Bacterial volatiles elicit differential olfactory responses in insect species from the same and different trophic levels

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Abstract

Insect communities consist of several trophic levels that have to forage for suitable resources among and within larger patches of non-resources. To locate their resources, insects use diverse stimuli, including olfactory, visual, acoustic, tactile and gustatory cues. While most research has focused on cues derived from plants and other insects, there is mounting evidence that insects also respond to volatile organic compounds (VOCs) emitted by microorganisms. However, to date only very little is known about the olfactory responses of insects to microbial VOCs within and across different trophic levels. In this study, using Y-tube bioassays and chemical analysis of VOCs we assessed how volatile compounds emitted by bacteria affect the olfactory response of insects of different trophic levels. Experiments were performed using two aphid species (*Amphorophora idaei* and *Myzus persicae* var. *nicotianae*), their most important primary parasitoid species (*Aphidius colemani*, *A. ervi*, and *A. matricariae*), and two of their hyperparasitoid species (*Asaphes suspensus* and *Dendrocerus aphidum*). Olfactory responses were evaluated for three bacterial strains (*Bacillus pumilus* ST18.16/133, *Curtobacterium* sp. ST18.16/085, and *Staphylococcus saprophyticus* ST18.16/160) isolated from the habitat of the insects. Results revealed that insects from all trophic levels responded to bacterial volatiles, but responses varied between and within trophic levels. All bacteria produced the same set of volatile compounds but often in different relative concentrations. For a number of these volatiles we found contrasting correlations between their concentration and the behaviour of the primary parasitoids and hyperparasitoids. Exploitation of such contrasting responses may lead to novel semiochemical-based strategies to improve biological aphid control.

Introduction

Insect communities typically consist of several trophic levels that have to forage for suitable resources that are commonly embedded within larger patches of non-resources (Aartsma et al., 2017). Herbivorous insects, for example, need to find their food plants among a diverse array of non-food plants, whereas predators and parasitoids (i.e. insects whose larvae live as parasites that eventually kill their insect hosts) have to find herbivore-infested plants as well as the actual prey or hosts on them. To locate their resources, insects use a variety of stimuli, including olfactory, visual, acoustic, tactile and gustatory cues (Visser, 1988; Little et al., 2019). The use of olfactory cues during long-range foraging is widely distributed among insects, although the precise cues attracting insects can be expected to differ between trophic levels (Bruce et al., 2005; Webster and Cardé, 2017; Aartsma et al., 2019). Whereas herbivorous insects predominantly use plant volatiles and colours to find suitable host plants during long-range foraging, higher trophic levels such as parasitoids mainly use herbivore-induced plant volatiles (HIPVs) and volatiles from herbivore faeces, as well as visual and mechanosensory cues to locate suitable hosts (Wäckers and Lewis, 1994; Fischer et al., 2001; van Oudenhove et al., 2017).

Most research on insect olfactory behaviour has focused on volatiles derived from plants and insects (Kaplan, 2012; Meiners and Peri, 2013). However, there is mounting evidence that insects also respond to volatile organic compounds (VOCs) emitted by microorganisms (Leroy et al., 2011; Davis et al., 2013;

Dzialo et al., 2017), and that these volatiles play an important role in mediating chemical interactions between plants and insects, as well as among insects (Schulz-Bohm et al., 2017; Weisskopf et al., 2021). For example, it has been shown that insects respond to microbial VOCs to locate suitable food sources, hosts or oviposition sites (Leroy et al., 2011; Davis et al., 2013; Dzialo et al., 2017). Most research in the field of insect-microbe interactions has focused on yeasts, which have generally been found to attract insects (Becher et al., 2018; Madden et al., 2018). This chemical communication between yeasts and insects is believed to be of mutual benefit, where the insects benefit from the microorganisms signalling suitable sugar resources and the yeasts profit from the insects by being transported to new habitats where they can continue to proliferate or complete their life cycle (Christiaens et al., 2014; Madden et al., 2018). So far, little is known whether a similar mutual benefit of chemical communication exists between bacteria and insects.

Recent research testing a large set of bacteria from diverse origins for their ability to mediate insect behaviour has shown that insect olfactory responses to bacterial volatiles can differ among bacterial species, varying between attraction and repellence (Goelen et al., 2020a; 2020b). These results indicate that insects can respond strongly to volatiles produced by bacteria that live on, in, or near food sources, hosts or preys (Leroy et al., 2011). Furthermore, recent Y-tube olfactometer bioassays indicate that insect responses to bacterial volatiles differ across trophic levels (Goelen et al., 2020a). However, as this study was only performed with two insect species (i.e. the primary parasitoid *Aphidius colemani* and the hyperparasitoid *Dendrocerus aphidum*), specificity of olfactory response to bacterial VOCs within and across multiple trophic levels remains largely unknown.

The objective of this study was to investigate the role of bacterial VOCs in mediating the olfactory response of insects of different trophic levels. Specifically, we asked whether VOCs emitted by bacteria elicit the same olfactory responses in insects from the same and different trophic levels and whether insect responses can be related to specific compounds or groups of compounds. To answer these questions, we here performed experiments with different species of the aphid-primary parasitoid-hyperparasitoid food web. This food web has been commonly used as a model system in ecological studies, partly because of the economic worldwide importance of aphids as pests and their natural enemies as biocontrol agents on a variety of agricultural crops, but also because of the relative ease of rearing aphids, their primary parasitoids, and hyperparasitoids. Olfactory response was evaluated for VOC blends from three bacterial strains isolated from the habitat of the insects. The composition of the volatile blends was analysed using gas chromatography-mass spectrometry (GC-MS) to identify compounds which may be correlated with insect olfactory response.

Materials & Methods

Study organisms. Experiments were performed using seven insect species belonging to three different trophic levels associated with the cultivation of sweet pepper (*Capsicum annuum*) or raspberry (*Rubus idaeus*). These included two aphid species, three primary parasitoids and two hyperparasitoids. Specifically, experiments were conducted using non-winged adults of *Amphorophora idaei* and *Myzus*

persicae var. *nicotianae* (both Hemiptera: Aphididae), adult females of their primary parasitoids *Aphidius colemani*, *A. ervi* and *A. matricariae* (Hymenoptera: Braconidae), and adult females of the hyperparasitoids *Asaphes suspensus* (Hymenoptera: Pteromalidae) and *Dendrocerus aphidum* (Hymenoptera: Megaspilidae). The European large raspberry aphid *A. idaei* is a specialist on *R. idaeus* plants (Blackman et al., 1977), while *M. persicae* var. *nicotianae* is a subspecies of the green peach aphid *Myzus persicae*, one of the most polyphagous aphid species. The subspecies *M. persicae* var. *nicotianae* is found mainly on tobacco and sweet pepper, although it can survive and reproduce on a relatively wide range of plant species (Clements et al., 2000). *Aphidius* spp. are generalist aphid parasitoids, commonly used in commercial biological control (Yano, 2006). *Dendrocerus aphidum* and *A. suspensus* are generalist, secondary idiobiont ectoparasitoids attacking pre-pupal and pupal stages of primary aphid parasitoids, such as *Aphidius* spp., inside mummified aphids (Walker and Cameron, 1981; Höller et al., 1993).

Initial aphid colonies of *A. idaei* and *M. persicae* var. *nicotianae* were obtained from the Research Centre for Fruit Cultivation (Sint-Truiden, Belgium) and NIOO-KNAW (Wageningen, the Netherlands), respectively. Aphids were reared and maintained under controlled conditions (22°C, 70% RH and a 16L:8D photoperiod) on *R. idaeus* var. “Kwanza” (Advanced Berry Breeding B.V., Hedel, Belgium) and *C. annuum* var. “Yolo Wonder” (Mexada DIY B.V., Sint-Agatha-Berchem, Belgium), respectively. Weekly, fresh plants were added to the colonies. All *Aphidius* spp. were obtained in the form of parasitized aphid mummies from Biobest (Westerlo, Belgium) (*A. colemani*: Aphidius-system®; *A. ervi*: Ervi-system®; *A. matricariae*: Matricariae-system®). Mummies were placed inside a nylon insect cage (17.5 cm × 17.5 cm × 17.5 cm, 96 × 26 grids per inch^2 – 680 µm aperture, BugDorm, MegaView Science Co., Ltd.) and kept under controlled conditions (22°C, 70% RH and a 16L:8D photoperiod) until parasitoid emergence. *Dendrocerus aphidum* and *A. suspensus* were reared in the laboratory on fresh (1 day old) *M. persicae* var. *nicotianae* mummies parasitized by *A. colemani*. Hyperparasitoids were allowed to hyperparasitize the mummies for a period of 48 hours. Subsequently, the mummies were placed in a fine-mesh nylon insect cage (24.5 cm × 24.5 cm × 24.5 cm, 150 × 150 grids per inch^2 – 160 µm aperture, BugDorm, MegaView Science Co., Ltd.) and kept under controlled conditions (22°C, 70% RH and a 16L:8D photoperiod) until hyperparasitoid emergence. All experiments were performed with < 24-hour-old, food- and water-deprived females.

