POTENTIAL OF INSECT-MICROBE CHEMICAL INTERACTIONS TO IMPROVE BIOLOGICAL CONTROL OF INSECT PESTS

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Preface – Voorwoord

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Tim

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"A man who dares to waste one hour of time has not discovered the value of life."

Charles Darwin

The Life & Letters of Charles Darwin (1887)

List of abbreviations

ANOVA	Analysis of variance
BCA	Biological control agent
BLAST	Basic local alignment search tool
CAFE	Capillary feeder
EAG	Electroantennography
FDR	False discovery rate
GC	Gas chromatography
GLMM	Generalized linear mixed model
HIPV	Herbivore-induced plant volatile
HS	Headspace
HSD	Honest significant difference
IPM	Integrated pest management
ML	Maximum-likelihood
MS	Mass spectrometry
mVOC	Microbial volatile organic compounds
NIOO-KNAW	Netherlands Institute of Ecology
NMDS	Non-metric multidimensional scaling
OD	Optical density
PC	Principal component
perMANOVA	Permutational multivariate analysis of variance
PI	Preference index
pPCA	Phylogenetic principal component analysis
PTFE	Polytetrafluoroethylene
rRNA	Ribosomal RNA
SE	Standard error of the mean
SMPE	Solid phase micro extraction
SRH	Scheirer–Ray–Hare
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VOC	Volatile organic compound

Samenvatting

Biologische controle waarbij natuurlijke vijanden zoals predatoren en parasitoïden worden ingezet, is een belangrijk alternatief voor de conventionele bestrijding van plaaginsecten. Wanneer natuurlijke vijanden echter in lage aantallen voorkomen of niet voldoende doeltreffend zijn, kan dit de efficiëntie van biologische controle sterk beperken. Daardoor worden naast de natuurlijk voorkomende nuttige insecten vaak bijkomend commercieel beschikbare natuurlijke vijanden uitgezet. Desalniettemin blijft het een grote uitdaging om deze uitgezette natuurlijke vijanden aan te trekken en in het gewas te houden, wat noodzakelijk is om hoge populatiedensiteiten te bereiken en zo plaaginsecten efficiënt te bestrijden wanneer deze in het gewas komen. Bijkomende middelen zoals het voorzien van voedselbronnen of het gebruikmaken van lokstoffen worden meer en meer ingezet om natuurlijke vijanden te ondersteunen. Dit blijkt een veelbelovende aanpak om de doeltreffendheid van biologische controle te verbeteren. Vaak zijn deze aanvullende suikerbronnen echter niet selectief voor natuurlijke vijanden en kunnen ze ook voordelig zijn voor schadelijke insecten zoals plaaginsecten en hyperparasitoïden. Deze laatste vormen een belangrijke groep van insecten die primaire parasitoïden parasiteren en op die manier biologische controle sterk kunnen verstoren.

Het foerageergedrag van natuurlijke vijanden wordt hoofdzakelijk beïnvloed door chemische signalen die vanuit de omgeving uitgezonden worden door andere insecten of planten. Deze chemische signalen worden ook wel “semiochemicaliën” genoemd. Hoewel het meeste onderzoek zich heeft gericht op signalen afkomstig van planten, is er steeds meer bewijs dat micro-organismen ook vluchtige componenten produceren die eveneens het gedrag van insecten kunnen beïnvloeden. Tot nu toe is er nog maar weinig bekend over hoe microbiële, vluchtige componenten het foerageergedrag van natuurlijke vijanden beïnvloeden en of deze aangewend kunnen worden om de biologische controle van plaaginsecten te verbeteren. De algemene doelstelling van deze doctoraatsstudie bestond erin om het potentieel te onderzoeken van selectief ondersteunende suikermengsels en microbiële vluchtige organische componenten (mVOCs) om de biologische controle van plaaginsecten te verbeteren. Hiervoor werden de bladluisparasitoïden *Aphidius colemani* Vierick and *Aphidius matricariae* Haliday (Hymenoptera: Braconidae) en één van hun hyperparasitoïden, *Dendrocerus aphidum* Rodani (Hymenoptera: Megaspilidae) als studieorganismen gebruikt. Beide *Aphidius*

soorten zijn solitaire, generalistische endoparasitoïden die verschillende bladluissoorten parasiteren, waaronder verschillende soorten van economisch belang.

In het eerste deel van deze studie (**Hoofdstuk 2**) hebben we het voedingsgedrag en de overleving van beide *Aphidius* soorten en hun hyperparasitoïde bestudeerd wanneer ze acht verschillende, individuele suikers werden aangeboden (fructose, galactose, glucose, melibiose, melezitose, rhamnose, sucrose en trehalose). Allereerst werd de suikerconsumptie over een periode van 9 uur opgevolgd aan de hand van een "capillary feeder (CAFE)" experiment. Vervolgens werd de overleving van de parasitoïden, die gevoed werden met deze suikers, verder opgevolgd. De resultaten toonden aan dat alle parasitoïden vooral suikers consumeerden die vaak voorkomen in honingdauw (sucrose, fructose, glucose en melezitose). Bovendien bleken ze ook het langst te overleven wanneer ze gevoed werden met deze suikers. Beide *Aphidius* soorten overleefden goed op melibiose, terwijl dit minder het geval was voor *D. aphidum*. Wanneer melibiose in een mengsel met glucose werd aangeboden, resulteerde dit in een sterk verminderde overleving van de hyperparasitoïde in vergelijking met enkel glucose. Dit effect was minder uitgesproken voor *Aphidius*, wat suggereert dat dit mengsel gebruikt kan worden om vooral *Aphidius* parasitoïden te ondersteunen.

In **Hoofdstuk 3** werd via Y-buis olfactometerexperimenten onderzocht hoe vluchtige componenten geproduceerd door bacteriën het gedrag van *A. colemani* en *D. aphidum* beïnvloedden. Hun gedrag werd getest aan de hand van geurmengsels afkomstig van bacteriën die geïsoleerd werden uit diverse omgevingen uit de leefomgeving van de parasitoïden, waaronder bladluizen, bladluismummies, honingdauw en de parasitoïden zelf. Uit de resultaten bleek dat de reactie van *A. colemani* op deze bacteriële geuren sterk varieerde, gaande van significante aantrekking, geen respons tot significante afstoting. Bovendien bleek dat *A. colemani* anders reageerde op deze geuren dan *D. aphidum*. Gaschromatografie-massaspectrometrie (GC-MS) analyses toonden aan dat de geurmengsels die een afstotend effect hadden op *A. colemani*, significant grotere hoeveelheden esters, organische zuren, aromatische componenten en cycloalkanen bevatten in vergelijking met de aantrekkelijke mengsels. De bacteriële geurmengsels die afstotend bleken voor *D. aphidum* bevatten significant grotere hoeveelheden alcoholen en ketonen in vergelijking met de aantrekkelijke mengsels, terwijl deze aantrekkelijke mengsels net hogere hoeveelheden monoterpenen zoals limoneen, linalool en geraniol bevatten. Verder toonden de resultaten aan dat *A. colemani* gelijkaardig reageerde op de

geurmengsels afkomstig van nauw verwante soorten van het geslacht *Bacillus*. Dit suggereert dat de samenstelling van de geurmengsels en, als gevolg, de reactie van *A. colemani* fylogenetisch geconserveerde eigenschappen zijn.

In **Hoofdstuk 4** werd deze hypothese in meer detail onderzocht, namelijk dat fylogenetische verwantschappen tussen micro-organismen de samenstelling van mVOCs en de reactie van insecten op deze geuren kunnen voorspellen. Uit de resultaten bleek dat nauw verwante *Bacillus* stammen gelijkaardige profielen van vluchtige componenten produceerden en bovendien een vergelijkbare reactie uitlokten in *A. colemani*. Analyses van de chemische samenstelling van de mVOCs toonden aan dat alle *Bacillus* stammen dezelfde set van vluchtige componenten produceerden, maar weliswaar in verschillende concentraties en verhoudingen. Benzaldehyde werd geproduceerd in grotere hoeveelheden door stammen die afstotend waren voor *A. colemani* in vergelijking met de stammen die aantrekkelijk waren, terwijl deze aantrekkelijke stammen grotere hoeveelheden acetoine, 2,3-butaandiol, 2,3-butaandione, eucalyptol en isoamylamine produceerden. Deze resultaten ondersteunen de hypothese dat verwantschappen tussen de bacteriën de mVOC samenstelling en de reactie van *A. colemani* op deze mVOCs beïnvloeden.

Ondanks een toenemend inzicht in de rol van mVOCs als semiochemicaliën voor insecten, is er maar weinig geweten over welke mVOCs of mVOC mengsels het gedrag van insecten beïnvloeden. Daarom beoogden we in **Hoofdstuk 5** specifieke componenten te identificeren in bacteriële geurmengsels die aantrekkelijk zijn voor *A. colemani*. Hiervoor werd gebruik gemaakt van een combinatie van gaschromatografie-elektroantennografie (GC-EAG), GC-MS, en Y-buis olfactometer experimenten met synthetische componenten. Vervolgens werd het meest belovende mengsel met aantrekkelijke synthetische componenten geëvalueerd in een kooi-experiment onder serreomstandigheden. Dit resulteerde in een selectie van componenten die significant aantrekkelijk of afstotend waren. Meer specifiek bleek een mengsel van 100 ng/µL styreen en 1 ng/µL benzaldehyde het meest aantrekkelijk, zowel in laboratorium als serre-experimenten. Alles bij elkaar genomen tonen deze resultaten aan dat een beperkte set van vluchtige componenten die vrijgegeven worden in specifieke concentraties een grote impact kan hebben op het gedrag van insecten. Bovendien biedt de verworven kennis nieuwe mogelijkheden om natuurlijke vijanden aan te trekken en te behouden in het gewas, en mogelijk ook om de biologische controle van plaaginsecten te verbeteren.

Globaal genomen heeft dit doctoraatsonderzoek tot betere inzichten geleid in de interacties tussen micro-organismen, parasitoïden en hyperparasitoïden door middel van vluchtige componenten. Deze kennis in combinatie met de selectief ondersteunende suikerbronnen kunnen benut worden om nieuwe strategieën te ontwikkelen om natuurlijke vijanden aan te trekken en in stand te houden, wat mogelijk leidt tot een verbeterde biologische bestrijding van plaaginsecten en bijgevolg duurzamere landbouwmethoden.

Summary

Biological control using natural enemies such as arthropod predators and parasitoids has become an important alternative way of pest management. However, the efficacy of biological pest control can be seriously hampered when naturally occurring enemies are not sufficiently abundant or effective. Therefore, naturally occurring beneficial insects are often complemented with the release of commercially-reared natural enemies. Despite these efforts, a major challenge in biological pest control remains to attract and retain the beneficial insects in the crop so that they reach high population densities in the crop and control the pest insects whenever needed. While the increasingly applied provisioning of supplemental food sources and attractants to lure and augment natural enemy populations appears to be a promising approach to increase biocontrol efficacy, these sugar sources are often not tailored to selectively support the natural enemies and may also benefit harmful insects like herbivores and hyperparasitoids. The latter constitute an important fourth trophic level of organisms that parasitize the primary parasitoids and therefore can disrupt biological pest control, ultimately leading to pest outbreaks.

The behaviour of natural enemies is largely determined by chemical cues released in the environment by insects or plants, so-called "semiochemicals". While most research in this field has focused on cues derived from plants, there is mounting evidence that microorganisms emit volatile compounds that also play a role in insect behaviour. However, so far little is known about how microbial volatiles affect the foraging behaviour of natural enemies, and whether they can be applied to improve biological control of insect pests. The overall aim of this PhD study was to investigate the potential of tailored sugar mixtures and microbial volatile organic compounds (mVOCs) to improve the biological control of insect pests. To this end, we used the aphid parasitoids *Aphidius colemani* Vierick and *Aphidius matricariae* Haliday (Hymenoptera: Braconidae) and one of their hyperparasitoids, *Dendrocerus aphidum* Rodani (Hymenoptera: Megaspilidae), as study organisms. Both *Aphidius* species are solitary generalist endoparasitoids that attack many aphid species, including numerous species of economic importance.

In a first part of this PhD study (**Chapter 2**), we investigated the feeding behaviour and longevity of both parasitoid species and their hyperparasitoid when provided with one of eight plant- and/or insect-derived sugars (fructose, galactose, glucose, melibiose, melezitose, rhamnose, sucrose and trehalose). We first evaluated sugar consumption over

a 9-h period of time by using a capillary feeder (CAFE) assay. Next, we studied survival of the parasitoids when fed with the different sugars. Results showed that the studied insect species consumed the largest amounts of sugars that are most commonly found in honeydew (sucrose, fructose, glucose and melezitose) and also survived best when feeding on these sugars. Both *Aphidius* spp. survived well on melibiose, whereas *D. aphidum* performed poorly on this sugar. When melibiose was offered in a mixture with glucose, a significant reduction in longevity was observed for *D. aphidum* when compared to glucose only, while this was less pronounced for *Aphidius*, suggesting that this mixture can be used to predominantly support *Aphidius* parasitoids.

In **Chapter 3,** we used Y-tube olfactometer experiments to assess how volatile compounds emitted by bacteria affected the olfactory response of *A. colemani* and *D. aphidum*. Olfactory responses were evaluated for volatile blends emitted by bacteria that were isolated from diverse sources from the parasitoid's habitat, including aphids, aphid mummies and honeydew, and from the parasitoids themselves. Results revealed that *A. colemani* showed wide variation in response to bacterial volatiles, ranging from significant attraction over no response to significant repellence. Interestingly, the olfactory response of *A. colemani* to bacterial volatile emissions was significantly different from that of *D. aphidum*. Gas chromatography-mass spectrometry (GC-MS) analyses revealed that the volatile blends repellent to *A. colemani* contained significantly higher amounts of esters, organic acids, aromatics and cycloalkanes than attractive blends. Bacterial volatile blends repellent to *D. aphidum* contained significantly higher amounts of alcohols and ketones, whereas the volatile blends attractive to *D. aphidum* contained higher amounts of the monoterpenes limonene, linalool and geraniol than the repellent blends. The results further showed that closely related species of the genus *Bacillus* elicited a similar olfactory response (attraction) in *A. colemani*, suggesting that volatile composition and, as a result, parasitoid attraction, are phylogenetically conserved traits.

In **Chapter 4**, we tested in more detail the hypothesis that phylogenetic relationships among microorganisms predict microbial volatile composition and the olfactory response of insects. Results revealed that phylogenetically closely related *Bacillus* strains emitted similar volatile blends and elicited a comparable olfactory response of *A. colemani* in Y-tube olfactometer bioassays, varying between attraction and repellence. Analysis of the chemical composition of the mVOC blends revealed that all *Bacillus* strains produced the same set of volatiles, but in different concentrations and

ratios. Benzaldehyde was produced in relatively higher concentrations by strains that repel *A. colemani* compared to strains that are attractive, while attractive mVOC blends contained relatively higher amounts of acetoin, 2,3-butanediol, 2,3-butanedione, eucalyptol and isoamylamine. Overall, these results support our hypothesis that bacterial phylogeny predicts mVOC composition and the olfactory responses of *A. colemani*.

Despite an increased understanding of the role of microbial volatile emissions as insect semiochemicals, at present it is not well known which microbial volatiles or blends of microbial volatiles define the insects' response. Therefore, in **Chapter 5** we aimed at identifying specific compounds in bacterial volatile blends that attract *A. colemani* by using a combination of gas chromatography-electroantennography (GC-EAG), gas chromatography-mass spectrometry (GC-MS), and Y-tube olfactometer bioassays using synthetic volatile compounds. Next, the most promising mixture of putatively attractive synthetic compounds was evaluated in two-choice cage experiments to investigate whether *A. colemani* parasitoids responded to the volatile blend under greenhouse conditions. Results revealed a number of compounds that were significantly attractive or repellent. In particular, a mixture consisting of 100 ng/µL styrene and 1 ng/µL benzaldehyde was most attractive for *A. colemani*, both in laboratory and greenhouse experiments. Overall, these results indicate that a limited number of volatiles released under particular concentrations can have an important impact on insect olfactory responses and therefore open new opportunities to attract or retain natural enemies of pest species in the crop and possibly to enhance biological pest control.

Altogether, this PhD study has provided a better understanding of volatile-mediated interactions between microorganisms, parasitoids and hyperparasitoids. This knowledge combined with a selectively supportive food source for natural enemies may be exploited to develop novel tools that attract, retain and sustain natural enemies of pest species, and potentially lead to improved biological control efficacy and consequently more sustainable agricultural practices.

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Chapter 1

General introduction

1.1 Biological control

Crop production is continuously threatened by weeds, pathogens and insect pests causing substantial crop losses. Figure 1.1 illustrates global variations in crop losses and production of five major food crops, including wheat, rice, maize, potato and soybean, showing crop losses from 17% to 30% worldwide (Savary et al., 2019). Despite major progress in agricultural productivity and economic well-being during the last decades, food insecurity continues to be a serious problem in many regions of the world with rapidly growing human populations (Sharma et al., 2017). In addition, this progress has inflicted serious damage to natural resources, depleting, degrading or polluting fresh water, soil quality and biodiversity. Insect pests are one of the major problems in today's agriculture causing significant economic losses by inflicting direct (e.g. feeding) and indirect (e.g. vectoring plant pathogens) damage to crops (Savary et al., 2019; Sharma et al., 2017). Quantitative data on damage caused by insect pests is only scarcely available (Oerke, 2006). Nevertheless, it is estimated that on average 18%-20% of annual crop production is lost worldwide to arthropod inflicted damage, estimated at an economic value of more than US$470 billion (Sharma et al., 2017).

Conventional pest control relies heavily on the use of chemical pesticides (Peshin & Zhang, 2014). However, the dependence on chemical means of control can have serious negative impacts such as fast resistance development against active components, and harmful effects on humans, the environment and other non-target organisms (Pimentel et al., 2005; Damalas & Eleftherohorinos, 2011; Geiger et al., 2010). Therefore, there is a growing need for more environmentally sound alternative methods replacing or reducing the use of chemical pesticides. In light of this, biological control that uses natural pest enemies (i.e. arthropods or entomopathogens) to reduce pest populations has become an important alternative way of pest management. Moreover, biological control represents an increasingly important part of Integrated Pest Management (IPM) programmes. In general, IPM is defined as a systematic approach to pest management where all possible tactics, including biological control, are implemented to prevent, monitor and control diseases and pests, ensuring that crop damage remains under defined economic thresholds. Chemical pesticides are only used as a last resort when all other tactics fail or are ineffective with care to minimize damage to the ecosystem (Benbrook et al., 1996; van Lenteren, 2012). Since January 2014, all EU professional growers are obligated to apply

IPM tactics according to EU Directive Sustainable Use (Directive 2009/128/EC), indicating that the use of biological control will only increase.

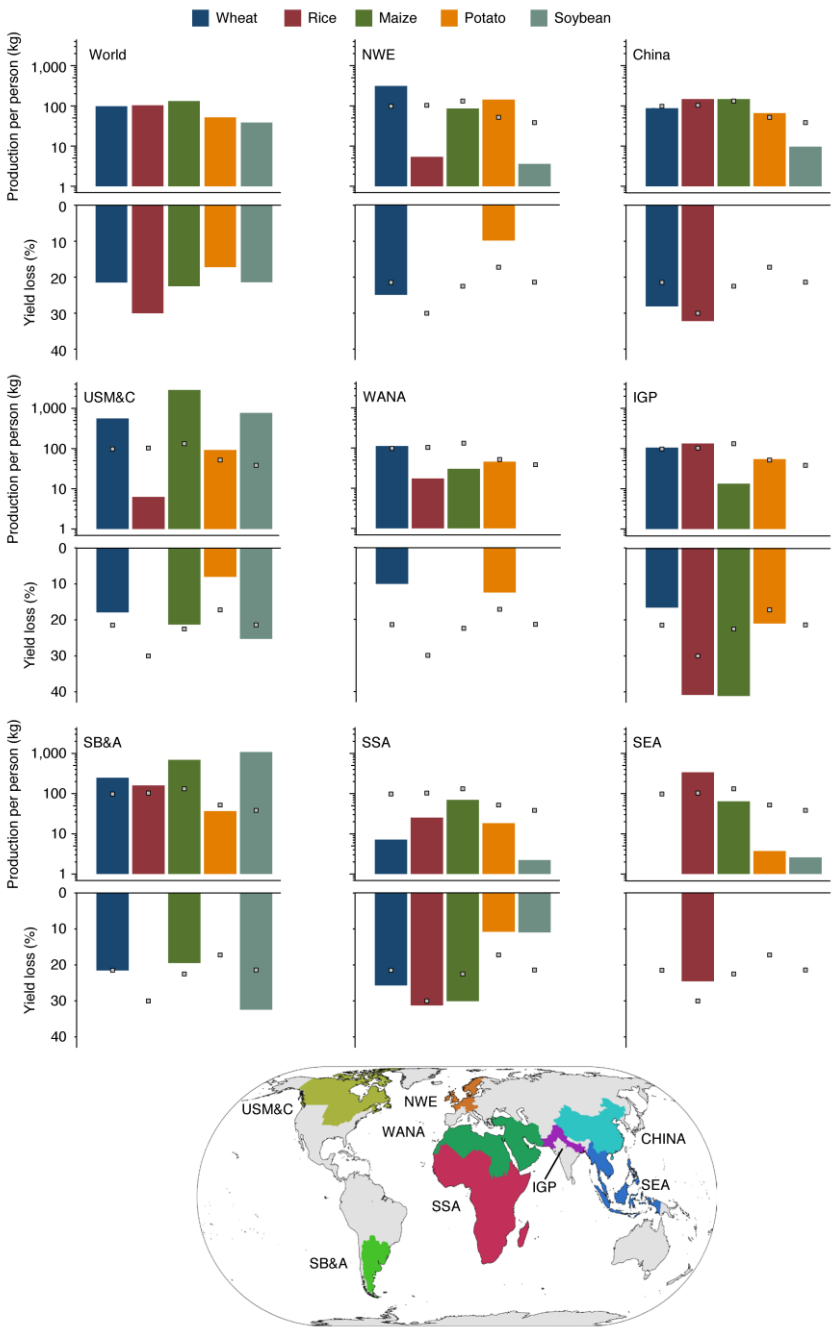

Figure 1.1: Global variations in crop losses and production. The top left chart illustrates losses and production of the major food crops wheat, rice, maize, potato and soybean at a global scale. The other charts are specific to the eight most important food security hotspots (i.e. most important regions with crop production with different levels of food security investigated in this study (Savary et al., 2019)): United States Midwest and Canada (USM&C); South Brazil, Paraguay, Uruguay and Argentina (SB&A); Northwest Europe (NWE); West Asia and North Africa (WANA); Sub-Saharan Africa (SSA); mainland China (China); the Indo-Gangetic Plain (IGP); and Southeast Asia (SEA). The upper portion of each chart shows the kilograms of crop production per person (2010–2014 averages) on a log10 scale. The lower portion shows the percentage yield losses across all reported pathogens and pests. Food security hotspot charts only show losses where there were sufficient survey responses to estimate the loss. The grey dots represent the world averages per crop. The global map shows the location of the eight food security hotspots (Savary et al., 2019).

Biological control can be defined as: "*The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be*" (Eilenberg et al., 2001). In this PhD study, we focus on the biological control of arthropod pests by natural enemies, also known as biological control agents (BCAs), which include predators (i.e. arthropods that feed on other arthropods), parasitoids (i.e. insects that lay their eggs in ("endoparasitoids") or on ("ectoparasitoids") other insects; the larvae then consume the host from the inside, eventually killing them) and entomopathogens (i.e. insect pathogenic viruses, bacteria, fungi and nematodes) (Bale et al., 2008). In nature, biological control occurs without human intervention in ecosystems all over the world. This is often referred to as "natural biological control", which is considered as an ecosystem service where naturally occurring beneficial organisms reduce pest organism populations (van Lenteren, 2012). This ecosystem service contributes substantially to crop production worldwide (Hill & Greathead, 2000; Oerke, 2006), as well as to forest production (Pimentel et al., 1997). Nevertheless, although the economic value of such biological control to society is clearly substantial (Costanza et al., 1997; TEEB, 2010), only few studies have actually estimated the value of natural biological control for farmers (Östman et al., 2003). In addition to natural forms of biological control, man started to use arthropod biological control around the year 300 AD by using predatory ants for control of pests in citrus orchards (van Lenteren & Godfray, 2005), and since then different types of biological control have been developed.

1.1.1 Types of biological pest control

There are different types of biological control in which man intervenes to reduce pest populations, but there is substantial variation in terminology depending on the categorising method used (Eilenberg et al., 2001). In general, three main types of biological control can be distinguished: (i) classical (also referred to as "inoculation" or "importation" biological control), (ii) augmentative (which includes two distinct types "inundative" and "seasonal inoculation") and (iii) conservation biological control (Orr, 2009).

CLASSICAL BIOLOGICAL CONTROL is the intentional release of an exotic natural enemy to control an invasive, non-native pest that has settled in a new geographical area (Myers & Cory, 2017). The expanding trade on a global scale has led to an increased number of invasive plants and insects establishing in new areas worldwide which have become targets for biological control (Hoddle and Syrett 2002). Generally, the exotic natural enemies (or sometimes related species) are collected in the area of origin of the pest and released in the new area with the aim to establish a permanent, self-sustaining population and ensure long-term control of the newly introduced pest (van Lenteren, 2012). This type of biological control is called "classical" control as it was the first type of biological control that was widely implemented (Debach & Rosen, 1991). Most successes were achieved in perennial crops (such as orchards and forests) where the ecosystem allows for long-term establishment of pest and natural enemy interactions (Hoddle et al., 2015). Despite its effectiveness, non-target impacts of introducing non-native natural enemies has received considerable attention lately, hindering further implementation of this type of biological control (Myers & Cory, 2017). Introducing non-native species can cause damage to native plants, vector harmful pathogens, cause biodiversity loss, and even replace or interfere with potential native natural enemies (De Clercq et al., 2011).

AUGMENTATIVE BIOLOGICAL CONTROL includes all activities in which natural enemies are periodically released to control native or non-native pest populations, usually depending on commercially mass-reared BCAs (Bale et al., 2008). van Lenteren (2012) provides a list of the most commonly used commercial natural enemies deployed in augmentative biological control. Hymenopteran parasitoids have been used most extensively in the past because, in comparison to predators, they are more specific, resulting in a more restricted host range and preventing unwanted non-target effects. However, more recently, generalist BCAs such as predatory mites and bugs are increasingly implementated for effective control measures. Within this type of biological control, a distinction is made between "inundative" and "seasonal inoculation". Inundative control refers to the method where mass-reared natural enemies are released in large numbers for immediate control or even local extinction of pest insects. Control is achieved mainly by the individuals that have been released rather than their offspring. This method is typically implemented in short-term annual crops where permanent establishment of natural enemies is impeded, and it often requires re-releases (Bale et al., 2008; van Lenteren, 2012). Seasonal inoculative control is a similar form of augmentation in which

natural enemies are mass reared and periodically released into short-term crops where many pest generations can occur in each growing season. As with inundative control, relatively large numbers of natural enemies are released to obtain immediate control, but in addition, a build-up of the natural enemy population occurs through successive generations during the same growing season (van Lenteren, 2000). In contrast to inundative control, seasonal control thus also involves control by the offspring of the natural enemies released. Examples include the control of whiteflies, leafminers, thrips, aphids and mites by parasitoids and predators in greenhouses (van Lenteren, 2012).

CONSERVATION BIOLOGICAL CONTROL is defined as the modification of the environment or management practices to protect and enhance the abundance or activity of natural enemies present in the environment (Eilenberg et al., 2001). These practices include modification of pesticide use, creation of sheltering and overwintering refuges, providing alternative hosts or prey during low pest levels, and providing essential food sources in the form of flowering plants or artificial food sources (Bale et al., 2008). This method is typically implemented to support classical and augmentative biological control in IPM programmes.

1.1.2 Advantages and challenges of biological control

Due to increasing resistance against chemical pesticides, increasing abandonment of chemical pesticides, increasing registration costs resulting in fewer successful compounds, and increased consumer awareness of residue impacts on human and environmental health, there is a growing demand for more environmentally sound alternatives of pest control (Gurr et al., 2012). Biological control can contribute to this demand as it is a more environmentally safe and even a more economically profitable alternative pest management method compared to chemical control (van Lenteren, 2012). One major advantage of biological control is that most BCAs have a specific host/prey range they attack (Bale et al., 2008), while chemical pesticides often also kill non-target organisms such as pollinators and natural enemies within and outside the agroecosystem (van Lenteren, 2012). Additionally, biological control can be carried out on a significantly large area with limited efforts when highly mobile BCAs are used, while chemical control is limited to the area where the pesticide is applied and often requires multiple applications. Further, natural enemies actively search for their host or prey and typically

can provide biocontrol activity over an extended period of time (Goldson et al., 2014). Nevertheless, there have been cases where non-target effects of BCAs were reported when non-native natural enemies were imported and released, although they are rare (Myers & Cory, 2017). For example, the introduction of the harlequin ladybird (*Harmonia axyridis*) in Europe as a natural enemy for aphids and scale insects has led to 30 – 44% decline of native coccinellid populations in a period of five years after the introduction. It is now considered as an invasive pest as it competes with and also attacks native coccinellid populations (Pyšek et al., 2017). Therefore, introducing non-native BCAs in biological control programmes has increasingly been under scrutiny, resulting in the impediment of new classical control initiatives (De Clercq et al., 2011; Myers & Cory, 2017). Currently, risk assessments are mandatory for the use of non-native natural enemies or the implementation is already restricted in several countries (De Clercq et al., 2011; van Lenteren, 2012).

The frequent and irresponsible use of chemical pesticides can lead to pest resistance through natural selection, which might render the product useless for future implementation (Bale et al., 2008). Resistance development to natural enemies is more rare or occurs much slower, but specific facultative endosymbionts of aphids and other insects have been shown to provide protection against the attack of hymenopteran parasitoids, sometimes conferring resistance close to 100% depending on the strain (Hoddle et al., 2015). The development time of new biological and chemical control products is practically the same, while the development costs of biological control (2 million US$) is only a fraction of that of chemical control (256 million US$), mainly due to toxicological evaluation costs. Moreover, compared to chemical control, the success ratio in finding new, applicable BCAs is very high (1:10 versus 1:140 000 for biological and chemical control, respectively) (Bale et al., 2008; van Lenteren, 2012). Furthermore, the benefit-to-cost ratio can be particularly high in biological control programmes (Myers & Cory, 2017).

One of the main limitations in biological control is that the pest insect is not immediately suppressed as is typically the case in chemical control. The natural enemies need a certain period of time to reach a density which can suppress the pest populations, and it may take several days for parasitized individuals to die (Bale et al., 2008). Furthermore, chemical control can ensure complete eradication of pests, while biological control can only reduce pest densities or realise local eradication (Bale et al., 2008). It has

to be noted, however, that pest reduction to levels below the economic threshold already brings about a satisfactory solution of the pest problem. Simultaneously, as natural enemies can maintain themselves on remaining populations, it may lead to long-term pest population suppression. However, success of a control measure is not only measured by decrease in pest population density. The application of biological control will further lead to reduced pesticide use, protection of human health, increased food quality, increased biodiversity and consequently maintenance of ecosystem services (Cock et al., 2010). Success in biological control is mainly dependent on reaching sufficient population densities of the natural enemy (Hoddle et al., 2015), but it remains a challenge to attract and retain these natural enemies into the crop where they are needed for control (Kaplan, 2012). Natural enemies tend to leave the targeted area when conditions are suboptimal (i.e. low host population density, high level of competition or un-preferred host plants), particularly in open fields (Boivin et al., 2012). Furthermore, the establishment of natural enemy populations can be hampered by the presence of the natural enemies of these groups, such as secondary parasitoids, i.e. parasitoids of immature stages of primary parasitoids which commonly occur in terrestrial food webs (also referred to as "hyperparasitoids") (Tougeron & Tena, 2019). Hyperparasitoids can considerably reduce primary parasitoid numbers because they develop at the expense of the primary parasitoid. As a result, they can seriously disrupt biological control programmes by reducing or completely eliminating control by the following parasitoid generations (Prado et al., 2015; Sullivan & Völk, 1999). Particularly high levels of hyperparasitism can be reached in greenhouse settings due to a temperature advantage for the hyperparasitoids (Prado et al., 2015). In fact, several studies have reported hyperparasitism levels reaching 90-100% (Acheampong et al., 2012; Bloemhard et al., 2014; Höller et al., 1993; Nagasaka et al., 2010). In addition, hyperparasitoids may not only affect primary parasitoid abundance and establishment through direct parasitism, but they can also act as intraguild competitors (i.e. facultative hyperparasitoids that exploit both herbivores and parasitoids) and induce dispersal and patch leaving by the emission of specific volatiles (Höller et al., 1994). Finally, many adult parasitoids used in biological control require non-prey food sources to meet their energetic and nutritional requirements to effectively control a certain pest (Wäckers, 2005), which will be discussed in more detail in the following section.

1.2 Biological control efficacy relies on availability of carbohydrate-rich food sources

An important aspect in the management of insect pests by natural enemies is the presence of non-prey food sources to cover the energetic and nutritional needs of the adult enemies (Jervis et al., 1993; Wäckers, 2005). Reports on the consumption of non-prey food sources, particularly plant materials, by predators and parasitoids are common throughout the literature (e.g. reviewed by Coll (1998) and Coll & Guershon (2002)). Predators belonging to a variety of orders and families are known to feed on pollen and nectar, and adult parasitoids acquire nutrients from honeydew and floral and extrafloral nectar. Feeding on carbohydrate-rich non-prey foods not only provides adult natural enemies with energy and nutrients, they also provide the natural enemies with additional benefits, such as enhanced flight activity, higher longevity and lifetime fecundity (i.e. total amount of eggs produced by a female natural enemy over the course of its lifetime), and improved foraging behaviour, thereby improving their biological pest control activities (Benelli et al., 2017; Tena et al., 2015; Winkler et al., 2006).

Depending on the life history stages of the natural enemies that feed on prey or hosts and non-prey food sources, a distinction can be made between true omnivores and life-history omnivores (Wäckers & van Rijn, 2005). On the one hand, in their adult stage parasitoid species often exclusively feed on non-prey food sources, while the larval stages feed on the host (life-history omnivores). Furthermore, female adults have to search for suitable hosts for oviposition. As a result, parasitoids have to frequently switch between food and host foraging behaviour, limiting their time spent on host location (Lewis et al., 1998; Azzouz et al., 2004). Moreover, non-prey food sites are often situated at different locations than their host sites (Lewis et al., 1998), especially in intensively managed agricultural systems where natural sugar sources are scarce. Traveling to these distant food sites decreases time and energy available for host searching, with possible negative effects on reproductive success (Jervis et al., 1996). Nevertheless, the distance between food sources and hosts can be considerably reduced if the parasitoid is associated with honeydew-producing hosts, as honeydew will always be in close vicinity of the host. However, abundance and quality of honeydew is variable in field conditions, as its production varies with the developmental stage of the aphid, the plant species infested, and its growth conditions (Azzouz et al., 2004; Lewis et al., 1998). On the other hand, some

parasitoid species can also obtain essential nutrients from direct host feeding (true omnivores) (Heimpel & Collier, 1996; Wäckers, 2005). Although such parasitoids need to shift less from host searching to food location, these species also need additional non-prey food sources to fully meet their energetic needs (Jervis et al., 1996). In the absence of suitable food sources, longevity and fecundity are often seriously hampered, compromising their potential to control pests (Heimpel & Jervis, 2005; Wäckers, 2003).

Host location abilities of parasitoids is greatly influenced by their physiological state. It has been demonstrated that a lack of suitable food sources can cause adult parasitoids to cease host location in favour of food foraging (Wäckers, 1994). Conversely, sugar-fed parasitoids have been shown to prefer host related cues over food related cues (Burrows et al., 2017; Wäckers, 1994), increasing their host location efficacy. Therefore, the availability of non-prey food sources are critical to parasitoids to perform biological pest control activities (Tena et al., 2015). Indeed, providing carbohydrate and protein sources to natural enemies prior to release has been demonstrated to increase their longevity, reproductive output and even to improve host finding in several parasitoid species (Heimpel & Jervis, 2005; Irvin et al., 2007; Irvin & Hoddle, 2007). To fuel their physical activity and metabolic upkeep, predators and parasitoids can exploit a wide range of carbohydrate sources, including floral and extrafloral nectar, fruits, plant sap exudates, and honeydew, which is the sugar-rich excretion product of phloem feeding arthropods (Wäckers, 2005). The suitability of a certain carbohydrate source depends on its availability, apparency, accessibility, nutritional composition and the foraging risks associated with its exploitation (Wäckers, 2005; Wäckers & van Rijn, 2012). The most important, natural sugar sources exploited by natural enemies, parasitoids in particular, are briefly discussed below.

In terms of availability, honeydew (Fig. 1.2) is the primary carbohydrate source in many ecosystems, certainly in agroecosystems where other carbohydrate sources like nectar are often scarce (Wäckers et al., 2008). Honeydew is a sugar-rich excretion product of phloem-sap feeders such as aphids, whiteflies, scales and mealybugs, and is primarily considered a waste product allowing phloem-feeders to dispose of excess carbohydrates (Wäckers et al., 2008; Wilkinson et al., 1997). Parasitoids often actively feed on honeydew in the field (associated with the crop itself, with weeds growing within the crop, or with surrounding vegetation), even when their hosts do not produce honeydew (Faria et al., 2008; Steppuhn & Wäckers, 2004; Tena et al., 2013b). Nevertheless, in comparison with

other carbohydrate sources such as nectar, honeydew may be of lower nutritional quality to parasitoids, e.g. due to unfavourable sugar composition, the presence of hostile plant-derived secondary metabolites and/or compounds synthesized by the honeydew-producing insects (Wäckers et al., 2008; Tena et al., 2016), which less support their energetic and nutritional needs. Further, the typical high viscosity of honeydew limits easy access and ingestion of the food source (Faria et al., 2008; Wäckers & Swaans, 1993; Wäckers, 2005). Furthermore, the nutritional value of honeydew can be highly variable, as it differs significantly in sugar composition and concentration depending on the plant and phloem-feeding insect species (Wäckers et al., 2008; Tena et al., 2018). Besides typical plant-derived sugars such as fructose, glucose, maltose and sucrose, honeydew can also contain more complex, insect-synthesised sugars like melezitose, raffinose, erlose and trehalose (Hogervorst et al., 2003, 2007b; Fischer et al., 2005; Wäckers, 2000, 2005). Parasitoids of honeydew-producing hosts typically have honeydew at their disposal in close vicinity to their host, which decreases effort and time required for searching for carbohydrate sources, minimising energy loss and risks associated with searching for other sources (Wackers et al., 2008). Despite the potential lower nutritional value of honeydew, it has been shown to increase parasitoid longevity and fecundity, although less in comparison with nectar (Tena et al., 2018). Finally, not only primary parasitoids, but also higher trophic levels such as hyperparasitoids are able to exploit and benefit from honeydew feeding, even for species not closely associated with honeydew producing insects (van Neerbos et al., 2019).

Figure 1.2: Honeydew produced by the aphid *Myzus persicae* (Hemiptera: Aphididae) on sweet pepper leaves (Photo credit: Tim Goelen).

Compared to honeydew, both floral and extrafloral nectar constitute more suitable carbohydrate sources to cover the energetic requirements of natural enemies. The terms 'floral' and 'extrafloral' nectar refer to the location of the nectaries where the nectar is produced (Hogervorst et al., 2007a; Wäckers, 2005). Floral nectar is a sweet, aqueous solution produced in the nectaries of flowers and mainly contains sugars and amino acids. Because floral nectar mainly consists of quickly digestible carbohydrates with high-energy content such as sucrose, glucose and fructose, it serves as the primary reward to recruit flower-visiting insects for their ecosystem services such as pollination and plant protection (Wäckers, 2005). In addition, lower concentrations of other carbohydrates, such as maltose, melibiose, galactose and raffinose have been found in floral nectar (Baker & Baker, 1983; Wäckers, 2001). Floral nectar can be particularly abundant in natural ecosystems, but it is generally limited in modern agroecosystems due to low availability or even complete absence of flowering plants (Wäckers & van Rijn, 2012). Despite the availability of floral nectar in natural ecosystems, it is often restricted by short periods of flowering and nectar production, which can range from a few hours to several days (Pacini & Nicolson, 2007; Wäckers & van Rijn, 2012). Furthermore, floral nectar may not be easily accessible to certain insect groups like parasitoids due to the short and less specialised mouthparts of many parasitoid species, particularly in plants with unsuitable flower structures such as deeper corolla flowers (Wäckers & van Rijn, 2012). In addition, the presence of other flower visitors can cause strong competition with parasitoids for floral nectar (Campbell et al., 2012; Wäckers & van Rijn, 2012). Nevertheless, availability of floral nectar has been shown to positively affect parasitoid longevity, fecundity and the ability to attack hosts compared to situations where these resources are lacking (Araj et al., 2006, 2008, 2009; Lee & Heimpel, 2008; Winkler et al., 2006). Some plant species are able to produce extrafloral nectar in various vegetative and reproductive structures of the plant outside flowers, such as leaves, stems and fruits, and therefore can serve as an additional food source for natural enemies (Koptur, 1992; Wäckers, 2005). Extrafloral nectar is often excreted by plants experiencing herbivory as a means to attract natural enemies that can defend the plant against the herbivore attack (Escalante-Pérez & Heil, 2012; Wäckers, 2005). The majority of extrafloral nectaries are easily accessible for natural enemies as a result of their exposed nature (Koptur, 1992; Wäckers, 2005). Furthermore, extrafloral nectaries often excrete high volumes of nectar and over larger periods of time than flowers (Wäckers & Bonifay, 2004). Research has shown that

parasitoids that feed on extrafloral nectar often show significantly higher longevity and fecundity (Géneau et al., 2012).

Because of the scarcity of flowering plants in modern agroecosystems and the advantages of nectar feeding on natural enemy fitness, flowering plants are increasingly implemented in conservation biological control programmes to improve the performance of natural enemies (Tschumi et al., 2015, 2016; Wäckers et al., 2008; Wäckers & van Rijn, 2012; Winkler et al., 2006, 2010). This can be achieved through establishing flower strips or margins alongside or through crop fields in which flowering plant species are sown or naturally regenerated (Fig. 1.3) (Haaland et al., 2011). Such flower strips not only increase plant diversity in the agricultural landscape, but also provide food resources, shelter and overwintering sites that may attract and sustain beneficial insects, including natural enemies that can potentially improve biocontrol of pests in the adjacent crops (Balzan et al., 2016; Balzan & Moonen, 2014; Olson & Wäckers, 2007; Tschumi et al., 2016). Although the advantages of nectar availability are clear, the effects of flower strips on biological control efficacy are not always consistent (Heimpel, 2019; Lee & Heimpel, 2005; Winkler et al., 2010). Nevertheless, studies have shown that the presence of flower strips may (locally) increase the abundance of natural enemies (Bianchi & Wäckers 2008), increase parasitism rates (Lee & Heimpel 2008), and even reduce pest populations and crop damage (Tschumi et al., 2015). On the other hand, it has also been shown that flower strips may harbour pest insects (Jacquemyn et al., 2019), which may disperse into the crop. It is therefore of utmost importance that the flower strips are equipped with species that are highly supportive for natural enemies, and not or less suitable for pest insects. Campbell et al. (2012) demonstrated that different beneficial insect groups show distinctly different responses to floral traits and trait diversity. This information can be used to adjust the species composition of the flower strips to selectively attract and support specific beneficial insect groups (Wäckers & van Rijn, 2012). However, still care should be taken that the selected flowers do not attract and support unwanted or harmful insects (Wäckers & van Rijn, 2012). It has been demonstrated that hyperparasitoids can benefit more from nectar availability than their hosts and hence lead to increased hyperparasitism rates (Araj et al., 2006, 2008, 2009), thereby negatively affecting natural enemy populations.

Figure 1.3: Nectar-producing plants in flower strips through or alongside the crop can provide carbohydrate-rich food sources to natural enemies (Photo credit: Tim Goelen).

Instead of providing nectar producing flowers, there is an increasing interest to use artificial sugar sources to increase the performance of natural enemies (Tena et al., 2015; Wade et al., 2008), especially in greenhouse environments where environmental conditions and available crop area impedes implementation of flowering plants. These supplemental food sources typically consist of carbohydrate- and/or protein-rich ingredients in liquid formulations, which can be provided in various forms. Artificial food sprays have been implemented in the past where the artificial food sources are applied to the foliage using a sprayer (Jacob & Evans, 1998; Tena et al., 2015; Wade et al., 2008), with the ultimate goal to attract, retain and support the natural enemies when non-prey food sources are lacking (Wade et al., 2008). Nevertheless, the use of food sprays can have serious drawbacks as the sticky sugar-rich solutions can stain the foliage with the risk of causing diseases on the sprayed plants (Mitsunaga et al., 2012). For example, sugar sprayed foliage can promote the growth of sooty moulds which results in decreased photosynthesis efficiency. Furthermore, the sugar sprayed foliage could attract insect pests that can further inflict herbivore damage (McEwan & Morris, 1998). An alternative method of providing artificial food sources to insects that limits these drawbacks, is the use of artificial feeding devices (Shimoda et al., 2014). Such artificial feeding devices are typically small cups or bottles containing the artificial food source, which is only accessible to insects on a limited area (Fig. 1.4) (Shimoda et al., 2014). However, a major disadvantage of such devices is that they are not easily found by natural enemies in tight

crops with high plant densities, as plain sugars do not produce a chemical signal (smell) (Tena et al., 2015). Nevertheless, this could potentially be alleviated by combining the device with natural enemy attracting colours and odours, as foraging natural enemies have been shown to use visual and olfactory cues when foraging for food (Kugimiya et al., 2010; Wäckers, 1994). The challenge, however, remains to develop specific food sources that selectively support natural enemies, without sustaining non-target insects such as pest insects and hyperparasitoids.

Figure 1.4: Bottle-type feeding device following the design of Shimoda et al. (2014) **(A) & (B)** comprised of a plastic bottle with plastic interior lid for storing sugar solutions, a capillary bundle of polypropylene fibres to supply the sugar solution to the top of the station and an attractively coloured cap (yellow) to visually attract parasitoids. **(B)** Photograph of yellow coloured cap with *Aphidius ervi* feeding on the sugar solutions supplied by the polypropylene capillary bundle (Photo credits: Dieter Baets).

1.3 Semiochemicals as mediators of insect foraging behaviour

1.3.1 Definitions

Semiochemicals are information-conveying chemical cues that mediate the interactions between two organisms by inducing a behavioural and/or physiological response in one or both of the involved organisms (Vet & Dicke, 1992). These chemical cues are an important source of information mediating ecological interactions between organisms either within or between species such as microorganisms, plants and insects (Beck et al., 2017). Depending on their ecological roles, semiochemicals can be categorised into two broad categories, i.e. pheromones and allelochemicals (Fig. 1.5) (Nordlund & Lewis, 1976; Vet & Dicke, 1992). Pheromones are semiochemicals that mediate the interactions between organisms of the same species (intraspecific interactions), which can be further subdivided based on the response they induce in the receiving individuals. These include aggregation pheromones, alarm pheromones, sex pheromones, territory marking

pheromones, and trail marking pheromones (Beck et al., 2017). Sex pheromones are widespread among insects and are emitted by males or females to elicit a behavioural response in the opposite sex, which might directly or indirectly lead to mating (Powell, 1999). Pheromones have been intensively studied over the years and have also been successfully implemented in IPM programmes for several pest species, particularly those of Lepidoptera (Witzgall et al., 2010). They can be implemented for both pest population monitoring and population reduction. The latter can be achieved by either mating disruption (i.e. causing disorientation and disrupting communication between sexes therefore reducing or preventing mating behaviour) or attract-and-kill techniques (i.e. attracting one or both of the sexes to a lure where they are eliminated by a killing agent) (Witzgall et al., 2010). Allelochemicals are semiochemicals that mediate the interactions between organisms of different species (interspecific interactions). Allelochemicals can be further subdivided in three categories based on the benefits of the emitter and the receiver: allomones are only favourable for the emitter; synonomes are beneficial for both the emitter and the receiver; and kairomones are only favourable for the receiver (Nordlund & Lewis, 1976; Vet & Dicke, 1992). However, this classification is context dependent rather than chemical dependent. For example, aphid alarm pheromones, which are emitted by aphids under attack to warn conspecifics of danger, can also be exploited by parasitoids to locate their aphid host, and therefore not only act as pheromones for their conspecifics but also as kairomones for the parasitoids (Micha & Wyss, 1996).

Semiochemicals can be volatile compounds that are detected by olfactory receptors up to long distances, or they can be non-volatile or low-volatile compounds, mainly acting as contact substances detected by gustatory receptors at a short range (Meiners & Peri, 2013). Therefore, semiochemicals play an important role in all major steps in volatile-mediated foraging behaviour of insects.

