Journal of Chemical Ecology (2025) 51:110
https://doi.org/10.1007/s10886-025-01662-7

RESEARCH

Bacterial Communities in Aphid Honeydew Provide Species-Specific Functional Chemical Cues in Aphid-Predator Interactions

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Received: 17 September 2025 / Revised: 29 October 2025 / Accepted: 31 October 2025

Abstract
The role of microbial communities in aphid honeydew in shaping multitrophic interactions remains uncertain, while bacterial diversity and its variation among aphid species and ageing are still underexplored. This study investigated variations in bacterial community structure and VOC profiles of honeydew between two aphid species (*Aphis fabae*, *Acyrthosiphon pisum)*, across time from fresh to 72-hour aged. We also assessed the behavioral responses of a natural enemy of aphids, the hoverfly *Episyrphus balteatus*, to different honeydew combinations. *Aphis fabae* honeydew harbored a more diverse bacterial taxonomic richness than *A. pisum*, including a significant shift in bacterial community composition over time that also extended to the semiochemical profiles. Further, 24-hour-aged honeydew from *A. fabae* emitted a higher concentration of volatile compounds than *A. pisum*. We assessed *E. balteatus* preferences for aged honeydew from both aphid species using wind tunnel assays, revealing a strong attraction to 48-hour-old *A. pisum* honeydew resulting in higher egg-laying activity. These findings underscore the role of microbial succession in aphid honeydew in shaping multitrophic interactions, suggesting potential biocontrol strategies by modulating microbial influences on aphidophagous beneficial behavior.

Keywords Insect–microbe interactions · Aphid · Honeydew · Bacterial diversity

Introduction

Aphids are sap-feeding insects comprising over 4,000 species, colonizing approximately 25% of all plant taxa (Blackman and Eastop 2017). As major agricultural pests, they contribute to yield losses ranging from 30% to total crop failure, depending on the species, crop type, region, and management practices (Gordy et al. 2021; Sharma et al. 2022). In addition to direct feeding damage, aphids transmit phytoviruses and excrete honeydew, which promotes sooty mold growth and interferes with plant physiological processes (Blackman and Eastop 2017; Gadhave et al. 2020). Honeydew, rich in carbohydrate and amino acid, also serves as an energy source for a variety of organisms, including mutualistic ants, pathogenic fungi, bacteria, and natural enemies of aphids, thereby generating a complex web of multitrophic interactions (van Neerbos et al. 2020; Álvarez-Pérez et al. 2024).

Beyond its nutritional role, honeydew also mediates chemical communication within these interactions. Volatile compounds (VOCs) released from honeydew may signal to other herbivores that a host plant is already colonized, thereby reducing further herbivore pressure. At the same time, these volatiles may attract natural enemies such as predators and parasitoids, which exploit them as indirect cues of prey presence (Liu et al. 2024; Fernández de Bobadilla et al. 2024). This phenomenon illustrates the concept of infochemical detours (Vet and Dicke 1992), whereby natural enemies exploit indirect chemical cues produced by associated organisms such as aphid honeydew or its

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Published online: 15 November 2025

bacterial community to locate their prey. Indeed, several studies have linked specific bacterial taxa to honeydew from distinct aphid hosts: *Mammaliicoccus sciuri* (Kloos et al. 1976) in *Acyrthosiphon pisum* (Harris, 1776), *Staphylococcus xylosus* Schleifer et Kloos 1975 in *Aphis fabae* Scopoli, 1763, and *Lysinibacillus fusiformis* MH1 (Priest et al. 1989) in *Sitobion miscanthi* (Takahashi, 1921) (Leroy 2011; Fisher 2015; Liu 2024). These microbial emissions act as infochemical detours exploited by aphid natural enemies or mutualistic partners for host location.

However, most previous work has characterized honeydew volatiles at a single time point, implicitly treating them as static chemical signals. Little is known about how microbial succession during honeydew aging influences VOC emission and, consequently, the responses of natural enemies. This gap limits our understanding of the reliability and temporal dynamics of honeydew-based infochemicals under natural conditions, where honeydew is continuously produced and altered as bacteria metabolize its sugars and amino acids potentially altering the cues available to natural enemies. These considerations highlight the importance of honeydew bacterial microbiota as active biochemical players, rather than passive contaminants, in shaping semiochemical landscapes. Yet, it remains unclear whether these bacterial communities are stable or dynamic, and how their succession affects VOC emissions and predator attraction.

Here, we investigate how bacterial succession and VOC emissions in aphid honeydew vary over time and between aphid species, focusing on a specialist aphid (i.e., *A. pisum*) and a generalist, myrmecophilous species (i.e., *A. fabae).* Specifically, we predict that (i) bacterial communities will shift as honeydew matures, (ii) these shifts will result in distinct volatile blends, and (iii) the behavioral response of the hoverfly *Episyrphus balteatus* (De Geer, 1776), a key aphid predator will reflect these temporal changes. By integrating bacterial microbiome, volatilome, and behavioral data, this study provides a mechanistic understanding of how bacterial succession shapes the chemical ecology of honeydew-based interactions, with potential applications for enhancing biological control.

Materials and Methods

Insects Rearing

Laboratory populations of *A. pisum* and *A. fabae* were reared on broad bean plants *Vicia faba L.* (BBCH 14 stage) major variety under controlled conditions (21 ± 2 °C, 60 ± 5% R.H., 16:8 h (l: d). For behavioral experiments, a laboratory population of *E. balteatus* was fed with fresh pollen, honey and sugar in netted flight cages (Bugdorm, Taiwan, 75 × 60 × 90 cm). To induce oviposition, *V. faba* infested with *A. pisum* were inserted into the cage for 24 h. Hoverfly larvae were maintained on these plants and fed with pea aphids ad libitum until they reached the pupal-instar stage.

