Functional Ecology

Volatiles of bacteria associated with parasitoid habitats elicit distinct olfactory responses in an aphid parasitoid and its hyperparasitoid

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ABSTRACT

1. To locate mating partners and essential resources such as food, oviposition sites and shelter, insects rely to a large extent on chemical cues. While most research has focused on cues derived from plants and insects, there is mounting evidence that indicates that microorganisms emit volatile compounds that may play an important role in insect behaviour.
2. In this study, we assessed how volatile compounds emitted by phylogenetically diverse bacteria affected the olfactory response of the primary parasitoid *Aphidius colemani* and one of its secondary parasitoids, *Dendrocerus aphidum*. Olfactory responses were evaluated for volatile blends emitted by bacteria isolated from diverse sources from the parasitoid's habitat, including aphids, aphid mummies and honeydew, and from the parasitoids themselves.
3. Results revealed that *A. colemani* showed wide variation in response to bacterial volatiles, ranging from significant attraction over no response to significant repellence. Our results further showed that the olfactory response of *A. colemani* to bacterial volatile emissions was different from that of *D. aphidum*. Gas chromatography-mass spectrometry (GC-MS) analysis of the volatile blends revealed that bacterial strains repellent to *A. colemani* produced significantly higher amounts of esters, organic acids,

aromatics and cycloalkanes than attractive strains. Strains repellent to *D. aphidum* produced significantly higher amounts of alcohols and ketones, whereas the strains attractive to *D. aphidum* produced higher amounts of the monoterpenes limonene, linalool and geraniol.

4. Overall, our results indicate that bacterial volatiles can have an important impact on insect olfactory responses, and should therefore be considered as an additional, so far often overlooked factor in studying multitrophic interactions between plants and insects.

Keywords:

Aphidius colemani, chemical communication, *Dendrocerus aphidum*, microbial odour, multitrophic interactions, natural enemy, semiochemical, VOCs

Introduction

Insects rely to a large extent on the detection of olfactory cues facilitating their most basic survival functions, including feeding, mating and oviposition (de Bruyne & Baker, 2008). While gustatory information may indicate the quality of food, mates or egg deposition sites after contact, both visual and olfactory cues provide information about where to search in the first place (Wäckers & Lewis, 1994). Likewise, several insects use olfaction to avoid threats and risks associated with toxic environments, pathogens or predators (Stensmyr et al., 2012; Yanagawa, Imai, Akino, Toh, & Yoshimura, 2015).

Previous studies have revealed that insect behaviour is strongly mediated by volatile organic compounds (VOCs) emitted by plants and insects when foraging for food, hosts or conspecifics (Meiners, 2015; Vet & Dicke, 1992). Volatiles from both aerial and underground plant parts play an important role in the communication between plants and insects (Meiners, 2015; Soler, Bezemer, & Harvey, 2013), while insect-derived volatiles have been intensively studied to better understand critical processes in insects such as mating, reproduction, aggregation and alarming conspecifics about danger (Wyatt, 2014). More recently, increasing evidence indicates that microorganisms like bacteria and fungi also emit volatile compounds that affect insect behaviour (Davis, Crippen, Hofstetter, & Tomberlin, 2013; Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017; Leroy et al., 2011b). Although production of microbial volatile organic compounds (mVOCs) is commonplace (Piechulla & Degenhardt, 2013), little is still known about their ecological role and how they interfere with volatile-mediated insect foraging behaviour. In spite of this, a growing body of literature suggests that responding to mVOCs benefits insects in various ways. Many insects employ mVOCs to locate appropriate resources such as suitable food sources or oviposition sites (Becher et al., 2012; Leroy et al., 2011a; Sobhy et al., 2018; 2019; Rering, Beck, Hall, McCartney, & Vannette, 2018). By contrast, some mVOCs have also been found to repel insects, e.g. when signalling unsuitable food sources, unsuitable hosts or hostile environments (Azeem, Rajarao, Nordenhem, Nordlander, & Borg-Karlson, 2013; Huang, Miller, Chen, Vulule, & Walker, 2006; Stensmyr et al., 2012). The advantage for the microorganisms is less clear, but it is reasonable to assume that they may benefit from being vectored to new habitats or get protection in the insects during unfavourable conditions (Christiaens et al., 2014; Pozo et al., 2018).

Recent research suggests that chemical signalling and insect attraction is a conserved trait in yeasts. Bioassays using the vinegar fly *Drosophila melanogaster* to assess odours of

nine phylogenetically and ecologically distinct yeast species revealed that the flies were attracted to all yeast species studied (Becher et al., 2018). So far, only very little is known whether these results are also representative for bacteria, which often produce different mVOCs or use other biochemical synthesis pathways than yeasts (Dzialo et al., 2017; Schulz & Dickschat, 2007). However, there is already some evidence that insects are attracted to bacteria that live on or near hosts or preys (Leroy et al., 2012a; Leroy et al., 2012b; Mazzetto et al., 2016) and that the mVOCs produced by these bacteria can be exploited by natural enemies to locate their hosts or preys (Boone, Six, Zheng & Raffa , 2008; Dillon, Vennard, & Charnley, 2000; Leroy et al., 2012a). Furthermore, little is known about whether and how mVOCs mediate insect behaviour across trophic levels. Previous studies on plant-insect interactions have shown that herbivore-induced plant volatiles (HIPVs) are an important source of information mediating multitrophic interactions (Dicke & Baldwin, 2010; van Oudenhove, Mailleret, & Fauvergue, 2017), not only attracting primary parasitoids but also mediating the behavioural response of secondary parasitoids (also referred to as "hyperparasitoids" having primary parasitoids as their host) (Cusumano, Harvey, Dicke, & Poelman, 2019; Poelman et al., 2012). Virtually nothing is known so far on the role of microbial volatiles in the chemical ecology of hyperparasitoids.

