Journal of Pest Science
https://doi.org/10.1007/s10340-023-01687-5

ORIGINAL PAPER

Chemical cues from honeydew-associated bacteria to enhance parasitism efficacy: from laboratory to field assay

Jiahui Liu[1,2] · Dianzhao Xiao[1] · Yulong Liu[1] · Yidi Zhan[1] · Frederic Francis[2] · Yong Liu[1]

Received: 29 March 2023 / Revised: 8 July 2023 / Accepted: 8 August 2023

Abstract

Honeydew from Hemipteran insects serves as carbohydrate source to beneficial insects but also to various microorganisms. Microbial volatile organic compounds (mVOCs) may play diverse roles in herbivore–microbe–natural enemy interactions. However, the functional significance of mVOCs from aphid honeydew remains largely unclear. In this study, a total of seven cultivable bacteria from *Sitobion miscanthi* honeydew have been isolated and identified based on 16S rRNA technique, which included *Lysinibacillus fusiformis*, *Erwinia aphidicola*, *E. tasmaniensis*, *Acinetobacter bereziniae*, *Klebsiella quasipneumoniae subsp. Similipneumoniae*, *Staphylococcus capitis* and *Bacillus safensis subsp. safensis*. One bacterial strain, *L. fusiformis* MH1, was found to be most attractive to *Aphidius gifuensis* parasitic wasp in Y-tube olfactometer. Two compounds, namely 1-ethyl-2-methylbenzene and 2-butyl-1-octanol, were emitted from *L. fusiformis* MH1 and were attractive to *A. gifuensis* and identified by using coupled gas chromatography–electroantennography and coupled gas chromatography with mass spectrometry. Application of bacterial and mVOCs formulations in crop field resulted in significant aphid abundance decrease associated with higher parasitism rates compared with control. Our results indicated that some microbes in aphid honeydew could manipulate the herbivore–natural enemy interactions and could be developed as a novel alternative for environmentally friendly biological control of aphids.

Keywords Behavioral manipulation · Biological control · Honeydew · Microorganism · Microbial volatile organic compounds

Key messages

- Seven bacteria were isolated and identified from *S. miscanthi* honeydew.
- *Lysinibacillus fusiformis* MH1 was the most attractive bacterium to *A. gifuensis*
- 1-ethyl-2-methylbenzene and 2-butyl-1-octanol were the functional compounds
- *Lysinibacillus fusiformis* MH1 and key compounds in field reduced aphid abundance by increasing parasitism rate.

Introduction

Hemipteran insects such as aphids, whiteflies, soft scales, mealybugs and plant hoppers have piercing–sucking mouthparts that extract host plant sap. In addition to stealing nutrients, many Hemiptera can transmit plant viruses and excrete honeydew inhibiting plant photosynthesis and causing global crop production losses (Douglas 2006; Quesada et al. 2020). Honeydew excreted by piercing–sucking insects plays an important role in multitrophic interactions between herbivores, microbes and their natural enemies in many ways. Honeydew is rich in nutrients such as sugars, organic acids, amino acids and some lipids that could serve as a food

Communicated by Jay Rosenheim.

✉ Frederic Francis
frederic.francis@uliege.be

✉ Yong Liu
liuyong@sdau.edu.cn

[1] College of Plant Protection, Shandong Agricultural University, No.61, Daizong Road, Taian 271018, Shandong, China

[2] Functional and Evolutionary Entomology, Gembloux Agro-Bio Tech, University of Liege, Passage des Déportés 2, 5030 Gembloux, Belgium

Published online: 29 August 2023

source exploited by many beneficial insects, including bees, ants, parasitic wasps and predators (Wäckers et al. 2008; Leroy et al. 2011; Tena et al. 2016). Natural enemies that feed on honeydew can increase their longevity and effectiveness as biological control agents (Tena et al. 2012; Watanabe et al. 2014; Rand and Waters 2020). Also, honeydew serves as host-location cues and\or oviposition stimuli for predators and parasitoids such as *Aphidius rhopalosiphi* De Stef. (Hymenoptera: Braconidae) (Wickremasinghe and van Emden 1992), *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiinae) (Choi et al. 2004; Watanabe et al. 2016) and *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) (Ayelo et al. 2022).

There is increasing evidence that microbial volatile organic compounds (mVOCs) can manipulate the behaviors of herbivores and associated natural enemies. In some cases, mVOCs attracted insects by signaling the presence of appropriate resources such as food sources and oviposition sites (Leroy et al. 2011; Becher et al. 2012; Rering et al. 2018). Besides locating food sources, mVOCs can also be exploited by natural enemies to locate hosts or prey, and even stimulate oviposition, thereby providing indirect pest control (Leroy et al. 2011; Francis et al. 2020).

Honeydew-inhabiting microorganisms have been found to play an important role in herbivore–microorganism–natural enemy interactions. *Staphylococcus sciuri* isolated from pea aphid honeydew strongly stimulated oviposition of *Episyrphus balteatus* (De Geer) (Diptera: Syrphidae) by mVOCs (Leory et al. 2011). Cues associated with mealybug honeydew bacterial volatiles allowed *Anagyrus dactylopii* (Howard) (Hymenoptera: Encyrtidae) to locate their hosts (Fand et al. 2020). Consequently, the use of honeydew-related cues can increase the calling of natural enemies and will open up new avenues for non-chemical pest management strategies.

Wheat is the most important food crop worldwide impacted by several aphid species representing major global pests (Liu et al. 2020). *Sitobion miscanthi* (formerly named *S. avenae)*, a phloem feeder and vector of plant viruses, damages approximately 14.6 million km^2 of wheat per year and causes up to 40% wheat yield loss in China (Zhou et al. 2016; Liu et al. 2021). To control *S. miscanthi*, conventional chemical insecticides were applicated leading to many well-known problems, such as target pest resistance and chemical residue occurrence in agro-ecosystems. Even integrated pest management (IPM)-recommended insecticides like thiamethoxam, imidacloprid and pymetrozine can harm beneficial insects through honeydew (Calvo-Agudo et al. 2019, 2020). Therefore, environmentally friendly pest management strategies are urgent needs to control aphids such as *S. miscanthi*. *Aphidius gifuensis*, a solitary koinobiont endoparasitoid, is a commonly augmented aphidophagous specialist regulating *S. miscanthi* populations (Liu et al. 2016). In China, this wasp has been widely bred and used as a biocontrol agent for more than four decades to control aphids (Li et al. 2021).