Honeydew Collection

Around 60 *V. faba* plants, each hosting population of either *A. fabae* or *A. pisum* with similar aphid densities, were placed 10 cm above four sterile micro-Tec borosilicate Petri dishes (20.2 cm ⌀) at a 45° angle, allowing honeydew droplets to naturally fall onto the Petri dish surface for 24 h under controlled conditions (21 ± 2 °C, 60 ± 5% R. H., 16:8 h (l: d)). After 24 h, in the same conditions, the aphid population was removed to avoid microorganism contamination. Honeydew was collected at four time point post-exposure to ambient air : 0 h (i.e. fresh), 24 h, 48 h and 72 h. To collect the honeydew, each Petri dish was rinsed with 600 µL of milliQ water and scraped using a sterile rake.

Bacterial Community

A total of 85 honeydew samples (46 from *A. fabae* and 39 from *A. pisum*; SI Appendix, Table S1) were collected. Bacterial DNA was extracted using the AllPrep DNA/RNA/Protein Kit (Qiagen, Valencia, CA) and amplified for sequencing on the Illumina MiSeq platform (2 × 250 nt). Detailed protocols for DNA preparation, normalization, amplification, and library construction are provided in the SI Appendix. Raw sequencing data were deposited in GenBank (PRJNA1295395). Paired end read files were processed in QIIME2 (Core 2020.11), where primer sequences were trimmed, and low-quality or chimeric reads were removed using DADA2. Amplicon Sequence Variants (ASVs) were generated and taxonomically assigned using database (SILVA). Singletons, chloroplasts, and mitochondrial contaminants, along with any ASVs detected in blank samples, were removed. Samples with fewer than 1500 reads were excluded from the analysis, leaving 77 honeydew samples for bacterial community profiling.

Volatile Organic Compounds Profile

The volatile compound profile of honeydew (three replicates per condition) was analyzed using solid-phase microextraction (SPME) sampling followed by gas chromatography coupled with mass spectrometry (GC-MS) (QP 2020 NX, Shimadzu, Kyoto, Japan). A 700 µL sample of honeydew was incubated in a water bath at 30 °C, and VOCs were collected for 24 h using a triphasic SPME fiber (DVB/CAR/PDMS), preconditioned at 275 °C for 30 min. Milli Q water has been used as control condition. After sampling, VOCs

were injected at 250 °C using a 1:3 split ratio. Volatiles were separated on an HP-5 MS capillary column (30 m × 250 µm × 0.25 µm, Agilent Technologies, Santa Clara, CA, USA), with an initial temperature of 40 °C for 3 min, followed by a temperature increase to 200 °C at 5 °C/min, then a 10 °C/min ramp to 300 °C, held for 3 min. VOCs were detected using a mass spectrometer set at 70 eV (scanning range: 35–500 m/z, ion source temperature = 200 °C). Volatile components were identified by comparing their mass spectra with reference libraries (FFNSC3, NIST17 1 and 2, and NIST17s), and identification was confirmed by calculating the retention index.

Behavioral Responses of Predators

To assess the impact of bacterial communities in honeydew (from different aphid species and at varying ages) on hoverfly behavior, 3 mL of honeydew samples and 3 ml of MilliQ water for controls were sprayed onto faba bean plants (BBCH14). Each honeydew-treated plant was placed 15 cm from a control plant in a Plexiglas wind tunnel (2.4 × 0.8 × 0.6 m, laminar flow at 0.4 m/s, 20 ± 2 °C, 60–70% RH, 2,300 lx). Ten gravid *E. balteatus* females (15–20 days old) were introduced 2 m from the plants. The number of eggs laid on each plant was recorded after four hours. Three replicates were performed for each plant pair (treatment/control).

Statistical Analysis

All statistical analyses were performed on R 4.4.1 (R Core Development Team, 2024). In all analysis, the significance level of $\alpha \leq 0.05$ was determined. The code analysis and the raw data used in this study can be found here: https://github.com/gregnoel/Aphid-Honeydew. Bacterial community analysis was performed using phyloseq R package integrating the ASVs community matrix, ASVs taxonomy, the phylogenetic tree and the associated metadata (McMurdie and Holmes 2013). The phyloseq object was then converted to MicrobiotaProcess object for further analysis (Xu et al. 2023). Rarefaction curves and the alpha diversity metrics (i.e., bacterial taxa richness or ASVs richness, Chao1's estimator and Shannon index) were estimated by a split number of 100 chunks using the mp_cal_rarecurve function (Xu et al. 2023).

To investigate how aphid species and honeydew aging influenced microbial and chemical profiles, we used a consistent multivariate framework. For both datasets, bacterial community composition and volatile organic compounds, we applied permutational multivariate analysis of variance (PERMANOVA) based on a Bray-Curtis dissimilarity matrix with 999 permutations, using Hellinger transformation. Community structure was visualized using Principal Coordinates Analysis (PCoA) for bacteria and nonmetric multidimensional scaling (NMDS) for VOCs. Relative abundances of dominant bacterial genera (top six or those identified via biomarker analysis) were analyzed using generalized linear mixed models (GLMMs) with a logit link function (glmer, *lme4* package), with honeydew age as a fixed effect and sample identity as a random factor. For the VOC dataset, compound specificity was explored by calculating Z-scores, visualized as heatmaps using the heat.map2 function from the *ggplot2* package. Differences in the relative peak areas of individual VOCs were assessed by first testing for normality (Levene's test); when assumptions were met, one-way ANOVA with Tukey's HSD was used, otherwise a Kruskal-Wallis test followed by Dunn's post hoc test was performed.

To assess the impact of aging and species on hoverflies oviposition, GLMM has been performed on the number of eggs found on plants. A Poisson distribution was employed for the oviposition preferences. Tunnel replicate was included as a random factor to prevent overdispersion and pseudo-replication impact. ANOVA Type III Wald's Chi-square test was performed on GLMM. For oviposition, pairwise post-hoc tests were performed directly on the fitted model using the lsmeans function (Lenth 2024), applying asymptotic *z*-tests with infinite degrees of freedom as standard. The full egg count data (means and standard deviations for each treatment group) are provided in the SI Appendix, Table S2.