Here, we asked the question whether mVOCs emitted by bacteria affect insect olfactory response, particularly parasitoids. Parasitoids constitute a very important group of natural enemies in the context of biological pest control, whose adult females lay eggs in or on other insects. The parasitoid larvae develop by feeding on the host bodies, eventually killing the host. Female parasitoids have to complete several foraging tasks during their adult lifetime to maximize reproductive success, including searching for suitable food sources, for a mating partner and for suitable hosts (Aartsma, Bianchi, van der Werf, Poelman, & Dicke, 2017; de Rijk, Dicke, & Poelman, 2013). Therefore, the olfactory response of female parasitoids and their efficiency in localizing and parasitizing hosts will have direct consequences on host-parasitoid population dynamics, and are hence key determinants of their effectiveness as biological control agents (Lewis, Vet, Tumlison, van Lenteren, & Papai, 1990; Mills, & Wajnberg, 2008). In previous research we have shown that mVOCs produced by nectar-inhabiting yeasts had a marked effect on the olfactory response of *Aphidius ervi* (Hymenoptera: Braconidae), a generalist primary parasitoid (Sobhy et al., 2018, 2019), but so far it is unclear whether bacterial odours elicit similar responses in parasitoids. Further, we asked whether mVOCs emitted by bacteria have similar effects on olfactory responses across

trophic levels. Experiments were performed using the primary aphid parasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) and one of its secondary parasitoids, *Dendrocerus aphidum* (Hymenoptera: Megaspilidae). Olfactory response was evaluated for mVOC blends emitted by bacteria isolated from diverse sources from the parasitoid's habitat, including hosts and host products (honeydew) and from the parasitoids themselves. The composition of the volatile blends produced by the bacteria was analysed using gas chromatography-mass spectrometry (GC-MS) to find out whether there were differences in mVOC profiles between attractive, neutral and repellent strains.

Materials and Methods

Study organisms

Insects

Experiments were performed using adult females of the primary parasitoid *A. colemani* and one of its hyperparasitoids, *D. aphidum*. *Aphidius colemani* is a generalist aphid parasitoid. *D. aphidum* is a generalist, secondary idiobiont ectoparasitoid attacking pre-pupal and pupal stages of hymenopteran primary parasitoids such as *Aphidius* spp. inside aphid mummies (Walker & Cameron, 1981). Both species preferentially feed on nectar and honeydew as a main source of sugars in their adult stage. *Aphidius colemani* was obtained in the form of parasitized aphid mummies from Biobest (Westerlo, Belgium) (Aphidius-system®). *Dendrocerus aphidum* was reared in the laboratory on fresh (1 day old) *Acyrthosiphon pisum* mummies parasitized by *A. ervi*. For both species, mummies were placed inside a nylon insect cage (20 cm × 20 cm × 20 cm, BugDorm, MegaView Science Co., Ltd., Taichung, Taiwan) and kept under controlled conditions (22°C, 70% relative humidity and a 16:8-h light:dark photoperiod) until parasitoid emergence. All experiments were performed with <24-h-old, food and water-starved females.

Bacteria

In total, 38 bacterial strains were used in this study (Table S1, Supporting Information). Strains were isolated from diverse sources sampled in greenhouses or a laboratory environment, including unparasitized aphids, aphid mummies, honeydew and *Aphidius* and *Dendrocerus* female adults. Studied strains represented a phylogenetically diverse collection of bacteria belonging to Actinobacteria, Firmicutes and Proteobacteria, which are typically associated with insects and insect-derived products (Engel & Moran, 2013, Grigorescu et al.,

2018; Leroy et al., 2011a; Luna et al., 2018). Further details on the isolation source (e.g. insect species or origin of honeydew) are given in Table S1 (Supporting Information). Honeydew was collected according to the procedure outlined by Leroy et al. (2011a). For the isolations from insect specimens, whole insects were used. Previous research has shown that insects can be attracted to volatiles produced by both symbiotic gut microorganisms and microbes on the exterior of the insects (Davis et al., 2013; Dillon et al., 2000; Leroy et al., 2011b; Mazzetto et al., 2016; Scheidler, Liu, Hamby, Zalom, & Syed, 2015). Insect specimens were homogenized with a motorized homogenizer (Precellys 24, Bertin Instruments, Montigny-le-Bretonneux, France) in 250 µL sterile physiological water (0.9% NaCl) with 0.01% Tween80 using 2 mm diameter glass beads, and then plated on tryptic soy agar (TSA; Oxoid, Hampshire, UK) supplemented with 0.3 g/L cycloheximide to prevent fungal growth. It has to be noted that this method not only samples bacteria that come in contact with the insect's environment, but may also yield endosymbionts living in specific host cells or compartments. Nevertheless, as such symbionts are generally not isolated through classical microbiological methods (Dale, Beeton, Harbison, Jones & Pontes, 2006), there is only a small chance that they were taken into account. For the isolations from honeydew, a 10-fold dilution series was plated on the same medium. Plates were incubated at 25°C for five days, and the most abundant morphotypes were purified and used for the study. Bacterial isolates were identified by amplifying and sequencing the 16S ribosomal RNA (rRNA) gene and comparison with the EzBiocloud 16S rRNA gene and whole-genome assembly database (Yoon et al., 2017). All isolates were kept in tryptic soy broth (TSB; Oxoid, Hampshire, UK) containing 25% glycerol at -80°C until further use.

