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PhD thesis

The exploitation of microbial volatiles for integrated pest management of spotted wing drosophila *Drosophila suzukii* Matsumura (Diptera: Drosophilidae)

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Foreword

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I am thankful for all people who contribute to my success, especially to my academic supervisors.

Walk away, down the alley of life

Stop and listen the harmony of an orchestra

where each player has a role

a small bird with music key in the beak

a lost yeast seeking a nectar on the wind

Sense the smell of the soil

whisper in the rain forest of the ecosystem of your life

Could you stop and embrace

all small creatures you unconsciously breading in

Would you stop and listen to the talk of trees

Enjoy glitter of the pebbles of the rain across prairies

Scream together in harmony of a symphony orchestra

on the path under million stars

framed with Alpine mountains and snow cover peaks

Could you stop, listen and observe

The fantastic call of nature to be discovered and understood.

Realise a small bacterial factory in a glass of your most tasty wine of life

—a small cues for friends and foes.

Listen if you could

Because the time is tide

and science of trinity of all beings calling

and waits for you

"In the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed." **– Charles Darwin**

List of abbreviations

Abbreviation	Definition
AL	Antennal lobe
ANOVA	Analysis of variance
BCA	Biological control agent
BPC	Biological pest control
EA	Ethyl acetate
EAD	Electro Antennographic Detector
EAG	Electro Antennogram
EAG	Electroantennography
EtOAc	Ethyl Acetate
EtOH	Ethanol
FDR	False Ionisation detector
FID	Flame Ionisation Detector
GC	Gas chromatography
GLMM	Generalised linear mixed model
HIPV	Herbivore-induced plant volatile
HS	Headspace
HSD	Honest significant difference
IA	Isoamyl acetate
IPM	Integrated pest management
ML	Maximum-likelihood
MS	Mass spectrometry
MVOC	Microbial volatile organic compounds
NMDS	Non-metric multidimensional scaling
OD	Optical density
OR	Odorant receptor
OSN	Olfactory sensory neuron
PC	Principal component
perMANOVA	Permutational multivariate analysis of variance
PI	Preference index
RT	Room temperature
SE	Standard error of the mean
SIM	Selected Ion Monitoring
SMPE	Solid-phase micro extraction
SPE	Solid Phase Extraction
SPME	Solid Phase Micro Extraction
SRH	Scheirer–Ray–Hare
VOC	Volatile organic compound

Sintesi

Il moscerino asiatico (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), originario dell'Asia orientale, è uno dei principali parassiti emergenti di colture pregiate in Europa e nelle Americhe. Nel 2008, si è verificata una rapida invasione di questa specie di fitofago dei frutti rossi in Europa e nelle Americhe. Di conseguenza, *D. suzukii* è attualmente uno degli insetti dannosi più rilevanti di colture pregiate, attaccando i frutti rossi e l'uva da vino, e causando milioni di dollari di danni ogni anno. A differenza delle altre Drosophilae, la SWD è in grado di penetrare la buccia della frutta in maturazione e di deporre le uova al suo interno, dove gli stadi larvali si nutrono, si sviluppano e causano danni. Questo piccolo moscerino della frutta, una specie strettamente imparentata con *D. melanogaster* e *D. simulans*, ha sviluppato un sofisticato sistema olfattivo che può rilevare l'odore della frutta e altri odori provenienti da un habitat potenzialmente adatto. Tali composti volatili sono mediatori a lungo raggio del suo comportamento. Lo studio del sistema olfattivo di *D. suzukii* può contribuire quindi a migliorare la nostra comprensione del comportamento e della fisiologia di questi insetti e ci permetterà di sviluppare soluzioni efficaci di controllo dei parassiti. Le attuali strategie di controllo si basano sull'uso massiccio di insetticidi, che hanno un impatto ecologico negativo e a lungo termine non sono né efficaci né sostenibili. Gli strumenti che l'ecologia chimica fornisce si adattano perfettamente ai programmi di gestione integrata (IPM) di *D. suzukii* e potrebbero offrire un approccio alternativo e più sostenibile per limitarne la diffusione e i danni.

In questa tesi di dottorato, abbiamo usato nelle nostre indagini saggi microbiologici, chimici, elettrofisiologici di laboratorio e studi in campo aperto. L'obiettivo generale di questo studio di dottorato è stato quello di indagare il potenziale di una miscela di vino, aceto di mele e canna da zucchero insieme a composti volatili microbici in una trappola innovativa progettata per migliorare l'azione attrattiva nei confronti di *D. suzukii* in campo aperto.

Approcci sostenibili per limitare la diffusione di *D. suzukii* e i danni che causa richiedono sempre esche e trappole efficaci. Il nostro obiettivo quindi è stato quello di sviluppare un'esca innovativa ed efficace da implementare in un sistema di trappole selettive per il controllo di SWD come parte dei programmi di gestione integrata dei parassiti (IPM) in campo aperto. Mentre la maggior parte delle ricerche su *D. suzukii* si è concentrata su composti volatili emessi dalla frutta o dai lieviti presenti sui frutti ospiti, c'erano poche prove su come altri microrganismi che emettono composti volatili possano giocare un ruolo nel comportamento di questo insetto. Inoltre, era poco conosciuto il ruolo di composti volatili batterici nel comportamento di ricerca degli ospiti di SWD e se quindi essi possano essere applicati per migliorare il controllo ed il monitoraggio di questa specie invasiva. Abbiamo usato Droskidrink®, un prodotto commerciale per la cattura di *D. suzukii*, come esca di base per il miglioramento e ulteriori indagini. Abbiamo dimostrato che l'aggiunta di batteri lattici al Droskidrink® nella prima settimana dopo la fermentazione migliora l'attrattività verso SWD. Abbiamo studiato i composti volatili chiave per SWD, emessi durante il processo di fermentazione del vino e dell'aceto mediato da batteri lattici. Inoltre, abbiamo trovato una relazione tra gli stessi composti volatili in grado di mediare il comportamento di *D. suzukii* e altri organismi a diversi livelli trofici. In particolare, abbiamo usato prodotti metabolici di Saccharomyces cerevisiae e il noto endoparassitoide di SWD *Trichopria drosophilae* Perkins (Hymenoptera; Diapriidae).

Nella prima parte di questa tesi di dottorato **(Capitolo 2)**, abbiamo studiato l'uso dei batteri come bio-catalizzatori dei processi metabolici che avvengono durante la fermentazione malolattica di una miscela di vino-aceto di mele-canna da zucchero attrattivo per *D. suzukii*. Abbiamo prima valutato l'attrattività dell'esca alimentare Droskidrink® integrata con diversi ceppi di batteri lattici. Questo esperimento è stato condotto in pieno campo in un vigneto commerciale. Abbiamo usato Droso-Trap® Biobest, e Droskidrink® integrato con *Oenococcus oeni*, *Pediococcus* spp e *Lactobacillus* spp. Inoltre, il rendimento dei ceppi batterici

attrattivi è stato studiato in condizioni di laboratorio. Successivamente, abbiamo studiato la risposta elettroantennografica di SWD ai ceppi di *O. oeni* più attrattivi, integrati con Droskidrink®. I risultati hanno mostrato che dei diversi batteri lattici studiati negli esperimenti di laboratorio e sul campo, tre ceppi di *O. oeni* erano i più attivi nei confronti di *D. suzukii*.

Nel **capitolo 3**, abbiamo eseguito l'estrazione dei composti volatili da miscele ottenute con diversi ceppi di *O. oeni* aggiunti al Droskidrink® per valutare come i composti volatili emessi dai batteri influenzassero la composizione chimica del Droskidrink®. Abbiamo testato l'influenza dei batteri lattici (due ceppi di *O. oeni* precedentemente selezionati come i ceppi più interessanti nelle prove sul campo) e la successiva fermentazione malolattica nelle miscele vino-aceto-zucchero, per un periodo di tre settimane. Per l'estrazione dei composti volatili, sono state utilizzate due diverse metodologie di estrazione, ovvero la raccolta diretta dallo spazio di testa e il metodo Closed-Loop-Stripping-Analysis (CLSA). La fermentazione è stata impostata in modo tale che i metaboliti sono stati estratti una, due e tre settimane dopo l'inizio della fermentazione malolattica. Per l'identificazione chimica dei composti altamente volatili, abbiamo utilizzato l'analisi diretta dello spazio di testa collegata a un gascromatografo con un rivelatore selettivo di massa. I composti volatili estratti nel solvente sono stati analizzati in un sistema GC-MS standard su due diversi tipi di colonna per aumentare il numero di composti identificati. Inoltre, sono stati utilizzati standard chimici sintetici per la co-iniezione e la conferma chimica. Successivamente, abbiamo studiato la risposta elettroantennografica dei composti volatili raccolti, dissolti in un solvente, su SWD femmina. L'esperimento comportamentale a scelta multipla è stato eseguito in condizioni di laboratorio e per sostenere la nostra ipotesi è stato anche testato in studi in campo aperto con l'uso di un innovativo sistema di cattura. I risultati hanno rivelato che la fermentazione malolattica con specifici ceppi di batteri lattici ha prodotto con una composizione specifica di composti volatili che ha reso la nostra miscela più attrattiva per *D. suzukii*. I nostri risultati hanno descritto la composizione chimica delle miscele emesse dal Droskidrink® dopo la fermentazione batterica. Questi composti volatili includevano alcuni nuovi composti elettrofisiologicamente attivi per SWD, come l'eugenolo e la triacetina. Inoltre, i risultati hanno mostrato una vasta gamma di composti organici volatili diversi che hanno fortemente influenzato il comportamento di SWD. È interessante notare che l'innovativa trappola progettata utilizzando solo 15 mL di una miscela di vino-aceto-zucchero di canna con un ceppo beta attrattivo di *O. oeni* ha aumentato di due volte la cattura della trappola quando è stata saggiata rispetto a un diverso attrattivo disponibile in commercio, ovvero Scentry® (miscela brevettata, Scentry Biologicals Inc., Billings, MT, USA). I risultati hanno ulteriormente confermato la teoria comunemente accettata dell'importanza dei composti volatili ubiquitari delle piante nell'attrarre gli insetti fitofagi.

Nonostante una maggiore comprensione del ruolo dei semiochimici per manipolare il comportamento di SWD, attualmente le tecniche basate su tali composti in campo aperto non sono ben consolidate per questa specie invasiva. Inoltre, la non selettività nella cattura di *D. suzukii* diminuisce l'efficacia dei sistemi di trappole sviluppati. Pertanto, nel **Capitolo 4**, abbiamo mirato a identificare composti specifici che possono repellere altre specie di drosofila e aiutare nella costruzione di sistemi di cattura più selettivi. Usando la gascromatografia-spettrometria di massa GC-MS, una combinazione di gascromatografia-elettroantennografia GC-EAD, e biosaggi in gabbia a scelta multipla con composti volatili sintetici, abbiamo cercato di trovare un composto repellente per le Drosophile catturate usando l'attuale sistema di trappola. Abbiamo condotto la nostra ricerca sulla specie sorella di *D. suzukii*, *Drosophila simulans* Sturtevant. Successivamente, è stata selezionata la miscela più promettente di composti putativamente repellenti. I risultati hanno rivelato che diverse miscele di composti erano significativamente non attrattivi a causa della presenza di composti repellenti. Nel complesso, questi risultati indicano che i composti: benzaldeide, eugenolo, etanolo, etile isovalerato, feniletil acetato, isoamil lattato, 1-octen-3-olo, etile caprolete, limonene, p-cimene, acido valerico erano significativamente repellenti.

Infine, l'uso di pesticidi tossici per combattere le specie invasive deve essere ridotto. I prodotti chimici non ecologici danneggiano gravemente l'ambiente. Sono stati usati insetticidi dannosi e tossici, non solo

per la salute umana ma anche per tutti gli organismi nell'habitat. Le sostanze chimiche tossiche ostacolano i nemici naturali degli insetti nocivi, i parassitoidi e i predatori. La gestione integrata dei parassiti (IPM) mira a bilanciare l'uso di buone pratiche agricole con la pianificazione strategica, il monitoraggio precoce, il controllo biologico e molte pratiche agricole diverse con un uso minimo di composti chimici che hanno dimostrato di essere pericolosi per la biodiversità. Il controllo biologico con l'uso di nemici naturali è una parte importante dell'IPM, in combinazione con altri metodi, ad esempio un buon monitoraggio e la cattura massale. Applicare molte strategie diverse allo stesso tempo potrebbe portare al raggiungimento dell'obiettivo. Proteggere e promuovere gli agenti di biocontrollo naturalmente presenti in un agroecosistema è quindi fondamentale (lotta biologica conservativa) insieme al rilascio razionale di quelli commercialmente disponibili (lotta biologica aumentativa). Comprendere il comportamento dei nemici naturali e la loro scelta degli insetti ospiti è quindi uno dei passi chiave nel miglioramento della lotta biologica. Il comportamento dei nemici naturali è determinato da composti chimici rilasciati nell'ambiente dagli insetti ospiti, dalle piante e dalla fonte di cibo dell'insetto ospite. I composti chimici provenienti dalla pianta ospite sono stati ampiamente studiati. Recentemente, l'attenzione si è concentrata sulle sostanze che sono prodotte durante il metabolismo dei microrganismi. Questi composti chimici sono chiamati composti organici volatili microbici, mVOCs. I mVOCs possono mediare il comportamento degli insetti e portare alla scelta dei siti di accoppiamento, ovodeposizione e alimentazione. Si sa poco su come i mVOCs influenzino il comportamento dei nemici naturali e sulla loro applicazione nell'IPM. Pertanto, nel **capitolo 5** abbiamo cercato di studiare uno dei principali endoparassitoidi di *D. suzukii*, *Trichopria drosophilae* e il suo comportamento nel contesto della ricerca dell'ospite. In questo capitolo, abbiamo studiato il comportamento di ricerca dell'ospite e l'utilizzo degli stessi segnali volatili nella Drosophila e nel suo parassitoide, emessi da una delle fonti primarie della dieta ricca di proteine della drosofila, *Saccharomyces cerevisiae*.

Nel complesso, questa tesi di dottorato ha fornito una migliore comprensione dell'interazione mediata da composti volatili tra i microrganismi responsabili della fermentazione di un attrattivo comune per *D. suzukii*, una miscela di vino-aceto-zucchero di canna. Dagli studi neurofisiologici, microbiologici e chimici, e attraverso studi comportamentali di laboratorio e di campo, abbiamo ottenuto importanti risultati innovativi. Queste conoscenze possono essere sfruttate per sviluppare un nuovo strumento per il monitoraggio di *D. suzukii* all'inizio del suo movimento nei campi agricoli dalle aree di rifugio invernali, e anche per la cattura massale durante il periodo di massima infestazione in estate. Inoltre, i risultati del nostro studio possono portare all'ottimizzazione dell'attuale gestione integrata di SWD e di conseguenza a pratiche più sostenibili per la gestione di le specie di insetti invasive. Abbiamo utilizzato i semiochimici specifici emessi da microrganismi legati alle piante ospiti dell'insetto per aumentare l'attrazione e come base per la progettazione di trappole innovative.

Summary

The spotted-wing Drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), native to Eastern Asia, is one of the main emerging pests of valuable crops in Europe and the Americas. In 2008, rapid invasion of this soft fruit pest species occurred across Europe and the Americas. Consequentially, *D. suzukii* is currently one of the most relevant pest of valued horticultural crops, attacking soft fruit and wine grapes, and causing millions of dollars of damage annually. In contrast with other Drosophilae, SWD is capable of penetrating the skin of ripening fruit and laying eggs inside, where larval instars feed, develop and cause damage. This small fruit fly, a closely-related species of *D. melanogaster* and *D. simulans*, has developed a sophisticated olfactory system that can detect fruit odour and other odours coming from a potentially suitable habitat. Volatile cues are long-range mediators of its behaviour. Investigation of the olfactory system of *D. suzukii* will thus develops our understanding of the behaviour and physiology of these insects and allows us to develop effective pest control solutions. Current control strategies rely on the heavy use of insecticides, which have a negative ecological impact, and in the long run are neither effective nor sustainable. The tools that chemical ecology provides fit perfectly into *D. suzukii* integrated pest management (IPM) programmes, and could offer an alternative, more sustainable approach to limit its spread and damage.

In this PhD thesis, we used microbiological, chemical, electrophysiological and laboratory bioassays and open field studies in our investigations. The overall aim of this PhD study was to investigate the potential of a tailored wine-apple cider-sugar cane mixture together with microbial volatiles in an innovative trap designed to improve the attractiveness of *D. suzukii* in the open field.

Sustainable approaches to limit the spread of *D. suzukii* and the damage it causes always require effective lures and traps. We, therefore, intended to develop an innovative and effective lure to be implemented in a selective trapping system for controlling SWD as part of integrated pest management (IPM) programmes in open fields. While most research investigating *D. suzukii* has focused on volatile cues derived from host fruit or yeast, there is little evidence about how other microorganisms emitting volatile compounds could play a role in this fly's behaviour. Moreover, little is known about how bacterial microbial volatiles affects the behaviour of SWD and whether they can be applied to improve integrated pest management control of this invasive species. We used Droskidrink®, a commercial product for catching *D. suzukii*, as a basic lure for improvement and further investigations. We demonstrated that adding lactic acid bacteria to Droskidrink® in the first week after fermentation improves attractiveness for SWD. We investigated key odourant cues for SWD, emitted by the wine-vinegar-lactic acid bacteria fermentation process. Furthermore, we found a connection between the same volatile cues capable of mediating the behaviour of *Drosophila suzukii* and other organisms on different trophic levels. For investigation across different trophic levels, we used *Saccharomyces cerevisiae* metabolic products and the well-known *D. suzukii* endoparasitoid *Trichopria drosophilae* Perkins (Hymenoptera; Diapriidae).

Volatiles extracted from different sources are used to develop simple attractants with the use of a small number of compounds in a special ratio and concentration. Currently, the most prominent lures contain volatiles isolated from Merlot wine, rice vinegar, wine vinegar, apple cider vinegar, apple juice, fermented apple juice, the surface of raspberries or crushed berry fruits, including blueberries, cherries and strawberries. Some volatile compounds are isolated from acetic acid bacteria grown in different liquid media, and volatiles from different yeast fermentations.

In the first part of the PhD study (**Chapter 2**), we investigated the use of bacteria as a bio-catalyser of metabolic processes occurring during malolactic fermentation of a wine-apple cider-sugar cane mixture attractive to *Drosophila suzukii*. We first evaluated the attractiveness of Droskidrink® food bait supplemented with different lactic acid bacteria strains. This experiment was conducted in open field studies in a commercial vineyard. We used Droso-Trap® Biobest, and Droskidrink® supplemented with *Oenococcus oeni*, *Pediococcus* spp and *Lactobacillus* spp. Moreover, the performance of attractive bacterial strains was investigated under laboratory conditions. Next, we studied the electroantennography response of SWD flies to the most attractive *O. oeni* strains, supplemented with Droskidrink®. The results showed that of the different lactic acid bacteria studied in laboratory and field experiments, three strains of *O. oeni* were most active to *Drosophila suzukii*.

In **Chapter 3**, we performed volatile extraction of the mixtures, with different *O. oeni* strains added to Droskidrink® to assess how the volatile compounds emitted by bacteria affected the chemical composition of Droskidrink®. We tested the influence of lactic acid bacteria (two strains of *O oeni* previously selected as the most attractive strains in the field trials) and subsequent malolactic fermentation in wine-vinegar-sugarcane mixtures, over a period of three weeks. For volatile extraction, two different extraction methodologies were used, namely Direct Headspace Collection and the Closed-Loop-Stripping-Analysis (CLSA) method. Fermentation was set up in such a way that volatiles were extracted one, two and three weeks after the beginning of malolactic fermentation. For chemical identification of highly volatile compounds, we used direct head-space analysis connected to a Gas-Chromatograph with a Mass Selective Detector. Volatile extracts in the solvent were analysed in a standard GC-MS system on two different types of column to increase the number of identified compounds. Moreover, synthetic chemical standards were used for co-injection and chemical confirmation. Next, we studied the electroantennographical response of the collected volatiles, dissolved in a solvent, on female SWD flies. The behavioural multi-choice experiment was performed under laboratory conditions and to support our hypothesis was also tested in open field studies with the use of an innovative trapping system. The results revealed that malolactic fermentation with specific LAB strains tuned VOC composition in a way that made our tested mixture more attractive to *D. suzukii*. Our results revealed the chemical composition of various volatiles emitted by Droskidrink® after bacterial fermentation. These volatiles included some newly electrophysiologically-active compounds for SWD, such as eugenol and triacetin. Additionally, the results showed a wide range of diverse volatile organic compounds that strongly mediated the behaviour of SWD. Interestingly, the field innovative trap designed using just 15 mL of a mixture of wine–vinegar-sugar cane with an attractive beta strain of *O. oeni* increased trap catch two-fold when tested compared to a different commercially available attractant, namely Scentry® (proprietary blend, Scentry Biologicals Inc., Billings, MT, USA). The results further confirmed the commonly accepted theory of the importance of ubiquitous plant volatiles in attracting insects.

Despite an increased understanding of the role of volatile emission as insect semiochemicals, and their use to manipulate SWD behaviour, at present semiochemically-based techniques in the open field are not well-established for this invasive species. Furthermore, non-selectivity and spillover in the catching of *D. suzukii* decreases the effectiveness of the trap systems developed. Therefore, **in Chapter 4**, we aimed to identify specific compounds that may repel other *drosophila* species and help in building more selective trapping systems. Using gas chromatography-mass spectrometry GC-MS, a combination of gas chromatography-electroantennography GC-EAD, and multi-choice cage bioassays with synthetic volatile compounds, we tried to find a repellent compound for untargeted Drosophilae caught using the current trap system. We conducted our research on the *Drosophila suzukii* sister species *Drosophila simulans* Sturtevant. Next, the most promising mixture of putatively repellent compounds was selected. The results revealed several compound mixtures were significantly not attractive because of repellent compound

presence. Overall, these results indicate that compounds: benzaldehyde, eugenol, ethanol, ethyl isovalerate, phenylethyl acetate, isoamyl lactate, 1-octen-3-ol, ethyl caprolete, limonene, p-cymene, valeric acid were significantly repellent.

Finally, the use of toxic pesticides to fight invasive species must be reduced. Environmentally unfriendly chemicals severely damage the environment. Insecticides that are harmful and toxic, not just for human health but also for all organisms in the habitat, have been used. Toxic chemicals impede naturally occurring enemies of pest insects, parasitoids and parasites. Integrated pest management (IPM) aims to balance the use of good agricultural practices with strategic planning, early monitoring, biological control and many different agricultural practices with minimal use of chemical compounds that have been proven to be dangerous for bio-diversity. Biological control using natural enemies is an important part of IPM, not just one aspect and strategy, as good monitoring and mass trapping could lead to a decline in the numbers of SWD in agricultural fields. Applying many different strategies at the same time could lead to achievement of the goal. Supplying the agro-environment with biological pest control (BPC) by boosting the natural population of parasitoids and predators is significant. In biological control (BC), naturally present beneficial organisms are supported with commercially reared natural enemies. One of the challenges in biological control is to maintain and attract beneficial insects to orchards (agricultural fields). Understanding the behaviour of natural enemies and their choice of host insects is one of the key steps in improvement of BC. The behaviour of natural enemies is determined by chemical cues released in the environment by host insects, plants, and the food source of the host insect. Chemical cues originating from the host plant have been widely studied. Recently, attention has been focused on chemical cues that are produced as products of the microorganism's metabolism. These chemical cues are called microbial volatile organic compounds, mVOCs. mVOCs can mediate insect behaviour and lead to the choice of the mating, oviposition and feeding sites. Little is known about how mVOCs influence the behaviour of natural enemies, and their application in IPM. Therefore, in **Chapter 5** we aimed to investigate one of main *Drosophila suzukii* endoparasitoids, *Trichopria drosophilae* and its behaviour in the context of host searching. In this chapter, we investigated host searching behaviour and utilisation of the same volatile cues in *Drosophila* and its parasitoid, emitted by one of the primary drosophila protein-rich diet sources, *Saccharomyces cerevisiae.*

Overall, this PhD study has provided a better understanding of volatile mediated interaction between microorganisms and fermentative effects on a worldwide homemade attractant for *Drosophila suzukii*, a mixture of wine-vinegar-sugar cane. From fundamental neurophysiological, microbiological and chemical studies, through laboratory insect behavioural studies applied to open field studies, we have obtained important findings. This knowledge, combined with applied studies in the open field, may be exploited to develop a novel tool that detects *D. suzukii* at the beginning of its movement to agricultural fields from winter shelter areas, and also for mass trapping during the peak infestation period, when farmers have not intervened during the bottleneck period of arrival of SWD in the field. Moreover, the results of our study potentially lead to improved integrated management control of SWD and consequently to more sustainable practices in dealing with invasive insect species. We utilised microbe host-specific semiochemicals for attraction and as a basis for innovative trap design.

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Chapter 1

General introduction

1.1 Climate change and invasive species

It is estimated that by 2050 there will be huge world demand for food, water and sustainable energy sources (FAO, 2009). Climate change has seen the global temperature rise annually at an average rate of 0.07°C (0.13°F) per decade since 1880, a level that is already hampering agricultural production worldwide (Hansen et al., 2006). Ironically, the most affected areas are the rural zones of the world, where the inhabitants have a lower carbon footprint. Intensive industrial development, agriculture and unsustainable transport are affecting the environment. Damage has been done to natural resources, exhausting, degrading and polluting freshwater, soil and air quality.

The green revolution after the Second World War also brought expansion in agricultural production and heavy industrial use of chemicals left over from the war. Nitrogen compounds were put to use for fertilisation, while DDT was used for pest control. DDT was advertised as a completely safe method for dealing with agricultural and house pests (Carson, 2002). Up to the time when Rachel Carson's book Silent Spring came out, governments made not a single move to combat and mitigate the adverse effects of numerous chemical toxic compounds on the environment, and directly and indirectly, the consequences for human health (von Hippel, 2020).
Agricultural production faces many attacks by pests, causing significant economic losses with direct feeding damage and indirect damage by vectoring plant pathogens and viruses. An estimated average of 18% to 26% of annual crops are lost worldwide due to insects, with an economic value of 400 billion euros (Culliney, 2014).

Among the challenges arising from climate change and the transformation of agroecosystems, agricultural production is heavily affected by invasive insect species. In the era of globalisation and climate change, a high level of trade between countries and continents has been added to a legacy of biological invasion. Cosmopolitan invasive insect pests come about as a result of globalisation and global trade. The invasion of new pest species is hampering biodiversity preservation and has led to severe issues for growers when an invasion occurs. New insects arrive in new ecological niches free from effective competitors or natural predators and parasitoids, which allows quick naturalisation in the invaded areas. Invasive insects can establish themselves in new areas, where their development can progress due to a suitable climate and lack of natural enemies (Hobbs, 2000). Farmers have few options to mitigate these attacks by insects. Approximately one third of worldwide food production is lost due to negative insect impact every year (Witzgall, Kirsch, & Cork, 2010).

Invasion by pest organisms is leading to a threat to native wildlife, agricultural production and ultimately human health. Understanding the biology and ecology of invasive species is a crucial step in mitigating the devastating effects of such insects. The biggest threat is seen as invasive species that are polyphagous and have high ecological plasticity. Invasive history and special analysis of one pest's movements and adaptation to a new environment are critical for understanding and organising management strategies (Cini, Ioriatti, & Anfora, 2012). The economic impact of invasive species is estimated to result in hundreds of billions of euros lost each year (Cini et al., 2012).

Environmentally-friendly measures are in high demand to reduce pesticide use in food production, as a vital action point of all research conducted on invasive species. Current control tactics using pesticides must be replaced with more sustainable methods to counter invasive insect species. Integrated pest management is a strategy adopting a systematic approach to dealing with pathogens and insect pests with many possible tactics, while reaching for pesticides is the last available option. The implementation of IPM mainly involves the use of pest monitoring for forecasting and thresholds of pest infestation, emphasising

prevention via cultivation methods vs treatments with insecticide, record keeping and documentation, the use of less toxic and least environmentally disruptive practices, resistance management, farmer education. Soil management, water use, nutrient and pest management complying with legal requirements, along with use of a systematic approach in applying all the above.

1.2 *Drosophila suzukii*

Drosophilas fruit flies (vinegar flies) are mainly considered as secondary parasites of rotten fruit, while *Drosophila suzukii* Matsumura (Diptera: Drosophilidae, SWD) is a pestiferous insect commonly known as spotted wing Drosophila, SWD, now already well-known as an invasive pest arriving in Europe and Americas in 2008. It is a highly invasive pest, threatening long-term sustainable production of commercial small fruits and the grapevine. Originally from South-East Asia, it has spread rapidly in the West (Cini et al., 2012; J. C. Lee, Bruck, Curry, et al., 2011). This characteristic rapidly spreading behaviour has caused enormous economic damage. Unlike the vinegar fly *Drosophila melanogaster,* which attacks rotten fruit, *D. suzukii* oviposits in healthy ripening soft summer fruit. This ability to sow inside the soft skin of the fruit is thanks to its large serrated ovipositor (Atallah, Teixeira, Salazar, Zaragoza, & Kopp, 2014; Crava, Sassù, Tait, Becher, & Anfora, 2019). When the larva emerges inside the fruit pulp, it starts to grow, feed and eat the pulp, destroying it (Walsh et al., 2011). The larva makes the fruit unmarketable and reduces processed fruit quality. SWD has short generation times, high reproductive levels, and high generational overlap, which is the most problematic issue for pest control. SWD reproductive potential is from 10 to 15 overlapping generations in a year. Survival of SWD in the environment is affected by fecundity, population dynamics, temperature, humidity and food availability. The population dynamics of SWD in the field is mostly influenced by temperature and reproduction survival (Walton et al., 2014). SWD prefers a moderate temperature climate, from subtropical to continental. *D. Suzuki* has a wide range of host plants in its native environment as well as in areas where it has been introduced, with berries being the preferred hosts (Alberto Grassi, Giongo, & Palmieri, 2011; J. C. Lee, Bruck, Curry, et al., 2011). The main hosts are soft fruit (raspberries, blackberries, strawberries and blueberries), stone fruit (cherries, peaches, apricots and plums), wine and table grapes, figs and kiwis (Walsh et al., 2011).

SWD opens the way for secondary damage caused by other insects, fungi and bacteria. The economic yield loss ranges from 30-40 % to 100% in different areas and crops (Cini et al., 2012). In the province of Trento in Italy, 400 ha of fruit production faced a loss of half a million euros in 2011 (Cini et al., 2012). In the USA, economic losses to berry farming were over 4,367 billion US dollars (Walton et al., 2014). In addition to the economic loss in terms of direct yield, it is necessary to add the cost of labour, monitoring, management and revenue losses because of the closure of fruit export markets from SWD-infested regions (Simoni et al., 2013). In Europe, the economic cost could reach €64,138,000 (Mazzi, Bravin, Meraner, Finger, & Kuske, 2017).

Currently, chemicals, traps, netting and insecticides are used to restrain and control the spread of this pest, along with early monitoring of the crops (Alnajjar, Collins, & Drummond, 2017; Cha, Loeb, Linn Jr, Hesler, & Landolt, 2018; Walsh et al., 2011). Early on in the invasion phase just two years after the SWD invasion, scientists from northern Italy developed a profile to investigate the invasive biology of SWD, which helped to direct effective management control (Cini et al., 2012). Pesticide application has been a key control tactic for growers, since other control measures represent a high risk for production. Pyrethroids, carbamates and spinosyns represent frontiers in insecticide control, but have started to weaken because of pest resistance. Preventive use of insecticide is unsustainable, because of the usual limitation, resulting in resistance to SWD, the destruction of non-target organisms and adverse effects on beneficial organisms

(parasitoids and predators). Organic production is particularly badly affected, because few effective organic pesticides have been approved. Since SWD attacks fruits in the ripening stage, and this time is close to harvesting, the short period between pesticide application and consumer use of fruits leads to the possibility of high pesticide residue levels. Good agricultural practices and cultural management practice are advised. One cultural management practice includes timely removal of fruits before the peak of attack by SWD (Walton et al., 2014). Other cultivation practices involve physical barriers (netting) and mass trapping. The microbiome associated with the insect pest may provide insight into combating SWD, and these studies have been carried out by (V. Vacchini et al., 2017).

1.3 Chemical ecology tools

Chemical ecology is an interdisciplinary area combining knowledge in the fields of chemistry, biology and ecology. Findings from these studies may produce a synthesis of semiochemicals important for pest insect species and may be utilised in chemical ecology research and for further practical applications. Research on chemical communication by insects began back in the late 1950s. In 1957 Schneider invented a methodology for electrophysiological studies based on elucidating the activity of chemical compounds. This method is called electroantennography (EAG). Then the discovery of silk moth pheromone started the development of chemical ecology (Schneider, 1957). One of the first books on chemical ecology was published in the late '60s (Brower, 1969). One of the first tools was developed a couple of years later, when insect antenna were combined with a gas chromatography-electroantennographic detector to detected chemical signals in the environment (GC-EAD). This tool allowed separation of several compounds on a high-resolution column, with insect antennae as the detector connected with a detecting system to computer software. To study the complex environment of insect habitat and interaction between all the participants in the ecosystem, tools are still being developed in analytical and synthetic chemistry, protein chemistry, molecular genetics, gene map coding, neurobiology and neurophysiology, ecology, evolution and phylogenetics. Analytical chemistry have progressed with a methodology and analysis purity. The biology of insects and related research have improved with GC-EAD, single-cell recordings and brain mapping techniques.

Prominent methods for combating pests in agricultural and forest areas have come from chemical ecology and the ability to manipulate insect behaviour, with monitoring, mass trapping, attract and kill systems, mating disruption, repelling, and push and pull methods. We will briefly explain some of these methods.

The first tool required in chemical ecology is the isolation of chemical cues that have an ecological and biological function in a natural system. These compounds, which have an ecological role, are usually present at a low concentration (ng and pg). To collect semiochemicals, a number of methods need to be tested for better determination and more accurate collection. Solid phase micro extraction (SPMA), a solvent-free method, allows absorption of a compound on polymer-coated fibre. This method is quick, and compounds from the air above the sample, soil, and water can be collected (Z. Zhang & YangM, 1994). After a suitable sampling time, the fibre collecting the compound is inserted into the inlet of a gas chromatograph for desorption of components. Another method for collection is headspace, which can be static or dynamic. Static collection is when a sample stands and the static volume of air above the sample is extracted, while dynamic collection takes place when an absorbent material is connected to one side of the sample while a pump circulates air or nitrogen through the sample for a certain period of time (Z. Zhang & YangM, 1994). Once a compound is collected, the next step is the use of analytical methods for identification, divided into destructive and non-destructive methods. One of the most commonly used destructive analytical methods is gas chromatography, which involves the separation of compounds using

their chemical properties on a CG column. This method is well explained in Chapter 3 of this thesis, since we used it for identification of the chemicals in our investigation. The electroanennographic detector (EAD) identifies compounds biologically active in insects. This method directly identifies compounds eliciting an antenna response. However, electroantennography cannot tell whether the compound of biological significance is attractive or repellent. Thus, the next step would be bioassay evaluation. Single-cell recording measures a response for a single cell or neuron to a specific compound on the antennae of an insect. The FID Flame Ionisation Detector (FID) measures the number of components present in a sample and synthetic references are used to obtain preliminary identification of semiochemicals. In this detector, the approximate number of carbons present in the molecule is measured. At the same time, mass spectrometry is the most sensitive detector in the range of 1-100 pg. MS is used for the structure of chemicals by comparing the compound to commercial libraries. We used an MS detector during our investigation in Chapter 3. Fourier Transform Infra Red Spectroscopy (FTIR) provides information about the functional groups in a molecule by means of infrared light. Quadrupole Time-Of-Flight Electro Spray Ionisation Mass Spectrometry, QTOF-ESI-MS is a destructive analytical method that provides very precise molecular weights and may provide a molecular formula. One of the non-destructive methods is Nuclear Magnetic Resonance Spectroscopy (NMR), which gives information about atom structure in molecules, usually with hydrogen and carbon nuclei. **Optical rotation** is a non-destructive analytical method that measures molecules with the chiral centres' ability to rotate light (De Hoffmann, Charette, & Stroobant, 1997).

Common methods used for investigation of insect behaviour in laboratory conditions in chemical ecology include the wind tunnel, Y-shape olfactometer, multi-choice arenas, ovipositional assays, semi-field and field experiments. In this thesis, we used some laboratory bioassays and open field studies to test the effects of semiochemicals on the invasive fruit fly *Drosophila suzukii*.