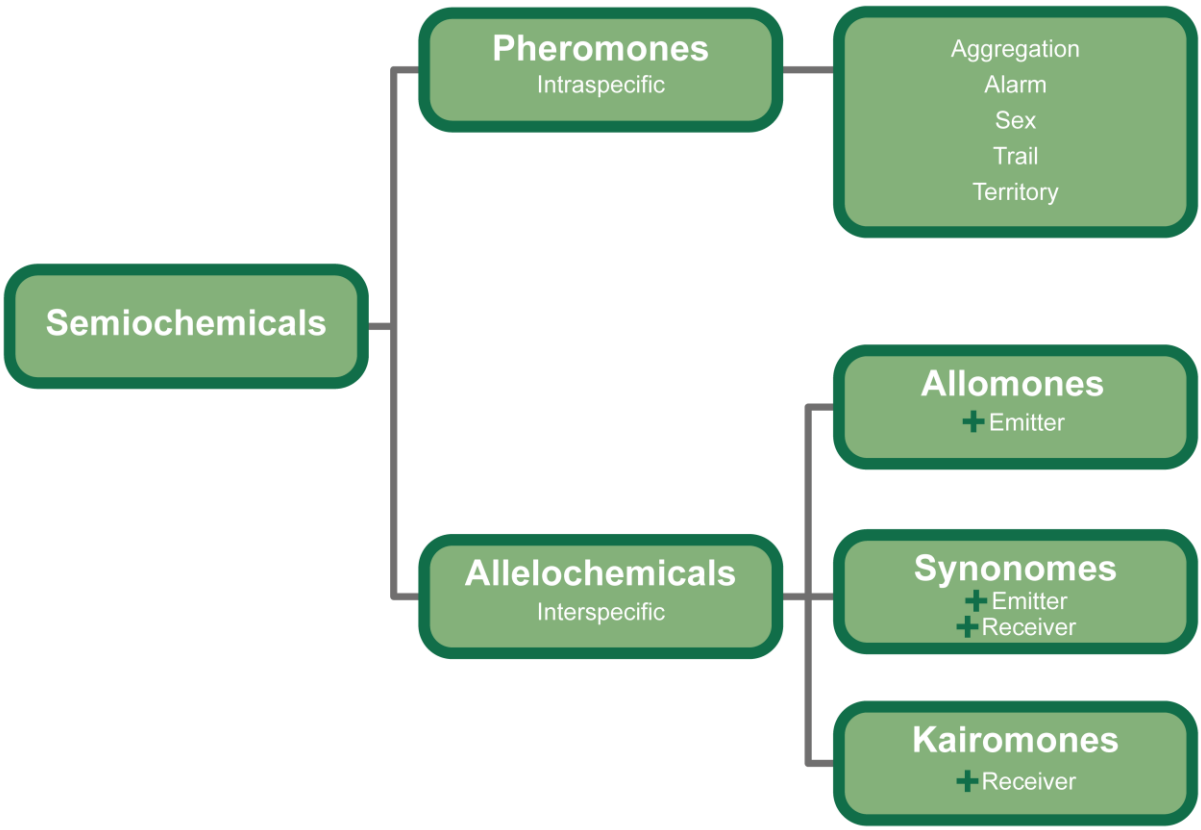

Figure 1.5. Classification of semiochemicals. Semiochemicals are classified according to the relationships between the organisms involved. Pheromones are secreted and released by an organism and cause a specific response in a receiving organism of the same species, whilst allelochemicals are produced by one species and cause a response in a different species. Allelochemicals are further subdivided in three categories based on the benefits of the emitter and the receiver: allomones are only favourable for the emitter; synonomes are beneficial for both the emitter and the receiver; and kairomones are only favourable for the receiver.

1.3.2 Volatile-mediated foraging behaviour in parasitoids

Insects live in highly complex environments of very different stimuli in which their resources are surrounded by several non-resources. Therefore, successful location of resources such as food, mates and oviposition sites requires the ability to efficiently detect these resources. Insects have evolved several sensory systems for perceiving stimuli from their environment, including olfactory, visual, acoustic, tactile and gustatory systems, and they use a combination of these cues to make foraging decisions (Bell, 1990; Schellhorn, et al., 2014). In parasitoids, olfactory cues play a major role in locating resources and other critical aspects of their life cycle (Bruce et al., 2005; Lima & Dill, 1990; Webster & Cardé, 2017). In their natural environment, parasitoids 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). These different messages not only arise from different senders or different biosynthetic

pathways (Wink, 2010; Wyatt, 2014a), but they can also differ by the way these signals are perceived by the sensory periphery and in the brain of the receiving insect (Ebrahim et al., 2015; Witzgall et al., 2012). During resource foraging, parasitoids use a wide variety of volatile cues originating from various sources. These sources can be classified into four main categories, including (i) cues originating from the habitat of the insect, the host microhabitat or the food plant, (ii) cues originating from the parasitoid itself, (iii) direct host-related cues, and (iv) indirect host-related cues. The first group of cues indicate a general area in which suitable resources are most likely to be found (Meiners, 2015). For example, green leaf volatiles can indicate the presence of a patch of host plants (i.e. plant species preferred by the parasitoids on which hosts are most likely to be found) in a complex landscape which also contains non-host plants (Webster & Cardé, 2017). Indeed, plant volatiles mainly act over long distances and are often good indicators for herbivore presence (Meiners & Peri, 2013). Further, parasitoids are known to respond to pheromones produced by both males and females. These include mainly sex pheromones, but also alarm, aggregation and marking pheromones are used by parasitoids, keeping them in certain areas or repel them out of these areas (Ruther, 2013). Direct host-related cues are highly reliable cues associated with a suitable host stage that are used to specifically locate feeding and/or oviposition sites (Webster & Cardé, 2017). These cues can be anything associated with the host, including sex, alarm and aggregation pheromones, feces, host cuticle or exuviae, honeydew, secretions and even associated microorganisms (Vet & Dicke, 1992; Meiners & Peri, 2013). Indirect host-related cues are indirectly associated with a suitable host stage as they arise from non-preferred host stages which can aid in locating suitable host stages (Meiners & Peri, 2013). For example, the egg parasitoid *Oomyzus gallerucae* (Hymenoptera: Eulophidae) is attracted by the faeces of both larvae and adult elm leaf beetle hosts, while it attacks the egg stage (Meiners & Hilker, 1997).

In terms of energy saving, host-searching parasitoids should maximize the rate at which they encounter appropriate hosts. Therefore, they must act on cues that directly or indirectly signal the presence of a suitable host. In this regard, parasitoids are faced with the "reliability-detectability problem" (Vet & Dicke, 1992). While foraging for resources, parasitoids can exploit easily detectable cues that are not directly associated with the resource, or use cues that are difficult to detect, but are directly associated to their resource. For example, host plant volatiles are typically present in high amounts because

of their high biomass, and are therefore easily detected by the parasitoid, especially when it is a crop plant growing in monoculture. However, during host searching they are less reliable since the plants may not be infested by herbivore hosts. In contrast, chemical cues associated with the host are very reliable for indicating host location, but usually they are low in concentration, and are therefore difficult to detect in a complex environment (Vet & Dicke, 1992). Parasitoid foraging behaviour can be divided into three main steps, including (i) habitat location, (ii) resource location within the habitat, and (iii) resource acceptance (Vinson, 1976). The latter can be further subdivided into host recognition and host acceptance (Muratori et al., 2006), in which parasitoids typically exploit contact cues, taste cues or ovipositor probing (Hatano et al., 2008). The first two steps are most often guided by olfactory cues, where the combination of different types of semiochemicals emitted by both the habitat and specific resources in the habitat are essential to locate food, hosts, or mates (Schröder & Hilker, 2008). Therefore, parasitoids use a hierarchical organisation in their foraging steps in which a different set of volatiles drives each step (Webster & Cardé, 2017).

Parasitoids can partly resolve the "reliability-detectability problem" by exploiting indirect information of herbivore presence such as the volatiles emitted by herbivore infested plants (Vet & Dicke, 1992). Plants attacked by herbivores emit an induced set of volatiles due to herbivore damage or oviposition that is significantly different from the set of volatiles emitted by uninfested plants (Hilker & Fatouros, 2015; Turlings et al., 1990). The emission of these herbivore-induced plant volatiles (HIPVs) can be exploited by parasitoids as reliable and well-detectable cues during host location to find infested host plants (Dicke & Baldwin, 2010). This phenomenon has been recorded for over 50 different plant species belonging to 25 different families attracting various natural enemies, including parasitoids and predators (Mumm & Dicke, 2010). For the HIPV emitting plants, the HIPVs function as an indirect defence mechanism by recruiting natural enemies to attack the herbivores (Dicke & Bladwin, 2010). However, this indirect plant defence tactic by releasing HIPVs can also be exploited by insects of higher trophic levels to locate their host. Recently, it has been shown that hyperparasitoids can respond to HIPVs in order to locate their primary parasitoid host (Poelman et al., 2012; Zhu et al., 2015). Moreover, the hyperparasitoids were able to distinguish between HIPVs released by plants infested with parasitized and unparasitized hosts (Poelman et al., 2012). HIPVs are mainly used by parasitoids for habitat location at long distances. Once in the vicinity of the habitat, they

engage in more localised foraging behaviour exploiting other short- to mid-range cues, which are typically host-related kairomones (de Rijk et al., 2013). This requires them to distinguish between, and act on, different semiochemicals during each step of the foraging process, which is realised by a high level of spatio-temporal resolution of their sensory systems (Meiners, 2015).

Parasitoids can exploit several chemical cues originating from their adult hosts to discriminate between infested and uninfested areas, and to locate suitable host stages. These kairomones can be detected at both long and short distances for habitat location and host location through induction of searching behaviour (Fatouros et al., 2008). Over long distances, parasitoids typically exploit various types of pheromones that are released by their hosts for habitat location (Colazza et al., 2009; Fatouros et al., 2008). Spying on host intraspecific communication through sex pheromone signals between female and male hosts by parasitoids in order to locate and attack their host is well documented for many parasitoid species (Huigens & Fatouros, 2013). For example, it has been demonstrated that the sex pheromone of the cabbage aphid *Brevicoryne brassicae* (Hemiptera: Aphididae) is attractive to both generalist and specialist natural enemies (Gabrys et al., 1997). Furthermore, some parasitoids are even able to bridge the temporal gap between nocturnal host mating and their oviposition activities by responding to pheromones adsorbed in epicuticular waxes of the host plant (Wäschke et al., 2013). Other intraspecific communication pheromones can also act as kairomones for parasitoids during foraging for hosts, including anti-sex (Huigens et al., 2009), aggregation (Nakamura et al., 2013) and alarm pheromones (Micha & Wyss, 1996). Other good sources of host location kairomones at both short- and long-range distances are host-derived by-products such as frass, honeydew, exuviae, secretions and scales (Fatouros et al., 2008, Meiners & Peri, 2013). The kairomones released from these by-products can even provide the parasitoid with specific information to discriminate hosts from non-hosts or even other suitable hosts, different stages of hosts, and hosts feeding on different plants (Battaglia et al., 2000; Chuche et al., 2006; Mattiacci & Dicke, 1995).

Once landed on a host plant, parasitoids mainly exploit contact and/or short-range kairomones of lower volatility that are closely associated to their host such as, for example, alarm and host-marking pheromones (Francis et al., 2005; Hoffmeister et al., 2000). Among other cues, chemical traces or “footprints” left behind by herbivores on the host plant play an important role in short-range host location (Rostás et al. 2008). These

chemical traces are typically high molecular weight compounds such as linear alkanes that probably originate from the herbivore cuticle, which are absorbed by the epicuticular wax layers of the leaves (Rostás & Wölfling, 2009). These kairomones can guide the parasitoid to suitable hosts on the plant and even allows for fine-tuning their host searching behaviour. Some egg parasitoids can use these "footprints" to discriminate between male and female hosts which allows them to efficiently locate potential host eggs (Colazza et al., 2007).

1.3.3 Microbial volatiles as insect semiochemicals

Interactions between plants and insects are not only driven by plant volatiles, but they can also be driven by volatiles released by other organisms (Beck et al., 2018). Microorganisms such as bacteria, fungi and yeasts are known to release a plethora of volatile organic compounds (microbial volatile organic compounds; further referred to as "mVOCs"), reaching the same complexity as seen in plants and insects (Schulz-Bohm et al., 2017). Up to now, over 2000 different mVOCs have been identified to be emitted by over 500 different microbial species (Effmert et al., 2012; Lemfack et al., 2017). mVOCs are typically small, odorous compounds (< C20) with low molecular mass (<300 Daltons), high vapour pressure, low boiling point and lipophilic moiety (Effmert et al., 2012; Schulz & Dickschat, 2007). In contrast with soluble metabolites, these properties facilitate evaporation and diffusion in below- and above-ground environments, allowing for long-distance transportation through the atmosphere (Tyc et al., 2017b). mVOCs are derived from several biosynthetic pathways (Peñuelas et al., 2014) and can be grouped into aromatic compounds, fatty acid derivatives, terpenoids, nitrogen- and sulphur containing compounds (Schulz & Dickschat, 2007). A number of chemical classes are especially wide-spread among microorganisms, among which the most important are acids, alcohols, aldehydes, alkenes, esters, ketones, terpenes, benzenoids, and pyrazines (Piechulla & Degenhardt, 2014; Schulz & Dickschat, 2007) (Fig. 1.6).

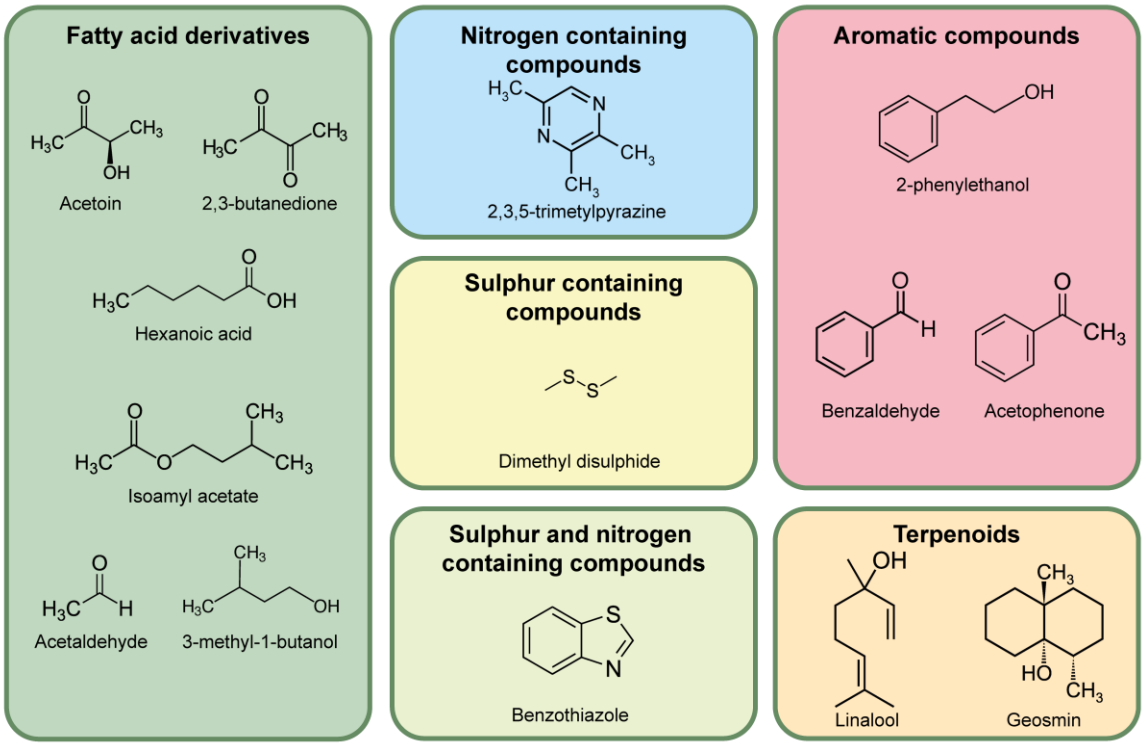

Figure 1.6: Classification of microbial volatile organic compounds (mVOCs) into fatty acid derivatives, nitrogen- and sulphur containing compounds, aromatic compounds, and terpenoids. Examples are provided which are commonly found in mVOC blends.

Although many volatiles are produced by various, unrelated microorganisms, there are also some unique volatiles that are only produced by specific microbial strains (Schulz & Dickschat, 2007; Garbeva et al., 2014). The composition of the mVOC blend, including concentration and relative ratios of the mVOCs, depends on several factors, including culturing conditions, the physiological state of the microorganism, oxygen availability, moisture, temperature, pH and presence of other microorganisms (Garbeva et al., 2014; Insam & Seewald, 2010; Romoli et al., 2014; Schulz-Bohm et al., 2015). Most mVOCs are considered to be side-products of primary and secondary metabolism, and are formed mainly by oxidation of glucose from various intermediates (Korpi et al., 2009). However, recent findings have shown that many mVOCs are not simply side-products, but display certain biological activities (Schmidt et al., 2015; Tyc et al., 2017a). Although the biological and ecological functions of mVOCs are not yet fully understood, several functions have been suggested. For example, it is assumed that mVOCs can act as (i) semiochemicals for inter- and intraspecific communication (Schulz-bohm et al., 2017); (ii) signals for cell-to-cell communication (Schmidt et al., 2015); (iii) means of disposing

waste or detoxification products (Claeson, 2007); or act as (iv) plant growth promoting or antibiotic agents (Kai et al., 2009). Several examples of ecological functions of mVOCs have been demonstrated recently, such as promoting plant growth and inducing systemic resistance to pathogens, mimicking plant hormones and inhibiting growth of competing microorganisms (Ryu et al., 2003, 2004). Additionally, there is mounting evidence that mVOCs may impact insect behaviour (Davis et al., 2013; Dzialo et al., 2017; Leroy et al., 2011b). 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 et al., 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 et al., 2013; Huang et al., 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 (Christiaens et al., 2014) or get protection in the insects during unfavourable conditions (Pozo et al., 2018). In addition, it can in some cases also aid them to complete their life cycle inside the insects (Burkepile et al., 2006; Pozo et al., 2018). The chemical communication between insects and microorganisms is thus believed to drive a mutualistic relationship, in which not only the insects benefit from the microorganisms, but also the microorganisms profit from the insects.

Several insect species have developed close associations with symbiotic microorganisms that provide essential nutrients or attract insects to suitable food or oviposition sites (Davis et al., 2013). A well-known example of microbial based insect attraction is the volatile-mediated attraction of *Drosophila melanogaster* (Diptera: Drosophilidae) (also known as the common fruit fly or vinegar fly) by yeasts occurring on rotting fruit on which the fruit flies can feed and lay eggs in. Moreover, the yeast itself constitutes a main food source for both adult and larval stages of *Drosophila* species (Stamps et al., 2012). Yeasts like *Saccharomyces cerevisiae* signal their presence by emitting a rich mVOC blend, which mainly contains fruity esters (Christiaens et al., 2014). *Drosophila* flies have been shown to exploit these mVOCs as semiochemicals to detect suitable food and oviposition sites and even use the mVOCs to discriminate between yeasts which best support their growth and survival (Scheidler et al., 2015). Recently, Becher et al. (2018) showed that volatile signalling and insect attraction is not limited to

a few yeast species, but instead is widespread and phylogenetically conserved in yeasts. Another example where yeast VOCs serve as important cues for location of suitable food sources includes floral nectar. Yeast volatile emissions in floral nectar have been shown to play a major role in the attraction of flower visiting insects, such as pollinators and natural enemies (Rering et al., 2018; Sobhy et al., 2018). Additionally, it has been suggested that volatiles produced by nectar yeasts serve as an excellent learning cue to locate suitable nectar sources (Sobhy et al., 2019). Furthermore, mVOCs emitted by bacteria colonising aphid honeydew have been shown to be attractive to tending ants, mediating ant-aphid interactions (Fischer et al., 2017b). Typically, microorganisms that can survive in the insect gut, on the outside of the insect body, and on plant surfaces or in plant secretions such as nectar release semiochemicals that affect insect behaviour (Beck et al., 2016).

Besides locating food sources, mVOCs can also be exploited by natural enemies to locate hosts or preys, and even stimulate oviposition (Davis et al., 2013; Leroy et al., 2011b). Parasitoids typically attack early life stages that most often do not emit pheromones which could be exploited to detect their host. Boone et al. (2008) showed that some hymenopteran parasitoids are able to bypass this obstacle by exploiting volatiles released by microbial symbionts associated with their host. In another example, bacteria associated with aphid hosts and honeydew have been shown to emit mVOCs that aid in the host location and even serve as an oviposition stimulant for hoverflies, increasing the number of eggs deposited on the host plant (Leroy et al., 2011a). While in these cases mVOCs elicit attractive behaviour in insects, some mVOCs have also been found to repel insects when signalling unsuitable food sources, unsuitable hosts or hostile environments (Azeem et al., 2013; Huang et al., 2006; Stensmyr et al., 2012). For example, geosmin, an earthly smelling microbial odour, precludes oviposition by *D. melanogaster*, preventing them from laying eggs on fruits colonized by harmful microorganisms (Stensmyr et al., 2012).

Furthermore, it has been demonstrated that microorganisms are able to manipulate intraspecific insect pheromone communication by altering the pheromones produced by the insects or directly producing pheromone compounds (Engl & Kaltenpoth, 2018). While pheromones have traditionally been regarded as insect-produced volatiles, mounting evidence indicates that host-associated microorganisms can have a major impact on the chemical communication between individuals of the same species (Engl &

Kaltenpoth, 2018; Keesey et al., 2017). For example, guaiacol is an aggregation pheromone of locusts, which seems to be mainly produced by bacteria present in the locust's gut and feces (Dillon et al., 2000). This indicates the mVOCs can play an important role in insect aggregation behaviour. In fact, a large number of insects that exhibit aggregation behaviours are strongly associated with specific microbial communities (Davis et al., 2013). This raises the question whether the semiochemicals that are now thought to be produced by plants, insects or their environment are not completely or in part the result of microbial volatile emission.

1.3.4 Use of semiochemicals in biological control

The potential of semiochemicals to affect the olfactory behaviour of insects has led to the development of novel tactics in biological control programmes to manipulate natural enemies by attracting and conserving them in the vicinity of the crops to be protected (Kaplan, 2012; Khan et al., 2008).

So far, research has mainly focussed on the application of insect- and plant-derived semiochemicals such as pheromones and HIPVs (Khan et al., 2008). Several tactics have been proposed for the implementation of HIPVs to affect natural enemy behaviour. One potential tactic is to synthetically produce HIPVs and release them into the crop separately or in a mixture, either by spraying them directly on the crop or using a slow-release dispenser (Simpson et al., 2011a; Uefune et al., 2012). Several field studies have shown that applying synthetic HIPVs can significantly increase natural enemy density and diversity within crops (Colazza et al., 2013; Simpson et al., 2013), as well as increase herbivore attack on infested plants (Uefune et al., 2012; Xu et al., 2018). Furthermore, synthetic HIPVs not only directly affect natural enemy behaviour, but they can also act as pheromones, inducing the production of endogenous HIPVs in the surrounding plants that could further recruit natural enemies (Rohwer & Erwin, 2008; Simpson et al., 2011a). Therefore, specific plant elicitors such as jasmonates have been sprayed on crops to induce HIPV emission, which in turn led to enhanced natural enemy attraction (van Poecke & Dicke, 2002; Sobhy et al., 2014). Finally, plants can be manipulated by selective breeding or genetic modification to produce and release specific HIPVs, leading to increased natural enemy recruitment (Gurr & You, 2016; Kos et al., 2009). When applying HIPVs in conservation biological control, care must be taken as they can have possible

negative effects such as increased possibility of intraguild predation or even attraction of hyperparasitoids, which can ultimately result in reduced pest control (Poelman et al. 2012; Poelman & Kos, 2016).

Besides using plant-derived volatiles, host-associated volatiles have been demonstrated to effectively attract natural enemies. Research mainly focussed on the exploitation of host sex and aggregation pheromones. Unlike HIPVs, these semiochemicals show high specificity and therefore provide accurate tools to enhance natural enemy efficacy (Meiners & Peri, 2013). Host sex and aggregation pheromones have been successfully used to enhance herbivore attack rates and reducing pest population in fields, but also for monitoring natural enemy populations (Mainali & Lim, 2012; Mansour et al., 2010). One example is the exploitation of the aphid sex pheromone, a blend of (4a*S*,7*S*,7a*R*)-(+)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-(-)-nepetalactol, as an effective lure to attract aphid parasitoids in the field (Hardie et al., 1991). However, the pheromone only showed strongest attraction in autumn as aphids typically only experience one sexual cycle in autumn. Therefore, the timeframe for application of this pheromone is limited (Powell et al., 1993). The above described tactics mainly exploit semiochemicals to increase the recruitment of natural enemies. However, semiochemicals can also serve as arrestants, decreasing emigration from release sites (Kelly et al., 2014). Parasitoid pheromones have proved to be promising tools for manipulation of parasitoid behaviour in this regard as well as for monitoring parasitoid populations (Hardy & Goubault, 2007; Kelly et al. 2014; Suckling et al. 2002).

Instead of affecting natural enemy behaviour, semiochemicals can also be implemented to directly affect the behaviour of pest insects. A popular tactic is mating disruption, in which a pest sex pheromone is used to capture or lure away (mostly) male conspecific pest insects and to disrupt the mating process, resulting in reduced pest populations (Ioriatti & Lucchi, 2016). Another common tactic is the attract and kill or mass trapping technique, in which semiochemicals are used to attract pests to an insect trap where the insects are killed by chemicals, drowning or dehydration (Gregg et al., 2018). In the case of attract and kill, a toxicant is added in the formulation in a gel or paste together with the semiochemical (Gregg et al., 2018). Repellent semiochemicals can be implemented to deter pest insects away from the crop (Borden et al., 2001). Repellents can further be deployed in a promising tactic such as the push-pull technique in which a combination of a repellent and an attractant of pest insects are used to simultaneously

deter the pest insects from the crop and lure them to other locations where they are less damaging, rather than killing the pest population (Kebede et al., 2018).

As insects can gain information and make choices based on microbial semiochemicals, mVOCs or even the microorganisms themselves may possibly be applied in future biological control programmes. Microbial semiochemicals can be applied in the same way as pheromones and HIPVs, and have already been implemented to either affect the behavioural responses of natural enemies or pest insects (Beck et al., 2016). However, so far, their implementation in biological control is still limited (Beck et al., 2016; Holighaus & Rohlfs, 2016; Leroy et al., 2011b). As another application, the combination of microbial semiochemicals and biological or chemical insecticides has been proposed as a promising tool to efficiently reduce pest populations (Knight & Witzgall, 2013; Knight et al., 2016; Mori et al., 2017). Additionally, semiochemicals that attract natural enemies may be coupled with specific rewards to increase natural enemy efficacy, a strategy known as "the attract and reward strategy". In this strategy, crops are treated with semiochemicals that specifically attract natural enemies to the crop, where they are provided with supplemental food sources (Rodriguez-Saona et al., 2012; Simpson et al., 2013). The main goal of this strategy is to attract and retain the natural enemies into the crop, and support them with supplemental food sources during low host/prey densities, which allows them to rapidly control pests when they arrive (Kaplan, 2012; Orre Gordon et al., 2013). It is assumed that attraction of natural enemies by itself is often not enough to enhance biological control efficacy when the natural enemies do not find essential resources such as hosts, food, or shelter. Furthermore, response to a semiochemical without a suitable reward or resource present might weaken or eliminate future responses to these semiochemicals (Blassioli-Moraes et al., 2019). So far, the attract and reward strategy is mainly used with synthetic HIPVs to attract natural enemies to nectar-producing flowers, providing them with essential food sources which serve as the reward (Orre Gordon et al., 2013; Simpson et al., 2011b,c). However, other types of semiochemicals and supplemental food sources such as artificial food sources can potentially be used (Tena et al. 2015; Wade et al., 2008). On the other hand, the combination of supplemental food sources and broadly attractive semiochemicals could lead to unintended attraction and support of harmful insects. Therefore, the ultimate goal is to define a combination of semiochemicals and supportive rewards that selectively recruit and sustain natural enemies, but not non-target organisms such as pest insects and hyperparasitoids (Orre Gorden et al., 2013).

1.4 Goals and objectives of this study

Despite different efforts to enhance biological control efficacy using natural enemies, a major challenge remains to attract and retain the beneficial insects in the crop so that they reach high population densities in the crop and control the pest insects whenever needed. While the increasingly applied provisioning of supplemental food sources and attractants to lure and augment natural enemy populations appears to be a promising approach to increase biocontrol efficacy, these sugar sources are often not tailored to selectively support the natural enemies and may also benefit harmful insects like herbivores and hyperparasitoids. In addition, most research on the development of attractants by which natural enemies can be effectively lured into the crop has focused on cues derived from plants. Nevertheless, there is mounting evidence that microorganisms emit volatile compounds that also play a role in insect behaviour. However, so far only little is known about how microbial volatiles affect the foraging behaviour of natural enemies, and whether they can be applied to improve biological control of insect pests.

The overall aim of this PhD study was to investigate the potential of tailored sugar mixtures and microbial volatile organic compounds (mVOCs) to improve the biological control of insect pests. To this end, we used the aphid parasitoids *Aphidius colemani* Vierick and *Aphidius matricariae* Haliday (Hymenoptera: Braconidae) and one of their hyperparasitoids, *Dendrocerus aphidum* Rodani (Hymenoptera: Megaspilidae), as study organisms. Both primary parasitoids are commonly used in the biological control of several aphid species of economic importance, particularly smaller species such as *Myzus persicae* Sulzer (Hemiptera: Aphididea), an important pest species of many crops worldwide (van Emden & Harrington; 2007). Aphids are plant phloem-feeding insects that pose one of the greatest threats to agriculture and horticulture worldwide, causing significant economic losses (Dedryver et al., 2010). They can cause direct damage by feeding on the plant phloem and by injecting phytotoxic compounds. However, more importantly, they can also cause indirect damage by vectoring a large variety of pathogenic plant viruses and by excreting honeydew, which promotes the growth of sooty moulds, decreasing photosynthesis efficiency. *Dendrocerus aphidum* is a secondary parasitoid that attacks parasitoid (pre)pupae within mummified aphids, and therefore can disrupt biological pest control, leading to pest outbreaks.

More specifically, we aimed to:

i. evaluate the feeding behaviour and longevity of *A. colemani*, *A. matricariae,* and *D. aphidum*, when provided with a variety of sugars to design a selectively supportive sugar mixture for *Aphidius* parasitoids;
ii. investigate the ability of bacterial mVOC blends to affect olfactory responses of primary (*A. colemani*) and secondary parasitoids (*D. aphidum*);
iii. Assess phylogenetic relationships among bacteria with variation in mVOC composition and the olfactory response of *A. colemani*; and
iv. identify physiologically active mVOC compounds that are attractive to *A. colemani*, and evaluate their potential to attract *A. colemani* under laboratory and greenhouse conditions.

A schematic overview of the thesis content is given in Figure 1.8. In **Chapter 2**, we investigated the feeding behaviour and longevity of both selected parasitoid species and their hyperparasitoid *D. aphidum*, when provided with one of eight plant- and/or insect-derived sugars. In **Chapter 3**, we investigated whether mVOCs emitted by bacteria affected the olfactory response of *A. colemani* and *D. aphidum*, using Y-tube olfactometer bioassays. 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. The most attractive and most repellent strains were used to determine whether the olfactory response of *D. aphidum* was differently affected. However, it should be noted that, although Y-tube olfactometer bioassays alone are not sufficient to draw profound conclusions regarding attraction or repellence (they rather demonstrate preference), in this PhD thesis positive olfactory responses are referred to as "attraction", while negative responses are referred to as "repellence". 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. Strikingly, results showed that closely related species of the genus *Bacillus* elicited a similar olfactory response in *A. colemani*, suggesting that volatile composition and, as a result, parasitoid attraction, are phylogenetically conserved traits. Therefore, in **Chapter 4,** we tested the hypothesis that phylogenetic relationships among microorganisms predict microbial volatile composition and parasitoid olfactory response. Subsequently,

in **Chapter 5**, using a combination of Y-tube olfactometer bioassays, gas chromatography-mass spectrometry (GC-MS) and gas chromatography-electroantennography (GC-EAG) we aimed at identifying specific compounds in bacterial volatile blends that attract *A. colemani*. Next, the most promising mixture of putatively attractive synthetic compounds was evaluated in two-choice cage experiments to investigate whether *A. colemani* parasitoids responded to the synthetic volatile blend under greenhouse conditions. Finally, **Chapter 6** summarizes the most important findings and provides general conclusions of this PhD study, as well as different perspectives for future research and applications.

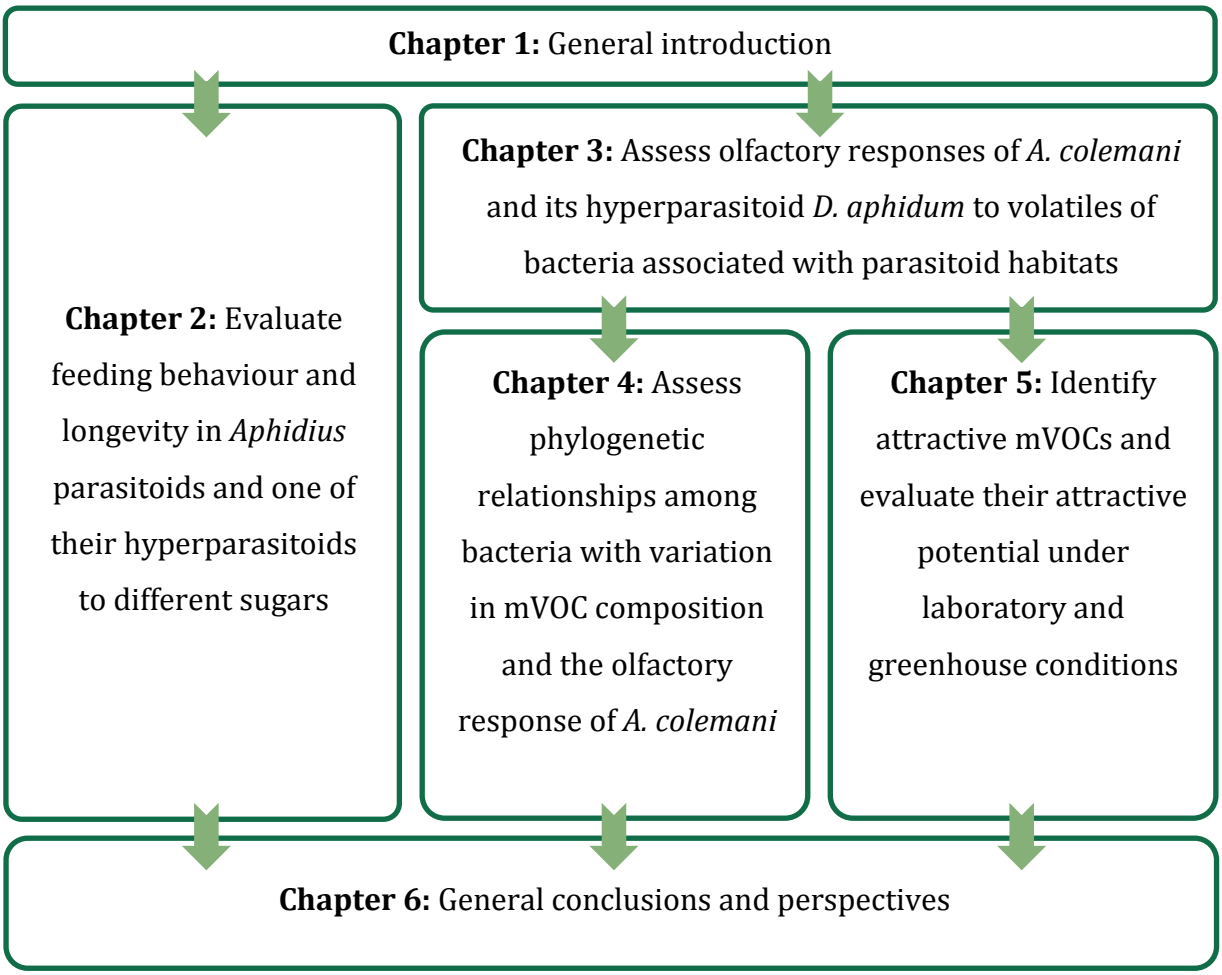

Figure 1.8: Schematic outline of the doctoral thesis. The overall aim of this PhD study was to investigate the potential of tailored sugar mixtures and microbial volatile organic compounds (mVOCs) to improve the biological control of insect pests.

Chapter 2

Gustatory response and longevity in *Aphidius* parasitoids and their hyperparasitoid *Dendrocerus aphidum*

This chapter is based on the following publication:
Goelen, T., Baets, D., Kos, M., Paulussen, C., Lenaerts, M., Rediers, H., Wäckers, F., Jacquemyn, H., & Lievens, B. (2018). Gustatory response and longevity in *Aphidius* parasitoids and their hyperparasitoid *Dendrocerus aphidum*. *Journal of Pest Science*, *91*, 351-360. DOI: 10.1007/s10340-017-0907-3

2.1 Introduction

Aphids constitute one of the most important threats to agriculture and horticulture worldwide, causing huge economical losses (Dedryver et al., 2010; van Emden & Harrington, 2007). Aphids harm crop plants directly by feeding on plant phloem sap and injecting phytotoxic compounds, or indirectly by spreading pathogenic viruses or excretion of sticky honeydew (Fig. 2.1A), which can irrecoverably tarnish fruits and promotes growth of sooty moulds making photosynthesis less efficient (Fig. 2.1B) (Nault, 1997; van Emden & Harrington, 2007). Aphids are commonly controlled by chemical means (Bass et al., 2014). However, due to potential hazards to human health and environment and the widespread development of insecticide resistance in numerous aphid species (Bass et al., 2014; Dedryver et al., 2010; Hillocks, 2012), there is a strong interest in environmentally sound alternatives of pest control. An important and widely used alternative is biological control, exploiting aphid natural enemies (Jones et al., 2003; van Lenteren, 2012).

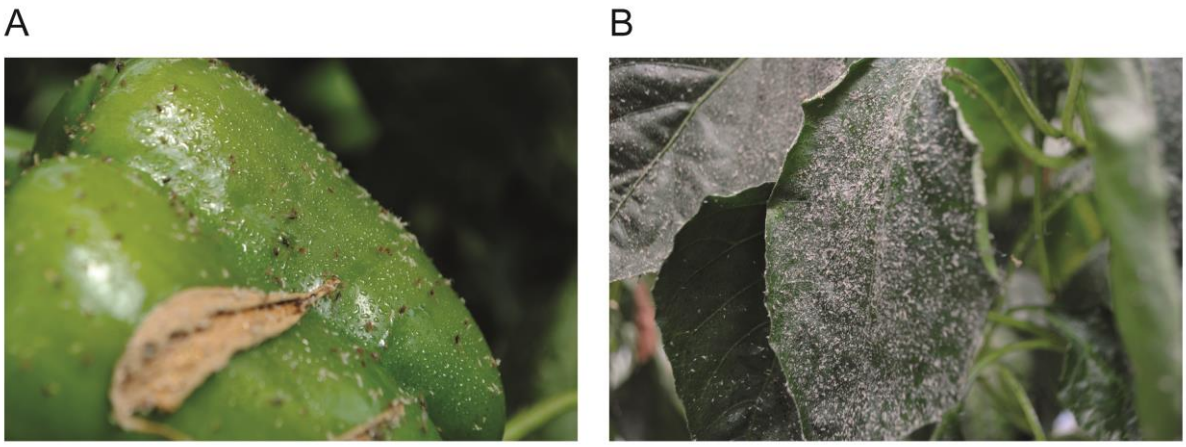

Figure 2.1: Indirect damage caused by aphid honeydew on sweet pepper plants. **(A)** Honeydew and aphid exuviae on sweet pepper fruits rendering them unsuitable for sale. **(B)** Sooty moulds growing in aphid honeydew on sweet pepper leaves making photosynthesis less efficient (Photo credits: Tim Goelen).

Among a wide array of natural enemies, parasitic Hymenoptera such as *Aphidius* spp. (Hymenoptera: Braconidae) have proven to be particularly effective in biological control programs against aphids (Hagvar & Hofsvang, 1991; Snyder & Ives, 2003; van Lenteren, 2012). However, their success in controlling pests largely depends on the availability of carbohydrate-rich food, which generally serves as their only adult energy source for maintenance and reproduction (Heimpel & Jervis, 2005; Jervis et al., 1993;

Wäckers, 2001). As these resources have become rare in intensified agricultural systems, nectar-producing plants are used more and more within or around the crop area to provide biocontrol agents with the necessary sugar resources (Wäckers & van Rijn, 2012). Although such an approach has been proven to effectively increase biological control efficacy (Heimpel & Jervis, 2005; Winkler, 2006), flowering period, nectar accessibility, chemical composition of the nectar, and competition with other nectar feeders represent important factors that can impede the efficacy of sugar supplementation (Campbell et al., 2012; van Rijn & Wäckers, 2016; Wäckers & van Rijn, 2012).

Instead of providing flowering vegetation, artificial food sources can be provided to support the energetic needs of beneficial insects (Tena et al., 2015). Such artificial food sources are typically comprised of carbohydrate and/or protein-rich ingredients in liquid formulation, and can be applied to the foliage (Tena et al., 2015) or provided through feeding stations (Shimoda et al., 2014). However, as the composition of the food source (mainly sugar composition and concentration) may affect the target parasitoids differently, the provided sugars should be carefully selected to match the parasitoids feeding preference and energetic needs (Benelli et al., 2017; Wäckers, 2001). This can be illustrated by the recent study of Lenaerts et al. (2016), who showed that *Aphidius ervi* (Hymenoptera: Braconidae), a commonly used parasitic wasp for controlling aphids in greenhouses, consumed relatively large amounts of sugars like sucrose, fructose and melezitose and survived best on these sugars, whereas intake of glucose or melibiose was considerably lower. Whether these trends also hold true for other *Aphidius* species that are used in the biological control of aphids remains to be investigated.

When developing artificial carbohydrate food sources for parasitoids it is important that the target parasitoids benefit more from the supplementary food than potentially harmful insects such as hyperparasitoids (Harvey et al., 2012). These secondary parasitoids constitute a highly evolved fourth trophic level, targeting already parasitized hosts and developing at the expense of the primary parasitoids (Sullivan & Völk, 1999). High levels of hyperparasitism have been observed in aphid-parasitoid populations within agroecosystems (Höller et al., 1993; Mackauer & Völkl, 1993), which can strongly impede the efficacy of biological aphid control (Araj et al., 2009; Gómez-Marco et al., 2015). As hyperparasitoids reside in the same habitat as the primary parasitoids it is reasonable to assume that hyperparasitoids have been adapted to the exploitation of the same sugar sources as those preferred by the primary parasitoids.

However, so far very little is known about the gustatory and longevity responses of hyperparasitoids to different sugars (Harvey et al., 2012).

The aim of this study was to investigate the gustatory response and longevity of two *Aphidius* species that are commonly used in the biological control of aphids (i.e. *Aphidius colemani* and *Aphidius matricariae*) and their hyperparasitoid *Dendrocerus aphidum* (Hymenoptera: Megaspilidae) when provided with one of eight plant and/or insect-derived sugars (fructose, galactose, glucose, melibiose, melezitose, rhamnose, sucrose, and trehalose). More specifically, we investigated whether there were differences in gustatory response and survival between the related primary parasitoids and their associated hyperparasitoid. Such differences could then be exploited in tailoring food sources to predominantly support the primary parasitoids, without benefiting the hyperparasitoids. To this end, we first evaluated sugar consumption over a nine hour period of time. Next, we studied survival of the parasitoids when fed with the different sugars. After identifying sugars that supported survival of *Aphidius* spp. but not of *D. aphidum*, we tested whether economically sound sugar mixtures could be designed that are beneficial to *Aphidius* spp. but not to the hyperparasitoid *D. aphidum*.

2.2 Materials and methods

2.2.1 Insects

Experiments were performed using adults of the aphid parasitoids *Aphidius colemani* (Fig. 2.2A) and *Aphidius matricariae* (Fig. 2.2B), and their hyperparasitoid *Dendrocerus aphidum* (Fig. 2.2C). Both *Aphidius* species are generalist, solitary aphid endoparasitoids that are widely used for biological control of aphid pests in greenhouses (van Lenteren, 2012; van Lenteren & Woets, 1988; Yano, 2006). *D. aphidum* is a secondary idiobiont ectoparasitoid, attacking pre-pupal and pupal stages of hymenopteran primary parasitoids such as *Aphidius* spp. inside aphid mummies (Walker & Cameron, 1981). *A. colemani* and *A. matricariae* were obtained from Biobest (Westerlo, Belgium) as mummies (Aphidius-system® and Matricariae-system®, respectively). Once received, the mummies were placed inside a nylon insect cage (BugDorm, MegaViewScience Co., Ltd, Taichung, Taiwan) and kept under controlled conditions (22 °C, 70 % relative humidity and 16:8 h light:dark photoperiod) until parasitoid emergence. Adult *D. aphidum* individuals were obtained from the Netherlands Institute of Ecology (NIOO-KNAW, Wageningen, The

Netherlands) where they are continuously reared on fresh (1 day old) *Acyrthosiphon pisum* mummies parasitized by *Aphidius ervi*, and maintained on potted broad bean plants (*Vicia faba*). Experiments were performed with <24h old, unfed and water-starved insects and included both males and females.

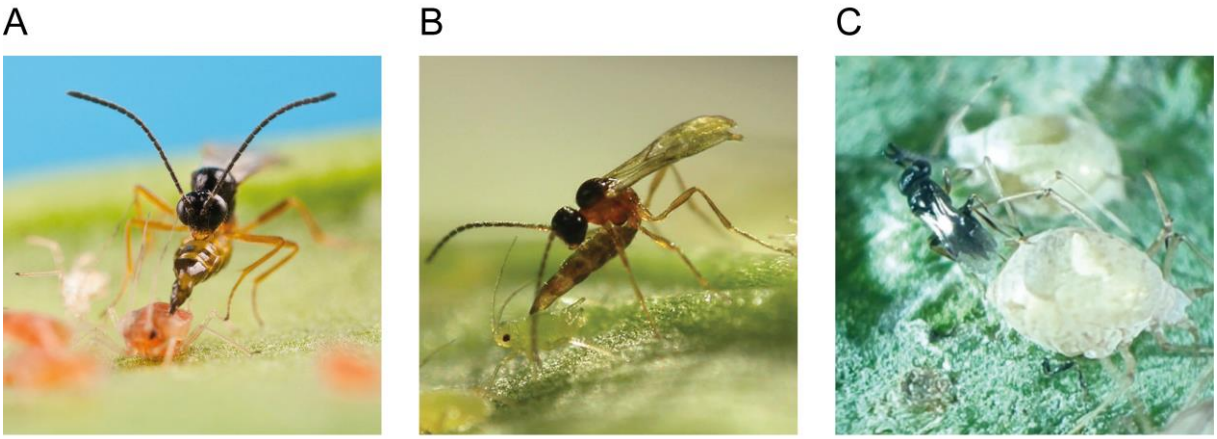

Figure 2.2: Studied parasitoid species. **(A)** *Aphidius colemani* (Hymenoptera: Braconidae) attacking an aphid host (Photo credit: Koppert Biological systems). **(B)** *Aphidius matricariae* (Hymenoptera: Braconidae) attacking an aphid host (Photo credit: Biobest Group NV). **(C)** *Dendrocerus aphidum* (Hymenoptera: Megaspilidae) parasitizing an aphid mummy (Photo credit: Tim Goelen).

2.2.2 Test sugars

The sugars used in the experiments represent a range of sugars that typically occur in natural sugar resources like nectar and honeydew, including fructose, galactose, glucose, melibiose, melezitose, rhamnose, sucrose and threhalose (Wäckers, 2001) (Table 2.1). Experiments were conducted using equiweight sugar solutions, having a mass:volume ratio of 180:1 (g/L) (corresponding to the molecular weight of glucose and fructose) (Table 2.1). When a glucose-melibiose mixture was tested, first sugars were combined in a 1:3 (w/w) ratio, after which the mixture was diluted with sterile demineralized water to obtain equiweight solutions with a mass:volume ratio of 180:1 (g/L). All sugar solutions were filter-sterilized (Rapid-Flow™ bottle top filter, pore size 0.2 µm, Nalgene™, Thermo Scientific™, Waltham, MA, USA) and stored in microcentrifuge tubes at -20 °C until required.

Table 2.1: Sugars used in this study

Sugar	Glycolytic linkage	Natural source[a]	Supplier	Tested mass:volume ratio (g/L)(molar concentration)
D(-)-fructose	-	Honeydew, floral and extrafloral nectar	Acros Organics	180:1 (1 M)
D(+)-glucose	-	Honeydew, floral and extrafloral nectar	Sigma	180:1 (1 M)
D(+)-melibiose	α-D-Galactopyranosyl-(1➔6)-D-glucose	Floral nectar	Sigma	180:1 (0.53 M)
D(+)-melezitose	α-D-Glucopyranosyl-(1➔3)-β-D-Fructofuranosyl-α-D-glucopyranoside	Honeydew, rare in floral and extrafloral nectar	Sigma	180:1 (0.36 M)
L(+)-rhamnose	-	Extrafloral nectar	VWR	180:1 (1.1 M)
D(+)-sucrose	β-D-Fructofuranosyl-α-D-glucopyranoside	Honeydew, floral and extrafloral nectar	Sigma	180:1 (0.53 M)
D(+)-trehalose	α-D-Glucopyranosyl-α-D-glucopyranoside	Honeydew	Sigma	180:1 (0.53 M)
D(+)-galactose	-	Honeydew, floral and extrafloral nectar	Sigma	180:1 (1 M)

[a] Wäckers, 2001.