Production of mVOCs

For production of mVOCs, the procedure of Sobhy et al. (2018) was followed with a few minor adjustments for bacteria. Briefly, bacterial stock cultures were plated on TSA and incubated at 25°C for 24h, followed by a re-streak on the same medium and incubation at 25°C for another 24h. Subsequently, single colonies were inoculated in 10 mL TSB and incubated overnight at 25°C in a rotary shaker at 120 rpm. Next, cells were washed twice in sterile physiological water (0.9% NaCl) and diluted in sterile physiological water until an optical density (OD 600 nm) of 1 was reached. Next, 1.5 mL of this cell suspension was used to inoculate a 250 mL Erlenmeyer flask containing 150 mL GYP25 medium prepared by filter-sterilizing (pore size 0.22 µm; Rapid-Flow™, Thermo Scientific, Waltham, USA) a medium of 5% w/v glucose (Sigma-Aldrich, Saint Louis, USA), 0.5% w/v peptone (Bacto™

Peptone, BD Biosciences, San Jose, USA) and 0.25% w/v yeast extract (Sigma-Aldrich, Saint Louis, USA). Erlenmeyer flasks were sealed with sterile silicone plugs and incubated at 25°C in a rotary shaker at 120 rpm for 48h. Each strain was cultivated in triplicate, and non-inoculated, blank medium was included as a negative control (also in triplicate). The GYP25 medium was selected to ensure abundant bacterial growth and mVOC production, while the medium itself had no significant effect on the parasitoid olfactory response. After incubation, the media were centrifuged for 15 min at 10,000 g, and subsequently filter-sterilized to obtain cell-free supernatants containing the produced mVOCs. The cell-free samples were then stored in small aliquots in sterile, amber glass vials at -20°C until further use.

Olfactometer bioassays

Insect olfactory response was evaluated using the Y-tube olfactometer bioassay described by Sobhy et al. (2018) (for details see Fig. S1, Supporting Information). The glass Y-tube olfactometer was placed on a table that was homogeneously illuminated by four high frequency 24W T5 TL-fluorescent tubes (16 x 549 mm, 1350 Lumen, 5500K; True-Light®, Naturalite Benelux, Ansen, The Netherlands) with a 96% colour representation of true daylight at a height of 0.45 m. To eliminate visual cues that could affect parasitoid responses, the olfactometer was fully enclosed with white curtains. Further, to improve parasitoid responsiveness, the olfactometer was positioned at a 20° incline to stimulate movement of the insects towards the bifurcation.

To test a given bacterial strain, 150 µL of the cell-free cultivation medium was loaded on a 37 mm-diameter filter paper (Macherey-Nagel, Düren, Germany) which was subsequently placed in one of the odour chambers, whereas in the second chamber another filter paper was placed on which 150 µL blank medium was added as a control. The bioassay was performed by releasing twelve consecutive cohorts of five adult females at the base of the olfactometer and evaluating their response 10 min after parasitoid release. Individuals that passed a set line at the end of one of the olfactometer arms (1 cm from the Y-junction) and remained there at the time of evaluation were considered to have chosen the odour source presented by that olfactometer arm. Parasitoids that did not make a choice at the time of evaluation were considered non-responding individuals and were excluded from the statistical analysis. New parasitoids were used for every release, and after every two releases the filter papers inside the odour chambers were renewed. To avoid positional bias, the odour chambers were rotated after every six cohorts. At the same time, the Y-tube glassware was also renewed by cleaned glassware. At the end of the assay, all olfactometer parts were

thoroughly cleaned with tap water, distilled water, acetone and finally pentane, after which the parts were placed overnight in an oven at 150°C. All bioassays were conducted at 21 ± 2°C, 60 ± 5% RH and performed between 09:00 and 16:00 h.

In a first experiment, bioassays were performed for *A. colemani* using one of the three medium replicates for all 38 bacterial strains investigated in this study. Further, to determine whether bacterial mVOC blends elicit the same response in primary and secondary parasitoids, a second experiment was performed for a subset of seven strains (see below). In this experiment, bioassays were performed using all three biological replicates with *A. colemani* and *D. aphidum*.

Chemical analysis of mVOCs

To determine the chemical composition of the mVOC blends, the cell-free cultivation medium of each biological replicate (n = 3) for the seven strains selected for the second experiment was analysed by headspace solid phase micro extraction gas chromatography followed by mass spectrometry detection (HS-SPME-GC-MS). The non-inoculated, sterile medium (n = 3) was used as a reference to find out how volatile composition changed by bacterial inoculation. GC-MS analyses were performed with a Thermo Trace 1300 system (Thermo Fisher Scientific, Watham, USA) fitted with a MXT-5 column (30 m length × 0.18 mm inner diameter × 0.18 µm film thickness; Restek, Bellefonte, USA) and a ISQ mass spectrometer (Thermo Fisher Scientific, Waltham, USA). 5 mL of each sample was supplemented with 1.75 g of NaCl and was kept at 60°C under constant agitation in a TriPlus RSH SMPE auto sampler (Thermo Fisher Scientific, Watham, USA). The HS-SPME volatile collection was conducted using an 50/30µm DVB/CAR/PDMS coating fibre (Supelco, Bellefonte, USA). Splitless injection was used with an inlet temperature of 320 °C, a split flow of 9 mL/min, a purge flow of 5 mL/min and an open valve time of 3 min. To obtain a pulsed injection, a programmed gas flow was used whereby the helium gas flow was set at 2.7 mL/min for 0.1 min, followed by a decrease in flow of 20 mL/min² to the normal 0.9 mL/min. The GC oven was programmed as follows: the temperature was initiated at 30°C, held for 3 min and then raised to 80°C at 7°C/min. Next, the temperature was raised to 125°C at 2°C/min, and finally the temperature was raised to 270°C at 8°C/min. Mass spectra were recorded in centroid mode using a mass acquisition range of 33 to 550 atomic mass units, a scan rate of 5 scans/s and an electron impact ionization energy of 70 eV. A mix of linear n-

alkanes (from C7 to C40, Supelco, Bellefonte, USA) were injected into the GC-MS under identical conditions to serve as external retention index markers.

