicates, with lowercase letters indicating statistically significant group differences. Taxa shown were identified as significant by MaAsLin2, and group comparisons were conducted using Kruskal-Wallis and Dunn's post-hoc tests. Additional taxa are shown in Figure S3; test results are in Table S7. Group labels are described in Fig. 1

Functional differences also allowed for spatial separation between workers and larvae (Figure S4B).

Discussion

In this study, we explored the drivers shaping gut microbiome colonization in eusocial species using a novel experimental design of reciprocal larval nursing across species under controlled common garden conditions. To our knowledge, this represents the first successful implementation of interspecies cross-fostering, providing a robust platform for evaluating host-environment interactions in microbial gut colonization at a broad phylogenetic scale. Given that this nursing behavior is a conserved evolutionary trait maintaining interdependence among nestmates, we leveraged this approach to show that gut symbionts are transferred during trophallactic interactions and may mediate life stage-specific nutritional requirements, thereby helping to sustain this evolutionarily conserved social behavior.

Microbiome importance in social insects

Life stage decoupling in eusocial insects is a critical mechanism supporting their social organization, particularly through differentiating nutritional requirements between larval and adult stages. Division of labor, among which is the nursing of larvae by workers through trophallaxis, is considered one of the hallmark of eusociality. Whereas the microbiomes of primitively eusocial species are typically less diverse and more dominated by environmentally acquired bacteria [42, 43], more complex societies such as highly social wasps (e.g., *Brachygastra mellifica* and the species analyzed in this study) harbor richer, more consistent microbial communities, characterized by bacteria supporting caste- and life-stage-specific roles [44]. Trophallactic interactions are thought to reinforce colony cohesion [21, 45], and bidirectional (mutualistic) feeding may result in larvae functioning as a specialized 'digestive caste,' solidifying their role within the colony – as observed in certain advanced eusocial