Honeydew has not been sufficiently studied in the context of conservative biological control (Tena et al. 2016) even if it was already found to enhance the performance of some parasitoids (Wäckers et al. 2008; Tena et al. 2016; Benelli et al. 2017; Ribeiro de Campos et al. 2020). *A. gifuensis* can locate *S. miscanthi* by plant-derived volatiles methyl salicylate (MeSA) and host-derived volatiles such as aphid alarm pheromone (E)-β-farnesene (EβF) in wheat fields (Wang et al. 2019; Liu et al. 2021). We hypothesize that *A. gifuensis* can use mVOCs from *S. miscanthi* honeydew to locate this host species. Therefore, the objectives of this study were to (1) determine whether *S. miscanthi* honeydew and associated microorganisms can attract *A. gifuensis*, (2) assess the attraction of honeydew-isolated bacteria to *A. gifuensis*, (3) identify mVOCs eliciting electrophysiological responses of *A. gifuensis*, (4) evaluate the attraction of single and blend of coupled gas chromatography-electroantennography (GC-EAG) active volatiles to *A. gifuensis*, (5) perform field experiments for biocontrol efficacy evaluation of bacteria and semiochemical formulations based on laboratory results.

Materials and methods

Plants and insects

Wheat (cultivar 'Jimai 22') used for rearing *S. miscanthi* was planted in a plastic basin (9 cm × 8 cm) in a glasshouse under controlled conditions: 22 ± 2 °C, 65 ± 5% relative humidity and 16 h light photoperiod. *S. miscanthi* and *A. gifuensis* were collected from wheat plants in the experimental farmland at Shandong Agricultural University (Shandong, China) and transferred to controlled chambers under similar conditions as described above. *A. gifuensis* was continuously reared in cages (75 cm × 60 cm × 90 cm) with wheat plants and aphids. After *S. miscanthi* parasitization, mummified aphids were collected individually and placed into small test tubes for hatching. Adult parasitoids were used for olfactometry tests within 1 day of emergence, with no previous oviposition experience or contact with plants.

Honeydew collection

Several wheat leaves were placed into sterile plastic Petri dishes (9 cm × 1.5 cm) with 1% agar solution to keep moisture, infested with *S. miscanthi* and covered with paraffin film before turning the Petri dish upside down. Honeydew droplets naturally fell on the paraffin film and were collected with a glass capillary tube after 24 h.

Isolation of honeydew microbial content

To investigate microbiota composition, 20 µL of *S. miscanthi* honeydew was diluted in sterile water to concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Then, 100 µL of each diluted honeydew samples was inoculated on 863 agar medium (2% of agar and 10 g each of glucose, yeast extract and casein peptone per liter) (Leroy et al. 2011). Each concentration was repeated three times, and sterile water was used as a control. Plates were incubated at 32 °C for 1 day. Most abundant bacterial morphotypes were selected, and colonies representing each morphotype were reinoculated onto 863 agar medium more than five times to obtain pure cultures of these honeydew bacteria. For sequencing the 16S ribosomal RNA (16S rRNA), three colonies were selected from each bacterial strain.

Primers used for PCR amplification of 16S rRNA sequences were universal primers 27F (5′-AGAGTTTGA TCCTGGCTCAG-3′) and 1492R (5′-TACGACTTAACC CCAATCGC-3′). The PCR mixture contained 2 × Accurate Taq Master Mix 22.5 uL, 27F 1 uL, 1492R 1 uL and bacteria 0.5 uL. Running parameters were 35 cycles of 40 s at 95 °C, 40 s at 53 °C, and 1.5 min at 72 °C, with a subsequent final extension at 72 °C for 10 min. Bacterial isolates were identified by amplifying and sequencing the 16S rRNA gene and comparison with the EzBiocloud 16S rRNA gene. All isolates were kept in 863 medium containing 25% glycerol at − 80 °C until further use.

Microbial volatile organic compound collection

For each biological replicate ($n = 3$), microbial volatiles were collected by air entrainment (dynamic headspace collection, Fig S1). The volatiles of medium inoculated by 7 bacteria strains isolated from *S. miscanthi* honeydew were individually collected. Non-inoculated-sterile medium ($n = 3$) was used as control. For each headspace collection, 5-mL bacteria-inoculated medium with an optical density (OD) of 1 was put in a 250-mL glass bottom. Air was purified by a passage through an activated charcoal filter and was pumped into the vessel through the inlet port at 600 mL min^{-1}. Air subsequently passed over the microbial in the glass bottom and headspace volatiles were adsorbed on 50 mg Porapak Q (Alltech Associates, Carnforth, Lancashire, U.K.) that were fitted on the outlet port through which air was drawn at a rate of 400 mL min^{-1}. The difference in the flow rate created a slight positive pressure to prevent unfiltered air from entering into the system. All connections were made with a poly (tetrafluoroethylene) (PTFE) tubing with brass ferrules and fittings were then closed with parafilm. Porapak Q-filled tubes were conditioned before use by washing with *n*-hexane (5 mL) and heating inside an oven (150 °C) under a stream of nitrogen for a minimum of 2 h. All connections in the air entrainment setup were sterilized. Volatiles were collected for 24 h on Porapak Q tubes inserted into the collection port on the top of the vessel and were subsequently eluted with 5 mL of fresh hexane and were stored at − 20 °C until further use.

Chemical analysis of microbial volatile organic compounds

Volatile patterns were analyzed on an Agilent 7890A gas chromatograph (GC, Agilent Technologies, Palo Alto, CA, USA) and a 7000D mass spectrometer (MS, Agilent Technologies, Palo Alto, CA, USA) using an HP-5 MS 5% diphenyl-95% dimethyl polysiloxane capillary column (30.0 m × 250 µm × 0.25 µm, Agilent Technologies). Before analysis, air entrainment samples were concentrated to 1 mL under an activated charcoal-filtered nitrogen stream. 1 µL of each headspace extract was injected into the GC. Helium (99.9% purity) was used as the carrier gas at a column flow rate of 1 mL·min^{-1} and a precolumn pressure of 7.0699 Psi. The column temperature regime was 40 °C for 2 min, followed by a 7 °C min^{-1} ramp-up to 150 °C for 5 min, and a 10 °C min^{-1} ramp-up to 250 °C which was maintained for 3 min. Ionization was performed by electron impact at 70 eV and 220 °C. The ion-source temperature was 250 °C. Mass spectra were recorded in centroid mode using a mass acquisition range of 35–350 atomic mass units, a scan rate of 5 scans/s. Peak identities were tentatively determined by manually comparing mass spectra with those from mass spectral databases using NIST MS Search software with the NIST library.