Results

Species-Specific Microbial and Volatile Profiles in Aphid Honeydew Highlight Distinct Dynamics in the Specialist Aphid. A total of 7,321,454 DNA sequences were obtained from all honeydew samples, with an average of 95,083.82 ± 591.19 (mean ± SEM; $n = 77$) sequences per sample. These sequences clustered into 446 amplicon sequence variants (ASVs) based on a 99% similarity threshold. Of these, 215 ASVs (48.21%) were shared between both aphid species, while 185 ASVs (41.48%) were unique to *A. fabae* honeydew and only 46 ASVs (10.31%) were specific to *A. pisum* (Fig. 1A). The bacterial communities differed significantly between aphid species (PERMANOVA: $F = 17.74$; d.f. = 1; $R^2 = 0.19$, $P < 0.01$; Fig. 1B), with *A. fabae* harboring over four times more unique ASVs than *A. pisum*. Rarefaction curves and alpha diversity indices confirmed that bacterial richness was significantly higher in *A. fabae* honeydew than in *A. pisum* honeydew (Observed taxa and Chao1 estimator: $P = 0.028$; d.f. = 1; Fig. 1C and D). However, both aphid species showed similar patterns in the

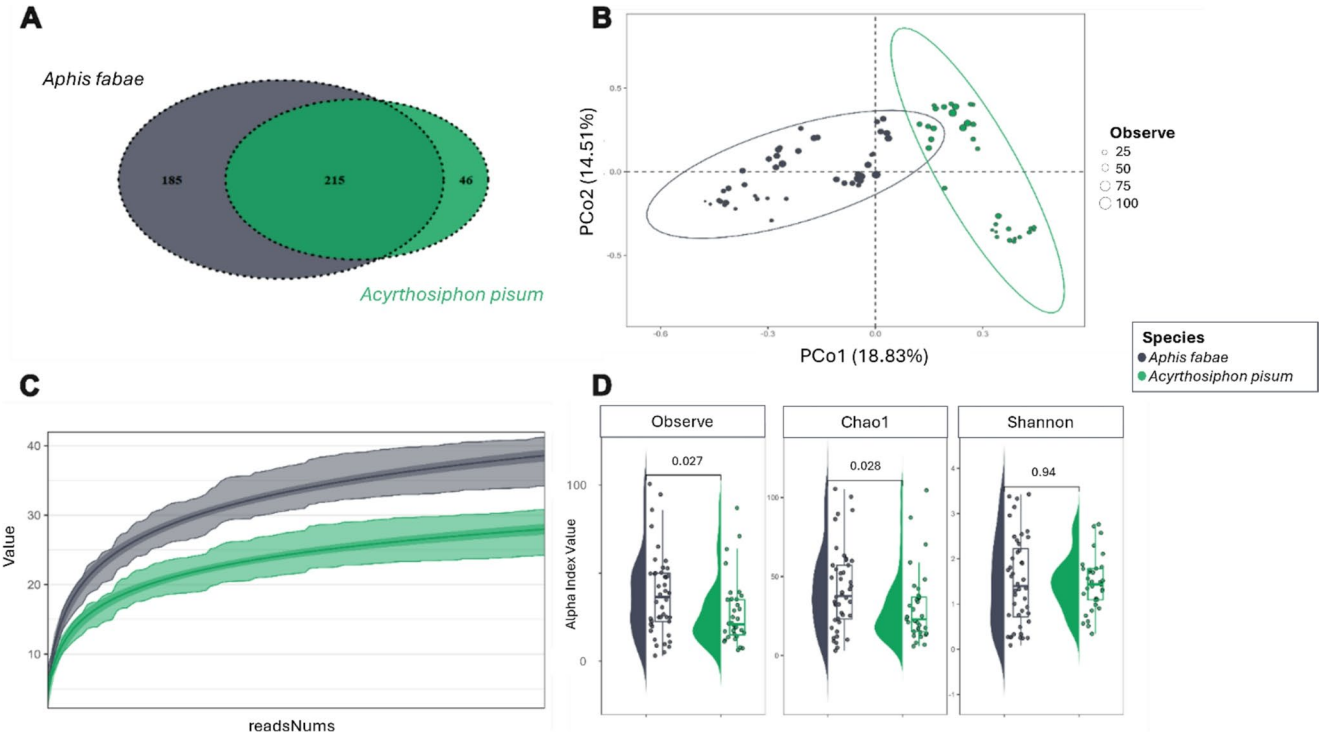


Fig. 1 Overview of the bacterial community structure and diversity in honeydew from two aphid species, *Aphis fabae* and *Acyrthosiphon pisum*. **A**. Venn diagram of the ASVs distribution ($n=446$) from the honeydew of both aphid species: *Aphis fabae* (dark grey) and *Acyrthosiphon pisum* (green). **(B)** Principal component analysis (PCoA) comparing bacterial communities from both aphid species honeydew: *Aphis fabae* (dark grey) and *Acyrthosiphon pisum* (green). The dot size is scaled according to the ASVs richness. **(C)** Rarefaction curves for *Aphis fabae* (dark grey) and *Acyrthosiphon pisum* (green) with 2,500 reads number as sampling to calculate the bacterial taxa richness. Shaded areas of both curves correspond to the 95% interval confidence. **(D)** Alpha diversity metrics comparing *Aphis fabae* (dark grey) and *Acyrthosiphon pisum* (green): bacterial taxa richness in ASVs (**A**), Chao1 richness estimator (**B**), Shannon's index (**C**)

relative abundance of dominant and rare bacterial taxa, suggesting that their microbial communities share comparable dominance–rarity structures ($P=0.96$; d.f. = 1; Fig. 1D).