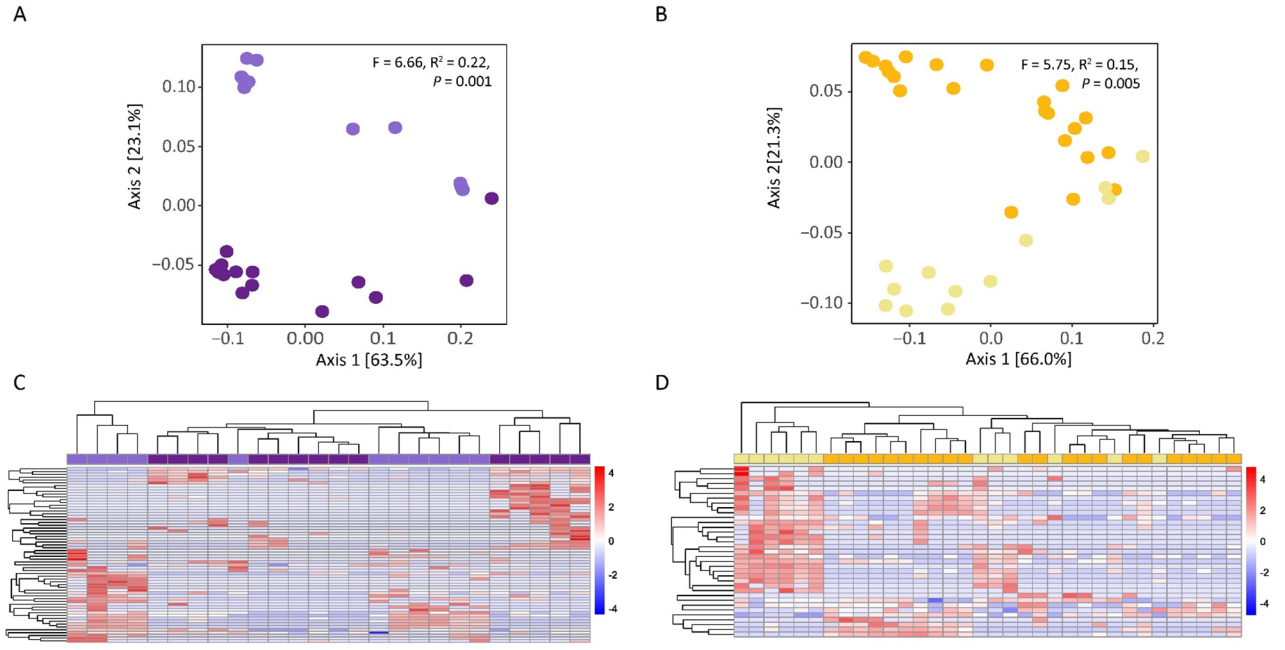

Fig. 5 Microbiome differences between larvae and nursing workers in two wasp species. (**A**–**B**) PCoA plots based on weighted UniFrac distances showing bacterial community differences between larvae and workers in *Vespa orientalis* (**A**) and *Vespula germanica* (**B**). (**C**–**D**) Heatmaps of normalized, $\log_2$-transformed abundances of bacterial taxa identified by MaAsLin2 analysis in larvae and workers of *V. orientalis* (**C**) and *V. germanica* (**D**). Taxa shown include those with nominal significance (raw $P < 0.05$) in at least one comparison and are presented to illustrate relative abundance patterns; statistical significance was evaluated using Benjamini–Hochberg (FDR)–adjusted P values (see Tables S8–S9). Rows correspond to taxa and columns to individuals. Data were z-score scaled by taxon. Color indicates abundance relative to the mean (blue = lower, red = higher). Larvae are shown in lighter purple/yellow shades and workers in darker shades for each species

lineages such as *V. orientalis* and *V. germanica* [10, 46, 47]. Our findings suggest that life stage- and caste-specific microbiomes, through reciprocal symbiotic nutrient metabolism, play a complementary role in supporting the division of labor in eusocial insects. We show that the microbiota in adult and larval stages in both species are effectively decoupled, similar to other eusocial species [48–50], but that the microbial symbionts are known for functions that benefit their respective hosts and are transferred during trophallaxis. Moreover, the functional signature of these microbiota includes pathways that make up the 'molecular toolkit' suggested to underlie the origins of eusociality in multiple eusocial lineages, such as ecdysteroid signaling and insulin signaling [51]. The contributing microbial gut symbionts may have evolved to aid in this process, or they may be a result of it; regardless, they likely aid in its conservation. Although it is unclear how much of their respective niche (e.g., diet) hosts can exploit using solely their own enzymes and the extent of the contribution their microbial symbionts provide, it is generally considered that gut symbionts allow their host to expand their potential niche [52–54]. The involvement of caste-specific microbiota in such conserved social behavior points to a likely coevolutionary mechanism shaping both microbial communities and host life history traits.

Life stage-specific drivers of microbiome composition

The distinct microbiomes observed in each larval treatment group and the importance of the identity of the nurses likely reflect the transfer of microbiota from workers to larvae during trophallaxis, in addition to species-specific symbionts previously obtained. The source of this species-specific variation could stem from the environment available to the larvae (e.g., cell wall or residual fecal material left by previous occupants) or previous interactions with nursing workers in the original colony. The larval gut microbiome is known to be more transient and perturbed than that of adults [50], and could therefore reflect differences in available sources for inoculation more readily. However, whether the distinct microbiome profiles pre-pupation affect post-pupation fitness remains to be tested and could provide more insight into host-microbiota interactions. One limitation of this study was the use of larvae instead of eggs as recipients of worker care. Eggs likely harbor a minimal microbial load [55, 56], and their use could have provided a clearer baseline to test colonization and establishment of gut symbionts. However, preliminary work using eggs resulted in workers either consuming them or neglecting their care, resulting in colony failure, thus leading us to use larvae.

Conversely, the similarity among worker microbiomes across treatments may reflect either environmental

dominance on microbiome establishment, or bacterial convergence driven by gut conditions and microbial interactions. Newly emerged workers, whose microbiome is depleted during metamorphosis [57, 58], provided a *tabula rasa* to test the establishment of a new gut microbiome. Many holometabolous insects show similar patterns of elimination of the larval gut and its contents as a meconium sac and subsequent acquisition of microbes from their environment, including mosquitos, honeybees, wasps and hornets [25, 58–60]. Berasategui et al. [61] found that several distinct species feeding on the same food source harbored highly similar microbiomes, suggesting that environmental factors played a dominant role in shaping gut microbial communities. Similar results were found in beetles [62] and among army ants and their associated beetle species [63], for which foraging habits and habitat structured the microbial communities and even brood parasites [64]. Moreover, beyond general similarity, our results show that workers sharing the same environment ("box") exhibited an even higher degree of similarity. Although we cannot rule out the effect of the experimental design, it is more plausible that worker-to-worker trophallaxis was the major driver of this result. Trophallaxis among siblings enables the transmission of beneficial microbes in the honeybee (*Apis mellifera*) [48, 49], a mechanism that can plausibly occur in both *V. orientalis* and *V. germanica* (we have observed worker-to-worker trophallaxis in both of our study species). Alternatively, the degree to which the gut community is acquired from the environment is primarily thought to be associated with host niche specialty [57]. It is possible that host gut physiology combined with microbial competition led to filtering of certain microbial species and resulted in a conserved microbiome composition optimized for the role of workers in the colony, such as detoxifying environmental toxins or regulating microbial entry, as seen in honey bees [50].