2.2.3 Capillary feeder (CAFE) assay

All experiments were performed using a previously developed capillary feeder (CAFE) assay (Lenaerts et al., 2016) (Fig. 2.3). Briefly, a cylindrical plastic container (height: 12.5 cm; diameter: 10.0 cm) was provided with four calibrated glass capillaries (5.0 µL, Blaubrand Intramark, Wertheim, Germany) that were all filled with 4.0 µL of a single sugar solution (no-choice) and covered with a mineral oil overlay (1.0 µL) to minimize evaporation. Microcapillaries were offered through the container lid via truncated 200 µL yellow pipette tips. The lid was provided with an opening (2.5 cm by 2.5 cm) covered with a fine mesh (mesh size 0.27 mm × 0.88 mm) to ensure ventilation and to prevent condensation. To cover the water requirements of the insects, a filter paper moisturized with 500 µL sterile water was placed at the bottom of the container at the start of every experiment, and supplemented with another 500 µL water daily in the longevity experiments (see below). For every experiment (see below), a total of 75 individuals were tested per treatment, divided over five replicate CAFE containers, after having experienced a dark period of eight hours. Subsequently, containers were put in a climate chamber at 22 °C, 70 % relative humidity and a 16:8 h light:dark photoperiod.

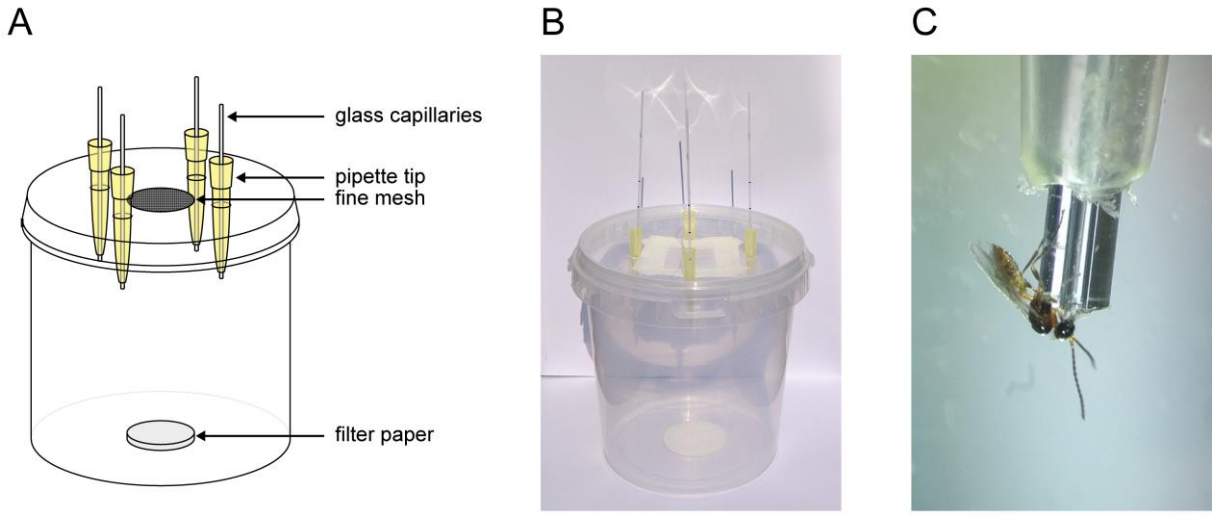

Figure 2.3: Capillary Feeder (CAFE) assay. **(A)** Schematic diagram of the CAFE assay. Test sugars (4.0 µL) were provided in four glass capillaries, topped with a mineral oil overlay (1.0 µL) to minimize evaporation. The glass capillaries were introduced through the lid via truncated 200 µL yellow pipette tips. To allow entry of air in the cage, the lid of the chamber was pierced and covered with fine mesh (2.5 × 2.5 cm; mesh size 0.27 × 0.88 mm). A water-imbibed filter paper was placed at the bottom of the chamber. For every experiment (see below), a total of 75 individuals were tested per treatment, divided over five replicate CAFE containers. **(B)** Photograph of a CAFE assay chamber used in this study. **(C)** *Aphidius colemani* parasitoid feeding on one of the glass capillaries filled with a certain sugar solution (Photo credits: Tim Goelen).

2.2.4 Experiment 1A: Assessing the effect of different sugars on sugar consumption

To assess differences in gustatory responses between the three studied insect species, sugar intake was monitored every hour for 9 hours in a row by measuring the sugar column in the microcapillaries using a digital caliper (Mitutoyo Digimatic, resolution 0.01 mm). Tested sugars included fructose, galactose, glucose, melibiose, melezitose, rhamnose, sucrose and threhalose. Per container (n = 5, each containing 15 insects) consumption values for the four capillaries were summed and subsequently averaged over the five replicates. To determine the exact starting point, we also measured the level of sugar solution right before the start of the experiment. For every sugar, an identical CAFE chamber devoid of parasitoids (blank) was included to establish the loss of sugar solution through evaporation during the course of the experiment. These values were subtracted from experimental readings in retrospect to account for evaporative losses. Data were analyzed for each species separately, by using a repeated-measures analysis of variance (ANOVA) with sugar as fixed factor and hourly sugar consumption as dependent variable. A Tukey HSD post hoc test was performed to investigate which sugars were consumed more than others. The Scheirer–Ray–Hare (SRH) extension of the Kruskal–Wallis test, a nonparametric equivalent of the two-way analysis of variance (ANOVA), was

used to test whether there was an overall difference in sugar consumption between the different species. In this analysis, total sugar consumption after nine hours was used as dependent variable and insect species and the tested sugars were used as independent variables.

2.2.5 Experiment 1B: Assessing the effect of different sugars on insect longevity

In a second analysis, the effect of the different sugars mentioned above on insect longevity was assessed. The experiment was performed using the same individuals surveyed in the first experiment (n = 75, equally distributed over five CAFE containers). Parasitoid longevity was assessed by counting and removing the dead individuals in each CAFE container daily, until the last individual had died. As a control, a water treatment was included in which the capillaries were filled with sterile water instead of a sugar solution. Capillaries were replaced daily to prevent microbial contamination. To test whether longevity differed for the different sugars, survival curves were generated for each species separately and compared using Kaplan-Meier estimates of the survival function. To determine if survival curves were significantly different between sugars, a log-rank statistic was performed followed by Holm-Sidak correction to account for each of the pairwise comparisons. To test whether there was an overall difference in longevity between the different species, the SRH test was performed with survival (days since the beginning of the experiment) as dependent variable for each sugar and insect species and the tested sugars as independent variables.

2.2.6 Experiment 2: Assessing the effect of a glucose-melibiose mixture on sugar consumption and insect longevity

Due to the poor performance of *D. aphidum* on melibiose, in a final experiment we assessed whether a sugar mixture based on melibiose and a cheaper sugar could be designed that was particularly beneficial to the *Aphidius* species, but not to the hyperparasitoid. The methodology was similar to the experiments described above, but here insects (75 individuals distributed over five CAFE containers, with 15 individuals each) were provided with a sugar mixture composed of glucose and melibiose in a relative concentration of 1:3 (w/w) (no-choice; all four capillaries contained the same sugar mixture). Treatments offering pure glucose or pure melibiose were included as controls.

Differences in sugar consumption were analysed for each species separately by ANOVA. With regard to the survival trial, the non-parametric Kruskall-Wallis test was used as the data did not conform to the criteria of normality and homogeneity of variance required for a parametric statistical test. To test whether there was an overall difference in sugar consumption and longevity between the different species the SRH test was performed as described above using R 3.1.1 (R Core Team, 2014). All other statistical analyses and evaluation of normality and homoscedasticity of the data were performed with SPSS (IBM SPSS Statistics for Windows, Version 23.0).

2.3 Results

2.3.1 Experiment 1A: Assessing the effect of different sugars on sugar consumption

Consumption of the different sugars tested varied significantly between the studied insect species and between the tested sugars (SRH test: insect species: $H_2 = 43.07$, $P < 0.001$; sugars: $H_7 = 79.44$, $P < 0.001$, respectively). Moreover, the interaction between insect species and sugar consumption was also significant ($H_{14} = 5.77$, $P < 0.001$), indicating that the effect of sugar compound on consumption differed between insect species. Whereas both *Aphidius* species consumed similar amounts of sugars within the nine hours of investigation, *D. aphidum* consumed much more (e.g. up to an average consumption of 2.4 µL for melezitose, which is approximately four times more than the maximum intake recorded for the *Aphidius* spp.) (Fig. 2.4). Additionally, within a single species, sugar consumption varied between the different sugars (*A. colemani*: $F_{7,32} = 5.32$; $P < 0.001$; *A. matricariae*: $F_{7,32} = 14.58$; $P < 0.001$; *D. aphidum*: $F_{7,32} = 51.13$; $P < 0.001$; Fig. A2.1, Appendix). Highest sugar consumption by *A. colemani* was observed for fructose, melezitose and galactose, while intake of sucrose, glucose, trehalose and melibiose was lower (albeit not significantly) (Fig. 2.4; Fig. A2.1A, Appendix). The related species *A. matricariae* consumed significantly more of the sugars fructose, galactose, sucrose, glucose and melezitose, while melibiose and trehalose were considerably less consumed (Fig. 2.4; Fig. A2.1B, Appendix). In contrast, the hyperparasitoid species *D. aphidum* clearly fed more on the honeydew sugar melezitose, whereas melibiose was only marginally consumed (8 times less than melezitose) (Fig. 2.4; Fig. A2.1C, Appendix). Rhamnose was only hardly ingested by the three insect species, although *A. colemani* seemed to consume it a bit more than the other insect species (Fig. 2.4; Fig. A2.1A, Appendix).

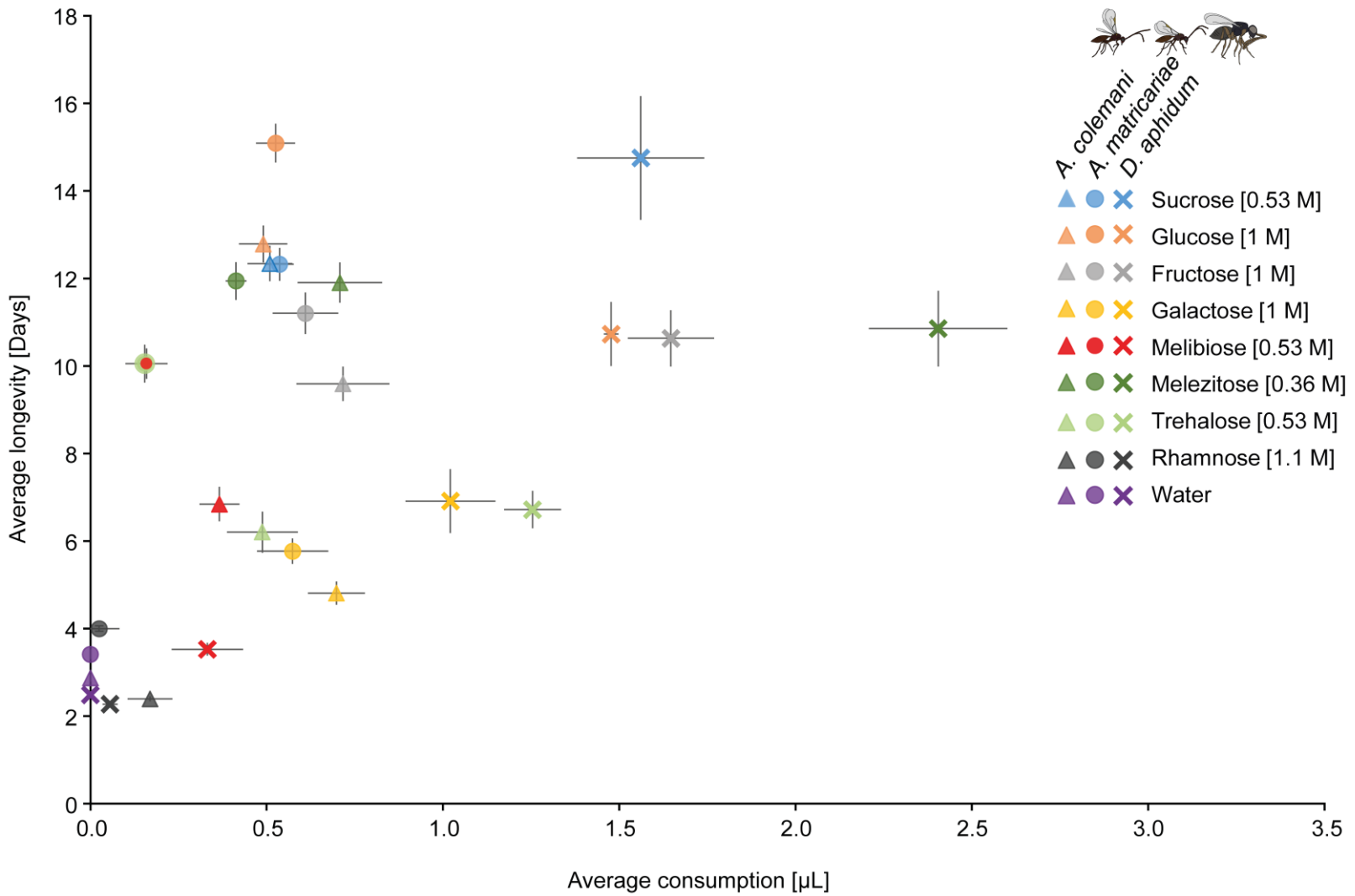

Figure 2.4: Scatter plot showing mean sugar consumption (±SE) after 9 h and mean longevity (±SE) when different individual sugars were provided at equal weight concentrations (mass:volume of 180:1 g/L) to unfed adult parasitoids. Experiments were performed using a capillary feeder assay (5 replicates; 15 individuals per replicate). The tested parasitoids were *Aphidius colemani* (triangles), *Aphidius matricariae* (circles) *and Dendrocerus aphidum* (crosses).

2.3.2 Experiment 1B: Assessing the effect of different sugars on insect longevity

Parasitoid longevity differed significantly among insect species and between the tested sugars (insect species: $H_2 = 45.72$, $P < 0.001$; sugars: $H_7 = 47.34$, $P < 0.001$, respectively). No significant interaction between insect species and sugar type was found (SRH test: $H_{14} = 3.80$, $P = 0.371$). Additionally, within each species, the various sugars tested differed considerably with regard to their effect on insect survival (*A. colemani*: $\chi^2 = 703.12$, $df = 8$, $P < 0.001$; *A. matricariae*: $\chi^2 = 735.70$, $df = 8$, $P < 0.001$; *D. aphidum*: $\chi^2 = 543.46$, $df = 8$, $P < 0.001$; Fig. A2.2, Appendix). In line with the limited consumption of rhamnose, rhamnose did not or only marginally affect the life span of the insect species tested (Fig. 2.4; Fig. A2.2, Appendix). In contrast, *A. colemani* survived longest when provided with glucose, sucrose and melezitose with an average survival time between 11.9 and 12.8 days, which is more than four times longer than when only water was provided. A less pronounced effect was observed for fructose, melibiose and trehalose, increasing the average parasitoid longevity by a factor 3.3, 2.3 and 2.1, respectively, when compared to the water treatment. For *A. matricariae*, the highest longevity was achieved when glucose was provided, increasing the average life span by a factor 4.4 compared to the water control. A significantly lower longevity was observed for the sugars sucrose, melezitose and fructose (increasing the average life span with a factor 3.3 to 3.6), followed by trehalose and melibiose (increasing the average life span with a factor 2.9 to 3.0, respectively). Compared to *A. colemani*, the latter two sugars ensured a longer average life span for *A. matricariae* (1.5 times longer). Remarkably, although galactose was one of the sugars that *A. colemani* and *A. matricariae* consumed the most, galactose only slightly increased their average life span (i.e. by a factor 1.7) (Fig. 2.4; Fig. A2.2, Appendix). For *D. aphidum*, highest longevity was observed when the species was provided with sucrose, increasing the average longevity with a factor 6.0 compared to the control. When the species was provided with melezitose, glucose or fructose, the average longevity increased by a factor 4.2 – 4.4. A significant lower longevity was recorded when *D. aphidum* was offered trehalose or galactose. Interestingly, whereas melibiose substantially increased the average and maximum life span of both *Aphidius* species tested (increase in average life span by a factor 2.3 (*A. colemani*) or 2.9 (*A. matricariae*); maximal survival of 17 (*A. colemani*) or 18 days (*A. matricariae*)), melibiose only slightly increased longevity of *D. aphidum* (increase in average longevity by a factor of 1.4 compared to the water treatment) with a maximum survival of 10 days. Furthermore, more than 97% of the *D.*

aphidum individuals had already died after 5 days of feeding on melibiose, compared to only 52% and 19% of the individuals of *A. colemani* and *A. matricariae*, respectively (Fig. 2.4; Fig. A2.2, Appendix). A 4.2 times lower life span on melibiose was observed for *D. aphidum* compared to its highest longevity on sucrose.

2.3.3 Experiment 2: Assessing the effect of a glucose-melibiose mixture on sugar consumption and insect longevity

Given the particular poor performance of melibiose in the consumption and longevity trial for *D. aphidum*, we hypothesized that an economically sound sugar mixture could be designed that was particularly beneficial to *Aphidius* spp. while being less supportive for *D. aphidum* by adding melibiose to a cheap, high-quality sugar such as glucose. Overall, a significant difference was observed between the tested species and between the tested sugar solutions for sugar consumption (SRH test: insect species: $H_2 = 23.93$, $P < 0.001$; sugars: $H_7 = 58.90$, $P < 0.001$, respectively) and parasitoid longevity (SRH test: insect species: $H_2 = 111.60$, $P < 0.001$; sugars: $H_7 = 3.66$, $P = 0.026$, respectively). For both sugar consumption and longevity a significant interaction between insect species and sugar was found (SRH test: $H_{14} = 8.05$, $P < 0.001$; $H_{14} = 9.71$, $P < 0.001$, respectively). Adding melibiose to glucose significantly affected sugar consumption and parasitoid longevity in comparison with the glucose-fed insects (Fig. 2.5). Moreover, melibiose significantly decreased the nutritional benefit of the suitable sugar with respect to insect survival (Fig. 2.5B). At a melibiose concentration of 75%, this effect was most pronounced for the hyperparasitoid *D. aphidum*, showing a decrease of 35% in average longevity compared to pure glucose. In contrast, the primary parasitoids were able to cope better with this mixture, showing a smaller decrease in longevity (average decrease of 20%) compared to pure glucose (Fig. 2.5).

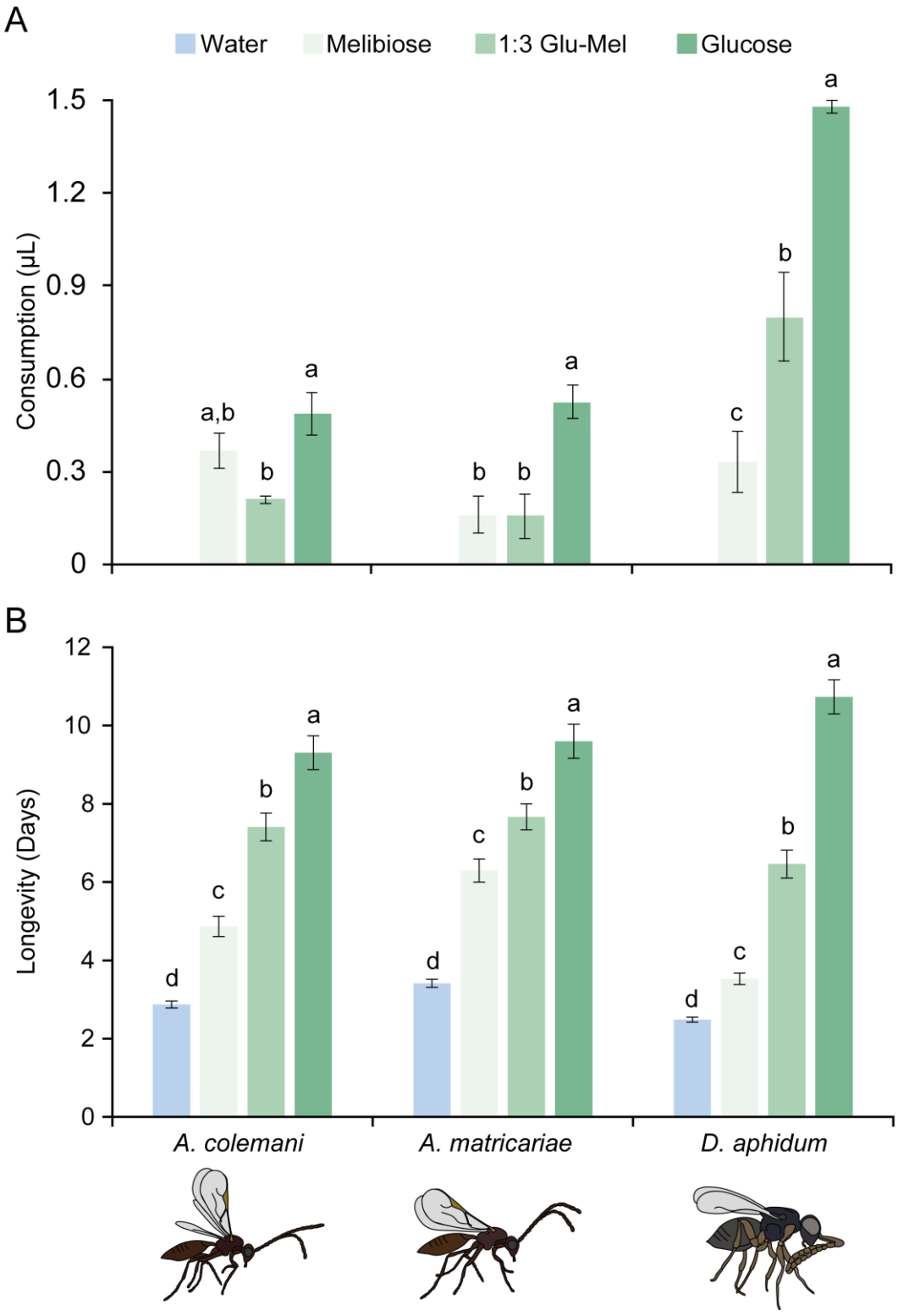

Figure 2.5: (A) Mean sugar consumption (±SE) after 9 h (n = 5 with 15 individuals tested per replicate) by unfed adult parasitoids and **(B)** mean longevity (n = 75) when different sugar solutions (glucose, 1:3 glucose:melibiose and melibiose) were offered at equal weight concentrations (mass:volume of 180:1 g/l). Water consumption was not evaluated, due to the water-imbibed filter paper in the CAFE-assay. The tested parasitoids were *Aphidius colemani*, *Aphidius matricariae*, and *Dendrocerus aphidum*. Statistical differences were assessed using ANOVA comparing consumption of the different solutions, and by using a non-parametric Kruskal-Wallis test to compare longevity. Different letters indicate statistical differences at the 95 % confidence level between different treatments

2.4 Discussion

In this study the gustatory responses and longevity of the aphid parasitoids *A. colemani* and *A. matricariae* and their hyperparasitoid *D. aphidum* were investigated for a number of sugars that occur in natural sugar resources like nectar and honeydew. Our results showed that both investigated *Aphidius* spp. consumed the largest quantities of sugars that are commonly present in honeydew (sucrose, fructose, glucose and melezitose) and also showed the highest longevity on these sugars (Fischer & Shingleton, 2001; Fischer et al., 2005; Hogervorst et al,. 2003, 2007b; Tena et al., 2013b; Wäckers, 2005). These findings confirm previous results for the related parasitoid species *A. ervi*, suggesting that parasitoids of honeydew producing hosts like aphids are adapted to sugars that are abundantly present in honeydew (Lenaerts et al., 2016), while parasitoids of non-honeydew producing hosts are not or less adapted (Williams & Roane, 2007; Winkler et al., 2005). However, it should be noted that glucose and melezitose can occur in highly variable concentrations in hemipteran honeydew depending on the plant and aphid species, including very low concentrations as well (glucose 2% - 30% ; melezitose 0% - 63%) (Fischer & Shingleton, 2001; Fischer et al., 2005; Hogervorst et al., 2003, 2007b; Tena et al., 2013b; Wäckers, 2005). Furthermore, *Aphytis melinus* (Hymenoptera: Aphelinidae), a parasitoid of armoured scale insects that do not produce honeydew, survives equally well on honeydew than on sucrose (Tena et al., 2013a). Therefore, further research comparing the gustatory response and survival of parasitoids of honeydew producing hosts and parasitoids of non-honeydew producing hosts is needed to draw firm conclusions. In comparison with the *Aphidius* species tested here, *A. ervi* performed less well on glucose (Lenaerts et al., 2016). Our results also indicated that *A. colemani* seemed to be a more generalist feeder, whereas *A. matricariae* was more selective in its feeding behaviour with a clear separation between highly consumed and less- or non-consumed sugars. A major difference between *A. colemani* and *A. matricariae* was observed for survival on the honeydew sugar trehalose. Whereas *A. matricariae* on average survived almost 10 days on trehalose, *A. colemani* survived for only 6 days when fed with this sugar. A similar poor performance on trehalose has also been reported for *A. ervi* (Lenaerts et al., 2016), as well as for other braconid species like *Anaphes iole* and *Cotesia glomerata*, and the ichneumonid wasp *Diadegma semiclausum* (Wäckers, 2001; Williams & Roane, 2007; Winkler et al., 2005).

Interestingly, both the hyperparasitoid species *D. aphidum* and its *Aphidius* host demonstrated similar patterns of sugar consumption and longevity, with average *D. aphidum* longevity ranging from 10.6 days (fructose) to 14.8 days (sucrose). Our results also indicated that *D. aphidum* consumed even greater quantities of the aphid-synthesized sugar melezitose, up to twice the amount of the other tested sugars. Altogether, these results suggest that parasitoids and hyperparasitoids inhabiting the same ecosystem may be adapted to exploit the predominant sugar sources occurring in their habitat. Further in-depth research, however, is needed to confirm this hypothesis.

Compared to other studies in which survival of *D. aphidum* on different plant nectars was evaluated (maximum survival of 57 days was recorded) (Araj et al., 2006, 2008), *D. aphidum* performed less on the pure sugars investigated in this study. This may suggest that other factors such as vitamins and amino acids that are also commonly found in natural sugar sources such as nectar (Carter et al., 2006) constitute important factors for survival of *Dendrocerus*. In contrast, no such discrepancy was found for *Aphidius* spp. when fed on nectar or pure sugars (Araj et al., 2006, 2008; Lenaerts et al., 2016). Rhamnose, which normally does not occur in floral nectar or honeydew (Wäckers, 2001; but see Akšić et al., 2015, Kwak et al., 1985, Truchado et al., 2008), was not or only marginally consumed by all species investigated and had no positive effect on longevity, as had been previously reported for other insect species as well (Lenaerts, et al. 2016; Wäckers, 1999).

Both *A. colemani* and *A. matricariae* survived relatively well on melibiose, prolonging the average survival by a factor 2.3 (6.8 days) and 3.0 (10.1 days) compared to the water control, respectively. In contrast, *D. aphidum* performed noticeably poorer on melibiose, reaching an average longevity of 3.5 days, which was only marginally higher than that for the water control. Strikingly, although *A. matricariae* wasps showed a low innate gustatory response to melibiose, consumption of this sugar has a substantial effect on insect survival. Such a low innate gustotary response has previously been observed for several other hymenopteran parasitoids, including *A. iole*, *C. glomerata* and *D. semiclausum* (Beach et al., 2003; Wäckers, 1999, 2001; Winkler et al., 2005). Given the poor performance of melibiose in the trials for *D. aphidum* compared to the tested *Aphidius* spp., together with the fact that melibiose is relatively expensive, we investigated the effect of melibiose in a sugar mixture with a cheap sugar such as glucose, aiming at the development of an economically sound sugar mixture that is particularly beneficial to

Aphidius and not or less supportive for *D. aphidum*. As expected, we found that adding a less nutritional sugar like melibiose to glucose decreased overall parasitoid longevity compared to pure glucose. Similar observations have been made for *C. glomerata* when rhamnose was added to glucose (Wäckers, 2001). However, effects were more pronounced in Wäckers (2001), reporting a reduction in longevity of almost 50% even at the lowest rhamnose concentration tested (20% rhamnose, 80% glucose). Nevertheless, we showed that the addition of melibiose to a glucose solution at a 3:1 ratio resulted in a strong reduction of *D. aphidum* survival (decrease of 35% in average longevity), while these effects were much less pronounced for *Aphidius* (decrease of 20% in average longevity).

Altogether, our study showed that *Aphidius* species and their hyperparasitoid *D. aphidum* consumed the largest quantities of sugars that are overrepresented in aphid honeydew (sucrose, glucose, fructose and melezitose) and survived best when feeding on these sugars. These sugars therefore represent promising constituents for future development of artificial food sources to cover the energetic needs of the beneficial insects. Our results with melibiose also showed that it was possible to develop a sugar composition that predominantly supports primary parasitoids, while being less supportive for harmful insects like *D. aphidum*. In the present study, we only investigated the impact of individual sugars and a mixture of two sugars. This leaves the question how interactions between several sugars and between sugars and non-carbohydrate compounds such as nitrogen sources and amino acids affect responsiveness of parasitoids. It also remains to be investigated whether the trends observed for longevity will also be reflected in other important life-history parameters such as parasitoid fecundity. Previous studies have shown that various sugar sources can affect parasitoid fecundity differently (Charles & Paine, 2016; Tena et al., 2013a). Future research is needed to investigate whether this is also the case for *Aphidius* and the sugars tested here.

These artificial sugar sources by themselves may not easily be found by parasitoids in agricultural settings (Shimoda et al., 2014). However, it has been shown the sugar source localisation by insects in nature strongly depends on volatile cues, such as flower odours which attracts pollinators to floral nectar (Wäckers et al., 1994). Therefore, the artificial sugar sources could be combined with attractive volatile cues to improve the sugar resource location. In **Chapter 3**, volatiles emitted by bacteria associated with the parasitoid's habitat are evaluated as possible mediators of parasitoid attraction.

Chapter 3

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

This chapter is based on the following publication:
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3.1 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 et al., 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 et al., 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, 2014b). More recently, increasing evidence indicates that microorganisms like bacteria and fungi also emit volatile compounds that affect insect behaviour (Davis et al., 2013; Dzialo et al., 2017; Leroy et al., 2011b). Although production of microbial volatile organic compounds (mVOCs) is commonplace (Piechulla & Degenhardt, 2014), 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; Rering et al., 2018; Sobhy et al., 2018, 2019). By contrast, some mVOCs have also been found to repel insects, e.g. when signalling unsuitable food sources, unsuitable hosts or hostile environments (Azeem et al., 2013; Huang et al., 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* (Diptera: Drosophilidae) 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 mVOC profiles 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., 2011a; Leroy et al., 2011b; 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 et al., 2008; Dillon et al., 2000; Leroy et al., 2011a). 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 et al., 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 et al., 2019b; 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 et al., 2017; de Rijk et al., 2013). Therefore, the olfactory response of female parasitoids and their efficiency in localising 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 et al., 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* (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.

3.2 Materials and Methods

3.2.1 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 3.1). 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 3.1. 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 et al., 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 et al., 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.

Table 3.1: Bacterial isolates used in this study.

Isolate identifier (GenBank Accession N°)[a]	Phylogenetic affiliation based on 16S rRNA gene sequence similarity[b]				Isolation origin[c]
	Phylum	Family	Closest match in EZBiocloud to identified species	Similarity (%)[d]	
ST18.17/034 (MK875132)	Actinobacteria	Corynebacteriaceae	*Corynebacterium sputi*	99.88	*Myzus persicae* var. *nicotianae**
ST18.16/042 MK875104)	Actinobacteria	Dermabacteraceae	*Brachybacterium rhamnosum*	99.69	*Acyrthosiphon pisum* mummy*
ST18.17/039 (MK875134)	Actinobacteria	Microbacteriaceae	*Leucobacter tardus*	99.24	*Myzus persicae* var. *nicotianae**
ST18.16/109 (MK875113)	Actinobacteria	Microbacteriaceae	*Microbacterium paraoxydans*	99.93	*Aphidius ervi**
ST18.16/085 (MK875112)	Actinobacteria	Micrococcaceae	*Curtobacterium* sp. (*C. flaccumfaciens, C. oceanosedimentum*)	99.66	*Myzus persicae* var. *nicotianae*
ST18.16/082 (MK875111)	Actinobacteria	Micrococcaceae	*Glutamicibacter halophytocola*	98.90	*Myzus persicae* var. *nicotianae*
ST18.17/002 (MK875125)	Actinobacteria	Micrococcaceae	*Glutamicibacter halophytocola*	99.40	Honeydew *M. persicae*/ *Capsicum annuum**
ST18.17/032 (MK875131)	Actinobacteria	Micrococcaceae	*Kocuria halotolerans*	99.88	*Myzus persicae* var. *nicotianae**
ST18.16/067 (MK875109)	Actinobacteria	Micrococcaceae	*Paeniglutamicibacter antarcticus*	99.36	*Myzus persicae* var. *nicotianae*
ST18.16/153 (MK875118)	Firmicutes	Bacillaceae	*Bacillus altitudinis*	99.27	*Macrosiphum euphorbiae*
ST18.16/150 (MK875117)	Firmicutes	Bacillaceae	*Bacillus circulans*	99.04	*Macrosiphum euphorbiae*
ST18.16/061 (MK875108)	Firmicutes	Bacillaceae	*Bacillus endophyticus*	99.93	*Myzus persicae* var. *nicotianae*
ST18.16/043 (MK875105)	Firmicutes	Bacillaceae	*Bacillus massiliosenegalensis*	99.51	*Dendrocerus aphidum**
ST18.16/044 (MK875106)	Firmicutes	Bacillaceae	*Bacillus megaterium*	99.92	*Myzus persicae* var. *nicotianae*
ST18.16/075 (MK875110)	Firmicutes	Bacillaceae	*Bacillus* sp. (*B. siamensis, B. velezensis*)	99.86	*Myzus persicae* var. *nicotianae*
ST18.16/020 (MK875099)	Firmicutes	Bacillaceae	*Bacillus* sp. (*B. subtilis, B. siamensis, B. velezensis, B. amyloliquefaciens*)	100	*Aphidius colemani**
ST18.16/188 (MK875121)	Firmicutes	Bacillaceae	*Bacillus velezensis*	99.41	*Macrosiphum euphorbiae*
ST18.16/133 (MK875116)	Firmicutes	Bacillaceae	*Bacillus zhangzhouensis*	99.56	*Aphidius ervi**
ST18.16/051 (MK875107)	Firmicutes	Leuconostocaceae	*Weissella soli*	99.69	*Myzus persicae* var. *nicotianae*
ST18.16/041 (MK875103)	Firmicutes	Paenibacillaceae	*Paenibacillus glucanolyticus*	100	*Myzus persicae* var. *nicotianae* mummy*

Isolate identifier (GenBank Accession N°)[a]	Phylogenetic affiliation based on 16S rRNA gene sequence similarity[b]				Isolation origin[c]
	Phylum	Family	Closest match in EZBiocloud to identified species	Similarity (%)[d]	
ST18.17/035 (MK875133)	Firmicutes	Staphylococcaceae	*Corticicoccus populi*	100	*Myzus persicae* var. *nicotianae**
ST18.16/119 (MK875115)	Firmicutes	Staphylococcaceae	*Staphylococcus hominis*	99.65	*Aphidius ervi**
ST18.16/160 (MK875120)	Firmicutes	Staphylococcaceae	*Staphylococcus saprophyticus*	99.50	*Macrosiphum euphorbiae*
ST18.16/012 (MK875098)	Firmicutes	Staphylococcaceae	*Staphylococcus sciuri*	99.90	*Myzus persicae* var. *nicotianae**
ST18.16/031 MK875101)	Firmicutes	Staphylococcaceae	*Staphylococcus sciuri*	99.71	*Aphidius colemani**
ST18.16/206 (MK875123)	Firmicutes	Staphylococcaceae	*Staphylococcus xylosus*	99.52	Honeydew *M. persicae*/ *Capsicum annuum*
ST18.16/207 (MK875124)	Firmicutes	Staphylococcaceae	*Staphylococcus xylosus*	99.55	Honeydew *M. persicae*/ *Capsicum annuum*
ST18.17/004 (MK875126)	Firmicutes	Staphylococcaceae	*Staphylococcus xylosus*	99.78	Honeydew *M. persicae*/ *Capsicum annuum**
ST18.16/116 (MK875114)	Proteobacteria	Acetobacteraceae	*Asaia lannensis*	100	*Aphidius ervi**
ST18.16/030 (MK875100)	Proteobacteria	Erwiniaceae	*Erwinia iniecta*	100	*Aphidius colemani**
ST18.16/032 MK875102)	Proteobacteria	Erwiniaceae	*Erwinia tasmaniensis*	99.89	*Aphidius colemani**
ST18.17/028 (MK875130)	Proteobacteria	Erwiniaceae	*Pantoea dispersa*	99.85	Honeydew *M. persicae*/ *Capsicum annuum**
ST18.16/159 (MK875119)	Proteobacteria	Moraxellaceae	*Acinetobacter lwoffii*	99.84	*Aphidius rhopalosiphi*
ST18.17/045 (MK875135)	Proteobacteria	Pseudomonadaceae	*Pseudomonas hunanensis*	99.87	*Myzus persicae* var. *nicotianae**
ST18.17/015 (MK875129)	Proteobacteria	Pseudomonadaceae	*Pseudomonas paralactis*	100	Honeydew *M. persicae*/ *Capsicum annuum**
ST18.17/008 (MK875127)	Proteobacteria	Pseudomonadaceae	*Pseudomonas soli*	100	Honeydew *M. persicae*/ *Capsicum annuum**
ST18.16/205 (MK875122)	Proteobacteria	Pseudomonadaceae	*Pseudomonas* sp. (*P. lurida, P. lactis, P. paralactis.* …)	99.87	Honeydew *M. persicae*/ *Capsicum annuum*
ST18.17/009 (MK875128)	Proteobacteria	Pseudomonadaceae	*Stenotrophomonas maltophilia*	99.14	Honeydew *M. persicae*/ *Capsicum annuum**

[a]Accession number of 16S rRNA gene fragements deposited in GenBank. Isolates indicated in bold were selected for the second experiment.
[b]Based on 16S rRNA gene sequencing and identification using the EZBiocloud database (November 2018). Only closest matches to type strains are reported.
[c]Samples were collected in greenhouses or from a laboratory environment (lab cultures; indicated with an asterisk).
[d]Average fragment length was 1017 bp.

3.2.2 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. Cell densities for each of the replicates were determined by plating serial dilutions directly after cultivation to determine whether cell densities were equal in each of the three replicates and if no contamination was present. Final cell densities ranged between 1.40×10^{7} cfu/mL and 3.83×10^{9} cfu/mL, except for ST18.16/109, ST18.17/002, ST18.17/004 and ST118.17/035 which ranged between 3.33×10^{2} cfu/mL and 2.87×10^{5} cfu/mL. 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.

3.2.3 Y-tube olfactometer bioassays

Insect behavioural response was assessed using the Y-tube olfactometer bioassay described by Sobhy et al. (2018). The glass Y-tube comprised of a 20 cm stem tube containing two 12 cm-long lateral arms at an angle of 60° at the Y-junction and had an internal diameter of 1.5 cm. Activated charcoal filtered, humidified air was supplied at a

rate of 400 mL/min in each arm of the Y-tube (controlled by separate flowmeters (Brooks Instrument, Hatfield, USA)) before passing through a glass odour chamber containing the test odours. The airflow was generated by an air pump (APS 300 Tetratec, Mella, Germany) containing two separate outlets. All connections in the olfactometer were made using polytetrafluoroethylene (PTFE) tubing (Fig. 3.1). 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.

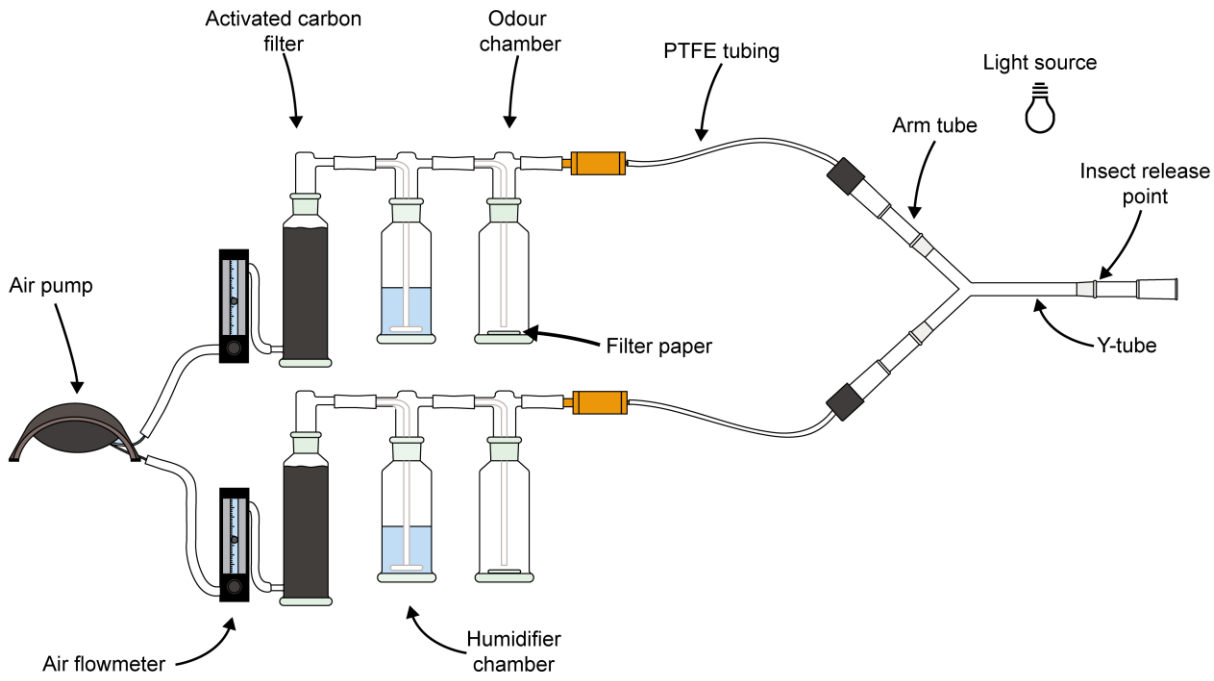

Figure 3.1: Schematic representation of the two-choice Y-tube olfactometer used in the bioassays. The olfactometer consisted of a 20-cm-long stem tube with 1.5 cm internal diameter and two 12-cm-long lateral arms with a 60° angle at the Y-junction. The detailed depiction of the various parts and connections is as follows: Air flow is generated by an electric air pump containing two separate outlets. The air flow was controlled at 400 mL/min for each arm of the Y-tube by separate flowmeters and subsequently purified and humidified by activated carbon filters, and humidifier chambers containing demineralized water, respectively. Next, the purified and humidified air passes through glass odour chambers containing the test odours. Finally, the air containing the test odours is directed to the glass Y-tube via polytetrafluoroethylene (PTFE) tubing connected with glass arm tubes. The glass Y-tube was homogeneously illuminated by four high frequency 24W T5 TL-fluorescent tubes (~10,000 lux) and positioned at a 20° incline to stimulate movement of the insects towards the bifurcation. During the bioassay, parasitoid individuals were introduced in the Y-tube at the base of the stem tube.

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. Moreover, an additional experiment was included where the parasitoids were offered blank medium in both arms of the Y-tube as a control to determine the presence of any positional bias. 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*.

3.2.4 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 determine 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/min^2 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 A3.1, Appendix).

3.2.5 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 (choice is binary: 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. To examine the preference of the investigated parasitoids for mVOCs produced by each of the tested bacterial strains, we tested the null hypothesis (H_0) that the parasitoids show no preference for any olfactometer arm (i.e. 50:50 response) by testing H_0: logit = 0, which equals a 50:50 distribution. In addition, 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 of all tested bacterial strains. 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 pheatmap function (pheatmap package in R). 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 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 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).

3.3 Results

3.3.1 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 (χ^2 = 74.71, df = 38, P < 0.001; Fig. 3.2). Three bacterial strains were found to significantly attract *A. colemani* (ST18.16/150: PI = 0.36, P = 0.011; ST18.16/043: PI = 0.31, P = 0.024; ST18.16/133: PI = 0.27, P = 0.046), while the mVOCs of three other strains were significantly deterrent (ST18.17/002: PI = -0.41, P = 0.004;

ST18.16/028: PI = -0.38, *P* = 0.006; ST18.16/160: PI = -0.30, *P* = 0.027). Volatile blends emitted by the other strains and the blank medium had no statistically significant effect on the olfactory response of *A. colemani* (Fig. 3.2). 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 strains isolated from honeydew had a significant influence on the olfactory response of *A. colemani* (χ^2 = 17.9, *df* = 2, *P* < 0.001). *Aphidius colemani* showed significantly lower PI-values when exposed to mVOCs produced by bacteria originating from honeydew.

3.3.2 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. 3.2). Results confirmed that the strains with the highest PI-values were significantly attractive to *A. colemani* (P ≤ 0.001), while the strains with the lowest PI-values significantly repelled *A. colemani* (P ≤ 0.001) (Fig. 3.3). Results also showed that insect response differed between the tested insect species (Fig. 3.3). 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* < 0.001; ST18.17/028: PI = -0.25, *P* = 0.003). 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, the volatile emissions of strain ST18.16/160 which were repellent to *A. colemani*, tended to attract *D. aphidum* (PI = 0.18, *P* = 0.035) (Fig. 3.3).

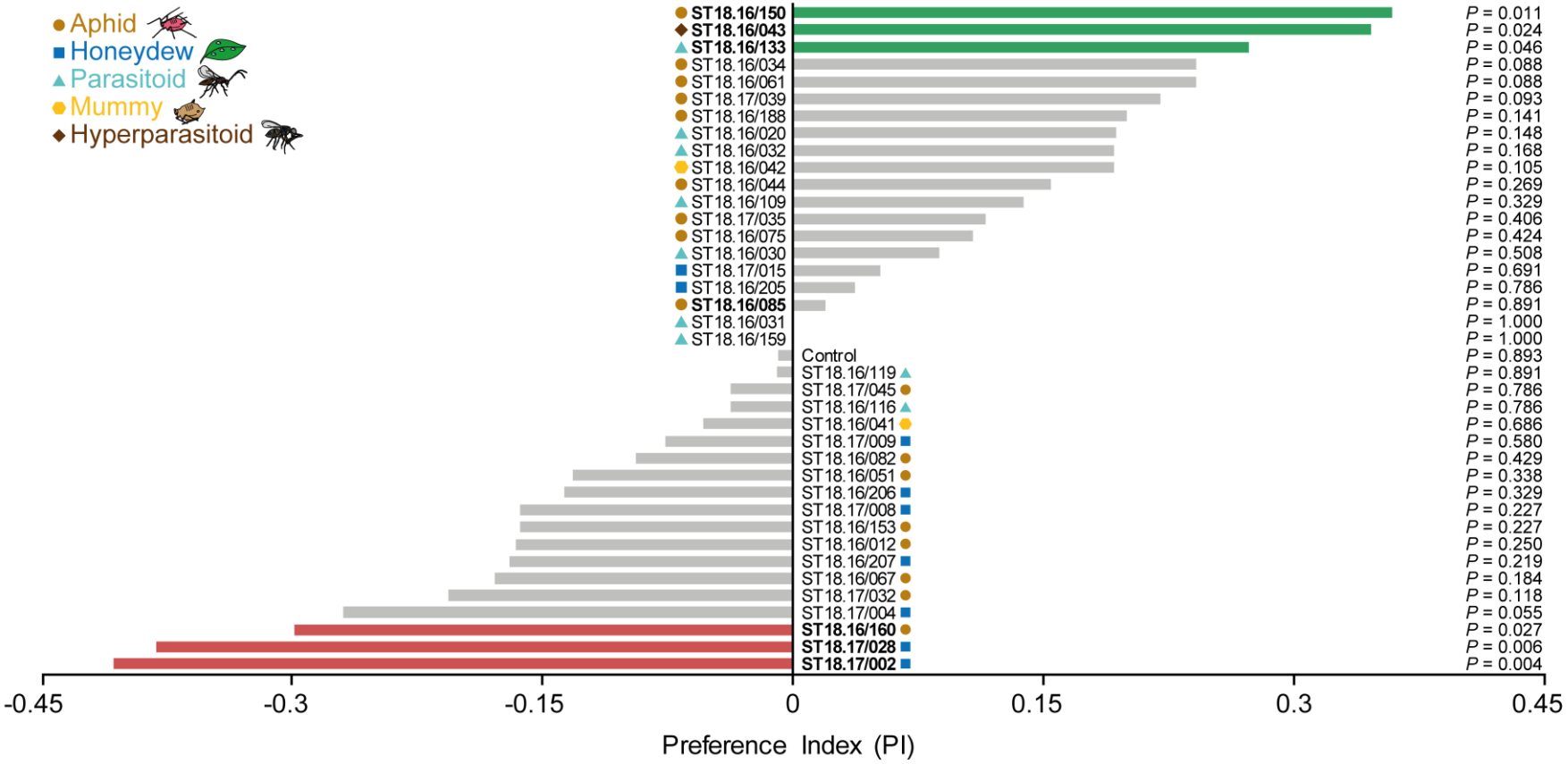

Figure 3.2: Olfactory response of adult *Aphidius colemani* females when given a choice between the odour of a test bacterium (n = 38) grown in GYP25 medium and the odour of the blank GYP25 medium in a Y-tube olfactometer. Insect response is expressed as the Preference Index (PI) which is calculated 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. In total, 60 individuals were tested (12 releases of 5 females). An additional experiment was performed as a control in which parasitoids were offered blank GYP25 medium in both arms of the Y-tube. 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 a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). 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.