Taxonomic composition also diverged significantly between species. At the phylum level, Proteobacteria dominated in both honeydew types, more so in *A. fabae* (86.37%) than in *A. pisum* (59.32%). In contrast, Firmicutes and Verrucomicrobiota were more prevalent in *A. pisum* honeydew (29.87% and 9.56%) compared to *A. fabae* (2.92% and 6.08%). At finer taxonomic scales, *A. fabae* honeydew was dominated by the Morganellaceae family and the genus Buchnera (55.89% and 55.86%, respectively), while *A. pisum* honeydew was dominated by Staphylococcaceae and *Staphylococcus* spp. (29.73% each). Details of the bacterial composition of both honeydews are provided in SI Appendix, Fig. S1.

Microbial succession over time revealed both shared and species-specific trends. In *A. fabae* honeydew, *Erwinia* spp. exhibited a significant peak at 24 h, while *Acinetobacter*, *Prosthecobacter*, and *Staphylococcus* spp. significantly increased over time. In contrast, *A. pisum* honeydew showed a more stable community, with only *Pseudomonas* and *Staphylococcus* spp. showing moderate increases. Despite these dynamics, core genera such as *Buchnera*, *Serratia*, *Massilia*, and *Carnimonas* spp. remained stable across time in both species, highlighting a persistent microbial core within aphid honeydew communities (Table 1; Fig. 2A). To further explore species-specific signatures, a differential abundance analysis identified 19 ASVs that significantly differed between *A. fabae* and *A. pisum* honeydew microbiota, including taxa from the genera *Buchnera*, *Acinetobacter*, *Serratia*, and others (SI Appendix, Supplementary Results and Fig. S2).

In parallel with these microbial differences, VOCs profiles also varied significantly between aphid species. Across all samples, 34 VOCs were detected, of which 25 showed significant variation across aphid species and aging treatments (SI Appendix, Table S2). The overall VOC composition differed significantly between *A. fabae* and *A. pisum* honeydew (PERMANOVA: d.f. = 1; $F=4.215$; $P=0.010$), and a significant interaction was detected between aphid species and honeydew aging (d.f. = 7; $F=2.584$; $P=0.034$), although aging alone had no significant effect (d.f. = 3; $F=2.308$; $P=0.074$). These differences were evident in the NMDS ordination plot, which separated honeydew samples primarily along the aphid species axis (Fig. 3A).

Table 1 Generalized Linear-Mixed models (GLMMs) parameters of the ageing effect on the abundance of the main bacterial genera. The statistical value of the GLMMs test (i.e., z-value) and the p-value are shown for the honeydew of *Aphis fabae* ($N=44$) and *Acyrthosiphon Pisum* ($n=33$) species. The significant models ($P<0.05$) are in bold

Aphid species	Bacterial Genus	z-value	*p*-value	Interpretation
Aphis fabae	*Buchnera* spp.	0.41	0.680	constant
	***Erwinia*spp.**	**82.38**	**<0.001**	**Significant peak at 24 h**
	Pseudomonas spp.	−0.54	0.590	constant
	***Prosthecobacter*spp.**	**2.85**	**<0.01**	**Increase along the experiment**
	***Acinetobacter*spp.**	**3.10**	**<0.01**	**Increase along the experiment**
	***Staphylococcus*spp.**	**2.35**	**<0.01**	**Increase along the experiment**
	Serratia spp.	0.53	0.600	constant
	Massilia spp.	0.48	0.630	constant
	Carnimonas spp.	0.041	0.970	constant
Acyrthosiphon pisum	*Buchnera* spp.	<0.01	1.000	constant
	Erwinia spp.	−0.14	0.890	constant
	***Pseudomonas*spp.**	**2.00**	**0.050**	**Increase along the experiment**
	Prosthecobacter spp.	−0.98	0.330	constant
	Acinetobacter spp.	1.36	0.170	constant
	***Staphylococcus*spp.**	**1.95**	**0.050**	**Increase along the experiment**
	Serratia spp.	−0.8	0.420	constant
	Massilia spp.	0.18	0.860	constant
	Carnimonas spp.	NA	NA	NA

VOCs profiling revealed that *A. fabae* honeydew exhibited higher chemical diversity and a higher number of unique compounds compared to *A. pisum*. Particularly, the 24-hour aged *A. fabae* honeydew had the most diverse profile, with 20 compounds present at significantly higher levels than in any other treatment. Fresh *A. fabae* honeydew was enriched in esters, specific alcohols (i.e., 1-heptanol, 2-methylbutan-1-ol), and terpenoid-derived compounds (i.e., 3-methylbut-3-en-1-ol, geraniol). In contrast, fresh *A. pisum* honeydew contained more acetals. At 48 h, *A. fabae* honeydew was enriched in undec-1-ene and 1 H-indole, while *A. pisum* honeydew contained higher levels of 3-methylbut-2-en-1-ol and 3-methyl-2-butenal. By 72 h, *A. fabae* honeydew was enriched in alcohols such as 1-dodecanol and 2-ethylhexan-1-ol, whereas *A. pisum* honeydew contained more (4-hydroxyphenyl)phosphonic acid (Fig. 3B).

Together, these results demonstrate that *A. fabae* honeydew differs substantially from *A. pisum* honeydew in both microbial community and volatile composition, suggesting a species-specific honeydew profile that contributes to differential attraction of natural enemies such as hoverflies.

Bacterial Succession is Key for the Functionalization of Honeydew in the Oviposition of Hoverflies