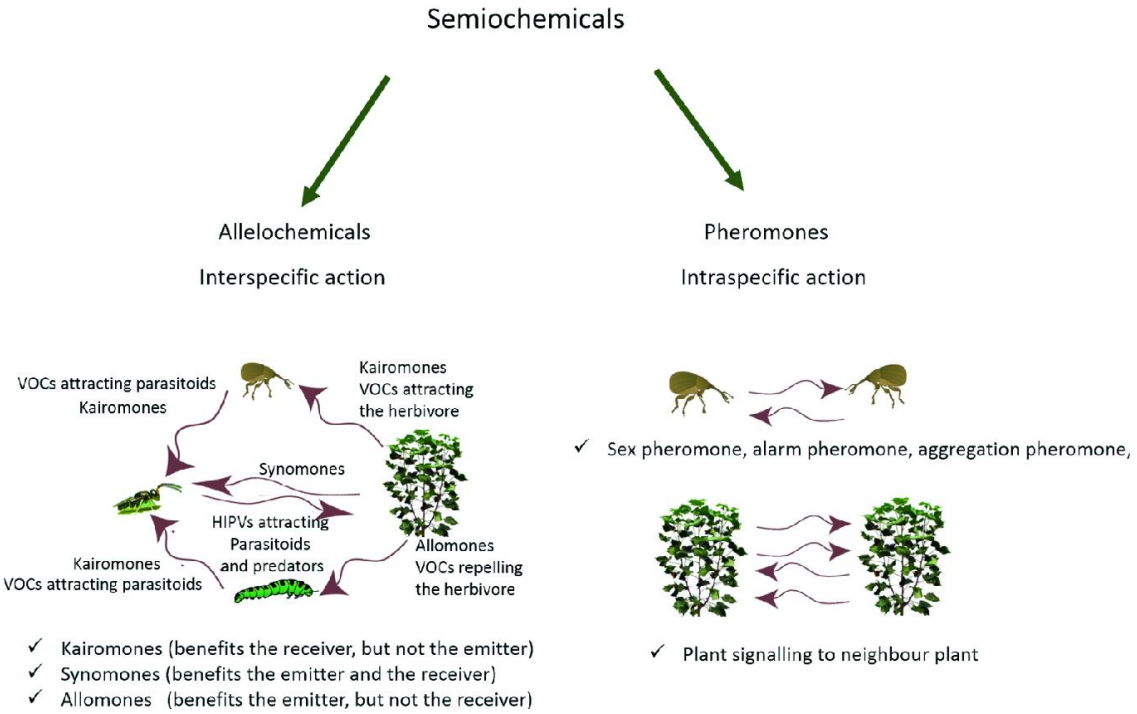
1.4 Semiochemicals as mediators of insect behaviour

1.4.1. Definitions

Arthropods live in a chemical world. Any chemicals that contain information of biological and ecological relevance for living organisms in the environment are called semiochemicals (Gk. Semeon, a signal). Semiochemicals include chemical cues that mediate the interaction between organisms (Scheme 1.1). Semiochemicals can be volatile compounds that act as pheromones. Semiochemicals are produced by various organisms and affect the behaviour and/or physiology of others. These chemicals mediate the integration between living organisms. These chemical signals can mediate interaction within a species or between different species. They affect host finding, food source searching, mate choice, and signalling of rivals or enemies. They can be volatile compounds acting as long-distance mediators detected by the olfactory sensory system, or non-volatile or low-volatile compounds used by direct contact with gustatory receptors at short range. Semiochemicals can be divided into two groups. If a chemical is emitted by an organism to attract a mate for reproduction, it is a **pheromone**, and this group of semiochemicals is intraspecific. Pheromones are active at very low levels, and they are not toxic to other organisms. Pheromones affect the behaviour of the releaser and primer. According to biological function, pheromones are divided into different groups and these groups have different chemical properties. Pheromones can affect insects at close or long range. Pheromones can fall into one of two chemical structural groups: firstly, compounds comprising straight-chain C10-C18 alcohols, aldehydes, and acetates, with 0-3 double bonds; and secondly compounds consisting of C17-C23 polyunsaturated hydrocarbons, or the corresponding mono- or diepoxides (Millar, 2005). They may serve as an alarm, sexual, aggregation, territorial or

ovipositional signal, trail signal, form of recruitment, kin-recognition, propaganda, or nested building signal. For instance, trail pheromones are compounds used by social insects to mark pathways for important life routes (way to the nest, food source). One of the most famous examples of an alarm pheromone is banana isoamyl acetate, or 2-heptanone for nest abandoning in honey bees. Primer pheromones are used for sexual reproduction, maturation, development, and regulation of other physiological states. Overall, pheromones affect insect behaviour.

On the other hand, if the production of **allelochemicals** benefits the sender, they are called allomones, used for defence and the production of repellent secretion. Allelochemicals are chemicals that interact between different species. Allelochemicals that benefit the receiver are called kairomones. Chemical cues that benefit both sender and receiver, as in the case of a floral scent when the flower benefits from sending a signal to the pollinator and the pollinator benefits from the flower for the collection of nectar, are **synomones (Marcel Dicke & Sabelis, 1992)**. **Kairomones** are used in a host, oviposition site or prey location (Hansson & Anton, 2000). This thesis deals mostly with kairomones: **apneumones** - when a compound is emitted from a non-living source and is beneficial for the receiver, with an example being the attraction of flies or insects attracted to the smell of dead fish. Moreover, if one species emits sex pheromone to attract the opposite sex, but a second species, for example, a parasitoid, uses that same chemical signal to locate the pheromone sender, this is a kairomone for the receiver (Marcel Dicke & Sabelis, 1992).



Scheme 1.1. Classification of semiochemicals. Semiochemicals are classified according to the interactions between the organisms in a given system. Allomones are produced by one species, and they convey information that causes a response in a different species. Pheromones are species-specific produced by one sex to convey information to the same species of the opposite sex or same-sex. Allelochemicals are further subdivided into three subcategories based on the benefits of the emitter and the receiver (Blassioli-Moraes, Laumann, Michereff, & Borges, 2019)

Semiochemicals are used in pest management strategies. They are adopted for pest population control, monitoring and decreasing the negative impact of pests. The most widely used are pheromones, for mating

disruption of lepidopteran species. Pheromones and other semiochemicals can be used for early monitoring, attract and kill, and push and pull strategies in the fight against notorious pest species (Witzgall et al., 2010). The advantages of producing and utilising these compounds in IPM is that they can be used in the dark, they can travel around obstacles without being reflected, low volatile chemicals can provide long-term signals, they can travel long distances, and they have a narrow channel of communication for specific organisms.
Depending on our knowledge of how insects use semiochemicals to communicate with each other and the environment, we can simulate and mimic their communication for our benefit. Semiochemicals can be used for monitoring, lure and kill methods, mass trapping, manipulation of behaviour or mating disruption, inhibition of aggregation, inhibition of ovipositional and alarm signals, and in the push and pull system. On the other hand, semiochemicals can be used for monitoring and manipulation of beneficial behaviour. Plants may produce herbivore-induced volatiles, and extensive research has been done in this area, allowing the use of HIPVs to manipulate plant defences.

Semiochemical use in agriculture is affected by many factors, such as the amount of the active blend, which depends on insect physiology, with sensing by insects resulting from insect physiology and insect behaviour. The diffusion coefficient of compounds in a given agricultural environment depends on chemical properties, trap design, and environmental conditions.

1.4.2. Insect perception of odour: morphology and physiology of the central and peripheral nervous system

Insects have an involved sensory system for detection of cues from the surroundings and host, and the nervous system is highly tuned with the ability to combine input from sensory neurons. Insects develop special olfactory circuit and class of numerous sensory neurons expressed in olfactory receptors that are specialised for detection of diverse upcoming olfactory cues from the environment and respond to ether positive or harmful properties of the habitat milieu (Stensmyr et al., 2012).The insect nervous system has developed to detect odours with a high level of spatial-temporal resolution (Meiners, 2015). Chemoreception plays a key role in regulation of behaviour in relation to the location of a host plant, site of food sources, and recognition of a suitable mating partner. Therefore, I will briefly explain the basics of the insect nervous system below.
The insect olfactory system contains odorant receptors (ORs), with narrowly and broadly tuned ORs. Narrowly tuned ORs are used to detect single olfactory cues, for which specialisation is not so common in insect world (Stensmyr et al., 2012). Single compound ORs are well-known in the case of pheromones. ORs that are tuned for recognition of olfactory blends are more abundant.

Olfactory sensory cell dendrites are located in sensilla on the antenna and in the maxillary palps of insects. There are three morphological types of olfactory sensilla: basiconic sensilla, trichoid sensilla, and coeloconic sensilla (Hansson, 1999). Odour is primarily detected with odorant receptors (ORs) hosted in the dendritic membrane of olfactory sensory neurons (OSNs) (Andersson, Löfstedt, & Newcomb, 2015). OSNs typically express only one type of OR. However, in some cases OSNs can also express up to three ORs. OSNs expressing the same types of OR go to a single glomerulus in the antennal lobe (AL) in the insect brain (Galizia & Sachse, 2010).

Dendrites, axons and synapses (neuropils) in the insect brain process olfactory information in a region called the antennal lobe (AL). The antennal lobe is the primary olfactory centre of the brain. The insect

antennal lobe is a structure in glomerular units, where OSN axons, local neurons and projection neurons interact. It is worth recalling that in insects neurons generally have their cell body outside the brain, and neural activities are accomplished in the somaless neuropil. Most OSNs have express receptor proteins Or83b in Drosophila. OSNs usually express ORs that are related to seven-transmembrane G–protein-coupled receptors (GPCR). Odour molecules contain information and the interaction of these molecules with specific receptor proteins in the dendritic membrane of OSNs activates ion channels, translating the molecular signal into an electrical response – action potential. Sensory neurons convert information from an odour stimulus into a frequency of action potentials and project their axons to the brain (Stengl, Hatt, & Breer, 1992). This action potential interacts between synapses with local interneurons and projection neurons in the glomeruli of the antennal lobes (Hansson, 1999). In the AL, chemical information is processed by local interneurons and moved on by projection neurons to higher brain areas. The main output area of the higher brain is mushroom body calyces and the lateral horn (Galizia, 2014).

Insect olfactory neurons have three types of chemoreceptors: ionotropic receptors (IRs), gustatory receptors (GRs), and odorant receptors (ORs). Recent work suggests that ORs, being younger than GRs and IRs, increase the detection spectrum of compounds but also sensitivity and speed of detection, which is important for odour-sensing during flight (Becher, Hagman, et al., 2018). The evolution of ORs would thus coincide with the evolution of early yeasts and bacteria (Becher, Hagman, et al., 2018). Some yeast volatiles are indeed known as OR ligands (Galizia, Sachse, Rappert, & Menzel, 1999; Münch & Galizia, 2016).

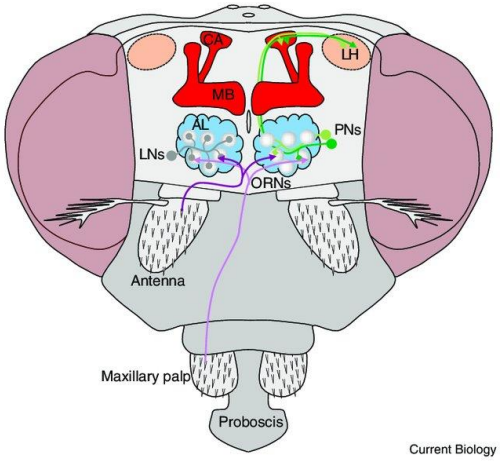


Figure 1.1 *Drosophila* olfactory organs, the antennae and the maxillary palps. Olfactory receptor neurons (ORNs) in sensillae on the 3 rd antennal segments and the maxillary palps project their axons bilaterally into individual glomeruli in the antennal lobe (AL). In these glomeruli, ORN input is integrated and processed by the action of mostly multiglomerular excitatory and inhibitory local interneurons (LNs). Processed odour information is then relayed to the calyx (CA) of the mushroom body (MB) and the lateral horn (LH) by uniglomerular projection neurons (PNs) (Perisse, Burke, Huetteroth, & Waddell, 2013).

1.4.3. Volatile-mediated interaction and use in integrated pest management

Stimuli used in habitat location are usually long-range olfactory cues, plant volatiles or herbivore pheromones, though other so-called short-range stimuli are non-volatile (colour)(Vet & Dicke, 1992).

In the complex environment in either natural or artificial ecosystems such as agricultural fields, crops and orchards, chemical cues are abundant. Successful location of the correct chemical signal is a crucial

survival ability for insects. The natural environment is full of volatile signals originating from different sources. VOCs are present in different concentrations and ratios, with different diffusion influenced by weather conditions, mixed with misleading signals of background odour, these presenting the main obstacles for insects in detecting food, mates and oviposition sites. The olfactory sensory system is therefore one of the most important factors in long-distance searching.

Volatile chemical compounds have physiochemical characteristics such as a low boiling point, high vapour pressure and low molecular weight (<300Da), the ability to evaporate in both gas and liquid phases, and the ability to diffuse in space, moreover they have an ecologically significant ability to carry important ecological information and act as informative chemicals. As chemical compounds, volatiles belong to acids, alcohols, aldehydes, alkenes, esters, ketones, terpenes, benzenoids and pyrazines (El-Sayed, 2020). Sources of volatile cues can come from insect, food or plant habitats, direct host-related cues or indirect host-related cues, such as background odour. Host plant VOCs are present in large amounts and are easily detectible by flying insects.

Volatiles are involved in two critical steps during insect foraging: firstly, location of a habitat where food can be found and mating can occur; and secondly, location of a habitat (fruit) for oviposition.

Semiochemicals affect insect behaviour and as such can be used for manipulation of insect behaviour. Novel tactics manipulating insect olfactory behaviour are used in biological control of invasive or destructive insects. Semiochemicals emitted by plants can affect or modify the behaviour or performance of insects (Loreto, Dicke, Schnitzler, & Turlings, 2014).

Volatiles produced by plants (HIPVs) under herbivore attack are important chemical cues for use by natural enemies in a tritrophic context (Marcel Dicke & Baldwin, 2010). Parasitoids exploit herbivore-induced plant volatiles as important cues in herbivore host location (Aartsma, Bianchi, van der Werf, Poelman, & Dicke, 2017). Research on herbivore-induced plant volatiles and their interaction at trophic level is well summarised in (Vet & Dicke, 1992). HIPVs are exploited in biological control to attract natural enemies. Sex and aggregation pheromones have been successfully used to reduce pest insect numbers, and in mating disruption of many insects, mostly lepidopterans. These pheromones are used to capture and lure male conspecific pest insects. Common tactics with pheromones are attract and kill systems, or mass trapping, which will be described in the following subsection. Repellent semiochemicals are implanted to deter pests. Repellent infochemicals are used in the push and pull technique, where an attractant is used simultaneously to push and lure the pest. mVOCs with an important ecological role for certain insect pests can be applied in trapping systems. In the form of synthetic volatiles, semiochemicals are used in applied studies developing new invasive trapping systems. The most behaviourally-active volatiles emitted by *D. suzukii* host fruit have been intensively investigated to develop more selective sustainable strategies (Revadi et al., 2015).

The discovery of the *Bombyx mori* sex pheromone (Butenandt, Beckmann, Stamm, & Hevker, 1959) was the first evidence of the ecological significance of insect chemical communication and started a chemical ecology revolution. Sexual pheromone dispensers are used for mating disruption of several pests, such as the codling moth *Cydia pomonella* in apple orchards (McGhee, Miller, Thomson, & Gut, 2016), *Spodoptera littoralis,* the Egyptian cotton leafworm, the Mediterranean flour moth, *Ephestia kuehniella,* the grapevine moth *Lobesia botrana (Claudio Ioriatti, Lucchi, & Bagnoli, 2008)*, and the pink bollworm, *Pectinophora gossypiella* (Saunders) (Witzgall et al., 2010). Volatiles that act as insect semiochemicals, pheromones, and kairomones are used to fight many pests in agriculture and horticulture, not only Lepidoptera but many Diptera as well (El-Sayed, 2020). Volatiles released from the apple fruits have been used for trapping of the apple maggot

fly *Rhagoletis pomonella* (A. Zhang et al., 1999). VOCs from cucumber have been used to attract *Bactrocera cucurbitae* (Siderhurst & Jang, 2010). *Bactrocera oleae* is attracted to the VOCs of the bacteria *Pseudomonas putida* (Liscia et al., 2013).

1.4.4. Microbial volatiles as insect semiochemicals

Interaction between plants and insects for food, habitat, mating and oviposition of *Drosophila suzukii* and other insects does not depend only on volatiles emitted by host fruit, plants and leaf mass. On the surface of a host plant, fruit and leaves there are microorganisms giving a smell to fruit and leaves, invisible to our eyes and sense of sell. The metabolic products of microorganisms give a smell to soil (Stensmyr et al., 2012). Microorganisms such as bacteria, fungi, and yeast release distinct and abundant different volatile organic compounds called microbial VOCs (mVOCs). These compounds are small odour molecules having less than 20 carbons in their structure, with a mass lower than 300 Daltons, high vapour pressure and a low boiling point (Piechulla, Lemfack, & Kai, 2017). mVOCs directly emitted by microorganisms are used by insects as valuable biological signals. Therefore, they influence insect choice and act as semiochemicals. In general, all mVOCs are the result of metabolic pathways, and they belong to aromatic compounds and fatty acid derivatives; terpenoids, nitrogen and sulphur compounds (Kakizaki, 2016). For example, fungi produce volatiles composed of eight-carbon alcohols (1-octen-3-ol), or other short alcohols, ketones and aldehydes (Combet, Henderson, Eastwood, & Burton, 2006). mVOCs have an ecological and biological role in ecosystems. Though their ecological role is not yet fully understood, it appears that complex tritrophic interaction can affect the production of mVOCs. mVOCs could signal host suitability and may indicate the time of optimal conditions for the use of a certain habitat (Beck & Vannette, 2017).

mVOCs may promote plant growth, induce systematic resistance against pathogens, mimic plant hormones and inhibit the growth of competitor organisms (Ryu et al., 2004). Moreover, they mediate insect behaviour and have an impact on mating attraction, oviposition sites and food resources, also affecting signalling avoidance in relation to possibly hazardous conditions (Davis, Crippen, Hofstetter, & Tomberlin, 2013). mVOCs attract the insect vector for dispersal (Christiaens et al., 2014) by evolving enzymes that produce the volatile directly responsible for insect attraction. mVOCs can be used to defend plant flowers under attack by a pathogenic bacterium (Huang et al., 2012). mVOCs signal food resources (Becher et al., 2012; Sobhy et al., 2018). The relationship between plant-insect and microorganisms is mutualistic, with both sides benefiting (Beck & Vannette, 2017). However, this relationship may be neutral, beneficial or even harmful for the receiver organism. The most famous example of mutualism between microorganisms is yeast and *Drosophila melanogaster*, with yeast being a source of food for Drosophila and used for ovipositional site finding (Scheidler, Liu, Hamby, Zalom, & Syed, 2015), while yeast uses Drosophila for its dispersal (Becher, Hagman, et al., 2018; Christiaens et al., 2014). Volatiles produced by the fungi *Puccinia monoica* Arth. mimic floral volatiles to attract insect pollinators to the buttercup, *Ranunculus inamoenus* Greene (Roy & Raguso, 1997).

Insects have fungal and bacterial symbionts that produce aggregation pheromones as an integral part of their body and gut (Davis et al., 2013). Fermented fruit volatiles are usually a product of mVOCs and are attractive to many insects. Additionally, these compounds are known as pheromone compounds: ethyl acetate, ethyl hexanoate, phenyethnol, for instance for *Cetatitis capitate*, the medfly (Diptera: Tephritidae)(Davis et al., 2013). (Becher et al., 2012) reported that yeast VOCs are responsible for *D. melanogaster* attraction.

Bacterial fermentation volatiles such as alcohols, pyrazines, ketones, acids and phenols are attractive to tephritid flies (Leroy, Sabri, Verheggen, et al., 2011). Volatiles emitted by aphid honeydew are influenced by bacteria inhabiting honeydew and are used by ants to distantly discriminate aphid species (Fischer et al., 2017). Yeasts of the genus *Metschnikowia* signal host plant finding and recognition to the codling moth *Cydia pomonella* (Tortricidae, Lepidoptera) (Witzgall et al., 2012).
Some mVOC cues can be used at different trophic levels, signalling food and prey location, an objective we investigate in Chapter 5.

During long evolutionary diversification, insects have developed a sensory system to detect plant mVOC compounds that are emitted from sources that may be harmful to their life or offspring. This ability gives them an adaptive advantage and allows more successful allocation of time and energy in search of valuable resources for offspring. The grapevine moth, *Lobesia botrana* Schiff (Lepidoptera: Tortricidae) is repelled by the *Botrytis cinerea* emitted 3-methyl-butanol volatile, which signals avoidance of sites with this fungus (Tasin, Knudsen, & Pertot, 2012). Entomopathogenic fungi produce repellent mVOC compounds that insects take advantage of to avoid contact with these fungi.

Additionally, if not influencing insect behaviour directly, mVOCs may attract or repel predators or parasitoids (Goelen et al., 2020). Moreover, microbial VOCs, from the plant surface or below ground, can improve, alter, mask, and interfere with plant volatiles (Marcel Dicke & Baldwin, 2010; Pineda, Zheng, van Loon, Pieterse, & Dicke, 2010).

Microbial VOCs may be ideal for use in agricultural applications, as they may be more species specific and improve current insect control and monitoring.

1.4.5. Use of semiochemicals to control *Drosophila suzukii*

Sex pheromones mediate the mating of many insect species (Witzgall et al., 2010). In *D. melanogaster* the male-produced pheromone, 11 cis-vaccenyl acetate (cVA), mediates aggregation of flies. At the SWD production site of this sex pheromone, the ejaculatory bulb is reduced, and two studies have produced different findings regarding this problem. While (I. W. Keesey et al., 2019) reported that SWD produces cVA in low amounts, another study explained that sensory neurons to perceive and react to this aggregation pheromone are conserved even if SWD does not produce the pheromone itself (Alkema, Dicke, & Wertheim, 2019; Dekker et al., 2015).

Odours that come from fermentation, yeast, fruit and leaf sources are attractive long-range cues utilised by SWD. mVOCs can be used in behavioural manipulation of insects because these volatiles are chemically distinct from the background plant volatiles (Witzgall et al., 2012). Commercial lures utilising the odours of SWD food sources have been utilised to develop attractants for application in the open field. Research has focused on chemical cues with broad-range attraction and repellence for SWD. A microbial volatile organic compound in use for attraction to SWD is well explained in the Chapter 3 discussion in this thesis.

The physiological status of the fly has to be taken into account when observing olfactory cues of SWD, since flies seek rotten fruit for food and to reach mates, while for oviposition ripening fresh fruit odours are used as the main oviposition location cues. In general, fruit flies use ubiquitous odours from fermentation products for their feeding purposes. Plant source (leaf) VOCs attracting SWD have been little investigated, as well as volatile cues coming from the mVOCs of microorganisms (Bolton, Piñero, & Barrett, 2019; Cloonan, Abraham, Angeli, Syed, & Rodriguez-Saona, 2018) stated that fruit and yeast volatiles might

interact in female attraction to ovipositition sites. Electrophysiologically active compounds may be produced by multiple sources. Single compounds can never be as attractive as a ubiquitous blend of different compounds in special ratios and adequate concentrations (Bruce, Wadhams, & Woodcock, 2005). The synergetic attraction of different fermenting odours is explained in the early years of SWD invasion (P. J. Landolt, Adams, Davis, & Rogg, 2012). They found a wine-vinegar combination and an ethanol-acetic acid combination to be better attractants that any of these tested singly. Following a series of studies by (Cha, Adams, Rogg, & Landolt, 2012; Cha et al., 2014b) on rice vinegar and Merlot wine, several compounds were selected for the creation of synthetic attractants. Acetic acid, ethanol, acetoin and methionol are components of commercially used attractants called Pherocon ® SWD Dual-Lure ™ (Trécé Ins., Adair, OK, USA) and Scentry® (proprietary blend, Scentry Biologicals Inc., Billings, MT, USA). Later on, the synergetic relation between acetic acid and acetoin was found (Becher, Jensen, Natsopoulou, Verschut, & Henrik, 2018; Cha, Landolt, & Adams, 2017). Knowledge about the synergistic effects of different attractive compounds could lead to improved lures for the attraction of SWD. Most of the compounds investigated in the last decade are the metabolic products of yeast formed in different yeast pathways. Acetoin, acetic acid and methionol are bioproducts of lactic acid bacteria, while ethanol is a bioproduct of yeast metabolism. Living yeast is a more attractive source than dead yeast for the drosophila parasitoid Leptopilina heterotoma (Thomson)(Hymenoptera: Eucoilidae) (M Dicke, Van Lenteren, Boskamp, & Van Dongen-Van Leeuwen, 1984) and for Drosophila itself (Becher, Hagman, et al., 2018).

Compounds produced or enriched during the fermentation process of apple juices were selected for a quinary blend using acetoin, ethyl octanoate, acetic acid, ethyl acetate and phenethyl alcohol. This blend showed detection of SWD at lower population levels before extensive fruit damage. Additionally, ethyl octanoate was found to increase the attraction of acetoin in the open field (Feng, Bruton, Park, & Zhang, 2018) synergistically.

Acetic acid bacteria associated with SWD gut belong to *Acetobacter, Gluconacetobacter and Gluconobacter* (V. Vacchini et al., 2017). (Hamby & Becher, 2016; Hamby, Hernández, Boundy-Mills, & Zalom, 2012) investigated microbial interaction and *D. suzukii*. Isobutyl acetate and isoamyl acetate are the main attractive VOCs produced by *Hamseniaspora uvarum* (Scheidler et al., 2015). SWD females uses yeast for its egg development and maturation in the period after mating (Mori et al., 2017). The adults feed on yeast, and fruit blossom and microbes present on blossom and nectar are important food for adult SWD during the early season in the absence of fruits (Mori et al., 2017; Tochen et al., 2016).

Currently, the most important synthetic attractants for *D. suzukii* contain the synthetic VOCs identified in the headspace of wine, vinegar, apple cider and rice vinegar. These compounds are produced as a result of yeast metabolic fermentation on or within host fruits (Cha et al., 2012; Cha et al., 2014b; Feng, Bruton, Park, & Zhang, 2018; Kleiber et al., 2014). Yeasts play a significant role in the attraction of *D. suzukii* (Barata, Malfeito-Ferreira, & Loureiro, 2012; Hamby et al., 2016). The most common and abundant yeast species found in the alimentary canal, midgut and larval frass is *Hanseniaspora uvarum* (Niehaus) Shehata et al. followed by *Issatchenkia terricola* (formerly Pichia) and *P. kluyveri* (Hamby et al., 2012), which together with other secondary species elicits olfactory responses and feeding behaviour, mainly in mated females (Mori et al., 2017; Scheidler et al., 2015). Further confirmation of the interaction between *D. suzukii* and yeasts was shown in field tests, where baits inoculated with *Saccharomyces cerevisiae* (Meyen ex E.C. Hansen) showed higher attraction than non-inoculated baits (A. L. Knight, Basoalto, Yee, Hilton, & Kurtzman, 2016; S. Knight & Goddard, 2015). As well as yeast influence on larval growth and development (Becher et al., 2012), gut bacteria can also influence mating behaviour, physiology and reproductive manipulation, increase longevity and other mutualistic mechanisms that have not yet been fully investigated (Hamby & Becher, 2016; Mazzetto et al., 2016)

Following the physiology of the female SWD, the fly searches for a carbohydrate-rich substrate as an egg oviposition site. At this stage, the female bases her search on the availability of fruit VOCs indicating the presence of a suitable fruit ovipositional site (Abraham et al., 2015). Further insight into intact fruit volatiles was provided by (Revadi et al., 2015). In the last few years, more attention has been given to leaf, stem and root volatiles (Bolton et al., 2019; I. W. Keesey, Knaden, & Hansson, 2015) and β-cyclocitral strawberry leaf terpenoid has emerged as a powerful attractive compound. The presence of fruit-based VOCs alone was not sufficient for strong behavioural attraction, and the addition of β-cyclocitral to fruit VOCs led to significant attraction for both SWD sexes (Bolton et al., 2019). It is important to note that SWD is able to detect different doses of compounds before *D. melanogaster*, such as isoamyl acetate, butyl acetate, hexyl acetate, methyl butyrate, and methyl isovalerate (I. W. Keesey et al., 2015).

A repellent compound could be implemented in a push-pull strategy, and for SWD several chemical cues from essential oils have been investigated; citronellol, geraniol, thymol, menthol, eugenol and vanillin (Corda et al., 2020; Renkema, Wright, Buitenhuis, & Hallett, 2016). mVOCs such as geosmin (Stensmyr et al., 2012) and 1-octen-3-ol (A. K. Wallingford, Hesler, Cha, & Loeb, 2016) have been discovered to elicit particularly strong repellent behaviour in SWD.

1.5 Biological control of *Drosophila suzukii*

There is growing demand for more environmentally friendly methods to replace or reduce the use of pesticides. Biological control is one of the key alternatives for controlling insect pests with natural enemies (bioagents) (parasitoids and predators). ''Biological control or biocontrol is a method of controlling pests such as insects, mites, weeds and plant diseases using other organisms'' (Wikipedia). In nature, biological control occurs as a consequence of the balance between organisms in an ecosystem. In agricultural areas overuse of mechanical and aggressive practices, rare crop rotation, lack of plant diversity and overuse of toxic pesticide has upset the natural balance and reduced the number of available natural enemies. The different types of biological control include: classical, augmentative and conservation biological control. **Classical biocontrol** represents the introduction of exotic natural enemies to control an invasive insect species in a new environment where these invasive species have invaded. These exotic natural enemies are collected from the areas of origin of the invasive pest. The problem that can arise with the introduction of non-native species is possible damage to native plants, harmful vector pathogens, pushing away and replacing native natural enemies. **Augmentative biological control includes** controls where natural enemies are periodically released to control insect pests in general. There are many commonly used commercial natural enemies (Van Lenteren, 2012). Hymenopteran parasitoids are usually preferred over generalist predators and hemipterans, because of the many advantages of this specific host range. In this type of biological control, there are two subtypes. If mass-reared natural enemies are released in huge numbers for immediate control, usually for annual crops, this is called **inundative control**. When mass-reared natural enemies are released periodically in the short-term to control many pest generations, this is called seasonal inoculative control (Biondi et al., 2017). This last type of pest control is achieved by the offspring, since they are introduced in large numbers periodically to control overlapping pest generations. **Conservation biological** control includes management practices to protect and enhance the number and activity of natural enemies native to the certain area. Conservation biological practices include reducing pesticide use, providing shelter and overwintering places, providing alternative artificial food when prey levels are low, and providing food sources in the form of flowering plant nectar for natural enemies.

The main advantages of biological control are that it is host specific and can be implemented in large areas because natural enemies are highly mobile, and lastly, it can take place over an extended period of time. However, in classical biocontrol, when bioagents are introduced, this can lead to a decline in native enemies naturally present. Therefore, risk assessment is mandatory before introducing non-native natural enemies into new areas. One of main drawbacks of biological control is that the pest is not immediately eliminated as with insecticide control. Biological control needs time to establish itself, and it takes several days for the pest organisms to die, the damage inflicted by the pest might continue for a few days. The second drawback is that biological pest control cannot totally eradicate pest organisms; it can only decrease the pest population. Successful biological control mostly depends on reaching adequate population densities of natural enemies (Rossi Stacconi, Grassi, Ioriatti, & Anfora, 2019). One of the drawbacks in terms of parasitoids is hyperparasitism, which can reduce the primary parasitoid population. Lastly, adult parasitoids use non-prey food to meet their energy and nutritional requirements. However, combined with other good agricultural practices, pests can be brought to levels below the economically significant threshold. Additionally, natural enemies can establish populations, but they must always be boosted, retained and supported by growers.

Currently, biological control of *D. suzukii* relies on pupal and larval parasitoids. However, standalone biological control strategies have a limited impact in decreasing the number of pests. The main families of parasitoids that attack *drosophilids* globally (50 species) belong to four families: Braconidea, Figtidae, Diapriidae and Pteromalidae (Walton et al., 2014). The most commonly found larval parasitoids include: Asobora and Leptopilina (*L. heterotoma* and *L. boulardi*). These species are not specialist. Most pupal parasitoid studies concern *Trichopria* sp, *Pachycrepoideus vindemiae,* and a larval parasitoid, *Leptopilina heterotoma (Thomson)* (M. V. R. Stacconi et al., 2017). In the early phase of SWD arrival in the Americas, the most promising parasitoid was *Asbara japonica* (Walton et al., 2014). Pupal ectoparasitoid, *Pachycrepoideus vindemiae* (Hymenoptera: Pteromalidae) is also investigated in Oregon and North Italy (R. Stacconi et al., 2013).

A generalist pupal parasitoid of Drosophila is *Trichopria drosphilae*. This parasitoid wasp is native to many regions where SWD invasion has occurred. Usually, conservational biological control is established with this parasitoid wasp in areas where they are already present. Like all other parasitoid wasps *Trichopria* forages and seeks its prey at medium and long distances, orienting itself with volatile chemical cues (Vet & Dicke, 1992). *T. drosophilae* parasitoids lay their eggs in pupae of *D. suzukii*. The larvae emerge from eggs laid inside pupae, then consuming the content of pupae from the inside and in this way killing its host. In Chapter 5 of this thesis, we focus on innovations that could be applied to the biological control of SWD with *Trichopria drosophilae*. Considering the investigation and findings from our previous studies, we try to look at our findings in the context of other trophic levels. Important chemical cues for SWD in search of food are those produced by mVOCs from its main food source, ether bacteria or yeast. Therefore, we also investigated the importance of some olfactory cues produced by microorganisms attractive to SWD and its parasitoid *T. drosophilae*.

1.6 Monitoring, mass trapping and efficient trap design

One of the ultimate goals of this PhD thesis was to develop a more sustainable system for decreasing and mitigating the effects of *Drosophila suzukii* invasive species. We have directed our work towards sustainable solutions, such as the implementation of biocontrol and good trapping systems, which would reduce calendar-based insecticide spraying in orchards and vineyards.

There are many successful applications of knowledge gathered from chemical ecology studies in general for suppressing the insect population in agricultural niches. One of the first steps in integrated pest management is **monitoring**. The aim of monitoring is to evaluate insect populations, their phenology, population dynamics and the risk of orchard damage (Hamby & Becher, 2016). The main importance of monitoring is in early detection of outbreaks, the establishment of emergence times, distribution mapping, and assessment of change in abundance and ultimately timing of insecticidal control measures if necessary. Monitoring with pheromones has been successfully applied to leafcutter ants with trail pheromones in citrus trees, the flour moth *Ephestia kuehiniella* female sex pheromone is used by in-store food producers, *Cydia pomonella*, a female sex pheromone, is used in pome fruits, and a male aggregation pheromone is used in palms to combat red palm weevil.

The scope of **mass trapping** is the removal of large populations, requiring a large number of traps to be distributed. This is a behaviour-based tool which uses attractive chemical cues or pheromones in a good trap to attract the highest possible number of target insects. Mass trapping is effective if both sexes are attracted, and if the species is oligophagous and univoltine. **Mating disruption** is widely used to manipulate herbivorous pest species and has practical implications. Using synthetic sex pheromones to disturb communication between sexes is a well-implemented strategy for many lepidopteran species. Other important tactics are the **attract and kill** system, similar to mass trapping as its scope is to kill insects after attracting them. With this trapping system, the killing component is usually an insecticide orentomopathogenic fungi.

The lack of a sexual pheromone in SWD, as well as the lack of an efficient species-selective trap system, is the current problem with SWD. Therefore, control measures for SWD depend on risk management. Monitoring using various baited traps could provide an idea of the seasonal abundance of SWD.

Monitoring techniques, trapping and early monitoring, provide some assistance as an early warning tool for SWD. Furthermore, extensive studies and research have been conducted to improve trap design and catching methods. Nevertheless, an improvement in the methodology for SWD trapping is still lacking. Effective determination of early fruit infestation is also scarce. Monitoring the number of flies arriving at the crops should help growers with management timing and the steps to be taken in plant protection planning. If monitoring and detection of first fly arrival in a crop is done promptly, it is possible to make optimal use of insecticides. Traps do not usually provide a reliable warning against *D. suzukii* attack. By the time the larvae are detected in fruit, it is too late to manage this insect, because the damage has already been done.

Population estimation with phenology models predicts life stages and management timing. Population modelling has been studied intensively (Asplen et al., 2015; Cini et al., 2012; Ørsted & Ørsted, 2019; Pfab et al., 2018; N. G. Wiman et al., 2016). Making a prediction of the intensity of attack is important for the timing of management strategies. In mixed population models, the major factors affecting the survival of SWD used are temperature, humidity and the presence of food, to explain population pressure and age structure (Nik G Wiman et al., 2014).

Assessment and evaluation of trap types in different seasons and on different special scales was carried out for SWD, as well as evaluation and optimisation of attractive lures, assessing the formulation, design, colour and size of the traps. Different types of trap have been designed for SWD and have improved in the last decade in terms of colour, size, aperture and materials. A good overview of the existing trap design is presented in (Cloonan et al., 2018).

In our investigation for this PhD thesis, although we focused attention on trap design, we mostly investigated lure and the efficiency of different lures based on wine-apple cider-sugar cane and lactic acid bacteria. Therefore, we will begin with a brief consideration of the factors to be taken into account when using a lure for insect attraction.

One promising approach to meet the demand for decreased use of pesticides in plant-protection strategies is the manipulation of insect behaviour and the use of pest-insect odour. Many aspects need to be taken into account when choosing an adequate chemical cue combination for luring SWD into traps. The physical and chemical processes by which volatile chemicals are distributed in space and time is well documented in (Conchou et al., 2019). Insects do not respond inflexibly to single elements in their odour but rather integrate several components of their environment (Conchou et al., 2019). Insect behaviour depends on how volatile compounds are distributed in space and time, and insect chemical ecology has paid attention to processes determining odour distribution in the atmosphere (Conchou et al., 2019). Changes in volatile emission in the open field depend on weather conditions, temperature, wind, and the vegetation surrounding the emitting source of VOCs, along with variations in the emission rate, physical transportation and interception on surfaces, and chemical degradation occurring over time. Therefore, spatial environmental conditions and the time distribution of VOCs from the source must be well-calculated. Environmental fluid mechanics is law for physical distribution of odours in a given space. VOCs spread through molecular diffusion and transportation by environmental factors such as wind and air flow. They can spread from a region with a high concentration to a region with a low concentration as a result of random air movements (Conchou et al., 2019).

Additionally, the behavioural response of the insect depends on the environment where it is found (Meiners, 2015). Insects are capable of sensing the smallest variation in the proportions or concentration of compounds, and successfully move towards their goal (host or food). Insect perception of odour depends on the physiological state of the insect itself and the context of the environment in a given habitat.

The odour signalling of a valuable food source to flies and other insects is always mixed with volatiles released by the vegetative parts of plants. The question is how and why insects respond to specific odour cues. Background odours have often been considered as a sensory disturbance in the detection of resource-indicating cues. Behavioural responses frequently depend on the addition of several stimuli interacting with each other synergistically or antagonistically. A sensory context is provided by volatiles, and it conveys spatial information helping insects to locate resources (Conchou et al., 2019). The insect is able to apply sensory adaptation to the background and has the ability to discriminate signals from this background. The background may make cues or signals appear less intense (Martelli, Carlson, & Emonet, 2013). VOCs may only make sense to a given insect when encountered in a specific context. Background odours interfere with VOC signal detection, but may provide a specific content recognisable by an insect.

The environment in which insects are present and contextual information from the environment influence insects' behaviour and response to signals. Individual signals are useful for understanding more about the ecological role of this signal, but these signals must be considered in the context of the complex environmental factors within an ecosystem. Some plant VOCs could be repellent or mask attractive compounds (Marcel Dicke & Baldwin, 2010). With a square of the distance from the VOC source, concentration decreases by several orders of magnitude. The insect needs time to obtain a reliable concentration average of odourscapes, and this is much longer than time need for navigation decisions once an adequate concentration of smell has been detected by the ORN. ORNs in *Drosophila* are highly precise and the behavioural response after detection of the VOCs ranges from 70-85 ms, while in contrast, moths take 150-200 ms (Conchou et al., 2019).