Three bacterial strains were used in this study. Strains were isolated from the aphids *Macrosiphum euphorbiae* (Hemiptera: Aphididae) and *M. persicae* var. *nicotianae*, and the primary parasitoid *A. ervi.* Based on 16S ribosomal RNA (rRNA) gene sequencing, strains were found to be ubiquitous environmental bacteria, and assigned to *Staphylococcus saprophyticus* (ST18.16/160), *Curtobacterium* sp. (ST18.16/085) and *Bacillus* sp. (ST18.16/133), respectively. Sequencing of the RNA polymerase B subunit gene (*rpoB*) classified ST18.16/133 as *Bacillus pumilus* (Goelen et al., 2020a). Previous Y-tube olfactometer bioassays using a small set of insects revealed that *A. colemani* females responded positively to VOCs from ST18.16/133, negatively to volatiles from ST18.16/160, and showed a neutral response to ST18.16/085. Further, it was found that *D. aphidum* showed neutral responses towards

strains ST18.16/133 and ST18.16/160, whereas ST18.16/085 was attractive (Goelen et al., 2020a). Strains were stored in tryptic soy broth (TSB; Oxoid) containing 25% glycerol at -80°C until use.

Production of VOCs. For production of VOCs, the procedure of Goelen et al. (2020a) was followed. Briefly, bacterial stock cultures were plated on tryptic soy agar (TSA; Oxoid) and incubated at 25°C for 24 hours. Subsequently, strains were re-streaked on the same medium and incubated at 25°C for another 24 hours. Next, a single colony was inoculated in 10 ml TSB and incubated overnight at 25°C at 120 rpm. After incubation, cells were washed twice and diluted in sterile physiological water (0.9% NaCl) until an optical density (OD 600 nm) of 1 was reached. Next, 1.5 ml of the cell suspension was inoculated in a 250 ml-Erlenmeyer flask containing 150 ml filter-sterilized GYP25 medium. Flasks were sealed with sterile silicone plugs and incubated for 48 hours at 25°C at 120 rpm. Fermentations were set up in triplicate, and non-inoculated, blank GYP25 medium was included as a negative control (also in triplicate). After incubation, the media were centrifuged for 15 min at 10,000 × g. Subsequently, collected supernatants were filter-sterilized to obtain cell-free test media containing the VOCs. The cell-free samples were then stored in small aliquots in sterile, amber glass vials at -20°C until further use.

Olfactometer bioassays. To investigate the olfactory response of the insects, a Y-tube olfactometer bioassay was performed as described previously (Goelen et al., 2020a). The Y-tube (stem: 20 cm; arms: 12 cm with a 60° angle at the Y-junction; inner diameter: 1.5 cm) was put on a table that was homogeneously illuminated by four 24W T5 TL-fluorescent tubes (16 × 549 mm, 1350 Lumen, 5500K, True-Light®, Naturalite Benelux) with a 96% colour representation of true day light at a height of 45 cm. Further, the Y-tube was mounted at a 20° incline to stimulate movement of the insects towards the bifurcation, and a charcoal-filtered air was led through each arm of the Y-tube at a speed of 400 ml min^{-1}. To eliminate any visual cues, the olfactometer was fully enclosed with white curtains. Olfactory response was investigated by loading 150 µl of the cell-free cultivation medium onto a filter paper (diameter: 37 mm; Macherey-Nagel, Düren, Germany) and subsequently placing it in one of the odour chambers, while a second filter paper loaded with 150 µl of the blank medium was placed in the second odour chamber. Insects were tested in 24 cohorts of five adult individuals and were released at the base of the stem section of the olfactometer. Olfactory response was evaluated 10 min after parasitoid or hyperparasitoid release (Goelen et al., 2020a), or 20 min after aphid release, which was found to be the optimal time point of evaluation in preliminary experiments. Individuals that had passed a set line in one of the olfactometer arms (1 cm from the Y-junction) at the time of evaluation were considered to have chosen the odour source presented by that olfactometer arm (Goelen et al., 2020a). All other insects were considered non-responding individuals and were eliminated from statistical analysis. Aphids were food- and water-deprived one hour prior to testing, while the tested freshly emerged parasitoids and hyperparasitoids did not receive any food or water before subjecting them to the bioassays. For every bioassay, new individuals were used. After every two releases the filter papers in the odour chambers were replaced with fresh filter papers with 150 µl medium. Furthermore, to compensate for unforeseen asymmetry in the setup, we swapped the odour chambers after releasing six cohorts. At the same time, the Y-tube glassware was replaced by clean glassware. At the end of the bioassay, all olfactometer parts

were cleaned with tap water, distilled water, acetone and finally pentane, after which the different parts were put in an oven at 150^{o}C until the next day. All bioassays were conducted at 23^{o}C ± 1^{o}C and 65 ± 5% RH between 08h00 and 17h00. As the VOC composition of all three biological replicates was highly similar, olfactory response was determined for one of the three biological replicates.

Chemical analysis of VOCs. For each biological replicate, the VOC composition was analysed by headspace solid phase micro-extraction gas chromatography followed by mass spectrometry analysis (HS-SPME-GC-MS). Volatile compounds were separated by a Thermo Trace 1300 GC system equipped with a MXT-5 column (30 m length × 0.18 mm inner diameter × 0.18 µm film thickness; Restek, Bellefonte, Pennsylvania, USA) and subsequently detected by a ISQ mass spectrometer. Five millilitres of each sample was supplemented with 1.75 g NaCl and kept at

-20^{o}C until analysis. Samples were thawed at room temperature and subsequently incubated at 60^{o}C under constant agitation in a TriPlus RSH SPME auto sampler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The HS-SPME volatile collection was conducted using a 50/30 µm DVB/CAR/PDMS coating fibre (Supelco, Bellefonte, Pennsylvania, USA). After five min of equilibration, the SPME fibre was exposed to the headspace sample for 30 min. The compounds trapped on the fibre were thermally desorbed in the injection port of the chromatograph by heating the fibre for 15 min at 270°C. Further volatile collection and separation conditions were as described by Goelen et al. (2020a). Compounds were identified and quantified as in Reher et al. (2019). Chromatograms were analysed with amdis v2.71 (Stein, 1999) to deconvolute overlapping peaks. Obtained spectra were subsequently annotated using the NIST MS Search v2.0g software, using the NIST2011, FFNSC and Adams libraries. This yielded a list of 244 tentatively identified compounds across all bacterial VOC blends and the blank medium. Peak areas of these compounds were compared to the background signal, which was identified by running a GC-MS with a sample of five milliliters of demineralized water and 1.75 g of NaCl. This background signal was then subtracted from the peak areas of the corresponding tentatively identified compounds, when the difference fell below a set threshold peak area of 1,000, they were eliminated from further analysis. This yielded a list of 66 different compounds. To further extract and integrate the compound elution profiles, a file was used with the identified compounds containing the expected retention times and spectrum profiles. Extraction was performed for every compound in every chromatogram over a time-restricted window using weighted non-negative least square analysis (Lawson and Hanson, 1995), and for every compound, the peak areas were computed from the extracted profiles. Finally, relative peak areas were computed as the ratio between the compounds' peak area and the total peak area per sample, and then multiplied by 100 (%). Relative peak areas were then used for further analysis, and summarized in a table (Table S1, Supporting Information).