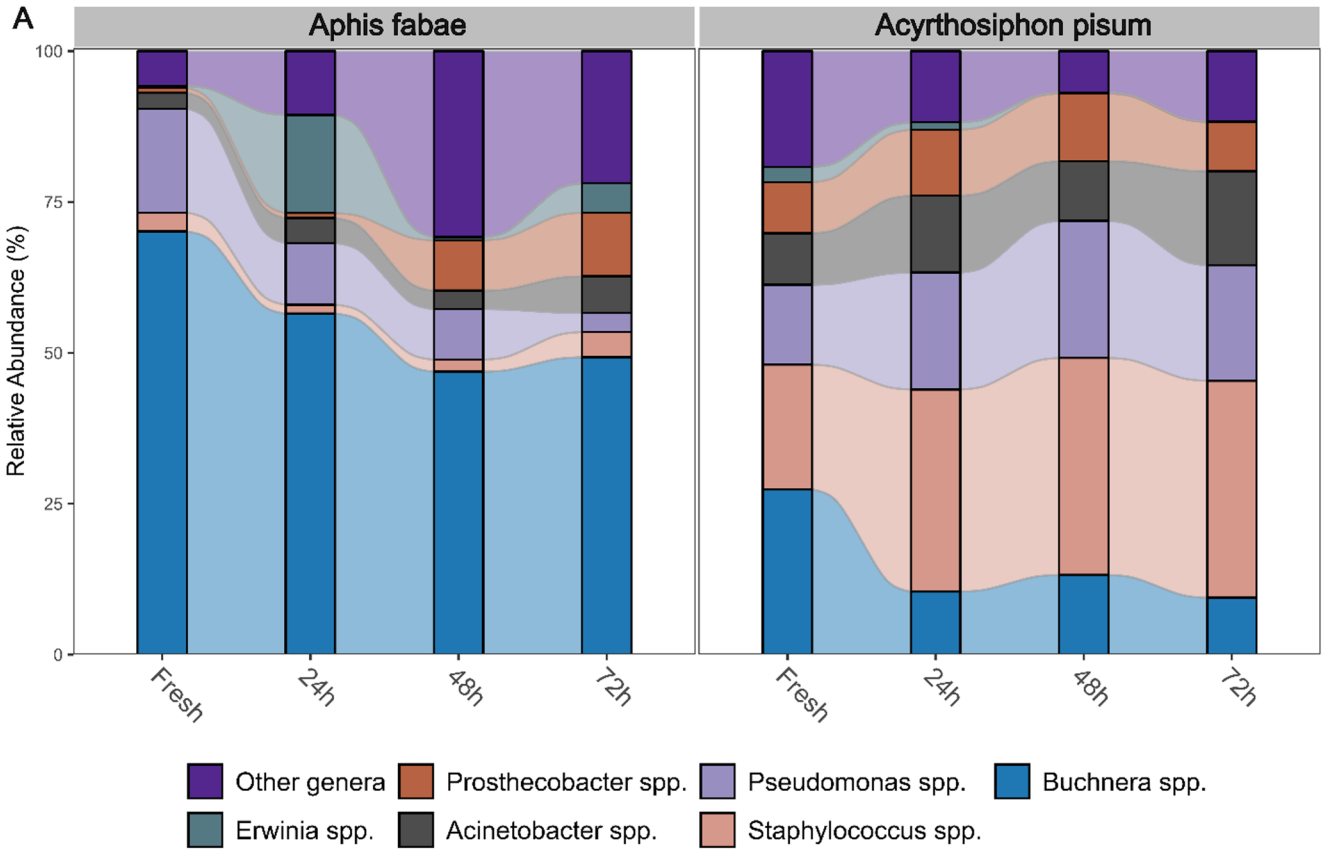
The impact of honeydew ageing on oviposition varied across aphid species (interaction: $\chi^2 = 24.31$; d.f. = 3; $P<0.001$; Fig. 4). The full egg count data (means and standard deviations for each treatment group) are provided in the SI Appendix, Table S3.

Control treatment resulted in the lowest egg counts (7.9 ± 7.2; mean ± SD), while the highest counts were observed under the 72-hour aged for *A. fabae* honeydew (341.0 ± 18.2; mean ± SD). Fresh *A. fabae* and *A. pisum* honeydew were not significantly different from each other (z = −1.096; d.f. = inf; $P=0.980$), but fresh *A. fabae* honeydew induced fewer eggs than 48-hour aged *A. pisum* (z = −4.48; d.f. = inf; $P=0.003$) and 72-hour aged *A. fabae* (z = −3.39; d.f. = inf; $P=0.024$).

A notable finding was the comparison between 72-hour aged *A. fabae* and sucrose, which were highly significantly different (z = −5.54; d.f. = inf; $P<0.001$) while 72-hour aged *A. pisum* did not differ significantly from sucrose (z = −2.2; d.f. = inf; $P=0.460$), indicating that both aphid species and the duration of honeydew ageing jointly determine oviposition responses.

Discussion

The microbial community in *A. fabae* honeydew exhibited a greater bacterial diversity than that of *(A) pisum*. The origin of those bacterial communities is a key factor in understanding this difference and their ecological roles. In any type of ecosystem, a keystone species is an organism that, even in low abundance, can stabilize microbial community composition by mediating strong biotic interactions, and their presence has been shown to reduce overall compositional turnover over time (Herren and McMahon 2018). These bacteria may originate from multiple sources, including the aphid itself, its gut microbiota, the plant phyllosphere, or the surrounding environment. Indeed, the aphid gut harbors a core microbiota essential for nutrient processing and the breakdown of plant-derived compounds, some of which may be excreted into the honeydew. *Buchnera aphidicola*, an obligate endosymbiont residing within aphid bacteriocytes, was detected in the honeydew of both aphid species and contributes to aphid nutrition by synthesizing essential amino acids that compensate for their nutritionally unbalanced phloem diet (Baumann 2005). *(B) aphidicola* was the only endosymbiont detected in our


A
Aphis fabae
Acyrthosiphon pisum
Relative Abundance (%)
100
75
50
25
0
Fresh
24h
48h
72h
Other genera
Prosthecobacter spp.
Pseudomonas spp.
Buchnera spp.
Erwinia spp.
Acinetobacter spp.
Staphylococcus spp.