Cydia pomonella males are attracted to blends of female sex pheromone and host plant volatile pear ester (Bengtsson et al., 2014). This is a case of synergy between host plant volatiles and pheromones, explained in neurophysiological and behavioural studies (Conchou et al., 2019). This synergy of two types of smell is necessary since pheromonal and host plant information is integrated by the moth antennal lobe, where input from pheromone ORNs is strongly activated by the blend of these two, and not by the pheromone alone. This tells us that it is important to take into account the co-evolution of insects and plants, when considering what drives their interaction. To add to this example, the aggregation pheromone and plant volatiles could also act synergistically in palm tree weevil species, where this insect congregates on host trees to feed and mate (Steinfeld et al., 2015). As a host plant, volatiles could also antagonise pheromone signals by masking the signal itself.

Individual olfactory receptor ORs detect only a small fraction of existing volatile chemicals. Volatiles have a different ecological function independently of their chemical nature.

Semiochemical-based traps must be taken into account. An ideal lure maintains attraction over a long period of time. The lure must be placed in an adequately shaped trap according to the target insect species, should not be non-inhibitory and must be omnidirectional. Challenges with a lure can happen because of the release rate of the blend and special diffusion. The trap must satisfy the visual, physical and chemical requirements. The colour of trap must be attractive and it should be positioned strategically, taking into special account the distribution of VOCs used inside the trap. The ideal trap should have a long life and durability despite various environmental challenges such as rain, UV radiation and low temperatures. The ideal trap should constantly catch insects during the phenological phase of the crop being attacked by pests, and catch the lowest possible number of non-target organisms. Trap installation and servicing should be kept to a minimum, reducing labour and additional costs. The ideal trap should have a low cost, but there could challenges in terms of manufacturing volume, complex design and short durability.

To summarise, the current lure and trapping systems are neither effective nor selective for SWD. Commercially available synthetic lures such as Pherocon and Scentry could provide information on SWD presence before it causes damage. On the other hand, a study by (D. M. Kirkpatrick, Gut, & Miller, 2018) suggested that use of these lures and initial detection of SWD flies in these lures showed that the SWD population in sweet cherry crossed over the damage thresholds. The current systems could not be effectively implemented in push-pull, attract and kill, or mass trapping systems. There is still a huge gap in the knowledge necessary to create efficient lure and trapping systems.

1.7 Goals and objectives of this study

The spotted wing Drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) is one of the main emerging pests of valuable crops, attacking soft fruits and wine grapes in Europe and the Americas. Current control strategies rely on the heavy use of insecticides, which have a negative ecological impact. Therefore, we focused our research on finding suitable and environmentally friendly approaches to limit its spread and damage. Numerous studies have been conducted on SWD to understand its biology, behaviour, and ecology in an attempt to build an effective monitoring and trapping system. A major challenge with trapping systems remains low effectiveness in terms of attraction, as well as a lack of specificity and a spillover effect in the field. So, with current trapping systems, SWD never reaches low population densities, below economic thresholds for the crop, and thus control of this invasive species must

be mitigated with insecticides. However, more importantly, insecticide control of this invasive insect species has caused indirect damage by damaging the ecosystem and human health (Hajek et al., 2016).

While the increasingly application of VOCs isolated from diverse sources to attract and lure SWD populations appears to be a promising approach to decrease the SWD population, these VOCs are not tailored to selectively catch large numbers of flies. In addition, most research on the development of attractants with which SWD can effectively be lured into traps has focused on cues derived from host fruit. In the last decade, there has been growing 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 population behaviour of invasive insects and whether they can be successfully applied to improve integrated pest management of *Drosophila Suzukii*.

The overall aim of this PhD study was to investigate the potential of microbial volatile organic compounds (mVOCs) to improve the attractiveness of insect lure early in the season and mass trapping systems to control SWD. Wine and vinegar are commonly used in the control of *Drosophila suzukii* as a homemade attractant to reduce damage in berry fruit. For this purpose, we focused our attention on improving existing commercial liquid baits for SWD Droskidrink® (DD), as the main subjects of study, together with the different strains of lactic acid bacteria, along with an attempt to develop innovative traps both for monitoring and mass trapping. *Trichopria drosophilae* was used to prove our hypothesis and the importance of the same volatile olfactory cues across different trophic levels.
More specifically, we aimed to:

1: Determine the trapping efficiency and specificity of the best food baits, improved with the selected species and strains of MOs (lactic acid bacteria);

2: Obtain chemical characterisation of malolactic fermentation metabolic products and understand the effects of the conversion of malic acid to lactic acid and the influence of these reactions on the volatile profile of wine-vinegar mixture;

3: Identify electrophysiologically active VOCs for *Drosophila suzukii*;

4: Increase selectivity of SWD traps by investigating other sister species;

5: Develop a prototype for a commercial liquid food trap with the addition of specific microorganisms (MOs) to improve attractiveness for *D. suzukii*;

6: Assess the behavioural response of Drosophila parasitoids *T. drosophilae* to microbial (*Saccharomyces cerevisiae*) volatiles and VOCs selected from Droskidrink.

A schematic overview of the thesis content is given in Figure 1.2. In **Chapter 2,** we focused our attention on improving existing commercial liquid baits for SWD Droskidrink® (DD) and attempted to develop an innovative trap both for monitoring and mass trapping. We conducted a series of bacterial fermentation tests under laboratory condition, and behavioural assays with SWD, where we tried to assess which lactic acid bacteria (LAB) had the best performance. Next, we studied the electroantennography response of SWD flies to the most attractive *O. oeni* strains supplemented by Droskidrink®. From laboratory experiments and field trials, we established which strain of LAB improved attractiveness to *D.suzukii*.

In **Chapter 3**, we investigated whether the mVOCs emitted by bacteria affected the olfactory responses of SWD better than wine-apple cider mixture alone. Additionally, the different mixtures of DD with LAB strains were chemically characterised. Various volatile extraction method were used, such as direct head-space analysis, solid-phase microextraction and closed-loop stripping analysis.

The chemical composition of the volatile blends produced by the bacteria was analysed using gas chromatography-mass spectrometry (GC-MS) to find out differences in the mVOC profiles in different strains of *O. oeni* and differences over a period of ageing of the mixture.

Furthermore, we carefully observed the quantitative and qualitative variations caused in the DD by adding LAB strains. In conclusion, we discovered a relatively important change in volatile microbial composition after malolactic fermentation with LAB bacterial strains. Strikingly, the results showed that in mixtures with bacteria, the presence of diverse and finely tuned complex chemicals explained the high level of attraction. Therefore, the results of chemical analysis supported the results of two-fold higher attractiveness of the lure with bacteria in open field studies. Overall, these findings from field and laboratory experiments highlighted the importance of *Oenococcus oeni* (*O.oeni*) for improvement of SWD attraction. Next, we explored which chemical compounds are directly responsible for SWD attraction. Olfactory responses were evaluated for volatile blends using the most attractive bacterial strains and wine-apple cider mixture isolated from direct head-space collection. Then we assessed the neurophysiology of SWD by means of gas chromatography coupled with electroantennographic detection (GC-EAD-FID). With this setup, we tested a volatile extract collected in solvent from the first year of our studies. Overall, the results of the chemical studies showed a very complicated relationship between bacterial strains and bait features. We found 32 electrophysiologically active compounds for *D. suzukii.* The compounds that elicited a constant and high electrophysiological response belonged to classes of acetate esters, esters, acids, short-chain alcohols, and ketones. Our findings further corroborate the commonly accepted theory that the absolute amount of ubiquitous volatiles is the critical factor mediating the recognition and the orientation of polyphagous insects such as SWD to feeding and oviposition sites. The quantitative variations induced in the DD through the addition of LAB were then carefully considered, as well as the interaction of the liquid trap mixture with environmental variables (temperature, humidity and non-target insect catches) during field experiments. The most attractive strains were used to determine whether the trap design could improve the attractiveness of bait.

Since the current drawback of commercially available lures for SWD is a lack of selectivity, in **Chapter 4** we attempted to investigate the odour receptor of non-target sister species of SWD, *D. simulans*. We selected the volatiles attractive to *D. suzukii* and *D. melanogaster* and tested them in an electrophysiological study on *Drosophila simulans*. We conducted a series of laboratory cage experiments in an attempt to find out which compound is repellent to *D. simulans* and finally to implement this knowledge with practical application for a SWD trapping system.

In **Chapter 5**, we tested the hypothesis that as an important pupal endoparasitoid of *Drosophila* flies, *Trichopria drosophilae* is attracted to the same olfactory cues as their hosts, aiding the localisation of suitable *Drosophila* hosts. Mutualism between microbes and *Drosophila* is a well-established evolutionary system, in which microbes produce a volatile organic compound that attracts its insect vectors, and insects benefit from a rich-protein source in return (Becher, Hagman, et al., 2018). We investigated similarities in volatiles released from bacterial and yeast fermentation to find a connection between the attraction of SWD and its natural enemies. From microbiological, genetic and chemical studies conducted in our laboratory and from previous available literature sources, we found that the volatiles common to yeast and bacterial fermentation belonged to the chemical group of acetate esters. We then focused our attention on

investigating the importance of these *acetate esters* produced in malolactic fermentation of DD with LAB strains, as well as from yeast metabolic activities, to the Drosophila generalist endoparasitoid *Trichopria drosophilae*. We genetically modified expression of the ATF1 gene responsible for the production of acetate ester and created mutants rendering the yeast unattractive to the parasitoid, with the lack of these genes. Parasitoid and SWD behaviour to different acetate esters was tested in a Y-tube olfactometer. We showed that at a certain concentration, yeast *acetate esters* mediate *T. drosophilae* attraction. Since biological control is one of the key components of integrated pest management (IPM), we wanted to fill the knowledge gap and show the connection between chemical cues mediating behaviour across trophic levels. These findings could be utilised in an efficient SWD trapping system and to boost biological control. Finally, **Chapter 6** summarises the most important findings and provides the general conclusion of this PhD study, as well as different future perspectives for research and applications.

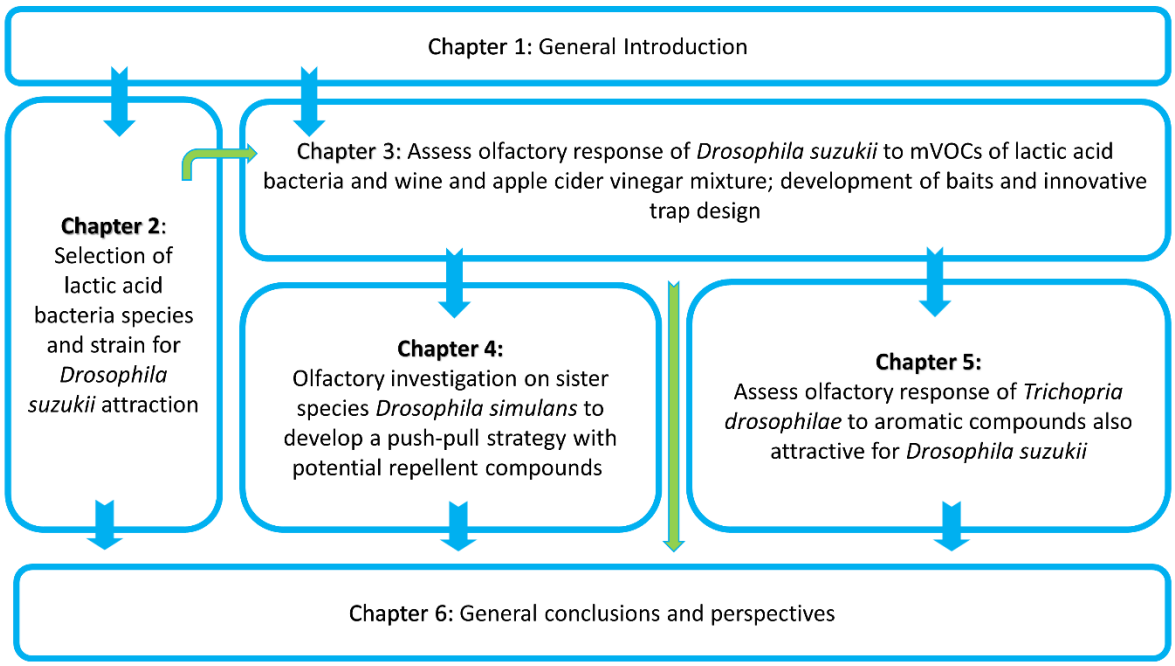


Figure 1.2. Schematic outline of the doctoral thesis. The overall of this PhD study was to investigate the potential of tailored mixture wine-vinegar and microbial volatile organic compounds emitted under lactic acid bacterial fermentation, to improve and enhance the trapping system of *Drosophila suzukii* (concept of figure (Goelen, 2020)).

Chapter 2

Selection of lactic acid bacteria species and strains for improvement of attractant for *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), the spotted-wing drosophila[1]

[1] This chapter is based on the following publication with modifications: Alawamleh, A.; Đurović, G.; Maddalena, G.; Guzzon, R.; Ganassi, S.; Hashmi, M.M.; Wäckers, F.; Anfora, G.; Cristofaro, A.D. Selection of Lactic Acid Bacteria Species and Strains for Efficient Trapping of Drosophila suzukii. Insects 2021, 12, 153. https://doi.org/10.3390/insects12020153

2.1 Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), the spotted-wing drosophila (SWD), is native to southeast Asia. However, since 2008, this insect has invaded both Europe and the Americas causing serious production losses to the soft fruit industry (Asplen et al., 2015; Cini et al., 2012; J. C. Lee, Bruck, Dreves, et al., 2011). Similar to the majority of *Drosophila* species, SWD is attracted to rotting and fermenting fruits for adult feeding (Cha et al., 2013; Walsh et al., 2011); however, unlike other species in the genus, SWD lays eggs in fresh fruit using a serrated ovipositor (Atallah et al., 2014; Karageorgi et al., 2017). Eggs and larvae have been detected in many cultivated fruits, such as sweet cherry, apricot, blueberry, strawberry, raspberry, blackberry, and wine grape (Bellamy, Sisterson, & Walse, 2013; I. W. Keesey et al., 2015; J. C. Lee, Bruck, Dreves, et al., 2011; Mazzi et al., 2017). High reproductive potential and adaptability to many environmental conditions have quickly made SWD a major agricultural pest in Western countries, capable of causing production losses greater than 90% (Cini et al., 2012). Current management of SWD relies mainly on insecticides (pyrethroids, spinosyns, and organophosphates), sanitation, net exclusion, and mulching of soil (Asplen et al., 2015; Beers, Van Steenwyk, Shearer, Coates, & Grant, 2011; Ebbenga, Burkness, & Hutchison, 2019; Farnsworth et al., 2017; Ian W Keesey et al., 2019; Shaw, Hemer, Cannon, Rogai, & Fountain, 2019). Pesticide applications have been the primary control technique against *SWD* in both North America and Europe (Sial et al., 2019; N. G. Wiman et al., 2016; Nik G Wiman et al., 2014). At present, each method of suppression has limitations and failures related to the pest population biology and dynamics, as well as social acceptability. Mass trapping and other alternative agronomical practices may also suppress populations (Alnajjar et al., 2017; Beers et al., 2011; Feng et al., 2018); however, currently used techniques do not sufficiently manage commercial infestations. Other environmentally safe methods, such as biocontrol with either resident or native natural enemies or microorganisms are still under investigation (Daane et al., 2016; Ibouh et al., 2019; Miller et al., 2015; Rossi Stacconi et al., 2019).

Monitoring SWD is the first step in an integrated pest management (IPM) program that determines where and when populations are present in the field, and when to enact control measures (D. H. Cha et al., 2018; D. Kirkpatrick, McGhee, Gut, & Miller, 2017). Currently, an accepted, and commonly used monitoring method for SWD involves the use of traps constructed from plastic cups with holes at the top for fly access (i.e. Droso Trap®, Biobest, Westerlo Belgium; Victor® Poison Free® yellow jacket & flying insect trap, Great Lakes IPM Inc., Vestaburg, MI, USA). Inside the cup, there may be a yellow sticky card and/or a mixture of apple cider vinegar (ACV), wine, vinegar and other fermenting liquids, and a surfactant (Asplen et al., 2015; Burrack et al., 2015; Clymans et al., 2019; P. J. Landolt et al., 2012; Tonina et al., 2018). However, these traps can only detect SWD after the insect population has already established i.e. when SWD numbers are generally high. Consequently, suppression of the pest population is a serious challenge (Bolda, Goodhue, & Zalom, 2010). Another drawback of these traps is that they are not species-specific, and hence there is a need to create a more sensitive trap. A species-specific trap containing odorants emitted by host fruit during ripening might attract SWD earlier in the season, allowing early detection and intervention. Such a trap would help to control the pest on a local level by suppressing population numbers (Abraham et al., 2015; Feng et al., 2018; I. W. Keesey et al., 2015; Revadi et al., 2015). A key goal of mass trapping is to capture the maximum number of insects possible before they reproduce or cause damage to crops. Effective trapping requires the use of lures that are able to attract fruit flies more effectively than natural sources; examples include food sources, calling virgin females, and mating aggregations. Furthermore, lures should be effective during the entire period of adult emergence and mating (El-Sayed, Suckling, Wearing, & Byers, 2006; Suckling et al., 2015). Accordingly, the traps must be visually attractive and capable of capturing and retaining flies long enough to provide a lethal dose of toxicant or prevent the escape by drowning or starvation (Lasa, Velazquez, Ortega, & Acosta, 2014). Understanding the biology and ecology of SWD is

therefore fundamental to setting up and optimising control techniques based on the interference of insect behaviour.

In many insects, volatile compounds released through interactions between their microbiota and target substrates have a key role in determining oviposition and feeding sites (Becher et al., 2012; Bruce & Pickett, 2011; Bruce et al., 2005; Hamby & Becher, 2016; I. W. Keesey et al., 2019). The behavioural activity driven by these volatile compounds can be exploited for the development of SWD control methods (A Grassi et al., 2015; Hamby & Becher, 2016). These volatile compounds are derived from fermentation – mediated by various microorganisms – of carbonaceous compounds present in such liquids (Barata et al., 2012; Becher et al., 2012; Gallardo-Chacon, Vichi, Urpi, Lopez-Tamames, & Buxaderas, 2010; McKenzie & Parsons, 1972). SWD are attracted to the fermenting substrates for feeding purposes (Dong H Cha et al., 2018; Clymans et al., 2019; Piñero, Barrett, Bolton, & Follett, 2019b). At present, the most effective traps for SWD are those baited with vinegar and wine, or synthetic compounds identified in the headspace of vinegar or wine (Asplen et al., 2015; Cha et al., 2014b; Feng et al., 2018; Piñero, Barrett, Bolton, & Follett, 2019a).

Yeast plays a significant role in attraction to SWD. The most common and abundant microbial species found in the alimentary canal, midguts, and larval frass of SWD is the yeast, *Hanseniaspora uvarum* (Niehaus) Shehata et al. (Hamby et al., 2012), which together with other secondary species elicits olfactory responses and feeding behaviour, mainly in mated SWD females (Mori et al., 2017; Scheidler et al., 2015). Further confirmation of the interactions between SWD and yeasts was shown in field tests, where baits inoculated with Saccharomyces cerevisiae (Meyen ex E.C. Hansen) showed higher attraction than uninoculated baits (A. L. Knight et al., 2016; S. Knight & Goddard, 2015).

However, it has been demonstrated that the attractiveness of food bait to SWD can be increased by the addition of different bacterial species. Recently, investigation of volatile organic compounds produced by bacterial fermentation has identified the key compounds in effectively attracting SWD flies. The attractiveness of bacterial volatiles has been investigated in this thesis in behavioural bioassays, along with chemical characterisation of volatile profiles. Strains and species of symbiotic acetic acid bacteria of commonly found genera in *D. suzukii* Italian populations (*Acetobacter*, *Gluconobacter* and *Komagataeibacter*) were tested in an olfactometer. Female flies showed a significant attraction for some strains of the *Gluconobacter* and *Komagataeibacter* species, which produced the most attractive volatiles and were proposed as a useful tool for developing sustainable control strategies(Mazzetto et al., 2016). The species with the highest frequencies in communities associated with SWD are Tatumella spp. (Enterobacteriaceae), Gluconobacter spp. and Acetobacter spp. (Acetobacteraceae) (Chandler, James, Jospin, & Lang, 2014). Certain species were shown to produce volatile metabolites capable of attracting SWD in olfactometer assays (Mazzetto et al., 2016). However, to date, there is little evidence that lactic acid bacteria involved in the fermentation of wine or vinegar increases attractiveness to SWD.

Lactic acid bacteria (LAB) are widespread microorganisms that can be found in any environment rich mainly in carbohydrates, such as plants and fermented foods. They are gram-positive and acid-tolerant microorganisms that grow anaerobically. LAB include several species, such as the genus *Lactobacillus*, as well as the genera *Pediococcus*, *Leuconostoc*, *Streptococcus* and *Oenococcus.* The fast-growing characteristics of LAB strains and their metabolic activities are the key to LAB benefits and applications (Zapparoli, Fracchetti, Stefanelli, & Torriani, 2012).

The primary metabolic activity is degradation of carbohydrates into different compounds, mainly lactic acid, through fermentation process (Hoefnagel et al., 2002). However, they share several unique

biochemical characteristics when compared to other bacterial groups (e.g. high malolactic activity, production of volatile compounds and growth rate) (Nielsen & Richelieu, 1999). *Oenococcus oeni* is the most well-adapted species to harsh wine conditions, due to its ability to tolerate low pH, a high ethanol concentration and sulphites (Capozzi et al., 2010). These characteristics of LAB species distinguish them from other bacterial species, making them good candidates for a more attractive and selective food bait.

The ability of LAB species to produce bioactive volatiles that attracts *Drosophila* flies to fruit has been evaluated in previous studies. The species *L. brevis* and *L. plantarum* were identified in the larval gut of *Drosophila melanogaster*, and the volatile compounds they emitted were evaluated for attraction to flies. Moreover, larval and adult flies showed significant attraction to volatiles emanating from food substrates that have been occupied by larvae, indicating that flies rely on microbial volatiles for long-distance attraction to suitable feeding sites (Venu, Durisko, Xu, & Dukas, 2014).

Additionally, *D. melanogaster* exhibited behavioural preferences for gut microbes. Both adults and larvae were attracted to volatile compounds associated with the flies' microbiome, such as *L. plantarum* in choice assays (Qiao, Keesey, Hansson, & Knaden, 2019). Similarly, symbiotic LAB species were identified in the gut of *D. suzukii,* i.e. *L. plantarum* and *L. brevis* (V. F. Vacchini, 2014). The attraction of *D. suzukii* to volatiles emitted by LAB species has not yet been assessed. There is a need to investigate the use of LAB species as a source of attraction for *D. suzukii* flies and their subsequent potential application as components of more attractive food bait.

The current study attempts to fill numerous gaps in knowledge regarding the attraction effect of volatiles emitted by LAB species on the preferences of *D. suzukii* flies, which will be helpful for developing effective tools for monitoring *D. suzukii*. Therefore, we aimed to improve the attractiveness of the commercially available food bait Droskidrink® based on the exploitation of active cultures of LAB species. In this study, we aimed to evaluate the attractiveness of different LAB species and strains for *D. suzukii* flies under field and laboratory conditions. The data obtained contributes to improving the food bait currently used to monitor *D. suzukii* and to developing new tools for efficient pest management, focusing on the exploitation of bioactive volatiles emitted by LAB strains.

2.2 Materials and Methods

2.2.1. Field assessment of the attractiveness of Droskidrink food baits inoculated with different lactic acid bacteria

A preliminary trapping experiment was carried out for approximately seven weeks during summer 2013 in a commercial vineyard at the bottom of the Adige Valley, Italy (San Michele all'Adige, 46°11'22.81" N 11°07'55.63" E). All baits were tested by using 200 mL of the adjusted DD in red plastic traps (Droso Trap ®, Biobest, Westerlo, Belgium). Treatments A, B, C, D, and E contained DD as an initial solution to which different strains of lactic acid bacteria, *O. oeni*, were added (Table S1). Treatment F was a positive control just with DD adjusted to pH 4.0. Treatment G was commercial DD with pH 2.5. Treatments H, I, and L were DD that had been sterilised at 70°C for 30 min to which *O. oeni* was then added; treatment I also contained 10 mL/L cycloheximide, and treatment L had 1 g of tetracyclin added. A drop of TritonTM X-100 (Sigma-Aldrich, St. Louis, MO, USA) drowning solution was added to each 200 mL of liquid bait to break the surface tension of the liquid. The nominal concentration of bacterial cells in DD traps inoculated with bacteria was adjusted at 10^6 cfu/mL. A randomised complete block design was used, with three replicate

blocks. During the seven-week trapping period, traps were assessed weekly as follows: insects were removed and counted, and the drowning solution was replaced.

The species of LAB were previously isolated from traditional Italian wine during the early phase of malolactic fermentation and were identified based on gene sequencing (Solieri, Genova, De Paola, & Giudici, 2010). LAB species were used as bio-catalysers for the production of biologically active compounds attractive to *D. suzukii*, and their performance was assessed based on biochemical changes in DD bait. Ten types of food baits were used with different compositions at a different pH value, as indicated in Table 1.

For each treatment, traps were set up along the rows of an orchard, with each trap having an experimental lure added. Six treatments were used in the first trial (Table 2.1). Two further trials were sequentially carried out in the same field, with extra treatments added at the start of each of trial. For all but the second assessment, traps were emptied and reset weekly.

At set-up, traps for one replicate were placed in treatment order, with the order of the treatments randomised for the second and third replicates (Figure 2.1). At each assessment, the number of male and female SWD were recorded for each trap.

Table 2.1. Composition of Droskidrink food bait tested in a preliminary field experiment in a var. Teroldego vineyard in San Michele all'Adige, Trento, Italy.

Type	Composition
Bait A	DD (pH 4.0) inoculated with *Oenococcus oeni* ATCC BAA-331
Bait B	DD (pH 4.0) inoculated with *Pediococcus* spp.
Bait C	DD (pH 4.0) inoculated with *Lactobacillus* spp. V223
Bait D	DD (pH 4.0) inoculated with *Lactobacillus* spp. V313
Bait E	DD (pH 4.0) inoculated with *Lactobacillus* spp. L308
Bait F	DD at pH 4.0
Bait G	DD at pH 2.5 (commercial version)
Bait H	DD at pH 2.5 pasteurised at 70°C for 30 min and inoculated with *Oenococcus oeni*
Bait I	DD at pH 4.0 pasteurised at 70°C for 30 min, mixed with 10 ml/l of cycloheximide aqueous solution (0.01%), and inoculated with *Oenococcus oeni*
Bait L	DD at pH 4.0 mixed with 1 g of tetracycline and inoculated with *Oenococcus oeni*

Figure 2.1. Layout and duration of trials comparing the attractiveness of Droskidrink food bait in a commercial vineyard

Trial 1 (16 Sep – 9 Oct 2013)									
A1	B1	C1	D1	E1	F1				
E2	C2	D2	F2	B2	A2				
C3	F3	B3	A3	E3	D3				
Trial 2 (9 Oct – 23 Oct 2013									
A1	B1	C1	D1	E1	F1	G1			
G2	E2	C2	D2	F2	B2	A2			
C3	F3	B3	A3	E3	D3	G3			
Trial 3 (23 Oct – 30 Oct 2013)									
A1	B1	C1	D1	E1	F1	G1	H1	I1	L1
L2	I2	H2	G2	E2	C2	D2	F2	B2	A2
H3	C3	L3	F3	B3	A3	E3	D3	I3	G3

Mean counts and percentages were obtained on the log/ logit scale along with associated 95% confidence limits: these were back-transformed. These were then divided by the number of days since set-up for a trial, to give mean catches per trap per day. All analyses were carried out with Genstat (Peter McCullagh, 2018).

2.2.2. Assessment of the Performance of *Oenococcus oeni* Strains in Droskidrink® Food Bait

Based on the findings of the preliminary field experiment (see Results section), *O. oeni* was selected as the most suitable bio-catalyser to improve the attractiveness of DD. Therefore, fourteen strains of *O. oeni* were used to determine their viability and fermentative metabolism in a standard media for LAB in terms of cultivation. Strains isolated from Italian wines were obtained from the bacterial culture collection of the Fondazione Edmund Mach laboratories in San Michele all'Adige, Italy.

The selected strains showed high resistance to wine-limiting factors (low pH, high ethanol concentration, and low fermentation temperature) and performed a reliable malolactic fermentation in previous work (R Guzzon, Anfora, Grassi, & Ioriatti, 2015). All strains were cultured in modified MRS broth medium MRSm (Oxoid, Milan, Italy) in a 96 micro volume (200 μL) plate (Starstedt, Germany) at 25°C. Thereafter, modified MRS broth medium was used to assay bacterial growth with the main values of the Droskidrink® parameters (pH value 4.0, acetic acid concentration 45 g/L, ethanol content 4% (v/v)) that would acti as limiting factors for the metabolism of *O. oeni* strains. In this case, strains were tested at a pH value of 4.0, with an acetic acid concentration of 45 g/l, ethanol content of 4% (v/v), and incubation temperature of 15°C, which is probably that encountered during field trapping. In each test, one DD parameter value was assessed with standard values of other parameters. In this experiment, three technical replicates and three biological replicates were used. Optical density (OD) measurements of bacterial cultures were performed in a PowerWave HT Microplate Spectrometer (BioTek, Winooski, VT, USA) with a Costar Flat Bottom 96-well plate with lid and 200 μL per well. Absorbance was measured at wavelength 480 nm and temperature 25 ◦C and the mean of three readings was taken. The increase in OD at 480 nm was measured. The OD at 480 nm of MRSm was used as blank. The incubation time was adjusted according to the methods recommended by the International Organization of Viticulture and Wine (OIV, 2014).

2.2.3. Electroantennography responses of *D. suzukii* females to volatile collections of *Oenococcus oeni* strains in droskidrink® food bait

On the basis of assessment of the performance of fourteen *O. oeni* strains in DD bait (see Results section), three strains were selected and used to determine the electrophysiological response of *D. suzukii* flies to volatile compounds released during the fermentation process. Then the responses of mated female *D. suzukii* flies (n=5) to the volatile compounds released by the three *O. oeni* strains strains (strain 2, strain 12, and strain 13) and the reference strain MRI 10000 were recorded for antennal activity using a standard Electroantennography (EAG) apparatus (Syntech).

Experimental flies were obtained from a laboratory colony established from fly populations collected in the Province of Trento in Italy. The colony was reared on a standard cornmeal-based artificial diet and maintained at 24±1°C, 65±5% relative humidity and with a (16:8 L:D) photoperiod. Volatile collection was obtained using a Closed Loop Stripping (CLS) approach (Salvagnin et al., 2018). A 50 ml DD sample was poured into a glass jar with a plastic top having two holes. One hole hosted a miniature 12 V vacuum graphite pump (Fürgut GmbH, Germany), which made the air circulate from the jar containing the liquid to the second tube loaded with a CLSA carbon filter (Brechbühler AG, Switzerland). After 60 minutes of

air flux, the volatiles concentrated in the CLSA filter were eluted with 100 μL of dichloromethane (Baker, Zhu, & Park, 2003).

Each stimulus, represented by the eluted samples obtained from CLS analysis, was prepared by adsorbing aliquots of the solution (25 μl) on 1.5 cm^2 filter paper and inserting them into Pasteur pipettes. The Pasteur pipettes were closed on the thinner side with a 1 ml blue tip. The solvent was allowed to evaporate for 10 min before starting the experiment. Three pipettes were used as a blank control (one empty pipette, one filled with paraffin oil solvent, and one filled with dichloromethane solvent). Before and after each recording, *D. suzukii* responses to dichloromethane solvent, 1-hexanol and 2-hexanal solutions, as standard stimuli, were recorded. These compounds are known to be common plant volatiles and effective in eliciting antennal responses in *D. suzukii*, hence they were used as reference stimuli (Revadi et al., 2015).

Each synthetic stimulus was prepared by absorbing 25 μl of dichloromethane solution at a concentration of 1 μg/μl on filter paper. A glass capillary indifferent electrode filled with Kaissling solution was inserted into the detached fly's head. The Kaissling solution was made as follows: we added KCL 0.175 g, NaCl 3.75 g, CaCl2 x 2 Molar water- 0.139 g, NaHCO3- 0.1g, PVP (of 40 PM 10 g / l) 416 arm 9 to 500 cc of water. The preparations were held in a continuous humidified, charcoal filtered air stream (1 L min-1) coming from a glass tube outlet which was positioned 5 mm from the preparation. The recordingelectrode was a similar capillary, brought into contact with the distal end of the antenna. After antenna preparation, the stimulus output was directly delivered to the antenna with the Pasteur pipette through the air flow, activated by operating an external pedal linked to the stimulus air control. Each stimulus pipette was replaced every three trials.

Data Analysis

The data obtained from experimental work to evaluate the performance of *O. oeni* strains were analysed using Principle Component Analysis (PCA). EAG responses were analysed with EAG2000 software (Syntech, Hilversum, Netherlands), and evaluated by measuring the maximum amplitude of negative deflection (mV) elicited by a given stimulus. EAG responses were compared across treatments by means of parametric one-way ANOVA, followed by Tukey's test for post hoc comparison of means. Homogeneity of variance had been determined previously with Levene's test (Statistica, version 9, Statsoft Inc., Tulsa, Oklahoma).

2.3 Results

2.3.1. Field Assessment of the Attractiveness of Droskidrink® Food Bait

The aim of this experiment was to characterise and exploit the lactic acid bacterium *O. oeni* as a biological catalyst in the production of volatile organic molecules attractive to SWD. We explored the attractive performance of different strains of lactic acid bacteria with regard to biochemical changes of the bait, such as the increase of the standard pH of DD (typically about 2.50) up to 4.0, a value recognised as ideal for the hetero-fermentative activity of wine lactic acid bacteria (Nielsen & Richelieu, 1999) . Overall, the DD baits inoculated with the different species of lactic bacteria caught a significantly higher number of *D. suzukii* adults compared with the other baits ($p<0.001$) (Appendix table A 2.1). The *O. oeni*-baited trap in treatment A caught a significantly higher number of *D. suzukii* adults than traps baited with the combination of DD and other genera of lactic acid bacteria and commercial DD alone. For all three trials, there were significant differences in the numbers of males and total SWD between the treatments ($p = 0.027$, 0.015, 0.003 for males and $p = 0.027$, 0.017, 0.001 for total SWD for Trials 1, 2, 3, respectively) (Figure 2.2). There was no significant difference in the numbers of females for Trial 1 ($p = 0.236$), but there were

significant differences in Trials 2 and 3 (p = 0.023, 0.001, respectively). The percentage of female SWD was similar for all treatments in Trials 1 and 3 (p = 0.828, 0.061, respectively), but there was a significant difference between treatments in Trial 2 (p = 0.039) (Appendix table A 2.1).

The mean numbers of females were strongly correlated with the numbers of males (r = 0.95, 0.96, 0.95 for the three trials), reflecting that the patterns between the means were very similar for both sexes, and thus also similar to patterns in the total SWD (Appendix table A 2.1 and Figure A 2.1).

For all three trials, the highest total SWD was in Treatment A (*O. oeni* alone) (Figure 2.2 and Apendix figure A 2.2). Of the original six treatments, Treatment C (*Lactoccocus* V223) had the lowest total SWD, followed by Treatment F (DD adjusted to pH 4.0). However, the total SWD in Treatment G (DD adjusted to pH 2.5) was lower than in C, and for Trial 3, the total SWD in Treatments H, I and L were lower than in Treatment G. Thus, of all the non-DD treatments, only Treatment C had a total SWD less than all DD treatments, with the exception of Treatment F (not all differences were significant). In addition, the effectiveness of *O. oeni* (C) was reduced following the addition of DD at pH 2.5 or 4.0 (treatments H and I compared with treatment A).

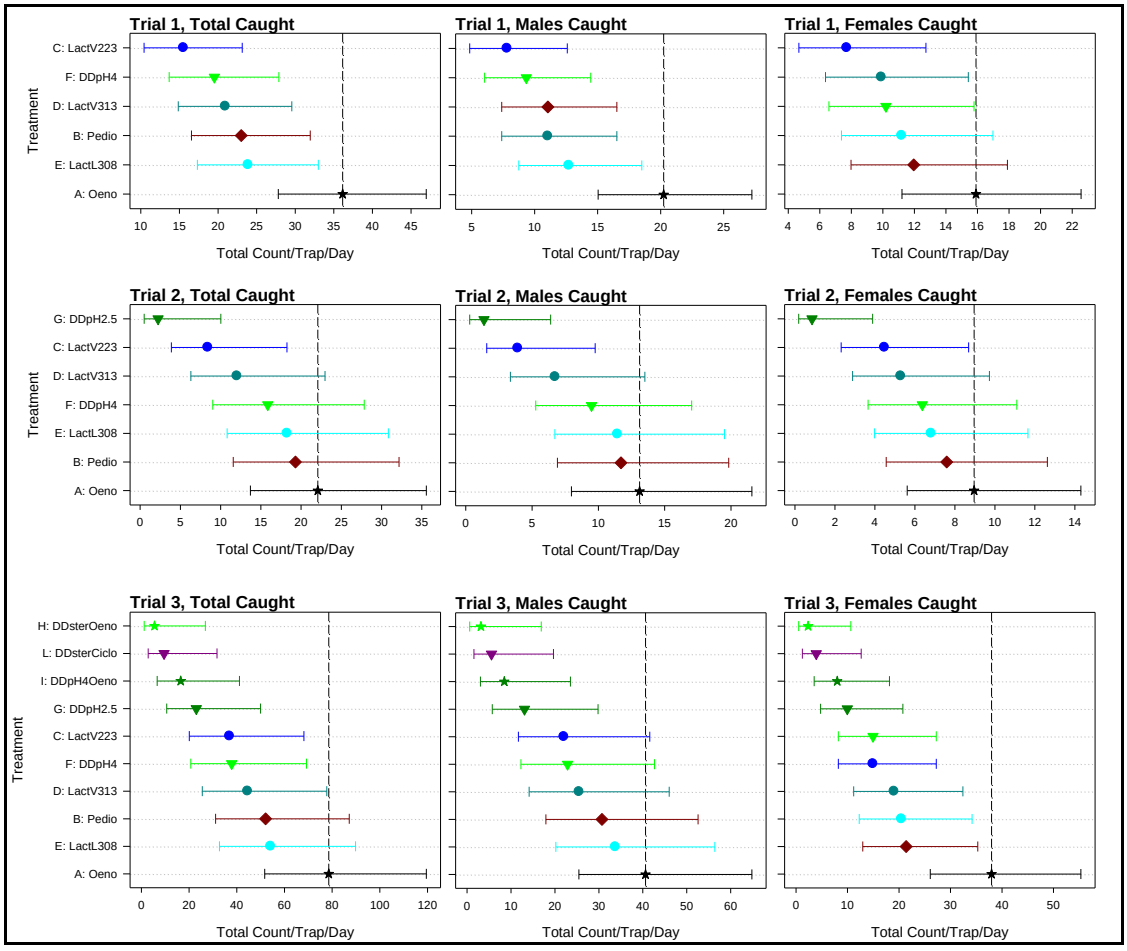


Figure 2.2 Mean numbers of *D. suzukii* male and female flies captured in each trap per day for three trials. Error bars show 95% confidence limits, and the vertical dashed line shows the mean for bait A. Note that where the order of the bait types differs between the graphs, the color/ symbol indicates bait type.