Statistical analysis. Insect olfactory response was analysed using a Generalized Linear Mixed Model (GLMM) based on a binomial distribution with a logit link function (logistic regression) using bacterial strain as fixed factor (performed in R using the glmer function from the lme4 package). Each release of one cohort of five insects served as a replicate (n = 24). To prevent pseudo-replication and to adjust for overdispersion, the release of each cohort was included in the model as a random factor. The number of

insects choosing either the control or the bacterial VOCs in each cohort was entered as a response variable. Insect preference was examined by testing the null hypothesis (H_o) that insects showed no preference for any olfactometer arm (i.e. 50:50 response) by testing H_o: logit = 0, which equals a 50:50 distribution. Results were presented by calculating the preference index (PI) by dividing the difference between the number of insects choosing the bacterial odours and the number of insects choosing the control odours, by the total number of responding insects. Furthermore, an analysis of variance Type III Wald chi-square test was performed on the GLMM to determine if there was an overall difference between the olfactory responses for the different VOC blends. A GLMM was also used to determine whether insect species or trophic level affected the olfactory response of the insects. Once again the number of insects choosing either the control or treatment side in each cohort was included as a dependent variable, insect species or trophic level were included as fixed factors. Finally, interaction GLMM models were created to determine the interaction effect of insect species or trophic level, and the different VOC blends on the olfactory response of the insects. The number of insects choosing either the control or treatment side in each cohort was included as a dependent variable, while the interaction between either insect species and the different VOC blends, or insect trophic level and the different VOC blends were included as fixed factors. The release of each cohort was included in the model as a random factor.

Differences in VOC composition were visualized by constructing a heatmap from the strain × relative peak area matrix of calculated Z-scores using the heatmap.2 function in the gplots package V3.1.0 in R (Warnes et al., 2016). For each compound in each sample, the Z-scores were calculated by subtracting the mean relative peak area of all samples for the respective compound from the relative peak area of that compound in the sample, and dividing that by the respective standard deviation. Furthermore, a non-metric multidimensional scaling (NMDS) plot was made from the strain × relative peak area matrix by using a Bray-Curtis distance matrix in the Vegan package in R (Oksanen et al., 2013). Additionally, a permutational multivariate analysis of variance (perMANOVA) was carried out to test for significant differences in the chemical composition of the VOC blends produced by the tested strains, based on 1,000 permutations. The analysis was performed using the adonis function from the Vegan package in R. Finally, differences in the relative peak area of different compound families were calculated by first checking for normality using a Levene's test. When the normality assumption was violated, a Kruskal-Wallis one-way analysis of variance (ANOVA), followed by a Dunn's test was performed. When the normality assumption was met, a univariate ANOVA followed by a Tukey's HSD test was performed.

Pearson correlation tests were performed to investigate correlations between insect olfactory response and the VOC composition. Calculations were performed using the data obtained from the biological replicate which was used for the olfactometer experiments and the VOC analysis. For each insect species and each VOC blend tested, we computed the PI values for each of the 24 cohorts of five insects, and correlated this to the relative peak area of each of the volatile compounds in the VOC blends (66 compounds × 4 relative peak areas (for 3 bacteria and 1 blank medium) in total). To control for multiple comparisons, the *P* values were adjusted using the Benjamini-Hochberg procedure, for the correlations of each individual species (Benjamini and Hochberg, 1995). This was done by multiplying the ratio between

the individual rank of the *P* value and the number of hypotheses (individual correlations per species = 66), by the false discovery rate. We defined the false discovery rate at 5% (Benjamini and Hochberg, 1995). In all analyses, a significance level of α = 0.05 was used to determine significant effects. All statistical analyses were performed in R 4.0.4 (R Core Development Team, 2019).

Results

Differences in olfactory response. Insect olfactory response to the volatile emissions of the tested bacterial strains and control varied significantly between the different treatments ($\chi^2_{(3)}$ = 10.636, *P* = 0.014). Olfactory response did not significantly differ between insect trophic levels ($\chi^2_{(2)}$ = 4.688, *P* = 0.096) or between insect species ($\chi^2_{(6)}$ = 6.774, *P* = 0.342). However, olfactory response did significantly differ for the interaction between treatment and insect trophic level ($\chi^2_{(11)}$ = 29.686, *P* = 0.002), as well as for the interaction between treatment and insect species ($\chi^2_{(27)}$ = 57.133, *P* < 0.001) (Table 1). *Amphorophora idaei* significantly preferred the VOC blend of the *Curtobacterium* sp. strain over the blank medium (PI = 0.284, *P* = 0.012). However, a neutral response was obtained when *A. idaei* was tested against the other bacterial strains. By contrast, *M. persicae* var. *nicotianae* significantly preferred the VOC blend of the *B. pumilus* strain (PI = 0.291, *P* = 0.011). A neutral response was found when *M. persicae* var. *nicotianae* was tested against the VOCs of the *S. saprophyticus* strain (PI = 0.012, *P* = 0.913) and the *Curtobacterium* sp. strain (PI = 0.215, *P* = 0.058) (Fig. 1; Table S1, Supporting Information). Out of the primary parasitoids tested, *A. colemani* showed a significant positive response to *B. pumilus* (PI = 0.308, *P* = 0.011) and a significant negative response to the *S. saprophyticus* strain (PI = -0.284, *P* = 0.016). Unlike *A. colemani*, *A. matricariae* showed a significant preference for the *Curtobacterium* strain (PI = 0.309, *P* = 0.006), while a neutral response was obtained for the two other bacterial strains. Similar results were obtained for *A. ervi*, although effects were less pronounced and not significant (Fig. 1). The hyperparasitoid *D. aphidum* showed a significant preference for the *Curtobacterium* (PI = 0.224, *P* = 0.041) and *S. saprophyticus* strains (PI = 0.266, *P* = 0.020) over the blank medium, but preferred the blank medium over the *B. pumilus* strain (PI = -0.224, *P* = 0.041). Finally, *A. suspensus* showed no significant response for any of the bacteria tested compared to the blank medium (Fig. 1; Table S1, Supporting Information).

Table 1
Statistical values for the different investigated generalized linear mixed models*.

Explanatory variable	Df	χ^2-value	*P*-value
VOC blend	3	10.636	0.014
Trophic level	2	4.688	0.096
Insect species	6	6.774	0.342
VOC blend x Trophic level	11	29.686	0.002
VOC blend x Insect species	27	57.133	< 0.001

*All generalized linear mixed models used the binary choice for treatment or control side of the Y-tube olfactometer tests as response variable and individual cohort of insects as random variable. All models were made using a binomial distribution with a logit link function.

VOC composition. The composition of the VOC blends differed significantly between the different treatments (perMANOVA: $F = 71.08$, $P = 0.001$). This is also displayed in the NMDS ordination plot of the VOC composition which separated all strains from the blank medium to a roughly equal degree along the first NMDS axis, while the strains were separated from each other along the second NMDS axis (Fig. 2). At class level, in comparison with the blank medium, overall bacterial strains produced higher amounts of ketones, organic acids and terpene-derived compounds (Fig. 3; Table S2, Supporting Information). The *Curtobaterium* sp. strain produced eight compounds (4-methyl-2-propyl-1-pentanol, nonan-2-ol, 2-phenylethanol, 3,5-dimethyl-benzaldehyde, decyl acetate, acetophenone, 3-methyl-2-buten-1-ol, and camphor) in a significantly higher relative concentration than the other strains and the blank medium. The *B. pumilus* strain produced two compounds (5,5-dimethyl-2,4-hexanedione and acetoin) in a significantly higher relative concentration than the other strains and the blank medium. The *S. saprophyticus* strain did not produce any compounds in a significantly higher relative concentration than the other strains and the blank medium. Finally, the blank medium emitted two compounds in significantly higher relative amounts compared to the bacterial treatments (isobutyryl chloride and 2-methyl-5-(1-methylethyl)-pyrazine) (Table S2, Supporting Information).