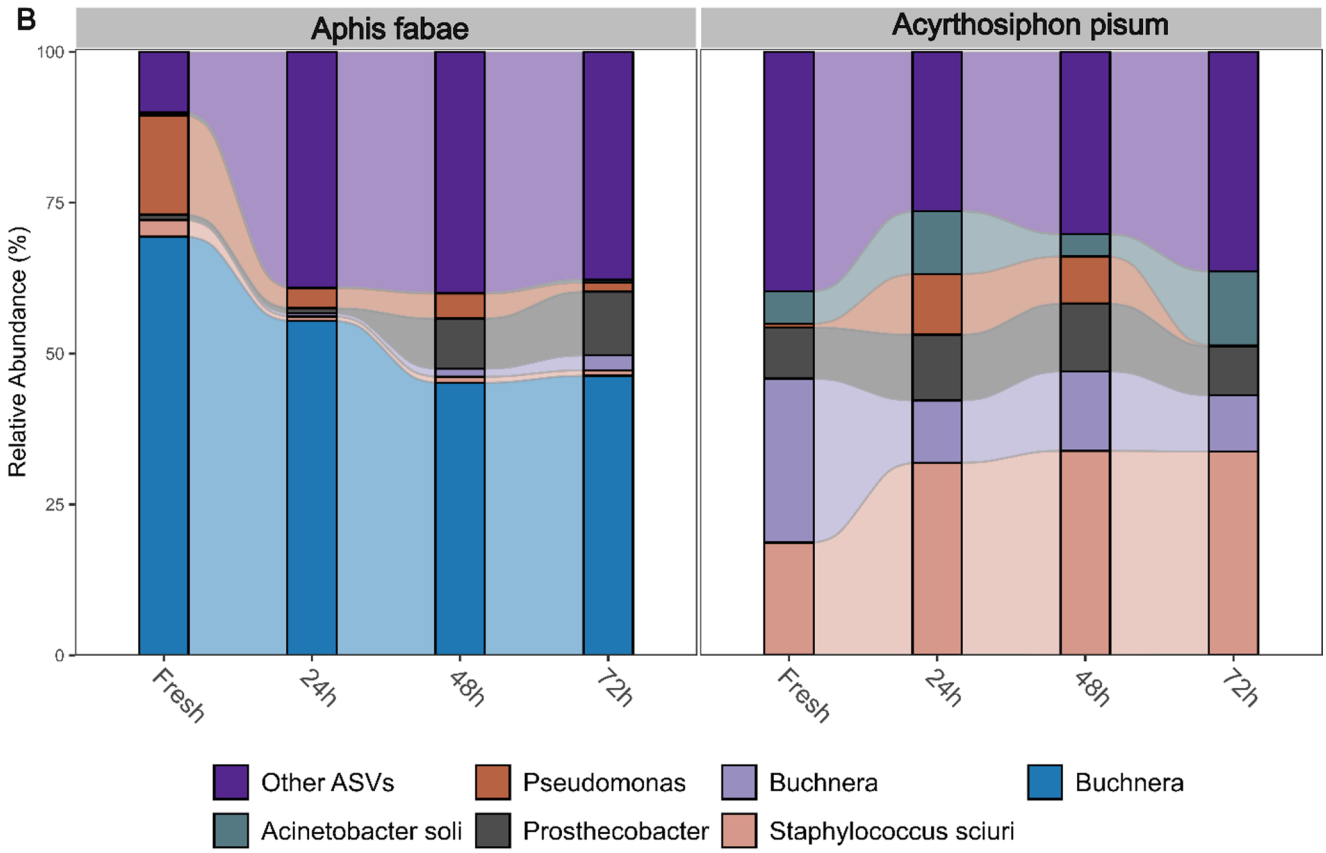

B
Aphis fabae
Acyrthosiphon pisum
Relative Abundance (%)
100
75
50
25
0
Fresh
24h
48h
72h
Other ASVs
Pseudomonas
Buchnera
Buchnera
Acinetobacter soli
Prosthecobacter
Staphylococcus sciuri

Fig. 2 Relative abundance patterns of dominant bacterial groups in honeydew over time for each aphid species. **(A)** Bar plots showing the relative abundance of the top six bacterial genera in honeydew samples from *Aphis fabae* and *Acyrthosiphon pisum* across different ageing time points. **(B)** Bar plots displaying the relative abundance of the top six taxa or ASVs in honeydew from *A. fabae* and *A. pisum* over the same ageing period

laboratory aphid populations from which the honeydew was collected.

While the host plant can influence microbial communities, both aphid species were reared on the same plant variety in our work. Thus, any observed differences are unlikely to be due to the host plant itself and may instead reflect species-specific feeding behavior and variation in honeydew composition. In particular, *A. fabae* demonstrates greater mobility and more diverse feeding behaviors, involving the exploitation of multiple feeding sites on the plant, in contrast to *A. pisum* (Salyk and Sullivan 1982), potentially exposing it to a broader spectrum of phyllosphere-associated microbes, thereby increasing its digestive tract bacterial diversity. During feeding, phyllosphere-associated microbes, such as *Xanthomonas* spp and *Erwinia* spp, may be deposited onto aphids, further enriching the diversity of bacteria in honeydew. These microbes, in turn, interact with the aphid's digestive processes and metabolic activity, further shaping the overall bacterial community (Wolfgang et al. 2023). The presence of highly connected microbial taxa, such as certain *Erwinia* and *Staphylococcus* strains, may reflect their role as potential keystone taxa, which influence whole-community composition by stabilizing microbial networks in honeydew ecosystems. This role may be especially important in explaining the temporal dynamics observed in our study.

Alternatively, differences in honeydew composition may also shape bacterial diversity. *Aphis fabae* honeydew has been reported to contain higher concentrations of melezitose and a greater total carbohydrate concentration compared to *A. pisum*, even when feeding on the same plant species (Luquet et al. 2021). This richer and more diverse carbohydrate profile could support a more diverse microbial community as reported in our study. However, an increased carbohydrate concentration and diversity may also elevate osmotic pressure in the honeydew, potentially imposing higher environmental stress that limits bacterial colonization (Álvarez-Pérez et al. 2024). As such, the bacterial communities present in *A. fabae* honeydew may not only be richer but also more adapted to these selective pressures.