Quantification of electrophysiologically active microbial volatile organic compounds

In order to determine electrophysiological active responses of *A. gifuensis* to mVOCs, GC-EAG analysis was performed three times, and for each replicate, a new antennal preparation was used. The GC-EAG techniques used were similar to those described by Smid et al. (2002). Briefly, the system was based on the above Agilent 7890A GC equipped with a polar HP-5 capillary column (30.0 m × 250 µm × 0.25 µm, Agilent Technologies, Palo Alto, CA, USA) split to a flame ionization detector (FID) and an electroantennogram (EAG) detector (Syntech, Hilversum, the Netherlands). A 4 µL aliquot of sample (3 µL for EAG, 1 µL for FID) was injected for a GC-EAG run and active peaks were identified by coupled gas chromatography with mass spectrometry (GC–MS) as above. The sample was injected in splitless mode, serving as the mobile phase, at a linear velocity of 40 cm s^{-1}, the oven initial temperature at 50 °C, ramped to 100 °C at 5 °C min^{-1}, held for 5 min, and then ramped-up to

250 °C at 10 °C min^{-1}. The compounds were carried to the antenna through a glass tube by a charcoal-filtered and humidified air stream at 0.5 m s^{-1}.

Parasitoids were first anaesthetized by chilling, and the head was isolated. The reference electrode was connected to the neck of the isolated head, while the recording electrode was connected to the antennal tip (with the last segment of antennas cut off). Chlorinated silver–silver chloride junctions were used to maintain electrical contact between electrodes and input of a 1 × preamplifier (Syntech®). The analog signal was detected through a probe (INR-II, Syntech®), captured and processed with a data acquisition controller (IDAC-4, Syntech®), and later analyzed with software (GC-EAD2010, Syntech®).

After mVOC identification with spectral data's from NIST Library, standard curves for 1-ethyl-2-methylbenzene and 2-butyl-1-octanol performed with a 0.01 µg mL^{-1} to 1000 µg mL^{-1} concentration range. Standard samples were dissolved in hexane and transferred to headspace. Concentrations of mVOCs were calculated from the regression equation.

Olfactometer tests

In order to test the attractiveness of mVOCs from *S. miscanthi* honeydew, associated bacteria and EAG-active volatiles, several samples–control couples were tested in Y-tube olfactometer bioassays following the protocol described in Table 1. A small glass Y-shape tube olfactometer (2 cm in diameter, 12 cm length of the arms and 14 cm length of the stem) with a 45° inside angle between two arms (described in detail by Fatouros et al. 2008) connected to an air pump producing a unidirectional airflow of 150 mL min^{-1} from the arms to the base. 10 µL of samples was placed on a 1 cm^2 filter paper and offered to insects. The filter paper was replaced each time. Females of *A. gifuensis* were individually released from the holding tube at the end of the Y-tube olfactometer. Each wasp was given 5 min to respond to the treatment. The first choice in one of the arms was recorded. The response was regarded as valid only if insects crossed half the arm, insects that did not make a choice within 5 min were considered as non-responding. Trials were replicated until 30 individuals had responded for each treatment. All

Table 1 Summary of samples and controls used in the behavioral assays

Olfactometer test	Sample	Control	Number of tested *Aphidius gifuensis*
S. miscanthi honeydew with/ without bacteria	10 µL Crude aphid honeydew	10 µL Distilled water	31
	10 µL Sterilized honeydew	10 µL Distilled water	31
	10 µL Crude honeydew	10 µL Sterilized honeydew	33
	10 µL Honeydewinoculated medium	10 µL Medium	31
	10 µL Medium	10 µL Distilled water	32
S. miscanthi honeydew associated bacteria	10 µL *Lysinibacillus fusiformis* MH1-inoculated 863 medium	10 µL Medium	35
	10 µL *Erwinia aphidicola* MH2-inoculated 863 medium	10 µL Medium	33
	10 µL *Erwinia tasmaniensis* MH3- inoculated 863 medium	10 µL Medium	36
	10 µL *Acinetobacter bereziniae* MH4- inoculated 863 medium	10 µL Medium	31
	10 µL *Klebsiella quasipneumoniae subsp. Similipneumoniae* MH6-inoculated 863 medium	10 µL Medium	33
	10 µL *Staphylococcus capitis* MH11-inoculated 863 medium	10 µL Medium	34
	10 µL *Bacillus safensis subsp. Safensis* MH12-inoculated 863 medium	10 µL Medium	31
EAG-active volatiles	10 µL 1-Ethyl-2-methylbenzene (2.73 µg mL^{-1})	10 µL *n*-Hexane	32
	10 µL 2-Butyl-1-octanol (2.43 µg mL^{-1})	10 µL *n*-Hexane	31
	10 µL Blend (2.73 µg mL^{-1} 1-ethyl-2-methylbenzene + 2.43 µg mL^{-1} 2-buty-1-octanol)	10 µL *n*-Hexane	33
	10 µL Blend (2.73 µg mL^{-1} 1-ethyl-2-methylbenzene + 2.43 µg mL^{-1} 2-buty-1-octanol)	10 µL 1-Ethyl-2-methyl-benzene (2.73 µg mL^{-1})	36
	10 µL Blend (2.73 µg mL^{-1} 1-ethyl-2-methylbenzene + 2.43 µg mL^{-1} 2-buty-1-octanol)	10 µL 2-Butyl-1-octanol (2.43 µg mL^{-1})	35

tests were conducted from 8:00 am to 5:00 pm at 22 ± 2 °C and 260 lx light intensity.

Five dual choices were conducted to determine the impact of the honeydew and sterilized honeydew on *A. gifuensis*. They were proposed as follows: (1) crude aphid honeydew versus distilled water, (2) sterilized honeydew versus distilled water, (3) crude honeydew versus sterilized honeydew, (4) 863 liquid medium inoculated with honeydew versus 863 liquid medium, (5) 863 liquid medium versus distilled water.