The mean percentage of the total SWD that was female varied from 44% to 52% in Trial 1, a relatively minor difference. In Trials 2 and 3, the variation across treatments was larger: 37–53% for Trial 2 and 38–49% in Trial 3. These results suggest that females are, in general, only slightly less likely to be caught than males (Appendix figure A 2.2).

2.3.2. Assessment of the performance of *Oenococcus oeni* strains in Droskidrink® food bait

All the strains of *O. oeni* showed differentgrowth rates in MRSs, with respect to the preliminary field experiment. (pH value 4.0, acetic acid concentration 45 g/L, ethanol content 4% (v/v), and incubation temperature 15 ◦C) allowed reasonable sorting of the *O. oeni* strains, according to the resistance to such limiting parameters. Figure 2.3 shows shows the difference in the mean absorbance of *O. oeni* strains growth. Strain 3 showed the highest tolerance to low pH (4.0), and strain 14 had a significant tolerance to high acetic acid concentration (45 g/L). When compared with the control, strain 3 and strain 14 exhibited higher growth than the control at the tested pH and acetic acid concentration. Likewise, strain 2 showed the highest growth at high ethanol content (4%) and strain 12 showed the highest growth at low temperature (15 ◦C). In comparison with the control, growth of strain 2 and strain 12 was higher than the control at the tested ethanol content and temperature. Strain 2 and strain 12 showed high tolerance to high ethanol content (4%) and low temperature (15 ◦C), respectively. Overall, strain 2 and strain 12 showed a significant tolerance to the main limiting parameters for *O. oeni* growth, i.e., high ethanol content and low temperature. Strain 13 showed the highest tolerance to three limiting parameters, i.e., low pH, high acetic acid concentration, and low temperature (Appendix table A 2.2). Thus, the three strains selected for further experimentation for the evaluation of the behavioral responses of *D. suzukii* to volatiles emitted during fermentation process were strain 2, strain 12, and strain 13.

2.3.3. Electroantennographic response of *D. suzukii* to volatile collection of *Oenococcus oeni* strains in Droskidrink bait

The EAG responses of *D. suzukii* females to the headspace collected from DD inoculated with different *O. oeni* strains are depicted in Figure 2.4. As expected, the reference compounds 1-hexanol and 2-hexanal elicited strong responses from *D. suzukii* antennae (Appendix figure A 2.3). There were no statistical differences between the samples (DD, strain 2, 12 and 13) and the reference strain (MRI 10000), but all the baits were statistically different from both the solvents and the blank control (ANOVA: d.f. = 47; F= 8.6; $p< 0.001$) (Appendix table A 2.3).

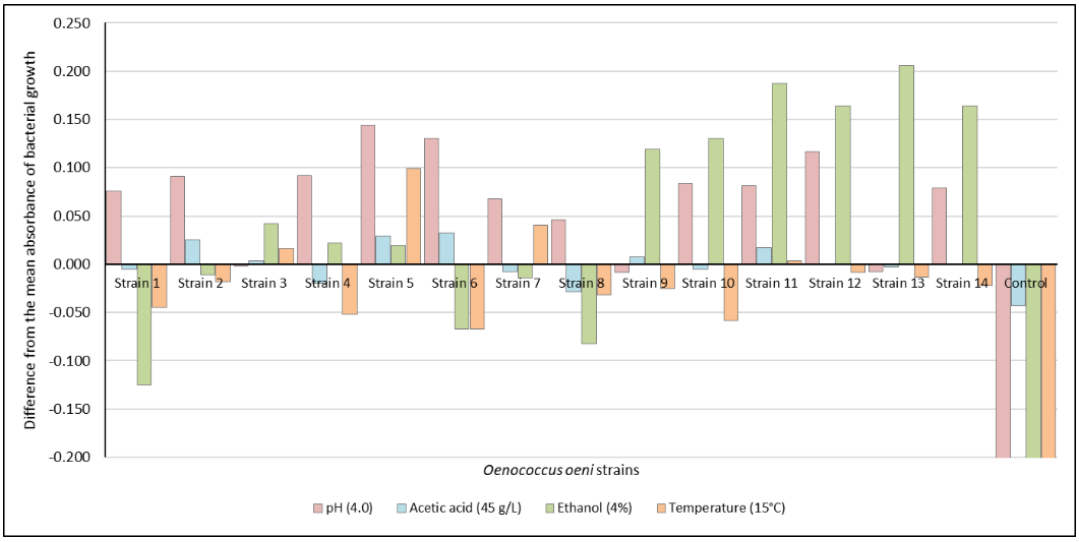


Figure 2.3 Differences in the mean absorbance of bacterial growth in synthetic media considering the four main DD limiting factors for O. oeni growth (pH 4.0), ethanol (4%), acetic acid (45 g/L), and temperature (15 ◦C).

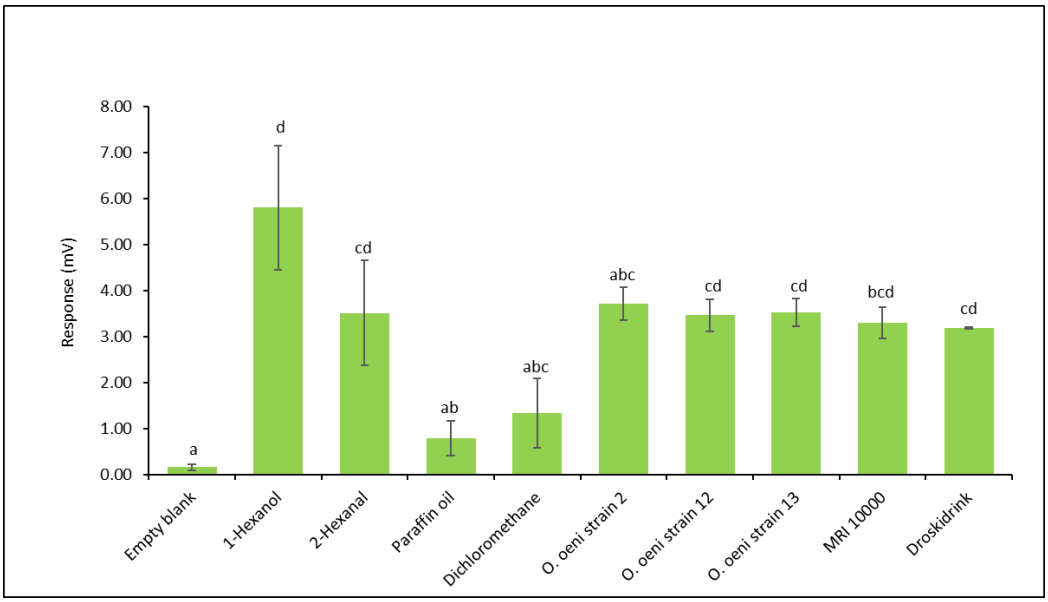


Figure 2.4. Mean electroantennography (EAG) responses (mV) of *D. suzukii* mated female antennae elicited by commercial DD and DD inoculated with different O. oeni strain 2, strain 12 and strain 13. Control stimuli: empty blank, paraffin oil, and dichloromethane solvent. Reference compounds: 1-hexanol, 2-hexanal. Baits: commercial DD, O. oeni reference strain (MRI 10000), strain 2, strain 12, and strain 13. The standard deviation of the means is reported. Bars represented by similar letters are significantly indifferent ($p > 0.05$).

2.4 Discussion

The attraction performance of different LAB species with regard to biochemical changes in the bait was explored, for example with an increase in the standard pH of DD (typically about 2.5) up to 4.0, since this value is recognised as ideal for hetero-fermentative activity in wine LAB (Liu, 2002; A. Lonvaud-Funel, 1999; Aline Lonvaud-Funel, 2001). Overall, the DD baits inoculated with the different LAB species attracted

a considerably larger number of flies in comparison to other baits. The highest attractiveness was observed in the bait inoculated with *O. oeni*, which coincides with the outcomes of recent research comparing different lures for their attractiveness for *D. suzukii* in sweet cherry orchards (Tonina et al., 2018). In contrast, pasteurised DD baits and DD bait mixed with tetracycline did not show any significant attractiveness, due to limited microbial activity in the liquid bait.

Our findings imply that the utilization of the selected LAB species in DD bait was carried out successfully in the preliminary field trials. This is not surprising considering that they are an extremely important group of industrially related lactic bacteria, and considering their behavior and robustness under stressful conditions. LAB strains were used in our study to ferment the liquid bait and produce bioactive volatile compounds that are associated with *D. suzukii* attraction. Therefore, the type and concentration of volatiles present in the DD bait are greatly affected by the LAB strain used. The results clearly confirm the hypothesis that volatiles produced by the fermentation metabolism of LAB are attractive to *D. suzukii*. Concerning the different species of LAB, it has been already mentioned that *O. oeni* have a remarkable hetero-fermentative activity and resistance in a low pH environment (Liu, 2002; A. Lonvaud-Funel, 1999; Aline Lonvaud-Funel, 2001; Sun, Chen, & Jin, 2018). Therefore, their enhanced biological activity and effectiveness in trapping *D. suzukii* is expected.

Recently, the addition of *O. oeni* to DD bait was evaluated in a comparative survey using Droso-Trap® for monitoring fly populations in sweet cherry orchards. It was found that DD with *O. oeni* was effective in attracting *D. suzukii* during the blooming and until the beginning of fruit ripening [20]. These findings support our results, which show that *O. oeni* contributed to the highest attractiveness of the DD baits. Despite the differences in DD composition, field temperature, fruit phenological phases and pest population density between this comparative survey and our study, *O. oeni* in DD showed a similar trend in attracting flies, indicating a consistent fly response towards *O. oeni*. Likewise, oenological trials showed that the pH value, adopted in the experiment, induces the shift of the metabolic activity of LAB from homo- to hetero-fermentation of sugars, increasing their energetic yield (Liu, 2002; Aline Lonvaud-Funel, 2001).

The field experiment in Italy was characterised by a low number of *D. suzukii* catches since it was carried out early in the season when population density in the fruit growing areas of Trento Province is low (N. G. Wiman et al., 2016). However, the aim of this trial was to evaluate the potential use of both DD and DD inoculated with *O. oeni* as an early warning tool to provide reliable information on the first *D. suzukii* infestation of cultivated tree fruits e.g. sour cherry. The numbers of insects caught in traps inoculated with bacterial strains did not differ from those recorded in standard DD or in traps baited with the commercial Pherocon SWD dispenser. Conversely, controls represented by either the pasteurised DD bait or the DD bait with antibiotic were far less attractive to *D. suzukii*, likely because of the inhibition of microbiological processes in the liquid bait. In this trial, we believe the low activity of the baits inoculated with bacteria can be explained by the fact that mean daily temperatures were generally below 15°C, which is considered unsuitable for adequate growth of *O. oeni* (Raffaele Guzzon et al., 2009). Consequently, biotransformation of the liquid substrate and the production of volatile metabolites attractive to *D. suzukii* was unlikely to have occurred. A similar observation was reported in yeast baits [11], which have comparable environmental needs for optimal growth. In general, all candidate microorganisms for the production of attractive volatile metabolites for *D. suzukii* (yeasts, acetic acid bacteria and lactic acid bacteria) are considered mesophilic, with an optimum of activity between 15 and 30 °C.

It is therefore crucial in future experiments to optimise trap architecture and bait components in order to keep the temperature of the liquid bait within the optimal range. It is well known that wine and vinegar substrates derived from the fermentation of yeasts and bacteria are highly attractive to SWD (Cha et al., 2014b; Kleiber et al., 2014; P. Landolt, Adams, & Rogg, 2012). The importance of the metabolites released during the fermentation of yeasts for SWD attraction has been previously examined (Becher, Hagman, et

al., 2018; Hamby & Becher, 2016; Mori et al., 2017), while the role of the bacterial component present in the above-mentioned mixtures has not yet been fully considered (Chandler et al., 2014; Mazzetto et al., 2016).

Interestingly, dissections of adult females captured during the dormant period (November to February) showed that less than 10% of the analysed females carried mature eggs in their ovaries (A. Grassi et al., 2018). This fraction nonetheless provides enough reproductive capacity for SWD to persist under extreme bottleneck periods such as imposed by winter conditions, allowing oviposition on early commercial crops (A. Grassi et al., 2018). Therefore, during the early portion of the season, food baits such as DD and *O. oeni*-inoculated DD could be particularly efficient, since gravid females would either be looking for feeding substrates before oviposition or for food substrates that are not yet available, such as ripening fruits (N. G. Wiman et al., 2016). A high level of adult removal in this early season should result in mitigation of a population outbreak, allowing reduced damage of the ripening crops (Rossi-Stacconi et al., 2016).

It has already been reported that *Oenococcus* spp. have remarkable hetero-fermentative activity (a type of fermentation of glucose in which the products include lactate, acetate and/or ethanol, and carbon dioxide) and resistance in a low-pH environment (J.-E. Lee, Hong, & Lee, 2009). Therefore, their enhanced biological activity and effectiveness in trapping *D. suzukii* is not unexpected. As a consequence, regulation of the pH in liquid bait is a key factor in improving trap efficacy. Furthermore, the obtained results show that the pH value in the liquid bait is a key point for the improvement of trap efficacy.The standard pH of DD (pH 2.5) is unsuitable for the development of most lactic acid bacteria species, therefore the pH level needs to be increased in order to provide them with an appropriate growing environment. On the other hand, excessively high pH values favour non-selective growth of contaminant microbes (yeasts, bacteria or mould) causing a rapid depletion of the nutrients contained in the bait. The pH value of 4.0 adopted in these experiments has been shown to induce a shift in the metabolic activity of lactic acid bacteria from homo- to hetero-fermentation of sugars in oenological trials, increasing their energetic yield (Liu, 2002; Aline Lonvaud-Funel, 2001). In our study, the selected LAB species were isolated from traditional Italian wine, indicating their ability as commercial starters to tolerate the stress conditions, i.e., acidic pH, encountered in the industrial winemaking pro cess. However, several studies reported the ability of wine LAB to develop stress-induced responses through various mechanisms (Papadimitriou et al., 2016; Spano & Massa, 2006). In earlier studies, *O. oeni* strains showed a high adaptive behavior to an acidic environment and a good malolactic performance at pH 3.5 and 3.0, respectively (Papadimitriou et al., 2016; Solieri et al., 2010). However, a pH of 4.0 represents the threshold level for optimal growth of both *Lactobacillus* spp. and *Pediococcus* spp., and this may partly explain their limited efficacy with respect to *O. oeni*. The poor performance of baits containing a very limited microbial community (if any) - pasteurised or antibiotic treated baits - further supported the hypothesis of the crucial role played by microbiota in regulating the attractiveness of feeding substrates for *D. suzukii*. Under such stress con ditions, the *Oenococcus*, *Lactobacillus*, and *Pediococcus* genera use the malolactic fermentation pathway to convert malic acid to lactic acid with the production of CO2. This pathway con tributes to LAB survival enhancement and promotes growth (Papadimitriou et al., 2016). In addition, it enhances their aroma profile, leading to a prevalence of the fruity aroma over the vegetative ones (Lerm, Engelbrecht, & Du Toit, 2010). Based on the previous findings, it is obvious that the LAB tested in our study were able to tolerate low pH and to produce fermentative volatiles that mediate fly attraction and significantly contribute to improving the attractiveness of the DD bait. Among all the tested LAB, O. oeni with DD was the most attractive bait. Overall variations in capturing levels between the tested LAB could be related to environmental conditions that may affect the fermentation process and, consequently, the volatile profiles qualitatively and quantitatively.

The non-linear increase of trap catches over time can be explained by the fact that the traps were only refilled every week to compensate for bait evaporation. The liquid bait was not completely replaced for the duration of a field test in order to maintain the original population of bacteria. In these conditions, the lactic acid bacteria added to DD were likely adapting to the harsh liquid bait conditions. We speculate that during the first weeks of field exposure, the effect of the initial high microbial concentration in the traps (about 10^6 cells/mL) allowed a satisfactory level of attraction. In the following weeks the composition [change] of DD could have initially resulted in a reduction of cell viability, and consequently a performance decrease of the traps baited with bacteria compared to the commercial DD and control baits. Adaptation of bacteria in the DD environment over the 5th week of exposure would have favoured considerable bacterial growth and the consequent recovery of the trapping capacity. As part of the stress response, LAB develop an adaptive response which enables them to survive and maintain their metabolic activities. During the fermentation process, the LAB can produce a series of biologically active compounds that affect fruit flies (Schulz & Dickschat, 2007). Consequently, volatile compounds that are most prevalent in the main components of DD (wine and vinegar), i.e., methanol, ethanol, acetic acid, and ethyl acetate, will be enriched by LAB fermentation. The results and observations reported in our study clearly suggest that the tested LAB strains contribute to improving the attractiveness of a commercial lure (DD) and recognize them as suitable agents for a more effective lure. *O. oeni*, the best-adapted species to the stress conditions(Papadimitriou et al., 2016), can be exploited as pest control agent in eco-friendly and sustainable management strategies. Therefore, the preliminary field experiment clearly indicated *O. oeni* as the most promising species of the tested LAB for improving the attractiveness of DD baits and it was thus selected for further laboratory tests.

In laboratory tests for evaluation of *O. oeni* performance in DD conditions, the use of bacteria as a bio-catalyser for the production of biologically active volatile compounds in DD is completely new. Therefore, assessment of the behaviour of different *O. oeni* strains in these specific conditions was needed. Four main DD characteristics (low pH, high ethanol content, high acetic acid concentration, and low temperatures during field application) that would reasonably act as limiting factors for the metabolism of *O. oeni* strains were considered. The outcome is particularly interesting, considering that acetic acid had a double influence in our experimental conditions. It was able to lower the pH level (2.5), but it was also an end-product of many metabolic reactions of *O. oeni*, and could inhibit bacterial activity unbalancing the ratio between substrates and catabolites (J.-E. Lee et al., 2009).

In contrast, the low selective pressure induced by ethanol is not surprising, at the values considered, since this outcome is in line with what has been previously observed as regards the interaction of bacteria and wine(R Guzzon et al., 2015; Nielsen & Richelieu, 1999). The laboratory tests identified three sub-populations of *O. oeni* strains that showed different adaptation to the specific composition of DD. These three groups of strains were selected and tested for their ability to produce volatile compounds with biological activity in relation to *D. suzukii* through the fermentative metabolism in DD. However, the significant differences between the three *O. oeni* strains warranted the carrying out of further field tests to select the most attractive bait for *D. suzukii* monitoring (Chapter 3) .

However, EAG analysis confirmed remarkable insect sensitivity to both DD and DD inoculated with *O. oeni*. Additional GC-EAD experiments were performed (Chapter 3) to understand which individual compounds can be perceived by the olfactory system of *D. suzukii*. The selection of single biologically-active compounds may pave the way for developing synthetic attractants.

To better explain *D. suzukii* preference for DD baits inoculated with *O. oeni* strains, chapter III of this work identifies the volatile profiles of the strains and determines their essential role in the preferences of flies. Although we could not provide an overall attraction pattern for *O. oeni* strains, we did show that three

strain groups of *O. oeni* produced the most attractive volatiles. A combination of the most effective strains was used to optimise the traps' efficiency in terms of pest management. To get a deeper insight into their nature, Chapter 3 of this work will determine the flies' response to the new DD baits in open field conditions using different trap designs. These field trials will be exploited to develop a new and efficient trapping system for *D. suzukii* management.

2.5 Conclusions

This chapter demonstrates that different strains of the LAB species *O. oeni* can improve the attractiveness of the commercial food bait DD, making it into a powerful tool for *Drosophila suzukii* control and monitoring. Hence, the results obtained from the series of preliminary field trials justify further investigation of the chemical and microbiological characteristics of DD in relation to the mechanisms of *D. suzukii* adult attraction, in order to produce an even more effective bait and a delivery device that is easy to manage in pest management strategies.

Additionally, it is anticipated that the findings provided in this study will pave the way for developing a new trap concept, in which the attractiveness of DD will be strongly increased by combination with LAB strains releasing biologically active volatiles for *D. suzukii*. Thus, **Chapter 3** will combine laboratory and open field trials, for chemical characterisation of volatiles produced in baits inoculated with *O. oeni* strains, providing optimal bacterial growth under open field conditions, in order to set up new and efficient traps.

Chapter 3

Chemical and electrophysiological characterisation of wine/vinegar with *Oenococcus oeni* and development of liquid baits and traps for *Drosophila suzukii*[2]

This chapter is based on the following publication with the addition of laboratory bioassays and experimental design explanations:

Đurović G, Alawamleh A, Carlin S, Maddalena G, Guzzon R, Mazzoni V, Dalton DT, Walton VM, Suckling DM, Butler RC, Angeli S, De Cristofaro A, Anfora G. Liquid Baits with *Oenococcus oeni* Increase Captures of *Drosophila suzukii*. *Insects*. 2021; 12(1):66. https://doi.org/10.3390/insects12010066 .

3.1 Introduction

Originally from south-east Asia, *Drosophila suzukii*(Diptera: Drosophilidae), spotted-wing drosophila (SWD), quickly established in its new range that had a suitable climate for rapid development and contained few parasitoids or predators (Cini et al., 2012). The evolutionary flexibility and fast life cycle have allowed *D. suzukii* a successful fast invasion and spread. The female has a sclerotized, serrated ovipositor, allowing it to deposit eggs under the skin of host fruits, while the male has a dark spot on the outer margin of each wing (Atallah et al., 2014; Cristina Maria Crava et al., 2019). These sexually dimorphic characteristics allow differentiation of *D. suzukii* from other species of *Drosophila* (Atallah et al., 2014; Rota-Stabelli, Blaxter, & Anfora, 2013). Developing larvae feed on the pulp of the fruit, resulting in economic damage. Oviposition activity further opens a route of infection for pathogenetic microorganisms (Walsh et al., 2011). The fruit becomes increasingly susceptible as harvest time approaches; thus, few chemical interventions are left as an option because of the likely presence of pesticide residues. A broad range of soft-skinned host fruits are available for attack by *D. suzukii* (C. Ioriatti et al., 2015; Karageorgi et al., 2017; Mazzi et al., 2017). Additionally, *D. suzukii* is a mobile pest that is able to shift environments from high woody bushes in winter to crops in warmer months during times of host fruit availability (Gabriella Tait et al., 2018; G. Tait et al., 2020).

A sister species of *D. suzukii*, *D. melanogaster* is attracted to overripe fermenting fruit for feeding, mating and oviposition (Becher et al., 2012). Host plant VOCs are important in oviposition site-selecting behaviour by pest insects. *Drosophila suzukii* is attracted by VOCs released from host plants, in particular soft berry fruit volatiles. Depending on the physiological status of SWD, it is attracted to different VOCs in different phases (I. W. Keesey et al., 2015). As an adult female at full maturity, this fruit fly searches for suitable fruit sites to mate and oviposit eggs. It is not only fruit volatiles that are attractive to SWD, but also VOCs released by fermenting products, which also play an important role. Adult flies are attracted to rotten fruit for feeding, as are other Drosopishila species. Fermenting wine-vinegar mixture emits volatiles that are attractive to *Drosophila suzukii*. In this chapter, we focused our studies on the volatiles emitted by the most well-known fermenting product used in current trapping systems for SWD, wine-vinegar, and its interactions with the olfactory system of *D. suzukii*. Previous investigations of SWD have focused on host fruit VOCs, yeasts, and the vegetative part of plants (Bolton et al., 2019; I. W. Keesey et al., 2015; Revadi et al., 2015).

D. suzukii is attracted to fermenting food sources, and recent studies have indicated that the combination of wine and vinegar is much more attractive to *D. suzukii* than wine or vinegar alone (Cha et al., 2014b). Numerous studies have shown the importance of host plant volatiles in food and oviposition site selecting behaviour by insect herbivores (Bruce et al., 2005; Bruce and Pickett, 2011). More recently, studies have clarified the contribution of microorganisms such as yeast from an additional trophic level in this process of attracting SWD. For example, Becher et al. (2012) reported that *Drosophila melanogaster* relies heavily on microbial rather than plant-related volatiles to locate feeding and oviposition sites, and that the flies reproduce and perform better when yeast is present (Cha et al., 2012).

Volatile organic compounds (VOCs) are abundantly produced by microorganisms, plants, insects and all organisms that are part of terrestrial or marine ecosystems (Loreto et al., 2014). Ecological roles of VOCs are broad, and yet their functions in chemical communication between organisms largely remain to be investigated (Bruce et al., 2005). VOCs mediate host preference (De Bruyne & Baker, 2008), mate location and ovipositional site choices of spotted-wing drosophila (SWD), *Drosophila suzukii* Matsumura(Becher et al., 2012; I. W. Keesey et al., 2015; Scheidler et al., 2015). The attractive VOCs are products of microorganisms that cohabitate on surfaces of host plants within the insect habitat (Bruce & Pickett, 2011; Bruce et al., 2005; Hamby et al., 2012; Mazzetto et al., 2016). In particular, microorganisms such as bacteria, fungi and yeast produce distinct and abundant microbial volatile organic compound (MVOC). MVOCs produce odors that influence complex behavioral interactions between insects and their habitats, yet little

is known about how organisms exploit such compounds as behavioral cues (Davis et al., 2013). Microbes make significant contributions to plant volatile blends. While the role of microbial volatiles in plant-insect interactions to some extend is summarized, an increasing field of research on MVOCs and their full potential in practical application is yet to come (Davis et al., 2013). Microorganisms present on surfaces of their host plants produce MVOCs that are important for the attraction of insects, also those volatiles affecting insects from other trophic levels (Bueno et al., 2019; Vet & Dicke, 1992).

Volatile compounds can be exploited for the development of SWD control methods (A Grassi et al., 2015; Hamby & Becher, 2016). SWD are attracted to the MVOCs from fermenting substrates for feeding purposes (Cloonan et al., 2018; Clymans et al., 2019). At present, the most attractive traps for *D. suzukii* are those baited with vinegar and wine. Such traps serve as one of the first alternatives for early detection and mass trapping (Asplen et al., 2015; Cha et al., 2014b; D. H. Cha et al., 2018; Feng et al., 2018). Monitoring is the first step in an integrated pest management (IPM) program, allowing determination of where populations are present in the field, and when to enact control measures (D. H. Cha et al., 2018; D. Kirkpatrick et al., 2017). Currently, an accepted and commonly used monitoring method for *D. suzukii* involves the use of traps constructed from plastic cups with holes at the top for fly access (i.e. Droso Trap®, Biobest, Westerlo Belgium; Victor® Poison Free® yellow jacket & flying insect trap, Great Lakes IPM Inc., Vestaburg, MI, USA). Inside the cup there may be a yellow sticky card and/or a mixture of apple cider vinegar (ACV), wine, vinegar and other fermenting liquids, and a surfactant(Asplen et al., 2015; Burrack et al., 2015; Clymans et al., 2019; P. J. Landolt et al., 2012; Tonina et al., 2018). However, these traps can only detect insect activity after the seasonal insect population has already established. Consequently, suppression of the pest population is a serious challenge (Bolda et al., 2010). Another drawback of these traps is that they are not species-specific, and hence there is a need to create a more sensitive trap. Understanding the biology and ecology of *D. suzukii* is therefore fundamental to setting up and optimizing control techniques, with a focus on early detection, based on the interference of insect behavior.

Previous knowledge from chemical ecology studies can be used for development of advanced trapping and early monitoring systems in the field. Furthermore, electrophysiological, neurological, analytical chemistry, laboratory behavioral studies in combination with open field studies may allow development of unique trapping systems targeting individual species (Scheidler et al., 2015; Schetelig et al., 2018; Witzgall et al., 2010).

Some information is known about bacterial volatiles and their influence on the attraction of *D. suzukii* (Bueno et al., 2019; Mazzetto et al., 2016). With regard to symbiotic bacteria, the species with the highest frequencies in communities associated with *D. suzukii* are *Tatumella* spp. (Enterobacteriaceae), *Gluconobacter* spp. and *Acetobacter* spp. (Acetobacteraceae) (Chandler et al., 2014). Certain bacterial species were shown to produce volatile metabolites capable of attracting *D. suzukii* in olfactometer assays (Mazzetto et al., 2016). However, to date, there is little evidence that lactic acid bacteria (LAB) involved in the fermentation of wine or vinegar increases attractivity.

There are several studies of sugar fermentation by both yeasts and LAB and their effects on wine chemistry. Though yeasts play a significant role in the attraction of *D. suzukii* (Barata et al., 2012; Hamby et al., 2016), there is not direct investigation on bacterial fermentation metabolites and their effect on *D. suzukii* behaviour. Currently, the most important synthetic attractants for *D. suzukii* contain the synthetic VOCs identified in the headspace of wine, vinegar, apple cider, rice vinegar; such compounds are produced as a result of yeast metabolic fermentation on or within-host fruits (Cha et al., 2012; Cha et al., 2014b; Feng et al., 2018; Kleiber et al., 2014). In particular, (Cordente, Curtin, Varela, & Pretorius, 2012; Nielsen & Richelieu, 1999) confirm the importance of the LAB species *Oenococcus oeni* (Garvie) in wine production. Cultures of *O. oeni* have been found in certain wines (Cordente et al., 2012; Nielsen & Richelieu, 1999). Large variability, in terms of resistance to environmental limiting factors and biosynthetic capacity, has also been reported between separate strains of *O. oeni* (Zapparoli et al., 2012). Most of the studies have focused on

yeast, and the potential of the use of bacteria is scarcely investigated. Therefore, we decided to conduct detailed investigative studies on MVOCs produced from fermentation by LAB.

Strains of LAB were selected based on their commercial use in the fermentation of wine and vinegar, and on their resistance to stressful environmental conditions present in the liquid baits (see part I of the present paper). We focused our attention on the interactions between *D. suzukii* and *O. oeni*, with the aim of developing a new trap capable of early fly capture.

The main objective of this study was to improve the existing trapping and monitoring system for *D. suzukii* in an open field setting, as stated in Chapter 1. Previous studies on the attraction of *D. suzukii* have focused on volatiles from yeast fermentation of host fruits. We focus on the VOCs produced as a result of bacterial fermentation. In Chapter 1, the bacterial strains were chosen under laboratory conditions and then further tested in preliminary field trials to determine the best strain of lactic acid bacteria to be used for furder investigation. In this study, the field trials conducted assessed the performance of different trap models baited with the selected commercial biotypes of *O. oeni*. Here, we investigate **microbial fermentation volatiles** involved in resource location by spotted wing Drosophila flies (SWD). Additionally, we aimed to identify the chemical cues from bait mixtures eliciting a strong attraction response in *D. suzukii*. To test these mixtures, we used an approach combining gas-chromatography and electrophysiology. Then the quantitative variations induced in Droskidrink (DD) (combination of 74.5% apple cider vinegar, 25% red wine, 5 g/L sugar cane) by the added bacterial stains were carefully considered, in the case of both increases and decreases in the initial amount of each volatile compound. The results of our work showed an unexpected set of chemical compounds that are products of bacterial fermentation. Field experiments showed that the attractiveness of DD was increased up to two-fold by the addition of commercially-available strains of *O. oeni*, when combined with an innovative trap design.

3.2 Material and methods

3.2.2. Trapping experiments with fermentation in an open field

Bacterial strains and composition of Droskidrink for *D. suzukii* trapping

We conducted three field experiments using droskidrink mixture with following composition: 20 g of sugar dissolved in 750 mL apple cider vinegar (Sysco Corporation, Houston, TX, USA), 250 mL Merlot red wine (Peter Vella, Modesto, CA, USA), and a drop of soap and ether Enoform Alpha or Enoform Beta of *O. oeni* (Lallemand Inc., Montreal, Canada). Mixtures were left to ferment for one week prior to use in the field trial.

After the preliminary experiments in Chapter I were conducted, three further experiments were carried out in a blueberry field site in Salem, Oregon, USA (44°54'34"N; 123°06'51"W). The objectives were to find an improved trap design and identify an effective lure for SWD control and early monitoring.

3.2.2.1. Trial 1: Comparison of new traps

The aim of this experiment was to search for a new trap model. Trap that could achieve better results than existing traps. In this trial, the fermentation process was carried out in the field using two commercial strains of *O. oeni* that showed the best growth performances in preliminary open field trials in Chapter I. In this trial, we wanted to determine which combination of *O. oeni* strain and trap performed best. In order to create an optimum environment for colonization and reproduction of the bacteria, the pH of the liquid lure was raised to 3.8 using potassium hydroxide (KOH) (monohydrate granular AR [ARS]). Eight treatments were tested in Trial 1 (Table 3.1).

Table 3.1. Trap type and composition of liquid added for three trials, with eight or six treatments per trial. Base solution: DD: Droskidrink+soap; CL: Cha-Landolt solution; pH: KOH added to adjust pH to 3.8. CA- Citric acid (1 g/L). Trap: Cup- Red cup with a white lid; DC- Delta trap with a cup without a lid, with two cotton balls; DIB- Delta trap with an insulated bottle; DUB- Delta trap with the uninsulated bottle (Appendix Picture A 3.1); y- Trap replaced weekly; n- not replaced weekly. Codes were created for better separation of treatments in results.

Code	Trial	Base Solution	pH	CA	Alpha/Beta	Alpha/Beta Rate g/L^{-1}	Trap	Amount of Liquid (mL)	Liquid Replaced weekly?
A: Cw	1	DD	2.6				Cup	200	y
A: Cw	2	DD	2.6				Cup	200	y
A: Cn	3	DD	2.6				Cup	200	n
B: Kw	1	DD	3.8				Cup	200	y
B: Kw	2	DD	3.8				Cup	200	y
B: Kn	3	DD	3.8				Cup	200	n
C: ChLn	1	CL	6.5				Cup	200	n
C: ChLw	2	CL	6.5				Cup	200	y
C: ChLn	3	CL	6.5				Cup	200	n
D: α5Kn	1	DD	3.8		Alpha	0.5	Cup	200	n
D: α2Kw	2	DD	3.8		Alpha	0.2	Cup	200	y
D: α2KΔn	3	DD	3.8		Alpha	0.2	DC	15	n
E: β5Kn	1	DD	3.8		Beta	0.5	Cup	200	n
E: β2Kw	2	DD	3.8		Beta	0.2	Cup	200	y
E: β2KΔn	3	DD	3.8		Beta	0.2	DC	15	n
F: β5KnCA	1	DD	3.8	y	Beta	0.5	Cup	200	n
F: β2KwCA	2	DD	3.8	y	Beta	0.2	Cup	200	y
F: β2KΔnCA	3	DD	3.8	y	Beta	0.2	DC	15	n
G: β5KΔIBn	1	DD	3.8		Beta	0.5	DIB	200	n
G: β5KΔCw	2	DD	3.8		Beta	0.2	DC	20	y
H: β2KΔUBn	1	DD	3.8		Beta	0.5	DUB	200	n
H: β2KΔCw	2	DD	3.8		Beta	0.2	DC	15	y

Treatment A represented the standard DD bait. Treatment B comprised the standard bait DD with the pH increased to 3.8. Treatment C was the commercially developed Cha-Landolt bait (Pherocon SWD dispenser, Trécé Inc., Adair, OK, USA). Treatments D and E were the same as B but instead inoculated with 0.5 gL^{-1} of Enoferm Alpha and Enoferm Beta, respectively. Treatment F was the same as Treatment E, but with the addition of 1.0 g gL^{-1} of citric acid (pellets AR [ARS]). Adding citric acid to the mixture results in malolactic fermentation that leads to increased production of acetoin, diacetyl (also called 2,3-butanedione) and 2,3-butanediol (Nielsen & Richelieu, 1999). These compounds may increase SWD attraction to the trap. Treatments G and H had the same composition as Treatment E, but the trap designs were different (Appendix Picture A 3.1 DIB). Treatment G had a delta tarp with an insulated bottle on the bottom; treatment H had a regular bottle.

For Treatments A–F, traps comprised a red plastic cup (532.3 mL) with a white lid (Appendix Picture A 3.1 c). Polystyrene cups were placed inside each of these to provide insulation. The temperatures of the liquid baits were monitored with portable data loggers (HOBO pendant loggers, Onset Computer Corporation, Bourne, MA, USA). To provide access for SWD, six holes (0.5 cm diameter) were punched into each cup. Cups were filled with approximately 200 mL of the appropriate treatment.

For Treatments G and H, corrugated plastic delta traps (Suterra LLC, Bend, OR, USA) containing a white sticky card were used. The delta traps were modified by creating a hole at the bottom centre of the traps, into which the head of a water bottle (Camelbak, Petaluma, CA, USA) was inserted (Pic Appendix 3.1 DIB). This bottle contained a pressure valve that allowed for the release of volatile compounds during fermentation. For Treatment G, insulated bottles were used; for Treatment H, the bottles were not insulated (Appendix Picture A 3.1.).

The treatments were replicated four times, and laid out in a randomised block design, with one replicate per row, and a distance of about 10 m between traps. The experiment was conducted in the centre of the blueberry field, avoiding the perimeters, so as to ensure a more homogenous olfactory environment. The experiment was conducted over four weeks (from 28th August to 25th September 2015) and checked on a weekly basis. Precipitation during the 28-day period totaled was 23.88mm. The traps were filtered, and contents returned to the lab in 70% EtOH. Sticky cards (Treatments G and H) were covered with plastic film and transported to the laboratory. Once in the laboratory, male and female SWD were identified and counted using a dissecting microscope.