Our results show that bacterial communities in aphid honeydew are highly dynamic and species-specific, influenced by ecological factors such as nutrient availability and interspecies interactions (Muzafar et al. 2024). *Erwinia* spp. were detected in both *A. fabae* and *A. pisum* honeydew. However they reached a significant peak at 24 h in *A. fabae*, while their abundance declined over time in *A. pisum*. In

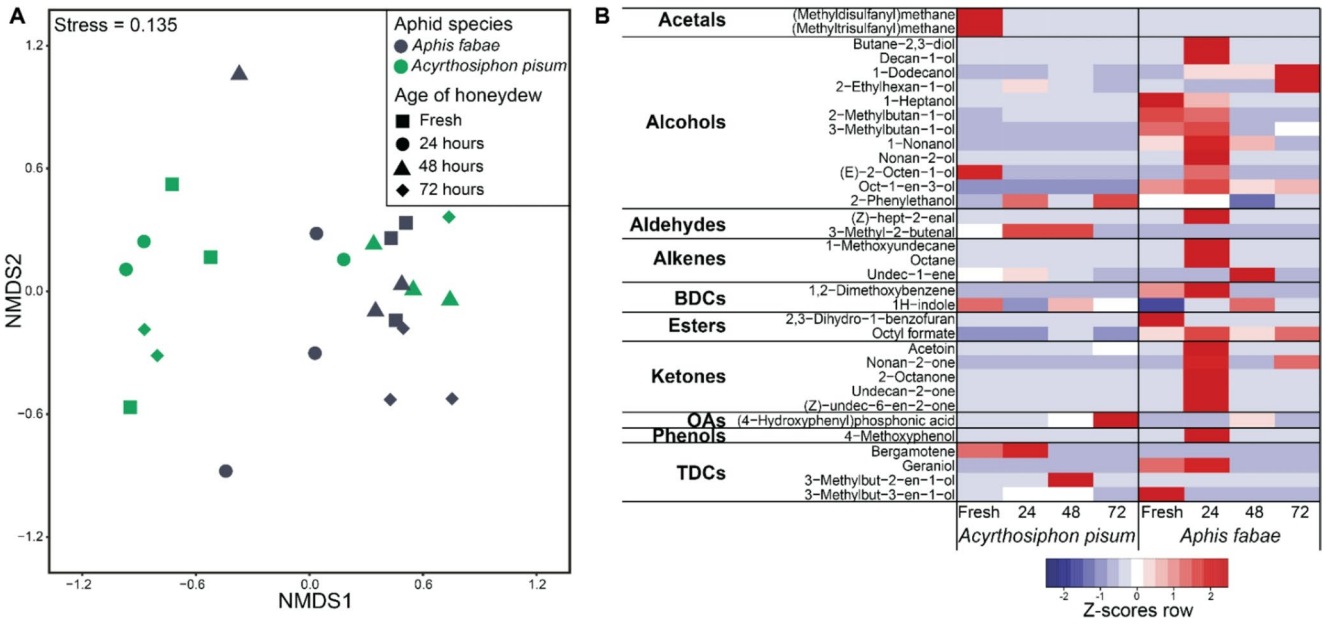


Fig. 3 Volatile organic compounds (VOCs) profile variation in honeydew from two aphid species across four ageing time points. **(A)** Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities of the relative peak areas of VOCs, illustrating differences in honeydew chemical profiles between *Aphis fabae* and *Acyrthosiphon pisum* and across four ageing durations (0 h, 24 h, 48 h, 72 h). **(B)** Heatmap representing the VOCs composition of honeydew samples from *A. fabae* and *A. pisum*, aged over the same four time points. Each identified compound is shown as a Z-score of the relative peak area, highlighting differences in abundance across aphid species and honeydew ageing

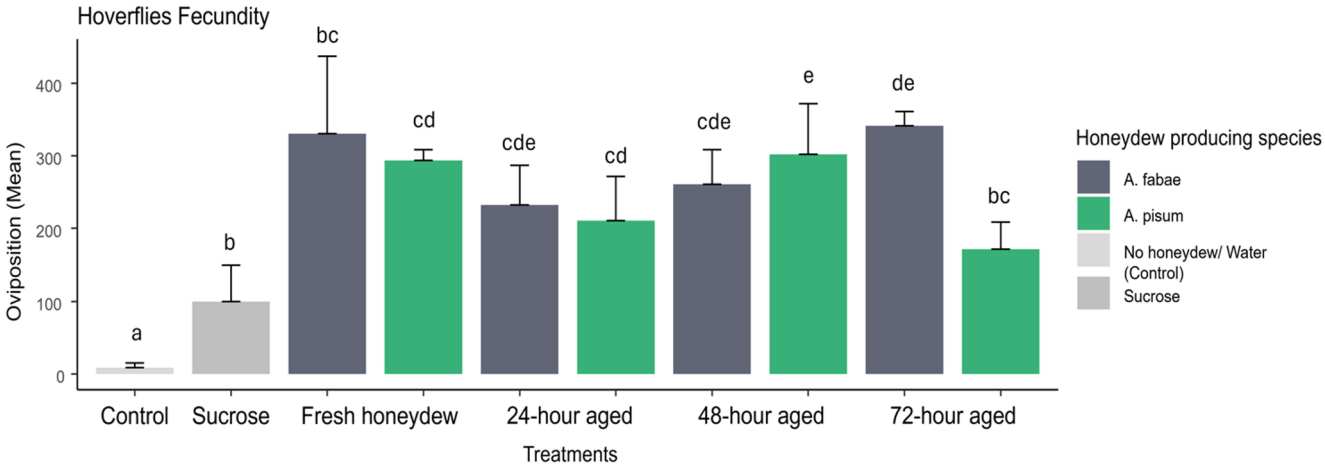


Fig. 4 Oviposition responses of hoverflies to treated and untreated plants. Number of eggs (mean + SD) laid on control plants (water), plants treated with sucrose and plants treated with aged honeydew (No ageing/Fresh, 24-hour aged, 48-hour aged, 72-hour aged) from two different aphid species. Different letters indicate significant differences among treatments