The second olfactometer test was performed to select the responses of *A. gifuensis* to 7 bacterial strains obtained from *S. miscanthi* honeydew, while 863 medium was consumed as the control.

For assessing parasitoid response to EAG-active compounds, the last group includes 1-ethyl-2-methylbenzene (2.73 μg mL^{-1}), 2-buty-1-octanol (2.43 μg mL^{-1}) each alone or blended (2.73 μg mL^{-1} 1-ethyl-2-methylbenzene + 2.43 μg mL^{-1} 2-buty-1-octanol) were tested. Concentrations were determined according to bacterial production from quantitative GC–MS analysis. Detailed information for quantification is in the Supplementary Information.

Field tests

Field experiments were performed in the experimental station of Shandong Agricultural University, Shandong Province, China (36° 09′ N, 117° 09′ E), in 2022. Treatments were applied as follows: (a) *L. fusiformis* MH1 inoculated in 863 medium; (b) 1-ethyl-2-methylbenzene release; (c) 2-butyl-1-octanol release; (d) 1-ethyl-2-methylbenzene + 2-butyl-1-octanol release and (e) untreated plot as control. Each plot measured 3 m × 3 m, with a 10-m interspace between each plot. A completely randomized design was used. Each treatment was replicated three times. Wheat (cv.'Jimai 22') was planted with a row space of 20 cm and was sown on October 15, 2021. No insecticide nor herbicide was used in the whole experimental area.

L. fusiformis MH1 in 863 medium was prepared as followed: 150 mL of agar solution at 40 °C was poured into 1 L of *L. fusiformis* MH1 inoculated in 863 medium (OD = 1), after it had solidified, transferred to the center of the plot for placement. The active compounds were formulated in paraffin oil at a concentration of 2.73 mg mL^{-1} and 2.43 mg mL^{-1} for 1-ethyl-2-methylbenzene and 2-butyl-1-octanol respectively, alone or in combination in an Eppendorf tube (2 mL) that was fixed to a trap stake in each treatment plot. All semiochemical release tubes were placed under a plastic roof (5 cm × 5 cm × 24 cm) to protect them from rain. All treatments were weekly changed. The first applications started on April 15, 2022.

Apterous and mummified aphids were sampled every week on five sampling dates. Each time, five sampling points were randomly selected along the bidiagonal lines in each plot, and 20 tillers in each point were selected. Aphid alates were collected using plastic yellow pan traps (27 cm in diameter and 10 cm in depth) set at the center of each plot and fixed 20 cm above the wheat canopy. Water with detergent was poured into pan traps that were emptied and refilled weekly. Collected insects were transferred to a tube containing 70% ethanol and brought back to the laboratory for identification. The parasitism rate was calculated by the formula [mummies/(aphids + mummies)].

Statistical analysis

The chi-square test was used to determine the significant difference in the wasp behavioral response in the Y-tube bioassay. Multivariate data analysis (Principal component analysis—PCA and orthogonal projection to latent structures discriminant analysis—OPSL-DA) was used to analyze peak areas of chemical compounds emitted by MH1 (attractive)/MH2, MH3, MH4, MH6, MH11, MH12 group (neutral) and medium. The measured peak areas were 1og transformed, using the comprehensive online tool suite MetaboAnalyst 5.0 (Cusumano et al. 2022). The results of the analysis were visualized in score plots, which reveal the sample structure according to model components, and loading plots, which display the contribution of the variables to these components. The ranking of the compounds that contribute the most in explaining statistical differences were identified based on the variable importance in the projection (VIP values). The difference between relative proportions of the volatile compounds, the effects of honeydew-associated bacteria and EAG-active compounds on aphids and parasitoids were analyzed by analysis of variance (ANOVA) followed by the least-significant difference test (LSD). The statistical analysis software used was SPSS v. 26.0 for Windows.

Results

Olfactory response of *Aphidius gifuensis* to aphid honeydew with/without bacteria

Females of *A. gifuensis* significantly preferred crude honeydew to distilled water or to sterilized honeydew. After being inoculated in 863 liquid medium for one day, the crude honeydew significantly attracted *A. gifuensis* females compared to the medium only (Fig. 1).

Olfactory responses of *Aphidius gifuensis* to *Sitobion miscanthi* honeydew bacteria

Seven strains of bacteria were isolated from *S. miscanthi* honeydew (Table 2). Attraction potential screening on *A. gifuensis* showed that only *L. fusiformis* MH1 was significantly active while other bacteria isolated from *S. miscanthi* honeydew had no effect (Fig. 2).

Multivariate analysis of mVOCs composition between attractive and neutral strains

A total of 16 mVOCs was detected in the headspace of tested honeydew bacterial isolates, whereas a total of 3 compounds were found in blank medium. These chemicals were generally assigned to chemical groups such as alcohol, aldehyde, aromatic and ketone (Supplementary Table S1). The PCA analysis revealed an overlap between bacteria volatiles and medium volatiles. Additionally, all of the blank medium

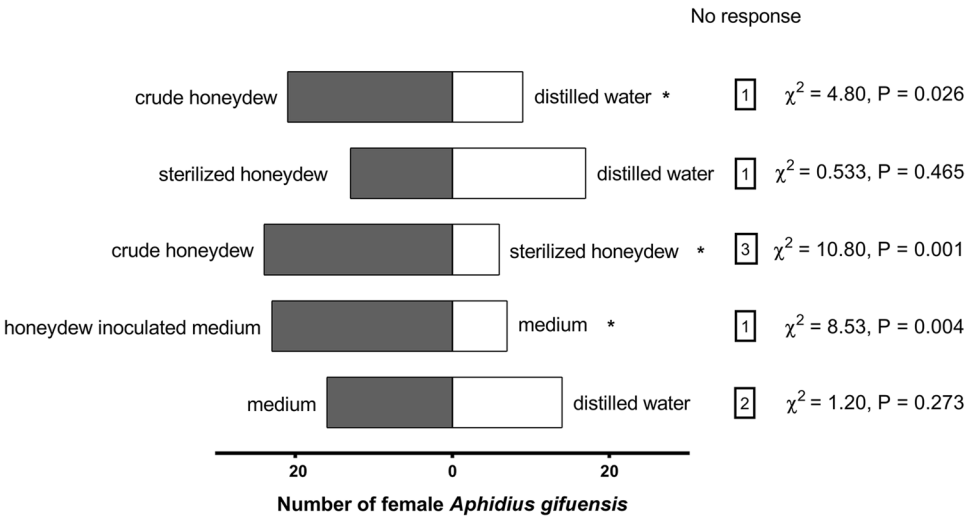

Fig. 1 Dual choice responses of *Aphidius gifuensis* females in Y-tube olfactometer. Significant differences ($P < 0.05$) between the two arms were indicated by * with statistical values