Volatile compounds were identified and quantified as in Reher et al. (2019). Briefly, chromatograms were analysed with AMDIS v2.71 (Stein, 1999) to deconvolute overlapping peaks, and obtained spectra were manually annotated using the NIST MS Search v2.0g software, using the NIST2011, FFNSC and Adams libraries, taking into account the expected retention time. This resulted in a list of 245 tentatively identified target compounds that were present in the samples. To extract and integrate the compound elution profiles, a file was used with all our target 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 & Hanson, 1995). Finally, the peak areas were computed from the extracted profiles and summarized in a table. For all chemical compounds, the mean and standard error (SE) were calculated for every bacterial strain (n = 3). A univariate ANOVA was performed on the peak areas of the individual compounds to test for differences in compound concentration between bacterial strains and the blank medium followed by a Tukey's HSD test with adjusted P-values as calculated after correcting for multiple comparisons. A Kruskal-Wallis test was used when the data did not conform to the criteria of normality and homogeneity of variance required for a parametric statistical test. Compounds that did not show a significant difference in relative concentration compared to the blank medium in at least one bacterial mVOC profile were considered not to be related to bacterial activity, and were removed from the table. This resulted in a total of 97 different compounds that were retained in the dataset (Table S2, Supporting Information).

Data analysis

Olfactometer bioassays

For each bacterial strain, parasitoid olfactory response was analysed using a Generalized Linear Mixed Model (GLMM) based on a binomial distribution (binary: choice for either control side or treatment side) with a logit link function (logistic regression) using bacterial treatment as fixed factor (performed in R with the glmer function from the lme4 package). Each release of one cohort of five individuals served as a replicate. To adjust for overdispersion and to prevent pseudoreplication, the release of each cohort (n = 12) was included in the model as a random factor. For the second experiment, in which all three biological replicates of the strains were tested, biological replicate was included as a random

factor as well. The number of parasitoids choosing for the control or treatment side in each cohort was entered as response variable. Parasitoid response in each treatment was compared to a control in which parasitoids were provided a blank medium in both arms of the olfactometer, using analysis of variance Type III Wald chi-square tests in the GLMM. Results were presented by calculating the Preference Index (PI) by dividing the difference between the number of parasitoids choosing for the bacterial odours and the parasitoids choosing for the control by the total number of responding insects. Additionally, a GLMM was used to determine whether the source of isolation of the bacterial strains (i.e. aphid, parasitoid or honeydew) had a significant influence on the olfactory response of *A. colemani*, by using the number of parasitoids in each cohort choosing for either the control or the treatment side of the Y-tube as a dependent variable, and source of isolation as fixed factor. The release of each cohort (n = 12) was again included in the model as a random factor. Strains originating from hyperparasitoids and aphid mummies were excluded from the analysis, due to the low numbers of strains representing these habitats.

Chemical analysis

To visualize the differences in the mVOC composition, a heat map was constructed from strain*volatile peak area matrix of the mean-centered, log transformed data, using the comprehensive online tool suite MetaboAnalyst 4.0 (Chong et al., 2018). Additionally, a non-metric multidimensional scaling (NMDS) was performed on the strain*volatile peak area matrix by using a Bray-Curtis distance matrix (Vegan package V2.4-6 in R). A permutational multivariate analysis of variance (perMANOVA) was carried out on the strain*volatile peak area matrix to test for significant differences in chemical composition of mVOCs produced by the tested strains, based on 1000 permutations. The analysis was performed by using the adonis function (Vegan package V2.4-6) in R. To further elucidate differences in mVOC composition at the level of compound classes, a univariate ANOVA followed by a Tukey's HSD test was performed on the summed peak areas of the compounds belonging to the same chemical class when strains were grouped according to olfactory response. Specifically, data were combined for strains evoking parasitoid attraction, repellence or a neutral response. Chemical classes generally induce similar responses in insects (Dzialo et al., 2017). However, caution should be taken when interpreting results as this is not always the case, e.g. for terpenes (Raffa, 2014). All statistical analyses and evaluation of normality and homoscedasticity of the data were performed in R 3.3.2 (R Core Team, 2014).

Results

Olfactory response of A. colemani *to bacterial volatile emissions*

Olfactory response of *A. colemani* varied significantly between the volatile emissions of the 38 bacterial strains tested ($\chi^2_{(38)}$ = 74.71, P < 0.001; Fig. 1). One bacterial strain (ST18.16/150) was found to significantly attract *A. colemani* (PI = 0.36, P = 0.048), while one other strain (ST18.17/002) was significantly deterrent (PI = -0.41, P = 0.039). Volatile blends emitted by the other strains and the blank medium had no statistically significant effect on the olfactory response of *A. colemani* (Fig. 1). Most strains having high PI-values belonged to the genus *Bacillus*, while strains belonging to the genus *Staphylococcus* showed relatively low PI-values. When evaluating the effect of origin, only honeydew had a significant influence on the olfactory response of *A. colemani* ($\chi^2_{(2)}$ = 17.9, P < 0.001). *Aphidius colemani* showed significantly lower PI-values when exposed to mVOCs produced by bacteria originating from honeydew.