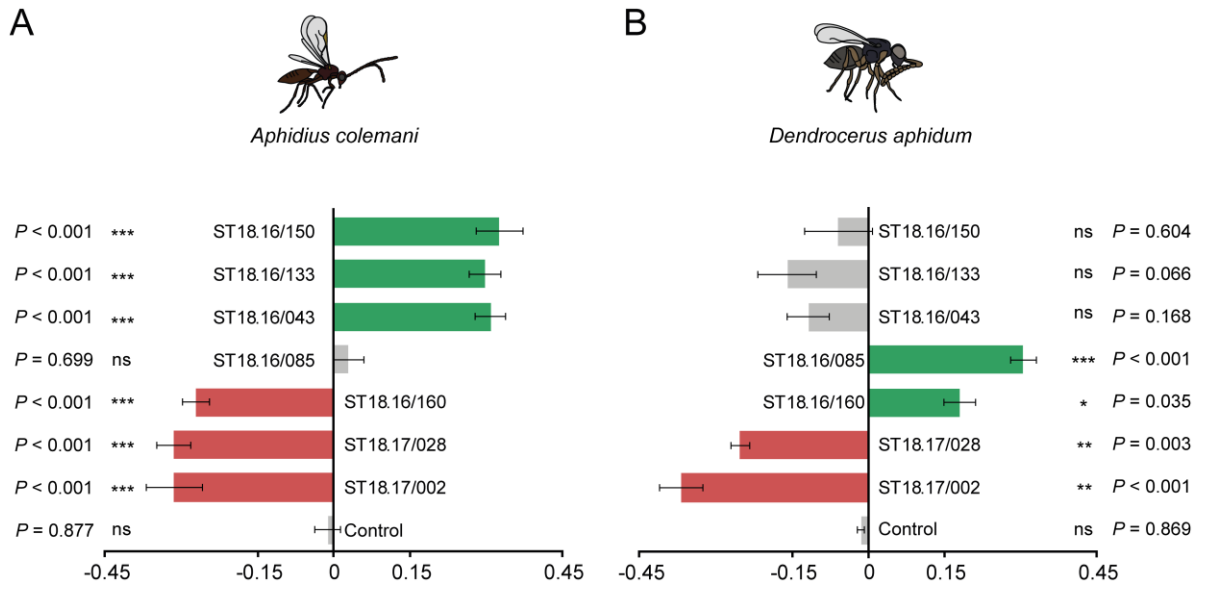

Figure 3.3: Different olfactory response of **(A)** adult *Aphidius colemani* females and **(B)** adult *Dendrocerus aphidum* females when given a choice between the odour of a test bacterium grown in GYP25 medium and the odour of the blank GYP25 medium in a Y-tube olfactometer. Insect response is expressed 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 a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). Overall parasitoid responsiveness was higher than 70%.

3.3.3 mVOC composition

The mVOC composition differed significantly between the seven bacterial treatments and the blank medium (perMANOVA: pseudo-$F_6 = 38.6$, $P < 0.001$). Overall, volatiles produced in the highest amounts belonged to alcohols, esters, ketones and organic acids (Fig. 3.4; Table A3.1, Appendix). 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 (Fig. 3.4; Table A3.1, Appendix). NMDS ordination of the mVOC composition (Fig. 3.5) 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. 3.5). Notably, these three strains elicited significant attraction in *A. colemani* (Fig. 3.5). 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. 3.5).

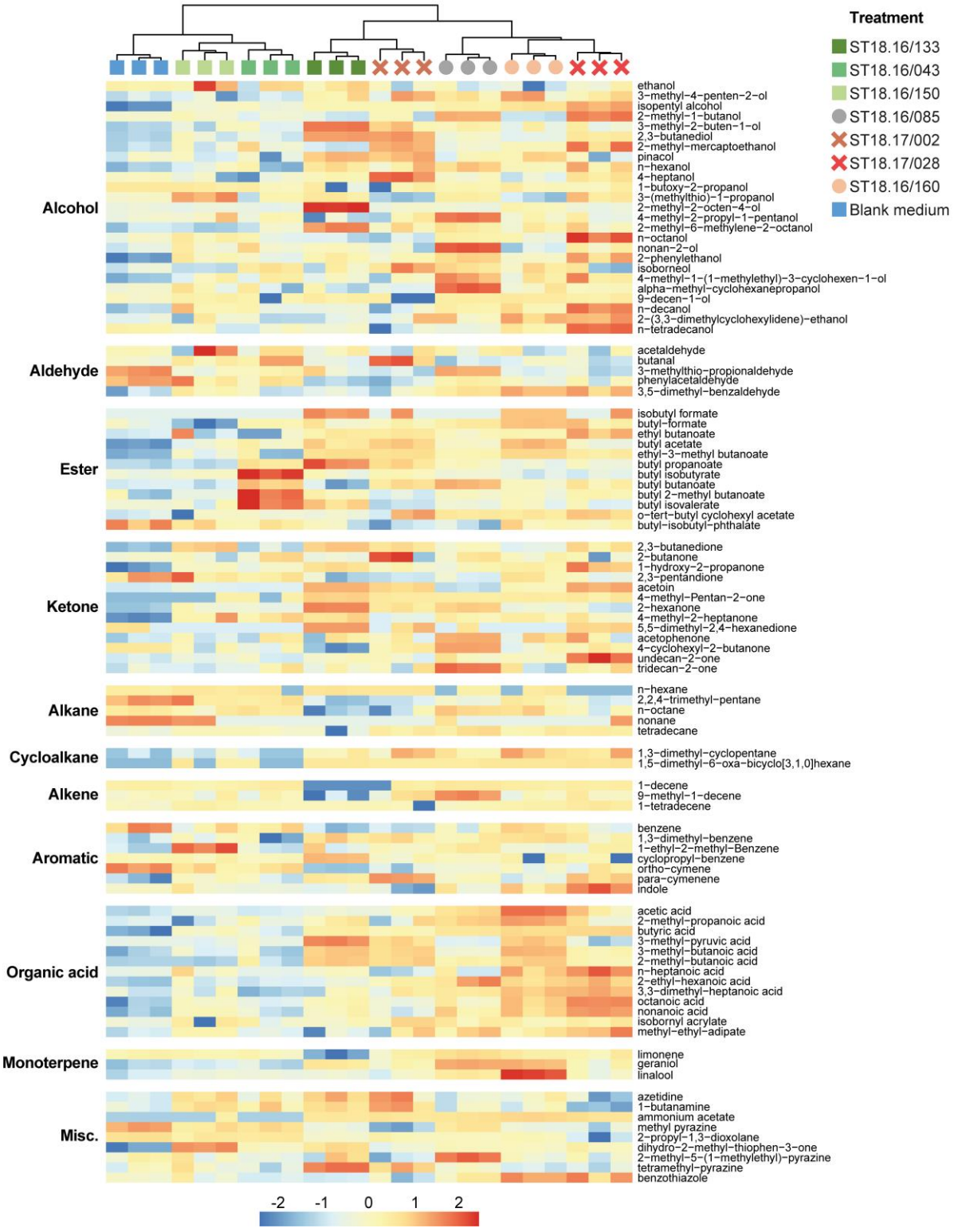

Figure 3.4: Heatmap of the mVOC composition of the selected seven bacterial strains investigated in this study. Data are presented in the form of mean centred, log transformed peak areas of compounds for each of the three biological replicates (n = 3). Bacterial strain treatments were clustered based on Euclidean distances by using Manhattan distances and a Ward.D clustering algorithm. mVOCs are grouped based on chemical classes. Symbol colours indicate the effect of the mVOCs 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 mVOCs on the olfactory response of the hyperparasitoid *Dendrocerus aphidum*, i.e. circle = attractive, square = neutral and cross = repellent, when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model).

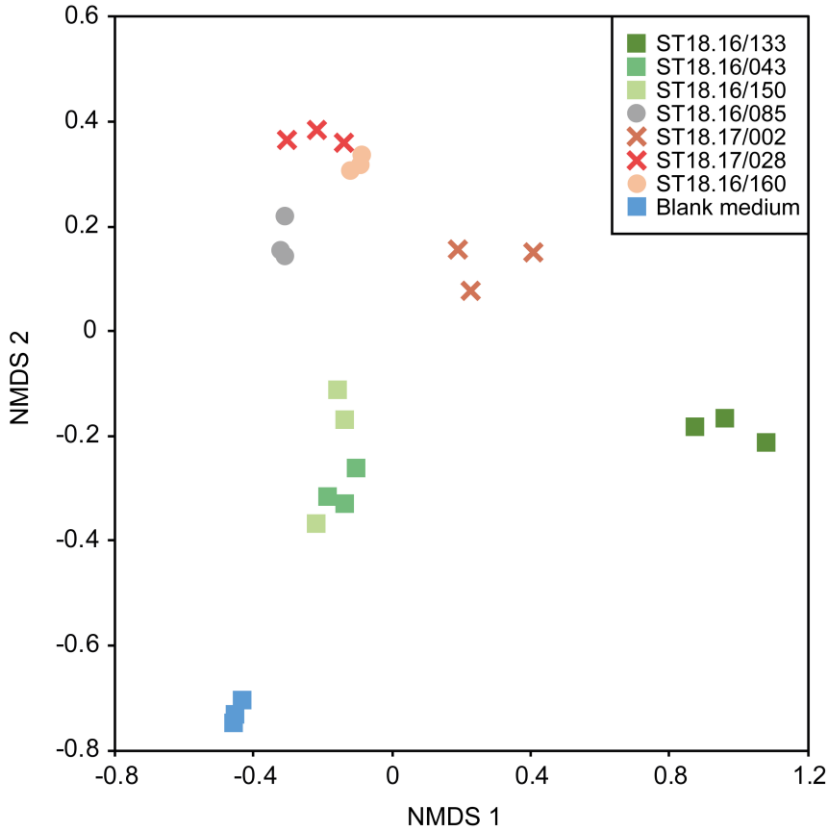

Figure 3.5: Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities of the mVOC composition of seven bacterial strains investigated in this study (n = 3) (stress value = 0.158). The mVOC composition differed significantly between the bacterial strains and the blank medium (perMANOVA: pseudo-F_6 = 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, when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model).

3.3.4 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. 3.4 and 3.6). 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 strains significantly attractive to *D. aphidum* (ST18.16/085 and ST18.16/160) produced higher amounts three monoterpenes limonene, linalool and geraniol (Fig. 3.4 and 3.7).

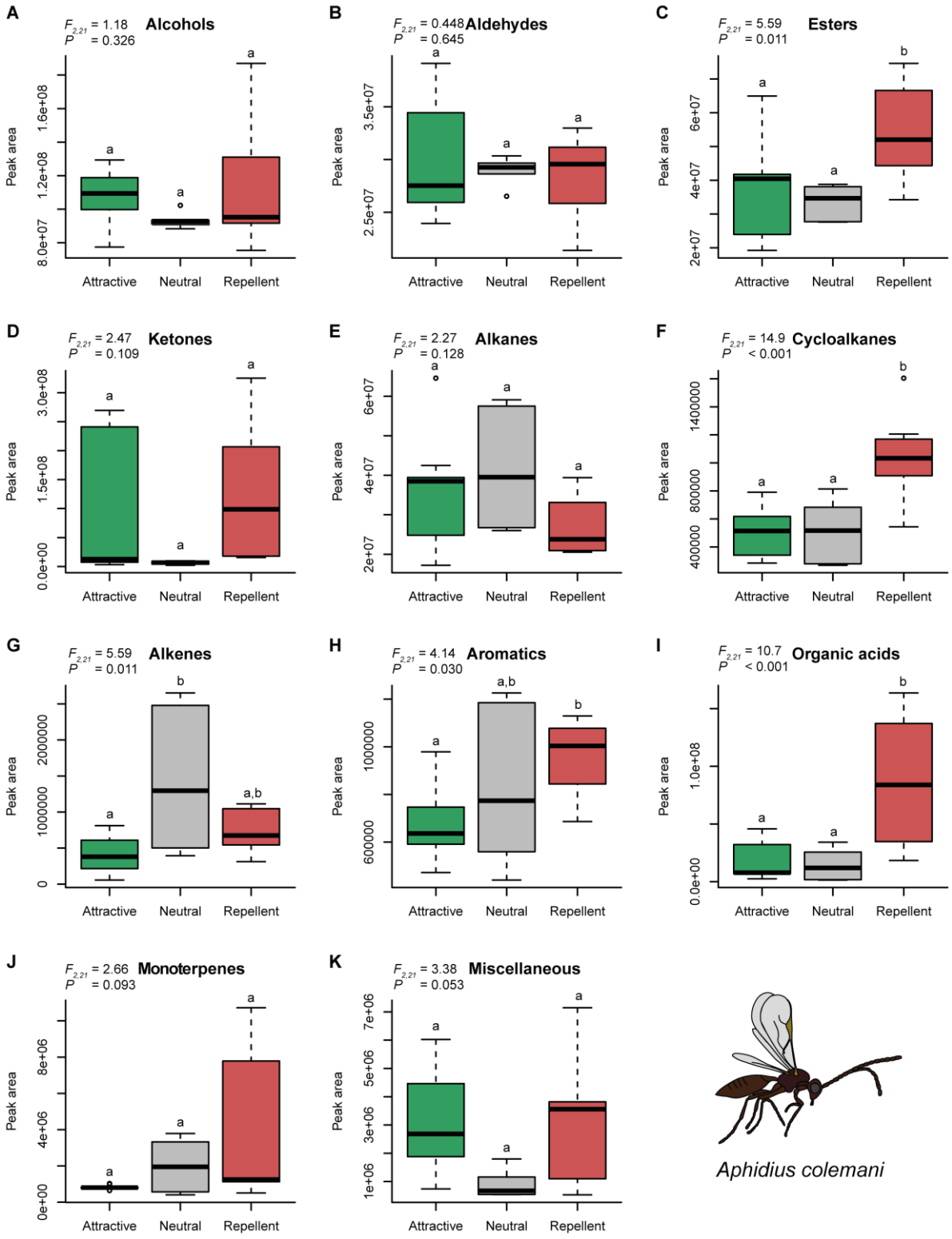

Figure 3.6: Linkage between the volatile composition of the cell-free bacterial cultivation media and the olfactory response of *Aphidius colemani*. Results are shown for **(A)** alcohols, **(B)** aldehydes, **(C)** esters, **(D)** ketones, **(E)** alkanes, **(F)** cycloalkanes, **(G)** alkenes, **(H)** aromatics, **(I)** organic acids, **(J)** terpenes, and **(K)** miscellaneous. 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.

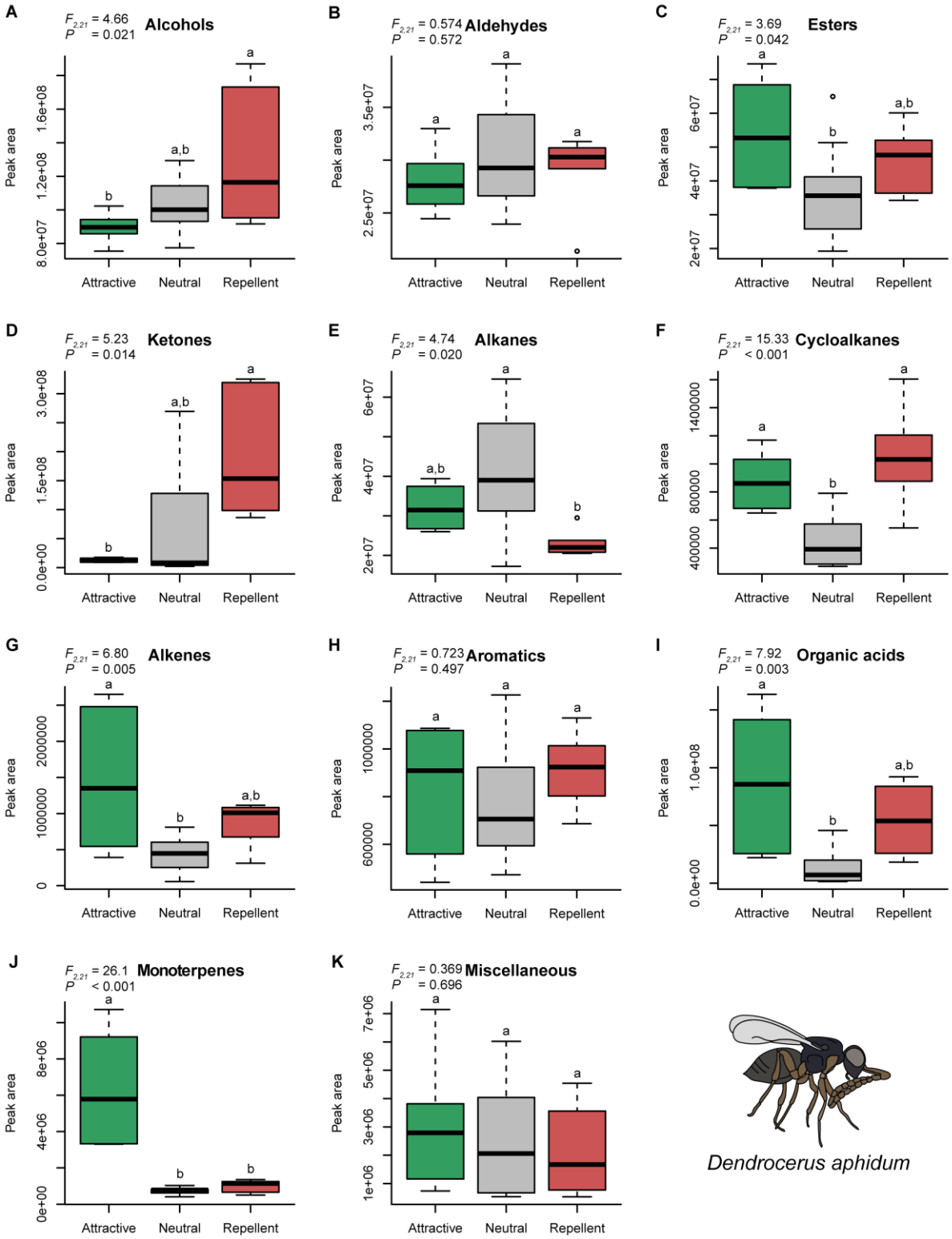

Figure 3.7: Linkage between the volatile composition of the cell-free bacterial cultivation media and the olfactory response of *Dendrocerus aphidum*. Results are shown for **(A)** alcohols, **(B)** aldehydes, **(C)** esters, **(D)** ketones, **(E)** alkanes, **(F)** cycloalkanes, **(G)** alkenes, **(H)** aromatics, **(I)** organic acids, **(J)** terpenes, and **(K)** miscellaneous. 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, ST18.16/160; Neutral = blank medium, ST18.16/150, ST18.16/133 and ST18.16/043; 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.

3.4 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 et al., 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.

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

Our results show that *A. colemani* females responded differently to the mVOCs of 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 et al., 2017). Additionally, a number of studies have shown that *Bacillus* volatiles may also affect insect behaviour. For example, both Rockett (1987) and Poonam et al. (2002) showed that volatiles produced by *Bacillus* species induced oviposition stimulation in *Culex quinquefasciatus* (Diptera: Culicidae) females. Furthermore, the melon fruit fly *Bactrocera cucurbitae* (Diptera: Tephritidae) was attracted to broth cultures of *Bacillus cereus* (Mishra et al., 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 et al., 2013, 2015). However, it is unclear so far whether there are also phylogenetic signals in mVOC composition and insect response.

3.4.2 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 produced mVOCs that were significantly repellent to *A. colemani* (ST18.16/160; putatively identified as *Staphylococcus saprophyticus*) induced a significantly attractive response in *D. aphidum*. Additionally, the strain that was neutral to *A. colemani* (ST18.16/085; *Curtobacterium* sp.) also 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., 2019b; Poelman et al., 2012).

3.4.3 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 et al., 2015). However, some compounds like geraniol, linalool, limonene, 2-phenylethanol, phenylacetaldehyde and acetophenone are also commonly reported as typical plant volatiles (Bruce & Pickett, 2011; Dudareva et al., 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 et al., 1994; Webster et al., 2010b).

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.

3.4.4 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 et al., 2005; Mumm & Hilker, 2005; Takemoto & Takabayashi, 2015; Webster et al., 2010a). 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 et al., 2000; McCormick et al., 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 et al., 2005; 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).

3.4.5 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). Therefore, we made a first attempt in **Chapter 5** to investigate whether *A. colemani* parasitoids can respond to volatile blends under more realistic, greenhouse conditions in the presence of plants. Furthermore, 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 et al., 2017b). 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. However, at present it is not well known which microbial volatiles or blends of microbial volatiles define the insects' response. Therefore, in **Chapter 5** we aimed at identifying specific compounds in bacterial volatile blends that attract *A. colemani*. Finally, our data seem to suggest that mVOC-mediated insect responses may be correlated with bacterial phylogeny. In **Chapter 4,** we tested in more detail the hypothesis that phylogenetic relationships among microorganisms predict microbial volatile composition and the olfactory response of insects.

Chapter 4

Bacterial phylogeny predicts volatile organic compound composition and olfactory response of an aphid parasitoid

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4.1 Introduction

Phenotypic complementarity and phylogenetic relationships fundamentally impact ecological processes and therefore have the potential to affect interactions between taxa (Ives & Godfray, 2006; Rezende et al., 2007). Whereas previous studies have shown effects of phenotypic complementarity in higher-order organisms (plants and animals) (Rezende et al., 2007), far less is known about the interactions involving microorganisms. Nonetheless, recent studies have indicated that the phylogeny of microorganisms may reflect phenotypic characteristics and key functional traits (Goberna & Verdú, 2016; Martiny et al., 2013, 2015), and therefore may affect interactions with other organisms.

Microorganisms release a plethora of volatiles (further referred to as mVOCs, microbial volatile organic compounds), many of which play an important role in intra- and inter-kingdom interactions (Schulz-Bohm et al., 2017). For example, it has recently been shown that mVOCs act as insect semiochemicals that affect insect behaviour (Becher et al., 2012; Davis et al., 2013; **Chapter 3**; Goelen et al., 2020, Sobhy et al., 2018, 2019). In some cases, mVOCs strongly attract insects by signalling the presence of suitable resources such as appropriate food sources or oviposition sites (Becher et al., 2012; Leroy et al., 2011b; Sobhy et al., 2018, 2019). This chemical communication between insects and microorganisms is believed to drive a mutualistic relationship, in which not only the insects profit from the microorganisms, but also the microorganisms benefit from the insects by being dispersed to new niches where they can continue to develop or complete their life cycle (Christiaens et al., 2014; Pozo et al., 2018; Um et al., 2013). Nevertheless, by contrast, some mVOCs have also been found to repel insects (Burkepile et al., 2006; Stensmyr et al., 2012).

Strikingly, results in **Chapter 3** showed that closely related species of the genus *Bacillus* elicited a similar olfactory response (attraction) in *Aphidius colemani* (Hymenoptera: Braconidae), which suggests that volatile composition and, as a result, parasitoid attraction, are phylogenetically conserved traits. In this study, we investigated in more detail the hypothesis that phylogenetic relationships among microorganisms predict microbial volatile composition and the olfactory response of insects. To test this hypothesis, we used a large set of *Bacillus* strains and the generalist aphid parasitoid *A. colemani* as our study organism.

The genus *Bacillus* represents a heterogeneous group of Gram-positive bacteria that occupy diverse ecological systems and have been isolated from various habitats, including soil, water, dust, air, insect guts and plant related environments such as roots, leaves, and nectar (Logan & De Vos, 2009). Up to now, 379 *Bacillus* species are on the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (http://www.bacterio.net/bacillus.html, last accessed December 10th, 2019), but it is anticipated that many more *Bacillus* species still need to be formally described (Maughan & Van der Auwera, 2011). Members of the genus *Bacillus* produce a wide array of volatiles (Kai et al., 2009), among which some that can promote plant growth without physical contact (Ping & Boland, 2004) or have antimicrobial activity (Gao et al., 2017). Furthermore, recent studies have shown that mVOCs produced by *Bacillus* strains may affect insect behaviour. For example, it has been shown that *Bacillus* volatiles stimulate oviposition in gravid *Culex quinquefasciatus* (Diptera: Culicidae) females (Pooneam et al., 2002; Rockett, 1987). Additionally, the melon fruit fly *Bactrocera cucurbitae* (Diptera: Tephritidae) and the parasitoid *A. colemani* have been shown to be attracted to mVOC blends emitted by *Bacillus* spp. (**Chapter** 3; Goelen et al., 2020; Mishra & Sharma, 2018).

The specific objectives of this study were (i) to assess whether phylogenetic relationships of *Bacillus* species are associated with variation in mVOC composition and olfactory response of *A. colemani*, and (ii) to identify the key mVOCs explaining any potential phylogenetic signal. First, we analysed the chemical composition of the mVOC blends produced by 40 phylogenetically diverse *Bacillus* strains using gas chromatography-mass spectrometry (GC-MS). Subsequently, we evaluated the olfactory response of *A. colemani* to these blends in a Y-tube olfactometer bioassay. Finally, we tested whether phylogenetic relationships between *Bacillus* strains could accurately predict mVOC composition and insect response using both univariate and multivariate tests for phylogenetic signal and trait correlations.

4.2 Materials and Methods

4.2.1 Study organisms

A collection of 40 *Bacillus* strains was used in this study, representing (at least) 20 different species (based on *rpoB* sequence similarity with identified strains), including ten type strains (Table 4.1). Strains were isolated from a variety of habitats, including insects, soil, soil conditioners, wooden barrels, and plant-related habitats such as floral nectar, fruits and honeydew (Table 4.1). All isolates were stored in tryptic soy broth (TSB; Oxoid, Hampshire, UK) containing 25% glycerol at -80°C until use.

Olfactory responses were investigated using female adults of *A. colemani* that had been obtained in the form of parasitized aphid mummies from Biobest (Westerlo, Belgium) (Aphidius-system®). 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% RH, 16L:8D-h) until parasitoid emergence. All behavioural experiments were performed with <24-h-old, food- and water-starved females.

4.2.2 Production of mVOCs

The mVOCs were produced according to the procedure described in **Chapter 3**. Cultivation for each strain was carried out in triplicate, and non-inoculated, blank medium was included as a control. The cell-free cultivation media containing the mVOCs were stored in small aliquots in sterile, amber glass vials at -20°C until further use.

Table 4.1: Bacterial isolates used in this study.

Isolate identifier[a]	16S rRNA gene Accession N°[b]	*rpoB* Accession N°[c]	Phylogenetic affiliation[d]				Source of isolation
			Phylum	Family	Closest match in GenBank to identified species (Accession N°)	Identity (%)	
ST14.14/060	MN220674	MN232848	Firmicutes	Bacillaceae	*Bacillus altitudinis* (CP024204.1)	99.82	Whisky barrel
ST12.14/235	MN220665	MN232841	Firmicutes	Bacillaceae	*Bacillus altitudinis* (CP024204.1)	99.64	Floral nectar *Centaurea cyanus*
ST14.14/007	MN220670	MN232844	Firmicutes	Bacillaceae	*Bacillus altitudinis* (CP024204.1)	99.91	Whisky barrel
ST14.14/047	MN220673	MN232847	Firmicutes	Bacillaceae	*Bacillus altitudinis* (CP024204.1)	99.46	Whisky barrel
ST14.14/029	MN220671	MN232846	Firmicutes	Bacillaceae	*Bacillus altitudinis* (CP024204.1)	99.73	Whisky barrel
ST14.14/046	MN220672	MN232845	Firmicutes	Bacillaceae	*Bacillus altitudinis* (CP024204.1)	99.00	Whisky barrel
ST04.14/017	MN220662	MN232843	Firmicutes	Bacillaceae	*Bacillus altitudinis* (CP024204.1)	99.28	Soil conditioner
LMG 24407#	EF114313	GCA_900101665.1	Firmicutes	Bacillaceae	*Bacillus aryabhattai* (GCA_900101665.1)	100.00	Cryogenic tube
DSM 105779#	QWVS01000027	GCA_003570725.1	Firmicutes	Bacillaceae	*Bacillus asahii* (GCA_003570725.1)	100.00	Soil
ST18.16/150	MN220680	MN232831	Firmicutes	Bacillaceae	*Bacillus circulans* (CP026033.1)	92.20	*Macrosiphum euphorbiae*
LMG 21715#	AF295302	GCA_900115845	Firmicutes	Bacillaceae	*Bacillus endophyticus* (GCA_900115845)	100.00	Cotton plants
ST18.16/061	MN220676	MN232836	Firmicutes	Bacillaceae	*Bacillus filamentosus* (CP026635.1)	99.28	*Myzus persicae* var. *nicotianae*
DSM 1316	MN220658	MN232837	Firmicutes	Bacillaceae	*Bacillus flexus* (CP040367.1)	99.10	Unknown
LMG 11155#	BCVD01000224	GCA_001591565.1	Firmicutes	Bacillaceae	*Bacillus flexus* (GCA_003184905.1)	100.00	Unknown
ST12.15/030	MN220666	MN232850	Firmicutes	Bacillaceae	*Bacillus halotolerans* (CP029364.1)	99.91	Dried figs
DSM 104297#	KY462210	GCA_003184905.1	Firmicutes	Bacillaceae	*Bacillus iocasae* (GCA_003184905.1)	100.00	Deep-sea sediment
DSM 16467#	LILC01000014	GCA_001274935.1	Firmicutes	Bacillaceae	*Bacillus koreensis* (GCA_001274935.1)	100.00	Rhizosphere
ST18.16/073	MN220660	MN232851	Firmicutes	Bacillaceae	*Bacillus licheniformis* (CP034569.1)	100.00	*Myzus persicae* var. *nicotianae*
ST12.15/036	MN220667	MN232829	Firmicutes	Bacillaceae	*Bacillus megaterium* (CP001982.1)	99.55	Dried figs
ST18.16/013	MN220675	MN232827	Firmicutes	Bacillaceae	*Bacillus megaterium* (CP001982.1)	99.64	*Myzus persicae* var. *nicotianae*
ST12.15/034	MN220686	MN232828	Firmicutes	Bacillaceae	*Bacillus megaterium* (CP001982.1)	99.64	Dried figs
ST01.11/095	MN220661	MN232839	Firmicutes	Bacillaceae	*Bacillus megaterium* (CP028084.1)	99.82	Activated sludge

Isolate identifier[a]	16S rRNA gene Accession N°	*rpoB* Accession N°	Phylogenetic affiliation[b]				Source of isolation
			Phylum	Family	Closest match in GenBank to identified species (Accession N°)	Identity (%)	
ST14.12/130	MN220669	MN232832	Firmicutes	Bacillaceae	*Bacillus megaterium* (CP001982.1)	99.19	Floral nectar *Epipactis palustris*
LMG 7127#	JJMH01000057	GCA_000832985.1	Firmicutes	Bacillaceae	*Bacillus megaterium* (GCA_000832985.1)	100.00	Unknown
LMG 20238#	LMBV01000055	GCA_001439925.1	Firmicutes	Bacillaceae	*Bacillus muralis* (GCA_001439925.1)	100.00	Mural
ST12.14/138	MN220664	MN232833	Firmicutes	Bacillaceae	*Bacillus mycoides* (CP009692.1)	99.55	Floral nectar *Symphytum officinale*
ST18.16/133	MN220679	MN232849	Firmicutes	Bacillaceae	*Bacillus pumilus* (CP029464.1)	100.00	*Aphidius ervi*
ST14.12/094	MN220668	MN232842	Firmicutes	Bacillaceae	*Bacillus pumilus* (LT906438.1)	99.91	Floral nectar *Epipactis palustris*
JCM 19454#*	JX293295	MN232838*	Firmicutes	Bacillaceae	*Bacillus qingshengii* (MN232838)	100.00	Weathered tuff surface
DSM 100650	MN220659	MN232853	Firmicutes	Bacillaceae	*Bacillus simplex* (CP017704.1)	96.56	Cleanroom facility
LMG 11160#	BCVO01000086	GCA_002243645.1	Firmicutes	Bacillaceae	*Bacillus simplex* (GCA_002243645.1)	100.00	Unknown
ST18.16/153	MN220681	MN232857	Firmicutes	Bacillaceae	*Bacillus* sp. (*B. altitudinis* [CP009108.1], *B. pumilus* [CP007436.1])	99.91	*Macrosiphum euphorbiae*
ST18.16/188	MN220683	MN232855	Firmicutes	Bacillaceae	*Bacillus* sp. (*B. velezensis* [CP029296.1], *B. amyloliquefaciens* [CP006845.1])	100.00	*Macrosiphum euphorbiae*
ST18.16/075	MN220677	MN232852	Firmicutes	Bacillaceae	*Bacillus* sp. (*B. velezensis* [CP029296.1], *B. amyloliquefaciens* [CP006845.1])	100.00	*Myzus persicae* var. *nicotianae*
ST04.13/022	MN220663	MN232854	Firmicutes	Bacillaceae	*Bacillus* sp. (*B. velezensis* [CP034176.1], *B. amyloliquefaciens* [CP007242.1])	99.91	Soil conditioner
ST12.14/237	MN220682	MN232840	Firmicutes	Bacillaceae	*Bacillus* sp. (*B. velezensis* [CP034176.1], *B. amyloliquefaciens* [CP007242.1])	98.91	Floral nectar *Centaurea cyanus*
ST18.16/043	MN220686	MN232830	Firmicutes	Bacillaceae	*Bacillus* sp. X1 (CP008855.1)	80.24	*Dendrocerus aphidum*
ST18.16/090	MN220678	MN232856	Firmicutes	Bacillaceae	*Bacillus subtilis* (CP035230.1)	100.00	*Aphidius ervi*
ST18.16/240	MN220684	MN232834	Firmicutes	Bacillaceae	*Bacillus wiedmannii* (CP024684.1)	99.73	*Myzus persicae* var. *nicotianae*/ *Capsicum annuum* honeydew
ST18.16/249	MN220685	MN232835	Firmicutes	Bacillaceae	*Bacillus wiedmannii* (CP024684.1)	99.00	*Myzus persicae* var. *nicotianae*/ *Capsicum annuum* honeydew

[a] Isolate identifiers of strains investigated in this study. 16S rRNA (1252-1262 bp) and *rpoB* gene (1102-1105 bp) sequences were obtaind by sequencing PCR products which were generated using the primers listed in Table 4.2.

#Type strains acquired from available culture collections (LMG, DSMZ and JCM). 16S rRNA gene sequences were obtained from the EZBiocloud database (referred to by EZBiocloud 16S rRNA gene accession numbers); *rpoB* sequences were extracted from full genome sequences obtained from the EZBiocloud database (referred to by EZBiocloud full genome accession numbers). *No full genome or *rpoB* sequence was available for this strain.

[b]Based on a BLAST search of the *rpoB* gene sequences against GenBank (July 2019). Only closest matches to identified species are reported.

4.2.3 Y-tube olfactometer bioassays

To determine the olfactory response of *A. colemani* to the mVOCs of the tested *Bacillus* strains, a Y-tube olfactometer bioassay was performed as described in **Chapter 3**, using cell-free cultivation media of the 40 *Bacillus* strains. As the mVOC composition of the three biological replicates was highly similar, olfactory response was determined for one of the three biological replicates. As described before, experiments were conducted with 60 female individuals, which were released in twelve cohorts of five individuals, and olfactory response was evaluated 10 min after their release. Parasitoids that did not make a choice within 10 min after release were considered as non-responding individuals and were eliminated from the statistical analysis.

4.2.4 Chemical analysis of mVOCs

For all samples, mVOC composition of the cell-free media was determined by headspace solid phase micro extraction gas chromatography followed by mass spectrometry detection (HS-SPME-GC-MS), as described in **Chapter 3**. Briefly, volatile compounds were separated by a Thermo Trace 1300 GC system (Thermo Fisher Scientific, Watham, USA) equipped with a MXT-5 column (30 m length × 0.18 mm inner diameter × 0.18 µm film thickness; Restek, Bellefonte, USA), and detected by a ISQ mass spectrometer (Thermo Fisher Scientific, Waltham, USA). An amount of 1.75 g NaCl was added to 5 mL of the samples and was kept at 60°C under constant agitation in a TriPlus RSH SMPE auto sampler (Thermo Fisher Scientific, Watham, USA). Volatile collection and separation conditions were as described previously (**Chapter 3**; Goelen et al., 2020), and identification and quantification of the compounds was performed as in Reher et al. (2019). Briefly, chromatograms were analysed using AMDIS v2.71 (Stein, 1999) to deconvolute overlapping peaks. The NIST MS Search v2.0g software in combination with the NIST2011, FFNSC and Adams libraries were used to manually identify the empirical spectra, taking into account the expected retention time. The compound elution profiles were extracted and integrated using a file with all our target compounds containing the expected retention times and spectrum profiles. The extraction was performed for every peak 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 single table.

4.2.5 Data analysis

Y-tube olfactometer bioassays

For all tested *Bacillus* strains, parasitoid olfactory response was analysed using a Generalized Linear Mixed Model (GLMM) based on a binomial distribution with a logit link function (logistic regression) using *Bacillus* strain as fixed factor and the number of parasitoids choosing for the control or treatment side in each cohort was entered as response variable. The GLMM was performed according to the procedure described in **Chapter 3**. Results were presented by calculating the Preference Index (PI) by dividing the difference between the number of parasitoids choosing for the bacterial volatiles and the parasitoids choosing for the control by the total number of responding insects.

Chemical analysis

To visualize the differences in the mVOC composition, a non-metric multidimensional scaling (NMDS) was performed on the strain*average volatile peak area matrix by using a Bray-Curtis distance matrix (Vegan package) in R 3.3.2 (R Core Team, 2019). To test for significant differences in chemical composition of mVOC profiles between bacterial clades (see further, six major clades were identified) and between groups of strains eliciting different olfactory responses in *A. colemani* (i.e. attractive, neutral or repellent), a two-way permutational multivariate analysis of variance (perMANOVA) was carried out on the strain*volatile peak area matrix. Volatile peak area was used as dependent variable, and bacterial clade and groups of strains evoking a similar olfactory response in *A. colemani* were used as independent variables. Statistical significance was estimated using 1000 permutations. This analysis was performed using the adonis function (Vegan package) in R 3.3.2 (R Core Team, 2019).

Testing for phylogenetic signal

Both multivariate and univariate analyses were performed to evaluate the presence of a phylogenetic signal in mVOC composition and insect behaviour. First, phylogenetic principal component analysis (pPCA) was used to evaluate the presence of phylogenetic signal in the complete, multivariate volatile composition dataset. pPCA is a method to summarise and visualise the phylogenetic resemblance of a multivariate trait dataset into two principal components (PCs) showing global or local phylogenetic structures (Jombart et al., 2010). The first PC denotes the global structure and reveals the mVOCs that are more

similar in related strains than in more distant strains. The local structure is depicted in the second PC and indicates the mVOCs that are more dissimilar in closely related strains (Jombart et al., 2010).

In order to test for statistical significance of the phylogenetic dependence of the PCs, we calculated the following indices: Pagel's λ (Pagel, 1999), Abouheif's C_{mean} (Abouheif, 1999) and Moran's I (Pavoine et al., 2008). All these indices have been developed to quantify and test for phylogenetic signal, but they are calculated in different ways, thereby capturing different aspects of phylogenetic signal. Particularly, these indices differ in their performance and their response to absence of branch length information, low sample sizes and degree of resolution of tree structure (Kamilar & Cooper, 2013; Münkemüller et al., 2012). Briefly, Pagel's λ is a quantitative measure of phylogenetic signal that assumes a Brownian motion model of trait evolution (i.e. in a random walk with constant trait variance over time (Fritz & Purvis, 2010)). It can continuously take values ranging from zero to one, where a value close to zero indicates phylogenetic independence, while a value of one corresponds to strong phylogenetic signal (Kamilar & Cooper, 2013; Münkemüller et al., 2012). Conversely, Abouheif's C_{mean} and Moran's I are autocorrelation indices not depending on a model of evolution and are unable to provide information on the strength of the phylogenetic signal. Therefore, these indices cannot be used to compare values between different phylogenies in contrast to Pagel's λ (Alonso et al., 2015; Münkemüller et al., 2012). The three indices are suited to correctly identify phylogenetic signal, even at moderate Brownian motion of traits and low sample sizes (Münkemüller et al., 2012). In addition, a Mantel test was used to test for correlations between the phylogenetic distance matrices and the distance matrices derived from the PC's from the pPCA. The distance matrices were calculated with Euclidean distances using the 'dist' function in R 3.3.2 (R Core Team, 2019), while phylogenetic distances were measured by selecting the complement of Abouheif proximity. This method allows for improved testing power when implementing the Mantel test for measuring phylogenetic signal (Hardy & Pavoine, 2012). Additionally, presence of phylogenetic signal was tested for each mVOC individually by calculating the same three indices and performing separate Mantel tests. To correct for multiple testing, *P*-values were adjusted using the false discovery rate (FDR) correction method (Benjamini & Hochberg, 1995). Finally, the same analyses were done to evaluate any

potential phylogenetic signal in the olfactory response of *A. colemani*, using the behavioural data expressed in PI-values as dependent variable.

In all calculations, a maximum-likelihood (ML) tree based on concatenated sequences of partial 16S rRNA gene (1252-1262 bp) and partial *rpoB* sequences (1102-1105 bp) was used. The tree was constructed by implementing the Kimura-2 model (gamma distributed with invariant sites (G+I)) with 1000 bootstraps. PCR amplification, sequencing and sequence alignment were performed as described previously (Bosmans et al., 2015), using the primers mentioned in Table 4.2. As the *rpoB* sequence could not be amplified using a single primer set, multiple primer sets were used (Table 4.2). The branch length information of the ML tree were kept in all analyses of phylogenetic signal and depiction of phylogenetic trees. All analyses were performed with functions in the packages 'adephylo' (Jombart et al., 2010), 'ape' (Paradis et al., 2004) and 'phytools' (Revell, 2012) in R 3.3.2 (R Core Team, 2019) using the volatile composition data in the form of mean centred, log transformed peak areas. *P*-values <0.05 were regarded as statistically significant. Finally, a phylogenetic heatmap was constructed using the ML tree and the volatile composition data in the form of mean centred, log transformed peak areas of compounds that showed phylogenetic signal in all three indices and the Mantel test, using the 'phylo.heatmap' function (Phytools package) in R 3.3.2 (R Core Team, 2019).

Table 4.2: Primers used for PCR amplification and sequencing.

Target	Primer[a]		Sequence	Spectrum (Clade)[b]	Reference
16S rRNA gene	27F	Forward	5′-AGAGTTTGATCMTGGCTCAG-3′	A,B,C,D,E,F	Lane (1991)
	1492R	Reverse	5′-GGTTACCTTGTTACGACTT-3′	A,B,C,D,E,F	Lane (1991)
rpoB	*rpoB*_r1f	Forward	5'-AGGTCAACTAGTTCAGTATGG-3'	A,C,D,E,F	Cai et al. (2017b)
	*rpoB*_r1fB	Forward	5'-AGGTCAACTAGTTCAATACGG-3'	B	This study
	*rpoB*_r1r	Reverse	5'-TAATTCAGCAAGCGGGTTCG-3'	A	Cai et al. (2017b)
	*rpoB*_r1rB	Reverse	5'-GTTAACTCTGCTAATGGGTTTG-3'	B,C	This study
	*rpoB*_r1rD	Reverse	5'-GAGTCAATTCAGCTAATGGATTTG-3'	D	This study
	*rpoB*_r1rEF	Reverse	5'-CAATTCGCCTAATGGATTCG-3'	E,F	This study

[a]Primers were combined to obtain amplicons for all strains studied.
[b]For the different clades, see Fig. 4.1.

4.3 Results

4.3.1 Olfactory response

Olfactory response of *A. colemani* to the volatile emissions of the tested *Bacillus* strains varied significantly between strains (χ^2 =116.75, *df* = 39, *P* < 0.001; Fig. 4.1; Table A4.1, Appendix). Female parasitoids significantly preferred the mVOC blends of ten *Bacillus* strains over the blank medium. By contrast, for five strains, parasitoids significantly preferred the blank medium (Fig. 4.1; Table A4.1, Appendix). Strain ST12.14/237 (having almost 99% *rpoB* sequence identity with *B. velezensis* and *B. amyloliquefaciens*) yielded the highest PI-value (PI = 0.42; *P* = 0.005), while *B. flexus* DSM 1316 elicited a strong significantly repellent response in *A. colemani* (PI = -0.55; *P* < 0.001). The mVOC blends of the remaining 25 strains had no statistically significant effect on the olfactory response of *A. colemani* and were further regarded as "neutral" responses. However, in general, strains belonging to clades A, B, C and D yielded positive PI-values (exception: ST12.14/138, PI = -0.05), whereas strains from clade E and clade F elicited a negative PI-value, except for *B. muralis* LMG 20238 (PI = 0.25) (Fig. 4.1).

4.3.2 mVOC composition

In total, 159 compounds were detected in the headspace of the tested cell-free media, including aldehydes, alkanes, amines, aromatics, esters, ketones, organic acids, pyrazines and terpenoids (Fig. 4.2). On average, the investigated *Bacillus* strains produced the highest amounts of acetoin, benzaldehyde and 3-methyl-butanoic acid. While most compounds were produced by all strains tested, allyl acetate, ammonium acetate and 4-methyl-pentan-2-one were only produced by a small subset of the investigated strains (Fig. 4.2). NMDS ordination of the mVOC composition showed clear clustering of the strains (Fig. 4.3), which corresponded well to their phylogenetic position in the six bacterial clades defined (Fig. 4.1). The mVOC composition differed significantly between the bacterial clades (perMANOVA: pseudo-F_5 = 10.7, *P* < 0.001). In addition, the perMANOVA revealed that there was a statistically significant difference in the mVOC chemical composition correlating with the response of *A. colemani* (pseudo-F_2 = 10.3, *P* < 0.001). Furthermore, the interaction between the independent variables olfactory response and bacterial clade was also significant (pseudo-F_2 = 4.06, *P* = 0.003), indicating that the response of *A. colemani* to *Bacillus* mVOCs depends on the bacterial clade.