Table 2 Bacteria isolated from *Sitobion miscanthi* honeydew

Strain ID	Phylogenetic affiliation based on 16S rRNA gene sequence similarity			GenBank accession
	Phylum	Family	Blast sequence with the highest identity	
MH1	Firmicutes	Planococcaceae	*Lysinibacillus fusiformis*	OR141139
MH2	Proteobacteria	Erwiniaceae	*Erwinia aphidicola*	OR141140
MH3	Proteobacteria	Moraxellaceae	*Acinetobacter bereziniae*	OR141141
MH4	Proteobacteria	Erwiniaceae	*Erwinia tasmaniensis*	OR141142
MH6	Proteobacteria	Enterobacteriaceae	*Klebsiella quasipneumoniae subsp. similipneumoniae*	OR141143
MH11	Firmicutes	Staphylococcaceae	*Staphylococcus capitis*	OR141144
MH12	Firmicutes	Bacillaceae	*Bacillus safensis subsp. safensis*	OR141145

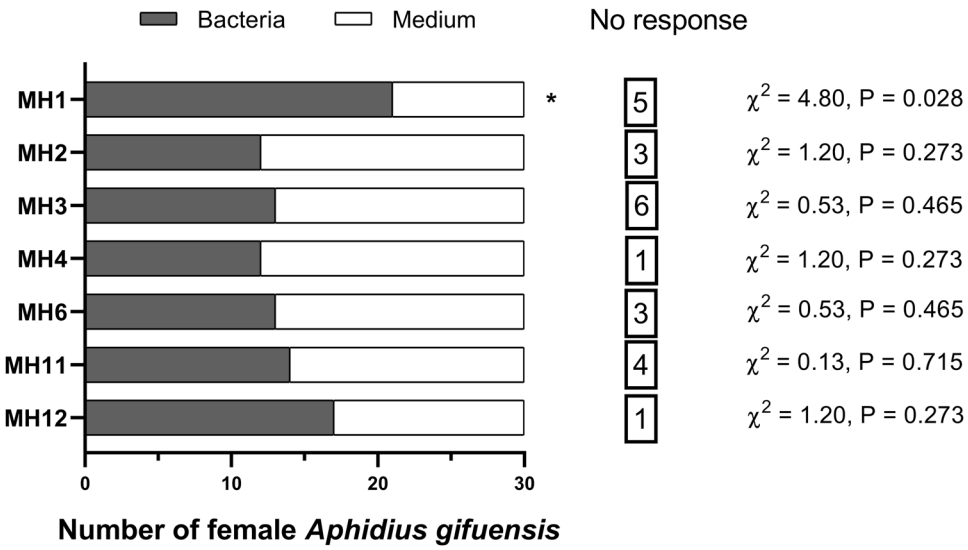

Fig. 2 Behavioral responses of *Aphidius gifuensis* females to bacteria isolated from honeydew in a Y-tube olfactometer. 863 liquid medium was used as control. Significant difference ($P < 0.05$) between bacteria and medium was indicated by *

compounds, including 3-ethyl-benzaldehyde, 1-(4-ethylphenyl)ethanone and 1-(4-acetylphenyl)ethanone, were detected in the honeydew-isolated bacteria volatiles. However, their concentrations in the medium were significantly higher compared to the bacteria. This observation suggests that certain compounds present in the culture medium might have undergone partial consumption or conversion during the cultivation process (Fig. 3A; Table S1). Based on the olfactory response of the parasitoid, the bacteria strains isolated from the honeydew were divided into two groups (attractive and neutral). To explore the difference among mVOCs composition obtained from GC–MS data for attractive and neutral bacteria strains, an OPLS-DA model of multivariate statistical analysis was carried out. The OPLS-DA score plot showed that the points of attractive bacteria samples (MH1) and neutral bacteria samples (others) were separated (Fig. 3B). The value of R^2 was 0.811, and that of Q^2 was 0.708, both of which were greater than 0.5, indicating that the model had suitable interpretation and prediction ability. The classification of different bacteria and discriminant volatile compounds are shown in Fig. 3C. In the VIP plot, five compounds had a VIP value > 1 indicating that these compounds strongly contributed to explaining the differences *A. gifuensis* behavior among attractive and neutral honeydew bacteria strains. These compounds were ID_8 (1-ethyl-2-methylbenzene), ID_10 (p-cymene), ID_6 (3-ethylbenzaldehyde), ID_11 (styrene) and ID_4 (2-butyl-1-octanol) (Fig. 3D).

EAG response of parasitoids to *Lysinibacillus fusiformis* MH1 volatiles

To further investigate the effective compounds, GC-EAG test in combination with GC-MS analysis showed that two compounds (A) 1-ethyl-2-methylbenzene, emitted only from MH1 ($F = 31.13$, $df = 7$, $P < 0.001$), and (B) 2-butyl-1-octanol, emitted from all isolated bacteria were identified. Moreover, MH1 produced the significantly higher amount of 2-butyl-1-octanol than the other bacteria ($F = 4.224$, $df = 7$, $P = 0.008$) (Fig. 4; Table S1).