Differences in olfactory response between A. colemani *and* D. aphidum

In order to test whether bacterial mVOC emissions elicited the same response in the primary parasitoid and one of its secondary parasitoids, olfactory responses of *A. colemani* and *D. aphidum* were compared for a selection of strains using three independent biological replicates for each strain. Selected strains included three strains having the highest PI-value when tested against *A. colemani* (ST18.16/150, ST18.16/043 and ST18.16/133), three strains with the lowest PI-value (ST18.17/002, ST18.17/028 and ST18.16/160) and one strain with a PI-value close to zero (ST18.16/085) (Fig. 1). Results confirmed that the strains with the highest PI-values were significantly attractive to *A. colemani* ($P \leq 0.005$), while the strains with the lowest PI-values significantly repelled *A. colemani* ($P \leq 0.017$) (Fig. 2). Results also showed that insect response differed between the tested insect species (Fig. 2). Volatile emissions from the strains that were significantly attractive to *A. colemani* had no significant effect on the olfactory response of *D. aphidum*. Further, the volatile emissions of two strains that were repellent to *A. colemani* were also significantly repellent to *D. aphidum* (ST18.17/002: PI = -0.37, P = 0.003; ST18.17/028: PI = -0.25, P = 0.043). By contrast, the mVOC mixture emitted by ST18.16/085, which was neutral to *A. colemani*, was significantly attractive to *D. aphidum* (PI = 0.31, P = 0.006). Additionally, strain ST18.16/160 which was

repellent to *A. colemani*, tended to attract *D. aphidum*, albeit not significantly (PI = 0.18, P = 0.102) (Fig. 2).

mVOC composition

The mVOC composition differed significantly between the seven bacterial treatments and the blank medium (perMANOVA: F = 38.6, P < 0.001). Overall, volatiles produced in the highest amounts belonged to alcohols, esters, ketones and organic acids (Table S2, Supporting Information; Fig. S2, Supporting Information). For a few compounds, concentrations were significantly higher in the blank medium compared to the bacterial treatments (i.e. phenylacetaldehyde, nonane, methyl pyrazine and 2-propyl-1,3-dioxolane), indicating that some compounds were partly consumed or converted during cultivation (Table S2, Supporting Information; Fig. S2, Supporting Information). NMDS ordination of the mVOC composition (Fig. 3) separated strain ST18.16/133 and to a lesser extent strain ST18.17/002 from the rest of the bacterial strains along the first NMDS axis. The second NMDS axis led to further separation of the strains, particularly separating the three *Bacillus* strains (ST18.16/133, ST18.16/043 and ST18.16/150) from the rest of the strains (Fig. 3). Notably, these three strains elicited significant attraction in *A. colemani* (Fig. 2). Additionally, the NMDS showed that the composition of the volatile blends of these strains (especially ST18.16/043 and ST18.16/150) was more closely related to the blank medium in comparison with the other strains (Fig. 3).

Differences in mVOC composition between attractive, neutral and repellent strains

Grouping the strains based on the effect of their mVOC blends on the olfactory response of the parasitoids (attraction, repellence or neutral response) showed that the strains repellent to *A. colemani* (ST18.16/160, ST18.17/028 and ST18.17/002) produced significantly higher amounts of esters, organic acids, aromatics and cycloalkanes, when compared to the attractive strains (ST18.16/150, ST18.16/133 and ST18.16/043) (Fig. 4; Table S2, Supporting Information; Fig. S2, Supporting Information). The strains repellent to the hyperparasitoid *D. aphidum* (ST18.17/028 and ST18.17/002) produced significantly higher amounts of alcohols and ketones, whereas the strain significantly attractive to *D. aphidum* (ST18.16/085) produced higher amounts of alkenes (especially 9-methyl-1-decene) and the three monoterpenes limonene, linalool and geraniol (Fig. 5; Table S2, Supporting Information; Fig.

S2, Supporting Information). These terpenes were also produced in high amounts by ST18.16/160 which yielded a relatively high PI-value as well (PI = 0.18), although not significantly attractive.

Discussion

In this study, we assessed the olfactory response of a generalist aphid parasitoid and one of its secondary parasitoids to mVOCs produced by phylogenetically diverse bacteria isolated from the habitat of the parasitoids. Further, we investigated whether the chemical composition of the mVOC blends differed between attractive, neutral and repellent strains. It has to be noted that the bacterial strains used here originated from samples collected from greenhouses and laboratory environments. Given the fact that insect microbiomes are partly acquired from their host's environment (Hannula, Zhu, Heinen, & Bezemer, 2019; Jones et al., 2018), it cannot be excluded that the strains investigated may not be representative for what the insects would carry in more natural situations. However, most of the bacteria investigated here were previously found in association with aphids, parasitoids and their environment (Grigorescu et al., 2018; Leroy et al., 2011a; Luna et al., 2018), reinforcing the robustness of our results.

Olfactory response of A. colemani *to bacterial VOCs depends on bacterial strain*

Our results show that *A. colemani* females responded differently to the various bacteria occurring in the parasitoid's habitat. Based on the experiments performed in this study, three significantly attractive and three significantly repellent strains to *A. colemani* were identified, while the majority of strains did not have a significant effect. Strikingly, all three attractive strains (as well as other strains with relatively high PI-values) belonged to the genus *Bacillus*. *Bacillus* species are ubiquitous in nature and are known to produce a wide array of volatiles (Kai et al., 2009), some of which can promote plant growth without physical contact (Ping & Boland, 2004) or have antimicrobial activity (Gao, Zhang, Liu, Han & Zhang, 2017). Additionally, a number of studies have shown that *Bacillus* volatiles may also affect insect behaviour. For example, both Rockett (1987) and Poonam and colleagues (Poonam, Paily, & Balaraman, 2002) showed that volatiles produced by *Bacillus* species exhibited oviposition stimulation in *Culex* females. Furthermore, the melon fruit fly *Bactrocera cucurbitae* was attracted to broth cultures of *Bacillus cereus* (Mishra, Sharma, & Subramanian, 2018). Strikingly, one of the attractive *Bacillus* strains (ST18.16/043) was isolated from the hyperparasitoid *D. aphidum*, which complicates predictions regarding the adaptive value of

responding to microbial volatiles. Given these observations, our results seem to suggest that mVOC-mediated insect responses may be correlated with bacterial phylogeny. Recent studies indicate that the phylogeny of microorganisms may reflect functional traits and ecological characteristics, pointing towards phylogenetic conservatism in phenotypic traits (Martiny, Jones, Lennon, & Martiny, 2015; Martiny, Treseder, & Pusch, 2013). However, it is unclear so far whether there are also phylogenetic signals in mVOC composition and insect response.