Baits for Treatments A and B were replaced weekly. Baits for Treatments C to H were left in the field for the duration of the experiment. The temperatures of the bait in these treatments were monitored with portable data loggers to check if the conditions were optimal for fermentation (mean = 19.1°C, min. = –0.4°C, max. = 56.9°C).

3.2.2.2. Trial 2: Fermentation in the open field and a different trap design

In the second stage of the fieldwork (Trial 1), the liquid bait was replaced on a weekly basis for all treatments. In addition, the amount of bacterial inoculum used for the preparation of the treatments was reduced from 0.5 g to 0.2 g L^{-1}. The fermentation of DD by *O. oeni* was conducted in the laboratory for one week prior to the field trials under a controlled temperature of 22 ± 2°C.

Eight treatments (see Table 3.1) were tested. The same trap designs were used for treatments A–F; traps for treatments G and H were modified to allow insertion of 30-mL cups (Dart Container Corporation, Mason, MI, USA) in the centre and the water bottles were removed. For Treatment G about 20 mL of DD was added to each trap. The cups were closed with specially prepared covers that excluded entry of insects but allowed volatile compounds to escape (Appendix Picture A 3.1 DC a). For Treatment H, approximately 15 mL of DD per trap was used, and two cotton balls were inserted inside the cups, which were left without lids (Appendix Picture A 3.1 DC b).

Trial 2 was carried out in the same blueberry field as Trial 1. Four replicates of each treatment were used and laid out in a randomised block design similar to Trial 1. The trial was conducted over four weeks (from 25thSeptember to 23rdOctober), traps were checked on a weekly basis, and captured species were identified in the laboratory. Precipitation over this trial period totaled 21.08mm.

3.2.2.3. Trial 3: Evaluation of the new trap design

Trial 3 was the last phase of the fieldwork and involved the assessment and development of a different trap as well as the improvement of traps used in previous trials. The aim of this trial was to assess whether this new model of the trap could further enhance the results achieved by the mixtures of DD inoculated with bacteria.

This trial used six treatments (see Table 3.1). Treatments A, B and C were as described previously, using the standard red cup trap with a white lid. Treatments D, E and F, used the same lures as for treatments D, E and F in Trial 2, but in this case, lures were used with the most effective trap from the previous studies: the plastic delta trap with a 30-mL cup inside (Appendix Picture A 3.1 DC b). This cup was equipped with two cotton balls and 15 mL of the test mixture. This trial took place over 5-weeks following the dry summer period, 30th October to 4th December 2015, (total precipitation was 128mm of rain, 35 days, 3.68mm/day). During this time, temperatures were cooler than in previous experiments (mean = 8.1°C with min. –10.3°C and max. 32.6°C), and, consequently, the population of SWD was relatively reduced. Once again, traps were checked weekly, and insects were counted. Three replicates of the treatments were used and laid out as a randomised block design, similar to previous trials.

3.2.2.4. Statistical Analysis

For all trials described in sections 2.2.1, 2.2.2 and 2.2.3, the number of SWD caught on a trap across all assessments was calculated for females, males and total (male + female). For each category (males, females and total), the numbers of SWD were analysed with a Poisson generalised linear model (Peter McCullagh, 2018) with a logit link. Proportions of females were analysed with a binomial GLM with a logit link. For all analyses, there was substantial over-dispersion; thus, the dispersion was estimated (i.e. quasi-Poisson and quasi-binomial GLM were used). An overall assessment for treatment differences was carried out using an F-test as part of the analysis of deviance within the analysis. Mean counts and percentages were obtained on the log/logit scale, along with associated 95% confidence limits; these values were back-transformed. The means and confidence limits were then divided by the number of days that had elapsed since trial set-up to give mean catches per trap per day. All analyses were carried out with Genstat (R. W. Payne, 2009).

3.2.3. Droskidrink preparation with *Oenococcus oeni* strain Pn4 (Alpha) and strain 31 (Beta) for VOCs collection.

Selected strains of *O. oeni* were added to DD at a rate of 1g per 400 ml liquid bait and homogenized by stirring. The pH of the mixture is normally ~ 2.5; for the trials, KCl was added until acidity reached 4.5 pH. The mixture was prepared in lidded cylindrical polyethylene boxes about 500 ml in volume. The mixture was left under aerobic conditions with a photoperiod of 16 : 8 light : dark (1000 lux during the light period) at 25±2°C and 60±5% relative humidity for 7, 14 or 21 days, after which elution of volatiles was performed.

We began investigating the two strains with the best results: *O.oeni strain* Pn4 and *O. oeni* strain 31. We created a mixture of wine-vinegar with these two strains separately, and as a control we used Droskidrink alone (hereafter referred to as DD I) (wine-vinegar-sugar cane, 30% red wine (Travello, Caviro, Italy, and 70% apple vinegar (Prantil; Italy and 5 g/L OF brown sugar cane)). In this chapter, DD I refers to one-week-

old Droskidrink, 31 I refers to one-week-old Droskidrink mixed with strain 31 of *O. oeni*, and PN4 I refers to one-week-old Droskidrink with the addition of the PN4 strain of *O. oeni*. The symbols II and III mean that mixture was two or three weeks old. We wanted to test the timescale for volatile emission eliciting the best response from SWD. We chose three time points for volatile elution. The first time point was when the mixture was 1 week old, the second after two weeks and third time point 21 days after making the mixture. These three time points for ageing of the above-mentioned mixtures were the main cases in our investigation. We chemically characterised the mixtures, then carried out electrophysiological studies, and finally tested the raw mixture of different ages with a laboratory multi-choice experiment.

Headspace collection and characterisation of DD inoculated with different strains by GC-MS

To characterise most volatile compounds of DD alone and DD with the addition of *O. oeni* reference strain MRI1000, Enoferm alpha, Enoferm beta, T1, S18, P1 was performed using dynamic DHA (direct headspace analysis) collection connected to gas chromatograph (GC). Samples of DD were inoculated with 1% v/v bacterial cultures and incubated at 25°C for one week. Prior to the incubation, 5 mL of DD were inserted into hermetically sealed glass vials (20 mL), and the volatile compounds were injected to the chromatograph using a multifunctional autosampler (Multi-Purpose Sampler MPS, Germany). Each vial was automatically introduced into an incubator at 38°C for 20 min, then 1 mL of air was removed from the headspace using a gas syringe preheated to 80°C and subsequently injected into the column of the GC. The GC-MS analysis was performed using a 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5ms column (length = 30 m, internal diameter = 0.25 mm, with a 0.25 μm film thickness) (Agilent Technologies), coupled with a 5975 inert XL mass selective detector (Agilent Technologies). The method was set to see ions in the range of 34 and 300 a.m.u. Sample introduction was performed by a multifunctional autosampler, and samples were prepared by the robot (MultiPurpose Sampler MPS for GC and GC/MS, Gerstel, Mülheim an der Ruhr, Germany). Helium was used as the carrier gas (flow rate of 1.2 Ml min^{-1}); the thermal cycle provided was 5 min at 30°C, a temperature ramp of 3.5°C/min to 240°C, and 2 minutes at 240°C. The total run time was 44.87 minutes, in splitless mode, total flow 54.2 $mlmin^{-1}$, purge flow to split vent 50 $mlmin^{-1}$ at 1 min temperature program 240 °C for 0 min. The volume injected 2.0 μl, acquisition mode was scan, scan parameters 35 to 400 a.m.u. Solvent delay of 4 min. The extracted ions are used for quantification of each single compound. Data acquisition and analysis were done by Mass Hunter Software (Agilent Technologies).

3.2.4. Volatile elution from Droskidrink and bacteria

Aeration and adsorption of volatiles from the tested mixtures

Aeration and absorption were carried out using a system called direct headspace collection. With this method, the volatiles in the air surrounding the odour source are trapped on an adsorbent. The odour source - Droskidrink and a mixture of Droskidrink with bacteria – were placed in an aeration chamber. Different adsorption materials are available, the choice depending on the compounds of interest and the desorption method used (Agelopolous & Pickett, 1998). We tried Tenax TA 60/80 and Porapak Q. We set up our method with Porapak Q 50/80 mesh size because it was shown to be best for our experiments. With this method, we eluted the volatiles with solvent dichloromethane (solvent desorption) and thus had a liquid sample which could be used a number of times in the process of compound identification (GC-MS, GC-DH-MS, GC-FID).

Absorbent material and storage of elutions: Droskidrink and different strains of *O. oeni* were enclosed in a glass vessel (24 cm diameter, 21 cm high). Air entering the vessel was pulled through a filter filled with activated charcoal and allochroic silica gel and was pumped out from the vessel for 48 h at 0,4 l/min through an adsorbent tube 5 mm internal diam. Volatiles were collected using 8 cm long absorbent glass tubes (5 mm internal diam.(ID)), packed with 500 mg Porapak Q polymer (80/100 mesh, Waters Corporation, Ireland) between silanised glass wool plugs. All connections were made with plastic tubing. Conditioning of equipment: before each entrainment or run of consecutive entrainments, all the equipment was conditioned to remove contamination (residual volatiles). Mostly new absorbent tubes were used. In replicates of elutions, Porapak Q tubes were washed through with at least 1 ml of redistilled diethyl ether and baked at 130°C in a flow of filtered nitrogen for at least two hours. Glassware was scrubbed with warm water, rinsed with acetone and baked along with the glass wool in an oven at 180°C for at least two hours. Activated charcoal filters (BDH 10-18 mesh) were conditioned by baking at 180°C in a filtered nitrogen flow for at least three hours. Once the equipment was conditioned, it was handled with cotton gloves to reduce contamination. All pumps were powered by batteries for small household aquariums. Volatiles were eluted from the Porapak Q trap with 1 mL dichloromethane. Three replications of the volatile collections from mixtures of 7-, 14-, and 21-day old fermentations were carried out for 48 h,in a climatic chamber under a 16 : 8 h light : dark photoperiod (1000 lux during the light period) at 25±2 °C and 60±5% relative humidity. Nine volatiles collections and three replicas of each sample were collected. This volatiles collection was further used for GC–MS, GC-EAD-FID analyses and behavioural experiments, with the addition of an internal standard that was subjected to quantitative GC–FID analysis. Volatile samples are stored in 2ml vials in a freezer (− 80 °C) until used for chemical analysis and electrophysiological experiments.

Volatiles collected by entrainment onto Porapak Q and eluted in dichloromethane were first analysed using Gas Chromatography (GC), coupled with a mass spectometerith use of a mass spectrometer detector.

3.2.5. Chemical characterisation of volatiles from Droskidrink and *O. oeni* strains.

3.2.5.1 Gas chromatography method

Gas Chromatography is a destructive analytical technique that involves the separation of compounds by their chemical properties on a column. The collected volatiles were separated and analysed using gas chromatography (GC) equipped with a capillary column. GC often requires only a small amount of analyte and hence it is a good starting point. In the column, the sample (a mixture of volatiles) is dispered through the column by a gas stream. The components of the mixture separate, as they partition differently in the mobile gas and in the stationary film covering the inside of the column, depending on volatility and chemical bonding. Separation of the analytes in a sample is important since overlapping peaks may make the interpretation of results difficult. It is recommended to run samples on different GC-columns, as we did, to ensure there are no such overlapping peaks.

The most volatile components and those with a low degree of chemical interaction reach the detector first. The components volatilising at higher temperatures and those interacting most with the stationary phase reach the detector last (Jennings, 1987). Consequently, the time required for a component to pass through the column can provide some information about molecular structure.

Various detectors can be used, depending on what information is of interest, and combining different detectors may yield a complete picture. Several detectors are available, and some of them are listed below.

The most commonly used detector in GC-analysis of organic compounds is the flame ionisation detector (FID), which is useful for quantification but does not provide details about molecular structures. A more specific detector is the mass spectrometer (MS). With GC-MS, the sample components are introduced into the ion source of the MS, where they are bombarded with electrons and thereby fragmented and ionised. The amounts of differently sized ions are continuously scanned. The recorded fragmentation

pattern, the mass spectrum, can be specific for the compound and analysed manually or by computerised comparisons with standard spectra in commercial or user-created mass spectra databases. The information from this detector provides structural information such as molecular weight and weights of fragments that can be related to specific chemical structures.

However, the GC-MS method can have the disadvantage of low sensitivity (only a fraction of the volatiles collected are analysed), while the solvent peak can mask compounds with short retention times in GC analysis (Agelopoulos and Pickett 1998).

After tentative identification, further identification was carried out using GC-MS and GC co-injection.

Direct headspace extraction combined with the GC-MS is a solvent-free method. This method can often reduce extraction times (Arthur and Pawliszyn 1990), and it is also possible to extract compounds from air, water and soil in the field, for example (Zhang et al. 1994). It allowed us to sample the most volatile compounds obtained in the tested mixtures. Headspace extraction consists of two potential methods: i) static collection, whereby a sample is allowed to equilibrate in a vial and a static volume of air is extracted for analysis; and ii) dynamic collection, whereby an absorbent material is connected to one side of the sample compartment while a pump circulates air or nitrogen through the sample for a specified time, and the compounds are subsequently extracted from the absorbent and subjected to analysis (Zhang et al. 1994). We used the first method - static collection. Samples were left to grow in anaerobic conditions, at 25 ± 2°C (as described 3.2.1.) before DHS-GC-MS analysis was performed. Before analysis, the samples were arranged in glass 20 ml vials with 5 ml of sample. Headspace volatiles from 5 ml of Droskidrink inoculated with 1g of each bacterial strain were collected in 20 ml hermetic glass vials (Sigma-Aldrich; St. Louis, MO, USA).

The samples were analysed using a Gas Chromatograph (7890A, Agilent Technologies, Santa Clara, USA) fitted with a Mass Selective Detector - MS (5975C, Agilent Technologies). The column used was a non-polar HP-5MS column (Agilent Technologies). The column was 30 m long and had an internal diameter of 0.25 mm with a 0.25 μm thick film. The utilized program was HS Drosky38C.M designed by Professor Angeli at the Free University of Bolzano and modified temperature ramp by Durovic. Quadrupole was scanned in the m/z range 34 the lowest mass to 300.0 highest mass at 2 scans per second. Sample introduction was performed by a multifunctional autosampler and sample preparation robot (MultiPurpose Sampler MPS for GC and GC/MS, Gerstel, Mülheim and der Ruhr, Germany). This robot was controlled from the Gerstel Maestro Software integrated with the Agilent Technologies ChemStation. Each vial was automatically taken and introduced in an incubator at 38°C for 20 min. The temperature was set to 38°C to see as more volatile compounds as possible without compromising their chemical structure. One ml of air was then removed from the headspace of each vial by a gas syringe at 80°C to be injected in intel, and from there, it is pushed into the column of the GC, by the carrier gas. The carrier gas, helium (He), flowed at a rate of 1, 2 ml/min and at an average velocity of 39.723 cm/s. The initial temperature of the oven was set at 30°C and held for 5 min. Temperature ramp 1 was at a rate of 3.5°C/min) until it reached 150, that temperature ramp 2 increase 25°C/min until 240 °C. The oven temperature was held at the final temperature of 240°C for 2 minutes in order to ensure the elimination of inpurities all compound from the column. Positive electron ionization (EI +) was used, with an electron energy level of 70eV Split mode was set 3:1. The total run time was 44.87 minutes. Data acquisition and analysis were done by ChemStation software (Agilent Technologies).

The activities were carried out wearing nitrile-coated gloves (VWR International BVBA, Leuven).

3.2.5.2 Chemical characterisation of volatiles from droskidrink and Oenococcus oeni strains.

3.2.5.3 GC-MS analysis

GC analyses were carried out with a Trace GC Ultra gas chromatograph coupled with a TSQ Quantum XLS Tandem mass spectrometer (Thermo Electron Corporation, Waltham, Massachusetts, USA) and equipped with a PAL Combi-xt autosampler (CTC Analytics AG, Switzerland). The separation module consisted of a ZB Wax PEG capillary column (30 m × 0.25 mm inner diameter × 0.25 μm film thickness; Phenomenex, Italy) programmed to increase from 60 °C (held for 3 min) at 8 °C min^{-1} to 220 °C (held for 10 min) and, finally, to 250 °C at 10 °C min^{-1} for 5 min. Helium was used as the carrier gas at a flow-rate of 1.2 mL min^{-1}. The temperature of the transfer line was 250 °C. The electron impact energy was 70 eV, and the filament current was 50 μA. Mass range was m/z 40-350 a.m.u. The source temperature was set to 200 ° C, the accelerating voltage to 4.6 kV and the filament current in the source set at 1 mA.

GC-MS analysis on an HP-5 MS non-polar column

In other to make our analysis and confirmation of the compounds emitted by Droskidrink and bacteria more reliable, we also analysed the same VOC extraction samples on a non-polar column. The GC-MS analysis with a liquid sample of extracted volatiles dissolved in dichloromethane was performed by a 7890A Gas Chromatograph (Agilent Technologies, USA) equipped with an HP-5MS column (Agilent Technologies, Santa Clara, USA) and coupled with a 5975 inert XL mass selective detector (5975C, Agilent Technologies). The column was 30 m long and had an internal diameter of 0.25 mm with a 0.25 μm thick film. The method was set to allow to see ions in the range between 34 and 300 a.m.u.. Helium was used as carrier gas (flow rate of 1.2 mL/min); The samples (2 μL) were injected in splitless mode with an initial thermal cycle provided 5 min at 30°C, then a temperature ramp of 3.5°C/min until it reached 240°C. The oven temperature was held at the final temperature of 240°C for 2 minutes. The total run time was 33.167 minutes, in splitless mode, total flow 54.2 ml/min, purge flow to split vent 50 ml/min at 1 min temperature program 240 °C for 0 min. The volume injected 2.0 μl, acquisition mode was scan, scan parameters 35 to 400 a.m.u... Solvent delay of 4 min. Data acquisition and analysis were done by ChemStation software (Agilent Technologies) and Masshunter Software.

3.2.6 Electrophysiological experiments

Insects

The culture of *D. suzukii* used in the laboratory experiments was established with field-collected populations from orchards in Alto Adige, Italy. *D. suzukii* was reared on a standard Drosophila semiartificial cornmeal-diet, at the temperature of 23-25° C, relative humidity (R.H) of 65±5% and with a 16L:8D photoperiod.

Electrophysiological experiments on insect antennae can be performed both as physiological studies on the function of the olfactory sense and as a tool in identifying behaviourally active odours. The most important techniques include the electro antennogram (EAG), Electroantennographic Detection (GC -FID-EAD), and single Sensilla recording SSR.

Electroantennography (EAG) is a neurophysiological technique that allows characterization of the perception of semiochemicals by an insect (Jones and Olham 1999). In this technique, the change of potential that occurs over the whole antenna following a chemical stimulus is measured, and it is thought to be the sum of all the receptor potentials elicited in all sensilla present on the antenna (Birch 1971). The antennal base and antennal tip are connected to the ground and a high impedance amplifier respectively (Hansson 1995).. The volatile stimulus is delivered in a purified and humidified air stream continuously

passing over the mounted antennae. Arecorded response provides no information about the effects on behaviour, it is not known does compund behave as repellent or atractant, the number of compounds to test in behavioural assays can be reduced with EAG experiments. This technique was first developed by Schneider in 1957 to measure electrophysiological responses from antennae of male *Bombyx mori* L. to volatile compounds from its conspecific female sex pheromones, and since its invention it has been widely used as a standard method in insect olfaction studies. The effectiveness of EAG is considerably increased by combining it with Gas Chromatography (GC), and Gas Chromatography-Electroantennodetection (GC-EAD) (Moorhouse et al. 1969). As a detector of GC effluents, EAG is a powerful analytical tool in the identification of behaviourally active compounds in complex blends (Roelofs 1977, Jones and Oldham 1999).

Gas chromatography -Flame Ionization Detection – Electroantennographic Detection (GC -FID-EAD) is an analytical procedure that couples a powerful technique (GC-FID) for separation and determination of the identity and relative abundance of compounds present in complex mixtures with the **electroantennographic detection** technique. During the GC-FID run, the antenna is continuously exposed to the eluting fractions. The signal from the antenna is monitored and recorded continuously and simultaneously with the signal from the GC-FID, and both signals are synchronized over time. **EAD-GC-FID** was used to measure insect antennal response to stimulation using behaviourally active chemical cues. We chose this method for our experiment because it allows rapid identification of compounds in complex mixtures that stimulate the olfactory receptors of an insect. This analytical procedure was used because it assigns priorities to compounds for behavioural bioassays and greatly speeds up identification of compounds that we could potentially use to manipulate *D. suzukii* behaviour.

The Flame Ionisation Detector (FID) measures the number of components present in a sample, and synthetic references are used to obtain preliminary identification of semiochemicals. The compounds are combusted in the detector, resulting in rough measurement of the number of carbons present in the molecule. The detection limit is in the upper picogram range (McNair and Miller 2011). FID is often combined with EAD.

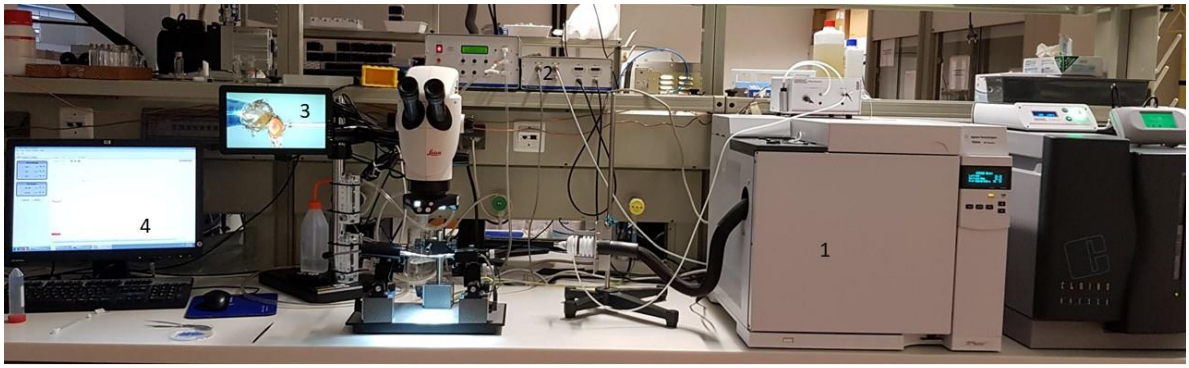
Samples obtained from DHS adsorption eluted in dichloromethane (as reported in 3.3.1) were used for EAD analysis. The procedure involved isolating an antenna (together with head and prothorax) (Picture 3.1), mounting it between two electrodes, blowing on it with an odorous stimulus from GC-FID and measuring the variation in electrical potential through an oscilloscope. The signals were passed through a high impedance amplifier (IDAC) and responses from the antennae measured in mV deflections. The indifferent electrode had a similar capillary, bringing it into contact with the distal end of the fly antenna. Compounds eluting from the capillary column were delivered to the antenna through a glass tube (12 cm× 8 mm) via a charcoal-filtered and humidified air stream. The recording electrode was connected to the IDAC and the signal was recorded with GC-EAD2017 software (with analysis carried out at the Fondazione Edmund Mach) and GC-EAG software (analysis performed at the Free University of Bolzano).

We used a glass capillary indifferent electrode filled with Kaissling solution for antenna mounting.

EAD-GC-FID was performed on the polar column at Fondazione Edmund Mach. Antennal signals were captured using a high-impedance AC/DC pre-amplifier (10x), sent to an IDAC-4 box, and stored on a PC hard disk using GC-EAD 32 (v. 4.6; 2017). IDAC 4 (Integrated Digital-Analog Converter) was set as follow: Chanel 1 (EAD) Low cutoff 0.1 Hz, range 15.6 mV, high cutoff 10 Hz, external amplification 7; channel 2 (FID): low cutoff DC, range 250 mV, high cutoff 10 Hz, external amplification.

The antennal signal and the FID signal were amplified and recorded simultaneously using Syntech software GC-EAD2017. Samples from volatile extracts were tested on the mated female *D. suzukii* females. Two microliters of the concentrated volatile extracts were injected in a Hewlett-Packard 5890 GC (Hewlett-Packard, Palo Alto, California) in splitless mode, with **a polar Innowax column** (30m× 0.32 mm; J&W Scientific, Folsom, California) programmed from 60 ∘C (hold 3 min) at 8 ∘C min−1 to 220 ∘C (hold 7min) with helium as the carrier gas and interfaced with the EAG apparatus (GC-EAD) (Riolo *et al*., 2012; Revadi

et al., 2015). A compound was considered electrophysiologically active when it elicited at least five antennal responses out of 10 electrophysiological recordings for each replication, which were different from background noise, as described in Anfora et al. (2009).



Picture 3.1. Electroantenographic set up: 1. GC-FID; 2: IDAC; 3. Enlarged look on recording electrode and inserted insect antennae; 4 EAD-2014 software

Additionally, we performed **EAD-CG-FID** using a non-polar **HP-5MS** column (Agilent Technologies 19091J-413) at the Free University of Bolzano, and all results presented here are from these experiments. EAD results from the polar column were used as additional confirmation of electrophysiologically active compounds. **HP-5MS** column was 30 m long and had an internal diameter of 0.32 mm with a 0.25 μm thick film, 7-inch cages. The method of EAD-GC-FID is based on the VOC1 method of the GC-MS. However, since this column has 230 i.d instead f 250 i.d., the oven program has been changed to fit the retention time of the VOC1 program. The samples (2 μL) were injected in splitless mode with an initial oven temperature of 50 ˚C, 1.8 minutes hold for 1.8 min, then the temperature was raised 7.3 ˚C/min up to 250 ˚C. Temperature was held on 250 ˚C for 1 min, total run 30.197 minutes. Helium was used as a carrier gas at a constant flow rate of 39.923 cm/sec flow to accelerate separation on the column (2.52 mL/minute). Max oven temperature was 300 ˚C. Inlet temperature was held constant at 250 ˚C. Split mode in ratio 5 to 1 with a flow of 12.648 ml/min. Flame ionization detector was set up at 350 ˚C, with hydrogen flow 50ml/min, airflow 500 ml/min, Helium flow 20 ml/min. Signal source of FID was 20Hz/01 min.. IDAC 2 was set up at maximum recording duration 51 min, voltage range 312,5 mV, channel 1 (EAD): low cutoff 0,05 Hz, offset 0, Ext. amp:23, digital 1 (trigger) initiates recording on, invert on. Chanel 2 (FID): low cutoff: DC, offset 0, ext. amp. 10, record signal data on. For analysing and recording, we used GC-EAG2014 Sintec software (Picture 3.1). 7-9-day-old mated female *D. suzukii* were used throughout all the experiments. The restrained fly was mounted upright on a glass slide, and the electrodes used were composed of silver wire inserted into drawn-out borosilicate glass capillaries filled with Kaissling saline. The cut inset on the prothorax was mounted on a recording electrode. The reference electrode was placed on the tip of insect antennae under a high magnification microscope. Eluted odour constituents from the GC column were added to this flow toward the insect antennae. The analysis was carried out using a customised software package enabling simultaneous recording of the EAG and FID responses (GC-EAG 2000 Syntech ®).

Hardware and software were from Syntech, Germany. The antennal signal was banded pass filtered between 3 kHz and 0.1 Hz, whereas the FID signal was not conditioned. Both signals were fed onto separate channels in the IDAC-2, and the digitized signal was fed into the PC. At least ten flies were tested for each extract odour, with up to ten recordings of the same odour per fly. Recording took place in the afternoon and typically extended into the evening. The absolute amplitude of the responses was measured (in μV) from the onset of depolarization (baseline) to the maxima of the deflection (Heinrich et al., 1975, De Cristofaro et al., 2000, Cha et al., 2012). A compound was considered electrophysiologically active (EAG)

when it elicited antennal responses at least three times greater than background noise (Zhang et al. 2001). Peaks were matched between GC-EAD and GC-MS with a retention index.

3.2.6.1 Data analysis

Data analysis from GC-MS and GC-EAD

Initial tentative identification of compounds from entrainment samples focused on peaks that gave a response in GC-EAD and was carried out using mass spectrometry (MS) as a detector with the Kovats Retention Index (RI) (Bartle 1993). To enable calculation of the RI, a series of known internal reference standards (alkanes, C7-C25 n-hydrocarbons at 1µl of 100 ngµl^{-1} diluted in distilled hexane purchased from Sigma Aldrich) were run on the GC columns prior to analysis of entrainment samples in the same conditions. The Kovats index was calculated and mass spectra were compared with libraries offered by XCalibur 1.2. The calculated Kovats index was compared with those (I. W. Keesey et al., 2015; I. W. Keesey et al., 2016; Revadi et al., 2015; G. Tait et al., 2020) describing electrophysiologically active compounds for SWD and other insect species.

All components collected from DHS extracts and analyzed on the GC-HD-MS were firstly tentatively identified after GC-MS analysis, by comparison of their mass spectra with those present in mass spectral databases. Each peak of interest detected in the gas chromatogram was analyzed and identified through its mass spectra (Riu-Aumatell, Castellari, López-Tamames, Galassi, & Buxaderas, 2004). Because many compounds present in the analyzed samples were concentrated in the first minutes of the x-axis of the chromatograms and were often overlapping, identifications were made on the non-polar column using the technique of single ion extraction, in order to allow a more precise and reliable identification. For quantification, one single ion per compound was "extracted" from the chromatogram and its area quantified for all the samples. The quantity of the detected components was expressed as a percentage to allow easy comparison of differences in VOCs emission between DD and DD + LAB.

We performed GC-EAD- to see which compounds elicit an antennal response in *D. suzukii*. Those that were identified as electrophysiologically active compounds were marked, and their linear retention index was calculated. Retention indices from GC-FID and GC-MS were compared, and if they coincided, the compounds were chemically confirmed. Identification of compounds tentatively identified using GC/ GC-EAD and GC-MS was confirmed or rejected by the use of co-injection with a laboratory standard on a polar column. The amount of synthetic standard added to the sample was aimed at doubling the area of the peak for that particular substance, without increasing its width. This was achieved by adding the appropriate concentration of a solution of the laboratory standard to the entrainment sample injected into the GC.

Compounds with the most prominent peaks in EAD-GC-FID with a non-polar column were chosen to be identified by mass spectra, utilising three mass spectra libraries (NIST11 (National Institute of Standards and Technology, 2011), Wiley7n, and W9N08, which is a combination of the previous and NIST08.). Kovats retention index times were used to evaluate the library search results. For each peak, the Kovats retention index was calculated and cross-referenced with retention times on similar GC-MS stationary phases on the NIST Chemistry WebBook, SRD69. Compounds which showed up in the blank control run with just hexane in the columns were discarded as deemed contaminations.

Tentative quantification of the amount (ng) of a compound in the injected sample was calculated by taking the mean amount of the alkanes (sum of the area of alkane peaks / number of alkanes)/ 100) and multiplying this by the area of each of the peaks. This provided quantifications from which it was possible to determine the approximate amount of compound. This figure was used to compare ratios in peak size before and after peak enhancement co-injection. Figure 3.1 shows the formula used for calculating the retention index (RI).

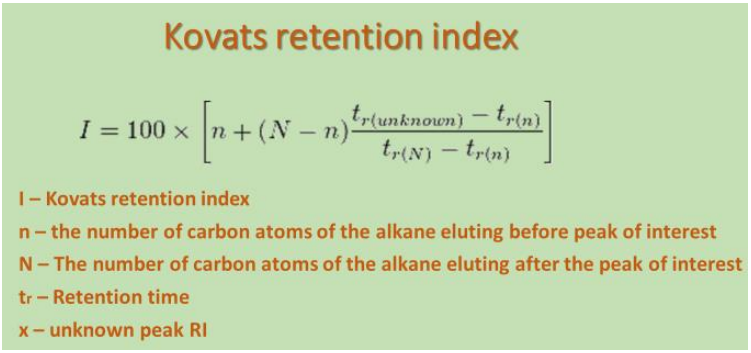


Figure 3.1. Kovats retention index formula used on both type of columns.

Identification of volatile wine-vinegar compounds was achieved by comparing the experimental linear temperature retention index (LTPRI) with retention indices reported in the literature.

We calculated the Kovatz index of targeted peaks on the both type of columns, and if the identification offered gave us 95% security, the tentative identification of the compound was confirmed. After identification of prominent peaks from CG-MS (with the non-polar column), we did not continue further confirmation of peaks. On the other hand, using the results from GC-MS with a polar column, after tentative identification, we selected peaks for co-injection. We performed EAD-GC-FID to see which compounds elicited an antennal response in *D. suzukii*. The compounds that had been identified as electrophysiologically active compounds were marked, and their linear retention index was calculated. The retention time was also confirmed for that type of column from previous publications. Retention indexes from GC-FID and GC-MS were compared, and if they coincided the compounds were chemically confirmed.

Peak enhancement co-injection (confirmation of compound identification)

Identification of compounds tentatively identified using GC/ GC-EAG and GC-MS was confirmed (or rejected) by using co-injection with a laboratory standard. The amount of standard added to the sample was aimed at doubling the area of the peak for that particular substance, without increasing its width. This was achieved by adding the appropriate amount (concentration) of a solution of the laboratory standard to the entrainment sample injected into the GC. This was carried out on a polar (ZB-Wax) column. Confirmation of compound identity was achieved by comparing the peak area with that of other nearby peaks in the sample before and after co-injection. Most of the electrophysiologically active compound in each extract was quantified by comparing its peak areas with those of the internal standard.

3.2.7 Bioassays

3.2.7.1 Y tube olfactometer bioassays

Evaluation of the olfactory responses of mated *D. suzukii* females to the mixture of Droskidrink and *O. oeni* and the mixture of the EAD-active compounds described in (Cha et al., 2012) was performed in a Y-tube olfactometer (stem 30 cm; arm length 20 cm; arm angle 60°; internal diam. 4 cm). Each arm of the Y-tube was connected to a Pyrex glass bulb (100 mL) with an open-end (φ = 2 cm). Activated charcoal-filtered and humidified clean air was pumped uniformly through the arms at 200 mL/min. Volatiles collected from a mixture of Droskidrink and PN4 *O. oeni* strains versus a control 5-component attractive bland were placed in two separate glass bulbs. For bioassays of volatile samples, 100 µL of solution was used to impregnate a piece of round filter paper (φ = 1.5 cm)/ or rubber, and the solvent was allowed to evaporate before the trials. Ten mated females were introduced into the olfactometer at the entrance of the main stem at one time and were observed until the fly made a 'choice' (entering the side arms) or until 15 min had elapsed. Flies that remained in the main stem were recorded as 'no choice'. After every trial, the olfactometer was washed and rinsed with distilled water and absolute ethanol, then hexane and baked overnight at 200°C. The experiments were conducted in a laboratory at 23±1 ∘C, with 60±5% relative humidity and 1000 lux for the duration of the experiment.

3.2.7.2 Multi-choice laboratory bioassay

In this laboratory experiment, we tried to assess the attractiveness of different lures to *D. suzukii*. In the first experiment we tested the same lures at the three different time points. We wanted to see whether the attractiveness changed with ageing of the bait. We tested Droskidrink alone at the three time points (DD I, DD II, and DD III) and 31 I; 31 II; 31 III, and PN4 I, PN4 II, and PN4 III separately. As a control in all experiments, we used the same amount of water as treatments. In one cage, we had three tests (DD I, DD II, and DD III) and one positive control water. The insects in cages were released from the side hole in the middle of the cage. The number of released insects was not precisely recorded, and the flies were different sexes, around 7-9 days old. The insects were left in the cage for 24h. Cages were kept inside the climatic chamber with a 16/8 light model and 70% humidity.

In the second trial, we tested a mixture of different lures at different time points. We had two water controls and the best performing Droskidrink from experiment 1 (DD I) in all cages. In one cage, we had two controls and 6 tests. DD I was also chosen to be tested to prove that adding *O. oeni* to Droskidrink increased attractiveness. The tested treatments were: 31 I, 31 II, 31 III, PN4 I, PN4 II, and PN4 III. The experiment ran for 24h, and the number of flies was not precisely recorded. The flies were mixed-sex and 7-9 days old. This experiment was designed to show which strain of *O. oeni* was most attractive and in which mixture age. Our hypothesis was that Droskidrink with the addition of one of the *O.oeni* strains (31 or PN4) would be more attractive to suzukii after a certain period of ageing.

Experimental data

Two trials were carried out to see the effect of modifications to the DroskiDrink Drosophila lure on the effect of *D. suzukii* catches.

First Experiment

Two new lures, *O.oeni* strain 31 ('31') and *O.oeni* strain PN4 ('PN4'), were used in conjunction with Droskidrink ('DD') aged one, two or three weeks (I, II, and III respectively). DD of the three ages was also used alone, and there was a water control. This involved 10 treatments in all. The trial was carried out in three runs, each using ten cages. Four traps per cage were used, each with a different lure. Each cage had a water lure in the trap, then one each of the same lure but with different DD ages (e.g. the four lures were water, PN4 I, PN4 II, and PN4 III), with DD lures alone used in one run, PN4 in another and 31 for the remaining run. The position of the lures within each cage was randomized.

Around 200 *D.suzukii* were released per cage. After 24 hours, the number of insects of each sex in each trap was counted.

Second Experiment

For this trial, water, DD (1 week old), PN4 with I, II and III week-old DD, and 31 with I, II and III week-old DD were used (8 treatments). All eight treatments were run similarly to the first trial. The entire trial was then repeated 2 days later. Nine cages, each with all 8 lures, were used on each day. The position of the lures within each cage was randomized.

3.2.7.2.1. Methods of analysis for the multi-choice experiment

The analysis methods were the same for both trials, and data from all days were used within single analyses. Thus, for the first trial, the data for the DD, PN4 and 31 lures were included within one analysis, allowing some comparison between these lure mixes.

For both trials, the data was 'multinomial response', in that each insect had a choice of which trap to land on.

We looked at the number caught in each type of trap as a percentage of the total caught across all the traps in a cage. This analysis is valid only if the percentage not in the traps is not affected by the lures in the cage or the trial run. Thus, the results, as presented, can be considered indicative only.

The catch per trap in relation to the total catch was analysed with a Poisson log-linear model approach for multinomial response data (P. McCullagh & Nelder, 1989). Since the data was over-dispersed, differences between the treatments were assessed using F-tests: these included some differences between the treatment groups, such as DD age. The numbers of females and males were each analysed similarly.

The percentage of females in each trap was analysed with a binomial generalised linear model (P. McCullagh & Nelder, 1989) with a logit link. Again, since the data were over-dispersed, contrasts between the treatments were assessed using F-tests.