Correlations between insect behaviour and VOC composition. To identify potential active compounds, a correlation analysis between the olfactory response of the insects and the relative peak area of the compounds in the VOC blends was performed. Out of the 66 identified VOCs, the relative peak area of 49 compounds was significantly correlated with the olfactory response of *A. idaei*, *M. persicae* var. *nicotianae*, *A. colemani*, *A. ervi*, *A. matricariae*, and *D. aphidum* (Pearson's correlation, $P < 0.05$, $r > 0.20$ or $r < -0.20$), while none of the compounds were significantly correlated with the response of *A. suspensus* (Fig. 4; Tables S3-S8, Supporting Information). Among these, 11 compounds were significantly correlated with the olfactory behaviour of one species, while 34 and four compounds were significantly correlated with two and three insect species, respectively (Fig. 4; Tables S3-S8, Supporting Information). Interestingly, all compounds that were significantly correlated with both the primary parasitoid *A. colemani* and the hyperparasitoid *D. aphidum* (11 compounds) showed a differential correlation, where

the behaviour of *A. colemani* was either positively correlated to the compound concentration and *D. aphidum* negatively, or vice versa. In total, nine compounds were positively correlated with the primary parasitoid, while they were negatively correlated with the hyperparasitoids. Among these, the three compounds with the highest difference in correlation coefficient with a positive correlation for *A. colemani* and a negative correlation for *D. aphidum* were n-hexanol (*A. colemani*: $P < 0.001$, $r = 0.363$; *D. aphidum*: $P < 0.001$, $r = -0.349$), 3-methyl-pyruvic acid (*A. colemani*: $P < 0.001$, $r = 0.348$; *D. aphidum*: $P < 0.001$, $r = -0.341$), and butyl propanoate (*A. colemani*: $P < 0.001$, $r = 0.350$; *D. aphidum*: $P = 0.001$, $r = -0.319$). The compounds with a significant positive correlation for *D. aphidum* and a negative correlation for *A. colemani* were linalool (*A. colemani*: $P = 0.005$, $r = -0.288$; *D. aphidum*: $P < 0.001$, $r = 0.345$) and acetic acid (*A. colemani*: $P = 0.005$, $r = -0.284$; *D. aphidum*: $P = 0.001$, $r = 0.329$) (Fig. 4; Tables S5 and S8, Supporting Information).

Discussion

In this study, we assessed the effects of three bacteria on the olfactory behaviour of seven insect species from three trophic levels. Results showed that insects responded to bacterial volatiles, but responses varied between and within trophic levels. For example, while the VOC blend produced by *S. saprophyticus* was repellent for the primary parasitoids (especially *A. colemani*), it was significantly attractive for the hyperparasitoid *D. aphidum* and to a lesser extent for *A. suspensus*. Likewise, the *B. pumilus* strain was significantly attractive for *M. persicae* var. *nicotianae* and its primary parasitoid *A. colemani*, but appeared significantly deterrent for *D. aphidum*. These findings are in agreement with a preliminary study where *A. colemani* and *D. aphidum* were tested against the same bacteria (Goelen et al., 2020a). By contrast, the *Curtobacterium* sp. strain evoked a neutral to positive response (with the exception of *A. suspensus* which yielded a slightly negative preference index) across all trophic levels. This result resembles earlier findings showing that the pupal *Drosophila* parasitoid *Trichopria drosophilae* is attracted to the same yeast odours that their adult hosts prefer (Đurović et al., 2021). *Drosophila* flies are consistently associated with a number of yeasts (Chandler et al., 2012) providing mutual benefits. Volatiles produced by the yeasts are a strong and reliable signal for the flies indicating the presence of an available resource like sugar-rich food. The otherwise immotile yeasts in turn benefit from getting dispersed to another patch of sugar or habitat (Becher et al., 2012; Christiaens et al., 2014; Madden et al., 2018). Whether such scenario also exists for bacteria remains unclear so far. Nevertheless, as parasitoids have great capability to learn to associate chemical cues with rewards (Petitt et al., 1992; Vet, et al., 1995; Olson et al., 2003), it is very likely that they could have evolved a specific response to volatiles from microbes that are associated with their hosts (Lewis and Lizé, 2015), which may lead to similar responses over trophic levels. Although *Curtobacterium* strains have been found in many hemipteran insects (Gai et al., 2011; Azevedo et al., 2016), including aphids (Goelen et al., 2020a; He et al., 2021), it is not yet clear whether such strong relationships exist between the *Curtobacterium* sp. and insect species investigated in this study. Our results also show that insect species from the same trophic level, and even from the same genus, responded differently to bacterial VOCs. This can be illustrated for the *B. pumilus* strain, evoking a positive or neutral response within the tested aphids and *Aphidius* parasitoids. Likewise,

the VOC blend was highly repellent for *D. aphidum*, while attractive (albeit not significantly; $P = 0.068$) to *A. suspensus*. Similar trends were observed for *S. saprophyticus*.

Although VOC composition differed significantly between bacterial strains, the tested strains emitted the same set of VOCs, but often in different relative amounts. The *Curtobacterium* sp. strain produced eight compounds in a significantly higher amount compared to the other strains and blank medium, whereas the *B. pumilus* strain produced two compounds in a significantly higher amount than the other strains and blank medium. By contrast, the *S. saprophyticus* strain produced no compounds in a significantly higher amount than the other strains. This suggests that the bacterial VOCs may elicit a different response in insects depending on the concentration of the VOCs and the composition of the blend, most probably determined by the presence of particular compounds at high concentrations or specific ratios between compounds (Bruce et al., 2005; Webster et al., 2010; Takemoto and Takabayashi, 2015; Liu et al., 2019). When zooming in at the VOC composition of the tested strains, 49 compounds were significantly linked to insect behaviour, among which 38 could be linked with the behaviour of more than one insect species. Interestingly, all compounds correlated with both *A. colemani* and *D. aphidum* behaviour showed an opposite pattern, where the behaviour of *A. colemani* was either positively correlated to the compound concentration while it was negatively correlated to the behaviour of *D. aphidum*, or vice versa. Two compounds, linalool and acetic acid, were significantly positively correlated with the olfactory response of *D. aphidum*, while negatively correlated with the olfactory response of *A. colemani*. Linalool is a volatile monoterpenoid alcohol that mediates interactions between plants and pollinators, herbivores, carnivores, and microbes (Raguso, 2016), and has been shown to be attractive or repellent to many insect species (McCormick et al., 2012; Takemoto and Takabayashi, 2015). Acetic acid is a volatile carboxylic acid and is a common bacterial fermentation product from the oxidation of ethanol (Sievers and Swings, 2005). It has been found to be an important attractant for *Drosophila* spp. and many other insect species when used in conjunction with other volatile compounds (El Sayed et al., 2005; Mansourian and Stensmyr, 2015). By contrast, nine compounds were significantly positively correlated with the olfactory response of *A. colemani*, while they were negatively associated with *D. aphidum*. Among these, butyl propanoate, a carboxylic ester, was found to be one of the compounds showing the biggest difference in correlation coefficient between both insect species. This compound is a common plant volatile (Pan et al., 2021) that is attractive for many insect species in the orders Hemiptera, Lepidoptera and Diptera (Chen et al., 2020; Liu et al., 2020; Pan et al., 2021).

Volatile compounds evoking a differential response in parasitoids and hyperparasitoids could be particularly interesting for improving present-day aphid biocontrol. Hyperparasitoids can disrupt biological aphid control by suppressing the populations of their parasitoid hosts, especially in confined environments such as greenhouses where augmentative biological control is commonly used (Acheampong et al., 2012; Yang et al., 2017). So far, there is no effective sustainable strategy that can be used to control hyperparasitoids. Volatile compounds that induce an attractive or repellent response in primary parasitoids and the reverse in hyperparasitoids offer a promising perspective in developing a so-called “push-pull” strategy. In such a strategy, hyperparasitoids could be “pushed” away from areas with their parasitoid hosts and potentially “pulled’ into traps. Primary parasitoids, on the other hand could be

“pulled” into the crop and “pushed” away from the hyperparasitoid traps, leading to enhanced biocontrol (Cusumano et al., 2020). Previous studies have shown that synthetic VOCs (pure compounds), especially when blended together in specific ratios, may be stronger candidates for push-pull systems than the microbial volatile blends in which they were identified (Goelen et al., 2021). Future studies are needed to further investigate the efficacy of synthetic VOCs as either “push” or “pull” components in field or greenhouse conditions.

Altogether, our results have shown that bacterial volatiles can elicit olfactory responses in insects from different trophic levels, and that responses vary between and within trophic levels, and even between species within the same genus. For a number of VOCs we found contrasting correlations between VOC concentration and the behaviour of primary and secondary parasitoids. This may lead to novel semiochemical-based strategies to manage hyperparasitoids.

Declarations

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Conflicts of interest/ Competing interests

The authors declare that no conflicts of interest exist.

Availability of data and material

Data and materials are available upon request.

Code availability

Codes for statistical analysis were written in R and are available upon request.

Author contributions

F.A.C.v.N, H.J. and B.L. conceived the ideas, designed the methodology, and led the writing of the manuscript. F.A.C.v.N. and P.D.W. collected the data. F.A.C.v.N, P.D.W., H.J. and B.L. analysed the data. T.W. contributed to equipment and reagents for the VOC analysis. F.W. provided all primary parasitoids required for the experiments. All authors contributed critically to the drafts of this manuscript and gave final approval for publication.