Olfactory response to bacterial VOCs differs between primary and secondary parasitoids

Primary parasitoids and their secondary parasitoids often forage for similar resources in the same habitat, and share part of their decision-making strategy in host finding (Aartsma et al., 2019). Therefore, it may be expected that generalist species such as *A. colemani* and *D. aphidum* respond similarly to olfactory cues occurring in their habitat. Our findings showed that responses of *A. colemani* were different from the responses of *D. aphidum*. Particularly, the *Bacillus* strains attractive to *A. colemani* did not elicit a significant olfactory response in its hyperparasitoid. Furthermore, it was found that one of the three strains that was significantly repellent to *A. colemani* (ST18.16/160; putatively identified as *Staphylococcus saprophyticus*) yielded a relatively high PI-value for *D. aphidum*, pointing towards attraction, albeit without statistical support. Additionally, the strain that was neutral to *A. colemani* (ST18.16/085; *Curtobacterium* sp.) elicited a significant attractive response in the hyperparasitoid. Hence, this suggests that the olfactory response of primary and secondary parasitoids towards mVOCs is different, as has also been found for HIPVs (Cusumano et al., 2019; Poelman et al., 2012).

Bacterial VOCs resemble plant and insect volatiles

The bacterial VOC blends comprised typical microbial fermentation products, such as methylated, low molecular weight alcohols and corresponding aldehydes and organic acids (Dzialo et al., 2017; Schmidt, Cordovez, de Boer, Raaijmakers, & Garbeva, 2015). However, some compounds like geraniol, linalool, limonene, 2-phenylechtanol, phenylacetaldehyde and acetophenone are also commonly reported as typical plant volatiles (Bruce & Pickett, 2011; Dudareva, Klempien, Muhlemann, & Kaplan, 2013). Moreover, certain compounds have been reported as insect pheromones, e.g. acetoin, 2,3-butanediol, 2-(3,3-dimethylcyclohexylidene)-ethanol, linalool and nonan-2-ol (Borg-Karlson et al., 2003; Löfstedt et al., 2008; Rochat et al., 2002). Nevertheless, it has to be noted that so-called "insect pheromones" are not necessarily produced by the insects themselves, but may also be

derived from the gut bacteria of insects (Dillon et al., 2000). This could also suggest that volatiles detected from plants are not necessarily (only) produced by the plants themselves, which may also explain the considerable variation in plant volatiles, even when exposed to similar conditions (Takabayashi, Dicke, & Posthumus, 1994).

Compared to plant and insect volatiles, still very little is known about the ecological role and biological function of mVOCs in the foraging behaviour of insects. However, there is increasing evidence that mVOCs signal important aspects of habitat or food suitability for foraging insects. For example, Leroy et al. (2011a) showed that aphid honeydew is particularly attractive to aphid natural enemies when it is contaminated with an aphid-associated bacterium like *Staphylococcus sciuri* producing mVOCs that act as effective attractants and ovipositional stimulants. However, in contrast with this study, our results suggest that *A. colemani* parasitoids are not attracted to, and can even be repelled by mVOCs produced by bacteria originating from aphid honeydew. Further research is needed to better understand the biological role of microbial volatiles in volatile-mediated foraging behaviour.

Differences in mVOC profiles between attractive, neutral and repellent strains

In general, tested strains emitted a similar set of volatile compounds, and most mVOCs produced by the strains that were attractive to *A. colemani* were also produced by the neutral and repellent strains, but often in lower concentrations and in significantly different ratios. This suggests that mVOCs may elicit a different response in insects depending on the concentration of the compounds and the composition of the blend, most probably determined by the presence of particular bioactive compounds or specific ratios of ubiquitous compounds (Bruce, Wadhams, & Woodcock, 2005; Bruce, Webster, Pickett, & Hardie, 2010; Mumm & Hilker, 2005; Takemoto & Takabayashi, 2015). More specifically, the mVOC blends of the strains attractive to *A. colemani* had lower concentrations of esters, aromatics, organic acids and cycloalkanes when compared to the composition of the mVOC mixtures emitted by the repellent strains. This might indicate that *Aphidius* parasitoids require lower concentrations of these compounds to become attractive or that the concentrations in the repellent mixture were too high and masked otherwise attractive compounds (Aartsma et al., 2017).

As was found for *A. colemani*, the chemical composition of the mVOC blends also differed between attractive and repellent strains for *D. aphidum*. In particular, strains attractive to *D. aphidum* produced significantly greater amounts of monoterpenes, while repellent strains emitted significantly greater amounts of alcohols and ketones. The monoterpenes produced included limonene, geraniol and linalool, which are known as typical

plant volatiles, many of which have been shown to be attractive to several insect species, including natural enemies (Koschier, De Kogel, & Visser, 2000; McCormick, Unsicker, & Gershenzon, 2012).

Surprisingly, the mVOC composition of strain ST18.16/133, which was attractive to *A. colemani*, and the repellent strain ST18.17/002 were quite similar, yet they elicited opposite olfactory responses. This suggests that changes in ratios of a select number of compounds can reverse the behavioural response of insects. Indeed, it has previously been shown that changing the concentration of certain compounds in an attractive blend of ubiquitous plant volatiles can disrupt attraction of herbivorous insects (Bruce & Pickett, 2011). Moreover, we have to take into account that often just a fraction of the volatile compounds present in the environment can be detected and subsequently cause a behavioural response in insects (Bruce & Pickett, 2011). Therefore, insect behaviour does not always reflect complete mVOC profiles, but rather the concentration and ratio of a select number of compounds that are detected by the insects (Conchou et al., 2019).