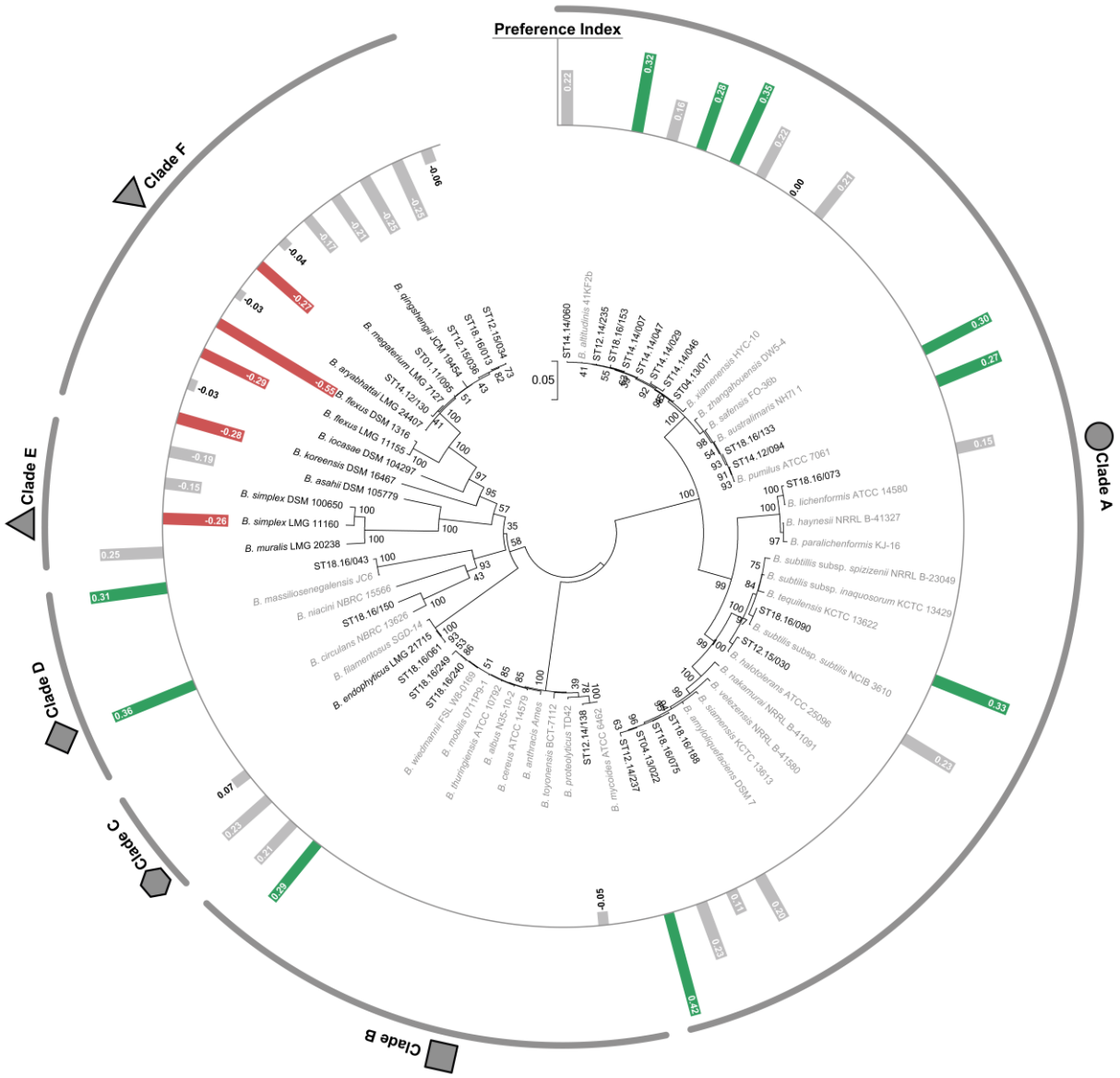

Figure 4.1: Maximum likelihood phylogenetic tree based on a concatemer of 16S rRNA gene and *rpoB* sequences of all 40 *Bacillus* strains investigated in this study (black). Additionally, the most closely related type strains (light grey) were added as a reference. Based on a sequence identity cut-off of 85%, six major clades can be distinguished. Bars depict the Preference Index (PI) for *Aphidius colemani* females when having the choice between the bacterial mVOCs and the blank medium. PI-values were calculated 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. Bar colours indicate the effect of the mVOCs on the olfactory response of *A. colemani*, i.e. green = significantly attractive ($P \leq 0.05$), grey = neutral ($P > 0.05$) , and red = significantly repellent ($P \leq 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). Overall parasitoid responsiveness was higher than 78%.

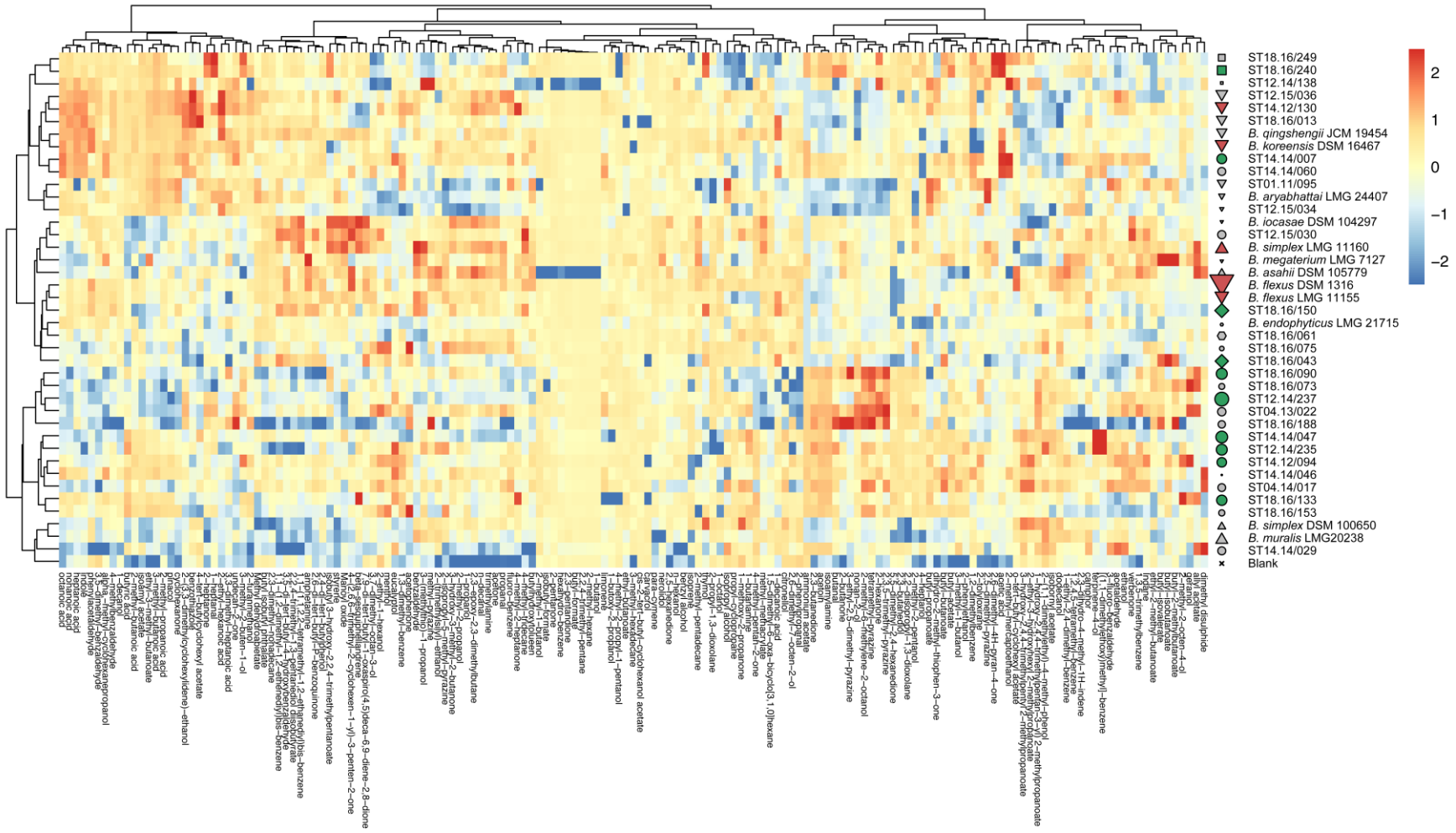

Figure 4.2: Heat map of the mVOC composition for the 40 *Bacillus* strains investigated in this study. Data are presented in the form of mean centred, log transformed average peak areas of compounds of three biological replicates (n = 3). mVOCs and *Bacillus* strain treatments were clustered by using Manhattan distances and a Ward.D2 clustering algorithm. Symbol shapes indicate the clade to which each of the strains belongs (see also Fig. 4.1): circle = clade A, square = clade B, hexagon = clade C, diamond = clade D, upward facing triangle = clade E, and downward facing triangle = clade F. Symbol colours indicate the effect of the mVOCs on the olfactory response of *Aphidius colemani*, green = significantly attractive ($P \leq 0.05$), grey = neutral ($P > 0.05$) , and red = significantly repellent ($P \leq 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). Symbol sizes are proportional to the absolute values of the Preference Index (PI) as determined in the olfactometer bioassay.

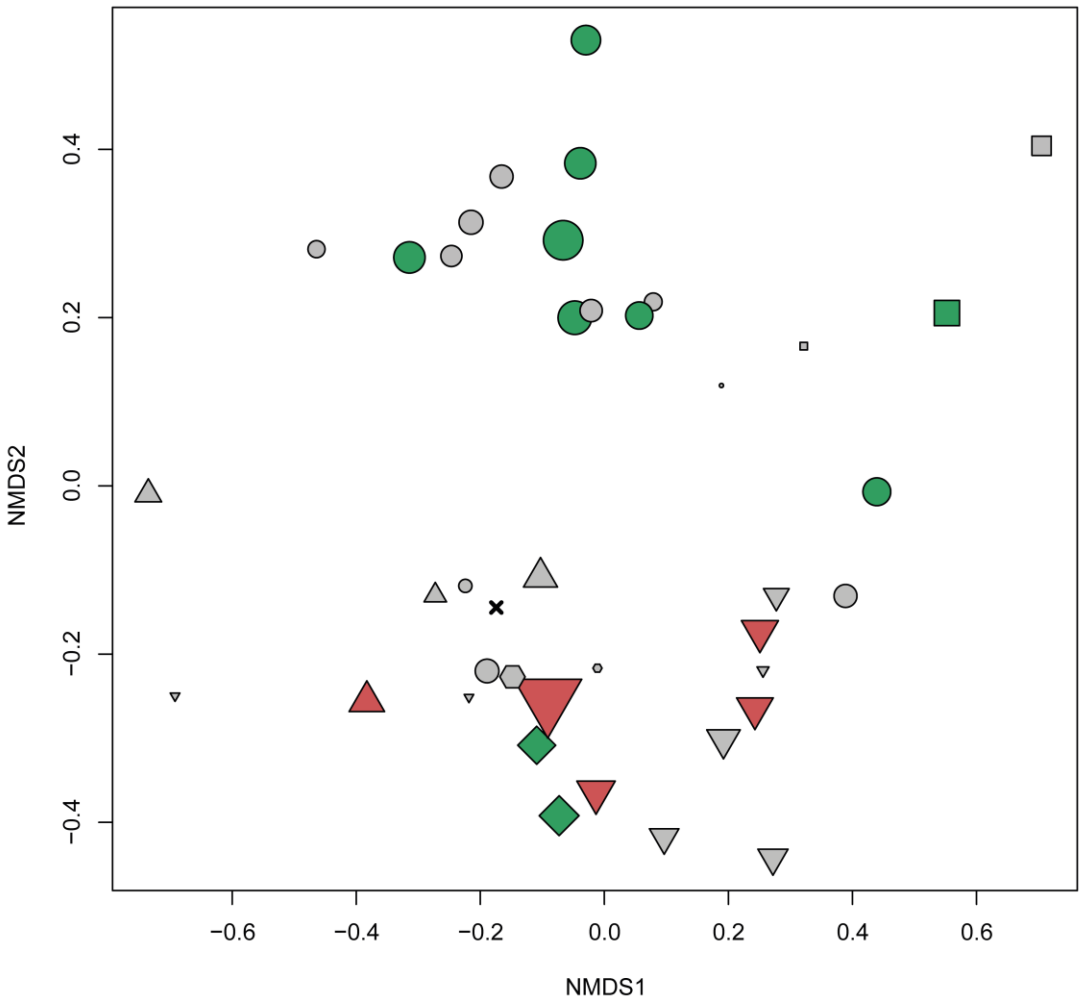

Figure 4.3: Non-metric multidimensional scaling (NMDS) ordination plot based on Bray-Curtis dissimilarities of the mVOC composition of the 40 *Bacillus* strains investigated in this study (n = 3) (stress value = 0.159). Symbol shapes indicate the clade to which each of the strains belongs (see also Fig. 4.1): circle = clade A, square = clade B, hexagon = clade C, diamond = clade D, upward facing triangle = clade E, and downward facing triangle = clade F. Symbol colours indicate the effect of the mVOCs on the olfactory response of *Aphidius colemani*, i.e. green = significantly attractive ($P \leq 0.05$), grey = neutral ($P > 0.05$) , and red = significantly repellent ($P \leq 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). Symbol sizes are proportional to the absolute values of the Preference Index (PI) as determined in the Y-tube olfactometer bioassay. The black cross refers to the blank medium. mVOC composition differed significantly between the bacterial clades defined (perMANOVA: pseudo-F_5 = 10.7, $P < 0.001$).

4.3.3 Phylogenetic signal analysis

In the pPCA of the 159 mVOCs detected, the global structure (PC1) was determined by a large number of compounds (Fig. 4.4). The most important compounds driving the global structure were acetoin, 2,3-butanedione, isoamylamine, nonan-2-ol, tetrametyl-pyrazine and 2,3-butanediol (highest positive loadings), and methyl-methacrylate, 2-ethyl-hexanoic acid, 2-methyl-propanoic acid, cyclohexanone and phenylacetaldehyde (highest negative loadings) (Fig. 4.4C; Table A4.3, Appendix). Strains from clade A showed the

highest scores of PC1, meaning that they produced the highest amounts of the compounds with highest positive loadings such as acetoin and 2,3-butanedione (Fig. 4.4A). Especially, strains related to *B. amyloliquefaciens* / *B. velezensis* produced the highest amounts of these compounds. Conversely, strains from clade E and F were found to have the highest negative scores for PC1. This clustering of positive and negative scores of PC1 into the different clades suggests a clear phylogenetic pattern in the mVOC emissions of *Bacillus*. Indeed, the univariate analysis of phylogenetic signal on PC1 was significant for all three indices as well as for the Mantel test (Table 4.3). This was further confirmed by the multivariate Mantel test on the complete mVOC dataset, which showed a significant correlation between phylogenetic distances and relative amounts of mVOCs produced by the *Bacillus* strains (Mantel $Z = 6.29\times10^{10}$, $P < 0.001$) (Table 4.3). Further, there was also a phylogenetic signal in the olfactory response of *A. colemani* to the volatile emissions of *Bacillus* strains (Table 4.3). When focusing on the individual compounds, it becomes clear that the phylogenetic position of the *Bacillus* strains reflects the production of certain compounds. More specifically, significant phylogenetic signal was found for 30.2%, 27.7%, 26.4% and 26.4% of the compounds detected for Pagels's λ, Moran's I, Abouheif's C_{mean}, and the Mantel Test, respectively (Table 1; Table A4.2, Appendix). In total, 15.7% of the mVOCs detected (25 compounds) were statistically significant according to all three indices and the Mantel test (Fig. 4.5).

Table 4.3: Overview of results of the phylogenetic signal tests on the behavioural response *of Aphidius colemani* and the mVOC profiles[a]

Variable	Index values	Phylogenetic signal index			
		Pagel's λ	Moran's I	Abouheif's C_{mean}	Mantel test
Behavioural response (40)		**0.775*****	**0.952*****	**0.603*****	**97.4*****
Chemical composition mVOCs (159)	Range	4.44×10^{-5} - 1.00	-0.21 - 0.85	-0.19 - 0.86	5.54 - 29.0
	Mean	0.27	0.17	0.14	20.73
	Significant compounds	**48 (30.2%)**	**44 (27.7%)**	**42 (26.4%)**	**42 (26.4%)**
	PC1	**0.61*****	**0.72*****	**0.72*****	**137*****
	PC39	0.00	-0.28	-0.30	91.2
	Mantel test		$\boldsymbol{Z = 6.29\times10^{10}}$	$\boldsymbol{P < 0.001}$	

[a]Pagel's λ, Moran's I, Abouheif's C_{mean}, were calculated and a Mantel test was performed using the preference index data (behavioural response of A. colemani) and mean centered, log transformed peak area data of every mVOC produced by the *Bacillus* strains (chemical composition mVOCs), in combination with a phylogenetic tree based on a concatemer of 16S rRNA gene and *rpoB* sequences. The same tests were performed on the eigenvectors (PC1 and PC39) resulting from the pPCA. Finally, a Mantel test was used to analyse the complete dataset of all mVOC produced by the *Bacillus* strains. Values in bold indicate a significant phylogenetic signal (***, P < 0.001).

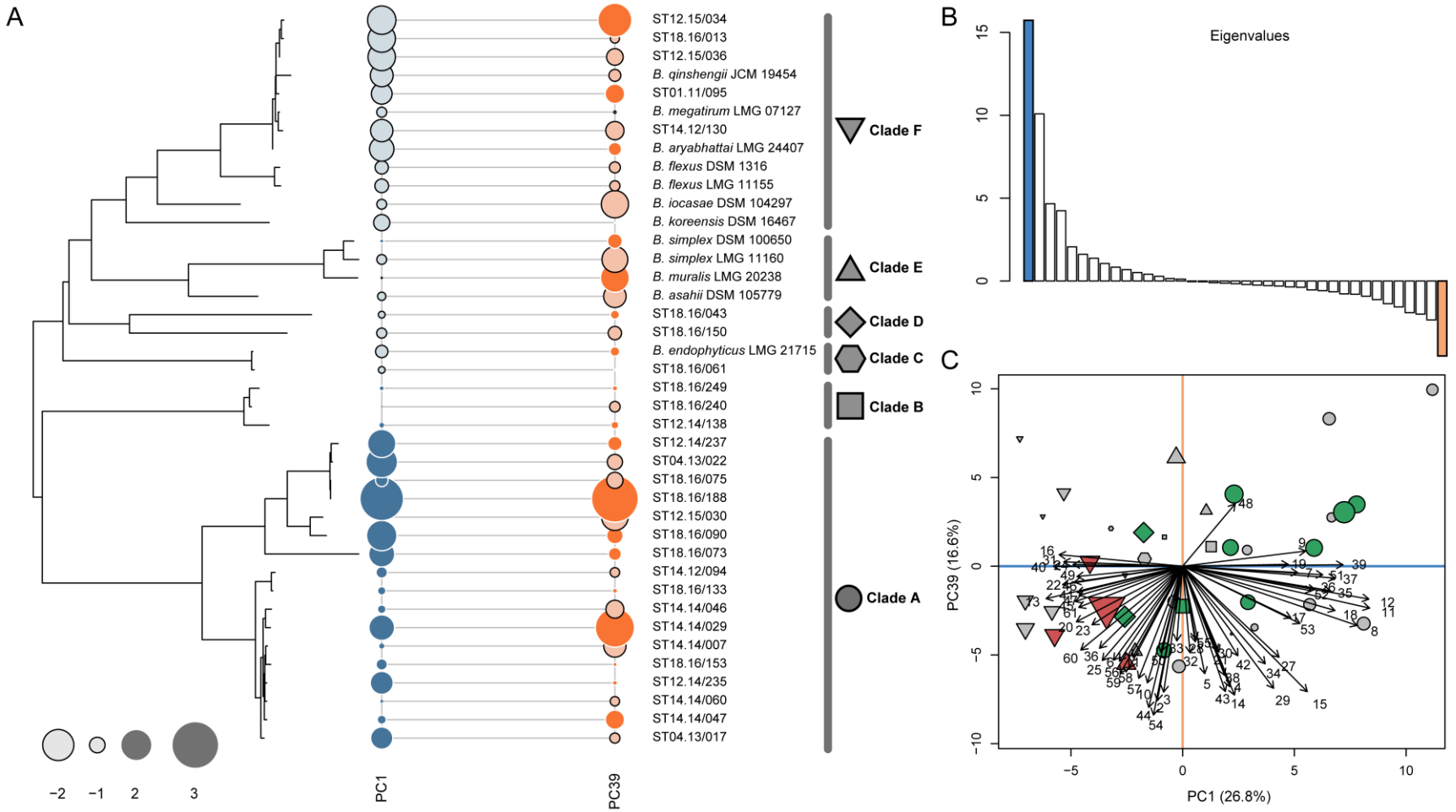

Figure 4.4: (A) Maximum likelihood phylogenetic tree based on a concatemer of 16S rRNA gene and *rpoB* sequences of all 40 *Bacillus* strains investigated in this study and results of the phylogenetic principal component analysis (pPCA) on the mVOCs produced by the strains. The phylogenetic tree was divided into six major clades based on a 85% sequence identity cut-off. Positive and negative scores on PC1 (global structure) and PC39 (local structure) are indicated by blue and orange circles, respectively. Circle size is proportional to the absolute score values. **(B)** Bar chart of eigenvalues of all PCs with PC1 (blue) and PC39 (orange). **(C)** pPCA biplot containing loadings of the mVOCs for global (blue axis) and local (orange axis) PCs. Only mVOCs with highest loadings (absolute value >0.1) on the PCs are shown. Symbol colours, shapes and sizes are as explained in Fig. 4.3. Vector numbers refer to the different mVOCs (see Table A4.3,Appendix).

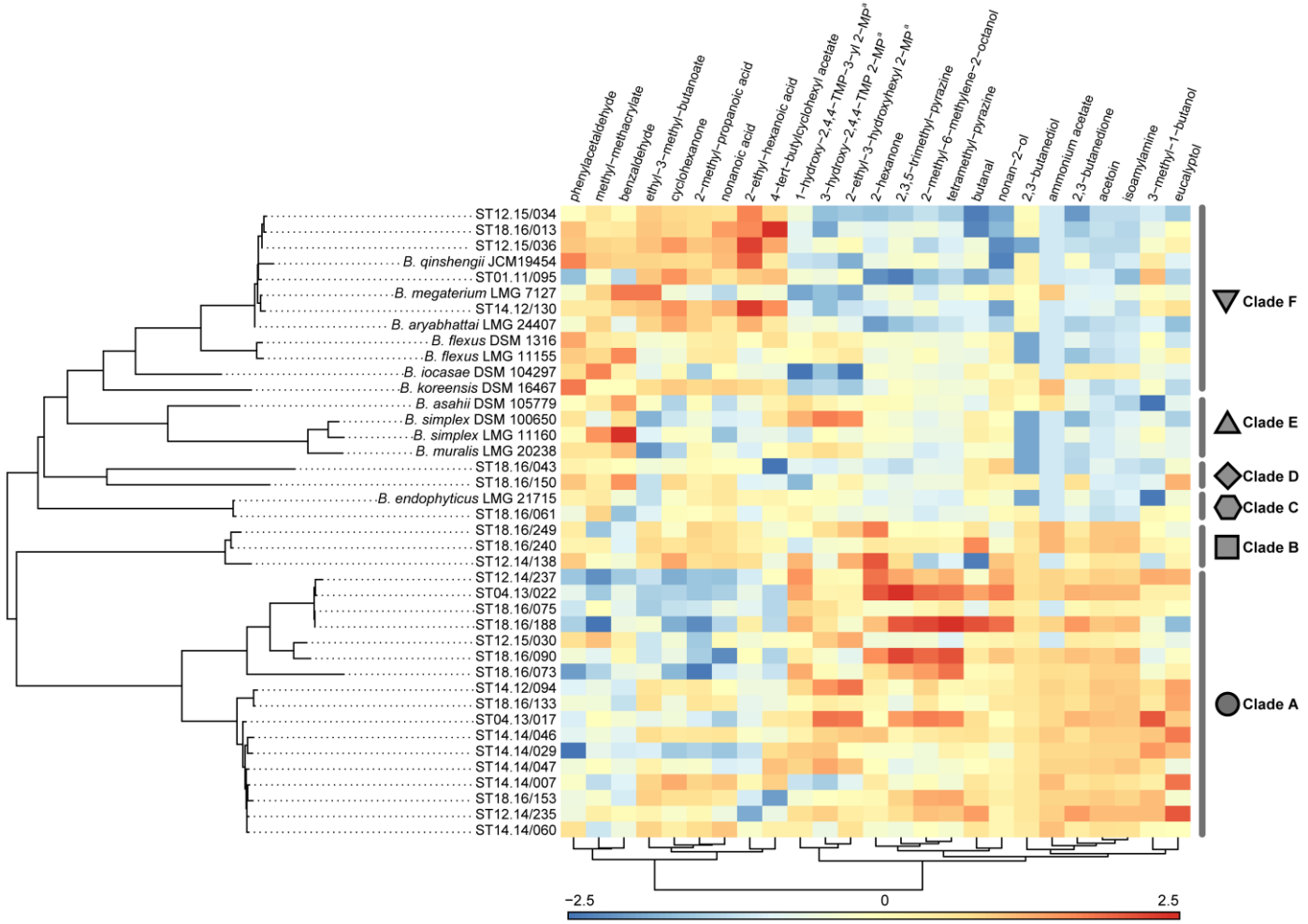

Figure 4.5: Phylogenetic heatmap using the maximum likelihood phylogenetic tree based on a concatemer of 16S rRNA gene and *rpoB* sequences of all 40 *Bacillus* strains used in this study. The phylogenetic tree was divided into six major clades based on a 85% sequence identity cut-off. The heatmap depicts 25 mVOCs that show a significant phylogenetic signal for all three indices calculated (Pagels's λ, Moran's I and Abouheif's C_{mean}) and the Mantel test. Data are presented in the form of mean centred, log transformed average peak areas of compounds (n = 3). mVOC compositions were clustered by using Manhattan distances and a Ward.D2 clustering algorithm. [a]TMP= trimethylpentan; MP = methylpropanoate.

4.4 Discussion

In this study, we have shown that phylogenetically related *Bacillus* strains tended to emit similar blends of mVOCs and elicited a comparable olfactory response in *A. colemani*, confirming earlier findings that phenotypic diversity associated with evolutionary history contributes to the interactions between different taxa. Furthermore, we identified the key mVOCs that significantly explained the phylogenetic signal.

4.4.1 Phylogenetic conservatism of mVOC profiles

In total, 15.7% of the detected mVOCs showed a significant phylogenetic signal for all statistical tests performed (i.e. Pagel's λ, Moran's I, Abouheif's C_{mean} and Mantel test). Among these, acetoin, 2,3-butanediol and 2,3-butanedione were among the most important drivers of the global structure in the pPCA. These compounds represent typical fermentation products, and are well known to be produced by *Bacillus* species (Asari, et al., 2016; Dettwiler et al., 1993). These compounds were particularly produced by a phylogenetically related subset of *Bacillus* strains, i.e. strains from clade A and B, whereas they were absent or only produced in low amounts by strains belonging to clade C to clade F. Our results further showed that several mVOCs significantly contributed to the local structure in the pPCA due to production of different relative amounts by closely related strains, which cannot be explained by the phylogeny. Instead, such patterns could arise from evolutionary events, such as gene loss, horizontal gene transfer and interaction with the environment, leading to random associations between phylogenetic and functional relatedness (Goberna & Verdú, 2016).

4.4.2 Insect response

Phylogenetic relationships of the investigated *Bacillus* strains predicted the olfactory response of *A. colemani*. While strains belonging to clade A, B, C and D produced attractive mVOC blends, strains belonging to clade E and F produced blends that were repellent to *A. colemani*. Closer inspection of the chemical composition of these mVOC blends revealed that all *Bacillus* strains produced the same set of volatiles, but often in significantly different concentrations and ratios. This suggests that mVOCs elicit a different response in the insects depending on the presence of background mVOCs and the mVOC

concentrations (Bruce et al., 2005; Mumm & Hilker, 2005). Benzaldehyde was produced in relatively high concentrations by strains that repel *A. colemani*, and in lower amounts by strains that elicit attractive behaviour, and is therefore an important candidate to explain the differences observed for both groups of bacteria. Previous research has shown that benzaldehyde can induce both electrophysiological and behavioural responses in several insects (James, 2005), including *Aphidius* and other parasitoids and aphid natural enemies (Han & Chen, 2002; Simpson et al., 2011a). A similar, albeit less pronounced, phylogenetic pattern was observed for phenylacetaldehyde, which has also been shown to affect insect behaviour (Bruce & Pickett, 2011; Dötterl et al., 2006). Attraction does not necessarily have to be caused by the most abundant compounds as has been shown for plant volatiles, where often minor constituents of volatile blends affect insect behaviour (McCormick et al., 2014). It has been shown that volatile concentration is a very important factor in affecting the behavioural response of insects, since the same compounds can evoke a different response depending on the concentration (Gadino et al., 2012). Therefore, concentrations of mVOCs produced by repellent strains can be too high and cause repellence or even mask otherwise attractive compounds (Aartsma et al., 2017). Moreover, certain compounds can induce or inhibit the response to other compounds (Turner & Ray, 2009). Conversely, attractive mVOC profiles, in particular the profiles emitted by strains belonging to clade A and B, contained relatively higher amounts of acetoin, 2,3-butanediol, 2,3-butanedione, eucalyptol and isoamylamine when compared to repellent mVOC profiles. All these volatile compounds have been shown to affect insect behaviour or even elicit insect attraction (Bengtsson et al., 2009; Kuhns et al., 2014; Rebora et al., 2017; Turner et al., 2011). Nevertheless, although our data suggest that these compounds may have contributed to the observed insect behaviour, additional experiments using GC-EAG and pure compounds are required to confirm the effects of these mVOCs on parasitoid olfactory response.

4.4.3 Parasitoids responding to ubiquitous bacteria

It is clear from our results that *A. colemani* response is strongly correlated with *Bacillus* phylogeny, suggesting that *A. colemani* also responds to bacteria which likely never encounter the parasitoid naturally. The question then rises why ubiquitous *Bacillus* species produce volatiles that affect parasitoids and why *A. colemani* responds to them.

Most mVOCs are considered as side-products of primary and secondary metabolism, and are formed mainly by oxidation of glucose from various intermediates (Korpi et al., 2009). The fact that they can act as insect semiochemicals could be a merely coincidental interaction as *Bacillus* species are known to produce volatiles that are typically associated with plants (e.g. eucalyptol, geraniol, limonene and phenylacetaldehyde) and insects (e.g. acetoin, 2,3-butanediol, nonan-2-ol) (Bruce & Pickett, 2011; Knudsen et al., 1993; Löfsteft et al., 2008) and thereby accidentally mimic host plant, food or insect host associated cues. This overlap between plant and microbial volatiles has recently also been observed in yeasts, which emit insect attracting volatiles typically associated with flowers (Ljunggren et al., 2019). However, there could also be a deeper ecological association between bacteria and insects, as is, for example, the case for yeasts. Collective evidence suggests that yeast volatiles mediate mutualistic interactions between yeasts and insects, in which the insects exploit the mVOCs as semiochemicals to detect suitable oviposition sites and food sources and even use them to discriminate between sources which best support their growth and survival (Scheidler et al. 2015, Becher et al. 2018, Madden et al. 2018, Rering et al. 2018). In turn, the yeasts may benefit from the insects as vectors to disperse to new habitats (Christiaens et al. 2014) or to survive unfavourable conditions (Pozo et al. 2018). However, further research is needed to investigate these scenarios for bacteria. Although microbial emissions may signal a number of advantages for insects, responding to ubiquitous microbes like *Bacillus* spp. may also pose potential caveats for parasitoids. Optimal foraging assumes that insects only respond to signals from which they benefit. Future experiments should therefore also be performed to investigate to what extent responding to semiochemicals from widespread microorganisms may pose potential trade-offs in parasitoid foraging success.

Not all *Bacillus* strains attracted *A. colemani*, and several strains were even repellent to the parasitoids. In this case, the adaptive value for both organisms is more difficult to explain. Nevertheless, it is known that, in some cases, insects can compete with microorganisms for the same resources (Burkepile et al., 2006; Holighaus & Rohlfs, 2016). For example, nectar-inhabiting microorganisms compete with insects for nectar resources, and may thereby negatively impact insect fitness (Lenaerts et al., 2017). Therefore, repelling flower visitors by the emission of mVOCs would ensure resource provision for the microorganisms, while the flower visitors can avoid less suitable nectars.

This hypothesis is in line with earlier observations that honey bees and bumble bees avoid nectar inoculated with specific bacteria (Good et al., 2014; Junker et al., 2014).

4.4.4 Conclusion

In conclusion, we have shown that the phylogeny of *Bacillus* species predicted both mVOC composition and the olfactory response of an aphid parasitoid, *A. colemani*. A specific subset of mVOCs was the main driver for the phylogenetic signal in *Bacillus*, which are possible candidates facilitating olfactory response in *A. colemani*, as these volatiles describe the difference between attractive and repellent clades. Nevertheless, although our data suggest that these compounds may have contributed to the observed insect responses, additional experiments, e.g. using gas chromatography-electroantennography (GC-EAG) and pure compounds, are required to confirm the effects of these mVOCs on parasitoid olfactory response, which is the main focus in **Chapter 5**. Finally, our results suggest that these volatiles could be part of the chemical communication driving the interactions between bacteria and insects.

Chapter 5

Identification and implementation of bacterial semiochemicals that attract a generalist aphid parasitoid under laboratory and greenhouse conditions

5.1 Introduction

Volatile organic compounds (VOCs) play a pivotal role in the communication between plants and insects (Bruce & Pickett, 2011; Cha et al., 2011; de Bruyne & Baker, 2008). Although most research studying the effects of volatiles on foraging behaviour of arthropod natural enemies has mainly focussed on plant- and insect-derived volatiles (Kaplan, 2012; Meiners & Peri, 2013), recent studies have demonstrated that microbial volatile organic compounds (mVOCs) released by microorganisms associated with the natural enemy or its resources are also able to affect the olfactory response of natural enemies (**Chapter 3;** Goelen et al. 2020; **Chapter 4**; Sohby et al. 2017, 2018). Despite an increased understanding of the role of microbial volatile emissions as insect semiochemicals (Beck & Vannette, 2016; Davis et al., 2013; Dzialo et al., 2017; Leroy et al., 2011b), so far only little is known about which microbial volatiles or combination of microbial volatiles is responsible for the insects' response. Furthermore, most studies, including the experiments performed in **Chapter 3** and **Chapter 4,** are typically performed under controlled conditions using laboratory bioassays in which the insects are exposed to only one odour source. However, in their natural environment, insects encounter numerous olfactory cues from many different sources and in different concentrations, from which they need to derive reliable information for accurate foraging decisions (Aartsma et al., 2017). Nevertheless, so far it is largely unknown how insects perceive and respond to volatile cues in more natural settings and how this affects their foraging behaviour (Aartsma et al., 2017; Morawo & Fadamiro, 2016; Simpson et al., 2011c). Such knowledge, however, is essential to fully grasp the potential of microbial volatiles to develop new semiochemical-based strategies to improve biological pest control efficacy.

Many natural enemy species depend on olfactory cues to locate resources such as food, mates and oviposition sites (de Bruyne & Baker, 2008). To this end, the insects typically exploit resource indicating volatiles associated with their host or prey (Bruce et al., 2005), their host plants and foraging habitat (Webster & Cardé, 2017), or the interaction of their host or prey with the infested plant (Dicke & Baldwin, 2010). These olfactory cues often consist of ubiquitous volatile blends with a characteristic volatile composition or, in some cases, of individual volatile compounds that are restricted to a narrow set of resources (Bruce et al., 2005; Bruce & Pickett, 2011). Besides the nature of each compound in the blend, the concentration and ratio of each compound can also affect

the response of foraging insects (Bruce & Pickett, 2011). Moreover, the interaction between volatiles in a blend can alter the perception of the blend (Ache et al., 1988; Schröder & Hilker, 2008; Liu et al., 2019). This implies that individual compounds can have a completely different effect and elicit different behavioural responses compared to blends of these compounds (Webster et al., 2010a). The response of insects to these volatiles can either be the sum of their responses to the single compounds or depend entirely on the mixture of volatiles inside the blend (Morawo & Fadamiro, 2014; Shiojiri et al. 2010; Takemoto & Takabayashi, 2015; Tasin et al., 2007; Webster et al., 2010a). Furthermore, there are examples demonstrating that simplified blends of synthetic volatiles, representing only a limited set of volatiles from the natural blend, can be as attractive as complex, natural volatile blends. This suggests that, despite the rich plethora of volatiles that is generally available from natural resources, only a select number of compounds evokes an olfactory response in the insects (Borrero-Echeverry et al., 2015; Frank et al., 2018; Morawo & Fadamiro, 2016).

In this study, we aimed to identify specific compounds in microbial volatile blends that had a significant impact on parasitoid olfactory response. Further, we aimed to develop a mixture of synthetic compounds by which parasitoids can be attracted. Experiments were performed under both laboratory and greenhouse conditions. Similar to our previous experiments (**Chapter 3** and **Chapter 4**), we used the generalist aphid parasitoid *Aphidius colemani* (Hymenoptera: Braconidae) as our study organism. First, we used coupled gas chromatography-electroantennography (GC-EAG) to determine which mVOCs in the cell-free cultivation medium of the seven bacterial strains investigated in **Chapter 3** elicited an electrophysiological response in *A. colemani*. Subsequently, five EAG-active compounds identified in the mVOC blend of an attractive strain were selected, and tested individually and in blends for their effect on parasitoid olfactory response in a Y-tube olfactometer bioassay. Finally, two-choice cage experiments in which part of the plants were treated with the most promising synthetic blend were performed to assess its attractive potential under greenhouse conditions. As a comparison, the cell-free cultivation medium of the attractive strain was included.

5.2 Materials and methods

5.2.1 Study organisms

Insects

Experiments were performed with adult females of the primary parasitoid *A. colemani*, which is a generalist endoparasitoid of several aphid species of economic importance (van Lenteren, 2012). *Aphidius colemani* was obtained in the form of parasitized aphid mummies from Biobest (Westerlo, Belgium) (Aphidius-system®). 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 behavioural experiments were performed with <24h-old, food and water-starved females.

Bacterial strains

To assess which bacteria-produced mVOCs that elicit a electrophysiological response in *A. colemani*, a selection of seven bacterial strains was used (Table 5.1). The effect of the mVOC blends emitted by the strains on the olfactory response of *A. colemani* was studied in **Chapter 3** using a Y-tube bioassay. Specifically, studied strains included three strains evoking attraction, three strains evoking repellence, and one strain evoking a neutral response (Table 5.1). Experiments were performed using the cell-free cultivation media (n = 3 (three biological replicates)) prepared in **Chapter 3** (stored at – 20°C until use).

Table 5.1: Bacterial strains used in this study.

Isolate identifier (GenBank Accession N°[a])	Olfactory response of *A. colemani*[b]	Phylogenetic affiliation based on 16S rRNA gene sequence similarity[c]			
		Phylum	Family	Closest match in EZBiocloud to identified species	Identity (%)[d]
ST18.16/133 (MK875116)	Attractive	Firmicutes	Bacillaceae	*Bacillus zhangzhouensis*	99.56
ST18.16/043 (MK875105)	Attractive	Firmicutes	Bacillaceae	*Bacillus massiliosenegalensis*	99.51
ST18.16/150 (MK875117)	Attractive	Firmicutes	Bacillaceae	*Bacillus circulans*	99.04
ST18.16/085 (MK875112)	Neutral	Actinobacteria	Micrococcaceae	*Curtobacterium* sp. (*C. flaccumfaciens, C. oceanosedimentum*)	99.66
ST18.17/002 (MK875125)	Repellent	Actinobacteria	Micrococcaceae	*Glutamicibacter halophytocola*	99.40
ST18.16/160 (MK875120)	Repellent	Firmicutes	Staphylococcaceae	*Staphylococcus saprophyticus*	99.50
ST18.17/028 (MK875130)	Repellent	Proteobacteria	Erwiniaceae	*Pantoea dispersa*	99.28

[a]Accession number of 16S rRNA gene fragements deposited in GenBank.
[b]Olfactory response of *A. colemani* parasitoids to the mVOCs produced by the strains in a Y-tube olfactometer bioassay as determined in **Chapter 3**.
[c]Based on 16S rRNA gene sequencing and identification using the EZBiocloud database (November 2018). Only closest matches to type strains are reported.
[d]Average fragment length was 1017 bp

5.2.2 mVOC collection

Headspace air entrainment was used to collect mVOCs from the bacterial cell-free cultivation media for use in subsequent electrophysiological and chemical analyses. Volatiles were collected for 1 h from 150 µL cell-free cultivation medium inside a 4 mL glass, screw top GC-vial (Thermo Scientific, Waltham, USA). In- and outlet ports were created by fitting Swagelock ports onto 19Gx2" syringe needles (Agani™, Terumo®, Leuven, Belgium) which were pierced through the 12 mm polytetrafluorethylene (PTFE)/silicone septum (Supelco, Bellefonte, USA) of the GC-vial. Activated charcoal filtered air was supplied through the inlet port at a rate of 400 mL/min. Air subsequently passed over the cell free-medium inside the GC vial and headspace volatiles were adsorbed on Porapak Q (0.05 g, 50/80 mesh; Supelco, Bellefonte, USA) filters that were fitted on the outlet port through which air was drawn at a rate of 300 mL/min. This created slight overpressure inside the vial to prevent outside odours from entering the enclosed headspace. Prior to entrainment, Porapak Q filters were washed with diethyl ether and conditioned by heating to 132°C in an activated charcoal filtered nitrogen stream for 2 h. Air entrainment of 150 µL of blank GYP25 medium was included as a control. All connections in the air entrainment set-up were made using PTFE tubing (Fig. 5.1). Entrained volatiles were eluted in 750 µL diethyl ether and were stored in 1.1 mL

glass microvials at -20°C until further use. Before analysis, air entrainment samples were concentrated to 50 µL under an activated charcoal filtered nitrogen stream. Entrainments were performed for all biological replicates (n = 3) of each of the seven strains.

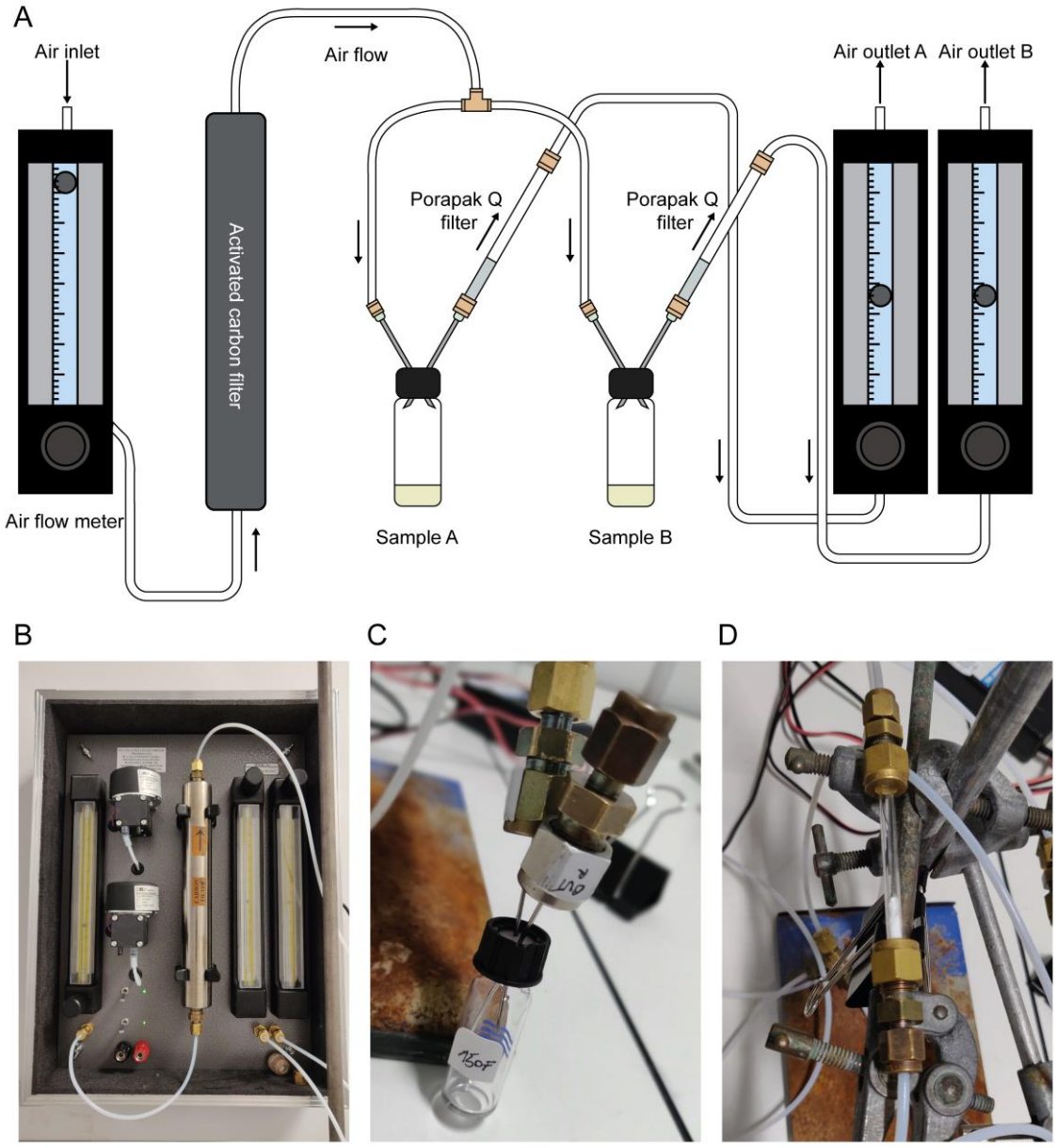

Figure 5.1: Air entrainment set-up used in the mVOC collection. **(A)** Schematic diagram of the air entrainment set-up used. A flow meter controls the air intake generated by an electric air pump to 800 mL/min after which it passes through an activated charcoal filter to remove impurities from the inlet air stream. Hereafter, the air stream is split in two separated streams of 400 mL/min which allows the set-up to trap the volatiles of two samples simultaneously. The charcoal filtered air passes through a 4 mL glass GC vial containing 150 µL of a sample, after which it passes through the outlet port over a Porapak Q filter to collect the mVOCs present in the vial headspace. The outlet airstream is controlled by two separate flowmeters set at 300 mL/min. All connections in the set-up were made using PTFE tubing. **(B)** Photograph of the air entrainment kit used which consisted of two electric air pumps generating the in- and outlet air streams, three flow meters controlling the in- and outlet air streams and an activated charcoal filter. **(C)** Close-up of a 4 mL glass GC vial which contained 150 µL of a sample during the headspace air entrainment. **(D)** Close-up of a Porapak Q filter collecting the mVOCs during the headspace air entrainment.

5.2.3 Gas Chromatography (GC)

Air entrainment samples were analysed using a 6890N GC machine (Agilent Technologies, Santa Clara, USA) equipped with a cold on-column injection system, and both a non-polar HP-1 capillary column (50 m; 0.32 mm internal diameter; 0.52 µm film thickness) and a polar DB-wax column (30m; 0.32 internal diameter; 0.50 µm film thickness). Separated compounds were detected by a flame ionisation detector (FID). The carrier gas was hydrogen. One µL of air entrainment sample was dual-injected into both columns of the GC machine. The oven temperature was initiated at 30°C and was maintained for 0.1 min, then raised to 150°C at a rate of 5°C/min, held for 0.1 min, then raised again at 10°C/min to 230°C and held for 25 min. Data were analysed using HP Chemstation software.

5.2.4 Coupled Gas Chromatography-Electroantennography (GC-EAG)

A GC-EAG was performed to determine which individual mVOCs present in extracts of the cell-free cultivation medium headspace elicited an electrophysiological response in female *A. colemani* antennas. GC-EAG analyses were performed with the air entrainment samples of one representative biological replicate of each of the seven bacterial strains and with antennal preparations of 1-2-day-old female *A. colemani* parasitoids (Fig. 5.2). This analysis was replicated three times in the GC-EAG. A new antennal preparation from a different insect was used in each replicate.

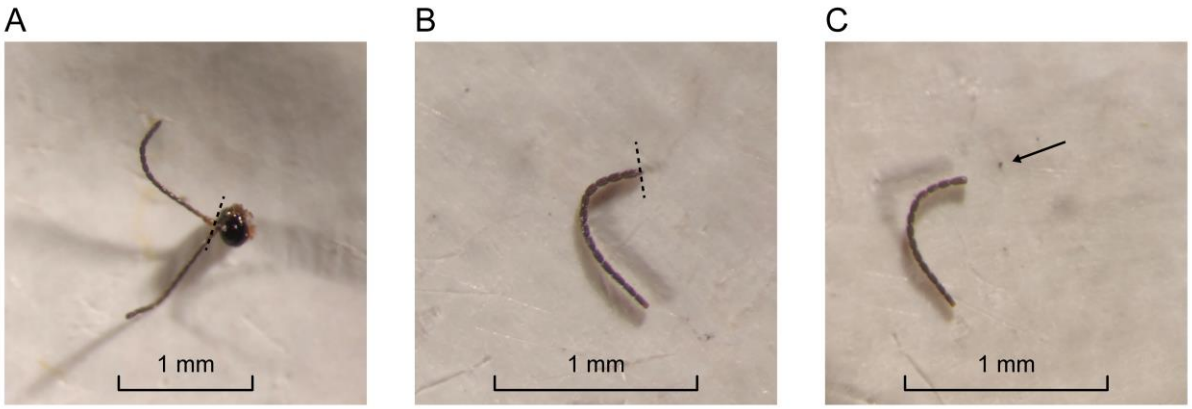

Figure 5.2: Preparation of an antenna of a female *Aphidius colemani* for coupled gas chromatography-electroantennography (GC-EAG) analysis. **(A)** Excised head of a female *A. colemani*. The antenna was removed by cutting the antenna with a scalpel as close to the head as possible (indicated by dashed line). **(B)** To ensure good contact with the recording electrode in the GC-EAG set-up, the tip of the last antennal segment was removed by cutting along the dashed line with a fine scalpel. **(C)** Excised antenna with removed tip (indicated by the arrow) (Photo credits: Tim Goelen).