Ethical note

Experimental manipulation of insects occurred according to the common and ethical requirements for animal welfare. All insects were carefully handled during experiments and maintained in the laboratory under appropriate conditions.

Consent to participate

Not applicable

Consent for publication

Consent for publication was granted by all co-authors

References

1. Aartsma Y, Bianchi FJJA, Werf W, Poelman EH, Dicke M (2017) Herbivore-induced plant volatiles and tritrophic interactions across spatial scales. New Phytol 216:1054–1063. https://doi.org/10.1111/nph.14475
2. Aartsma Y, Cusumano A, Fernández de Bobadilla M, Rusman Q, Vosteen I, Poelman EH (2019) Understanding insect foraging in complex habitats by comparing trophic levels: insights from specialist host-parasitoid-hyperparasitoid systems. Curr Opin Insect Sci 32:54–60. https://doi.org/10.1016/j.cois.2018.11.001
3. Acheampong S, Gillespie DR, Quiring D (2012) Survey of parasitoids and hyperparasitoids (Hymenoptera) of the green peach aphid, *Myzus persicae* and the foxglove aphid, *Aulacorthum solani* (Hemiptera: Aphididae) in British Columbia. J Ent Soc Br Columb 109:12–22

4. Azevedo JL, Araújo WL, Lacava PT (2016) The diversity of citrus endophytic bacteria and their interactions with *Xylella fastidiosa* and host plants. Genet Mol Biol 39:476–491. https://doi.org/10.1590/1678-4685-gmb-2016-0056
5. Becher PG, Flick G, Rozpędowska E, Alexandra S, Hagman A, Lebreton S, Larsson MC, Hansson BS, Piškur J, Witzgall P, Bengtsson M (2012) Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. Funct Ecol 26:822–828. https://doi.org/10.1111/j.1365-2435.2012.02006.x
6. Becher PG, Hagman A, Verschut V, Chakraborty A, Rozpędowska E, Lebreton S, Bengtsson M, Flick G, Witzgall P, Piškur J (2018) Chemical signaling and insect attraction is a conserved trait in yeasts. Ecol Evol 8:2962–2974. https://doi.org/10.1002/ece3.3905
7. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol 57:289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x
8. Blackman RL, Eastop VF, Hills M (1977) Morphological and cytological separation of *Amphorophora* Buckton (Homoptera: Aphididae) feeding on European raspberry and blackberry (*Rubus* spp.). Bull Entomol Res 67:285–296. https://doi.org/10.1017/S000748530001110X
9. Bruce TJA, Wadhams LJ, Woodcock CM (2005) Insect host location: a volatile situation. Trends in Plant Sci 10:269–274. https://doi.org/10.1016/j.tplants.2005.04.003
10. Chandler JA, Eisen JA, Kopp A (2012) Yeast communities of diverse *Drosophila* species: comparison of two symbiont groups in the same hosts. Appl Environ Microbiol 78:7327–7336. https://doi.org/10.1128/AEM.01741-12
11. Chen L, Tian K, Wang G, Xu X, He K, Liu W, Wu J (2020) The general odorant receptor *GmolOR9* from *Grapholita molesta* (Lepidoptera: Tortricidae) is mainly tuned to eight host-plant volatiles. Insect Sci 27:1233–1243. https://doi.org/10.1111/1744-7917.12725
12. Christiaens JF, Franco LM, Cools TL, De Meester L, Michiels J, Wenseleers T, Hassan BA, Yaksi E, Verstrepen KJ (2014) The fungal aroma gene *ATF1* promotes dispersal of yeast cells through insect vectors. Cell Rep 9:425–432. https://doi.org/10.1016/j.celrep.2014.09.009
13. Clavijo McCormick A, Unsicker SB, Gershenzon J (2012) The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. Trends in Plant Sci 17:303–310. https://doi.org/10.1016/j.tplants.2012.03.012
14. Clements KM, Sorenson CE, Wiegmann BM, Neese PA, Roe RM (2000) Genetic, biochemical, and behavioral uniformity among populations of *Myzus nicotianae* and *Myzus persicae*. Entomol Exp Appl 95:269–281. https://doi.org/10.1046/j.1570-7458.2000.00666.x
15. Cusumano A, Harvey JA, Bourne ME, Poelman EH, de Boer JG (2020) Exploiting chemical ecology to manage hyperparasitoids in biological control of arthropod pests. Pest Manag Sci 76:432–443. https://doi.org/https://doi.org/10.1002/ps.5679
16. Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK (2013) Microbial volatile emissions as insect semiochemicals. J Chem Ecol 39:840–859. https://doi.org/10.1007/s10886-013-0306-z

17. Đurović G, Van Neerbos FAC, Bossaert S, Herrera-Malaver B, Steensels J, Arnó J, Wäckers F, Sobhy IS, Verstrepen KJ, Jacquemyn H, Lievens B (2021) The pupal parasitoid *Trichopria drosophilae* is attracted to the same yeast volatiles as its adult host. J Chem Ecol 47:788–798. https://doi.org/10.1007/s10886-021-01295-6
18. Dzialo MC, Park R, Steensels J, Lievens B, Verstrepen KJ (2017) Physiology, ecology and industrial applications of aroma formation in yeast. FEMS Microbiol Rev 41(Supp1):S95–S128. https://doi.org/10.1093/femsre/fux031
19. El-Sayed AM, Heppelthwaite VJ, Manning LM, Gibb AR, Suckling DM (2005) Volatile constituents of fermented sugar baits and their attraction to lepidopteran species. J Agr Food Chem 53:953–958. https://doi.org/10.1021/jf048521j
20. Fischer S, Samietz J, Wäckers F, Dorn S (2001) Interaction of vibrational and visual cues in parasitoid host location. J Comp Physiol A 187:785–791. https://doi.org/10.1007/s00359-001-0249-7
21. Gai CS, Dini-Andreote F, Dini Andreote F, Lopes S, Luiz JR, Araújo W (2011) Endophytic bacteria associated to sharpshooters (Hemiptera: Cicadellidae), insect vectors of *Xylella fastidiosa* subsp. *pauca*. J Plant Pathol Microbiol 02:109–117. https://doi.org/10.4172/2157-7471.1000109
22. Goelen T, Sobhy IS, Vanderaa C, Boer JG, Delvigne F, Francis F, Wäckers F, Rediers H, Verstrepen KJ, Wenseleers T, Jacquemyn H, Lievens B (2020) Volatiles of bacteria associated with parasitoid habitats elicit distinct olfactory responses in an aphid parasitoid and its hyperparasitoid. Funct Ecol 34:507–520. https://doi.org/10.1111/1365-2435.13503
23. Goelen T, Sobhy IS, Vanderaa C, Wäckers F, Rediers H, Wenseleers T, Jacquemyn H, Lievens B (2020) Bacterial phylogeny predicts volatile organic compound composition and olfactory response of an aphid parasitoid. Oikos 129:1415–1428. https://doi.org/10.1111/oik.07301
24. Goelen T, Vuts J, Sobhy IS, Wäckers F, Caulfield JC, Birkett MA, Rediers H, Jacquemyn H, Lievens B (2021) Identification and application of bacterial volatiles to attract a generalist aphid parasitoid: from laboratory to greenhouse assays. Pest Manag Sci 77:930–938. https://doi.org/10.1002/ps.6102
25. He B, Chen X, Yang H, Cernava T (2021) Microbiome structure of the aphid *Myzus persicae* (Sulzer) is shaped by different solanaceae plant diets. Front Microbiol 12:1765. https://doi.org/10.3389/fmicb.2021.667257
26. Höller C, Borgemeister C, Haardt H, Powell W (1993) The relationship between primary parasitoids and hyperparasitoids of cereal aphids: an analysis of field data. J Anim Ecol 62:12–21. https://doi.org/10.2307/5478
27. Kaplan I (2012) Attracting carnivorous arthropods with plant volatiles: The future of biocontrol or playing with fire? Biol Control 60:77–89. https://doi.org/10.1016/j.biocontrol.2011.10.017
28. Lawson CL, Hanson RJ (1995) Solving least squares problems, vol 15. SIAM
29. Leroy PD, Sabri A, Heuskin S, Thonart P, Lognay G, Verheggen FJ, Francis F, Brostaux Y, Felton GW, Haubruge E (2011) Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. Nat Commun 2:348. https://doi.org/10.1038/ncomms1347