Concluding remarks

Although our study has greatly contributed to our understanding of the role of mVOCs in insect behavioural ecology, the next challenge is to study their ecological role and influence under more natural conditions. In this study, experiments were performed under controlled conditions in a clean environment using laboratory bioassays. However, in their natural environment, insects encounter numerous volatile signals, from different sources and in different concentrations, from which they need to derive reliable information for accurate behavioural decisions (Aartsma, et al., 2017). It has been shown that background odours can have different effects on volatile-mediated foraging behaviour. Background odour can be irrelevant and not interact with foraging behaviour, or may mask resource-indicating target cues, thereby reducing the response of insects to attractants. Additionally, there is some evidence that background odours may also enhance insect response to cues indicating the presence or suitability of resources (Schröder & Hilker, 2008). Emission of mVOCs, including their chemical composition, is also dependent on a variety of factors, including growth stage of the microbes, nutrient availability, temperature, oxygen availability, pH, etc. (Tyc, Song, Dickschat, Vos, & Garbeva, 2017). Future experiments should therefore be performed to investigate to what extent the mVOCs measured here mimic those that are emitted under more natural conditions, and how parasitoids will experience mVOCs in more natural settings, in combination with food, host or habitat odours, like HIPVs.

Altogether, we have shown that insect responses to bacterial volatile emissions depend on the bacterial strain. Further, we have shown that the olfactory response of an aphid parasitoid and one of its hyperparasitoids to bacterial VOCs is different, and that mVOC composition differed between attractive, neutral and repellent strains. Future research should focus on how these mVOCs influence insect behaviour when perceived with other cues related to food and hosts.

Authors' contributions

TG, ISS, FW, HR, HJ and BL conceived the ideas and designed methodology. TG and CV collected the data. FD, FF, KJV and TW contributed to equipment and reagents, and contributed to the mVOC analysis. JDB and FW provided insects required for experiments. TG, CV, TW, HJ and BL analysed the data. TG, HJ and BL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. The authors have declared that no competing interests exist.

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Data accessibility

16S rRNA gene sequences of the studied bacteria have been deposited in GenBank under the Accession Numbers MK875098– MK875135. Further, underlying experimental data can be found at the Dryad Digital Repository https://doi.org/10.5061/dryad.fj6q573q9 (Goelen et al., 2019).

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Bacterial isolates used in this study

Table S2 Microbial volatile organic compound (mVOC) composition of the cell-free cultivation media used in this study

Figure S1 Schematic representation of the two-choice Y-tube olfactometer used in the bioassays

Figure S2 Heat map of the mVOC composition of seven bacterial strains investigated in this study

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Figure captions

Figure 1. Olfactory response of adult *Aphidius colemani* females when given a choice between the odour of a test bacterium (n = 38) grown in GYP25 medium and the odour of the blank GYP25 medium in a Y-tube olfactometer. Insect response is expressed as the Preference Index (PI). In total, 60 individuals were tested (12 releases of 5 females). Non-responders were excluded from the statistical analysis. Grey bars indicate non-significant olfactory responses ($P > 0.05$), green bars indicate significant attractive responses ($P \leq 0.05$) and red bars indicate significant repellent responses ($P \leq 0.05$) when compared to the control in which parasitoids were offered GYP25 medium in both arms of the Y-tube olfactometer. Error bars represent standard error of the mean. Overall parasitoid responsiveness was higher than 80%. Coloured symbols indicate the source of isolation. Strains that were selected for the remainder of the study are indicated in bold

Figure 2. Different olfactory response of adult *Aphidius colemani* females (A) and adult *Dendrocerus aphidum* females (B) when given a choice between the odour of a test bacterium grown in GYP25 medium and the odour of the blank GYP25 medium in a Y-tube olfactometer. Insect response is expressed as the mean Preference Index (PI) obtained for three biological replicates (n = 3; per replicate, 60 individuals were tested in 12 releases of 5 females). Non-responders were excluded from the statistical analysis. Grey bars indicate non-significant olfactory responses ($P > 0.05$), green bars indicate significant attractive responses ($P \leq 0.05$) and red bars indicate significant repellent responses ($P \leq 0.05$) when compared to the control in which parasitoids were offered blank GYP25 medium in both arms of the Y-