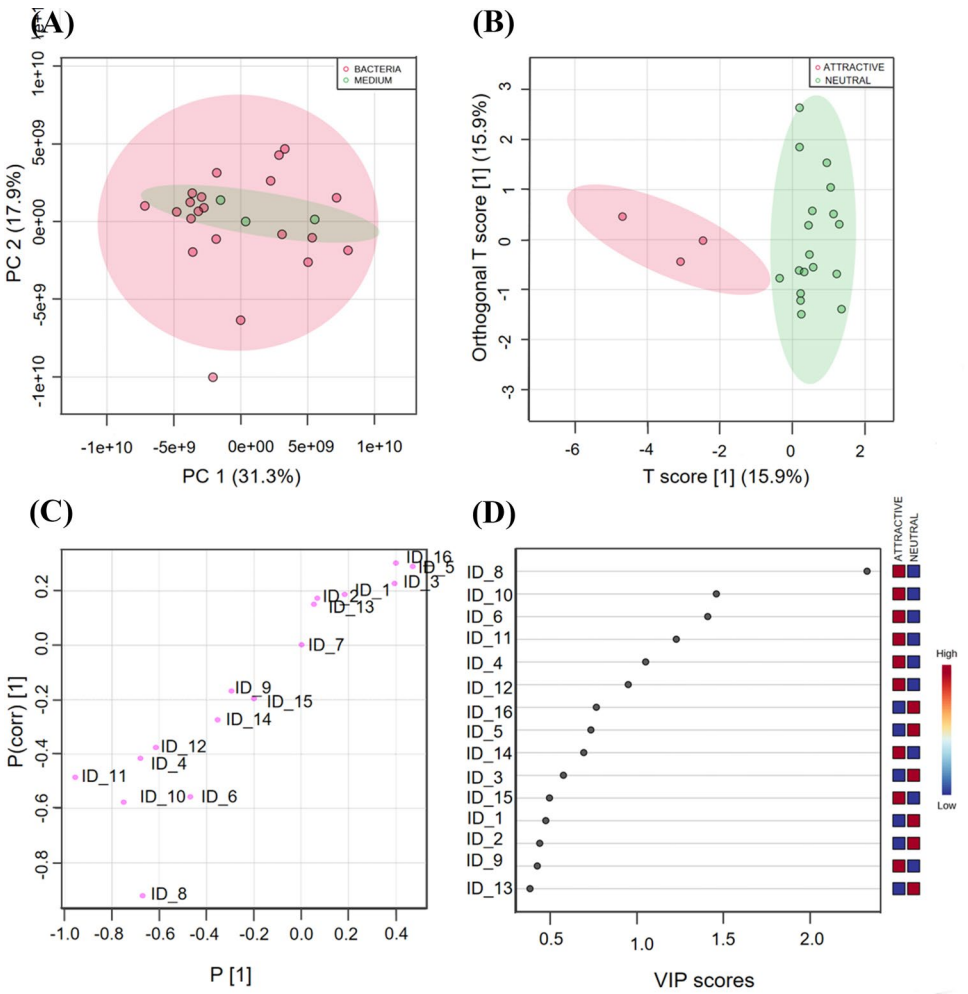

Fig. 3 Principal component analysis (PCA) of volatile profiles produced from honeydew bacteria (**A**) and Orthogonal partial least squares discriminant analysis (OPLS-DA) of the volatile profiles produced from the attractive and neutral bacteria (**B**–**D**). **B** Score plot, **C** S-plot, **D** Variable important for the projection (VIP) plot. VIP with a value > 1 are ID_8 (1-ethyl-2-methylbenzene), ID_10 (p-cymene), ID_6 (3-ethylbenzaldehyde), ID_11 (styrene) and ID_4 (2-butyl-1-octanol). See Table S1 for the list of compound IDs

Olfactory responses of *Aphidius gifuensis* females to EAG-active compounds

A. gifuensis showed significant responses to 1-ethyl-2-methylbenzene, 2-butyl-1-octanol and the blend compared with the solvent *n*-hexane. No significant differences were found between the blend and each compound (Fig. 5).

Effects of honeydew-associated bacteria and functional compounds on aphid and parasitoids

No significant difference was found in the total abundance of aphid alatae ($F = 1.60$, $df = 4$, $P = 0.249$) (Fig. 6A). Whereas the total abundances of apterous aphids significantly decreased in bacteria-treated plots and semiochemical-treated plots compared with control ($F = 5.23$, $df = 4$, $P = 0.015$). No significant difference was found between treatments of bacteria and semiochemicals (Fig. 6B).

Treatments had a significant effect on mummified aphid abundance and parasitism rate ($F = 9.07$, $df = 4$, $P = 0.002$), while there was no significant difference in mummified aphids abundance between *L. fusiformis*-treated plots and untreated plots ($P = 0.183$). Among each treatment, compared to *L. fusiformis* MH1, the blend significantly increased the abundance of mummified aphids ($P = 0.012$) and parasitism rate ($P = 0.031$) (Fig. 6C, D).

Discussion

Semiochemicals are essential to guide natural enemies to locate and recognize suitable hosts or prey. Most studies focused on plant- and host-derived semiochemicals (Wang et al. 2019; Liu et al. 2021; Xiao et al. 2022), while little thought has been given to mVOCs as cues to manipulate natural enemy behaviors (Davis et al. 2013). In this study, we found that bacteria-containing honeydew from *S. miscanthi*

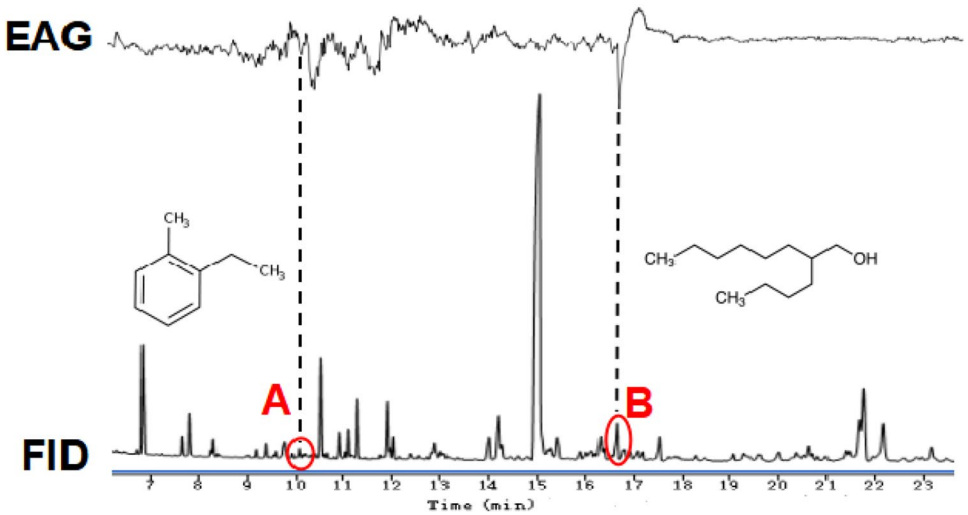

Fig. 4 Illustration of gas chromatography–electroantennography test on *Aphidius gifuensis* to volatiles from *Lysinibacillus fusiformis* with active compounds: **A** 1-ethyl-2-methylbenzene; **B** 2-buty-1-octanol