30. Lewis Z, Lizé A (2015) Insect behaviour and the microbiome. Curr Opin Insect Sci 9:86–90. https://doi.org/10.1016/j.cois.2015.03.003
31. Little CM, Chapman TW, Hillier NK (2019) Considerations for insect learning in integrated pest management. J Insect Sci 19:6–20. https://doi.org/10.1093/jisesa/iez064
32. Liu C-M, Matsuyama S, Kainoh Y (2019) Synergistic effects of volatiles from host-infested plants on host-searching behaviour in the parasitoid wasp *Lytopylus rufipes* (Hymenoptera: Braconidae). J Chem Ecol 45:684–692. https://doi.org/10.1007/s10886-019-01088-y
33. Liu Y, Cui Z, Wang G, Zhou Q, Liu Y (2020) Cloning and functional characterization of three odorant receptors from the chinese citrus fly *Bactrocera minax* (Diptera: Tephritidae). Front Physiol 11:246. https://doi.org/10.3389/fphys.2020.00246
34. Madden AA, Epps MJ, Fukami T, Irwin RE, Sheppard J, Sorger DM, Dunn RR (2018) The ecology of insect–yeast relationships and its relevance to human industry. Proc Royal Soc B 285:20172733. https://doi.org/10.1098/rspb.2017.2733
35. Mansourian S, Stensmyr MC (2015) The chemical ecology of the fly. Curr Opin Neurobiol 34:95–102. https://doi.org/10.1016/j.conb.2015.02.006
36. Meiners T, Peri E (2013) Chemical ecology of insect parasitoids: essential elements for developing effective biological control programmes. Chemical Ecology of Insect Parasitoids. John Wiley & Sons, Ltd, pp 191–224. https://doi.org/10.1002/9781118409589.ch9
37. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H (2017) Package 'vegan.' In Community ecology package, version (Vol. 2, Issue 9, pp. 1–295)
38. Olson DM, Rains GC, Meiners T, Takasu K, Tertuliano M, Tumlinson JH, Wäckers F, Lewis WJ (2003) Parasitic wasps learn and report diverse chemicals with unique conditionable behaviors. Chem Senses 28:545–549
39. Pan H-S, Xiu C-L, Williams L, Lu Y-H (2021) Plant volatiles modulate seasonal dynamics between hosts of the polyphagous mirid bug *Apolygus lucorum*. J Chem Ecol 47:87–98. https://doi.org/10.1007/s10886-020-01236-9
40. Petitt FL, Turlings TCJ, Wolf SP (1992) Adult experience modifies attraction of the leafminer parasitoid *Opius dissitus* (Hymenoptera: Braconidae) to volatile semiochemicals. J Insect Behav 5:623–634. https://doi.org/10.1007/BF01048009
41. R core team (2019) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org
42. Raguso RA (2016) More lessons from linalool: insights gained from a ubiquitous floral volatile. Curr Opin Plant Biol 32:31–36. https://doi.org/10.1016/j.pbi.2016.05.007
43. Reher T, Van Kerckvoorde V, Verheyden L, Wenseleers T, Beliën T, Bylemans D, Martens JA (2019) Evaluation of hop (*Humulus lupulus*) as a repellent for the management of *Drosophila suzukii*. J Crop Prot 124:104839. https://doi.org/10.1016/j.cropro.2019.05.033

44. Schulz-Bohm K, Martín-Sánchez L, Garbeva P (2017) Microbial volatiles: Small molecules with an important role in intra-and inter-kingdom interactions. Front Microbiol 8:2484. https://doi.org/10.3389/fmicb.2017.02484
45. Sievers M, Swings J (2005) Family II. *Acetobacteraceae*. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of synthetic bacteriology, vol 2. Springer, New York, NY, pp 41–95
46. Stein SE (1999) An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry data. J Am Soc Mass Spectr 10:770–781. https://doi.org/https://doi.org/10.1016/S1044-0305(99)00047-1
47. Takemoto H, Takabayashi J (2015) Parasitic wasps *Aphidius ervi* are more attracted to a blend of host-induced plant volatiles than to the independent compounds. J Chem Ecol 41:801–807. https://doi.org/10.1007/s10886-015-0615-5
48. van Oudenhove L, Mailleret L, Fauvergue X (2017) Infochemical use and dietary specialization in parasitoids: a meta-analysis. Ecol Evol 7:4804–4811. https://doi.org/10.1002/ece3.2888
49. Vet LEM, Lewis WJ, Cardé RT (1995) Parasitoid foraging and learning. In: Carde RT, Bell WJ (eds) Chemical ecology of insects 2. Springer, Boston, MA, pp 65–101. https://doi.org/https://doi.org/10.1007/978-1-4615-1765-8_3
50. Visser JH (1988) Host-plant finding by insects: Orientation, sensory input and search patterns. J Insect Physiol 34:259–268. https://doi.org/10.1016/0022-1910(88)90056-X
51. Wäckers F, Lewis WJ (1994) Olfactory and visual learning and their combined influence on host site location by the parasitoid *Microplitis croceipes* (Cresson). Biol Control 4:105–112. https://doi.org/10.1006/bcon.1994.1018
52. Walker GP, Cameron PJ (1981) The biology of *Dendrocerus carpenteri* (Hymenoptera: Ceraphronidae), a parasite of *Aphidius* species, and field observations of *Dendrocerus* species as hyperparasites of *Acyrthosiphon* species. NZ J Zool 8:531–538. https://doi.org/10.1080/03014223.1981.10427979
53. Warnes MGR, Bolker B, Bonebakker L, Gentleman R, Huber W (2016) Package 'gplots.' In Various R programming tools for plotting data
54. Webster B, Bruce TJA, Pickett J, Hardie J (2010) Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. Anim Behav 79:451–457. https://doi.org/10.1016/j.anbehav.2009.11.028
55. Webster B, Cardé RT (2017) Use of habitat odour by host-seeking insects. Biol Rev 92:1241–1249. https://doi.org/10.1111/brv.12281
56. Weisskopf L, Schulz S, Garbeva P (2021) Microbial volatile organic compounds in intra-kingdom and inter-kingdom interactions. Nat Rev Microbiol 19:391–404. https://doi.org/10.1038/s41579-020-00508-1
57. Yang F, Xu L, Wu Y-K, Wang Q, Yao Z-W, Žikić V, Tomanović Ž, Ferrer-Suay M, Selfa J, Pujade-Villar J (2017) Species composition and seasonal dynamics of aphid parasitoids and hyperparasitoids in wheat fields in northern China. Sci Rep 7:1–9. https://doi.org/https://doi.org/10.1038/s41598-017-14441-6

58. Yano E (2006) Ecological considerations for biological control of aphids in protected culture. Popul Ecol 48:333–339. https://doi.org/10.1007/s10144-006-0008-2