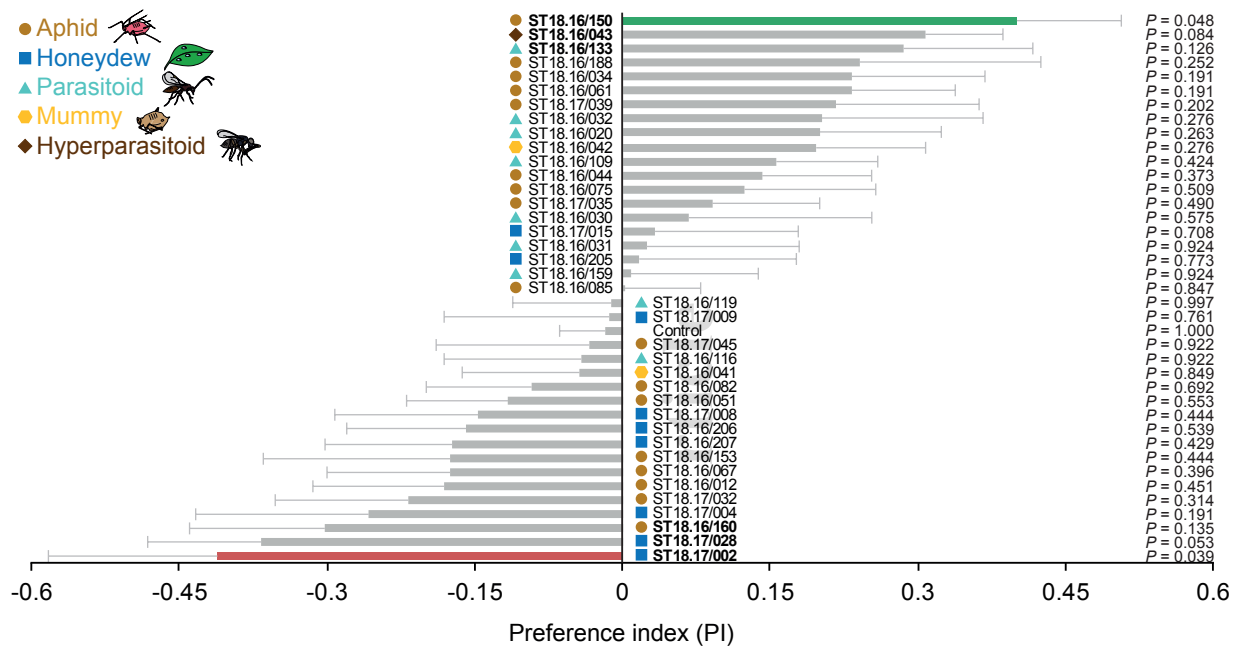
tube olfactometer. Error bars represent standard error of the mean. Overall parasitoid responsiveness was higher than 70%

Figure 3. Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities of the mVOC composition of seven bacterial strains investigated in this study (n = 3) (stress value = 0.158). The mVOC composition differed significantly between the bacterial strains and the blank medium (perMANOVA: F = 38.6, P < 0.001). Symbol colours indicate the effect of the mVOC blends on the olfactory response of the primary parasitoid *Aphidius colemani*, i.e. green = attractive, grey = neutral, and red = repellent. Blue refers to the blank medium. Symbol shapes indicate the effect of the mVOC blends on the olfactory response of the hyperparasitoid *Dendrocerus aphidum*, i.e. circle = attractive, square = neutral and cross = repellent

Figure 4. Linkage between the volatile composition of the cell-free bacterial cultivation media and the olfactory response of *Aphidius colemani*. Results are shown for alcohols (A), aldehydes (B), esters (C), ketones (D), alkanes (E), cycloalkanes (F), alkenes (G), aromatics (H), organic acids (I), terpenes (J), and miscellaneous (K). Presented values are the sum of peak areas of corresponding compounds per chemical class as detected by the MXT-5 equipped GC-MS, and the result from three biological replicates (n = 3). Bacterial strains are grouped by the effect of their mVOCs on the olfactory response of the tested parasitoid: Attractive = ST18.16/150, ST18.16/133 and ST18.16/043; Neutral = blank medium and ST18.16/085; Repellent = ST18.17/002, ST18.17/028 and ST18.16/160. Different letters indicate significant differences ($P \leq 0.05$) between bacterial strains based on an univariate ANOVA or Kruskal-Wallis non-parametric test

Figure 5. Linkage between the volatile composition of the cell-free bacterial cultivation media and the olfactory response of *Dendrocerus aphidum*. Results are shown for alcohols (A), aldehydes (B), esters (C), ketones (D), alkanes (E), cycloalkanes (F), alkenes (G), aromatics (H), organic acids (I), terpenes (J), and miscellaneous (K). Presented values are the sum of peak areas of corresponding compounds per chemical class as detected by the MXT-5 equipped GC-MS, and the result from three biological replicates (n = 3). Bacterial strains are grouped by the effect of their mVOCs on the olfactory response of the tested parasitoid: Attractive = ST18.16/085; Neutral = blank medium, ST18.16/150, ST18.16/133, ST18.16/043 and ST18.16/160; Repellent = ST18.17/002 and ST18.17/028. Different letters

indicate significant differences ($P \leq 0.05$) between bacterial strains based on an univariate ANOVA or Kruskal-Wallis non-parametric test


Aphid
Honeydew
Parasitoid
Mummy
Hyperparasitoid
ST18.16/150 P = 0.048
ST18.16/043 P = 0.084
ST18.16/133 P = 0.126
ST18.16/188 P = 0.252
ST18.16/034 P = 0.191
ST18.16/061 P = 0.191
ST18.17/039 P = 0.202
ST18.16/032 P = 0.276
ST18.16/020 P = 0.263
ST18.16/042 P = 0.276
ST18.16/109 P = 0.424
ST18.16/044 P = 0.373
ST18.16/075 P = 0.509
ST18.17/035 P = 0.490
ST18.16/030 P = 0.575
ST18.17/015 P = 0.708
ST18.16/031 P = 0.924
ST18.16/205 P = 0.773
ST18.16/159 P = 0.924
ST18.16/085 P = 0.847
ST18.16/119 P = 0.997
ST18.17/009 P = 0.761
Control P = 1.000
ST18.17/045 P = 0.922
ST18.16/116 P = 0.922
ST18.16/041 P = 0.849
ST18.16/082 P = 0.692
ST18.16/051 P = 0.553
ST18.17/008 P = 0.444
ST18.16/206 P = 0.539
ST18.16/207 P = 0.429
ST18.16/153 P = 0.444
ST18.16/067 P = 0.396
ST18.16/012 P = 0.451
ST18.17/032 P = 0.314
ST18.17/004 P = 0.191
ST18.16/160 P = 0.135
ST18.17/028 P = 0.053
ST18.17/002 P = 0.039
-0.6
-0.45
-0.3
-0.15
0
0.15
0.3
0.45
0.6
Preference index (PI)