The GC-EAG system (Fig. 5.3), in which the separated compounds from the GC column are simultaneously directed to the antennal preparation and the flame ionization detector (FID) inside the GC, was described previously by Wadhams (1990). The GC-EAG system was equipped with a 6890N GC machine (Agilent Technologies, Santa Clara, USA) fitted with a cold on-column injection system, and a non-polar HP-1 capillary column (50 m; 0.32 mm internal diameter; 0.52 µm film thickness), using an FID. The carrier gas was helium. The oven temperature was initiated at 30°C and was maintained there for 2 min and then raised to 250°C at a rate of 5°C/min. The GC column effluent was split equally (1:1) between the FID detector and the heated transfer line which delivered the separated compounds onto the antennal preparations through an activated charcoal filtered, humidified air stream (Fig. 5.3). Antennal preparations were created by first anesthetising the female parasitoid by placing it on ice for 1 min, excising the head, removing one complete antenna, and by finally removing the tip of the last antennal segment to ensure good contact with the recording electrode (Fig. 5.2). The excised antenna was brought into contact with the Ag-AgCl ground electrode by inserting the antennal base into a glass capillary housing the electrode and filled with saline solution (composition as in Maddrell (1969), but without the glucose). The distal-end was brought into contact with the recording electrode in a similar way. Detected signals, voltage differences over the antenna ends, were amplified by a high impedance amplifier (UN-06; Ockenfels Syntech GmbH, Kirchzarten, Germany) and analysed using customized Syntech software. Outputs from the FID and the EAG amplifier were analysed simultaneously with the custom software. Only volatiles with a consistent electrophysiological response peak in all three replicates were considered as EAG-active.

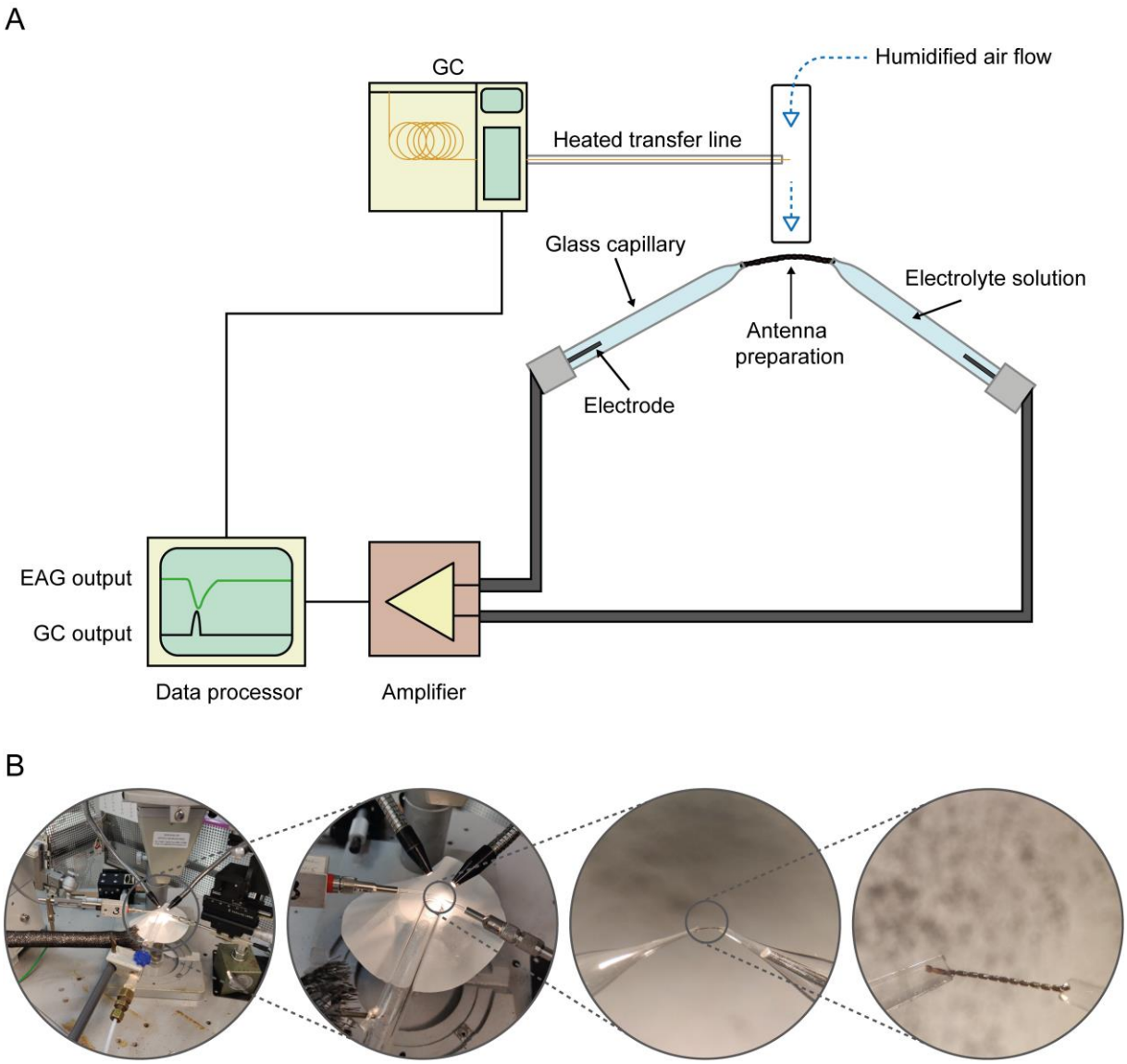

Figure 5.3: Coupled gas chromatography-electroantennography (GC-EAG) set-up. **(A)** Schematic diagram of the GC-EAG set-up used to determine which mVOCs elicited a physiological response in the antenna of *A. colemani*. A GC machine separates the injected mVOC blend in individual peaks. The GC is equipped with a capillary column in which the effluent is split equally (1:1) between the flame ionization detector (FID) and a heated transfer line, which allows the separated compounds to be simultaneously detected by the FID and to be transferred to the parasitoid antenna preparation. Activated charcoal filtered, humidified air carries the separated compounds from the heated transfer line onto the antenna preparation. When a compound elicits a physiological response in the antenna, a voltage difference occurs across the Ag-AgCl glass electrodes which is amplified and subsequently detected by the data processor that simultaneously plots the GC-chromatogram and the EAG response (schematic is not to scale). **(B)** Photographs at different positions of the GC-EAG set-up and antenna preparation (Photo credits: Tim Goelen).

5.2.5 Coupled Gas Chromatography-Mass Spectrometry (GC-MS)

Peaks associated with electrophysiological activity in *A. colemani* were tentatively identified by coupled GC-MS using 4 µL of air entrainment samples on a Waters Autospec Ultima mass spectrometer (VG Autospec; Fisons Instruments, Manchester, UK), coupled to an Agilent 6890 GC (Agilent Technologies, Santa Clara, USA; cold on-column injector, 50 m × 0.32 mm internal diam, 0.52 µm film thickness HP-1- column). Ionization was performed by electron impact at 70 eV and 250 °C. The GC oven temperature was initiated at 30°C and maintained for 5 min and then raised to 250°C at 5°C/min. Helium was the carrier gas. Peak identities were determined by manually comparing mass spectra with those collected in mass spectral databases using NIST MS Search v2.0g software with the NIST 2011 library.

5.2.6 Y-tube olfactometer bioassay

To determine the olfactory response of *A. colemani* to EAG-active volatiles and blends thereof, a Y-tube olfactometer bioassay was performed as described in **Chapter 3**, using (mixtures of) synthetic volatile compounds instead of cell-free cultivation media. In total, five synthetic volatile compounds were included in the experiment, each of which elicited a physiological response in *A. colemani* in the GC-EAG. Tested compounds were identified in an mVOC blend attractive to *A. colemani* which showed the highest number of EAG-active compounds that could be identified, i.e. *Bacillus* strain ST18.16/133. Tested compounds included benzaldehyde (≥99.5%); butyl acetate (99.7%); 1,3-diacetyl benzene (97.0%); styrene (≥99.0%) (all purchased from Sigma-Aldrich, Saint Louis, USA) and 1,2-dimethyl benzene (*o*-xylene) (≥99.0% Fluka, Bucharest, Romania). Compounds were dissolved in diethyl ether prior to loading 10 µL of the mixture on a 37 mm-diameter filter paper (Macherey-Nagel, Düren, Germany) which was left for 30 sec to allow the diethyl ether to evaporate. Subsequently, the filter paper was placed in one of the odour chambers of the olfactometer set-up, whereas in the second chamber another filter paper was placed on which 10 µL diethyl ether was added as a control. As described before, experiments were conducted with 60 female individuals, which were released in twelve cohorts of five individuals, and olfactory response was evaluated 10 min after their release. Parasitoids that did not make a choice within 10 min after release were

considered as non-responding individuals and were eliminated from the statistical analysis.

In a first experiment, the different test compounds were diluted in diethyl ether in nine different concentrations, resulting in nine different doses, i.e. 1, 10, 50 and 100 ng, and 1, 10, 50, 100 and 250 µg. Each dose was individually tested for each compound in the Y-tube olfactometer. In a second experiment, two distinct synthetic volatile blends were tested, which are further referred to as "Blend 1" and "Blend 2". Blend 1 consisted of two compounds to which *A. colemani* showed significant preference in the first experiment, i.e. benzaldehyde and styrene. More particularly, the blend was created by combining both compounds in their most attractive dose as determined in the first experiment (i.e. 10 ng for benzaldehyde and 1 µg for styrene), resulting in a total of 1.01 µg pure compounds (1.0% benzaldehyde and 99.0% styrene). In addition, four other doses of the blend were tested (ranging from 0.53 µg to 2.02 µg) to assess the dose-response range of this blend, resulting in a total of five doses tested (Table 5.2).

Table 5.2: Composition (µg) of the five different doses of Blend 1[a] tested in the Y-tube olfactometer bioassay.

Tested dose	Composition		
	Styrene	Benzaldehyde	Total
2x	2.000	0.020	2.020
1.5x	1.500	0.015	1.515
1x[b]	1.000	0.010	1.010
0.75x	0.750	0.008	0.758
0.5x	0.500	0.005	0.505
Relative amount	99.0%	1.0%	100%

[a]Blend 1 consisted of two volatile compounds which elicited a significant EAG-response in the GC-EAG analysis.
[b]The 1x dose contains the sum of doses of styrene and benzaldehyde at which they were most attractive individually in the Y-tube olfactometer bioassay. In all doses tested relative composition of the compounds was kept constant, i.e. 1.0% benzaldehyde and 99.0% styrene.

Blend 2 consisted of five physiologically active compounds identified in the GC-EAG analysis. The blend was created by adding the different compounds in respective relative concentrations resembling the headspace composition of the ST18.16/133 cell-free medium as detected by the GC, and was tested at five different doses (ranging from 0.083 µg to 82.64 µg; Table 5.3) in the Y-tube olfactometer.

Table 5.3: Composition (µg) of the five different doses of Blend 2[a] tested in the Y-tube olfactometer bioassay.

Tested dose	Composition[b]					
	***o*-Xylene**	**Styrene**	**Benzaldehyde**	**Butyl acetate**	**1,3-Diacetylbenzene**	**Total**
1x	18.10	10.00	10.74	34.05	9.75	82.64
0.5x	9.05	5.00	5.37	17.02	4.88	41.32
0.1x	1.81	1.00	1.07	3.40	0.98	8.26
0.01x	0.18	0.10	0.11	0.34	0.10	0.83
0.001x	0.018	0.010	0.011	0.034	0.010	0.083
Relative amount	21.9%	12.1%	13.0%	41.2%	11.8%	100%

[a]Blend 2 consisted of five volatile compounds which elicited a significant EAG-response in the GC-EAG analysis.
[b]The relative amount of each of the five compounds of Blend 2 resembled the headspace composition of these compounds in the ST18.16/133 cell-free medium as detected by the GC.

For all individual compounds and blends, parasitoid olfactory response was analysed using a Generalized Linear Mixed Model (GLMM) based on a binomial distribution with a logit link function (logistic regression) on each compound or blend, using compound or blend dose as fixed factor. The GLMM was performed according to the procedure described in **Chapter 3**. Results were presented by calculating the Preference Index (PI) by dividing the difference between the number of parasitoids choosing for the volatile compounds and the parasitoids choosing for the control by the total number of responding insects.

5.2.7 Cage experiments

To evaluate the potential of bacterial mVOCs to affect parasitoid foraging behaviour under greenhouse conditions, two separate two-choice cage experiments were performed. Experiments were performed in a greenhouse compartment (average temperature 22 ± 4 °C) in a 2x3x2 m cage that was closed at all sides with a fine mesh. Eight-to-ten-week-old sweet pepper plants (*Capsicum annuum* cv. IDS) were placed onto elevated platforms (height: 40 cm) in each corner of the cage (Fig. 5.4). Plants were treated by spraying them (using a vaporizer) with either Blend 1 (1 ng/µL benzaldehyde and 100 ng/µL styrene in diethyl ether) or the cell-free cultivation medium of strain ST18.16/133 ("Treatment") or a control solution (diethyl ether or non-inoculated GYP25 medium) ("Control"). To do so, the leaves of the plants were sprayed by applying 20 puffs onto the leaves of the plants. In this way, on average 2.5 mL was deposited onto the leaves. This resulted in a total amount of 250 µg volatile compounds per treatment plant for synthetic Blend 1. Treatment and control plants were always placed diagonally relative to each other. To evaluate the ability

of the volatile mixtures to affect the behavioural response of *A. colemani*, 30 min after spraying the plants, 60 females, were released from an elevated platform (height: 40 cm) in the centre of the cage (Fig. 5.4), followed by recording of the parasitoids' preference. To this end, directly behind each plant a transparent glue plate (40x25 cm; Biobest, Westerlo, Belgium) was placed to trap the parasitoids that were attracted to this site of the cage (Fig. 5.4). We chose to use transparent glue plates to prevent bias from the plate colour which could affect the behavioural responses of the parasitoids. Parasitoid response was evaluated by counting the number of parasitoids on the glue plates 48 hours after parasitoid release. The experiment was replicated eight times on four different experimental days. For each replicate, plants were renewed, and treatment and control plants were switched sides to exclude positional bias inside the cage.

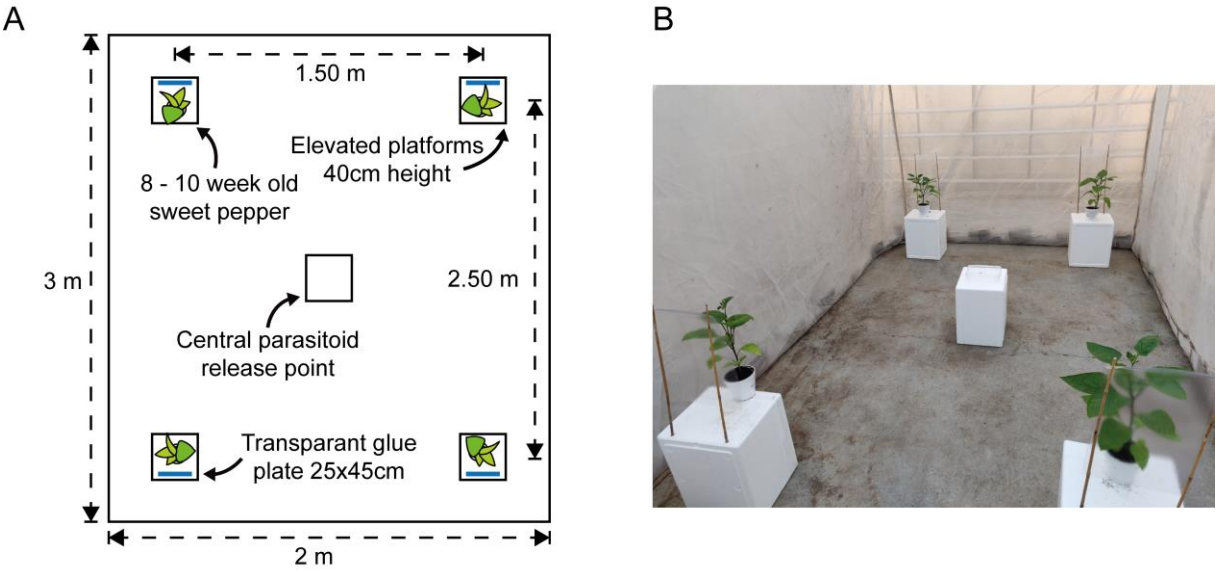

Figure 5.4: Experimental set-up of the cage experiments under greenhouse conditions. **(A)** Schematic diagram of the experimental set-up used in the cage experiments, which were performed in a 2x3x2 m cage fitted with a fine mesh. Eight-to-ten-week-old pepper plants were placed on elevated platforms in each corner of the cage. Plants were treated by spraying them with a mVOC solution or control solution. Control and treatment plants were placed diagonally relative to each other inside the cage. Directly behind each plant a transparent glue plate was placed to catch attracted parasitoids. In each experiment, 60 *Aphidius colemani* females were released from a central release point on an elevated platform. **(B)** Photograph of the experimental set-up of the cage experiments inside a greenhouse compartment (Photo credit: Tim Goelen).

Parasitoid behavioural response was analysed using a GLMM based on a binomial distribution with a logit link function (logistic regression) using blend type (Blend 1 or cell-free cultivation medium) as fixed factor, and the total number of individuals trapped at control and treatment plants as dependent variable. The GLMM was performed as described in **Chapter 3**. Results were presented by calculating the Preference Index (PI).

5.3 Results

5.3.1 Electrophysiological responses of *A. colemani* to mVOCs

Each of the bacterial strains and the blank GYP25 medium elicited similar EAG responses within the three tested replicates in *A. colemani*. Among the different mVOC extracts, the lowest number of physiological responses were observed for ST18.16/085 (the number of responses was 4) and ST18.16/160 (4), while the highest number of responses were observed for ST18.16.133 (9) and ST18.17/002 (10) (Fig 5.5). In total, 31 different EAG-active compounds were retrieved (showing consistent EAG-responses in all three replicates), among which thirteen compounds could be identified by GC-MS (Table 5.4). Due to co-elution and small peak areas the remaining compounds could not be identified and were therefore labelled as "unknown". While most of the EAG-responses were elicited by compounds unique to a certain strain, twelve EAG-active compounds originated from the mVOC extracts of more than one strain (Table 5.4). A number of compounds could be associated with the olfactory behaviour of *A. colemani*. More specifically, 3-methyl-3-pentanol, isobutyric acid, 4-hydroxy-4-methyl-2-pentanone, ethylbenzene and six unidentified compounds (unknown 4, 10, 11, 12, 15 and 17) were only found in the mVOC blends of repellent strains. By contrast, benzaldehyde, ethyl cyclohexane and six unidentified compounds (unknown 1, 6, 7, 9, 14 and 16) were associated with mVOC blends of attractive strains only. Moreover, ethylbenzene and benzaldehyde were associated with two of the three repellent strains and two of the three attractive strains, respectively (Table 5.4). Further, a number of EAG-active compounds could be associated with strains that elicited a different olfactory response in *A. colemani* (Table 5.4). These included heptane, butyl acetate, cyclohexanone, styrene, *o*-xylene, 1,3-diacetylbenzene and three unidentified compounds (unknown 2, 8, and 13).

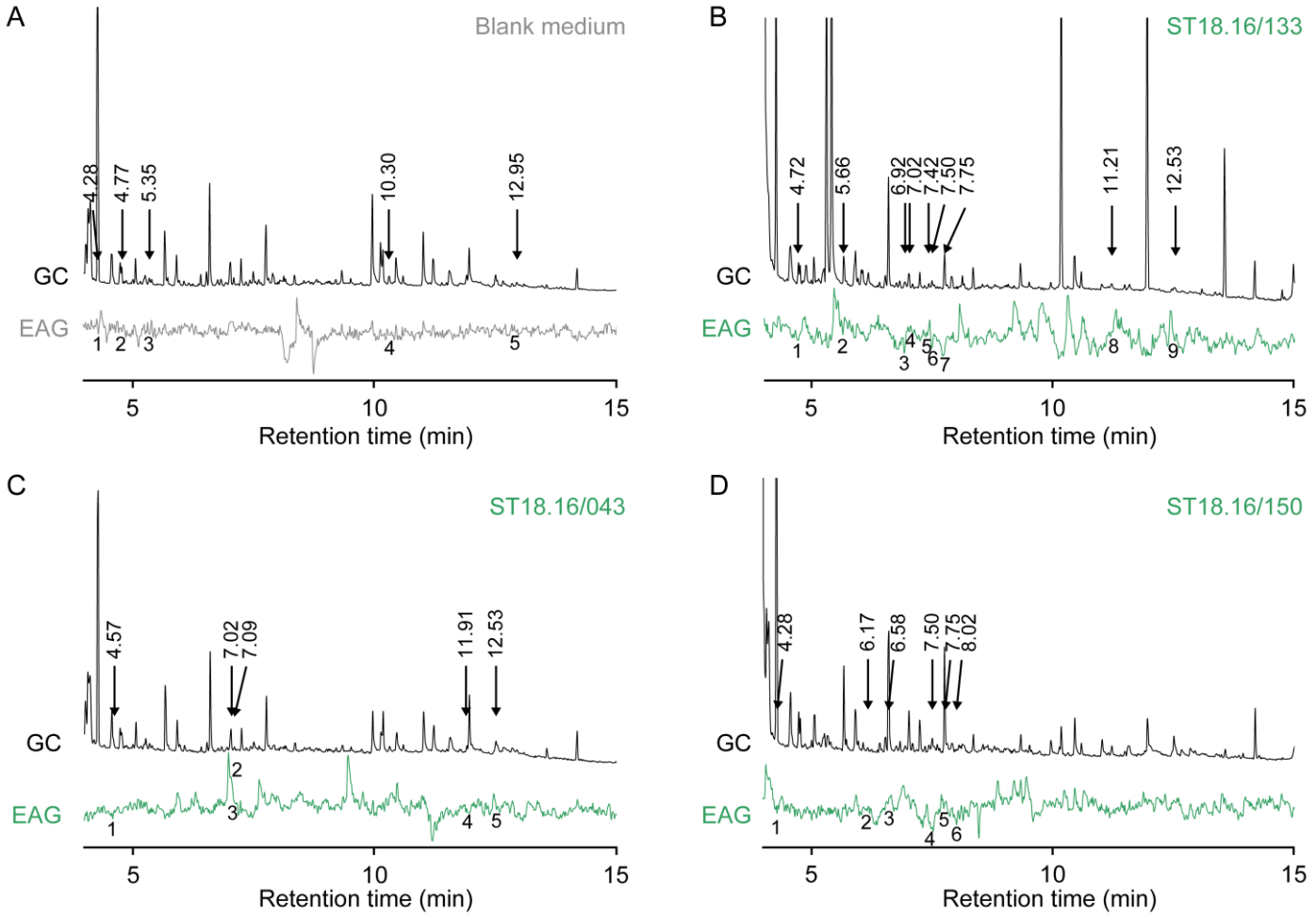

Figure 5.5: Coupled GC-EAG with female *Aphidius colemani* antennae on volatiles entrained from the cell-free cultivation medium of the seven tested bacterial strains and the blank medium. Upper trace: result of the GC analysis; lower trace: EAG-response. Major EAG-active peaks (found in all three replicates tested) are marked and associated retention times (min) on the GC chromatogram are indicated (for more details, see Table 5.4). The colour of the electroantennogram indicates the effect of the volatile blend of the tested strain on the olfactory response of *Aphidius colemani*, i.e. green = attractive, grey = neutral, and red = repellent in a Y-tube olfactometer bioassay as determined in **Chapter 3**.

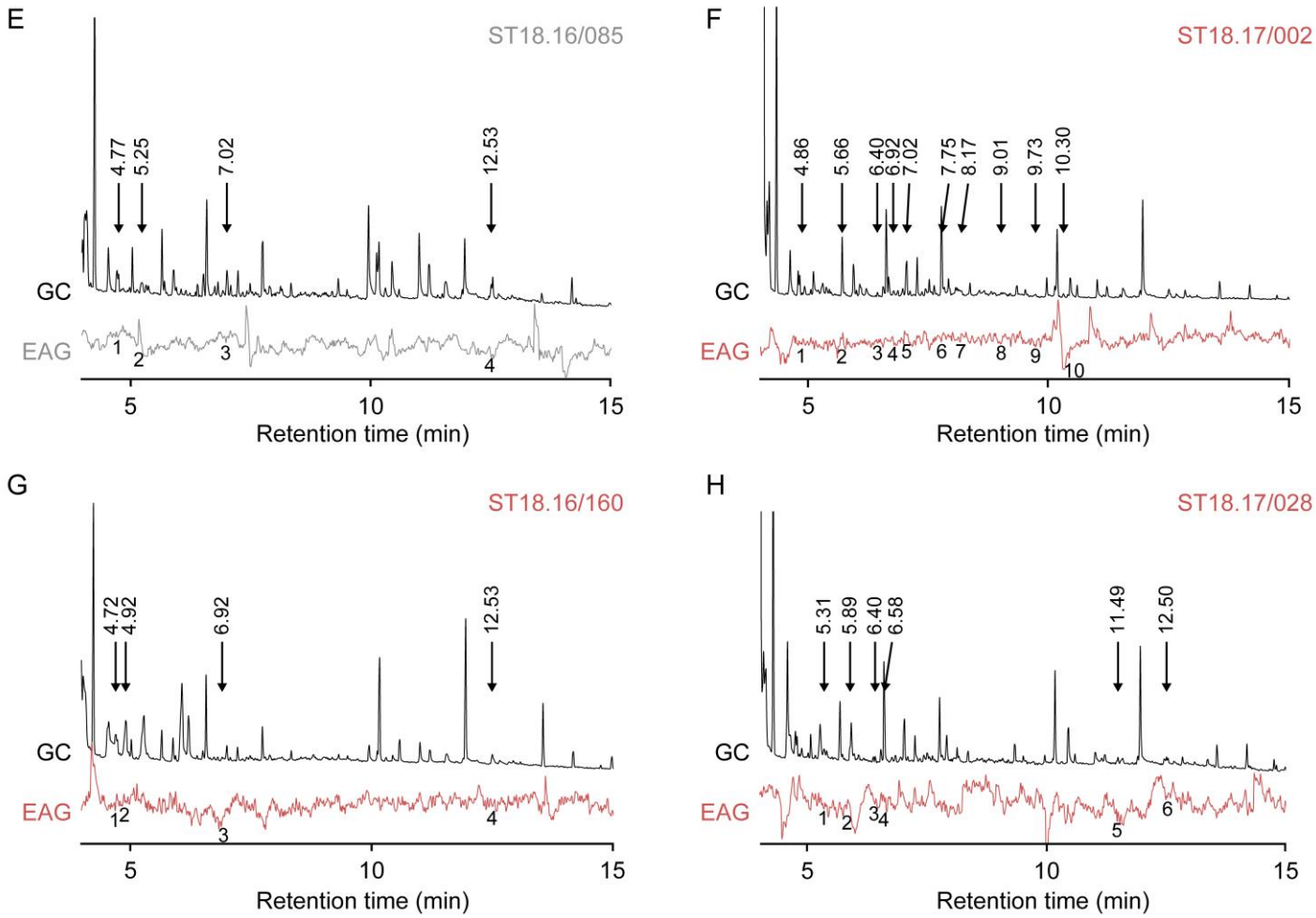

Figure 5.5 (continued): Coupled GC-EAG with female *Aphidius colemani* antennae on volatiles entrained from the cell-free cultivation medium of the seven tested bacterial strains and the blank medium. Upper trace: result of the GC analysis; lower trace: EAG-response. Major EAG-active peaks (found in all three replicates tested) are marked and associated retention times (min) on the GC chromatogram are indicated (for more details, see Table 5.4). The colour of the electroantennogram indicates the effect of the volatile blend of the tested strain on the olfactory response of *Aphidius colemani*, i.e. green = attractive, grey = neutral, and red = repellent in a Y-tube olfactometer bioassay as determined in **Chapter 3**.

Table 5.4: Compounds[a] identified in the coupled GC-EAG with female *Aphidius colemani* antennae from volatiles entrained from the cell-free cultivation medium of the seven tested bacterial strains and the blank medium.

				Neutral	Attractive			Neutral	Repellent		
EAG response[b]	**RT (min)[c]**	**RI[d]**	**Compound**	**Blank medium**	**ST18.16/133**	**ST18.16/043**	**ST18.16/150**	**ST18.16/085**	**ST18.17/002**	**ST18.16/160**	**ST18.17/028**
A1, D1	4.28	705	heptane	33.1			53.6				
C1	4.57	727	unknown 1			15.4					
B1, G1	4.72	738	unknown 2		1.7					2.7	
A2, E1	4.77	741	2,4-dimethyl hexane	1.4				1.4			
F1	4.86	748	3-methyl-3-pentanol						0.5		
G2	4.92	752	isobutyric acid							12.2	
E2	5.25	773	unknown 3					0.9			
H1	5.31	777	unknown 4								0.9
A3	5.35	780	unknown 5	0.8							
B2, F2	**5.66**	**798**	**butyl acetate**		**4.2**				**8.0**		
H2	5.89	816	4-hydroxy-4-methyl-2-pentanone								2.8
D2	6.17	837	ethyl cyclohexane				1.1				
F3, H3	6.40	853	ethylbenzene						0.8		0.8
D3, H4	6.58	868	cyclohexanone				18.9				7.9
B3, F4, G3	**6.92**	**890**	**styrene**		**1.2**				**1.3**		**1.1**
B4, C2, E3, F5	**7.02**	**896**	**o-xylene**		**2.2**	**3.2**		**4.0**	**5.5**		
C3	7.09	901	unknown 6			0.9					
B5	7.42	929	unknown 7		1.0						
B6, D4	**7.50**	**935**	**benzaldehyde**		**1.3**		**1.6**				
B7, D5, F6	7.75	956	unknown 8		4.6		11.3		13.0		
D6	8.02	976	unknown 9				0.7				
F7	8.17	987	unknown 10						0.5		
F8	9.01	1059	unknown 11						0.3		
F9	9.73	1121	unknown 12						0.6		
A4, F10	10.30	1175	unknown 13	2.7					1.1		
B8	11.21	1263	unknown 14		1.1						
H5	11.49	1290	unknown 15								1.6
C4	11.91	1335	unknown 16			0.9					
H6	12.50	1396	unknown 17								0.5
B9, C5, E4, G4	**12.53**	**1399**	**1,3-diacetylbenzene**		**1.2**	**3.4**		**1.8**		**4.0**	
A5	12.95	1447	unknown 18	1.2							

[a]Peak areas of each compound that elicited a EAG-response are shown for each strain as determined by an HP-1 equipped GC. Compounds indicated in bold were selected for further experiments.
[b]Letter and number combinations refer to the different panels and marked EAG-active peaks in Figure 5.5.
[c]Retention times of associated compounds as identified in the GC-EAG analyses.
[d]Retention indices (Kováts index) relative to retention times of C7-C22 n-alkanes on an HP-1 GC column.

5.3.2 Parasitoid olfactory responses to synthetic EAG-active compounds and blends thereof

In order to investigate the olfactory response of identified EAG-active compounds, parasitoids were subjected to Y-tube olfactometer bioassays in which five synthetic compounds were tested in different doses and blends thereof. All tested compounds were found in the volatile extract of attractive strain ST18.16/133, which showed the highest number of identified EAG-active compounds, and included butyl acetate, styrene, *o*-xylene, benzaldehyde and 1,3-diacetylbenzene.

Parasitoid olfactory response was significantly affected by two of the five synthetic volatiles. The analysis showed that the concentration of styrene (GLMM: χ^2 = 23.33, *df* = 8, *P* = 0.003) and benzaldehyde (GLMM: χ^2 = 18.73, *df* = 8, *P* = 0.016) significantly affected parasitoid olfactory response (Fig. 5.6). *Aphidius colemani* females had a significant preference for 1 µg of styrene (PI = 0.38, *P* = 0.005), and 50 ng (PI = 0.29, *P* = 0.035) and 10 ng (PI = 0.31, *P* = 0.011) of benzaldehyde. The parasitoid olfactory response to 10 or 50 ng benzaldehyde resembled that of the response to the mVOCs of ST18.16/133 (PI = 0.30), while the response to 1 µg styrene was considerably higher (Fig. 5.6). Results for benzaldehyde also suggest that doses equal or higher than 1 µg elicited a negative response in *A. colemani* (Fig. 5.6). The parasitoid olfactory response was overall not significantly affected by the dose of the three other compounds tested. Nevertheless, according to the analysis 10 ng of butyl acetate was significantly repellent to *A. colemani* (PI = -0.36; *P* = 0.011) (Fig. 5.6).

Based on these results, 1 µg styrene and 10 ng benzaldehyde were combined in a synthetic volatile blend (Blend 1, dose 1x) to test for potential synergistic effects on the olfactory response of *A. colemani*. Additionally, two lower and two higher doses of the blend were tested, while maintaining the relative amounts of both compounds (99.0 % styrene and 1.0% benzaldehyde). Furthermore, the effect of all five EAG-active compounds in one blend was tested at relative amounts in which they occurred in the mVOC blend of strain ST18.16/133 (Blend 2: 41.2% butyl acetate; 12.1% styrene; 21.9% *o*-xylene; 13.0% benzaldehyde; and 11.8% 1,3-diacetylbenzene). Again, the compound mixture was tested in different doses, while maintaining the relative amounts of the different compounds.

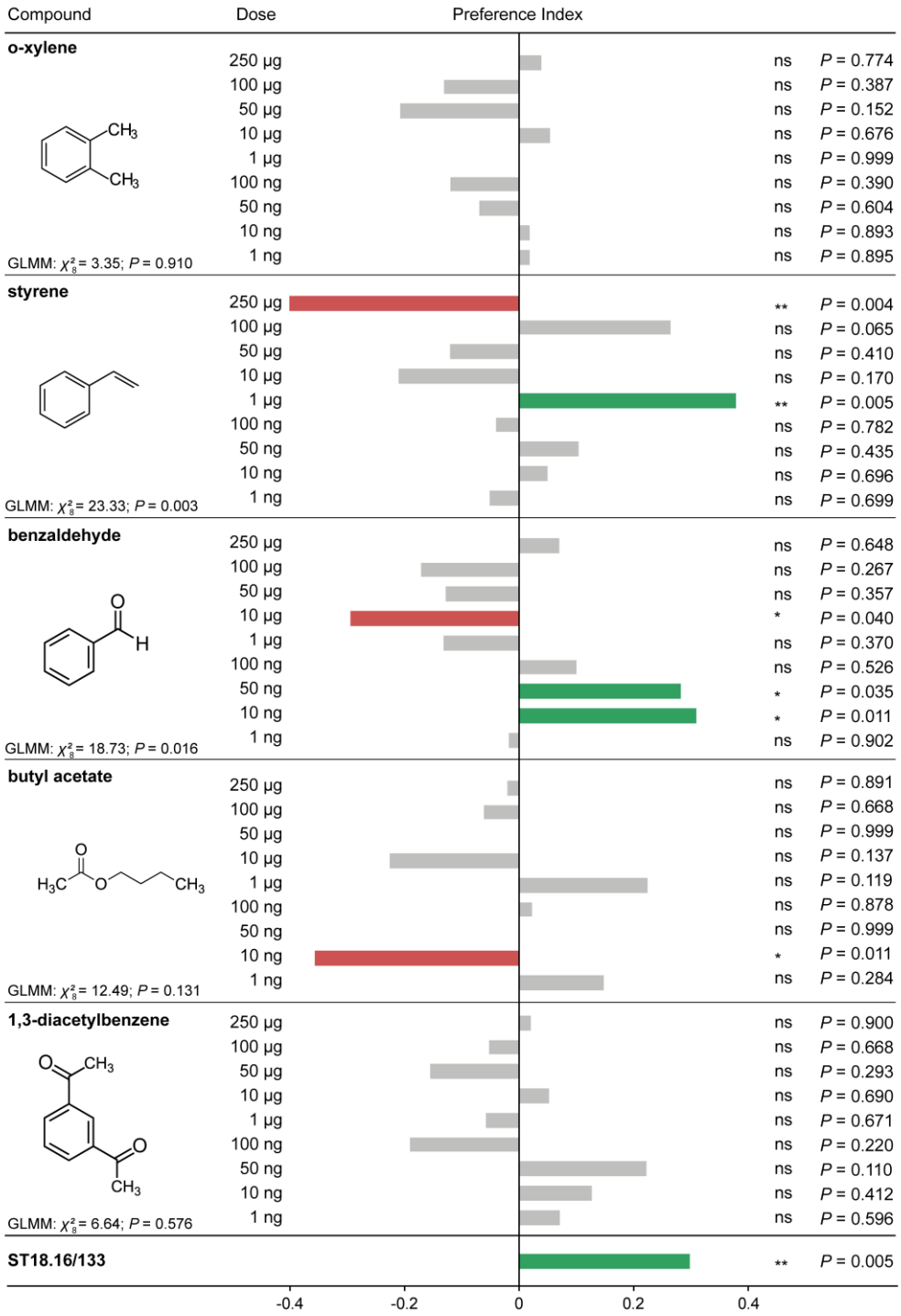

Figure 5.6: Olfactory responses of adult *Aphidius colemani* females when given the choice between one of nine different doses ranging from 1 ng to 250 µg of five synthetic volatile compounds (i.e. *o*-xylene, styrene, benzaldehyde, butyl acetate and 1,3-diacetylbenzene) and a diethyl ether blank in a Y-tube olfactometer bioassay. Parasitoid response is expressed in the form of a Preference Index (PI), calculated by dividing the difference between the number of parasitoids choosing for synthetic volatiles and the parasitoids choosing for the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females; n = 12) for each dose. Non-responders were excluded from the statistical analysis. Olfactory response of *A. colemani* to the mVOCs of ST18.16/133, as determined in **Chapter** 3, is included as a reference. 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 a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). ** 0.001 ≤ P < 0.01; * 0.01 ≤ P ≤ 0.05; ns, non-significant. Overall parasitoid responsiveness was higher than 67%.

From the two synthetic blends tested, Blend 1 showed a statistically significant effect on the olfactory response of *A. colemani* (GLMM: $\chi^2 = 21.15$, $df = 4$, $P < 0.001$), while the effect of Blend 2 was not significant in none of the doses tested (GLMM: $\chi^2 = 5.90$, $df = 4$, $P = 0.207$) (Fig. 5.7). *Aphidius colemani* females were significantly attracted by the 0.75x (PI = 0.32; $P = 0.043$), 1x (PI = 0.50, $P < 0.001$) and 1.5x dose (PI = 0.28, $P = 0.022$) of Blend 1, while they were significantly deterred by the 2x dose (PI = -0.28, $P = 0.046$) (Fig. 5.7). The combined effect of 1 µg styrene and 10 ng benzaldehyde in Blend 1 elicited a considerably stronger attractive response (PI = 0.50) in comparison to the responses to the individual synthetic compounds ($PI_{styrene} = 0.38$, $PI_{benzaldehyde} = 0.31$) and the mVOCs of ST18.16/133 (PI = 0.30).

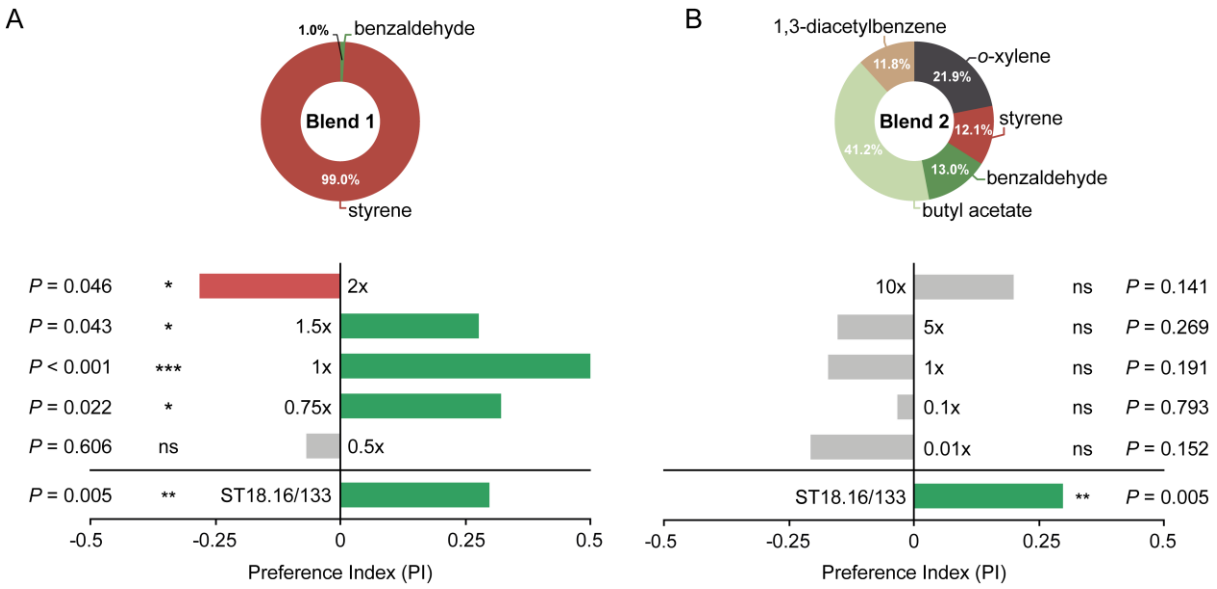

Figure 5.7: Olfactory responses of adult *Aphidius colemani* females when given the choice between one of five different doses of a synthetic volatile blend and a diethyl ether blank in a Y-tube olfactometer bioassay. Synthetic blends tested included **(A)** Blend 1, consisting of two compounds and **(B)** Blend 2, consisting of five compounds. The volatile composition of the synthetic blends tested is illustrated by the pie charts. Parasitoid response is expressed in the form of a Preference Index (PI), calculated by dividing the difference between the number of parasitoids choosing for the volatile blend and the parasitoids choosing for the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females; $n = 12$) for each dose. Non-responders were excluded from the statistical analysis. Olfactory response of *A. colemani* to the mVOCs of ST18.16/133, as determined in **Chapter** 3, is included as a reference. 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 a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). *** $P < 0.001$; ** $0.001 \leq P < 0.01$; * $0.01 \leq P \leq 0.05$; ns, non-significant. Overall parasitoid responsiveness was higher than 80%.

5.3.3 Parasitoid behavioural response under greenhouse conditions

Parasitoid behavioural response in the two-choice greenhouse experiment varied significantly between synthetic Blend 1 and the mVOC blend of strain ST18.16/133 (GLMM: $\chi^2 = 5.75$, df = 4, $P = 0.016$). Plants treated with Blend 1 attracted significantly more *A. colemani* females than the control plants (PI = 0.35, $P < 0.001$). All but one replicate showed consistently more trapped individuals on the glue plates behind the treatment plants after 48 h, with attraction ranging from 50% to 80% of the total amount of trapped individuals (Fig. 5.8). Blend 1 showed an average PI = 0.35, meaning that it on average attracted 67.7% of the trapped individuals. Plants treated with the cell-free cultivation medium of ST18.16/133 elicited no statistically significant behavioural response in *A. colemani* females (PI = 0.03, $P = 0.677$). In the experiment with Blend 1, on average 20 out of the 60 released insects (33.0%) were caught on the sticky plates, whereas this was considerably lower in the experiment with the mVOC blend, where on average 11.5 insects (19.2%) were caught (Fig. 5.8).

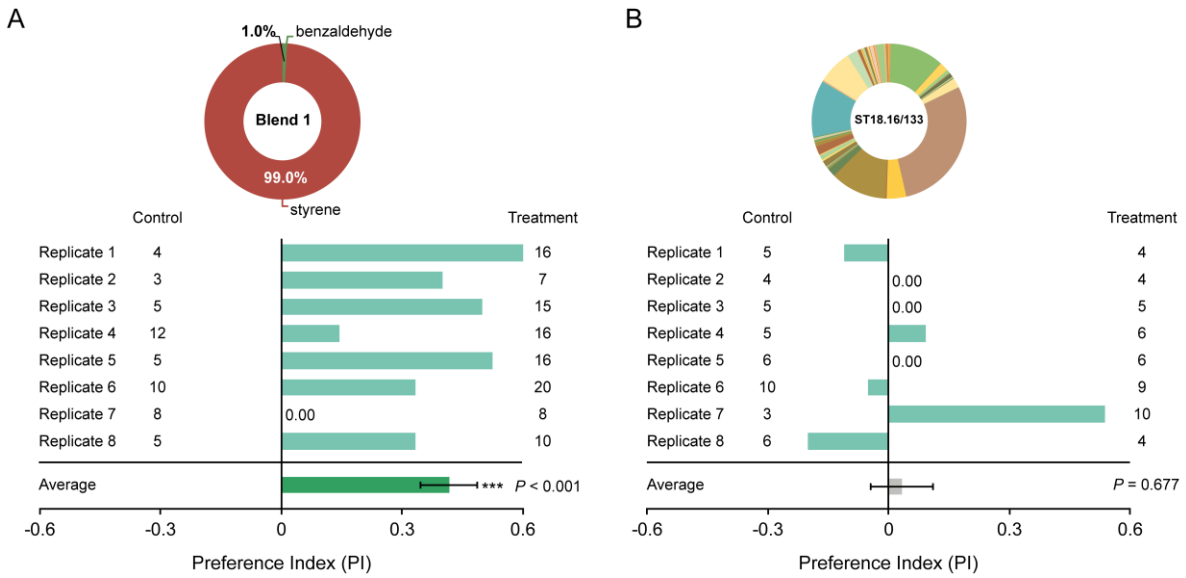

Figure 5.8: Olfactory responses of adult *Aphidius colemani* females under greenhouse conditions when given the choice between two sweet pepper plants treated with a volatile blend and two control plants. Experiments included application of **(A)** Blend 1 and with diethyl ether as a control, and application of **(B)** the cell-free cultivation medium of ST18.16/133 and with blank GYP25 medium as a control. The volatile composition of the blends tested is illustrated by the pie charts. Blend 1 was composed of 100 ng/µL styrene and 1 ng/µL benzaldehyde. Parasitoid response is expressed by calculating the Preference Index (PI) for each replicate and the average PI (±SE) of all replicates ($n = 8$). In total, 60 individuals were released in each replicate, and parasitoid response was evaluated 48h after insect release. The total number of individuals trapped on the transparent glue plates behind the pepper plants were used to evaluate parasitoid behavioural response. On the average PI values, the green bar indicates an average significant attractive response ($P \leq 0.05$), while the grey bar indicates an average non-significant olfactory response ($P > 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). *** $P < 0.001$; ns, non-significant. Average responsiveness with Blend 1 was 33.0% and with the mVOC blend of ST18.16/133 was 19.2%.

5.4 Discussion

In the previous chapters we have shown that mVOCs released by bacteria can affect the olfactory response of parasitoids. In this study, using a combination of GC-EAG analyses and Y-tube olfactometer bioassays with synthetic volatile compounds, we aimed to identify the compounds that had a significant impact on parasitoid attraction. Further, we aimed to develop a synthetic compound mixture by which *A. colemani* parasitoids can be attracted. In this regard, we also investigated whether *A. colemani* parasitoids can respond to these volatile blends under more realistic, greenhouse conditions in the presence of plants.

5.4.1 Bacterial mVOCs elicit physiological responses in *A. colemani*

The results of the GC-EAG analyses showed that *A. colemani* females were able to detect several, but not all mVOCs produced by the bacterial strains tested, including three attractive strains, three repellent strains and one neutral strain. In total, physiological responses to 31 different mVOCs were recorded. This indicates that only a fraction of the complex plethora of volatiles in bacterial mVOC blends is perceived by the parasitoid. These results further suggest that only certain compounds play a key role in affecting parasitoid olfactory responses. This is in concordance with results on plant- and host-associated volatiles, which has shown that only part of the emitted volatiles are ecologically relevant for natural enemies (Bruce et al., 2005; Du et al., 1998; McCormick et al., 2014; Morawo & Fadamiro, 2016). Similarly, attraction of *Drosophila suzukii* fruit flies to yeast fermented foods could be replicated by a synthetic blend of only four yeast-produced volatiles (Cha et al., 2015).

Although GC-EAG analyses allow to determine electrophysiologically active compounds, an EAG-response does not necessarily indicate behavioural activity (Park et al., 2001). Strikingly, several compounds that elicited a physiological response such as *o*-xylene, styrene and 1,3-diacetylbenzene, were emitted by several bacterial strains, some of which induced an attractive olfactory response in *A. colemani*, while others were repellent. This suggests that the type of olfactory response to mVOCs may depend on the compositions and ratios of constituents in a blend, rather than on the individual compounds (Bruce et al., 2005; Bruce & Pickett, 2011; Mumm & Hilker, 2005). These results are in line with previous research that has shown that differences in

concentrations and ratios of ubiquitous plant volatiles are essential cues for host recognition by natural enemies (Bruce & Pickett, 2011; Cha et al., 2011; de Bruyne & Baker, 2008).