Figures

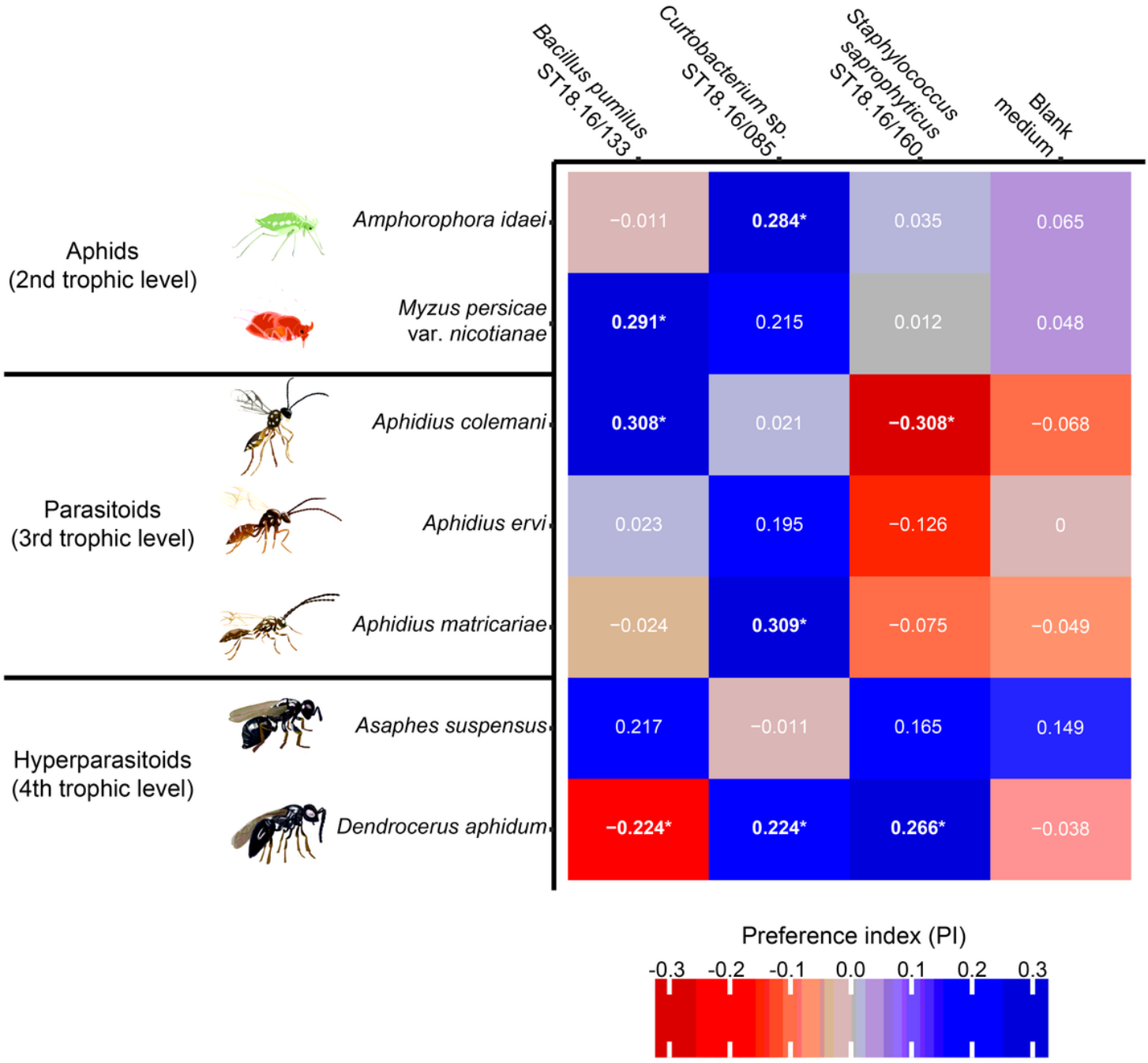

Figure 1

Olfactory response of the aphid species *Amphorophora idaei* and *Myzus persicae* var. *nicotianae*, their primary parasitoids *Aphidius colemani*, *A. ervi* and *A. matricariae* and the hyperparasitoids *Asaphes suspensus* and *Dendrocerus aphidum* when given a choice between the odour of a bacterial test strain

grown in GYP25 medium and the odour of the blank GYP25 medium in a Y-tube olfactometer. Bacterial strains tested included *Bacillus pumilus* ST18.16/133, *Curtobacterium* sp. ST18.16/085 and *Staphylococcus saprophyticus* ST18.16/160. Insect response is expressed as the preference index, which is calculated by dividing the difference between the number of insects choosing the bacterial odours and the number of insects choosing the control odours, by the total number of responding insects. In total, for each insect species 24 releases of 5 individuals were tested. Non-responders were excluded from the statistical analysis. Colours in the matrix are allocated according to sign and strength of the preference index. Values expressed in bold and marked with an asterisk indicate a significant olfactory response ($P < 0.05$) when compared to a 50:50 distribution. Overall insect responsiveness was 71.0%, and ranged between 67.7% and 72.3%.

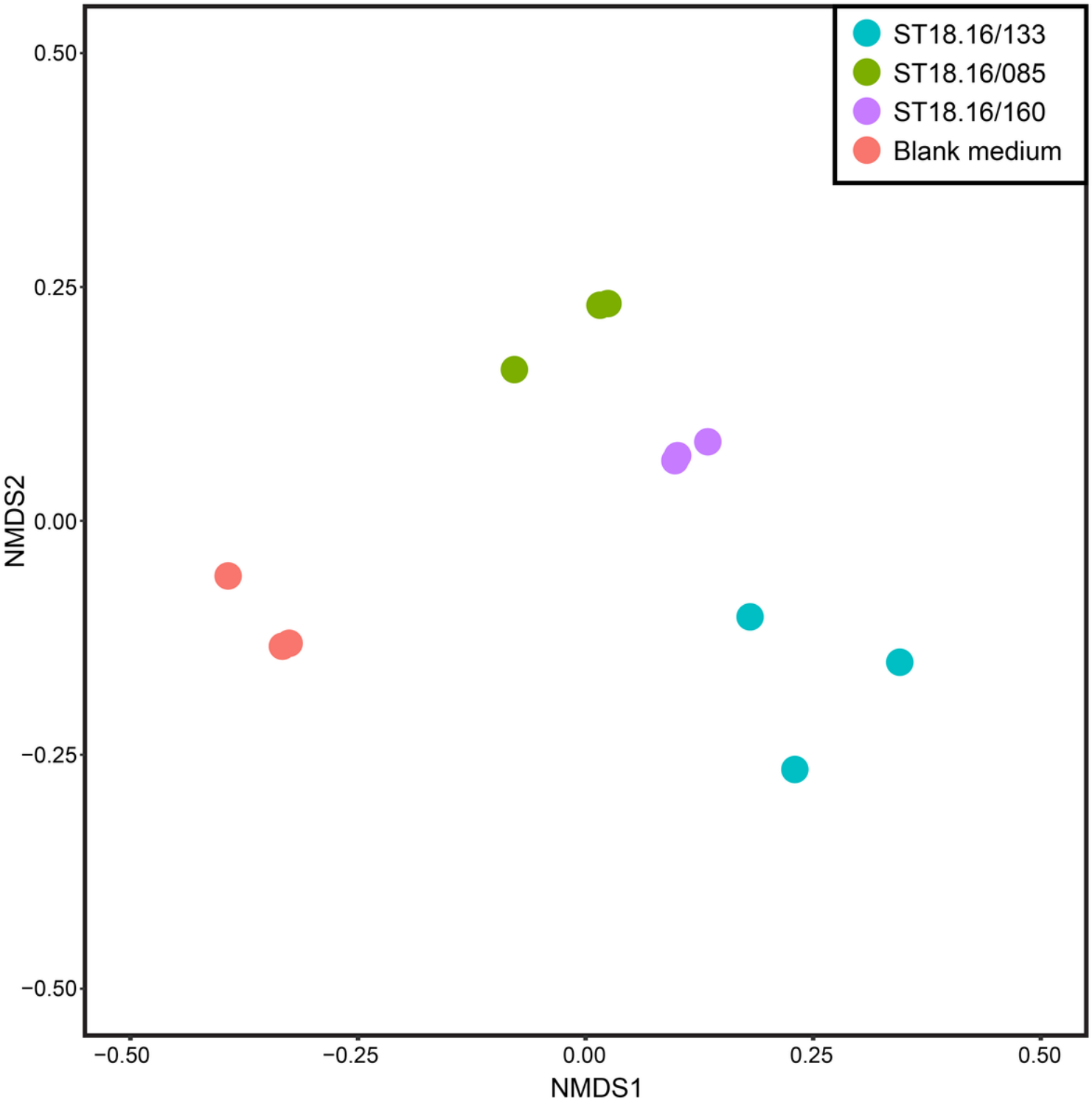

Figure 2

Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities of the relative peak area of the VOC composition of the three bacterial strains cultivated in GYP25 medium and the blank GYP25 medium (n = 3, stress value = 0.020). Investigated strains included *Bacillus pumilus* ST18.16/133, *Curtobacterium* sp. ST18.16/085 and *Staphylococcus saprophyticus* ST18.16/160. The VOC composition of the different treatments differed significantly from one another (perMANOVA: F = 71.08, P = 0.001).