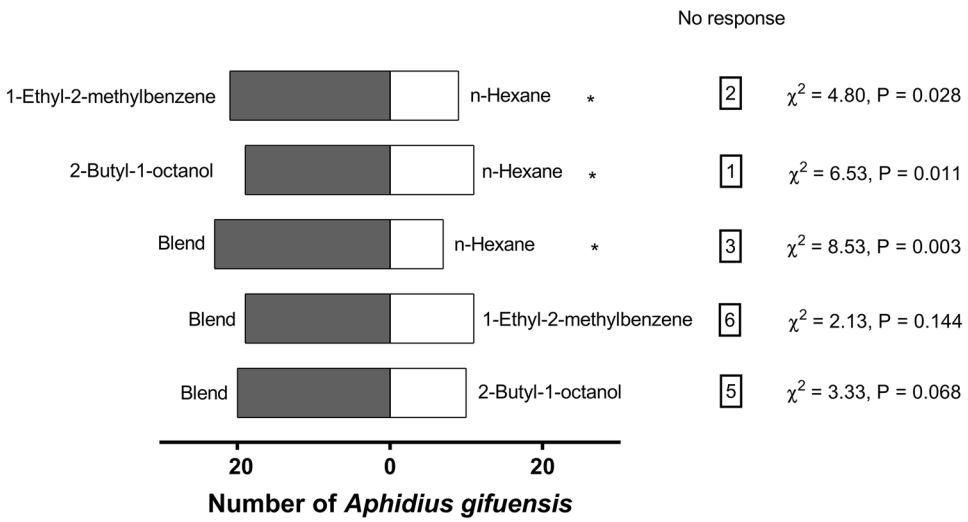

Fig. 5 Olfactory responses of *Aphidius gifuensis* females when given the choice between various combinations using 1-ethyl-2-methylbenzene and 2-butyl-1-octanol. Significant difference ($P < 0.05$) between the two arms was indicated by *

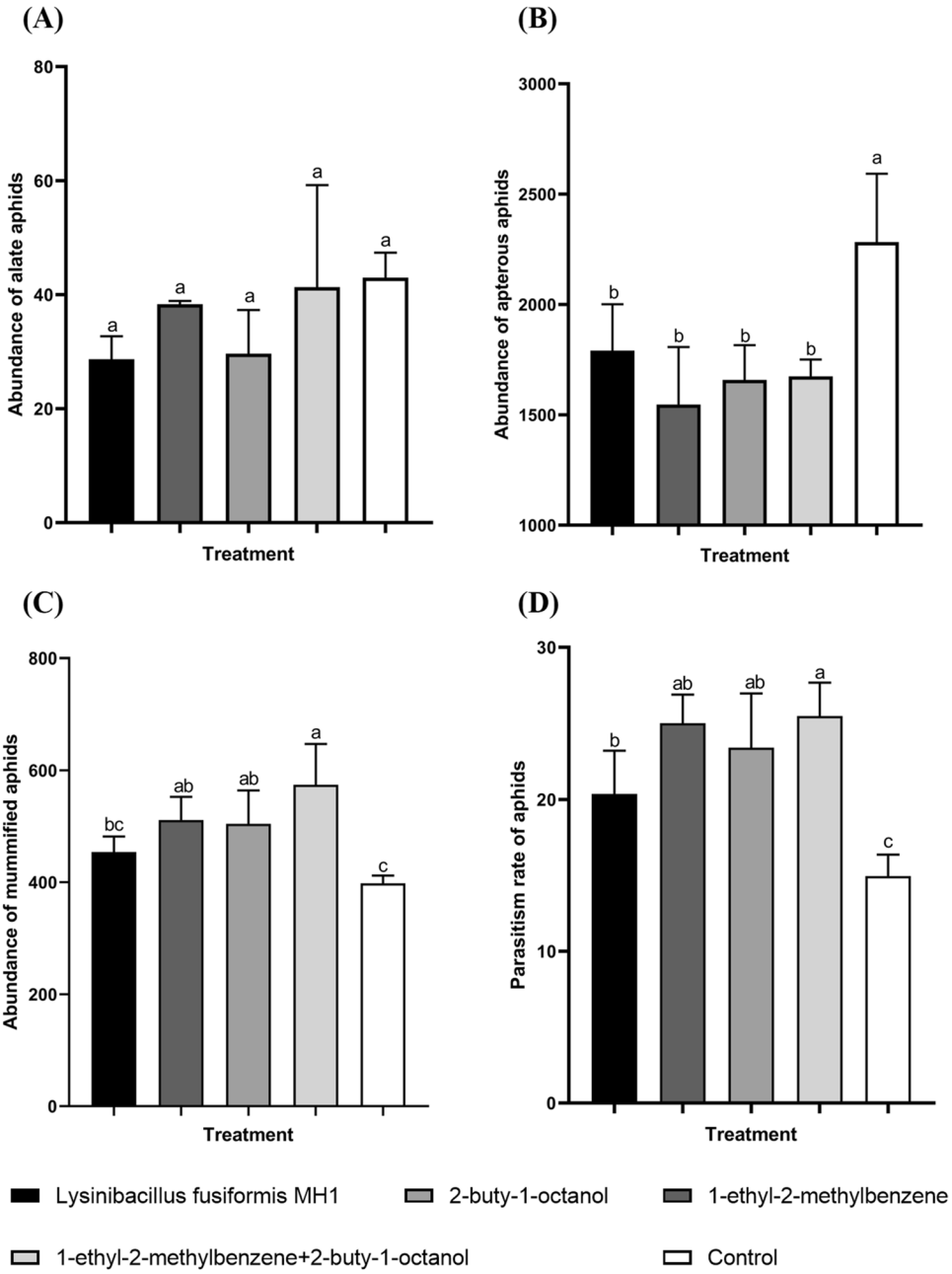

Fig. 6 Effects of honeydew bacteria *Lysinibacillus fusiformis* MH1 and EAG-active compounds 1-ethyl-2-methylbenzene and 2-butyl-1-octanol alone and in association on **A** alate aphids, **B** apterous aphids, **C** mummified aphids and **D** the parasitism rate for the entire duration of the experiment in 2022. Data are mean ± SE ($n = 3$). Different letters on bars indicated significant differences (One-way ANOVA, $P < 0.05$)

significantly attracted the aphid parasitoid *A. gifuensis*. In the field experiment, the bacteria *L. fusiformis* MH1 and semiochemicals 1-ethyl-2-methylbenzene and 2-butyl-1-octanol emitted from bacteria could enhance the efficacy in reducing the abundance of *S. miscanthi* and increasing the parasitism rate. Our findings represent an evident case of a honeydew-associated bacterium attracting parasitoids through the emission of mVOCs in wheat crops.