5.4.2 Volatile compounds affect parasitoid olfactory responses in a dose-dependent manner

Olfactometer experiments using different doses of EAG-active synthetic volatile compounds showed that single compounds strongly affect the olfactory response of *A. colemani* females in a dose-dependent manner. More specifically, styrene and benzaldehyde were able to significantly affect parasitoid olfactory response, inducing both attractive and repellent responses depending on the dose. The variation in responses to different doses clearly confirms that volatile concentration is an important factor in affecting the behavioural response of insects, since the same compound can evoke an opposite response depending on the concentration (Gadino et al., 2012). This also suggests that mVOCs can carry different units of information at various dose ranges, resulting in different behavioural responses. This has previously also been observed for herbivore-induced plant volatiles (HIPVs) in a related *Aphidius* species (*A. ervi*) and a predatory mite species (Gadino et al., 2012; Takemoto & Takabayashi, 2015). At a dose of 1 µg of styrene and 10 ng or 50 ng of benzaldehyde, the synthetic compounds elicited a similar or even greater attractive response in *A. colemani* than the mVOC blend of ST18.16/133. Similar behavioural responses have been observed in a parasitoid species of the family Tephritidae, which were more or equally attracted to individual synthetic infested-fruit volatiles than to the infested fruits themselves (Benelli et al., 2013).

Previous research has indicated that benzaldehyde, a chemical with an almond-like smell, is an important volatile organic compound that can elicit both electrophysiological and behavioural responses in insects (James, 2005), including parasitoids like *Aphidius* and other aphid natural enemies (Han & Chen, 2002; Simpson et al., 2011a). Our results clearly confirm this for *A. colemani.* In addition, this corroborates the suggestion made in **Chapter 4** that benzaldehyde, one of the compounds driving the presence of a phylogenetic signal in the mVOC composition in volatile blends produced by *Bacillus* species, might explain the differences observed in olfactory response of *A. colemani* to mVOC blends produced by *Bacillus* species. More specifically, results obtained in

Chapter 4 suggested that higher concentrations of benzaldehyde were correlated with a negative reponse, while relatively lower concentrations evoked parasitoid attraction, which is in agreement with the results obtained in this Chapter.

The effects of styrene, a chemical with a sweet smell, on insect behaviour have only rarely been documented so far. In one study, Azeem et al. (2013) showed that two volatiles produced by the fungus *Penicillium expansum*, styrene and 3-methylanisole, reduced pine weevil attraction to its host plant. The three other physiologically active compounds identified in the attractive mVOC blend of ST18.16/133 (o-xylene, butyl acetate and 1,3-diacetylbenzene) had no significant effects on the olfactory response of *A. colemani*. However, individual compounds do not have to elicit an olfactory response to be able to exert an effect in a blend of volatiles. Previous research has demonstrated that insects that are attracted to a specific blend can be unaffected by or even repelled by the individual compounds of that blend (Shiojiri et al. 2010; van Wijk et al., 2011; Webster et al., 2010a). Thus, volatiles that are individually unattractive or have no effect may still contribute to the olfactory perception of the blend by having a synergistic effect on the attraction of other volatiles in the blend (D'Alessandro et al., 2009). This also indicates that insects respond to a blend of volatiles as a whole rather than to the sum of the effects of the individual compounds (Bruce & Pickett, 2011; van Wijk et al., 2011). However, it is important to note that only a limited range of concentrations was evaluated because no absolute concentrations were determined. Therefore, there is the possibility that the concentration in which a certain compound is active was not tested. Furthermore, the compounds detected were only tentatively identified through GC-MS analysis. GC peak enhancement with co-injection of authentic standards should be performed to achieve absolute identification and quantification of these compounds.

5.4.3 A blend of volatiles is more attractive than the single volatiles

Blend 1, which contained a synthetic mixture of styrene (99.0%) and benzaldehyde (1.0%), induced a significantly attractive response in *A. colemani* over a range of three doses, reaching the strongest response at a combined dose of 1.01 µg (1x). Strikingly, the olfactory response at this dose was considerably higher than the responses to the individual compounds. More particularly, Blend 1 attracted 75% of the responding individuals (PI = 0.50), which is comparable to levels of attraction obtained with synthetic

plant volatiles and volatiles from aphid infested plants in *Aphidius* species (Du et al., 1998; Liu et al., 2009; Takemoto & Takabayashi, 2015; Storeck et al., 2000; Yang et al., 2009). Such a synergistic response has also been observed in the related species *Aphidius ervi* to a synthetic blend of plant volatiles (Takemoto & Takabayashi, 2015). While in that study the attraction was maintained at a broad range of doses (between 0.001 and 5 ng), here attraction to Blend 1 was only induced within a narrow dose range (between 0.758 and 1.515 µg). In addition, the response to Blend 1 was significantly higher than the response to the mVOC blend of ST18.16/133, which attracted 10% less individuals (PI = 0.30). Several examples exist where the response to a blend containing a select number of synthetic compounds exceeds the response to the natural blend (Anfore et al., 2009; Cai et al., 2017a; Cha et al., 2014; D'Alessandro et al., 2009; Frann et al., 2018). This suggests that additional compounds within the mVOC blend of ST18.16/133 have a masking or inhibitory effect on the key compounds responsible for the attractiveness of the blend (Cha et al., 2012, 2014; Verschut et al., 2019).

Blend 2, which consisted of five identified EAG-active compounds with relative proportions as found in the attractive mVOC blend of ST18.16/133, did not induce an attractive response in *A. colemani*. Despite the fact that the original bacterial blend was attractive, and styrene and benzaldehyde elicited an attractive response in Blend 1, they did not do so in the synthetic mixture of five compounds. This suggests that one or more of the other compounds may have masked or inhibited the attractive effect of a combination of styrene and benzaldehyde (Cha et al., 2012; Verschut et al., 2019). Nevertheless, it has to be noted that the amounts and proportions of both compounds in both blends were different, which may also affect insect behaviour (Bruce & Pickett, 2011; Verschut et al., 2019). Alternatively, it is reasonable to assume that one or more key compounds that were present in the bacterial blend were absent in the mixture of five compounds. Previous research has shown that removing certain key compounds from an attractive volatile blend can disrupt attraction to that blend (Liu et al., 2019; Morawo & Fadamiro, 2016; Tasin et al., 2007). Therefore, it is not unreasonable to assume that one or more of the unidentified EAG-active compounds in the ST18.16/133 headspace are essential to facilitate an attractive response in *A. colemani*. However, additional research is required to identify these other EAG-active compounds and their effect on parasitoid olfactory response, both individually and in mixtures.

5.4.4 Parasitoids are attracted to host plants treated with EAG-active volatile compounds under greenhouse conditions

The two-component mixture of styrene and benzaldehyde (Blend 1) attracted significantly more *A. colemani* females in both laboratory and cage experiments than control treatments. Surprisingly, given its attractiveness in Y-tube olfactometer bioassays, plants treated with the cell-free cultivation medium of ST18.16/133 did not significantly attract parasitoids in the cage experiments. In addition, the total number of individuals responding to these treatments was significantly lower in comparison with Blend 1, reinforcing the attractiveness of Blend 1 as more individuals showed oriented flights and successful landings near the Blend 1 treated plants. The discrepancy between laboratory and greenhouse experiments with the bacterial volatile blend confirms previous research showing that results from laboratory experiments cannot always be extrapolated to more realistic environments and over longer distances (Anfora et al., 2009; Cai et al., 2017a; Guerrieri et al., 1993; Knudsen et al., 2007; Xu et al., 2017). It is assumed that the background odour from the environment competes with the attractant, thereby reducing the signal-to-noise ratio, and altering the perception of the attractant subsequently disturbing the attractive response (Cai et al., 2017a; Xu et al., 2017). In agricultural environments, natural enemies are confronted with a wide array of volatiles that mainly originate from crop plants. These crop volatiles can mask potential attractants, thereby disrupting insect attraction (Schröder & Hilker, 2008). Certainly, when the compounds of a faint attractant overlap with the compounds present in the environment, it may not provide a distinct signal (Riffel et al., 2014; Wist et al., 2014; Xu et al., 2017). Consequently, efficient attractants must stand out and must be able to compete with odours from the environment, e.g. by implementing higher concentrations of the attractant or increasing its specificity (Cai et al., 2017a; Szendrei & Rodriguez-Saona, 2010). In this regard, the exploitation of mVOCs in new strategies to enhance biological pest control, e.g. by attracting natural enemies to the crop field, may have a significant advantage over plant-derived volatiles, as mVOCs tend to be significantly different from plant odours and thus differ from odours in the direct environment. This distinction from plant volatiles allows even small amounts to be highly detectable by insects (Cai et al., 2017a; Cha et al., 2015; Szendrei & Rodriguez-Saona, 2010). Additional benefits of exploiting synthetic blends over complex fermentation blends are that they may be easier to produce and implement

in a consistent way, and that they are more selective as they might attract less non-target insects (Cha et al., 2015).

The complexity of the mVOC blend of ST18.16/133 and its overall interaction with the plant odours may have resulted in the loss of its attractiveness. It has also to be noted that the concentrations of the attractive compounds within this blend might have been too low to elicit significant responses when applied on plants and in cages where insects are released at a longer distance from the odour sources. It has been shown that applying the same concentration of volatile blends that were proven to be attractive in a laboratory experiment lost their attractiveness in the field (Xu et al., 2017). Certainly when the active compounds overlap with the volatiles of crop plants or the environment, they will not stand out from background volatile noise (Cai et al., 2017a). Furthermore, it is important to note that Y-tube experiments are not always comparable to behavioural experiments such as wind tunnel and cage experiments, as these also involve flight which is not possible within a Y-tube (Guerrier et al., 1993). It has been shown that *A. ervi* was attracted to healthy plants, aphids and aphid infested plants in a Y-tube olfactometer, but only aphid infested plants could elicited oriented flights and successful landings in wind tunnel experiments (Guerrier et al., 1993). Also, the overall responsiveness of tested individuals in such behavioural experiments tends to be considerably lower, which explains the lower responsiveness observed in our cage experiments (Cai et al., 2017a; Du et al., 1998; Guerrier et al., 1993). Altogether, this further confirms that results from small scale laboratory experiments cannot always be extrapolated to more realistic environments. Furthermore, it has to be noted that our method used to trap the insects (glue plates behind treated plants) may have underestimated the numbers of parasitoids that were actually attracted to each of the two treatments. Further research is needed to establish whether our experimental set-up and method to measure responsiveness can be improved.

5.4.5 Conclusion

Altogether, this study provides a better understanding of the role of mVOCs in insect behavioural ecology, and their ecological role and impact under more natural conditions. We have demonstrated that mVOCs emitted by bacteria can elicit electrophysiological responses in *A. colemani* parasitoids. Furthermore, we have shown that the olfactory

response of *A. colemani* is dependent on the dose of the volatiles, and may vary among different blends of volatiles. Moreover, we have shown that synthetic volatile blends can be composed by which *A. colemani* parasitoids can be attracted under greenhouse conditions, while this was not the case for the original complex bacterial mVOC blend. Altogether, these results indicate that bacterial volatiles can have an important impact on insect olfactory responses and therefore open new opportunities to attract or retain natural enemies of pest species with the aim to enhance biological pest control at the greenhouse or field scale. However, further investigations are required to determine the effects of attracting natural enemies with mVOCs on biological control efficacy.

Chapter 6

General conclusion and perspectives

6.1 Main results of this study

Aphidius *species and their hyperparasitoid* Dendrocerus aphidum *consume most of and survive best on honeydew-associated sugars*

Aphid parasitoids are commonly used in the biological control of aphids, which constitute one of the most important threats to agriculture and horticulture worldwide (Dedryver et al., 2010; van Lenteren et al., 2012). However, successful biological control using parasitoids largely depends on the availability of carbohydrate-rich food as an energy source for maintenance and reproduction (Heimpel & Jervis 2005). Therefore, as these resources have become rare in modern agricultural systems, external sugar sources like flowering plants or artificial sugar solutions are increasingly used to provide the biocontrol agents with the necessary sugars (Tena et al., 2015; Wäckers & van Rijn, 2012). When developing such artificial food sources, it is essential to carefully select the sugars that best support the target parasitoids without benefiting non-target insects, such as pest insects or hyperparasitoids (Benelli et al., 2017; Harvey et al., 2012).

In **Chapter 2** we investigated feeding behaviour and longevity of two commonly used aphid parasitoids (*Aphidius colemani* and *Aphidius matricariae*) and one of their hyperparasitoids (*Dendrocerus aphidum*) when provided with one of eight plant- and/or insect-derived sugars (fructose, galactose, glucose, melibiose, melezitose, rhamnose, sucrose and trehalose). As hyperparasitoids reside in the same habitat as their host parasitoids, we hypothesised that hyperparasitoids have been adapted to the exploitation of the same sugar sources as those preferred by their host parasitoids. Our results showed that the studied *Aphidius* species consumed the largest amounts of sugars that are most commonly found in honeydew (sucrose, fructose, glucose and melezitose), while the consumption of sugars that are underrepresented in honeydew (rhamnose, melibiose and trehalose) was significantly lower. Similar results for sugar consumption were observed in the hyperparasitoid *D. aphidum*. However, overall consumption by *D. aphidum* was significantly higher in comparison to the *Aphidius* species, which could potentially be attributed to the higher mobility of *D. aphidum* inside the CAFE-containers. Strikingly, *D. aphidum* consumed significantly more melezitose, a typical aphid-synthesised honeydew sugar, reaching up to twice the amount consumed of the other sugars tested. Furthermore, the primary parasitoids and the hyperparasitoid showed similar patterns in longevity on the tested sugars. All tested species survived best on the typical honeydew sugars increasing their longevity by a factor ranging from 3.3 to 6.0 compared to the control

treatment in which no sugars were provided. The tested *Aphidius* species reached highest longevity on glucose, while *D. aphidum* survived best on sucrose. Rhamnose, a sugar that does not occur in honeydew (Wäckers, 2001), was not or only marginally consumed and did not increase longevity of any of the tested species. Interestingly, despite low innate gustatory responses both *Aphidius* spp. survived well on melibiose, whereas *D. aphidum* performed particularly poorly on this sugar. This difference can possibly be explained by the parasitoid's physiology. Some sugar polymers require to be hydrolysed before the monosaccharide components can pass through the gut wall (Weil, 1987). Therefore, enzymatic breakdown in the parasitoid's gut is essential for efficient sugar conversion into energy. It is possible that the ezyme required for meliobiose hydrolysis in *D. aphidum* has a low activity resulting only in partial conversion. Furthermore, it is also possible that melibiose or its components is either poorly absorbed through the gut wall or that the metabolism is insufficient, resulting in poor longevity on this sugar (Hausmann et al, 2005). When melibiose was offered in a mixture with glucose (3:1 ratio), a significant reduction in longevity (35%) was observed for *D. aphidum* when compared to glucose only, while this was less pronounced for *Aphidius* spp., suggesting that this mixture can be used to predominantly support *Aphidius* parasitoids. While the sugar concentrations tested in this study represent typical concentrations found in floral nectar and honeydew, also higher concentrations should be further evaluated as higher concentrations typically significantly increase parasitoid longevity (Azzouz et al., 2004).

mVOCs emitted by bacteria associated with parasitoid habitats elicit distinct olfactory responses in **Aphidius colemani** *and one of its hyperparasitoids,* **Dendrocerus aphidum**

To locate mating partners and essential resources such as food, oviposition sites and shelter, insects rely to a large extent on olfactory cues (de Bruyne & Baker, 2008). While most research on volatile-mediated foraging behaviour has focused on cues derived from plants and insects (Kaplan, 2012; Meiners & Peri, 2013), there is mounting evidence that also microorganisms emit volatile compounds that play a role in affecting insect behaviour (Davis et al., 2013; Dzialo et al., 2017; Leroy et al., 2011b). Nevertheless, despite the fact that microbial volatile emission is commonplace (Piechulla & Degenhardt, 2014), still little is known about the ecological and biological functions of bacterial mVOCs and their impact on volatile-mediated foraging behaviour in arthropod natural enemies. In

addition, virtually nothing is known about the role of microbial volatiles in the chemical ecology across trophic levels. Therefore, in **Chapter 3** we assessed how volatile compounds emitted by phylogenetically diverse bacteria affected the olfactory response of *A. colemani* and one of its hyperparasitoids, *D. aphidum*. Olfactory responses were evaluated in a Y-tube olfactometer bioassay using 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. Results revealed that *A. colemani* showed wide variation in response to bacterial volatiles, ranging from significant attraction over no response to significant repellence. Strikingly, despite the fact that aphid natural enemies are known to respond to kairomones emitted by honeydew (Leroy et al., 2011a), our results suggest that *A. colemani* parasitoids are not attracted to, and can even be repelled by mVOCs produced by bacteria isolated from aphid honeydew. Potentially, this can be explained by the difference in chemical composition of honeydew and the cultivation medium used in this study. Previous research has shown that medium composition can have a strong impact on volatile profiles when fermented by microorganisms and subsequently on parasitoid foraging behaviour (Sobhy et al., submitted). Therefore, further research with more isolates and media mimicking the natural environment of the bacteria is required to robustly determine the effect of microbial habitat or isolation origin on parasitoid volatile-mediated foraging behaviour.

As previously observed for herbivore-induced plant volatiles (HIPVs) (Cusumano et al., 2019b; Poelman et al., 2012), we also found that the olfactory response of primary parasitoids to bacterial volatile emissions was significantly different from that of their hyperparasitoids. This suggests that, although primary parasitoids and their hyperparasitoids share several resources within the same environment, their olfactory response towards plant and microbial volatiles may be different. Gas chromatography-mass spectrometry (GC-MS) analysis revealed that volatile blends attractive to *A. colemani* generally contained significantly lower amounts of volatiles, while the volatile blends attractive to *D. aphidum* contained significantly higher amounts of the monoterpenes limonene, linalool and geraniol. A preliminary Y-tube olfactometer bioassay with synthetic volatile compounds confirmed that the olfactory response of *D. aphidum* can be affected by these three monoterpenes, indicating the robustness of our results (De Boer & Goelen; data not shown). Further, volatile blends repellent to *A. colemani* contained 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 than attractive strains. The results further showed that closely related species of the genus *Bacillus* evoked a similar olfactory response, suggesting that volatile composition, and thus parasitoid attraction, are phylogenetically conserved traits.

Bacterial phylogeny predicts emitted mVOC composition and olfactory response of **Aphidius colemani**

Despite an increased understanding of the role of mVOCs as insect semiochemicals, little is known about whether phylogenetic relationships among microorganisms predict mVOC composition and the olfactory response of insects. In **Chapter 4**, we tested this hypothesis in detail using 40 *Bacillus* strains from diverse origins and the primary aphid parasitoid *A. colemani*. Results revealed that phylogenetically closely related *Bacillus* strains emitted similar blends of mVOCs and elicited a comparable olfactory response of *A. colemani* in Y-tube olfactometer bioassays, varying between attraction and repellence, pointing towards significant phylogenetic conservatism. Analysis of the chemical composition of the mVOC blends revealed that all *Bacillus* strains produced the same set of volatiles, but often in different concentrations and ratios. This suggests that different concentrations and ratios of specific mVOCs determine parasitoid olfactory responses, rather than single or unique compounds (Bruce et al., 2005; Mumm & Hilker, 2005). In addition, our results indicated that specific mVOC compositions could be linked with bacterial phylogeny. In total, 15.7% of the detected mVOCs showed a significant phylogenetic signal in all statistical tests performed (i.e. Pagel's λ, Moran's I, Abouheif's C_{mean} and Mantel test). Acetoin, 2,3-butanediol, 2,3-butanedione and benzaldehyde were important drivers of the phylogenetic signal. The same compounds were found to be linked with parasitoid olfactory response. Benzaldehyde was produced in higher amounts in mVOC blends that repel *A. colemani* compared to attractive mVOC blends, while attractive mVOC blends contained relatively higher amounts of acetoin, 2,3-butanediol, 2,3-butanedione, eucalyptol and isoamylamine. Therefore, as the ratios between these volatiles define the difference between attractive and repellent mVOC blends, they constitute promising candidates to be exploited in semiochemical-based management strategies.

Bacterial volatiles attract* Aphidius colemani *parasitoids under laboratory and greenhouse conditions

Although the previous chapters (**Chapter 3** and **Chapter 4**) clearly increased our understanding of volatile-mediated interactions between parasitoids and bacteria, potential active compounds were only suggested based on linkage of obtained datasets (i.e. mVOC composition and olfactory response). Results of the GC-EAG analyses performed in **Chapter 5** showed that *A. colemani* females only detect a fraction of the mVOC blends produced by the bacteria, suggesting that only certain mVOCs play a key role in affecting parasitoid olfactory responses (Bruce et al., 2005; Du et al., 1998; McCormick et al., 2014; Morawo & Fadamiro, 2016). Furthermore, although several EAG-active compounds could be linked to a particular set of strains evoking the same insect response (attraction or repellence), a number of EAG-active volatiles such as *o*-xylene, styrene and 1,3-diacetylbenzene were found in both attractive and repellent mVOC blends. This reinforces earlier observations that insect olfactory response strongly depends on the chemical composition of the entire mVOC blend, including specific ratios of ubiquitous mVOCs, rather than on the individual compounds (Bruce et al., 2005; Bruce & Pickett, 2011; **Chapter 3;** Goelen et al., 2020; **Chapter 4**; Mumm & Hilker, 2005).

Y-tube olfactometer bioassays using synthetic compounds for a number of EAG-active mVOCs identified in a bacterial mVOC blend that was attractive to *A. colemani* (ST18.16/133), revealed compounds that were significantly attractive or repellent. In particular, a mixture of 100 ng/µL styrene and 1 ng/µL benzaldehyde was most attractive to *A. colemani*. While benzaldehyde regularly has been identified to affect insect olfactory responses (James, 2005; Han & Chen, 2002; Simpson et al., 2011c; Xu et al., 2017), this is to our knowledge one of the first times that styrene has been shown to affect insect responses (but see Azeem et al., 2013; Schiebe et al., 2019). Remarkably, when a mixture containing all identified EAG-active compounds in relative amounts as they occurred in the bacterial mVOC blend was tested, no significant olfactory response was recorded. This suggests that, most probably, other, unidentified EAG-active compounds within the mVOC blend of ST18.16/133 play a crucial role in the overall attractiveness of this blend. Furthermore, results indicated that volatile compounds affect parasitoid olfactory responses in a dose-dependent manner. In particular, compounds such as styrene and benzaldehyde, and a blend thereof, tended to elicit negative responses at higher doses, while positive responses were observed at lower doses. These results corroborate the

suggestions made in **Chapter 4** about the possible effect of benzaldehyde concentrations on parasitoid olfactory responses. This observation also raises the question whether differences in olfactory responses may be caused by differences in final cell densities in the fermentation media. Although initial inoculum density was controlled (OD_{600nm} = 1), fermentation media were not corrected for potential differences in final cell densities before testing them in the olfactometer assays. However, strains eliciting positive and negative olfactory responses showed both high and low cell densities at the end of cultivation. Therefore, no direct association can be made between cell density and induced parasitoid olfactory response. Nevertheless, preliminary experiments in which cell-free cultivation media were diluted and tested in a Y-tube olfactometer demonstrated that dilution of the mVOC blends can affect the olfactory response.

In line with the results obtained in the Y-tube olfactometer assays, application of the synthetic blend of styrene and benzaldehyde on sweet pepper plants in two-choice cage experiments, confirmed the attractiveness of the blend under greenhouse conditions. Surprisingly, given its attractiveness in the Y-tube olfactometer bioassays, plants treated with the cell-free cultivation medium of ST18.16/133 did not attract parasitoids in the cage experiments, confirming previous findings that Y-tube olfactometer results cannot always be extrapolated to behavioural experiments under more realistic conditions. Indeed, it is not unreasonable to assume that natural background odours from the environment can compete with released mVOCs, altering the perception of the mVOC signal and subsequently disturbing the response of the insect (Cai et al., 2017a; Xu et al., 2017). Altogether, our results indicate that specific microbial volatile compounds released in particular concentrations can have an important impact on insect olfactory responses. Exploitation of such volatile compounds may lead to new strategies to attract and retain natural enemies in the crop. However, further study is required to determine the effects of attracting natural enemies with mVOCs on their biological control efficacy.

6.2 Perspectives

Overall, this PhD study has provided a better understanding of sugar feeding in *Aphidius* parasitoids and one of their hyperparasitoids (*D. aphidum*). Further, this PhD study provided new insights in the chemical communication between bacteria, parasitoids and hyperparasitoids. Although the obtained knowledge may lead to improved biological control using microbe-based insect semiochemicals and artificial sugar sources, to lure and support the parasitoids in the crop, respectively, additional research is required before implementation in real-world applications. Below, a number of potential routes for further investigation are outlined.

6.2.1 Designing selectively supportive artificial sugar mixtures

In this study, we have shown that *Aphidius* species and the hyperparasitoid *D. aphidum* are well adapted to consume and survive on sugars which are typically present in honeydew, one of the most predominant sugar sources in their habitat. Interestingly, it became clear from our results that specific sugar mixtures can be developed by which particular insects can be supported. Specifically, our results suggest that a 1:3 mixture of glucose and melibiose can be developed that predominantly benefits the primary parasitoids, while being less supportive for harmful insects like *D. aphidum*. However, further research with more insect species is needed to thoroughly assess the selectivity of the sugar mixture. Further, it would be worthwhile to investigate the effect of additional sugars that may be added to the mixture to further increase its selectivity. Amongst several others, these sugars may include mannose, lactose, maltose or more complex sugars such as raffinose, erlose and stachyose, which all can occur in the natural habitat of the target parasitoids, e.g. floral nectar and/or honeydew (Tian et al., 2019; Wäckers 2001). Alternatively, selectivity may be enhanced by adding one or more compounds that are toxic or deter non-target insects. In this regard, is has been shown that adding the polyol erythritol to sugar mixtures or food sources can significantly reduce longevity of *Drosophila* fruit flies (Choi et al., 2017; Sampson et al., 2017). Furthermore, van Neerbos et al. (2019) recently suggested that the concentration of the sugar alcohol sorbitol in honeydew was negatively correlated with the longevity and fecundity of hyperparasitoids, including *D. aphidum*. Although sorbitol has been detected in parasitoids feeding on various honeydews (Hogervorst et al. 2007a; Wyckhuys et al.,

2008), the individual effect of sorbitol on the longevity of both primary and secondary parasitoids still remains to be evaluated. In the event that compounds that negatively affect unwanted organisms, and that have no or only a minor effect on the target insects are identified, they can be used to optimize the sugar mixtures in such a way that their selectivity for beneficial insects is increased.

In this PhD study, we focused on the impact of sugars and sugar mixtures on parasitoid longevity, as longevity represents one of the most important life-history parameters of parasitoids. Increased longevity allows to maintain control for longer periods of time, which generally translates into higher parasitism rates (Heimpel & Jervis, 2005). However, it is also important to study whether and how supplemented sugars and sugar mixtures affect other life-history parameters such as fecundity and oviposition frequency, as well as to investigate their ultimate effects on biological control efficacy (Benelli et al., 2017; Harvey et al., 2012; Olson et al., 2005; van Neerbos et al., 2019). Previous studies have indicated that sugar sources can affect parasitoid fecundity differently (Tena et al. 2013a; Charles & Paine 2016). Providing suitable food sources may either directly improve female reproductive mechanisms such as egg load and egg maturation (fecundity) or prolong the adult lifespan and increase oviposition behaviour, consequently increasing reproductive rate (lifetime fecundity) (Benelli et al., 2017). *Aphidius colemani* is considered a proovigenic species (Jandricic et al., 2014), whose females emerge with a set of (almost) completely matured eggs (Jervis et al., 2001). Therefore, providing supplemental food sources to proovigenic species has an almost insignificant effect on fecundity (Jervis et al., 2001), but it will affect lifetime fecundity (Benelli et al., 2017). Conversely, *D. aphidum* is considered a synovigenic species, whose females emerge with no or a limited set of mature eggs (Araj et al., 2006, 2008; Jervis et al., 2001). As a result, they have to keep maturing eggs during their adult life, on which they need to spend energy and nutrients (Chapman, 1998). Therefore, in synovigenic parasitoid species access to food sources affects fecundity (Araj et al., 2008). Additionally, it has been shown that food sources may affect oviposition rates, and therefore directly affect the parasitoids' ability to control aphid pests (Araj et al., 2008, 2009; Charles & Paine, 2016; Jado et al., 2019). However, it has to be noted that most studies in this field have focused on natural food sources such as nectar and honeydew, while effects of individual sugars still have to be determined.

6.2.2 Elucidating ecological roles of mVOCs

The results obtained in this PhD study provide a better understanding of the volatile-mediated interactions between bacteria, primary parasitoids and hyperparasitoids. Our results clearly indicate that parasitoid olfactory response to bacterial mVOCs may vary substantially among bacterial strains. Nevertheless, it is also clear from our results that mVOC composition, and thus insect olfactory response, is strongly associated with bacterial phylogeny, rather than source of isolation. Indeed, even bacteria originating from environments which are not typically associated with insects were found to strongly attract *A. colemani*, while other bacteria which were associated with the habitat of the parasitoid (e.g. honeydew) were not. The question arises whether the volatile blends obtained here are also produced in the natural habitat of the microorganisms. Indeed, the bacteria investigated in this study were cultivated in a synthetic, relatively rich growth medium to ensure sufficient bacterial growth, allow for sufficient mVOC production and allow for comparison between the different bacterial strains. However, emission of mVOCs, including their chemical composition, is dependent on a variety of factors, including growth medium, growth stage of the microbes, nutrient availability, temperature, oxygen availability, pH, etc. (Schmidt et al., 2019; Sobhy et al., submitted; Tyc et al., 2017b). Therefore, it is not unreasonable to assume that the volatile emissions by these strains may be different under more natural conditions. Future experiments should therefore be performed to investigate to what extent the mVOCs measured here mimic those that are emitted under more natural conditions. However, previous research has shown that mosquitoes are attracted to specific bacterial strains grown on both human sweat and classical artificial growth media (Braks & Takken, 1999, Verhulst et al., 2009). This suggests that similar volatile blends can be produced on both natural and artificial media, even if they do not mimic natural conditions (Verhulst et al., 2010). Moreover, these attractive volatiles could be identified and applied in a mVOC blend to attract mosquitoes in the field (Verhulst et al., 2011). In addition, in this PhD study bacterial strains were cultivated as monocultures. However, under natural conditions, bacteria rather occur in microbial communities consisting of many microorganisms that occupy the same physical location and that interact with each other. As a result, volatile blends that result from microorganisms in a microbial community may be different from those emitted by the same strains in a monoculture. For example, Fischer et al. (2017a) demonstrated that *Drosophila* fruit flies prefer a yeast-bacterium co-culture over their

individual cultures or even the mixture of individual cultures. They showed that when the yeast and bacterium were allowed to interact with each other, they exchanged metabolites leading to the production of new compounds which could only be produced in a co-culture. This thus suggests that microbial interactions may lead to new mVOCs which may affect insect olfactory responses.

Despite the ubiquitous and often massive appearance of microorganisms, so far only little is known about the biological or ecological role of mVOCs. As most mVOCs are considered side-products of primary and secondary metabolism (Korpi et al., 2009), the volatile-mediated interaction between bacteria and insects could be merely coincidental. During the course of this PhD we discovered that volatiles emitted by bacteria display a certain degree of overlap with plant- and insect-associated volatiles, which has recently also been shown between yeasts and plants (Ljunggren et al., 2019), and between insects and plants (Beran et al., 2019). As a result, response to mVOCs may be predominantly driven by the insect's innate behaviour to respond to plant or insect volatiles. However, there could also be a deeper ecological association between bacteria and insects, as is, for example, the case for yeasts (Becher et al., 2018; Christiaens et al., 2014). Several bacterial species, including many species tested in this study, are known as insect symbionts, providing their hosts several benefits, including contribution to nutrition, protection from parasites, pathogens or even pesticides (Gilliam et al., 1990; Itoh et al., 2018; Um et al., 2013). Therefore, it is not unreasonable to assume that insects like *A. colemani* could have developed specialised systems to detect, pick-up and maintain these mutualistic bacteria (Salem et al., 2015). The advantage for the bacteria is less clear, but it is reasonable to assume that they may benefit from being vectored to new habitats or being dispersed over large distances (Christiaens et al., 2014) or get protection in the insects during unfavourable conditions (Pozo et al., 2018).

6.2.3 Limitations and challenges of insect semiochemicals in biological pest control

The increasing body of knowledge on how to manipulate the behaviour of natural enemies by the use of semiochemicals has opened up new opportunities which may lead to efficient, environmentally sustainable pest control strategies. Previous research has demonstrated that the implementation of semiochemicals can lead to increased recruitment of natural enemies, to natural enemy arrestment, decreasing emigration from

release sites and increased pest control services (Colazza et al., 2013; Kelly et al., 2014; Simpson et al., 2013). Despite the promising results on the application of semiochemicals, the methods and techniques that have been developed previously and are still being developed may include potential limits and risks of using these compounds in the field (Kaplan, 2012; Meiners & Peri, 2013; Rodriguez-Soana et al., 2012; Pappas et al., 2017). A major constraint is that the use of semiochemicals to manipulate parasitoids' behaviour is done in environments that are, by definition, highly complex where various factors, including divers background odours, can affect and disturb the response to otherwise physiologically active semiochemicals (discussed in more detail below). In our experiments, it was observed that application of a cell-free cultivation medium which was attractive in the Y-tube olfactometer bioassay to *A. colemani* (ST18.16/133) did not result in parasitoid attraction when sprayed on the leaves of sweet pepper plants. By contrast, when applying a mixture of two attractive compounds (i.e. styrene (100 ng/µL) and benzaldehyde (1 ng/µL)), parasitoids were significantly attracted to the treated plants. So far, it remains unclear why the bacterial volatile blend lost its attractiveness in the plant assay. However, it is reasonable to assume that concentrations of the active compounds were too low when applied in the cage experiment to attract the parasitoids (22 cm distance between place of release and place of evaluation in the olfactometer bioassay vs. 1.90 m in the cage experiment), especially in combination with volatiles emitted by the plants. Despite the attractiveness of the styrene-benzaldehyde mixture under both laboratory and greenhouse conditions, it cannot be guaranteed that the effects observed in the plant assay were only caused by the applied blend of synthetic volatiles. The volatiles released by the plants could have caused a synergistic effect on the perception of the volatile blend, providing additional context to the perceiving parasitoids, thereby increasing its attractiveness (Schröder & Hilker, 2008). In this scenario, the plants provide a background odour that is imperative for the parasitoids to respond to the volatile compounds applied. Another possibility is that spraying the compounds onto the leaves induced a response in the plants which altered their volatile emission, thereby enhancing parasitoid attraction. It has been shown that plants exposed to HIPVs of other plants can become induced to increase their volatile emissions (Kaplan, 2012; von Mérey et al., 2011; Ton et al., 2007). However, additional research should be performed to determine the exact differences in the volatile emission of treated and control plants. Furthermore,

further research is needed to assess how long the observed effects would remain after application of the blend.

In addition to interference with background odour, deploying semiochemicals to attract beneficial insects throughout a greenhouse or field can have negative impacts on the searching efficiency of natural enemies. Application of semiochemicals may decrease the foraging efficiency of parasitoids when they are attracted to locations with no hosts, decreasing the time actually spent on host-killing (Powell & Pickett, 2003). Moreover, the application of semiochemicals in certain areas within a field can also lure parasitoids away to these specific locations, leaving other areas unprotected (Meiners & Peri, 2013). Another potential risk that should be taken into account is that semiochemicals might attract non-target arthropods, including pest insects and hyperparasitoids. While the synthetic blend of styrene and benzaldehyde seems promising to attract *Aphidius* parasitoids under greenhouse conditions when applied on plants, it still remains to be investigated how non-target insects such as their hyperparasitoid *D. aphidum* responds to these volatiles.

6.2.4 Foraging in a complex environment: an important constraint to be overcome

Insects forage in complex environments where their resources are found among non-resources. In addition to their resources also non-resources emit volatile compounds, which makes it challenging for foraging insects to filter information about their desired resources in a plume of background odour (Aartsma et al., 2017). In the past, background odour was first considered to be irrelevant to resource-indicating odours. Although situations have been found in which this is indeed the case (Dicke et al., 2003), background odour can have serious influence on the perception of resource-indicating semiochemicals in the environment (Schröder & Hilker, 2008). On the one hand, the presence of repellent or inhibitory volatiles in the background odour can mask the attractiveness of resource-indicating semiochemicals, thereby reducing the response of insects to attractants (Mauchline et al., 2005; Riffell et al., 2014; Thiery & Visser, 1986). On the other hand, in some cases resource-indicating semiochemicals might require background odours to provide the necessary context to make sense to a given insect (Borrero-Echeverry et al., 2018; Mozuraitis et al., 2002; Mumm & Hilker, 2005; Shröder & Hilker, 2008). Overall, when microbe-produced semiochemicals will be used in biological

pest control, plant volatiles will probably contribute most to the volatile composition of the background odour, as plants often make up most of the biomass present in an agricultural environment (Conchou et al., 2019). However, it is important to note that only a fraction of the volatiles that insects encounter can be readily detected by their olfactory receptors (Bruce et al., 2005). Furthermore, parasitoid species differ in their ability to detect volatile compounds due to differences in specificity and sensitivity of their olfactory receptors, which determine their capacity to differentiate between volatile blends (Aartsma et al. 2017; Gouinguené et al., 2005). Further research is needed to investigate whether microbe- and plant-released volatiles are differently perceived in insects, and therefore would not interfere when they are both present in an odour plume.

The distribution of emitted volatiles is also dynamic and exhibits large spatial and temporal variation in composition in natural environments and agricultural landscapes (Carrasco et al., 2015), making it even more complex for foraging insects to navigate towards their resources. After an odour leaves the emitting resource it forms a volatile plume that consists of discrete odour filaments that will be carried away in the direction of the wind (Murlis et al., 1992; Beyaert & Hilker, 2014). Besides wind, other air currents, turbulence and degradation of volatiles by reactive compounds such as ozone reduce the volatile concentration and consequently alter the composition of the plume (Aartsma et al., 2017; Conchou et al., 2019). In addition, obstacles such as plant structures may affect plume transport and mixing with other odour plumes, resulting in an assemblage of odour plumes which do not form a continuous gradient pointing towards its resource (Celani et al., 2014). These factors finally determine the shape, concentration, composition and spatial extent of the odour plume and its odour filaments, which can alter the perception by insects (Aartsma et al., 2017). This makes volatile-mediated resource location at large distances a challenging task. Nevertheless, many insects are particularly proficient at pursuing these plumes (Murlis et al., 1992; Cardé & Willis, 2008; Bau & Cardé, 2015). Flight capacity will determine the spatial scale at which a foraging insect can respond to these odour plumes (Roland & Taylor, 1997) and they may change their navigational strategy at certain distances from the odour emitting resource (Bau & Cardé, 2015). Primarily, insects use wind direction as a directional cue to point them towards the emitting resource (Conchou et al., 2019), during which more complex navigational strategies can be used such as flying in a zigzag motion upwind to catch an olfactory trail of a certain resource (Cardé & Willis, 2008). Insects can display various modes of

movement, allowing them to travel distances ranging from several meters up to kilometres (Kristensen et al., 2013; Murlis et al., 1992). Future experiments should therefore be performed to investigate how parasitoids will experience mVOCs in more natural settings, in combination with food, host or habitat odours, and other disturbing factors.

6.2.5 Potential applications of the research results to improve biological pest control

While the provisioning of supplemental food sources and attractants to lure and augment natural enemy populations in the crop appears to be a promising approach to increase biocontrol efficacy, a major challenge in biological pest control remains to attract and retain the beneficial insects in the crop so that they can control the pest insects whenever needed. In this PhD study, we have shown that mVOCs are able to (selectively) attract beneficial insects, particularly *A. colemani*. Additionally, we have developed a sugar solution that predominantly supports *Aphidius* parasitoids, while being less supportive for *D. aphidum*. As performed in **Chapter 5**, in future applications an attractive blend of synthetic volatiles could be sprayed on crop plants to induce parasitoid attraction, while supporting them in the crop with sugar-providing feeding stations (Shimoda et al., 2014). Alternatively, both findings could potentially be combined in the development of a parasitoid-attracting feeding station providing the parasitoids with a suitable sugar solution, while simultaneously releasing selective mVOCs to attract the beneficial insects. The use of attractive volatile compounds in combination with a feeding station can be considered a form of an "attract and reward strategy", where the volatiles attract and retain the natural enemies and the sugar solution acts as a reward and simultaneously sustains them. The combination of both elements is believed to be important, as it is assumed that attraction of natural enemies by itself is often not enough to enhance biological control efficacy when the natural enemies do not find essential resources such as hosts, food, or shelter. Furthermore, response to a semiochemical without a suitable reward or resource present might weaken or eliminate future responses to these semiochemicals due to mechanisms such as habituation (Blassioli-Moraes et al., 2019, Meiners & Peri, 2013), especially in parasitoids. Parasitoids are known for their ability of associative learning (i.e. the ability to learn associations between a stimulus and a positive

or negative experience) (Sobhy et al., 2019; Wäckers & Lewis, 1999). Conversely, associative learning will allow parasitoids to learn the olfactory cues associated with the food reward (Wäckers & Lewis, 1994, 1999). Subsequently, they will be able to use this acquired information in the future to modify their innate parasitoid behaviour, which will allow them to find the food sources more efficiently, and therefore increase their reproductive success (Smid & Vet, 2016). So far, the attract and reward strategy is mainly used with synthetic HIPVs to attract naturally-occurring natural enemies to nectar-producing flowers near the crops, but it can also be applied in augmentative biological control. In several studies, these strategies have been proven to effectively attract natural enemies and improve pest suppression (Rodriguez-Saona et al., 2012; Simpson et al., 2011b,c, 2013). However, obtained results have not always been consistent (Orre Gordon et al., 2013), which stresses the need for further investigation of the efficacy of such a strategy. Furthermore, it remains to be investigated how this approach of attracting and feeding natural enemies affects pest suppression.

First of all, one crucial prerequisite that should be investigated before implementing such strategy is that the parasitoids used are sugar limited in the agricultural system. Only then sugar provisioning will significantly affect parasitoid life-history traits and improve their fitness compared to situations without sugar provision (Tena et al., 2013b, 2015). This can be achieved by checking the level of food deprivation in the parasitoids and by closely monitoring available sugar sources in the environment. However, it can be assumed that parasitoids in modern agricultural systems will be sugar limited due to scarcity or absence of common sugar sources in these systems. Furthermore, in order for the feeding station to efficiently provide sugars several parameters require optimisation, such as the feeding efficiency of the parasitoid, sugar concentration and the amount supplied, replenishment interval, and density and positioning of the feeding stations. Replenishment will most likely depend on the sugars and concentration used in the feeding station, as some sugars tend to crystallise faster than others (Wäckers, 2001). Moreover, microbial contamination of the sugar source might require timely replenishments. The placement of the feeding stations can also be important, as it has been shown that the height of a feeding station can strongly affect the utilisation efficiency of the parasitoids (Mitsunaga et al., 2012). Furthermore, adding an attractive colour to the station may improve its localisation in addition to the applied volatiles (Mitsunaga et al., 2012).

Besides identifying selective semiochemicals, also careful considerations should be made on how the volatiles should be applied in combination with the sugar source. Additional research is required to determine optimal density and concentration of the applied semiochemicals. The atmospheric concentration of these volatiles is dictated by the total amount released in the environment. This will be determined by the density of feeding stations applied and by how much compound is released over time per feeding station (Kaplan, 2012). The latter is determined by the method of volatile release, for which several options are available. Semiochemicals have already been applied by either spraying them directly on the crop or using a slow-release dispenser (Simpson et al., 2011a, Uefune et al., 2012). Our results indicated that volatile concentration can be an important factor. In this scenario, releasing too little volatiles may not induce sufficient attraction, whereas releasing to much might disturb attraction or even induce repellence (Whitman & Eller, 1992). However, recent evidence suggests that the risk of releasing too high concentrations seems relatively low (Kaplan, 2012). When volatiles are sprayed on the plants there is no control over the release rate of the volatiles and this might induce additional reactions in the plant, which possibly can affect parasitoid behaviour. A slow-release dispenser will allow controlled, uniform release of the volatiles that have shown to effectively attract the parasitoids. Several formulations and dispenser types have been developed with different slow-release properties (Heuskin et al., 2011). The choice of dispenser type will depend on the type of volatiles to release and the desired release rate. In addition, the density of feeding stations should also be based on the flight capacity of the target natural enemy, because this affects the spatial scale at which a foraging natural enemy can respond to the emitted volatiles (Roland & Taylor, 1997).

The results obtained in this PhD study can also be implemented in other strategies to potentially improve biological control programmes. While semiochemicals have widely been used for the monitoring of pest insects (Witzgall et al., 2010) in integrated pest management, the microbial volatiles discovered here could also be implemented to monitor the establishment and population density of *Aphidius* parasitoids. This information will aid in early detection of population declines of the parasitoids and therefore determine moments at which additional parasitoids should be released. Furthermore, volatiles selectively attractive to hyperparasitoids can aid in the monitoring and early discovery of hyperparasitoid presence. In addition, our results on the hyperparasitoid *D. aphidum* open up new opportunities to develop novel semiochemical-

based strategies to manage hyperparasitoids. Currently, very little is known about the chemical ecology of hyperparasitoids, which limits the semiochemical toolbox available to combat this understudied group of pest species (Cusumano et al., 2019a). In this study we have discovered two interesting strains which were either neutral (ST18.16/085) or repellent (ST18.16/160) to *A. colemani* whereas they were both attractive to *D. aphidum*. Additionally, preliminary data on the olfactory response of *D. aphidum* to individual compounds revealed that linalool was significantly attractive (de Boer & Goelen; data not shown). Recently, it has been shown that *Aphidius ervi* can be repelled by linalool at certain concentrations (Takemoto & Takabayashi, 2015), which makes it even more promising to be used in the management of *D. aphidum*. In this regard, a push-pull strategy could be developed in which repellent volatiles deter hyperparasitoids from the crop with a minimum effect on primary parasitoid behaviour, while attractive volatiles could pull them into traps, eventually killing them. More research is needed to determine whether this is a plausible scenario.

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Appendix

Appendix to Chapter 1: No supplementary files for Chapter 1.

Appendix to Chapter 2:

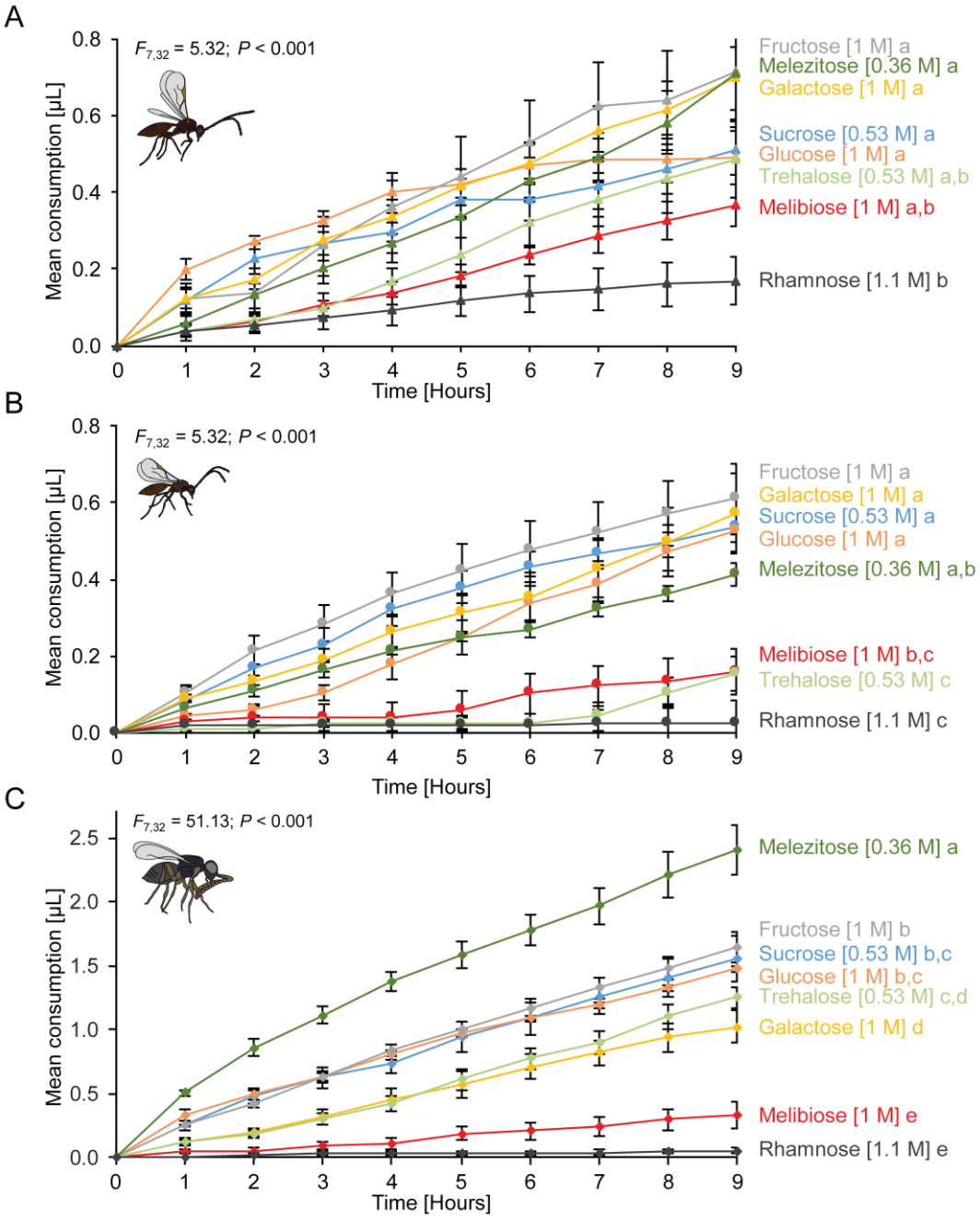

Figure A2.1: Mean sugar consumption (±SE) when single sugars were provided at equal weight concentrations (mass:volume of 180:1 g/l) to unfed adult parasitoids. The tested parasitoids were **(A)** *Aphidius colemani*, **(B)** *Aphidius matricariae* and **(C)** *Dendrocerus aphidum*. Experiments were performed using a capillary feeder (CAFE) assay (5 replicates; 15 individuals per replicate). Statistical differences were assessed using repeated measures ANOVA comparing consumption of the different solutions over the course of 9 h. Different letters indicate that sugar consumption was significantly different at the 95 % confidence level.