Differences in microbiome assembly and functionality across life stages

The core bacterial taxa associated with the nursing workers was dominated by taxa in the genera *Lactococcus* and *Proteus*, as well as multiple lineages within the family Enterobacteriaceae, possibly playing an important role in assisting in the metabolism of nutrients in the workers' carbohydrate-rich diet. Members of the genus *Lactococcus* produce lactic acid by fermenting glucose, which is one of the primary nutrients in the diet of adult hornets and wasps [65], whereas some *Proteus* species were shown to hydrolyze cellulose and xylan [66–68]. The order Enterobacterales was also found to be one of the two most dominant orders in *Vespa mandarinia* and, to a lesser extent, in *Vespa simillima* [55]. Although studies detailing hornet and wasp microbiomes are still scarce, our results correspond with taxa shown to be abundant in closely related species with similar diets, such as *Lactococcus*, *Fructobacillus*, and *Leuconostoc* [25, 55, 56]. In larvae, the pattern of abundant and prevalent taxa was more diverse and included the species *Enterococcus G sulfureus* and *Convivina intestini* in *V. orientalis* and the mostly opportunistic genera *Proteus* and *Staphylococcus* in *V. germanica*. This pattern likely reflects a more permissive and transient gut environment in larvae, shaped by underdeveloped immune development, and exposure to nest or prey-derived microbiota as observed in other eusocial species [12, 69]. In addition, the genus *SerratiaL 726,994*, that was widely prevalent among both larvae and workers in both species, was notably less abundant in workers. This predominantly opportunistic pathogen is typically rare in adult guts but can proliferate in immunocompromised hosts or larvae [70], and its decreased abundance in workers supports the maturation of their immune system or stabilization of their gut microbiome.

Although correlative, functional analysis provides additional support for the decoupling of life stages in both species. Larvae exhibited enrichment in energy-intensive metabolic pathways, including the TCA cycle, oxidative phosphorylation, fatty acid degradation, and steroid biosynthesis, consistent with their role in digesting protein-rich food and converting it into carbohydrates for worker nutrition. The relatively higher predicted presence of steroid biosynthesis pathways further suggests a role in larval development, as ecdysteroid hormones regulate insect molting and metamorphosis [51]. In contrast, workers' microbiota showed higher predicted abundance of pathways related to antimicrobial compounds production (e.g., polyketide, streptomycin, and vancomycin biosynthesis), detoxification (e.g., dioxin and cyanoamino acid metabolism), and nutrient sensing (e.g., insulin signaling and phosphotransferase systems). This functional profile reflects their social roles in the colony as caretakers and gatekeepers, even in the experimental conditions in this study that limited their environmental exposure. Caste-specific microbiome profiles ('metabolic division of labor') have been linked to behavioral tasks in other eusocial species [12, 47, 71]. Here, we support those findings by showing that life stage-specific functions are maintained independently of external conditions, suggesting that microbial contributions to life stage physiology may be conserved across taxa. The persistence of these distinct metabolic signatures under controlled conditions further implies strong host-microbiota coadaptation, where microbiome functions are coupled with developmental and social behavior.

Our findings highlight the pivotal role of gut microbiota in mediating life stage metabolic division of labor in eusocial species, thereby reinforcing social interdependence in complex eusocial systems. By demonstrating

that life stage-specific microbial signatures and functions persist even under cross-species nursing and are maintained by social interactions, we provide experimental support for the hypothesis that host-microbiota associations are tightly linked to developmental and social roles. We propose that microbial communities co-evolved with their hosts to support colony cohesion by adapting to host-specific functional roles. The extent to which hosts rely on their microbiome to maintain their metabolic roles within the colony, and whether such mechanisms are conserved or have evolved independently across phylogenetically and ecologically diverse social lineages, remains to be determined. These insights and other evidence of host-microbiome symbiosis emerging from other species [72, 73], contribute to a broader understanding of the evolution of social microbiomes, positioning microbial symbionts as key players in the emergence and maintenance of eusociality.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-026-00560-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions
TMC and EL conceptualized the idea for the study; TMC, LB, ST, EF, AC, SB and ET conceived and executed the experiments and subsequent analyses; EL and OK supervised; TMC, LB, ST, EF and AC wrote the original draft; TMC, LB, ST, EF, AC, SB, ET, OK and EL edited the draft.

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Data availability
The dataset generated and analyzed during the current study is available in the European Bioinformatics Institute (EBI) – European Nucleotide Archive (ENA) data repository at https://www.ebi.ac.uk/ena/browser/view/PRJEB72672, and in the qiita repository, https://qiita.ucsd.edu/public/?study_id=15406.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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