For all analyses, percentages are presented along with 95% confidence limits. For all analysed variables, these were estimated from a binomial GLM on the logit scale, and back-transformed for presentation. For the main analyses (male, female and total per trap), the binomial analyses were carried out for each treatment separately, using the over-dispersion as estimated as part of the multinomial analyses, and the total caught in a cage as the binomial totals. This is legitimate, since binomial analyses are sub-models of the multinomial model.

The analyses were all carried out with Genstat (R. Payne, Murray, & Baird, 2017), and the graphics were produced with SigmaPlot or Genstat.

3.3 Results

3.3.1. Trapping experiments with fermentation in an open field

3.3.1.1 Trial 1: Comparison of new traps

In this trial, we assessed the effectiveness of select strains of *O. oeni* in combination with different trap designs. Overall, total numbers of *D. suzukii* increased from the first to the final assessments (Figure 3.3a-i). Numbers of males, females, total *D. suzukii*, and the percentage of captured females all varied between treatments ($p < 0.001$, $F_{7,24} = 37.8, 33.0, 37.3, 8.3$, respectively; Figure Appendix 3.1 a,b,c, Table Appendix 3.1).

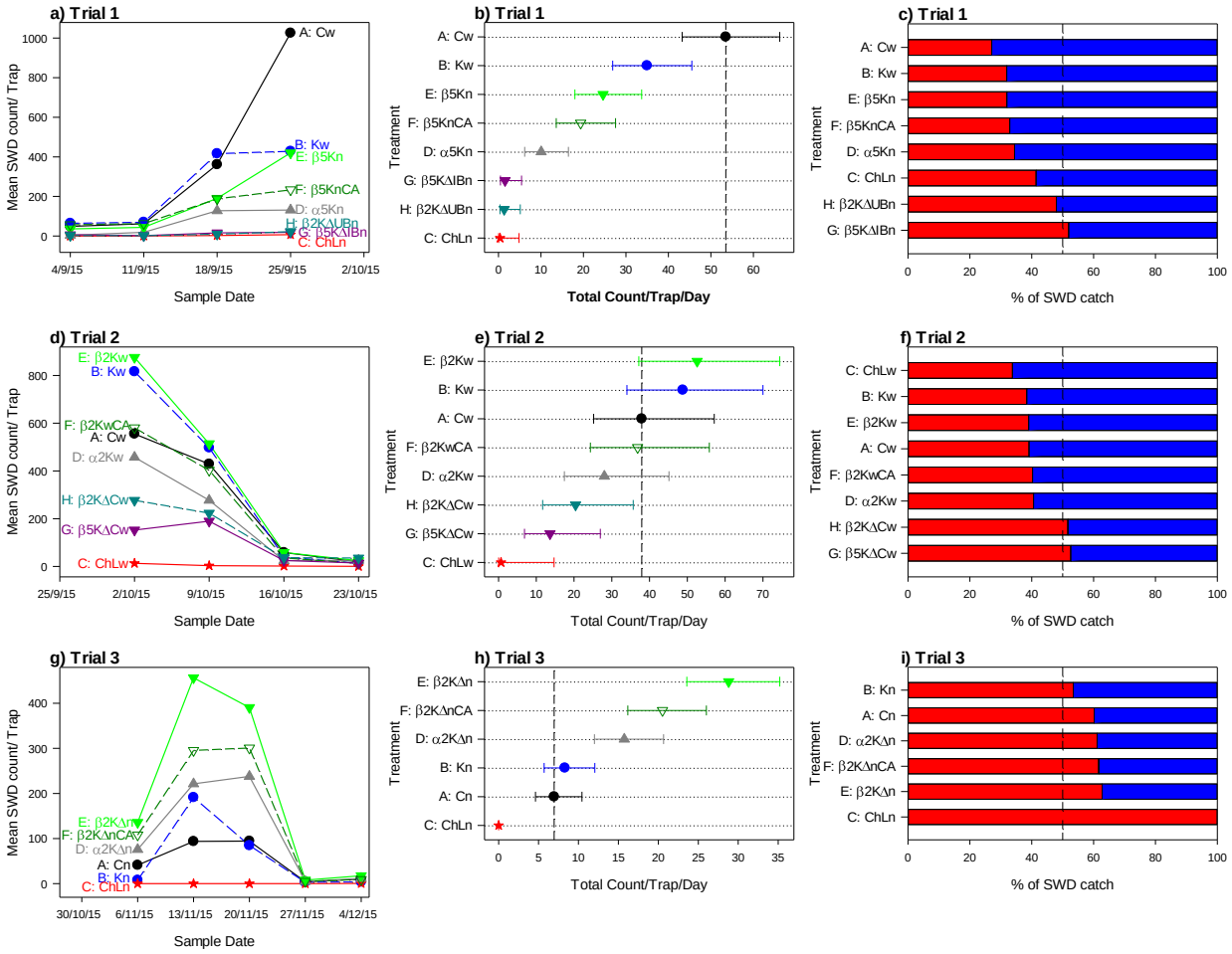


Figure 3.2: For each treatment in trials 1-3: Mean *Drosophila suzukii* (SWD) total catches per trap at each assessment (a, d, g); dot plots of mean total catch/trap/day with 95% confidence limits, sorted by the means, with dotted vertical line at the mean for Treatment A (control) (b, e, h); and percentage of the total catch that was female (blue) or male (red) (c, f, i). Note that for h, the upper confidence limit for a mean of 0 is not shown. For description of treatments, see Table 1: Traps are: C = cup with a lid; Δ= delta trap; IB = insulated bottle; UB = uninsulated bottle. Delta traps without a bottle had a cup without a lid and supplied with two cotton balls. Liquid components are: K = KOH added; α= Alpha; β = Beta; 2 = rate 0.2; 5 = rate 0.5; CA = citric acid added. n = no liquid replacement; w = liquid replaced weekly.

Differences in the numbers of males and females caught were similar across all treatments. The lowest total *D. suzukii* capture rate (six per trap) was for Treatment C, which used the Cha-Landolt bait. The mixtures inoculated with LAB (Treatments E, D, F) were significantly less attractive than standard DD (Treatment A) and DD with pH adjusted to 3.8 (Treatment B). The mixtures inoculated with strains of *O. oeni* were left in the field throughout the 4-week period of the trial, whereas the baits in the control treatments A, B and C were refreshed weekly. This helped to assess the ability of bacteria to perform malolactic fermentation directly under field conditions. In an attempt to create optimal temperatures for malolactic fermentation (20°C), polystyrene cups were used to buffer against temperature changes. Although the inoculated treatments did capture flies, their poor performance relative to the non-inoculated treatments, treatments A and B, may be linked to the temperatures reached by the liquid during the night and during the hottest hours of the day. The temperatures of the mixtures fluctuated by as much as 50°C over the course of the 4-week trial, reaching a minimum temperature of –0.4°C and a maximum

temperature of 56.9°C. These temperatures are far outside the range required by bacteria to carry out malolactic fermentation.

3.3.1.2. Trial 2: Fermentation in the open fields and different trap design

In the second trial, all attractants were replaced on a weekly basis. As in Trial 1, numbers of females, males, total *D. suzukii* and the percentage of females varied significantly between the treatments ($p<0.001$, $F_{7,24} = 9.4, 8.6, 9.2, 7.0$ for the four variables, respectively; Figure 3.2 d, e, f). However, numbers caught per trap per day were generally higher in Trial 2 than in Trial 1 (29.9 individuals trap^{-1} day^{-1} c.f. 18.2 for Trial 1). As in Trial 1, the lowest catch (19 individuals trap^{-1}) was for Treatment C, which used the Cha-Landolt bait. However, in Trial 2, total catches decreased from the first to the last week (Figure 3.2 d) (Fig A. 3.1 d,e,f).

In Trial 2, mixtures inoculated with bacteria, particularly Treatment E, had better capture performances than in Trial 1. Treatment E had the highest catch of all treatments, both overall and weekly, with the exception of the last week during which all treatments had low trap captures (Figure 3.2d). The data suggest that microbial activity, and particularly the activity of LAB, increases the attractiveness of the base DD mix.

Compared to Trial 1, total catches in Treatments G and H were higher, although still well below those in the control Treatments A and B. The effective capture that characterizes this model of trap bodes well for its use in future monitoring efforts.

3.3.1.3. Trial 3: Evaluation of the new trap design

The aim of Trial 3 was to assess whether a novel trap design could further enhance the results achieved by the mixtures of DD inoculated with LAB. Overall, total catches increased from week 1 to weeks 2 and 3, and then dropped to almost zero for the final 2 weeks of the trial (Figure 3. 2g). Numbers of males, females, total SWD, and the percentage of females caught all varied substantially between the treatments (Figure 3. 2 g, h, i; $p<0.001$, $F_{5,12} = 34.4, 43.7, 40.6$, $F_{5,10} = 5.2$) for the four variables, respectively. Two traps had no catches (total SWD = 0). Differences in the number of trapped males and females was similar between treatments. The lowest catch (0.3 trap^{-1}) was for Treatment C.

For Trial 3, the modified delta trap (as used in treatment H of Trial 2) was evaluated using different modifications of the DD mixture. When compared to the controls (treatments A, B, and C), all treatments caught on average at least twice as many insects. This differs from results in Trial 1 where the control (treatment A) caught the largest numbers, and in Trial 2 where only treatments B and E caught more insects than the control. Thus, the results of Trial 3 provide further support that trap design has been improved. Mixtures inoculated with LAB provided excellent results throughout the five weeks of the trial. In Trial 3, more than 50% of the catch was female for all treatments. This is in contrast to the first two trials, where more males than females were caught for almost all treatments. Additionally, in this new trapping system, the time required for identification and counting of insects was one third of the time needed for the previous standard traps. The best results were achieved by the use of treatment E, but all treatments inoculated with LAB outperformed control treatments that used the standard cup trap (Figure 3. 2 h). This same pattern of increased capture with the new trap design was present each week where catches were non-negligible (Figure 3. 2 g) (Figure A 3.1 g,h,i).

3.3.2. Headspace characterisation of DD inoculated with different *O. oeni* strains by GC-MS

We selected the six strains of *O. oeni* (MRI1000, alpha, beta, T1, S18, P1) for analysis of volatile organic compound (VOC) release from the mixture of DD and *O. oeni* strains, which we compared with a standard

unfermented DD sample (Table 3. 2). The aim was to identified peaks of thethe most volatile compounds in the mixtures that with standard GC-MS analysis methods were hidden behind the solvent peak. The MRI1000 strain was analyzed as a reference strain of *O.oeni*. Thirteen compounds were detected (Table 3. 2); however, no qualitative differences were found among the baits, and the same compounds were detected in most samples. Interestingly, the relative quantity of compounds varied between samples. The T1 strain was characterized by a high concentration of acetic acid and ethanol. The beta and S18 strains showed a considerable reduction of acetic acid emission (–45% of DD release), while ethanol increased by about 30%. T1 and P1 strains produce a very high quantity of acetic acid, 874% and 669% more, respectively, than the DD alone. These also showed higher ethanol content than the other samples. For the alcohol 3-methyl-1-butanol, all baits showed a content equal to or higher than 100%, except the S18 sample (85%), compared to DD alone. The bacterial strains T1 and P1 produced 79% and 44% less acetoin, respectively, compared to DD alone. For the compound acetidin, the content of all bacterial baits was very low compared to that of DD. The beta strain has a content of 7% and alpha 6%, while strains T1, S18 and P1 are around 4%, relative to DD alone.

Table 3.2. Relative quantification of volatiles emitted by baits inoculated with different strains of *Oenococcus oeni* (MRI1000, alpha, beta, T1, S18, P1). Data are expressed as the percentage (%) with respect to the amount measured in the standard, unfermented Droskidrink (DD).

Volatile Compound	Retention Time	Extracted Ions	Droskidrink	MRI1000	α	β	T1	S18	P1
	Min	m/z				%			
Acid									
Acetic acid	2.2	60	100	47.78	55.75	55.9	874.39	64.88	669.25
Alcohols									
Ethanol	1.55	45	100	126.07	129.25	138.499	165.14	119.49	154.68
Isoamyl alcohol	4.7	41	100	123.66	136.69	188.1	115.98	99.73	109.61
Aldehyde									
Acetoin	3.78	88	100	149.45	169.16	184.76	78.7	126.11	44.61
Esters									
Ethyl acetate	2.27	88	100	5.04	5.53	6.98	3.65	4.33	3.89
2-Butyl acetate	5.55	87	100	114.63	105.54	112.49	70.92	81.63	68.71
Isobutyl acetate	6.17	56	100	70.72	69.19	106.79	48.35	51.06	43.88
Ethyl butyrate	7.36	71	100	64.95	62.2	90.78	52.81	46.44	51.73
Isoamyl acetate	10.84	43	100	40.16	37.22	42.18	31.41	33.42	29.5
Ethyl caproate	16.73	88	100	95.83	92.47	147.36	72.36	57.51	50.62
Ethyl octanoate	25.35	88	100	143.35	135.62	241.42	97.7	91.79	72.75
Ketone									
2-Butanone	2.13	72	100	139.39	149.26	164.71	75.32	110.97	84.98

3.3.3. Chemical characterisation of volatiles from Droskidrink and *O. oeni* strains.

The compounds have different affinities for the two different stationary phases in the columns we used, allowing us to identify the highest possible number of compounds. Gas Chromatography (GC) on the polar column: GC analysis of the entrainment samples of DD and LAB revealed a complex mixture of the volatile compounds that change over time and by the use of different bacterial strains. The experiments revealed complex changes in the quality of the VOCs released from tested mixtures during a three-week period. We tentatively identified about 80 peaks from GS-MS data sets using the non-target approach with data analysis software Xcalibur 1.2 (data available upon request). GC co-injection with synthetic chemical standards confirmed the identity of target compounds present in the entrainment samples in areas of 24 peaks. The remaining 7 peaks that elicited response in electrophysiological studies were only tentatively identified because of the absence of laboratory synthetic standards or presence of different isomers.

It is evident that bacteria increased the quantity of some VOCs in the mixture (Figure A 3.3 a, b, c). The complex volatile blend in the mixtures changed not only in the presence of LAB, but also with ageing of the mixture. Most of the compounds of interest were not detectable 20 days after the addition of the bacterial strains. In general, the amount of total alcohols in DD with LAB increased in the second week, then decreased by the third week. Acid compounds decreased toward the second week, and then later increased. Addition of *O. oeni* strain 31 to DD increased the total amount of acid compounds together with alcohols, while esters and ketones were reduced (Figure A 3.4). *Oenococcus oeni* strains 31 and PN4 showed a considerable reduction in acetic acid and acetoin in the second week (Figure A 3.3 b, c). The emission rate of 3-methyl-1-butanol, reported as a "non-target" attractive volatile for a wide range of moths (Landolt, Adams, Zack, & Crabo, 2011), was higher than in standard DD in the case of O. oeni strains 31 and PN4.

The intensity of the volatile compounds produced by wine vinegar changed with addition of strains of *O.oeni*, and most of the compounds decreased with ageing of the mixture. It is evident that bacteria increased the quantity of some compounds in the mixture, (Table 3.3, Figure A 3.2 a,b,c). The complex volatile blend inside mixtures changed with bacteria but also with ageing of the mixture. In one-week-old Droskidrink alone we tentatively identified 42 peaks with a high percentage of matching with library and Kovats indexes. On the other hand, the number of peaks in two and three week-old Droskidrink was 28 and 32 respectively. In Droskidrink with *O.oeni* strain 31, the number of tentatively identified peaks was 29 in the first week, 33 in the second, and 36 in the third. This is telling, as these compounds changed within the timeline. The number of tentatively identified compounds in Droskidrink with *O. oeni,* the Pn4 strain, was 32 in the first week, then 23 in the second week and with 30 peaks in three-week-old extracts.

Most of the compounds of interest were no longer produced after 20 days ageing of the mixture. With the addition of *O. oeni* to Droskidrink, some compounds were significantly reduced, such as acetoin, acetic acid, 1-Hexanol, 2-ethyl, β-Phenethyl acetate, Decanoic acid, hexanoic acid, and butanoic acid 3 methyl (Figure A. 3.3).

The relative quantity of compounds showed significant differences. 31 I samples were characterised by a high concentration of acetic acid (+560% more than standard DD). The 31 strain and PN4 strain showed a considerable reduction in acetic acid and acetoin. It is likely that acetoin is one of the key compounds for the attraction of *D. suzukii* to DD (Cha et al., 2012; R Guzzon et al., 2015). **Acetoin** (3 hydroxy buton-2-one) was one of prominent peaks with the addition of the *O. oeni* strain that was produced significantly less in the first week with the addition of the PN4 and 31 strains (Figure A 3.5a and Figure 3.4.). The amount of this molecule showed a decrease of 55% in 31 II, 31 III and PN4 II, and PN4 III group headspace when compared to DD I, 31 I, PN4 I. Acetoin is described in the literature as one of the critical compounds for the attraction of *Drosophila suzukii* (Cha et al., 2012; Feng & Zhang, 2017).

The difference in GC-MS chromatographs was in peak abundance for the different ages, as well as between the control and Droskidrink with the addition of the PN4 and 31 strains of *O. oeni* (Figure A 3.4 a, b, c).

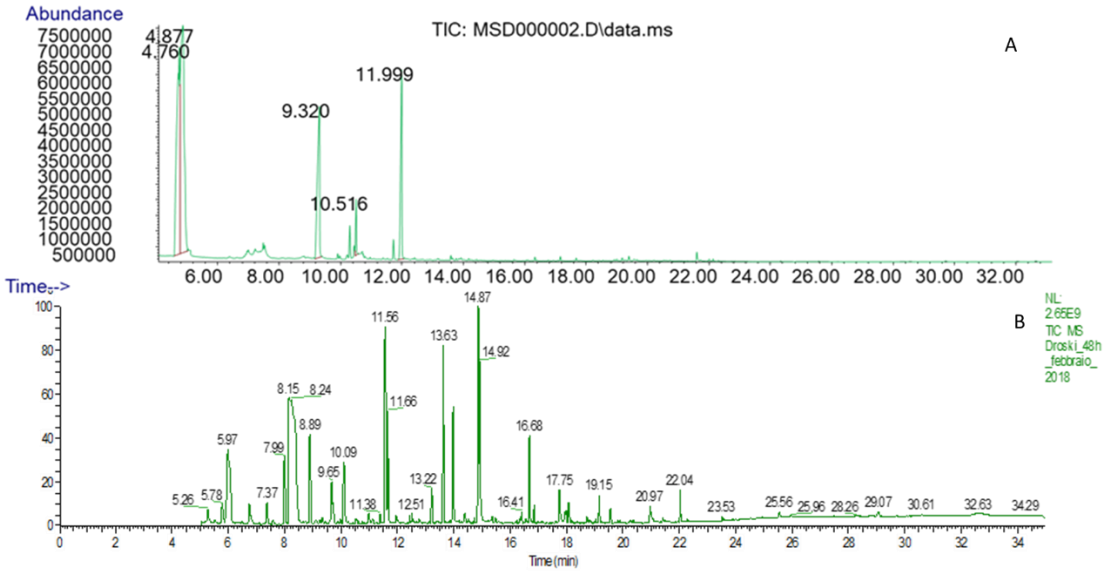


Figure 3.3 a, b Typical GC traces from A) a non-polar column (HP-1), and B) a polar column (ZB-wax) of 2 µl of an entrainment sample (elution) of headspace volatiles collected from Droskidrink alone

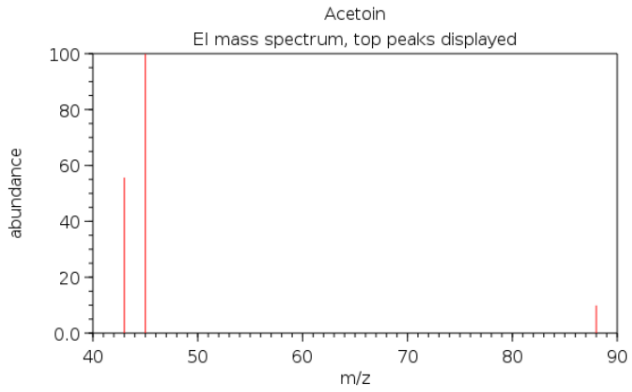


Figure 3.4. Mass spectra of acetoin, one of the most important compounds for *Drosophila suzukii* attraction

3.3.4. Results from electrophysiological experiments

GC-EAD and compound identification by GC-MS and GC-co-injection: GC-EAD analysis of antennal responses was constant and significantly higher than background noise in 32 EAG peaks in the entrainment samples of DD and DD + *O. oeni* strains 31 and PN4 (Table 3.3). Confirmation of the identity of electrophysiologically active compound with RT 12.42 on the non-polar column could not be made. This compound according to mass spectra is monoterpene, but it did not match any library search. Also, the compound on the non-polar column that elicited a response with RT 7.006 could not be identified, although a NIST11 (National Institute of Standards and Technology, 2011), Wiley7n, and W9N08library search with 90 % of match offers identification as 3-Hexenoic acid, ethyl ester. The laboratory standard of this compound is not available. Interestingly, some of the compounds did not elicit an antennal response in one-week-old extracts, which indicates that these compounds may only be biologically active in certain

amounts (Fig. 4). GC-EAD-FID identified around 18 active EAD peaks in the different samples of DD and samples with LAB (Table 3.3).

Subsequently, behavioural bioassay evaluation with significant compounds will uncover which combination of different VOCs is most attractive to SWD. Behavioural bioassays will help us to build an ideal laboratory combination of VOCs that can be used in traps for testing in open field studies.

Table 3.3. Compounds that are electrophysiologically active from all eluted mixtures, RT- retention time for compounds eluted on the polar column, retention index from the polar and non-polar column.

			RT	Retention Index	
	Compound Chemical Name	**CAS**	**ZB-WAX**	**ZB-WAX**	**HP-5 MS**
1	Acetic acid	64-19-7	8.32	1480	600
2	2-Butanone, 3-hydroxy	513-86-0	6.02	1288	662
3	3-Methyl-1-butanol	123-51-3	4.9	1117	720
4	Ethyl butyrate	105-54-4	10.58	1591	788
5	Ethyl lactate	97-64-3	6.76	1338	781
6	Butanoic acid	107-92-6	11.15	1630	843.7
7	1-Butanol, 3-methyl-, acetate	123-92-2	4.05	1148	879.8
8	3-(Acetyloxy)-2-butanone	4906-24-5	7.36	1376	899.1
9	1-Hexanol	111-27-3	6.86	1344	862
10	Grape butyrate	5405-41-4	10.11	1518	907
11	Benzaldehyde	100-52-7	9.58	1534	947
12	Ethyl hexanoate	123-66-0	5.26	1234	980
13	Butanoic acid, 3-methyl-	503-74-2	11.57	1668	913.1
14	3-Hexenoic acid, ethyl ester	2396-83-0	6.08	1293	956.8
15	Nonanal	124-19-6	7.6	1392	1074
16	1-Hexanol, 2-ethyl	104-76-7	8.9	1486	1034.9
17	Hexanoic acid	142-62-1	13.98	1833	908.0
18	1- Octanol	111-87-5	10.02	1553	1050
19	Acetic acid, hexyl ester	142-92-7	5.78	1271	1018.1
20	Sorbic Acid	110-44-1	17.74	2133	1089.8
21	Acetic acid, phenylmethyl ester	140-11-4	12.43	1719	1167.3
22	Phenylethyl Alcohol	60-12-8	14.87	1900	1120.1
23	Benzenemethanol, benzyl alcohol	100-51-6	14.4	1865	1039.0
24	Acetic acid, 2-phenylethyl ester	103-45-7	13.63	1793	1262.5
25	Benzeneacetic acid, ethyl ester	101-97-3	13.21	1777	1250.3
26	Eugenol	97-53-0	18.07	2162	1361.5
27	Triacetin	102-76-1	16.85	2059	1354.4
28	Diethyl succinate	123-25-1	11.66	1674	1182.8
29	Benzothiazole	95-16-9	15.62	1960	1225.5
30	Octanoic acid, ethyl ester	106-32-1	8.14	1428	1198.0
31	Benzoic acid, 2-hydroxy-, methyl ester	119-36-8	13.12	1769	1197.8
32	Phenol, 2,4-bis(1,1-dimethylethyl)	96-76-4	19.57	2293	1511.8

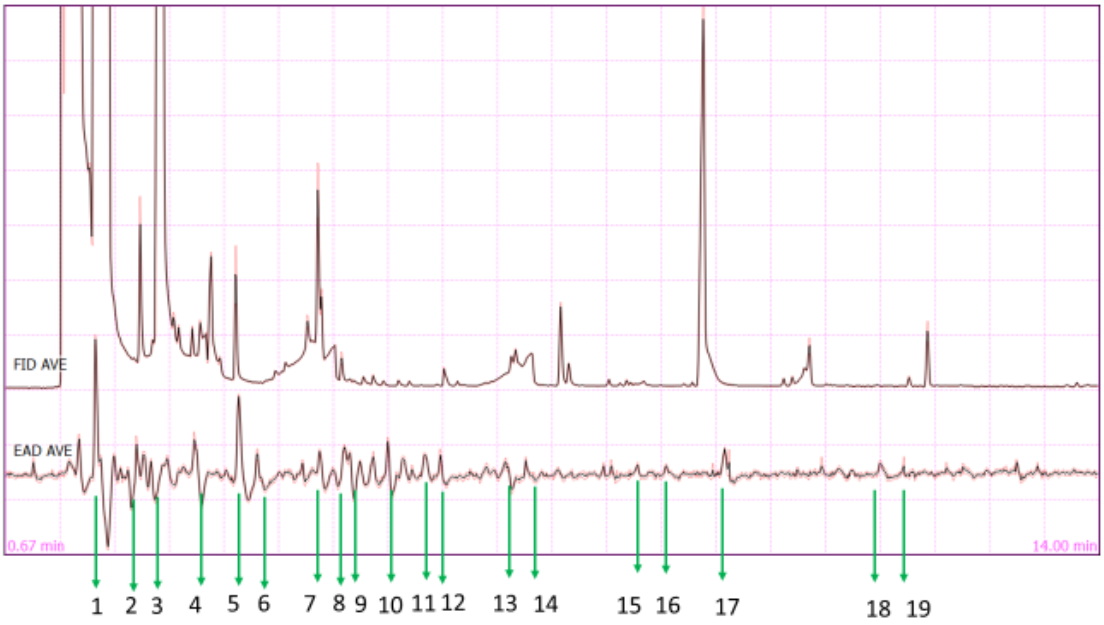


Figure 3.5. Example of a coupled GC-EAG trace of antennal response of a *Drosophila Suzuki* mated female to an entrainment sample of headspace volatiles collected from Droskidrink mixed with the *O.oeni* strain PN4. The top trace corresponds to the FID detector on the GC. The bottom trace corresponds to the antennal response of the insect preparation. Green arrows indicate antennal responses and corresponding areas of the GC trace of interest. Peak numbers correlate to compounds (listed in Table 3.4.1.a), some of which were identified by GC mass spectrometry (GC-MS) and peak enhancement co-injection with authentic laboratory standards. The GC-EAG experiment was repeated with up to 10 recordings, same mixture was presented to 10 different insects with corresponding EAD peaks. Compounds identified: 1. Solvent peak, 2. Acetic acid, 3. Isoamyl alcohol , 4. Acetoin, 5. Ethyl butyrate, 6. Ethyl lactate, 7. 1-butanol, 3-methyl-,acetate, 8. Butanoic acid, 9. 3-acetyloxy-2-butanone, 10. Benzaldehyde, 11. Hexanoic acid, 12. Hexanoic acid, ethyl ester, 13. Ethyl hexanoate, 14. 1-Hexanol, 2-ethyl, 15. Diethyl succinate, 16. Benzeneacetic acid, ethyl ester, 17. Acetic acid, 2-phenylethyl ester, 18. Triacetin, 19. Eugenol

Compaund name	DD i	DD ii	DD iii	31 i	31 ii	31 iii	PN4 i	PN4 ii	PN4 iii
Acetic acid									
Acetoin									
3-Methyl-1-butanol									
Ethyl butyrate									
Ethyl lactate									
Butanoic acid									
Isoamyl acetate									
Acetoin acetate									
1-Hexanol									
Grape butyrate									
Benzaldehyde									
Ethyl hexanoate									
Isovaleric acid									
3-Hexenoic acid, ethyl ester									
Nonanal									
1-Hexanol, 2-ethyl									
Hexanoic acid									
1- Octanol									
Ethyl butyrate									
Acetic acid, hexyl ester									
Sorbic Acid									
Phenylmethyl acetate									
Phenylethyl Alcohol									
Benzyl alcohol									
Phenethyl acetate									
Ethyl phenylacetate									
Euginol									
Triacetin									
Diethyl succinate									
Benzothiazole									
Ethyl caprylate									
Methyl salicylate									
Dimethylacetophenone									
Prodox 146									

Table. 3.4. CG-EAD response to active electrophysiological peaks, the number of responses in ten consequent runs. The colours indicate the percentage of averaged responses. A darker colour represents a higher absolute amplitude of the responses in μV from the onset of depolarization (baseline) to the maxima of the deflection.

3.3.5. Behavioural bioassays

We performed Y tube olfactometer bioassays with Droskidrink with the lure mixture (Cha et al., 2012), where we proved that the *D. suzukii's* response rate was higher than 90 per cent in mated females, followed by virgins and then males (Figure 3.6).

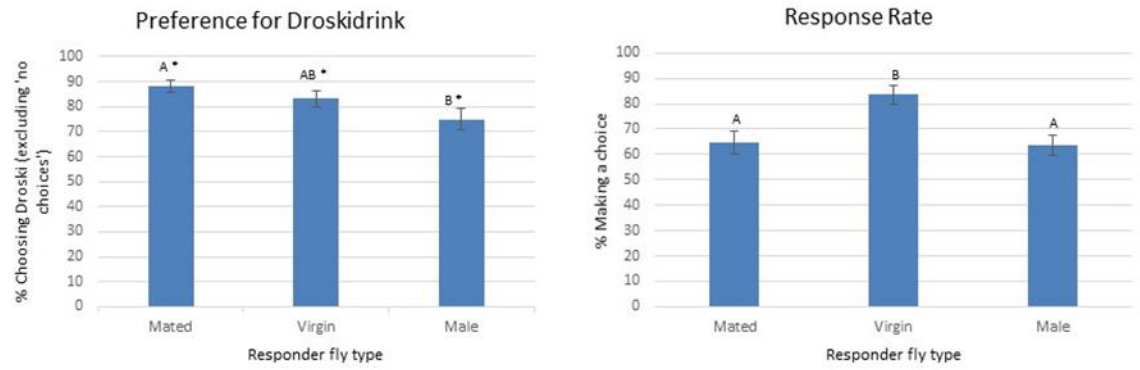


Figure 3. 6. a) Preference towards Droskidrink. The average percentage of flies choosing Droski over the control in the y-tube (the group of 10 flies in chohort per trial). Binomial GLM for differences between groups. Different letters indicate a difference in preference between groups at $p < 0.05$. Asterix (*) denotes significant deviation from 50% (no preference) at $p < 0.001$ (t-test and Wilcoxon signed-rank test). Error bars represent s.e.m. b) Percentage of flies making a choice. The average percentage of flies making a choice in the y-maze (a group of 10 flies per trial). Quasibinomial GLM for differences between groups. Different letters indicate a difference in preference between groups at $p < 0.01$. Error bars represent s.e.m.

3.3.6. Results from the multi-choice experiment

<u>First Experiment</u>

The numbers of males were fairly well correlated with the numbers of females. Thus, the pattern of catches across the lures is reasonably similar. 51% of the total flies caught were males.

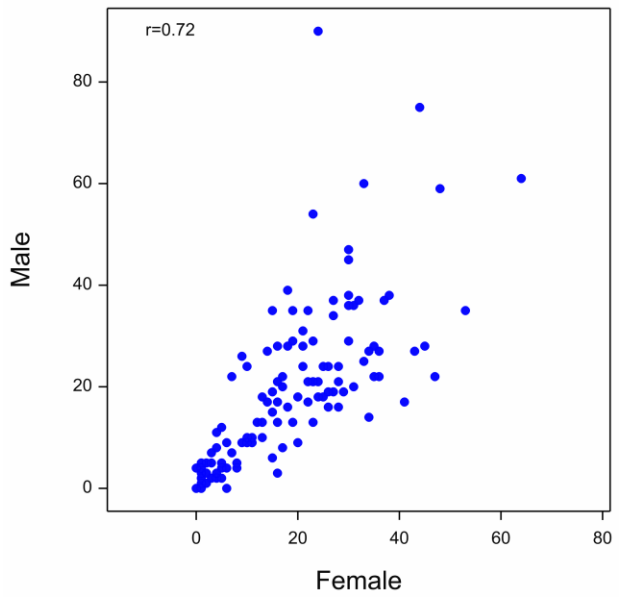


Figure 3.7. First Trial. Relationship between the number of males and the number of females per trap.

The main features were the generally lower catches for water and a small number of outliers. However, these outliers were at least partly related to the variable number of flies in the cage. The total numbers of flies caught varied substantially (Figure A 3.8), even in the same run of 10 cages, indicating that the total number of flies released is also likely to have varied substantially.

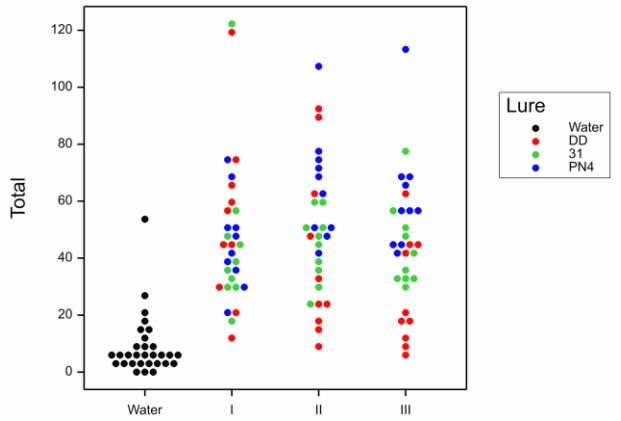


Figure 3.8. First Trial. Number of flies caught in each trap for each run, plotted against lure age (I, II, III).

For males, females and total flies, the main differences in total catch % were between the water traps and the other traps ($p<0.001$ for each), with a much lower percentage of flies caught in water than with the other traps. However, there were some smaller differences between lures, with the changes in lure age varying with the lure type ($p=0.069$; 0.020, 0.025 for males, females and total flies, for the lure by week of interaction). The percentage caught decreased with age for the DD lures alone, remaining fairly similar for the 31 lures, and increasing then decreasing slightly for the PN4 lures (Table A 3.5). The percentage of flies caught in the water traps was fairly similar for the three runs, with overall means of 6.5, 5.0 and 5.8 % for males, females and total flies. The percentage of female flies caught in each trap did not vary strongly between lures ($p=0.271$).

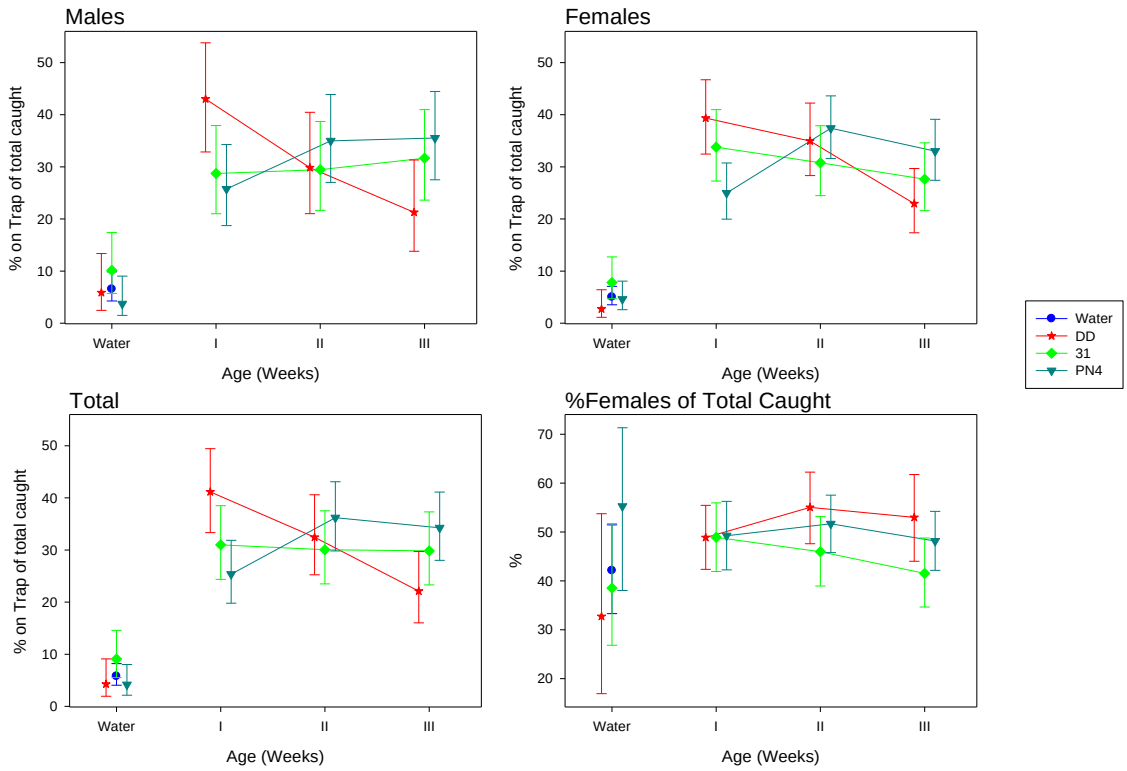


Figure 3.9. First Trial. Percentage of total catch in the traps of each lure type. Error bars are 95% confidence limits. Note that the confidence limits were calculated using the dispersion for the model, fitted with the water estimate averaged overruns. This estimate for water is shown (in blue) along with individual estimates from each run.

Second Trial

The numbers of males caught were moderately correlated with the numbers of females caught. Thus, the pattern of catches across the lures is relatively similar. 49% of the total catch was males (Figure A 3.9).

The main features were the generally lower catch for water and a small number of outliers. However, these outliers were at least partly related to the variable number of flies in the cage: the total numbers of flies caught varied substantially (Figure A 3.10), even in the same run of nine cages, indicating that the total number of flies released was also likely to have varied substantially.