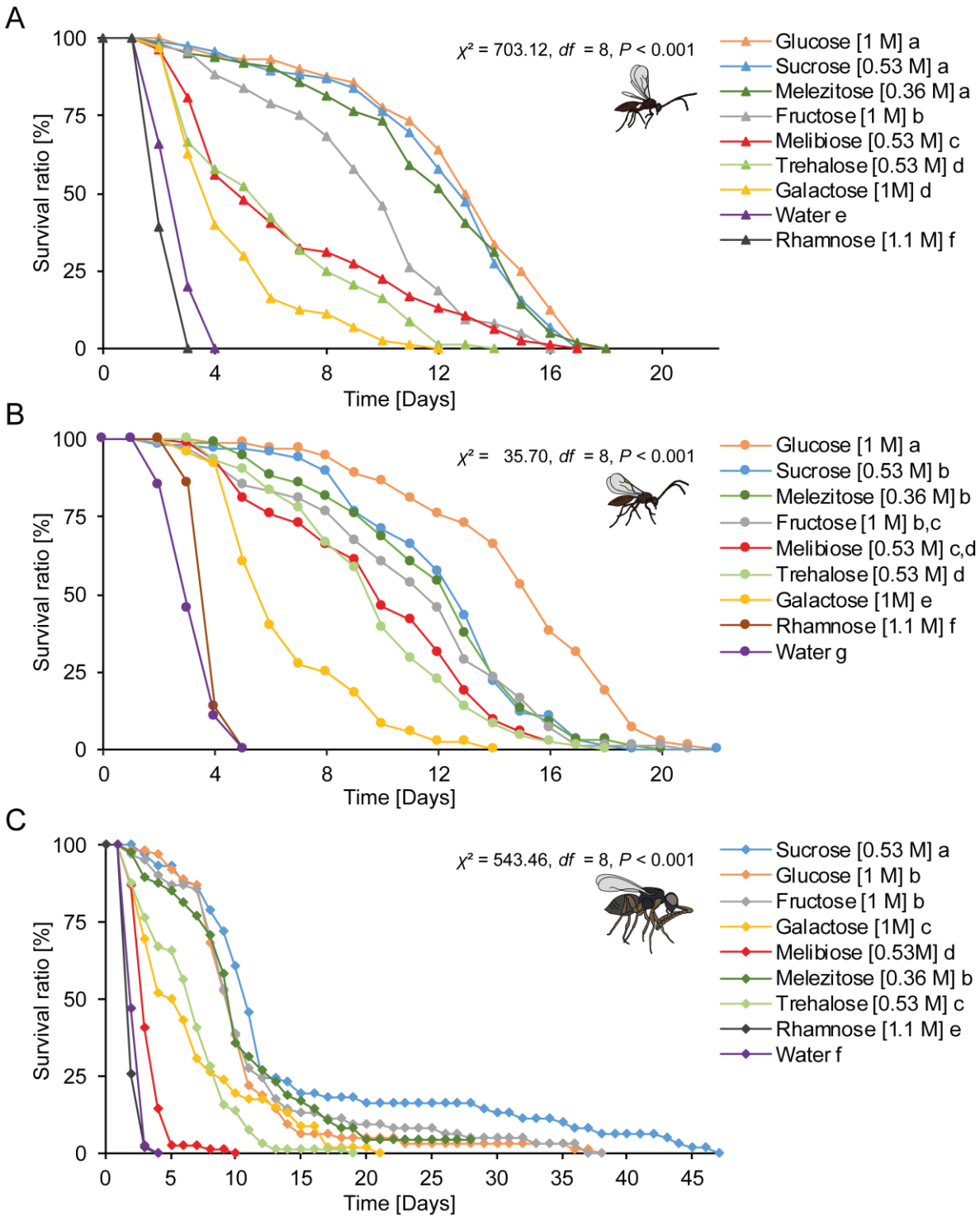

Figure A2.2: Survival time curves of adult parasitoids supplied with different sugars at equal weight concentrations (mass:volume of 180:1 g/l). The tested parasitoids were **(A)** *Aphidius colemani*, **(B)** *Aphidius matricariae* and **(C)** *Dendrocerus aphidum*. Experiments were performed using a capillary feeder (CAFE) assay in which a total of 75 individuals were examined (distributed over 5 containers with 15 parasitoids each). Feeding capillaries were replaced daily to prevent microbial contamination. Different letters indicate that treatments were significantly different at a 95% confidence level.

Appendix to Chapter 3:

Table A3.1: Microbial volatile organic compound (mVOC) composition* of the cell-free cultivation media used in this study.

Class	Compound	Retention index	Peak area (×10⁴) Blank medium	ST18.16/150	ST18.16/133	ST18.16/043	ST18.16/085	ST18.17/002	ST18.17/028	ST18.16/160
Alcohol	ethanol	578	8796 ± 53[a]	10206 ± 948[a]	9129 ± 369[a]	8762 ± 841[a,b]	7776 ± 431[a,b]	7836 ± 527[a,b]	8323 ± 296[a,b]	6943 ± 597[b]
	3-methyl-4-penten-2-ol	686	0.31 ± 0.06[b]	0.38 ± 0.17[a,b]	1.8 ± 0.51[a,b]	0.30 ± 0.04[a,b]	1.9 ± 0.42[a,b]	3.3 ± 1.5[a,b]	1.9 ± 0.49[a,b]	3.7 ± 1.5[a]
	isopentyl alcohol	729	0.23 ± 0.06[b]	60 ± 13[b]	199 ± 5.2[b]	58 ± 25[b]	310 ± 6.4[b]	174 ± 23[b]	5030 ± 914[a]	914 ± 85[b]
	2-methyl-1-butanol	731	1.3 ± 0.10[b]	2.2 ± 0.98[b]	0.21 ± 0.11[b]	0.69 ± 0.24[b]	125 ± 4.6[b]	2.3 ± 1.1[b]	578 ± 123[a]	0.09 ± 0.01[b]
	3-methyl-2-buten-1-ol	750	1.2 ± 0.12[b]	3.6 ± 1.5[a,b]	60 ± 1.1[a]	3.7 ± 1.3[a,b]	8.9 ± 1.1[a,b]	18 ± 5.4[a,b]	6.1 ± 2.69[a,b]	5.6 ± 2.7[a,b]
	2,3-butanediol	752	0.15 ± 0.03[b]	4.7 ± 3.3[a,b]	533 ± 42[a]	1.2 ± 0.50[a,b]	4.1 ± 1.0[a,b]	515 ± 140[a]	41 ± 5.2[a,b]	1.2 ± 0.16[a,b]
	2-methyl-mercaptoethanol	819	0.17 ± 0.04[b]	1.2 ± 0.65[a,b]	0.22 ± 0.04[a,b]	5.0 ± 4.07[a,b]	0.92 ± 0.20[a,b]	31 ± 3.1[a]	74 ± 32[a]	2.5 ± 0.01[a,b]
	pinacol	849	21 ± 5.0[c]	18 ± 5.1[c]	86 ± 7.9[a]	16 ± 6.4[c]	27 ± 7.3[b,c]	86 ± 9.4[a]	16 ± 6.9[c]	58 ± 11[a,b]
	n-hexanol	869	18 ± 1.5[d]	33 ± 4.7[b,c,d]	64 ± 4.1[b,c,d]	28 ± 12[c,d]	74 ± 4.9[a]	60 ± 16[b,c,d]	74 ± 14[a]	45 ± 0.83[b,c,d]
	4-heptanol	894	5.6 ± 0.21[b]	2.1 ± 1.2[b]	9.1 ± 1.1[b]	1.8 ± 0.98[b]	5.0 ± 1.3[b]	502 ± 160[a]	27 ± 5.9[b]	1.5 ± 0.70[b]
	1-butoxy-2-propanol	951	212 ± 14[a]	133 ± 52[a,b]	37 ± 37[b]	29 ± 28[b]	8.1 ± 1.4[b]	28 ± 26[b]	29 ± 28[b]	46 ± 45[b]
	3-(methylthio)-1-propanol	989	20 ± 1.4[b]	158 ± 32[a]	48 ± 4.0[a,b]	25 ± 9.8[b]	42 ± 1.6[a,b]	3.3 ± 0.44[b]	72 ± 20[a,b]	5.0 ± 1.5[b]
	2-methyl-2-octen-4-ol	1028	17 ± 0.72[b]	16 ± 1.4[b]	1262 ± 206[a]	14 ± 1.0[b]	18 ± 1.5[b]	22 ± 3.2[b]	17 ± 1.3[b]	43 ± 5.1[b]
	4-methyl-2-propyl-1-pentanol	1033	37 ± 3.1[b]	71 ± 29[b]	20 ± 8.7[b]	52 ± 8.7[b]	246 ± 9.5[a]	52 ± 20[b]	45 ± 15[b]	47 ± 9.1[b]
	2-methyl-6-methylene-2-octanol	1077	71 ± 9.5[b]	64 ± 5.2[b]	371 ± 21[a]	105 ± 17[b]	196 ± 14[b]	173 ± 35[b]	175 ± 44[b]	164 ± 25[b]
	n-octanol	1078	61 ± 10[b]	125 ± 22[b]	29 ± 5.1[b]	63 ± 25[b]	123 ± 3.3[b]	25 ± 7.9[b]	948 ± 219[a]	70 ± 22[b]
	nonan-2-ol	1111	2.1 ± 0.46[b]	7.2 ± 3.2[b]	6.0 ± 0.52[b]	11 ± 6.6[b]	140 ± 6.9[a]	2.7 ± 1.2[b]	10 ± 2.8[b]	1.5 ± 0.52[b]
	2-phenylethanol	1144	0.03 ± 0.02[b]	2.9 ± 1.3[a,b]	20 ± 5.3[a,b]	1.01 ± 0.37[b]	67 ± 4.1[a]	16 ± 4.9[a,b]	28 ± 9.7[a,b]	1.3 1.1 0.29[b]
	isoborneol	1162	11 ± 0.92[b]	8.5 ± 0.09[b]	12 ± 2.3[b]	17 ± 0.70[a,b]	18 ± 0.74[a,b]	22 ± 2.8[a]	11 ± 3.5[b]	16 ± 1.9[a,b]
	4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol	1183	9.0 ± 0.54[c]	20 ± 3.5[a,b,c]	18 ± 1.8[a,b,c]	23 ± 4.1[a,b,c]	43 ± 3.6[a]	17 ± 3.9[a,b,c]	35 ± 8.1[a,b]	24 ± 4.2[a,b,c]
	α-methyl-cyclohexanepropanol	1249	0.95 ± 0.09[b]	3.0 ± 1.8[b]	0.94 ± 0.78[b]	1.5 ± 1.3[b]	64 ± 6.5[a]	0.41 ± 0.05[b]	1.6 ± 0.11[b]	2.7 ± 1.6[b]
	9-decen-1-ol	1260	0.01 ± 0.01[b]	0.59 ± 0.19[b]	1.4 ± 0.72[a,b]	0.22 ± 0.12[b]	1.5 ± 0.15[a,b]	0.59 ± 0.59[b]	1.3 ± 0.82[a,b]	3.9 ± 0.96[a]

Class	Compound	Retention index	Peak area (×10⁴) Blank medium	ST18.16/150	ST18.16/133	ST18.16/043	ST18.16/085	ST18.17/002	ST18.17/028	ST18.16/160
	n-decanol	1280	4.0 ± 1.0^{b}	107 ± 58^{b}	17 ± 1.4^{b}	27 ± 14^{b}	62 ± 5.7^{b}	12 ± 7.3^{b}	561 ± 98^{a}	73 ± 30^{b}
	2-(3,3-dimethylcyclohexylidene)-ethanol	1288	11 ± 0.86^{b}	14 ± 5.5^{b}	10 ± 1.5^{b}	9.4 ± 2.3^{b}	22 ± 6.8^{a,b}	19 ± 8.3^{a,b}	39 ± 3.7^{a}	37 ± 4.3^{a}
	n-tetradecanol	1675	3.0 ± 0.23^{b}	0.85 ± 0.44^{b}	0.63 ± 0.10^{b}	0.67 ± 0.37^{b}	0.34 ± 0.04^{b}	0.21 ± 0.18^{b}	233 ± 29^{a}	3.8 ± 1.7^{b}
Aldehyde	acetaldehyde	578	2494 ± 19.0^{b}	2894 ± 499^{a}	2457 ± 115^{b}	2602 ± 177^{b}	2481 ± 113^{b}	2309 ± 223^{b}	2285 ± 190^{b}	2257 ± 211^{b}
	butanal	612	86 ± 7.9^{b}	159 ± 26^{b}	62 ± 5.6^{b}	330 ± 90^{a,b}	40 ± 8.9^{b}	610 ± 189^{a}	50 ± 15^{b}	79 ± 15^{b}
	3-methylthio-propionaldehyde	915	44 ± 3.3^{a}	8.8 ± 1.3^{b}	14 ± 6.4^{b}	14 ± 1.4^{b}	36 ± 1.4^{a}	4.7 ± 0.95^{b}	5.0 ± 1.6^{b}	5.9 ± 0.56^{b}
	phenylacetaldehyde	1048	335 ± 29^{a}	276 ± 110^{b,c}	40 ± 3.5^{b,c}	178 ± 36^{b}	155 ± 8.5^{b,c}	47 ± 12^{b,c}	105 ± 30^{b,c}	91 ± 12^{b,c}
	3,5-dimethyl-benzaldehyde	1228	2.9 ± 1.3^{c}	60 ± 19^{c}	9.1 ± 1.9^{c}	11 ± 7.9^{c}	116 ± 20^{b,c}	21 ± 11^{c}	365 ± 123^{a}	343 ± 56^{a,b}
Ester	isobutyl-formate	670	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.11 ± 0.08^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.04 ± 0.04^{a,b}	0.02 ± 0.02^{a,b}	0.00 ± 0.00^{b}
	butyl-formate	672	2571 ± 117^{b,c}	1751 ± 157^{c}	2890 ± 216^{b,c}	3006 ± 10^{b,c}	3178 ± 46^{a,b}	2919 ± 82^{b,c}	3250 ± 235^{a,b}	3634 ± 17^{a}
	ethyl butanoate	757	0.05 ± 0.01^{b}	19 ± 19^{a,b}	2.3 ± 0.63^{a,b}	0.11 ± 0.11^{b}	0.61 ± 0.14^{a,b}	2.7 ± 0.44^{a,b}	20 ± 7.1^{a}	1.1 ± 0.06^{a,b}
	butyl acetate	780	6.81 ± 0.66^{d}	190 ± 67^{d}	1314 ± 447^{b,c}	411 ± 146^{c,d}	267 ± 12.6^{d}	2063 ± 202^{a,b}	183 ± 49^{d}	2937 ± 234^{a}
	ethyl-3-methyl butanoate	850	0.00 ± 0.00^{b}	0.13 ± 0.09^{b}	65 ± 9.8^{a}	1.8 ± 1.8^{b}	2.1 ± 0.23^{b}	57 ± 6.5^{a}	10 ± 2.6^{b}	100 ± 3.9^{a}
	butyl propanoate	916	0.86 ± 0.12^{b}	1.1 ± 0.16^{b}	331 ± 151^{a}	29 ± 4.8^{b}	9.4 ± 1.3^{b}	43 ± 9.1^{b}	5.9 ± 1.9^{b}	3.7 ± 0.95^{b}
	butyl-isobutyrate	961	51 ± 2.6^{b}	64 ± 1.6^{b}	63 ± 11^{b}	434 ± 71^{a}	56 ± 0.85^{b}	77 ± 4.0^{b}	42 ± 4.6^{b}	42 ± 4.7^{b}
	butyl-butanoate	1000	8.3 ± 0.64^{e}	7.7 ± 1.3^{e}	5.1 ± 2.8^{e}	102 ± 13^{a,b}	133 ± 8.2^{a}	70 ± 9.5^{b,c}	49 ± 11^{c,d}	25 ± 2.3^{d,e}
	butyl 2-methyl butanoate	1042	8.2 ± 5.4^{b}	8.5 ± 0.06^{b}	18 ± 2.6^{b}	168 ± 58^{a}	14 ± 1.1^{b}	6.6 ± 1.1^{b}	20 ± 0.78^{b}	13 ± 1.9^{b}
	butyl Isovalerate	1050	1.5 ± 0.03^{b}	2.3 ± 1.1^{b}	15 ± 3.1^{a,b}	116 ± 51^{a}	1.3 ± 0.18^{b}	0.38 ± 0.05^{b}	1.5 ± 0.93^{b}	2.1 ± 0.68^{b}
	o-tert-butyl cyclohexyl acetate	1295	29 ± 3.2^{c}	37 ± 17^{b,c}	44 ± 2.8^{b,c}	48 ± 3.2^{b,c}	96 ± 2.6^{b,c}	135 ± 55^{a}	133 ± 11^{a}	95 ± 5.9^{b,c}
	butyl-isobutyl-phthalate	1886	220 ± 23^{a}	145 ± 15^{a,b}	94 ± 14^{a,b}	137 ± 13^{b}	67 ± 11^{b}	61 ± 9^{b}	117 ± 7.2^{a,b}	135 ± 20^{a,b}
Ketone	2,3-butanedione	611	8.8 ± 1.06^{b}	602 ± 60^{a}	585 ± 34^{a}	27 ± 13^{b}	108 ± 16^{b}	361 ± 59^{a}	379 ± 99^{a}	41 ± 4.0^{b}
	2-butanone	612	22 ± 1.7^{b}	20 ± 9.0^{b}	17 ± 1.1^{b}	73 ± 27^{a,b}	59 ± 19^{a,b}	420 ± 213^{a}	21 ± 11^{b}	34 ± 9.4^{b}
	2,3-pentanedione	679	188 ± 56^{a}	202 ± 129^{b,c}	14 ± 7.0^{b,c}	75 ± 13^{b,c}	20 ± 5.2^{b,c}	12 ± 0.69^{b,c}	26 ± 7.1^{b,c}	81 ± 15^{a,b}
	1-hydroxy-2-propanone	684	0.52 ± 0.13^{b}	4.97 ± 0.26^{b}	13 ± 0.76^{a,b}	4.0 ± 0.61^{b}	5.6 ± 1.2^{b}	5.5 ± 1.1^{b}	24 ± 7.8^{a}	8.4 ± 0.53^{b}
	acetoin	714	58 ± 3.9^{b}	134 ± 35^{b}	22607 ± 781^{a}	85 ± 9.4^{b}	36 ± 6.1^{b}	8466 ± 710^{b}	27333 ± 3836^{a}	1124 ± 144^{b}
	4-methyl-pentan-2-one	734	0.00 ± 0.00^{b}	0.01 ± 0.01^{b}	98 ± 24^{a}	0.02 ± 0.01^{b}	0.11 ± 0.03^{b}	0.31 ± 0.05^{b}	0.89 ± 0.56^{b}	0.2 ± 0.03^{b}

Class	Compound	Retention index	Peak area (×10⁴) Blank medium	ST18.16/150	ST18.16/133	ST18.16/043	ST18.16/085	ST18.17/002	ST18.17/028	ST18.16/160
	2-hexanone	755	2.5 ± 0.26^{d}	21 ± 7.0^{c,d}	277 ± 7.0^{a}	12 ± 1.6^{d}	94 ± 2.8^{b}	55 ± 8.7^{b,c}	8.9 ± 4.2^{d}	13 ± 1.2^{d}
	4-methyl-2-heptanone	948	8.0 ± 0.32^{b}	146 ± 63^{a,b}	136 ± 14^{a}	119 ± 23^{a,b}	86 ± 9.8^{a,b}	35 ± 3.6^{a,b}	81 ± 24^{a,b}	91 ± 11^{a,b}
	5,5-dimethyl-2,4-hexanedione	1020	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	1465 ± 72^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	4.5 ± 4.5^{b}	0.13 ± 0.03^{b}	0.00 ± 0.00^{b}
	acetophenone	1073	56 ± 7.8^{b}	105 ± 37^{b}	178 ± 76^{a,b}	182 ± 68^{a,b}	469 ± 3.4^{a}	150 ± 49^{a,b}	263 ± 145^{a,b}	252 ± 86^{a,b}
	4-cyclohexyl-2-butanone	1237	3.7 ± 0.19^{b}	0.69 ± 0.39^{b}	0.00 ± 0.00^{b}	0.50 ± 0.41^{b}	41 ± 1.8^{a}	2.2 ± 0.74^{b}	2.1 ± 1.0^{b}	0.55 ± 0.40^{b}
	undecan-2-one	1298	3.2 ± 0.46^{b}	5.2 ± 0.60^{b}	9.0 ± 1.5^{a,b}	4.7 ± 1.9^{a,b}	14 ± 5.5^{a,b}	7.0 ± 2.2^{a,b}	223 ± 127^{a}	9.2 ± 0.77^{a,b}
	tridecan-2-one	1487	1.2 ± 0.23^{b}	1.1 ± 0.10^{b}	1.9 ± 0.39^{b}	2.4 ± 0.71^{b}	7.9 ± 0.39^{a}	2.2 ± 0.84^{b}	3.0 ± 0.50^{b}	1.7 ± 0.88^{b}
Alkane	n-hexane	611	3.5 ± 1.1^{b}	38 ± 16^{a,b}	137 ± 57^{a}	40 ± 24^{a,b}	0.81 ± 0.41^{b}	69 ± 10^{a,b}	0.00 ± 0.00^{b}	18 ± 14^{b}
	2,2,4-trimethyl-pentane	674	5501 ± 306^{a}	4672 ± 868^{a}	1880 ± 243^{b}	3929 ± 157^{a}	2716 ± 120^{a,b}	2095 ± 94^{b}	2440 ± 256^{b}	3629 ± 198^{a,b}
	n-octane	760	15 ± 0.8^{a,b,c}	11 ± 2.4^{b,c}	1.9 ± 0.55^{c}	16 ± 1.6^{a,b}	22 ± 1.6^{a}	4.90 ± 2.9^{b,c}	8.0 ± 2.3^{b,c}	18 ± 3.5^{a,b}
	nonane	882	7.0 ± 0.99^{a}	0.50 ± 0.29^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.07 ± 0.07^{b}	0.00 ± 0.00^{b}
	tetradecane	1403	0.01 ± 0.00^{b}	0.11 ± 0.00^{b}	0.01 ± 0.00^{b}	0.05 ± 0.03^{b}	11 ± 5.4^{a}	1.6 ± 0.66^{b}	1.4 ± 0.32^{b}	1.0 ± 0.79^{b}
Cycloalkane	1,3-dimethyl-cyclopentane	687	31 ± 3.6^{b}	43 ± 4.9^{b}	65 ± 5.4^{a,b}	32 ± 3.8^{b}	69 ± 5.7^{a,b}	86 ± 17^{a}	85 ± 12^{a}	96 ± 8.3^{a}
	1,5-dimethyl-6-oxa-bicyclo[3,1,0]hexane	728	0.00 ± 0.00^{b}	3.4 ± 3.3^{b}	4.3 ± 0.48^{b}	0.06 ± 0.06^{b}	2.9 ± 1.6^{b}	0.90 ± 0.19^{b}	38 ± 11^{a}	8.0 ± 0.96^{b}
Alkene	1-decene	996	2.2 ± 0.42^{b}	0.37 ± 0.13^{b}	0.00 ± 0.00^{b}	6 ± 2.4^{b}	19 ± 2.8^{a}	1.7 ± 1.2^{b}	4.8 ± 0.75^{b}	3.2 ± 1.0^{b}
	9-methyl-1-decene	1033	46 ± 4.6^{b}	51 ± 13^{b}	10 ± 5.2^{b}	48 ± 12^{b}	218 ± 17^{a}	79 ± 25^{b}	56 ± 12^{b}	43 ± 8.2^{b}
	1-tetradecene	1396	0.38 ± 0.14^{b}	8 ± 1.1^{b}	1.1 ± 0.09^{b}	1.5 ± 0.30^{b}	1.43 ± 0.95^{b}	0.86 ± 0.50^{b}	31 ± 9.4^{a}	7.8 ± 0.54^{b}
Aromatic	benzene	679	87 ± 18^{a}	35 ± 12^{b}	9.1 ± 2.1^{b}	42 ± 7.8^{b}	24 ± 6.8^{b}	11 ± 1.1^{b}	22 ± 6.9^{b}	51 ± 5.2^{a,b}
	1,3-dimethyl-benzene	860	3.3 ± 0.99^{c}	12 ± 3.5^{b,c}	42 ± 9.9^{a,b}	6.5 ± 5.9^{c}	21 ± 5.2^{b,c}	36 ± 7.7^{a,b}	22 ± 6.2^{b,c}	42 ± 2.8^{a}
	1-ethyl-2-methyl-benzene	984	1.23 ± 0.18^{b}	12 ± 1.3^{a}	3.1 ± 1.4^{a,b}	1.68 ± 0.35^{a,b}	4.2 ± 0.22^{a,b}	2.8 ± 0.19^{a,b}	3.3 ± 0.65^{a,b}	2.2 ± 0.07^{a,b}
	cyclopropyl-benzene	1028	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	3.3 ± 0.72^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
	para-cymene	1060	1.2 ± 0.48^{d}	7.7 ± 0.99^{d}	14 ± 0.88^{c,d}	4.02 ± 1.5^{d}	7.6 ± 0.84^{d}	39 ± 3.6^{a}	22 ± 3.1^{b,c}	3.6 ± 0.67^{d}
	ortho-cymene	1063	11 ± 1.3^{a}	4.1 ± 0.81^{b,c}	0.43 ± 0.02^{c}	4.2 ± 0.74^{b}	1.5 ± 0.03^{b,c}	0.89 ± 0.26^{b,c}	2.7 ± 0.94^{b,c}	1.7 ± 0.34^{b,c}
	indole	1290	1.3 ± 0.06^{b}	2.0 ± 1.0^{b}	0.75 ± 0.16^{b}	0.79 ± 0.02^{b}	1.9 ± 1.1^{b}	0.30 ± 0.20^{b}	20 ± 6.0^{a}	4.8 ± 1.3^{b}
Organic acid	acetic acid	647	8.5 ± 2.3^{b}	32 ± 2.5^{b}	44 ± 7.6^{b}	17 ± 2.6^{b}	706 ± 108^{b}	109 ± 32^{b}	619 ± 403^{b}	7598 ± 441^{a}
	2-methyl-propanoic acid	746	102 ± 4.0^{c}	87 ± 24^{c}	200 ± 23^{b,c}	140 ± 21^{b,c}	262 ± 10^{a,b}	149 ± 58^{b,c}	165 ± 37^{b,c}	334 ± 12^{a}

Class	Compound	Retention index	Peak area (×10⁴) Blank medium	ST18.16/150	ST18.16/133	ST18.16/043	ST18.16/085	ST18.17/002	ST18.17/028	ST18.16/160
	butanoic acid	769	0.77 ± 0.32^{e}	65 ± 15^{d,e}	170 ± 11^{c}	16 ± 8.9^{e}	280 ± 10^{b}	95 ± 14^{d}	494 ± 20^{a}	433 ± 15^{a}
	3-methyl-pyruvic acid	777	17 ± 0.70^{b}	16 ± 1.9^{b}	462 ± 40^{a}	23 ± 5.5^{b}	13 ± 1.0^{b}	131 ± 18^{a,b}	11 ± 5.3^{b}	176 ± 14^{a,b}
	3-methyl-butanoic acid	855	1.2 ± 0.57^{c}	17 ± 11^{c}	1488 ± 224^{b}	23 ± 22^{c}	28 ± 2.8^{c}	1035 ± 193^{b}	157 ± 30^{c}	2610 ± 202^{a}
	2-methyl-butanoic acid	868	1.2 ± 0.09^{b}	1.2 ± 0.17^{b}	1155 ± 84^{a}	73 ± 70^{b}	142 ± 14^{b}	846 ± 208^{a}	108 ± 21^{b}	1015 ± 115^{a}
	n-heptanoic acid	1093	3.5 ± 0.04^{b}	14 ± 8.0^{b}	7.6 ± 0.67^{b}	3.2 ± 0.07^{b}	6.7 ± 4.7^{b}	1.7 ± 0.64^{b}	107 ± 31^{a}	41 ± 11^{a,b}
	2-ethyl-hexanoic acid	1135	1.0 ± 0.17^{c}	7.6 ± 3.3^{c}	6.41 ± 1.1^{c}	3.4 ± 0.40^{c}	133 ± 55^{a}	23 ± 4.9^{c}	80 ± 18^{a,b}	52 ± 6.3^{a,b}
	3,3-dimethyl-heptanoic acid	1152	0.63 ± 0.34^{b}	2.4 ± 1.4^{b}	2.9 ± 1.4^{b}	0.12 ± 0.04^{b}	11 ± 0.69^{b}	7.6 ± 3.8^{b}	53 ± 8.8^{a}	36 ± 5.2^{a}
	octanoic acid	1194	9.5 ± 3.6^{c}	117 ± 11^{c}	187.1 ± 25^{c}	42 ± 6.9^{c}	191 ± 92^{c}	68 ± 7.8^{c}	2308 ± 63^{a}	779 ± 142^{b}
	nonanoic acid	1288	21 ± 6.7^{c}	373 ± 31^{c}	379 ± 104^{c}	82 ± 44^{c}	931 ± 429^{b,c}	118 ± 43^{c}	4156 ± 308^{a}	1658 ± 402^{b}
	isobornyl acrylate	1376	0.00 ± 0.00^{b}	2.5 ± 1.5^{b}	0.12 ± 0.09^{b}	0.48 ± 0.47^{b}	3.99 ± 2.6^{a,b}	34 ± 17^{a}	4.0 ± 2.7^{a,b}	0.86 ± 0.74^{b}
	methyl-ethyl-adipate	1389	1.3 ± 0.25^{b}	8.5 ± 0.09^{b}	1.8 ± 0.93^{b}	2.1 ± 0.28^{b}	22 ± 7.6^{a,b}	13 ± 8.0^{a,b}	45 ± 17^{a}	11 ± 3.3^{a,b}
Terpene	limonene	1031	17 ± 1.2^{b}	16 ± 1.0^{b}	0.77 ± 0.32^{b}	13 ± 2.3^{b}	35 ± 1.5^{a}	16 ± 3.7^{b}	18 ± 3.7^{b}	21 ± 2.0^{b}
	linalool	1106	34 ± 4.6^{c}	61 ± 2.8^{b,c}	63 ± 3.7^{b,c}	59 ± 3.09^{b,c}	150 ± 6.6^{b}	65 ± 14^{b,c}	79 ± 15^{b,c}	784 ± 68^{a}
	geraniol	1262	0.51 ± 0.13^{c}	1.3 ± 0.18^{c}	31 ± 4.2^{b}	1.3 ± 0.75^{c}	163 ± 8.4^{a}	12 ± 3.8^{b}	11 ± 4.8^{b}	119 ± 16^{a}
Misc.	azetidine	610	1.8 ± 0.27^{b}	24 ± 4.2^{a,b}	38 ± 11^{a,b}	11 ± 6.7^{a,b}	3.1 ± 1.0^{a}	66 ± 33^{a}	1.7 ± 1.2^{b}	7.9 ± 3.8^{a,b}
	1-butanamine	611	8.1 ± 2.3^{b}	82 ± 12^{a,b}	81 ± 12^{a,b}	64 ± 32^{a,b}	14 ± 5.2^{b}	187 ± 65^{a}	1.4 ± 0.28^{b}	17 ± 7.3^{b}
	ammonium acetate	649	0.00 ± 0.00^{b}	0.03 ± 0.03^{b}	2.7 ± 0.80^{b}	0.00 ± 0.00^{b}	37 ± 26^{b}	13 ± 9.3^{b}	47 ± 37^{b}	404 ± 115^{a}
	methyl pyrazine	808	5.6 ± 0.21^{a}	3.7 ± 0.27^{b,c}	2.2 ± 0.06^{c}	4.1 ± 0.19^{b}	3.85 ± 0.12^{b}	1.2 ± 0.15^{c}	1.9 ± 0.30^{c}	2.2 ± 0.08^{c}
	2-propyl-1,3-dioxolane	834	16 ± 0.44^{a}	3.9 ± 1.7^{b}	0.02 ± 0.01^{c}	4.8 ± 1.4^{b}	0.10 ± 0.02^{c}	0.46 ± 0.29^{c}	0.00 ± 0.00^{c}	0.27 ± 0.09^{c}
	dihydro-2-methyl-thiophen-3-one	987	0.28 ± 0.08^{c}	370 ± 42^{a}	75 ± 12^{b}	14 ± 2.5^{b,c}	17 ± 1.5^{b,c}	6.9 ± 1.7^{b,c}	21 ± 6.6^{b,c}	15 ± 1.5^{b,c}
	2-methyl-5-(1-methylethyl)-pyrazine	1057	17 ± 1.7^{b}	16 ± 2.8^{b}	19 ± 5.0^{b}	12 ± 0.26^{b}	36 ± 1.5^{a}	9.2 ± 1.2^{b}	14 ± 2.2^{b}	14 ± 1.2^{b}
	tetramethyl-pyrazine	1096	3.1 ± 1.1^{c}	6.2 ± 3.6^{b,c}	65 ± 6.9^{a}	1.4 ± 0.49^{c}	3.0 ± 0.43^{c}	20 ± 6.6^{b}	1.3 ± 0.40^{c}	0.95 ± 0.16^{c}
	benzothiazole	1226	5.0 ± 0.23^{b}	6.1 ± 2.4^{b}	2.4 ± 0.06^{b}	2.6 ± 0.19^{b}	9.8 ± 2.1^{b}	3.0 ± 1.4^{b}	29 ± 9^{a}	30 ± 4.2^{a}

*Peak areas and retention indices were recovered from a MXT-5 equipped GC-MS. Presented values are means (±SE) of three biological replicate fermentations (n = 3) with the tested bacterial strains. Different letters in each row indicate statistically significant differences between treatments ($P \leq 0.05$), based on a univariate ANOVA 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.

Appendix to Chapter 4:

Table A4.1: Results of the Y-tube olfactometer bioassay.

Isolate identifier	Preference Index (PI)[a]	*P*-value
ST14.14/060	0.22	0.107
ST12.14/235	0.32	**0.027**
ST14.14/007	0.28	**0.043**
ST14.14/047	0.35	**0.027**
ST14.14/029	0.22	0.106
ST14.14/046	0.00	0.995
ST04.14/017	0.21	0.112
B. aryabhattai LMG 24407	-0.04	0.794
B. asahii DSM 105779	-0.19	0.176
ST18.16/150	0.33	**0.011**
B. endophyticus LMG 21715	0.07	0.601
ST18.16/061	0.25	0.078
B. flexus DSM 1316	-0.55	**<0.001**
B. flexus LMG 11155	-0.29	**0.030**
ST12.15/030	0.23	0.099
B. iocasae DSM 104297	-0.03	0.796
B. koreensis DSM 16467	-0.28	**0.038**
ST18.16/073	0.15	0.244
ST12.15/036	-0.25	0.078
ST18.16/013	-0.22	0.079
ST12.15/034	-0.06	0.681
ST01.11/095	-0.18	0.212
ST14.12/130	-0.27	**0.046**
B. megaterium LMG 07127	-0.04	0.791
B. muralis LMG 20238	0.25	0.064
ST12.14/138	-0.05	0.699
ST18.16/133	0.30	**0.027**
ST14.12/094	0.27	**0.046**
B. qingshengii JCM 19454	-0.21	0.119
B. simplex DSM 100650	-0.15	0.280
B. simplex LMG 11160	-0.26	**0.049**
ST18.16/153	0.16	0.229
ST18.16/188	0.20	0.112
ST18.16/075	0.11	0.423
ST04.13/022	0.23	0.113
ST12.14/237	0.42	**0.005**
ST18.16/043	0.31	**0.025**
ST18.16/090	0.32	**0.018**
ST18.16/240	0.29	**0.030**
ST18.16/249	0.21	0.152

[a]Results are 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. *P*-values in bold indicate statistically significant differences when the response was compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model).

Table A4.2: Summary of phylogenetic signal indices and Mantel test used to analyse the mVOC profiles for the presence of a phylogenetic signal in individual compounds.

Compound	Pagel's λ	Moran's I	Abouheif's C_{mean}	Mantel test
butanal	**0.21**	**0,25**	**0.28**	**24.2**
2,3-butanedione	**0.70**	**0.65**	**0.66**	**28.4**
ammonium acetate	**0.57**	**0.44**	**0.46**	**25.5**
acetoin	**0.95**	**0.85**	**0.86**	**28.8**
isoamylamine	**0.88**	**0.83**	**0.83**	**29.0**
methyl-methacrylate	**0.35**	**0.31**	**0.32**	**24.6**
3-methyl-1-butanol	**0.52**	**0.32**	**0.35**	**22.4**
2-methyl-propanoic acid	**0.71**	**0.49**	**0.51**	**24.3**
2,3-butanediol	**0.75**	**0.44**	**0.47**	**21.9**
2-hexanone	**0.56**	**0.53**	**0.54**	**25.2**
ethyl-3-methyl-butanoate	**0.83**	**0.58**	**0.59**	**24.7**
cyclohexanone	**0.60**	**0.42**	**0.44**	**25.3**
benzaldehyde	**0.51**	**0.42**	**0.44**	**24.4**
2,3,5-trimethyl-pyrazine	**0.35**	**0.34**	**0.35**	**23.3**
eucalyptol	**0.29**	**0.28**	**0.29**	**25.4**
phenylacetaldehyde	**0.29**	**0.32**	**0.33**	**25.5**
2-methyl-6-methylene-2-octanol	**0.39**	**0.42**	**0.44**	**26.3**
tetramethyl-pyrazine	**0.42**	**0.45**	**0.46**	**25.6**
nonan-2-ol	**0.70**	**0.64**	**0.64**	**25.1**
2-ethyl-hexanoic acid	**0.58**	**0.51**	**0.52**	**25.3**
nonanoic acid	**0.36**	**0.32**	**0.33**	**24.7**
4-tert-butylcyclohexyl acetate	**0.37**	**0.26**	**0.27**	**24.2**
1-hydroxy-2,4,4-trimethylpentan-3-yl) 2-methylpropanoate	**0.62**	**0.38**	**0.40**	**25.2**
3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	**0.34**	**0.41**	**0.42**	**25.6**
2-ethyl-3-hydroxyhexyl 2-methylpropanoate	**0.40**	**0.26**	**0.27**	**24.4**
butyric acid	**0.62**	**0.38**	**0.40**	23.6
3-methyl-butanoic acid	**0.75**	**0.52**	**0.53**	24.6
2-methyl-butanoic acid	**0.97**	**0.25**	**0.27**	20.9
2-heptanone	**0.90**	**0.36**	**0.38**	22.5
butyl-propanoate	**0.70**	**0.37**	**0.39**	24.1
2,6-dimethyl-4H-pyran-4-one	**0.76**	**0.36**	**0.38**	17.9
5,5-dimethyl-2,4-hexanedione	**0.87**	**0.38**	**0.39**	19.0
4-methyl-2-propyl-1-pentanol	**0.49**	**0.36**	**0.38**	10.8
2-phenylpropanal	**0.42**	**0.28**	**0.30**	21.3
3,5-dimethyl-benzaldehyde	**0.24**	**0.26**	0.27	**23.2**
2,2-diisopropyl-1,3-dioxolane	**0.67**	**0.33**	0.34	15.4
butyl isobutyl phthalate	**0.31**	0.21	0.22	**22.6**
Methyl dehydroabietate	**0.27**	0.18	0.19	**23.3**
2-methyl-hexane	**1.00**	-0.05	0.03	6.2
isobutyl-formate	**1.00**	-0.01	0.03	9.7
butyl-formate	**1.00**	-0.05	-0.01	5.5
1-butanol	**1.00**	-0.05	0.03	6.2

Compound	Pagel's λ	Moran's I	Abouheif's C_{mean}	Mantel test
2,2,4-trimethyl-pentane	**1.00**	-0.06	0.02	6.5
2-pentanone	**0.99**	-0.05	0.03	8.5
2,3-pentandione	**1.00**	-0.07	-0.03	6.7
3,7-dimethyl-octan-3-ol	**0.49**	0.19	0.22	21.5
2-phenylethanol	**0.72**	0.15	0.18	19.3
3-methyl-hexadecane	**0.99**	0.02	0.05	8.2
ethyl-butanoate	0.13	**0.52**	**0.52**	**14.4**
pinacol	0.21	**0.27**	**0.28**	**23.0**
dimethyl disulphide	0.00	**0.25**	**0.26**	21.3
butyl-acetate	0.51	**0.36**	**0.38**	23.7
ethyl-2-methyl-butanoate	0.44	**0.26**	**0.29**	21.9
2-methyl-2-octen-4-ol	0.00	**0.21**	**0.23**	20.0
geraniol	0.00	**0.29**	**0.31**	23.2
isobornyl acetate	0.09	**0.32**	**0.33**	20.5
2,3-epoxy-2,3-dimethylbutane	0.26	0.18	0.19	**24.8**
1,5-dimethyl-6-oxa-bicyclo[3,1,0]hexane	0.15	0.12	0.14	**17.0**
2-methyl-2-pentanol	0.23	0.12	0.15	**23.8**
styrene	0.19	-0.03	-0.01	**24.4**
4-methylbenzaldehyde	0.13	0.06	0.07	**17.4**
2,3-dihydro-4-methyl-1H-indene	0.23	0.18	0.19	**20.7**
alpha,-methyl-cyclohexanepropanol	0.15	0.13	0.14	**22.7**
1-decanol	0.09	0.07	0.08	**14.2**
indole	0.18	0.13	0.14	**25.3**
2-(1,1-dimethylethyl)-4-methyl-phenol	0.25	0.14	0.16	**24.9**
1,1-(1,1,2,2-tetramethyl-1,2-ethanediyl)bis-benzene	0.12	-0.03	-0.02	**23.2**
PC1	**0.61*** **	**0.72*** **	**0.72*** **	**137*** **
PC39	0.00	-0.28	-0.30	91.2
Mantel test		$Z = 6.29 \times 10^{10}$	$P < 0.001$	

Pagel's λ, Moran's I and Abouheif's C_{mean} were calculated and a Mantel test was performed using normalized peak area data of every mVOC produced by each of the *Bacillus* strains and a phylogenetic tree based on a concatemer of the 16S rRNA gene and *rpoB* sequences. The same tests were performed on the eigenvectors (PC1 and PC39) resulting from the pPCA. Only the compounds showing a significant phylogenetic signal in one or more indices, or test are shown. A Mantel test was used to analyse the complete dataset of all mVOC produced by the *Bacillus* strains. Values in bold indicate a significant phylogenetic signal (***, $P < 0.001$).

Table A4.3: Vector numbers referring to the mVOCs with highest loadings for PC1 and PC39 (absolute value >0.1) in the pPCA biplot.

Vector number[a]	Compound	Loading PC1	Loading PC39
1	propanal	-0.06	-0.13
2	trimethylamine	-0.03	-0.19
3	acetone	-0.02	-0.18
4	2-methyl-2-propanol	0.05	-0.17
5	1-propanol	0.02	-0.15
6	2,3-epoxy-2,3-dimethylbutane	-0.08	-0.13
7	butanal	0.13	-0.01
8	2,3-butanedione	0.20	-0.08
9	ammonium acetate	0.14	0.02
10	fluoro-benzene	-0.04	-0.16
11	acetoin	0.21	-0.06
12	isoamylamine	0.21	-0.05
13	methyl-methacrylate	-0.15	-0.05
14	3-hydroxy-3-methyl-2-butanone	0.06	-0.18
15	2-methyl-2-pentanol	0.14	-0.18
16	2-methyl-propanoic acid	-0.14	0.02
17	2,3-butanediol	0.12	-0.07
18	2-hexanone	0.17	-0.06
19	ethyl-butanoate	0.12	0.00
20	pinacol	-0.12	-0.08
21	1,3-dimethyl-benzene	0.04	-0.12
22	cyclohexanone	-0.13	-0.03
23	2,5-hexanedione	-0.10	-0.08
24	benzaldehyde	-0.12	0.00
25	styrene	-0.09	-0.13
26	2,3,5-trimethyl-pyrazine	0.15	-0.03
27	eucalyptol	0.11	-0.13
28	limonene	0.01	-0.11
29	2-ethyl-1-hexanol	0.10	-0.17
30	indane	0.04	-0.12
31	phenylacetaldehyde	-0.13	0.01
32	*p*-cymene	-0.01	-0.11
33	acetophenone	0.09	-0.14
34	2-methyl-6-methylene-2-octanol	0.17	-0.03
35	4-methylbenzaldehyde	-0.09	-0.12
36	tetramethyl-pyrazine	0.17	-0.02
37	3,7-dimethyl-octan-3-ol	0.05	-0.15
38	nonan-2-ol	0.18	0.00
39	2-ethyl-hexanoic acid	-0.14	0.00
40	2,3-dihydro-4-methyl-1H-indene	-0.12	-0.04
41	menthol	0.06	-0.13
42	terpinene-4-ol	0.02	-0.07
43	verbenone	0.05	-0.18

Vector number	Compound	Loading PC1[b]	Loading PC39[c]
44	n-decanal	-0.04	-0.20
45	3,5-dimethyl-benzaldehyde	-0.12	-0.05
46	indole	-0.12	-0.02
47	nonanoic acid	-0.12	-0.04
48	cis-2-tert-butyl-cyclohexanol acetate	0.06	0.10
49	4-tert-butylcyclohexyl acetate	-0.12	-0.01
50	isobutyl 3-hydroxy-2,2,4-trimethylpentanoate	-0.02	-0.12
51	1-hydroxy-2,4,4-trimethylpentan-3-yl) 2-methylpropanoate	0.16	-0.01
52	3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	0.14	-0.03
53	2-ethyl-3-hydroxyhexyl 2-methylpropanoate	0.13	-0.08
54	2,6-di-tert-butyl-P-benzoquinone	-0.03	-0.21
55	4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-penten-2-one	0.01	-0.10
56	2,2,4-trimethyl-1,3-pentanediol diisobutyrate	-0.07	-0.13
57	1,1-(1,2-dimethyl-1,2-ethenediyl)bis-benzene	-0.05	-0.16
58	3,5-di-tert-butyl-4-hydroxybenzaldehyde	-0.06	-0.14
59	1,1-(1,1,2,2-tetramethyl-1,2-ethanediyl)bis-benzene	-0.07	-0.15
60	butyl isobutyl phthalate	-0.11	-0.12
61	methyl dehydroabietate	-0.12	-0.06

[a]Vectornumbers belonding to the biplot arrows in the pPCA biplot in Fig. 4.4C. Only mVOCs with highest loadings for PC1 and PC39 (absolute value >0.1) are shown.
[b]Loadings on the horizontal axis, principal component 1 (PC1; global structure in pPCA).
[c]Loadings on the vertical axis, principal component 39 (PC39; local structure in pPCA).

Appendix to Chapter 5: No supplementary files for Chapter 5.

Appendix to Chapter 6: No supplementary files for Chapter 6.

Publication list

Articles in internationally reviewed academic journals

Goelen, T., Sobhy, I.S., Vanderaa, C., de Boer, J.G., Delvigne, F., Francis, F., Wäckers, F., Rediers, H., Verstrepen, K.J., 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. *Functional Ecology*, in press. DOI: 10.1111/1365-2435.13503

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Patent applications

de Boer, J.G., **Goelen, T.,** Lievens, B., Rediers, H., & Jacquemyn, H. (2019). Aphid hyperparasite attractant. ZL919074. Submitted October 21, 2019.

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Goelen, T., Rediers, H., Jacquemyn, B., & Lievens, B. (2019). Innovative tools to improve biological control of aphids: development of a parasitoid attracting feeding device based on microbial infochemicals. 71st International Symposium on Crop Protection (ISCP). Ghent, Belgium, 21 may 2019. [Oral presentation]

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Sobhy, I., Baets, D., **Goelen, T.,** Herrera, B., Van den Ende, W., Verstrepen, K., Wäckers, F., Jacquemyn, H., & Lievens, B. (2017). Floral nectar yeasts enhance parasitoid foraging and maintenance. The annual meeting of British Ecological Society (BES), Ecology Across Borders. Ghent, Belgium, 11-14 December 2017. [Poster presentation]