Our results suggested that bacteria in wheat aphid honeydew could be a cue for *A. gifuensis* to locate the host in cereal crops. From the *S. miscanthi* honeydew, we identified seven bacterial strains that caused different behavioral responses (attractive/neutral) to *A. gifuensis*. The attractive strain *L. fusiformis* MH1 strain has been identified as a plant-beneficial microbe living in association with the roots of wheat (Sharma et al. 2019), apple trees (Bulgari et al. 2012), citrus (Trivedi et al. 2011) and tomato (Rahmoune et al. 2017), and it could be enhanced the wheat root and shoot biomass and seed grain yield (Sharma et al. 2019). It is the first case of *L. fusiformis* strain MH1 acting as beneficial bacteria to attract parasitic wasps and then has the potential to be used for pest control. Some facultative endosymbiotic bacteria were able to protect the aphid from parasitoid attack (Oliver et al. 2005). In this case, aphid-associated bacteria

L. fusiformis MH1 could be used as a beneficial bacteria to attract parasitic wasps, this highlights the complexity of multitrophic systems and the potential for intricate coevolutionary relationships among plant, aphid, microbial and parasitoid.

The wheat plant may be considered as the source of the *L. fusiformis* MH1, since MH1 has been found from wheat (Sharma et al. 2019), during aphid probing on the host plant, *L. fusiformis* MH1 can pass through the stylets food canal before adhering to the luminal face of intestinal epithelia (Grenier et al. 1994) and then acquired by aphids, finally being partially excreted in the honeydew. The latter on plant leaves constitutes an excellent growth medium promoting the rapid development of several mVOCs release widespread in nature. These findings may reveal an evolutionary scenario in which plants recruit beneficial bacteria to inhabit the nutrient-rich honeydew produced by aphids, ultimately leading to the emission of volatiles that act as chemical cues for attracting natural enemies.

According to our OPLS-DA model, the compounds which have the higher VIP values are most likely correlated with parasitoid attraction. It was reported that styrene and benzaldehyde could emit from parasitoid habitat-associated bacteria to attract parasitoid *A. colemani* (Goelen et al. 2021). *p*-Cymene could be a plant volatile to attract parasitoids (Xiao et al. 2022). Nevertheless, it has been noted that plant volatile is not only produced by the plant themselves but may also be derived from the plant bacteria, which may also explain the considerable variation in plant volatiles, even when exposed to similar conditions (Takabayashi et al. 1994). In the olfactory response, the females were tested as the key determinant in locating and parasitizing hosts to induce direct repercussions on host-parasitoid population dynamics (Aartsma et al. 2017). Coupled GC-EAG analysis and olfactory test in our study located physiologically and behaviorally active components 1-ethyl-2-methylbenzene and 2-butyl-1-octanol in attracting parasitoids. It is the first report of these molecules in modifying insect behavior. Odorant-binding proteins (OBPs) play crucial roles in various aspects of odor perception, including feeding, host searching, mating and oviposition. The interaction between OBPs and odor molecules is the first step in insect recognition of chemicals (Leal 2012). Based on the 3D modeling of *A. gifuensis* OBP (AgifOBP) and molecular docking, it was observed that compound 2-buty-1-octanol exhibited a stronger affinity with AgifOBP compared to 1-ethyl-2-methyl-benzene (Unpublished data). This finding may explain the higher EAG response observed for 2-buty-1-octanol when compared to 1-ethyl-2-methyl-benzene (Fig. 4). However, in the field test, 2-buty-1-octanol exhibited a similar effect on aphid control and attraction of parasitoids when compared to 1-ethyl-2-methyl-benzene. This similarity can be attributed to the fact that the amount of semiochemicals used in the wheat field was determined based on the olfactory results and relative quantification. It is important to note that the release rates of these synthetic compounds may differ from those naturally occurring in honeydew bacteria, and the field environment itself is inherently complex. Therefore, further optimization of the optimal quantities will be necessary in the future.

In this study, mVOCs were collected using Porapak Q from the bacteria-inoculated medium, it is essential to acknowledge that Porapak Q has a limited capacity to adsorb substances with fewer than six carbon atoms. As a result, some small molecules of mVOCs may not be effectively adsorbed by Porapak Q and could potentially go undetected. Additionally, it should be noted that the precise composition of volatile blend emitted by microorganisms may depend on the nutrient medium and collection method (Gonzalez et al. 2019). Analysis ideally should be repeated for different absorbent, medium and collect methods to investigate how microbial culturing and managing conditions affect the composition of mVOC blend and the associated responses of parasitoids.

Understanding the nature of these chemicals and their ecological role in multitrophic interactions is essential to design more sustainable strategies for pest management, including biological control. Honeydew-associated bacteria and mVOCs have ecological significance in their behavioral manipulation of *A. gifuensis*. Meanwhile, the species has the ability to parasitize other aphid species (e.g., *M. persicae*, *Lipaphis erysimi*, *Aphis glycines* and *A. gossypii*), making it a potential candidate for deployment in various ecosystems (Song et al. 2021). To maintain ecological balance in wheat fields, we suggest combining these compounds with other strategies such as release commercially reared natural enemies, intercropping or planting flowering plants around wheat fields provides alternative food sources and shelter for beneficial insects, acting as a natural enemies' bank. We suggest that entomologists, agronomist, ecologists, chemist, government officials, and farmers could work together to optimize the combination of these strategies. Evaluating their impact on aphid control, ecological balance and crop productivity in wheat fields could develop an effective and sustainable approach to control aphids.

Author contributions

JL, FF and YL conceived research. JL, DX, YZ and YL conducted experiments. JL analyzed data and conducted statistical analyses. JL, FF and YL wrote the manuscript. All authors read and approved the manuscript.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10340-023-01687-5.

Funding This work was supported by grants from the Natural Science Foundation of Shandong Province (ZR2020MC121).

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This article does not contain any studies with vertebrates performed by the authors.

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