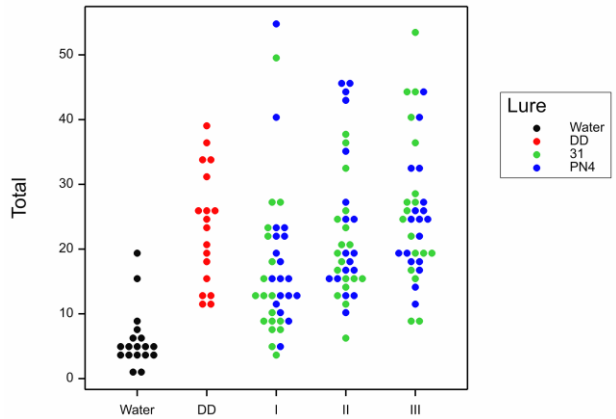


Figure 3.10. Second Trial. The number of flies caught in each trap for each run is plotted against lure age (I, II, III).

For males, females, and total flies, the main differences in total catch % were between water traps and the other traps ($p<0.001$), with a much lower percentage of flies caught in water than in the other traps. The catch with DD alone was substantially higher than the catch with water ($p<0.001$ for males, females and total). The catch with DD was relatively similar to the catch using other lures, with little difference on average between the three lure types ($p=0.771$, 0.103, 0.246 for males, females and total flies). However, on average for the PN4 and 31 lures, the total catch percentage increased with lure age, from around 10% of all flies caught for I, to about 14% for II and about 16% for III (Table A 3.5). The percentage of female flies caught in each trap did not vary strongly between lures ($p=0.795$).

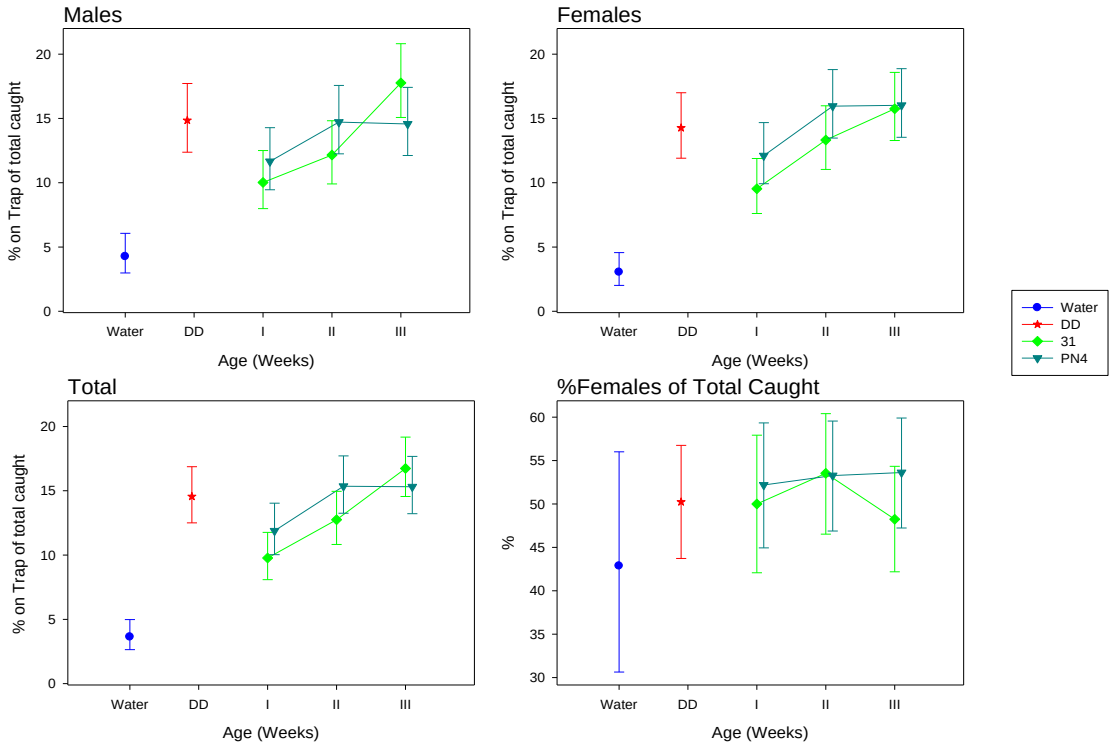


Figure 3.11. Percentage of total catch in the traps of each lure type. Error bars are 95% confidence limits.

3.4 Discussion

This study found that crucial volatiles for the attractiveness of SWD released as a products of malolactic fermentation in the wine-vinegar mixture in presence of LAB, are commonly found also in host fruits (Becher et al., 2012; Becher, Hagman, et al., 2018; I. W. Keesey et al., 2015; Revadi et al., 2015). However, some compounds like phenylethyl alcohol, methyl salicylate and eugenol are also commonly reported as typical plant-green leaf volatiles (Bruce & Pickett, 2011; I. W. Keesey et al., 2015). Moreover, certain compounds have been reported as insect pheromones, e.g. acetoin and 2,3 butanediol (El-Sayed, 2020; Rochat et al., 2002). Compounds that elicited constant and high electrophysiological response common to all samples belong to classes of acetate esters, esters, acids, short-chain alcohols, and ketones.

Our results show the basis for improved attraction of spotted wing Drosophila using lactic acid bacteria (LAB) fermentation acting on a complex mixture of apple cider vinegar, red wine and sugar cane. The addition of fermentation by-products from yeasts has been reported to improve the attraction of lures for *D. suzukii* (A. L. Knight et al., 2016; S. Knight & Goddard, 2015), but the potential for improvement from bacterial fermentation is new and offers an exciting line of inquiry for the future. Bacterially induced

fermentation resulted in fine tuning of certain MVOCs to a level that is highly attractive for SWD. Overall, the chemical studies results show a very complex relationship between bacterial strains and bait features.

In GC analysis on the HP- 5 non-polar column, the solvent peak may have masked some low sensitivity volatiles, as only a fraction of the volatiles are absorbed and analysed. This difficulty we faced was overcome with additional analysis using direct headspace collection combined with GC-MS analysis. In DH- GC-MS compounds of importance were identified. Compounds with a relatively low Kovats index on the GC-MS non-polar column are covered by solvent peaks. These compounds are acetic acid, acetoin, 3 methyl 1 butanol, ethyl butyrate, ethyl lactate, Butanoic acid, 3-hydroxy-, and ethyl ester.

Difficult identification of some peaks of interest may be due to absorbent choice. The lack of sensitivity of the absorbent may be overcome by a comparison of 2 different absorbents. At the beginning of our volatile collection, we used Tenax as an absorbent, but it proved not to be suitable for dilution with polar solvents (dichloromethane, acetone). We then used Porapak Q, mash size 50/80.. Initially, in the first trials we performed aeration of Porapak Q columns for 24 h, but consecutive GC-MS analysis did not give many peaks, suggesting that we may have missed some of the heavy molecules that could be important in the behaviour of *Drosophila suzukii*. Success was achieved by increasing the time period and the quantity of Droskidrink entrained, thus helping us to collect a greater quantity of headspace volatiles.

We found a number of electrophysiological active compounds for *D. suzukii* (Table 3). Our findings are in line with other electrophysiological studies (Feng et al., 2018; I. W. Keesey et al., 2015; Mazzetto et al., 2016; Revadi et al., 2015). Typical yeast volatiles present in DD include: ethanol, isobutanol, 3-methyl butanol, 3-hydroxy-2-butanone (acetoin), acetic acid, isobutyric acid, ethyl hexanoate, ethyl octanoate, and phenylethyl alcohol (Arguello, Sellanes, Lou, & Raguso, 2013; Becher et al., 2012). VOCs of fermenting products and fruit that elicited high and constant electrophysiological responses in our study are: isoamyl acetate, ethyl acetate, isopentyl acetate, hexyl acetate, ethyl hexanoate, benzyl acetate, benzaldehyde, ethyl octanoate, and acetoin (I. W. Keesey et al., 2015; I. W. Keesey et al., 2016). Moderate electrophysiological responses resulted from: grape butyrate, 2-phenylethanol, methionol, isoamyl lactate and diethyl succinate, although it is important to remark that none of these volatiles are species-specific. These odours are also well known for eliciting electrophysiological responses in *Drosophila melanogaster* and other drosophilids (I. W. Keesey et al., 2015; Revadi et al., 2015; Scheidler et al., 2015).

An excessive concentration of some compounds characterizing DD could induce positive or negative responses from the olfactory system of the SWD. For example, earlier studies (Cha et al., 2012; Cha et al., 2014b) reported that several EAD-active compounds released from wine and vinegar had deterrent effects on *D. suzukii* attraction at high concentrations. One of these compounds is isoamyl acetate, likely released by the epiphytic community on fruit surfaces as well as by fermenting substrates (Revadi et al., 2015). When it was tested, isoamyl acetate was attractive only within a concentration range similar to that emitted by fresh fruit, whereas the 100-fold higher release rates from wine and vinegar were behaviourally repellent (Cha et al., 2012; Revadi et al., 2015). In our experiments, the release rate of isoamyl acetate was remarkably reduced after bacterial inoculation and lactic fermentation, which could be one of the reasons for the increased attractivity to *D. suzukii*. It is already established that acetic acid and ethanol are key volatiles for the attraction of *D. suzukii* to wine and vinegar(Cha et al., 2014b).

Chemical compounds that elicited the response in the EAD system may not be significantly important as a single attractant. We need to evaluate the mixture according to the actual proportion in the volatile collection. Furthermore, their deflection must first be investigated with EAG experiments, building a dose-response curve from those that are significant. The dose-response curve may help us to build future bioassays.

The compound acetoin, reported to be one of the main attractive compounds (Zang et al., 2018, Cha et al., 2014), significantly decreased with the addition of lactic acid bacteria and ageing of our tested mixtures. However, acetoin, one of the key compounds for the attraction of *D. suzukii* to DD (Feng et al., 2018; R Guzzon et al., 2015; Romano & Suzzi, 1996), showed a decrease of about 55% in samples inoculated with LAB. Acetoin is a compound known to be present both wine and vinegar, and is a fermentation product of LAB (Cha et al., 2012). The evidence from earlier studies on SWD commonly supports the theory that the absolute amount of VOCs is important for insects' choice of mating and oviposition sites. Isoamyl acetate decreases attraction when added to acetic acid and ethanol under laboratory and field conditions (Cha et al., 2012)

All electrophysiologically active compounds had been carefully evaluated, and significant compounds must be selected to obtain a suitable mixture for testing in bioassays.

Ageing of the mixture and the addition of different *O.oeni* strains have contributed to the changes in odour profile. These induced changes in odour profile often affect the odour-induced behaviour of the insect, our experiment proving that attractiveness increased with lactic acid bacteria.

Thirty-two peaks were identified as active using GC-EAD, and the probable identity of twenty-four of these peaks was established. For two of the peaks that elicited a constant response (RT 7.006 and RT 12.42), we could not confirm identity. The GC and GC-EAG traces indicated that the headspace volatiles emitted by Droskidrink and *O. oeni* are a complex mixture. Most of these compounds identified as biologically-active are present at low concentrations, accompanied by a few large amounts of active compounds, among relatively small amounts of numerous inactive compounds.

Therefore, the quantitative variations induced in the DD through the addition of LAB should be carefully considered, as well as the interactions of the trap mixture with environmental variables (temperature, humidity, not-target insect catches) during field ageing of lures.

We show that the emission rate of 3-methyl-1-butanol, reported as a "non-target" attractive volatile for a wide range of moths (P. J. Landolt, Adams, Zack, & Crabo, 2011), is higher after addition of strains of *O. oeni* than in the standard DD. The impact of bacterial activity on the release rate of ester compounds appears more uniform, with a generalized decrease in comparison to DD, except for a slight increase of ethyl octanoate production.

During the first field trial, Trial 1, total catches of *D. suzukii* generally increased from week 1 to week 4. In particular, there was a substantial weekly increase in catch for the positive control, Treatment A. This confirms the importance of microbial activity for the attractiveness of the mixture. In the treatments inoculated with LAB and left in the field for the entire period of the experiment, liquid bait temperatures appear to have inhibited the development of bacterial flora. Across the four weeks of the trial, Treatments E and F, which contained *O. oeni* Beta, caught more SWD than Treatment D, which contained *O. oeni* Alpha. This suggests that the Beta strain is better adapted to high temperatures than the Alpha strain. Adding citric acid to the mixture in Treatment F resulted in malolactic fermentation leading to increased production of acetoin, diacetyl and 2,3-butanediol (Nielsen & Richelieu, 1999). These compounds might increase attractivity to *D. suzukii*. However, traps baited with *O. oeni* and citric acid did not show any change in attraction. The results obtained from Treatment A are most likely due to the weekly replacement of liquid bait making it possible to maintain a higher concentration of autochthonous bacteria. Treatments G and H, which included DD + LAB but had different trap designs, showed very low numbers of captured flies.

We optimized trap architecture and bait components in order to keep the temperature of the liquid bait within the optimal range. Different trap designs were tested in the field in order to provide bacteria with optimal growing conditions and easier, more rapid release of volatile compounds. Indeed, an innovative trap architecture in combination with the DD + LAB bait produced a powerful attraction and enabled the capture of a greater number of *D. suzukii* compared with other traps, particularly of females and during the cold periods of low *D. suzukii* population density. This activity could be further improved by calibrating the concentration of *O. oeni* bacterial strains and better controlling the factors that limit the

development of these bacteria. We also confirmed that the malolactic fermentation by strains of LAB added to variants of DD appears to be instrumental in boosting catches of *D. suzukii*. It is also clear that, in addition to all other limiting factors such as pH and SO_2 concentration, a certain temperature must be maintained to ensure that adequate fermentation takes place. For this reason, it is necessary that the mixtures, once prepared, are kept under controlled temperatures and that they are maintained so as not to suffer contamination by other microbial species that could trigger undesirable fermentation. Our results show that the commercial bacterial strain Enoferm Beta provides the best results, and that the addition of citric acid is a limiting factor for this type of bait. Moreover, a great advantage of the trap developed in this work is that it is capable to be quickly and easily serviced, which helps in planning the use of insecticides and other control strategies in an IPM program approach against SWD.

Contrary to our results, earlier studies suggested that the presence of *O. oeni* cultures did not significantly improve the attraction to DD (Tonina et al., 2018). However, in these cases, commercial plastic cup traps (Droso Trap®) were used, which are highly susceptible to temperature fluctuations that likely inhibited bacterial metabolic activity, as shown in our field trials. Furthermore, the studies were carried out under different environmental and seasonal conditions, and used different strains of *O. oeni*.

Monitoring of the target insect is the first step to an IPM program. Ideally, a species-specific trap containing odorants emitted by host fruit during ripening might attract *D. suzukii* earlier in the season, allowing early detection and intervention. Such a trap would help to control the pest on a local level by suppressing population numbers (Abraham et al., 2015; Feng et al., 2018; I. W. Keesey et al., 2015). The trap could also be utilized in a mass trapping system later in the season. A key goal of mass trapping is to capture the maximum possible number of insects before they reproduce or cause damage to crops. Effective trapping requires the use of lures that are more attractive than natural sources, such as food, female sex pheromones, and mating aggregations. Furthermore, lures should be effective during the entire period of adult emergence and mating (El-Sayed et al., 2006; Suckling et al., 2015). Accordingly, the traps must be visually attractive and capable of capturing and retaining flies long enough to provide a lethal dose of toxicant or prevent the escape by drowning or starvation (Lasa et al., 2014).

Catching a large proportion of insects during the early season is crucial to reduce the damage caused by *D. suzukii* to the fruit (El-Sayed et al., 2006; Rossi-Stacconi et al., 2016). Our results suggest that the use of the *O. oeni*-baited DD trap may provide an important contribution to significantly reduce populations of *D. suzukii*, especially during bottleneck phases under relatively cold climatic conditions of the early growing season. The presence of VOCs in our mixture that are also commonly reported as typical fruit volatiles helps achieve this goal. We hypothesized that population control methods based on behavioral manipulation over a wide territorial scale, such as mass-trapping, attract-and-kill and push -pull, should be maximized near winter shelter areas, as well as in unmanaged environments flanking fruit production areas susceptible to *D. suzukii* infestations (Gabriella Tait et al., 2019; Gabriella Tait et al., 2018). In addition, mass trapping methods targeting mainly gravid females and carried out before the beginning of the flowering and fruiting season have the potential to be extremely effective because of the lack of competition between natural sources and bait traps. Further studies are needed to corroborate the results obtained here, in particular under various climatic and environmental conditions, to confirm the possible reduction of *D. suzukii* populations and associated crop damage.

3.5. Conclusions

Overall from chapter 2 and chapter 3, our results showed that the attractiveness of wine-vinegar liquid bait for SWD was increased up to two-fold by the addition of commercially available Enoform Beta strain of *O. oeni* when combined with an innovative trap design. Findings from our studies are currently utilized for early detection and mass trapping of *D. suzukii* in Trentino and Alto Adige Valleys, Italy. Future directions will focus on building a better trapping system through the utilization of important chemical

cues released from *O. oeni* fermentation of wine-vinegar mixtures. The goal is to build a simpler system of attraction in the form of a dispenser with utilization not only of long-range volatiles identified in our study, but also VOCs originating from the leaf canopy of host plants.

Chapter 4

Identification of repellent compounds to minimize bycatch in the traps for *Drosophila suzukii;* a case study on *Drosophila simulans*

4.1 Introduction

In nature, plants emit a huge quantity of VOCs into the surrounding air; these compounds are related to plant ecology, physiology and environmental chemistry (Guenther et al., 1995). Many of these compounds act as semiochemicals involved in plant-plant, plant-animal, and plant-microorganism interactions (Penuelas & Llusià, 2001) and these interactions have been well studied (Szendrei & Rodriguez-Saona, 2010). Some of the VOCs produced by plant or microorganisms may act as defensive/averse compounds against insect pest and plant pathogens (Penuelas & Llusià, 2001). There are a few examples of effective control with the use of repellent chemical cues in IPM. This is partly because of a poor understanding of the biological significance of repellents on insect behaviour. By definition, a repellent is any chemical that elicits an avoiding reaction in an organism (Dethier, Browne, & Smith, 1960).

Drosophila suzukii (Matsumura) (Diptera: Drosoppilidae) or spotted wing drosophila (SWD) is a highly polyphagous pest of fresh fruit. It is the only Drosophila with a strongly serrated ovipositor, which enables it to oviposit in unwounded fresh fruits, thereby making them unmarketable (Cini et al., 2012). Almost a decade ago, *D. suzukii* invaded Europe, and it has heavily affected the fruit industry (Rota-Stabelli et al., 2013). It is likely that in the future this invasive species will also spread to New Zealand, Oceania and other unaffected areas, since it has already invaded the USA and Europe. Current control strategies rely on the heavy use of insecticides, which have a negative ecological impact, and in the long run, are neither effective nor sustainable. The tools that chemical ecology provides fit perfectly into integrated pest management programmes for *D. suzukii*, and could offer more sustainable approaches to limit its spread and damage. Economic damage is estimated at more than $2 billion in North America, over $4 billion in Europe and $500 million in Asia. The economic costs do not only include yield losses but also increased labour and chemical input costs for monitoring and management, including the losses which can occur when fruit from *D. suzukii*-infested regions is banned from trading on foreign markets. There have been several attempts to control *D. suzukii* since it has become a pest in Europe and the United States of America, first through the use of insecticides, while recently many other ecologically friendly techniques have been tested in order to counteract invasion and damage by this insect. Some of the most promising methods are good record-keeping and monitoring of insect behaviour, preventive measures, the possible introduction of parasitoids, the use of protective netting, investigation of pheromones, improvements in the attract and kill method, and research into the role of volatile compounds on SWD behaviour in order to select attractant compounds that could be used as bait for mass trapping control strategies. Host plant VOCs are important in oviposition site-selecting behaviour by insect herbivores (Szendrei & Rodriguez-Saona, 2010). No efficient monitoring and control tools of *D. suzukii* have been developed yet. However, in previous laboratory trials at the Plant and Food Research Centre in New Zealand conducted on different *Drosophila* spp., they found that the biological activity of food baits towards *Drosophila spp.* dramatically increased in the third week of trapping with different USDA lures. We assume that this higher attractiveness from the third week onwards is due to the evaporation of repellent VOCs from the lure mixture, which is known to attract SWD in USA (Feng et al., 2018).

Chapter 3 deals with the development of a lure that has relatively good selectivity in relation to *Drosophila* species and caught a large number of SWD and some other *Drosophila* spp. in field trials. A more selective and efficient trap is required. Finding out about repellent compounds and understanding their possible synergistic relationship and interaction with different *Drosophila* species could increase trap selectivity. Compounds that are deterrent to other *Drosophila* species but neutral to target species may increase the selectivity of commercial *D. suzukii* lures by excluding other *Drosophila* by-catches.

The use of repellents in a trap system for Drosophila would increase trap selectivity or could be implemented in a push and pull system in the open field. Therefore, one main objective of this study was

to find out about repellent compounds by subtracting a different number of VOCs selected from a review of the literature and commercial lures for Drosophilae.

In the first part of the research covered in this chapter, we tested different commercial lures and their efficacy on *Drosophila simulans*, a sister species of SWD. The main objective of this chapter was to investigate two Drosophila species: *Drosophila suzukii* and *Drosophila simulans*, and their behaviour towards select volatiles. Specific understanding of the differences in volatile perception for these two species could be useful and implemented in a trapping system. Our hypothesis is that volatiles perceived by SWD may not be perceivedby its sister species and different mode of perception could be involved . Finding a difference in perception and the effects of these volatiles on fly behaviour could be used to implement the different types of technique in open field studies. If we could find a compound attractive/neutral to *D. suzukii* but repellent to *D. simulans* ultimately, we would have a design for a highly selective trap/trapping system. On the basis of these considerations, the aim of this study was first to investigate the peripheral sensitivity of male and female adults of *D. simulans* toward volatile compounds that have been shown to exert an attractive or repellent action in other Drosophila, by means of an electrophysiological and behavioural study. Secondly, the aim was to design a mixture of active compounds detected using an electrophysiological approach and test it in lab bioassays to characterise the biological significance and thus to find repellent compounds.

In preliminary field trials conducted in a New Zealand orchard where the SWD has not yet arrived, the most abundant species in lures for different Drosophila was *D. simulans*. Therefore, we chose this *Drosophila* species as the main subject of our research. Chemical compounds in commercial lures were tested with gas-chromatography following two different timelines. The first tests took place in the first week of lure exposure in semi-field conditions to identify repellent VOCs. The second tests followed in the third week, aiming to find attractive volatile compounds in the lure. Extraction of volatiles from existing lures from PFR for *Drosophila* took place with direct headspace VOC collection analysis. We tested the chemical compounds in different lures with gas-chromatography mass spectrometry. The neurophysiology of *Drosophila simulans* on different VOCs was assessed by means of gas chromatography coupled with electro-antennographic detection (GC-EAD) and GC-sensillogram recordings. This experiment led to partial identification of the compounds that are physiologically active in *Drosophila simulans*. In the second part of the work, we created a blend of VOCs, selected according to their biological activity in our electrophysiological studies and a review of the literature. Different VOC blends were tested with pitfall behavioural experiments (in laboratory conditions - multi-choice) but time restrictions did not allow testing in semi-field conditions as had been planned. The compounds showing repellence activity were benzaldehyde, eugenol, ethanol, ethyl isovalerate, phenylethyl acetate, isoamyl lactate, 1-octen-3-ol, ethyl caprolete, limonene, p-cymene, and valeric acid. Use of compounds with a repellent effect for untargeted insect capture would allow the design of more selective innovative trapping concepts for plant protection technologies. The final goal of this chapter is to improve the attractiveness of baits against *D. suzukii* and to develop new trapping systems for the early detection and management of this pest. The conditions and substrates to maximise trapping efficiency will be selected based on the potential future use of baits and VOCs under field conditions, based on the knowledge obtained from experiments conducted in Chapters 3 and 4.

4.2 Material and methods

4.2.1 Insects

The insect colony in the laboratory was established from field-collected *D. simulans* flies in Christchurch. The insects were reared in laboratory conditions in natural light in New Zealand

summertime conditions, with a 16:8 photoperiod, and temperature of 23˚C. Diet: potato flakes 128g, casein (protein) 28 g, powdered sugar 20g, brewer's yeast 8g, methyl paraben 0,6g, ascorbic acid 1,3 g, benzoic acid 0,6 g.

Chemicals. Most synthetic standards were purchased from Sigma/Aldrich (St. Louis, Missouri, USA). The purity of compounds ranged from 98% to 99.5%. Some compounds were purchased from Fluka™ Fisher Scientific (Waltham, Massachusetts, USA) with 95% chemical purity.

Compound selection and mixture preparation

We selected stand out compounds from a list known to be biologically active for D. *melanogaster* and *D. suzukii* (Table 4.1) and compounds identified in GC-MS from commercial lures for Drosophila. We made up the mixtures and ran the compounds first using the CG-MS system. The selected list of tested compounds included more than 44 chemicals. Because some of the compounds had very similar RT times, there would be considerable co-elution of peaks and time would be needed at the end to distinguish which chemical was effectively causing *Drosophila simulans* to respond in EAD-GC-FID. We, therefore, needed to divide the number of compounds into six mixtures according to their Kovats index and polarity, for testing on the non-polar and polar column separately. Compounds with Kovats greater than 900 were mixed together, so we made up 3 mixes each with ~15 compounds, A, B and C, each compound being at a 0.02mg/mL concentration in the mixture. The A, B and C mixtures were tested on a non-polar HB5-MS column and should elute on the chosen column with Kovats greater than 900 (hexane is 600 Kovats)(Table 1.). For the other compounds, for which the Kovats retention index is less than 900, we made up mixes D, E and F at a 0.02mg/mL concentration per compound in the mixture and ran them on a DB-wax polar column (Table A. 4.1).

Suffix	Compound	Synonym	CAS	Kovats NP	Kovats P	Mix
2	Heptanol		543-49-7	901	1320	a_non polar
	Methyl hexanoate		106-70-7	925	1184	a_non polar
	Hexanoic acid	Caproic acid	143-62-1	990	1846	a_non polar
α	Phellandrene		99-83-2	1005	1167	a_non polar
	Ethyl butyrate	Ethyl butanoate	105-54-4	1013	1445	a_non polar
	Ethyl sorbate		2396-84-1	1097	1501	a_non polar
2	Phenethyl alcohol	(beta)	60-12-8	1116	1906	a_non polar
	Benzyl acetate	Acetic acid, phenylmethyl ester	140-11-4	1164	1720	a_non polar
	Diethyl succinate		123-25-1	1182	1680	a_non polar
	Benzeneacetic acid, ethyl ester	Ethyl phenylacetate	101-97-3	1246	1783	a_non polar
	Decanoic acid		334-48-5	1373	2276	a_non polar
	Myristic acid	Tetradecanoic acid	544-63-8	1768	2694	a_non polar
Z9	Tricosene		27519-02-4	2278	2320	a_non polar
	Pentanoic acid	Valeric acid	109-52-4	903	1733	b_nonpolar
	Benzaldehyde		100-52-7	962	1520	b_nonpolar
6	Methyl-5-hepten-2-ol	Sulcatol	1569-60-4	994	1465	b_nonpolar
Z3	Hexenyl acetate		3681-71-8	1005	1315	b_nonpolar
P	Cymene		99-87-6	1025	1272	b_nonpolar
1	Octanol		111-87-5	1071	1557	b_nonpolar
	Linalool		78-70-6	1099	1547	b_nonpolar
	Methyl octanoate		111-11-5	1126	1385	b_nonpolar
4	Ethyl phenol	Phenol, 4-ethyl	123-07-9	1169	2187	b_nonpolar
	Methyl salicylate		119-36-8	1192	1765	b_nonpolar
	Phenethyl acetate	Acetic acid, 2-phenylethyl ester	103-45-7	1258	1813	b_nonpolar
	Ethyl decanoate	Ethyl caprate	110-38-3	1396	1638	b_nonpolar
α	Humulene		6753-98-6	1454	1667	b_nonpolar
	Palmitoleic acid		373-49-9	1951	2926	b_nonpolar
1	Octen-3-ol		3391-86-4	980	1450	c_nonpolar
	Ethyl caproate	Ethyl hexanoate	123-66-0	1000	1233	c_nonpolar
	Hexyl acetate.		142-92-7	1011	1272	c_nonpolar
	Limonene		138-86-3	1030	1200	c_nonpolar
	Pentyl butyrate	Amyl butyrate	540-18-1	1077	1305	c_nonpolar
	Methyl benzoate		93-58-3	1094	1612	c_nonpolar
	Nonanal		124-19-6	1104	1391	c_nonpolar
E2	Nonenal		31502-14-4	1176	1713	c_nonpolar
	Ethyl octanoate	Ethyl caprylate	106-32-1	1196	1435	c_nonpolar
	Eugenol		97-53-0	1357	2169	c_nonpolar
E	Caryophyllene		87-44-5	1419	1595	c_nonpolar
β	Lonone		14901-07-6	1491	1971	c_nonpolar
	Palmitic acid		57-10-3	1968	2931	c_nonpolar

Table 4.1. Compounds tested on the GC-MS non-polar column, semi-standard nonpolar column.

We first made up the chemicals of all Kovats indexes as stock solutions with 1 mg/mL solutions in hexane, except for acetoin, which is soluble in water.For acetoin we needed a different preparation, since this compound is not soluble in hexane or ethanol. For acetoin, a 1 ml volume of the desired mixture with a concentration of 1 mg/ml was diluted in demi water, and the final concentration was adjusted to 0.02 mg/ml of volume 1 ml (Feng et al., 2018).

4.2.2 GC-MS and EAD-CG-FID

Before the EAD approach, we ran all the compound mixtures through GC-MS with an H-P 5890/DB-5 mass selective detector. The GC-MS was equipped with either a DB-5 column identical to the one used in the CG-EAD system described below or with an HP-5 column. The temperature program was the same as that described below for CG-EAD analysis. Mass spectra were recorded from 30 to 550 a.m.u with electronic impact ionisation at 70 eV. Identification of compounds was confirmed by comparison of retention indexes and mass spectra with those of authentic standards.

To identify bioactive volatile compounds from the mixtures with *D. simulans* antenna and palps, we used an Agilent 7890A gas-chromatograph (GC) equipped with a 30 m by 0.32 mm i.d using a 0.25μm HP-5 5890 capillary column (Agilent Technologies, CA, USA) equipped with a flame ionisation detector (FID), coupled to an electro-antennogram detector (EAD, IDAC 4, Syntech Research and Equipment, Hilversum, Netherlands). A 1 μl aliquot of the poral mixture extract was injected into the GC port and passed through the column at 1 mL/min with helium carrier gas which was split between the insect antennae and the FID. The injector was set at 250oC and was in splitless mode at 0.5 minutes. The detector was set at 300°C, and the GC oven temperature programmed from 60°C, held for 1 minute, then increased to 250°C at 20°C/min and held for 10 minutes. The fly was anaesthetised using carbon dioxide gas, then the head of the female/male of *D. simulans* was excised and positioned between two sharpened glass electrodes, one containing electrode gel (Spectra 360, Parker Laboratories Inc, Fairfield, New Jersey, USA) used as the reference electrode and one containing Ringers solution (ref) as the recording electrode. Each glass electrode held a length of 1-mm silver wire that electrically connected the preparation to the recording unit's preamplifier. The EAD exit port temperature was maintained at 250°C, and the antennal preparation was placed in a charcoal-filtered and humidified air system (400 ml/min). The recording software GC-EAD (2014 Version 1.2.5, Syntech Research and Equipment, Hilversum, Netherlands) was used to record the antennal response and FID response. Kovats retention indexes (KI) (Kováts and Weisz 1965) were calculated for the antennally active compounds and compared to the KI calculated as well from the same samples run on the gas-chromatograph coupled to the mass-spectrometer (GC-MS).

We ran the mixture of compounds over female and male *D. simulans* antennae and palps. The EAD was connected with autospike software to record antennal response. The GC-FID for testing mixtures A, B and C had an HP-5 non-polar column. The system had a micromanipulator, which helps with precise movement of the recording electrode on the antenna or insect palps.

Mixtures D, E and F were tested in the same system but with a DB-wax 30 mm column, 0.0250 mm diameter, and film thickness of 0.5 μm. The program was set with an initial oven temperature set at 40°C for 3 min, then the temperature was ramped by 7 °C per minute up to 150 C°, ° hold time, then ramped by 20° C to 200° C, with a hold time of 1 minute.

4.2.3 Laboratory bioassay

All trapping tests were conducted in Bug-Dorm cages (40 x 40 x 40 cm) in the laboratory at room temperature (23°C± 3°C) and in daylight. About 200 mixed-sex, mixed-age flies (age difference no more than 2 days) were released in a cage containing randomly placed traps with different treatments. The traps were constructed with 18 ml clear plastic cups (Fill-Rite Inc., Newark, New Jersey, USA) covered by a white paper lid with a hole (2.5 mm diam.) made in the centre. For experiments with synthetic compounds, a medical-dental cotton wick (2 cm long) was used as a dispenser. The blends were mixed in hexane and 1 ml of water (water was critically important for vinegar fly attraction; no flies were attracted to traps loaded with chemicals alone) and were placed at the bottom of the cup. Compounds that were not water-soluble were loaded onto a cotton wick and placed in a treatment cup with a second wick dosed with 1 ml of water. Controls contained only water.

<u>Choosing a positive control</u>

When searching for a positive control in our multi-choice experiment, we tested 3 attractive lures, with the addition of negative control water and positive control hexane, all in small traps in the mesh cage. The number of replicas was 10. We chose three lures to be tested. The first mixture was a lure prepared according to (Feng et al., 2018), and we call that lure Zhang (Table 4.2). The second lure was as in Baker patent 1, with a standard blend of ethanol, acetic acid, and 2-phenylethanol, plus other compounds added

as in (Baker et al., 2003). The third lure contained modified Baker patent 2 (Zhu, Park, & Baker, 2003). Our standard blends Baker 1 and 2 (Table 4.2), and they comprised 100 mg of ethanol, acetic acid, and 2-phenylethanol (in a ratio of 1:22:5) in 1 ml of water (water was critically important for fly attraction; no flies were attracted to traps loaded with chemicals alone), was placed at the bottom of the cup.

Source	Control	Chemical	Concentration	Ratio		Type of solution
Zhang	1	Acetic acid	neat	15		In drowning solution with dishwash liquid
Zhang	1	Ethyl acetate	neat	5		In drowning solution with dishwash liquid
Zhang	1	2-phenylethanol	neat	1		In drowning solution with dishwash liquid
Zhang	1	ethyl octanoate	neat	1		separate lure with acetoin
Zhang	1	Acetoin	neat	1		separate lure with ethyl octanoate
Baker	2	ethanol		0.1 - 30	3 mg	ethanol solution
Baker	2	indole		0.1 - 100	330 µg	ethanol solution
Baker	2	Sucrose		10 - 1000	340 mg	aqueous solution
Baker	2	Acetic acid		10 - 300	67 mg	aqueous solution
Baker	2	2-phenylethanol		0.1 - 100	10 mg	aqueous solution
Baker	2	water		10 - 1000	1 mL	aqueous solution
Baker	3	methanol		0.1 - 30	3 mg	ethanol solution
Baker	3	trimethylamine		0.1 - 100	330 µg	ethanol solution
Baker	3	Sucrose		10 - 1000	340 mg	aqueous solution
Baker	3	propionoic acid		10 - 300	67 mg	aqueous solution
Baker	3	benzyl alcohol		0.1 - 100	10 mg	aqueous solution
Baker	3	water		10 - 1000	1 mL	aqueous solution
Baker	3	b-caryophyllene		0.1 - 10	0.5 mg	aqueous solution

Table 4.2. Lures and composition with exact concentration and ratio for choice of a positive control

We had intended to have another positive control from (Cha et al., 2012), but lack of ethyl-lactate, grape-butyrate and methionol made this impossible (Cha et al., 2014a). Zhang attractant was 10 times more diluted than it is in (Feng et al., 2018). The lure position was different in each replica. The flies released in the experiment were 2-10 days old, numbering around 200 of mixed-sex. After 24 hours at room temperature and with light provided for the whole night, we selected the best positive control for *Drosophila simulans*. The cage experiment for positive control had 5 replicas repeated twice, one day after the next. The male and female flies could be differentiated by the presence of sex combs in males on the prothorax legs. In this experiment, we did not count the sex ratio of capture.

Testing a single compound against the best lure from the positive control

In order to test the attractiveness or repellence of individual compounds that showed electrophysiological activity in EAD or EPD, we conducted pitfall cage experiments. The concentration of single compounds was 100 mg/ml. We first had a compound with a concentration of 1 mg/ml (as in the GC-MS experiment). From this, we then made a 100 mg/ml solution of each compound. In the multi-choice experiment, we added a volume of 10 µl with a cotton wick of the single compound at a concentration of 100mg/ml stock solution. We used Zhang lure as a positive control in the individual compound experiment: ml 15:5:1 compound ratio.

Testing a mixture of compounds with the exclusion of one compound per mixture

In the next experiment, we tested a mixture of compounds, subtracting one compound per mixture. This mixture was made from single compounds at a concentration of 100mg/ml. The first mix had 18 compounds, all eliciting a response in D. *simulans* to mixtures A, B and C. The concentration of this solution

was 1/200 = 0.5 %. For the second and subsequent blends, the number of the compounds was 17, dissolved in hexane. One of the compounds in each blend was excluded, as shown in Table 4.