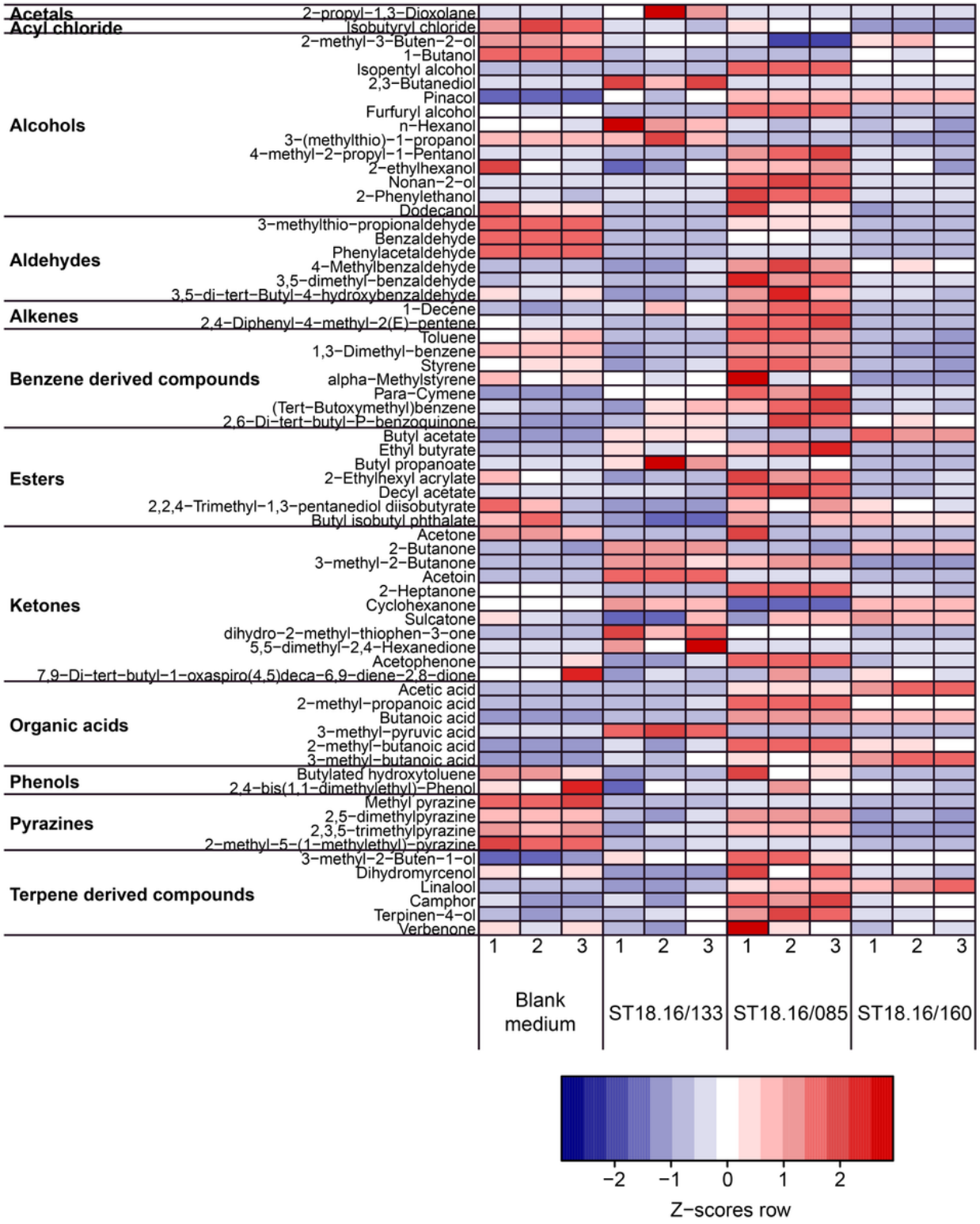

Figure 3

Heatmap of the VOC composition of the three bacterial strains cultivated in GYP25 medium and the blank GYP25 medium (*n* = 3). Investigated strains included *Bacillus pumilus* ST18.16/133, *Curtobacterium* sp. ST18.16/085 and *Staphylococcus saprophyticus* ST18.16/160. For each compound, relative peak area is represented as the calculated Z-score. Compound families are ordered alphabetically. Compounds within the families are ordered along their retention index.

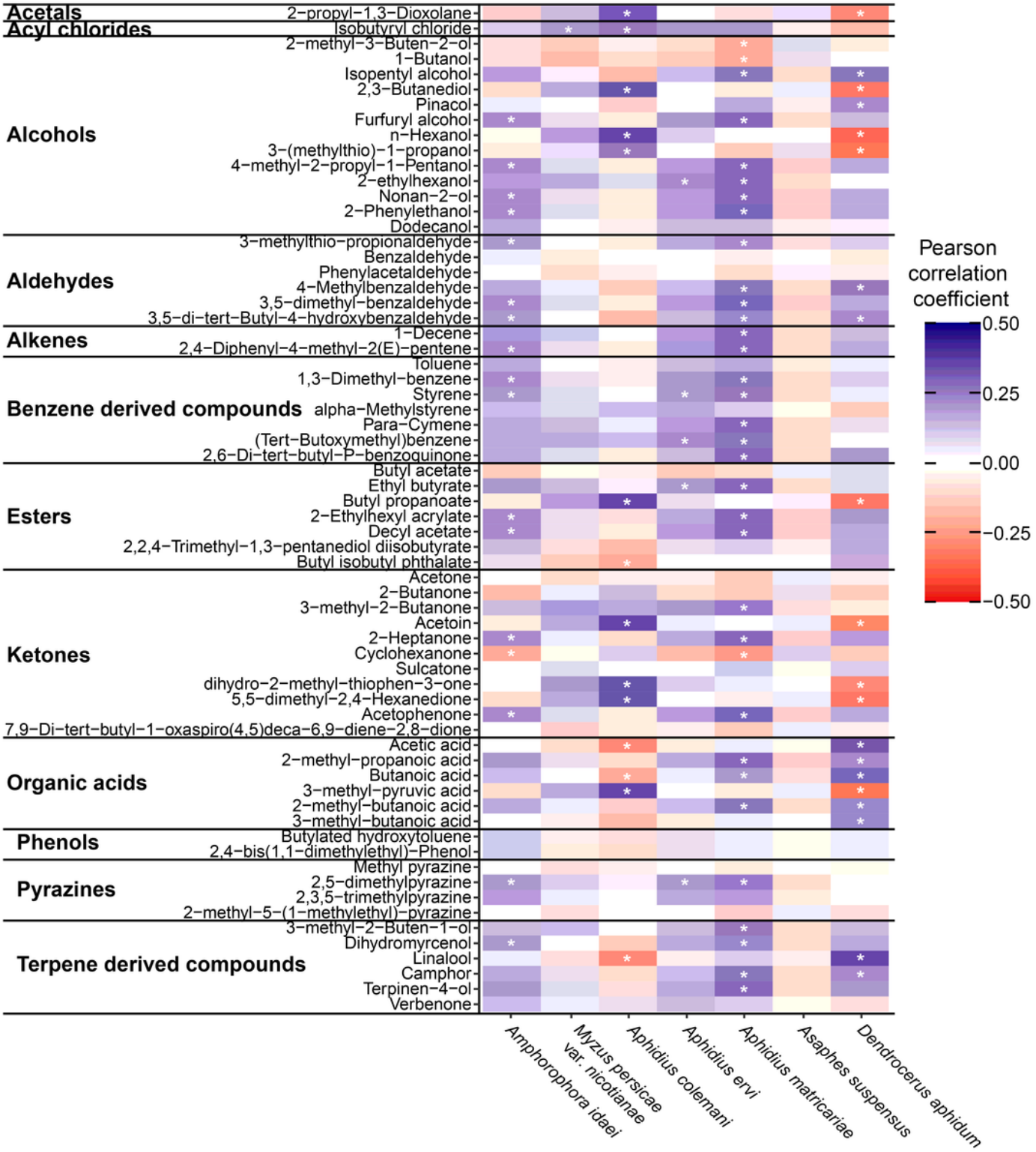

Figure 4

Correlation matrix between insect olfactory response (preference index) and the VOCs detected in the cell-free media of the three tested bacterial strains and the blank GYP25 medium (n = 4). Investigated insect species included the aphid species *Amphorophora idaei* and *Myzus persicae* var. *nicotianae*, their primary parasitoids *Aphidius colemani*, *A. ervi*, and *A. matricariae*, and their hyperparasitoids *Asaphes suspensus* and *Dendrocerus aphidum*. The investigated bacterial strains were *Bacillus pumilus* ST18.16/133,

Curtobacterium sp. ST18.16/085 and *Staphylococcus saprophyticus* ST18.16/160. Correlations between the calculated preference index of each insect cohort and the relative peak area of each compound in the volatile blends were calculated using Pearson's correlation coefficients (r). Significant correlations ($P < 0.05$) are indicated with an asterisk. Corrected P-values and Pearson's correlation coefficients (r) for significant correlations are given in Tables S3-S8 (Supporting Information).

Supplementary Files

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- SupplementaryinformationvanNeerbosetal.pdf