contrast, *Pseudomonas* spp. and *Staphylococcus* spp. steadily increased only in *A. pisum*. Across both aphid species, core genera including *Pseudomonas*, *Acinetobacter*, *Prosthecobacter*, and *Staphylococcus* were consistently present from the earliest time point and persisted throughout the 72-hour ageing period, despite differences in relative abundance and dynamics. These shifts align with niche construction theory, where bacterial interactions, including cross-feeding and metabolic adaptations, create conditions for distinct bacterial populations to emerge by occupying specific metabolic niches, defined by their unique biochemical roles and resource use (San Roman and Wagner 2018; Nguyen et al. 2021; Lopez and Wingreen 2022). The early peak of *Erwinia* spp. in *A. fabae* may reflect an initial abundance of available nutrients or a strong competitive advantage. Conversely, the increasing dominance of *Pseudomonas* and *Staphylococcus* spp. in *A. pisum* could be associated with resource shifts or altered microbial architecture (Tian et al. 2017; Loxdale et al. 2020). The absence of similar trends in *A. fabae* may suggest that microbial succession in this honeydew variant depletes key resources more rapidly, eliminating the metabolic niches necessary for the expansion of these later-stage bacteria.

As higher bacterial richness in aphid honeydew may indicate a higher quality diet for hoverfly larvae, this richer bacterial profile might also signal food resource stability, as microbial diversity has been linked to ecosystem resilience and productivity (Beyter et al. 2016). Such resilience may be driven, at least in part, by the stabilizing influence of keystone microbial taxa, which buffers the community against rapid compositional turnover and contribute to long-term functional consistency (Herren and McMahon 2018).

Aphids exhibiting a more diverse microbiota may offer more attractive oviposition sites, potentially due to more stable or abundant resources for predatory larvae. In support of this, the higher bacterial richness observed in *A. fabae* honeydew aligns with the increased hoverfly egg counts recorded. Specific bacterial taxa may further contribute by emitting volatile compounds that attract hoverflies. For example, *S. sciuri* and *S. xylosus*, found in the honeydew of *A. fabae*, *A. pisum*, and *Myzus persicae* (Sulzer, 1776), produce VOCs known to influence aphid predator and parasitoid behavior (Leroy et al. 2011; Fischer et al. 2015). Then, microbial volatiles may not only reflect resource quality but also actively shape oviposition preferences. In our study, *E. balteatus* exhibited a strong preference for 48-hour-old honeydew from *A. pisum*, as well as for 48- and 72-hour-old honeydew from *A. fabae*. These honeydews exhibited higher abundances of several volatile compounds, including 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, undec-1-ene, 1 H-indole, 2-ethylhexan-1-ol, octyl formate, nonan-2-one, and 1-dodecanone. Previous findings revealed that these volatiles may not only originate from the plant (Erb et al. 2015; Almeida et al. 2023), but also from the aphid itself (Krohn et al. 1992; Schlamp et al. 2005) or from the microbial community which may be present in the aphid honeydew (van Neerbos et al. 2023; Kemmler et al. 2025). Although the exact origins of these volatiles are difficult to disentangle, their ecological roles are well documented. For instance, previous work has shown that compounds like 3-methyl-2-butenal and nonan-2-one can attract hoverflies, increasing their visitation and oviposition behavior (Leroy et al. 2011). Our detection of these compounds supports their potential importance as chemical cues mediating interactions between aphids and their natural enemies. These results support again the concept of an infochemical detour (Vet and Dicke 1992). In this context, volatiles originating from honeydew bacteria may act as reliable

indicators of aphid colony presence, even when direct aphid cues are weak or variable. Such infochemicals can therefore extend the sensory landscape available to predators like *E. balteatus*, facilitating efficient foraging and reinforcing the tri-trophic linkage between plants, aphids, and their natural enemies (Francis et al., 2004; Leroy et al. 2011; Almohamad et al., 2007). Given the presence of microbial volatiles and the higher bacterial richness in *A. fabae* honeydew, it is plausible that this increased microbial diversity contributes to the elevated egg counts observed in our study. However, it is important to note that the predators were reared exclusively on pea aphids, which could have influenced their preferences. Interestingly, this pattern contrasts with previous literature, which generally reports that specialization or rearing on a single aphid species often leads to reduced performance or preference when encountering alternative aphid species (Rana et al. 2002). Our results suggest a more complex interaction, potentially mediated by microbial community differences in honeydew, that warrants further investigation.

This study offers valuable insights into microbial dynamics and VOC profiles in aphid honeydew, demonstrating the intricate relationship between microbial communities, aphid species, and predator behavior. Our findings highlight the significant temporal shifts in bacterial communities and VOCs. Honeydew plays a central role in these interactions, acting as both a medium for microbial communities and a source of chemical cues that mediate multitrophic interactions. Future research should focus on identifying specific microbial taxa responsible for VOC production, their interactions, and the ecological implications of these compounds. Beyond their ecological relevance, such infochemical detours could also be harnessed for biological control strategies. By identifying and synthetizing key honeydew- or microbe-derived volatiles that attract natural enemies, it may be possible to develop novel semiochemical-based tools to enhance predator activity and improve aphid management in agroecosystems. These insights could enhance our understanding of multitrophic interactions emphasizing the complexity of microbial and chemical signals in ecosystems.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10886-025-01662-7.

Acknowledgements Acknowledgments - We thank Emilie Bera for her valuable microbiological support throughout this study and Frédéric Dresen for his technical assistance. Their contributions were instrumental to the successful completion of this work.

Author Contributions F.F., L.G. and G.N. designed research; L.G. and G.N. performed research; L.G., G.N., C.M. and F.A.C.V.N. analyzed data; L.G., G.N., C.M., F.A.C.V.N., F.D. and F.F. revised manuscript; and L.G., G.N., C.M., F.A.C.V.N. and F.D. wrote the paper.

Funding - The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data Availability The code analysis and the raw data used in this study can be found here: https://github.com/gregnoel/Aphid-Honeydew.Raw sequencing data were deposited in GenBank (PRJNA1295395)All the other data are provided within the manuscript or supplementary information.

Declarations

Competing interests The authors declare no competing interests.

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