Tests	EAD	cas number	B 1	B2	B 3	B 4	B 5	B 6	B 7	B 8	B 9	B 10	B 11	B 12	B 13	B 14	B 15	B 16	B 17	B 18	B 19
1	Ethyl butyrate	105-54-4	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
2	**2-heptanol**	543-49-7	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o		o
3	**methyl hexanoate**	106-70-7	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o		o	o
4	Ethyl sorbate	2396-84-1	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o		o	o	o
5	**Phenylethyl alcohol**	60-12-8	o	o	o	o	o	o	o	o	o	o	o	o	o	o		o	o	o	o
6	Benzyl acetate	140-11-4	o	o	o	o	o	o	o	o	o	o	o	o	o		o	o	o	o	o
7	Ethyl phenyl acetate	101-97-3	o	o	o	o	o	o	o	o	o	o	o	o		o	o	o	o	o	o
8	**Sulkatol**	1569-60-4	o	o	o	o	o	o	o	o	o	o	o		o	o	o	o	o	o	o
9	1-octanol	111-87-5	o	o	o	o	o	o	o	o	o	o		o	o	o	o	o	o	o	o
10	Methyl octanoate	111-11-5	o	o	o	o	o	o	o	o	o		o	o	o	o	o	o	o	o	o
11	4-ethyl phenol	123-07-9	o	o	o	o	o	o	o	o		o	o	o	o	o	o	o	o	o	o
12	Methyl salicylate	119-36-8	o	o	o	o	o	o	o		o	o	o	o	o	o	o	o	o	o	o
13	**Phenethyl acetate**	103-45-7	o	o	o	o	o	o		o	o	o	o	o	o	o	o	o	o	o	o
14	**1-octen-3 ol**	3391-86-4	o	o	o	o	o		o	o	o	o	o	o	o	o	o	o	o	o	o
15	**Ethyl caproate**	123-66-0	o	o	o	o		o	o	o	o	o	o	o	o	o	o	o	o	o	o
16	Hexyl acetate	142-92-7	o	o	o		o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
17	**Eugenol**	97-53-0	o	o		o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
18	**b-caryophyllen**	87-44-5	o		o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

Table 4.3 Compounds eliciting a strong response in the EAD (electrophysiological active compounds) experiment and chosen to be tested as single compounds in bioassays. An empty place indicates which compound was excluded from a mixture. Compound concentrations were 100mg/ml from a stock solution. The mixture blend was selected for testing in the multi-choice experiment by excluding one compound in each blend.

4.2.3.1 Methods of analysis for the multi-choice experiment.

The analysis methods were the same for both trials, and data from all days were used within single analyses. For both trials, the data was 'multinomial response', in that each insect had a choice of which trap to land on. We looked at the number caught in each type of trap as a percentage of the total catch in all the traps in a cage. The catch per trap in relation to the total catch was analysed with a Poisson log-linear model approach for multinomial response data (P. McCullagh & Nelder, 1989). Since the data was over-dispersed, differences between the treatments were assessed using F-tests: these included some differences in the treatment groups. For all analyses, the percentages are presented along with 95% confidence limits. All the analysed variables were estimated from a binomial GLM on the logit scale, and back-transformed for presentation. For the main analyses (male, female and total per trap), the binomial analysis was carried out for each treatment separately, using over-dispersion as estimated as part of the multinomial analysis, and the total catch in a cage as the binomial totals. The analysis was all carried out with Genstat (R. Payne et al., 2017), and the graphics were produced with SigmaPlot or Genstat.

4.3 Results

4.3.1 Results of EAD-CG-FID

We found 18 compounds electrophysiologically active for *D. simulans* (Table 4) from the mixture with a Kovats index greater than 900 and tested on a semi-standard non-polar column. The following compounds had a strong response on the antennae of males and females with high depolarisation (voltage deflection (μV)) on the baseline of EAD signal: methyl hexanoate; 2,4 hexadienoic acid, ethyl ester; phenylethyl alcohol; benzyl acetate; sulkatol; octanoic acid, methyl octanoate; methyl salicylate; phenethyl acetate; 1-octen-3 ol; ethyl hexanoate (Tables 4.4, 4.5 and 4.6).

	EAD	EAD	EAD	EAD	EAD	EAD	EPD	EPD	EPD	EPD	EPD	EPD	EPD
Mix A	Male	Male	Female	Female	Female	Female	Male	Male	Male	Female	Female	Female	Female
Compound chemical name	190116-6	190116-7	190104-4	181226	190116-5	190106-4	190114-5	190114-8	190115-7	190115 ds	190107-3	190115-1	190105-5
Ethyl butyrate			ooo	ooo	ooooo							ooooo	ooo
2-heptanol			ooooo	oooo	ooooo	oooo				oo	ooo	ooo	
Methyl hexanoate (Coproate)	ooooo	ooo	oooo	oooo	oooo	oo	oo	ooo	ooo				ooo
Hexanoic acid (Caproic acid)											ooo		
a- phellandrene	ooo	oo	ooo						oo	o	o	oo	oo
m-Cymene					ooo	ooooo							
2,4 hexadienoic acid, ethyl ester	ooo	ooo	oooo	oo	oooo	oo				ooooo	oo	oo	
Phenylethyl alcohol	ooooo	ooooo	oooo	oooo	ooo	ooo						ooo	ooooo
Benzyl acetate	ooooo	ooo	ooo	ooo	ooooo	ooo							
Diethyl succinate			ooo			oo						oo	
Ethyl phenyl acetate	ooo		ooooo	oooo	oooo	ooo			oooo	oo	ooo	o	
Decanoic acid	oo				oo								
Myristic acid													
Z-9-Tricosene													

Table 4.4. Mixture A and the response recorded in males and females on antenna with EAD and palps with EPD; the compounds in bold were selected for cage experiments

The compounds that elicited a high response only in female fly antenna were: ethyl butyrate; 2-heptanol; m-Cymene; diethyl succinate; ethyl phenyl acetate; 1-octanol; b-caryophyllen. The response of certain compounds perceived on insect palps was also interesting. The following elicited a strong response on the palps: methyl hexanoate (coproate); ethyl butyrate; 2,4 hexadienoic acid, ethyl ester; ethyl phenyl acetate; 4-ethyl phenol; phenethyl acetate; 1-octen-3 ol; hexyl acetate; ethyl hexanoate; eugenol; b-caryophyllen.

Surprisingly, in general females had more sensitivity on the palps than males. Methyl salicylate was not perceived by palps in either males or females (Table 4.5). On the other hand, compounds such as methyl hexanoate (coproate); octanoic acid, methyl octanoate, benzoaldehyde; 1-octanol; palmitoleic acid, were only perceived in males, or their response was higher in male palps than in female palps. In appendix A 4.1, results for electrophysiologically active compounds for *D. simulans* are presented for mixture D,F,E.

	EAD	EAD	EAD	EAD	EAD	EPD	EPD	EPD	EPD	EPD	EPD	EPD	EPD
	Male	Female	Female	Female	Female	Male	Male	Male	Female	Female	Female	Female	Female
Mix B	190116-7	190104-3	181226-2	181226-5	190106-3	190107-2	190114-7	190115-6	190114 ds	190114-2	190115-2	190116-10	190116-11
Compound chemical name													
Pentanoic acid (valeric acid)	oo			ooooo		oo				oo			
Benzoaldehyde							ooooo						
Sulkatol	ooooo	oooooo	ooo	ooooo	ooooo					ooo		ooo	
Cis-3-hexenyl acetate		oo				oooo				ooo			
p-cymene													
1-octanol	oo	oooo	oo	ooo	ooo	ooo	oooo	oo					
Linalol						oo							
Octanoic acid, methyl octanoate	ooooo	oooo	oo	ooo		o	oooo	oooo					
4-ethyl phenol	ooooo					ooo	oooo	oooo	oooo	ooooo	ooo	ooooo	ooooo
Methyl salicylate	oooo	ooooo	oooo	oooo	ooooo								
Phenethyl acetate		ooooo	oooo	oooo	ooo	ooooo	oooo	ooooo	ooooo	ooooo	ooooo	ooooo	ooooo
Ethyl decanoate(wthyl caprote)											ooo		
Humaline	oo	o											
Palmitoleic acid							ooo	oo					

Table 4.5. Mixture B and the response recorded in males and females on antenna with EAD and palps with EPD; the compounds in bold were selected for cage experiments.

	EAD	EAD	EAD	EAD	EAD	EAD	EAD	EAD	EAD	EPD	EPD	EPD	EPD	EPD
Mix C	Male	Male	Male	Male	Male	Female	Female	Female	Female	Male	Male	Male	Female	femele
Compound chemical name	190106-1	190116-3	190116-1	190115-9	190116-2	181226-4	190104-1	190104-2	190116-4	190106-2	190114-6	190107-1	190115-3	190114-1
1-octen-3 ol	ooooo	ooooo	ooooo	oooo	ooooo	ooooo	ooooo	ooooo	ooooo		ooo	ooo		
Ethyl hexanoate	ooooo	oooo	ooooo	oooo	oooo	ooooo	oooo	ooooo	oooo			oo		
Hexyl acetate	ooo	ooo	ooo	ooo		ooooo	ooo	ooooo	ooo	oo	oo	oo	ooo	
Limonene				oo	ooo									
Pentyl butanoate	ooo					ooo	ooo	ooo						
Pentyl butanoate (one of isim	ooo	ooo	ooo	oo		ooo	ooo	ooo	ooo	ooooo				
Methyl benzoate		ooo	ooo		ooo				ooo					
Nonanol											oo			
E-2-NONANOL	ooo	ooo				oo		ooooo						
Ethyl octanoate														
Eugenol	oo					oooo	ooo	oooo	ooooo	ooooo	oooooo	ooooo		oooooo
A-copaene		ooo												
b-caryophyllen	ooo			ooooo	ooo	oooo	oooo	oooo	ooooo				ooo	oo
Humulene (a-caryophyllene)														
b-Ionone														
Palmitic acid							ooo			ooooo		oo		

Table 4.6. Mixture C and the response recorded in males and females on the antenna with EAD and palps with EPD; the compounds in bold were selected for cage experiments.

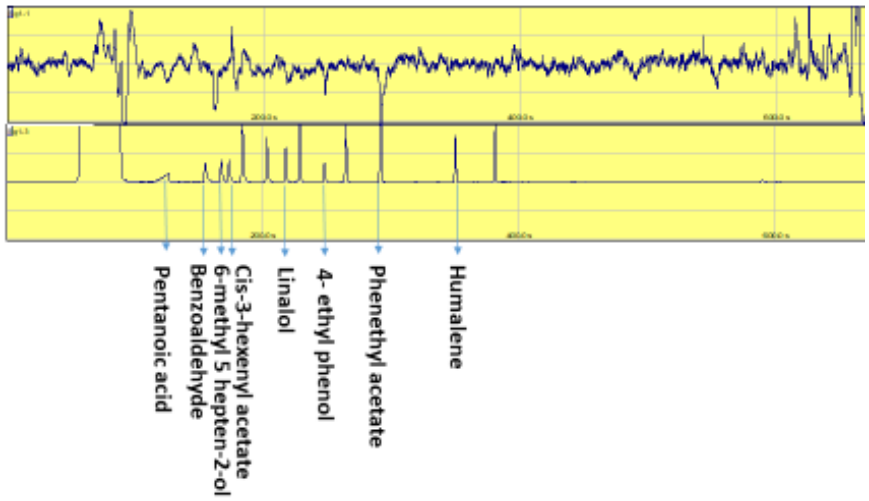


Figure 4.7. Electropalpogram recording of *D. simulans* with mixture B

4.3.2 Results of laboratory bioassays

Choosing a positive control

In the positive control test, the highest catch was with Zhang lure. The control traps caught a lower number of flies, while a lower catch was also recorded in Baker lure 1 and Baker lure 2.

Testing of the individual compounds against the best lure from the positive control

The lowest catch in the single compound lures of the multi-choice test suggests the following as possible repellent compounds: E caryophyllene; eugenol; benzaldehyde; 1-octanol-3-ol; ethyl sorbate; sulcatol; benzyl acetate.

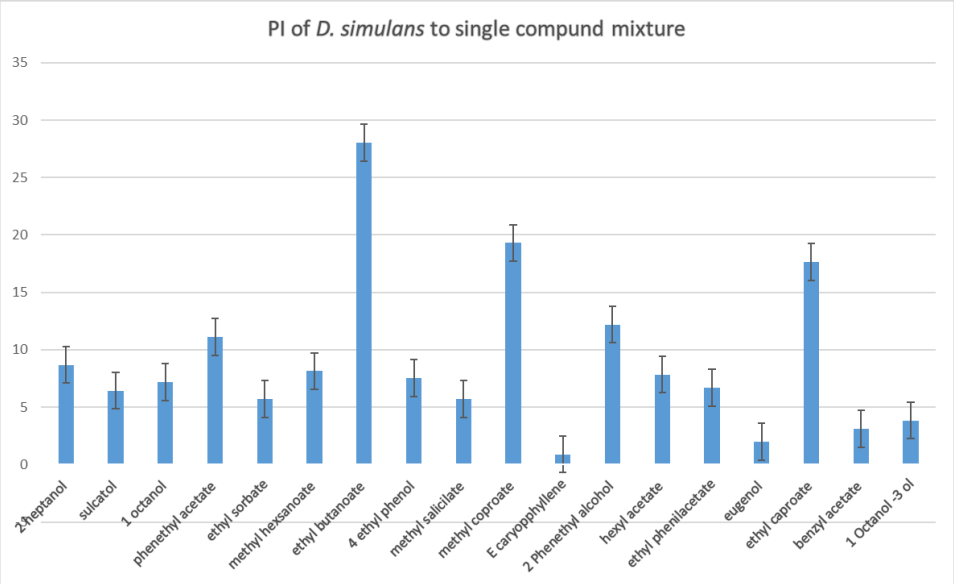


Figure 4.8. The Preference index of both males and females caught in the traps with a single compound dissolved in hexane.

Testing a mixture of compounds with the exclusion of one compound per mixture

The lowest catch was recorded with blends 4, 8, 9, 10, 11, 14 and 16. The highest catch was recorded in blends 2, 5, 6, 7, 12, 15 and 17.

From this experiment, the total catch in traps was significant for some blends, with a p-value >0,001. The traps with significantly high catch indicated missing compound repellence in relation to the folloiwng: b-caryophyllene, eugenol; ethyl caproate; 1-octen-3 ol; phenylethyl alcohol; methyl hexanoate (coproate). In addition, sulkatol, phenethyl acetate, and ethyl phenyl acetate acted as a mild repellent, with a difference in total catch of p >0,05.

4.4 Discussion

To date, the *Dropshila suzukii* fruit fly has represented a problem for the production of soft and berry fruit, as current trapping systems have to deal with a spill-over effect and a high number of non-target species. Therefore, the main objective of this study was improvement of an existing trapping system for SWD by finding repellent compounds for non-target sister species and other insects. The aim was to evaluate the olfactory sensitivity of *D. simulans* females and males to different volatile organic compounds and to further this scope a range of laboratory experiments was set up to select repellent compounds. The second aim of the study was to investigate the behaviour of chemical compounds, alone or combined. The evidence strongly suggests that the following compounds have a repellent and deterrent effect on *D. simulans*: benzaldehyde; eugenol; ethanol; ethyl isovalerate; phenylethyl acetate; isoamyl lactate; 1-octen-

3-ol; ethyl caprolete; linalool; limonene; p-cymene; valeric acid; b-caryophyllene; phenylethyl alcohol; methyl hexanoate (coproate); sulkatol, phenethyl acetate and ethyl phenyl acetate. We showed that repellent compounds mixed with other compounds are not influenced by the synergetic activity of other compounds. Additionally, in the mixture with strong attractant compounds, the repellent compound did not lose its repellent characteristics. This study analyses a numerous number of volatile organic compounds used in commercial chemical lures and compounds described in previous research as electrophysiologically active for Drosophila. Our study tackles the effects of VOCs in the context of the synergetic effectiveness of the mixture in laboratory and semi-field conditions. The research further narrows down the electrophysiologically active compounds we found when testing the antennae and palps of *D. simulans* with an EAD/EPD-GC-FID approach. The study builds on and contributes to work in the search for repellent compounds to develop a better push-pull trapping system for invasive insect species. The findings from this study could be directly utilised in practical applications to improve mass trapping of *drosophila suzukii*.

Chemoreception plays a key role in regulating behaviour such as host fruit localisation, discrimination of host plants for mating, and oviposition. For SWD and other *Drosophila,* some of the well-known repellent compounds found are geosmin, 1-octen-3-ol, bezaldehyde, octenol, phenol (Becher, Bengtsson, Hansson, & Witzgall, 2010; Knaden, Strutz, Ahsan, Sachse, & Hansson, 2012; Mansourian et al., 2016; Stensmyr et al., 2012; Anna K Wallingford, Cha, Linn Jr, Wolfin, & Loeb, 2017; A. K. Wallingford et al., 2016). Compounds such as limonene, linalool, and plant monoterpenes did not elicit a response in EAD on *D. simulans*, although they are known to be *Drosophila* repellents or to repel other insects (Corda et al., 2020; Maia & Moore, 2011). Repellent compounds are defined as any chemical that elicits an avoiding reaction in insects. They can be categorised into five different types: a true repellent; attraction inhibitors; contact irritants; deterrent compounds and visual masking (Dethier et al., 1960). In our study we found repellents acting as a true repellent, where the flies moved away from the source of the stimulus without coming into contact with our lures. In our experimental design repellent, the compounds caused aversion behaviour independently of the presence of attractive odours inside the created lure mixture. These compounds (in our findings) are also attraction inhibitors because they reduced the attractiveness of our mixtures, when the repellent compound was present. In our research on *D. simulans,* ethyl octanoate did not elicit any electrophysiological response, which makes this compound neutral. Ethyl octanoate is a short-range compound that synergetically increases the attractiveness of acetoin in the lure applied in open field studies on *D. suzukii* (Feng et al., 2018). As an insect attractant and component of pheromones (El-Sayed, 2020), ethyl acetate showed different behaviour in our experimental setup, while in (Feng et al., 2018) it synergistically increased the attractiveness of the lure for SDW. In support of our findings on the behavioural activity of ethyl acetate (Cha et al., 2012) found that this compound acts as a repellent and reduces the attraction of blends of ethanol and acetic acid. Therefore, we can speculate that some compounds in combination with others could act as repellents in certain combinations.

While (Feng et al., 2018) designed lure for SWD and reported higher selectivity of the lure of up to 72% in terms of total flies caught in the trap, in our experiment this particular lure caught an extremely high quantity of *D. simulans*. The high reported success in terms of the percentage of *Drosophila suzukii* specified in the total catch (Feng et al., 2018) may be due to the absence of other drosophilae in the tested field area. In all our trials, this lure was a positive control and was excluded from formal analysis, since it caught ten times more flies than other individual compounds or mixtures. This should be added to the fact that a smaller amount of compounds could be a better attractant (Cha et al., 2015).

In our electrophysiological study, the findings were quite interesting. α- phellandrene was electrophysiologically active, but we did not choose this compound for further laboratory experiments because its electrophysiological activity was low and limited to males. M-cymene only had an

electrophysiological effect on female antennae. Diethyl succinate was also not included for further studies because of a weak response on female antennae alone. Sesquiterpene β-caryophyllene had a stronger response on female antennae but modest voltage deflection on female palps. Benzaldehyde and palmitoleic acid were only perceived by male palps in *D. simulans*. Therefore we did not select them for further studies in bioassays. Benzaldehyde is a known repellent compound for *Drosophila (I. W. Keesey et al., 2015)*.

Methyl benzoate, a compound naturally occurring in many plants as a volatile organic compound, is toxic to various insect pests, including SWD (Feng & Zhang, 2017). This volatile is reported to be 5 to 20 times more toxic than conventional insecticides. Plant mineral oil also has an adverse effect on SWD, as do its essential compounds: α-terpinene, γ- terpinene, terpineol, cineole, α-pinene, cis-jasmone, eugenol, vanillin, and menthol (Corda et al., 2020; Dam, Molitor, & Beyer, 2019) but they have not proven as effective in the field. When 20% 1-octen-3-ol was applied, an oviposition deterrent effect of 49.5% fewer offspring of SWD was reported (Anna K Wallingford et al., 2016). (Anna K Wallingford et al., 2016) have suggested that there is potential for the use of repellents to decrease pest pressure and reduce the number of foliar insecticide applications and intervals. Additionally, the same ovipositional deterrent was tested as a push element in combination with a mass trapping pull (attractant) element, and the combination of both was reported to be a better strategy than when applied individually (Anna K Wallingford, Cha, & Loeb, 2018).

Plant volatiles are usually utilised as tools for pest management and are applied as an attractant, while just 3% of field studies have investigated volatiles as repellents (Szendrei & Rodriguez-Saona, 2010). Thus our study contributes towards research on repellent compounds. The inclusion of repellent compounds for other insect species might help to reduce non-target insect capture (Cloonan et al., 2018). Our results show that the presence of a repellent compound in a multi-choice cage experiment actually reduces the flies attracted to that lure. Finding a compound that acts as a repellent for non-target species is a challenging task, since it could take numerous laboratory experiments prior to testing in the field. Furthermore, the behavioural activity of this possible repellent compound in the natural complex chemical environment may not be the same as in laboratory assays (Feng et al., 2018). In addition, our results show that the OSNs responsible for odour perception are also located on *D. simulans* palps. Moreover, some of the compounds were sensed only by OSNs present on the palps of this Drosophila. In particular, we found that compounds such as 4-ethyl phenol, methyl salicylate, phenethyl acetate, and eugenol evoked an EAP response higher than the response on antennae. This could be due to higher specialisation for detecting this compound in OSNs in palps.

Development of additional strategies for SWD population control and crop protection is of particular importance. One of these techniques could be the push-pull strategy (Cook, Khan, & Pickett, 2007). On the basis of our preliminary results, we could offer more environmentally friendly options for SWD control by taking advantage of olfactory knowledge about averse compounds.

The avoiding response observed in our laboratory studies with repellent compounds was not robust enough to be put to immediate practical use. Thus field trials must be conducted before using such a potential reverse odour as a tool for crop protection. One additional drawback in the use of repellent compounds in crops could be the maintenance of a relatively high concentration of a certain compound in the open field for a long period of time (Anna K Wallingford et al., 2016).

However, future experiments need to focus on narrowing down a mixture of tested compounds in laboratory studies to understand synergistically attractive compounds and their ratios, and the influence of repellent compounds in such an attractive mixture. Narrowing down compounds and understanding their synergistic activity could lead to a better understanding of the effect of repellent or neutral compounds in a mixture. This narrowing down would take place with the exclusion of two or three compounds from the mixtures tested in our second bioassay. Later on, when there are fewer compounds, the next step will be experimentation with a specific ratio of the compound. Once an attractive combination is found and compared with attractiveness to SWD, then it will be possible to reach conclusions about

specific lures. Following all the results from Chapter 4, repellent compounds could be used in push and pull trapping systems.

4.4.1 Concluding remarks

Behaviour-based strategies to manage invasive insect species are needed in order to avoid the use of harmful and unsustainable toxins and chemicals. Through extensive research has been done, there is still a huge gap in our understanding of the behaviour and mate/food-seeking of *Drosophila suzukii*. An additional knowledge gap also regards our understanding of specific habitats that emit attractive or aversive chemical cues. Using reverse cues against D. *suzukii* could enable the creation of a better push-pull system (A. K. Wallingford et al., 2016), or these cues could be directly applied in orchards (Feng & Zhang, 2017).

Understanding how repellent compounds could be efficiently applied would ultimately lead to sustainable management systems in the fight to reduce the negative effects of invasive insect species, and in this way, the disadvantages of repeated insecticide treatments, along with the negative effect on natural enemies, development of outbreaks of secondary pests, reduction of preharvest intervals. Furthermore, the high resistance to insecticides could be reduced, if not eradicated.

Chapter 5

Yeast-derived aromatic volatiles mediate insect behaviour across trophic levels: a case study on the *Drosophila* parasitoid *Trichopria drosophilae*[3]

[3] This chapter is based on the publication Gordana Đurović [a, b, c, d, #], Francine A.C. Van Neerbos [a, b, #], Sofie Bossaert [a, b], Beatrice Herrera-Malaver [e, f], Jan Steensels [e, f], Judit Arnó [g], Felix Wäckers [d, h], Islam S. Sobhy [a, b, i], Kevin J. Verstrepen [e, f], Hans Jacquemyn [b, j], Bart Lievens [a, b, *]:"The pupal parasitoid *Trichopria drosophilae* is attracted to the same yeast volatiles as its adult host". Submitted to Journal of Chemical Ecology.

5.1 Introduction

Microorganisms produce many secondary metabolites, including diverse volatile organic compounds (VOCs) that act as olfactory cues and mediate interactions among organisms involved in an ecosystem. Mutualism between microbes and their insect vectors is a direct evolutionary survival system (Christiaens et al., 2014). The importance of microorganisms, particularly fungi and yeasts, as a source of a rich protein diet is well known for many insects. Microbes, in turn, take advantage of insects as vectors for dispersal (Christiaens et al., 2014). The mechanisms that maintain these mutualisms can be diverse; the production of secondary metabolites that attract insect vectors is a common one. However, there is a lack of information on how microbial-derived olfactory cues could affect higher trophic levels (i.e. parasitic wasps or predators), over and beyond the attraction of insect vectors. Therefore understanding how volatile cues mediate behaviour on different trophic levels could be used in applied entomology to mitigate the harmful effects of invasive insect species.

The chemical environment which insects inhabit is quite complex, dynamic and depends on many factors. Host plants, surrounding plants, insect herbivores, and sap-sucking feeders; their friends and foes are connected with conspecific and heterospecific relations arranged on different trophic levels. The selective pressure of the intricate environment and complex interactions among organisms across trophic levels force the insect to adapt to diverse and dynamic changes. Infochemicals are the main mode of interaction between multiple trophic levels. Insects have evolved diverse sensory systems for orientation in their environment, such as vision, hearing, smell and vibration sensing, and a combination of all this sensory information is used for making foraging decisions (Aartsma, Bianchi, van der Werf, Poelman, & Dicke, 2017). Olfaction is the most important for food searching, mate finding, avoidance of enemies and competition. Chemical cues could trigger avoidance behaviour and activate the olfactory circuit in the insect, detecting toxic microbes and an unsuitable environment (Stensmyr et al., 2012). Complex odour bouquets within a trophic level can be positive for insects and their parasitoids, negative for both or just effective on one trophic level (Meiners, 2015). Understanding chemical ecology and the evolution of plant-insect-microbe interaction in the context of a multitrophic perspective is a challenging task. Chemical ecology studies, combined with new molecular approaches and genetic engineering, could better explain the mechanism behind some multitrophic interactions. Studying mutant organisms that have acquired changes or deletions in their genome has the advantage of determining gene functions in a very efficient way, particularly in a community context, to clarify the ecological functions of genes without affecting other genome features (Christiaens et al., 2014; Degenhardt, Gershenzon, Baldwin, & Kessler, 2003; Marcel Dicke & Baldwin, 2010). As yet, there is no knowledge of fully understood interaction mediated by infochemicals' influence on different trophic levels (Vet & Dicke, 1992). Volatiles produced by plants under herbivore attack are important chemical cues for use by natural enemies in a tritrophic context (Marcel Dicke & Baldwin, 2010). On the other hand, some of the volatile metabolic products from microorganisms (mVOCs) present in the insect habitat are important for higher trophic levels, such as parasitoid wasps. mVOCs mediate attraction to insects that could be used as vectors for their dispersal (Christiaens et al., 2014). Very little research has been conducted on microbial sources of odour, their ecological role, and how organisms initially evolved to utilise microbial volatile organic compounds (mVOCs) (Davis, Crippen, Hofstetter, & Tomberlin, 2013). mVOCs have distinctive scent profiles that mediate host finding and food location. For example, bacteria from the pea aphid *Acyrthosiphon pisum* honeydew, *Staphylococcus sciuri*, influence ovipositional preferences for the natural enemy, the hoverfly *Episyrphus balteatus* (De Geer) (Diptera: Syrphidae) (Leroy, Sabri, Heuskin, et al., 2011).

In some cases, mVOCs resemble volatiles of their insect vector host plants. *Letopilina heterotoma, a* parasitic wasp of *D. melanogaster*, uses VOCs produced by yeast as the main mediator in host habitat search (M Dicke et al., 1984). It is well known that yeast fermentation products stimulate larval survival and the development of adults in Drosophila flies (Becher et al., 2012). Factors causing attraction are also mainly

products of live yeast metabolism (Dicke, Van Lenteren, Boskamp, & Van Dongen-Van Leeuwen, 1984). Yeast volatiles cues and the ecological relevance of those cues to insects are a phylogenetically ancient trait in *Drosophila* (Becher et al., 2018).

Moreover, the relationship between insects and yeasts evolved before the emergence of flowering plants. The presence of shared volatiles emitted by yeast and flowers may suggest an evolutionary contribution of yeast-insect communication to the evolution of insect pollination in flowers (Becher et al., 2018). Furthermore, for its dispersal, yeast that inhabits floral nectar produces fermentation metabolic VOCs to attract floral visitors such as the insect parasitoid *Aphidius ervi* (Braconide) (Sobhy et al., 2018). Additionally, yeast enhances the quality of nectar by changing nectar chemistry, the production of distinct aromatic volatile blends, and tuning amino acid concentration and composition to attract floral visitors, and does not compromise their life history parameters (Sobhy et al., 2018). The importance of acetate esters - ethyl acetate and isoamyl acetate produced by the alcohol acetyl transferase (ATF1) gene in *Saccharomyces cerevisiae* yeast - for dispersal by drosophila flies has already been studied (Christiaens et al., 2014), in a system with the use of null mutant yeast where both alleles of ATF1 were deleted.

In this study, we hypothesize that *Drosophila* parasitoids like *Trichopria* are attracted to the same olfactory cues as their hosts, aiding the localisation of suitable *Drosophila* hosts. The ATF1 gene encodes production of the aromatic-fruity compounds ethyl acetate, isoamil acetate, and phenylethyl acetate from the central carbon and amino acid metabolism in common brewer's yeast *Saccharomyces cerevisiae* (Christiaens et al., 2014). These olfactory cues help to attract *Drosophila melanogaster* flies that serve as vectors and promote dispersal of *S. cerevisiae*. Deletion of ATF1 orthologs in *S. cerevisiae* drastically reduces the attraction of *D. melanogaster* and therefore limits yeast dispersal. There is a gap in knowledge about the ecological importance of acetate esters on higher trophic levels. Therefore, we focused our attention on the importance of yeast acetate esters for the well-known drosophila endoparasitoid *Trichopria drosophilae* (Hymenoptera: Diapriidae). We expected that *T. drosophilae* would choose a *Saccharomyces cerevisiae* strain that produces more acetate esters and would be neutral to a null mutant strain that lacks the AFT1 gene. Our hypothesis would be in agreement with previous research performed on another *Drosophila* parasitoid (*Leptopilina heterotoma*), which is attracted to long-range mediating chemicals such as ethanol (5%), ethyl acetate (10−2, 10−3%), and acetaldehyde (1%) produced by brewer's yeast (M Dicke, Van Lenteren, Boskamp, & Van Dongen-Van Leeuwen, 1984). We used a null mutant of *Saccharomyces cerevisiae*, where the AFT1 gene was knockout, an ATF1 overexpression mutant and the wild type Y182 of *S. cerevisiae*. Strains were grown in 10% YPD broth and fermented for seven days. Volatile profiles were determined from 4 replicas of tested yeast and 3 blank controls with gas-chromatography, coupled with mass spectrometry. Parasitoid behaviour was tested in a Y-tube olfactometer. We showed that olfactory cues produced by the ATF1 gene of *Saccharomyces cerevisiae* mediated *Trichopria* attraction. Also, by restoring acetate esters in the deleted mutant, we proved the importance of acetate esters for *Trichopria* attraction. Our study reveals the significance of the same volatile cues across trophic levels. Knowledge that the same volatile cues mediate the behaviour of the host organism and its parasitoid in the search for a suitable habitat could be utilised for the attraction of a sufficient number of natural enemies to crop fields for effective top-down control of pest insect populations.

5.2 Material and Methods

5.2.1 Study organisms

Insects

Experiments were performed using adult females of the parasitoid *Trichopria drosophila*. *Trichopria* was obtained in the form of pupae from Biobest (Westerlo, Belgium) (*Trichopria drosophilae*®). Pupae 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 naïve females.

Yeast

When tested, *S. cerevisiae* S288c, commonly used in the laboratory studies, produces less aroma compared to wild strains, which could significantly explain the importance of aroma compounds when yeast is present in a natural environment (Verstrepen, Van Laere, et al., 2003). Therefore, we used the wild Y182 strain of *S. cerevisiae*, because of its suitability for genetic transformation and genetic engineering, which enabled ATF1 gene deletion (Christiaens et al., 2014) and its overexpression in our study. The wild strain was isolated from vineyards in Belgium (Christiaens et al., 2014). The study strains represented three different groups of the same yeast *Saccharomyces cerevisiae.* These included the wild type strain Y182 (WT-Y182) and two ATF1 mutants of Y182, in one of which the ATF1 gene was deleted (DEL-KV3734) and one in which the ATF1 gene was overexpressed (OE-KV3735). It is well known that laboratory strains of *Saccharomyces cerevisiae* lose their attractiveness and production of acetate ester (San 2014), which is why we chose to test a wild type of this yeast. Two were genetically modified; in the first the AFT1 alleles were deleted, and we called this group deletion mutant KV3734. Alcohol acetyl transverase (ATF1) was knockout using deletion cassettes based on pUG6, conferring resistance to either the antibiotic Hygromycin B or G-418 disulfate. Both markers were removed using the Cre/LoxP technique with pSH65. Deletions, as well as marker removal, were confirmed through (lack of) growth on selective media, as well as PCR. The second was the strain in which ATF1 gene was overexpressed, K*V3735*.

Preparation of the fermentation yeast medium

Yeast fermentations were performed as outlined in Christiaens et al. (2014). Briefly, fermentations were started by inoculating the yeasts from a YPD 2% plate into a test tube with 5 mL YPDB 2%, and incubating the tubes at 30°C on a rotary shaker at 100 rpm. After one night, 300 µL was inoculated into 50 mL YPDB 4% in a 250-mL Erlenmeyer flask, which was then sealed with a water lock and incubated overnight at 30°C (100 rpm). Subsequently, the OD_{600} was measured, and the preculture was used to inoculate a 250-mL Erlenmeyer flask containing 150 mL YPD 10% at a final OD_{600} of 0.5. Flasks were sealed with a water lock, and fermentations were allowed to continue for seven days at 30°C while shaking at 100 rpm. Afterwards, fermentation media were spun down at 4,500 g for 5 min and subsequently filtered (pore size 0.22 µm; Nalgene, Waltham, MA, USA) to obtain cell-free cultures. Obtained media were then stored in small aliquots in sealed sterile dark glass vials (Fagron, Nazareth, Belgium) at -20 °C until further use. For each yeast strain, three independent fermentations were performed, and a medium without yeast inoculation was included as a control (sterility of the blank medium was confirmed after the incubation period).

5.2.2 Chemical analysis of mVOCs

To determine the chemical composition of the mVOC cell-free yeast fermentation media, the fermentation medium of each biological replicate (n = 4) and three blank controls with just free cell medium was analysed in a Headspace Gas Chromatography system, coupled to a Flame Ionization Detector (HS-GC-FID) as described previously (Christiaens et al., 2014). The GC was calibrated for 15 important yeast specific volatiles, including esters (ethyl acetate, isobutyl acetate, propyl acetate, isoamyl acetate, phenyl

ethyl acetate, ethyl propionate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate), higher alcohols (isoamyl alcohol, isobutanol, butanol, and phenyl ethanol) and acetaldehyde as described in (Gallone et al., 2016). The GC was fitted with a DB-WAX column (30m length × 0.32mm inner diameter × 0.5µm film thickness, Agilent Technologies, Santa Clara, CA, USA). 5ml samples of fermented yeast were collected in 15ml glass tubes containing 1.75 g of sodium chloride each. These tubes were immediately closed and cooled to minimise evaporation of volatile compounds. The injector port of the GC instrument was held at 250◦C via a headspace auto sampler (PAL system; CTC Analytics, Zwingen, Switzerland). N2 was used as the carrier gas. The GC oven temperature was programmed at 50˚C for 5 min, after which it was increased to 80˚C at 5˚C min−1. Next, the temperature was increased to 200˚C at 4˚C min−1 and held at 200˚C for 3 min, followed by a final ramp of 4˚Cmin−1 till 230˚C. The results were analysed with GC Solution software version 2.4 (Shimadzu, Kyoto, Japan).

Volatile compounds were identified and quantified as in Reher et al. (2019). Briefly, the 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 tentatively identified target compounds (Table 5.1 in results) 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). Firstly, the peak areas were computed from the extracted profiles and summarised in a table. For all chemical compounds, the mean and standard error (SE) were calculated for every yeast fermenting media n=4 and blank media n=3. 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 Tukey's HSD test with adjusted P-values, as calculated after correcting for multiple comparisons. The 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.

The chemical composition of the yeast fermentation products was compared by employing principal component analysis (PCA), followed by analysis of variance (ANOVA). In PCA, each compound within the mVOC profile of the fermentation products was treated as a separate variable (Rencher, 2003). Prior to analysis with PCA, data were normalized by subtracting the mean of the variable and dividing that by the standard deviation. The normalized data were then analysed by calculating a matrix of "scores", which provided the location of each sample on each PC, and a matrix of "loadings", which indicated the strength of correlation between individual compounds and each PC. This was achieved by employing the factoMineR and factoextra packages (Lê et al. 2008) (Kassambara & Mundt, 2017) for R version 4.0.3 (Team, 2013). Next, to gain a better understanding of the changes in the mVOC profile of the fermentation products, data were analysed using one-way ANOVA for each individual compound. Data were first checked for normality and homogeneity of variance using Shapiro-Wilk and Levene's test, respectively. If the compound was not normally distributed, it was analysed using Kruskal-Wallis one-way analysis of variance. The critical value was adjusted for multiple testing using the Bonferonni correction.

Secondly, changes in chemistry (MVOCs) resulting from genetical manipulation on different yeasts were visualised using principal component analysis (PCA), incorporating each compound as a variable according to (Rencher, 2003). We used two types of output: a matrix of "scores," which provided the location of each sample on each PC, and a matrix of "loadings", which indicated the strength of correlation between individual compounds and each PC. Prior to analysis, data were normalized by sum, cube root transformed and mean-centred, and divided by the standard deviation of each variable, using the comprehensive online tool suite MetaboAnalyst 3.0 (Xia, Mandal, Sinelnikov, Broadhurst, & Wishart, 2012). Next, to gain better insight into the changes in yeast profiles upon yeast inoculation, data were analysed using one-way ANOVA for each individual compound. Data were first checked for normal distribution

and homogeneity of variance with Shapiro–Wilk and Levene's test, respectively. The obtained P-values were adjusted for multiple testing using the Benjamini and Hochberg (BH) step-up procedure to control the false discovery rate (FDR) (Benjamini & Hochberg, 1995).

5.2.3 Y-tube olfactometer bioassays

Insect behavioural response was assessed using the Y-tube olfactometer bioassay described by (Sobhy et al., 2018) (Diagram 5.1). The glass Y-tube comprised 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 700 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. 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 the 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 45° incline to simulate the movement of the insects towards the bifurcation.

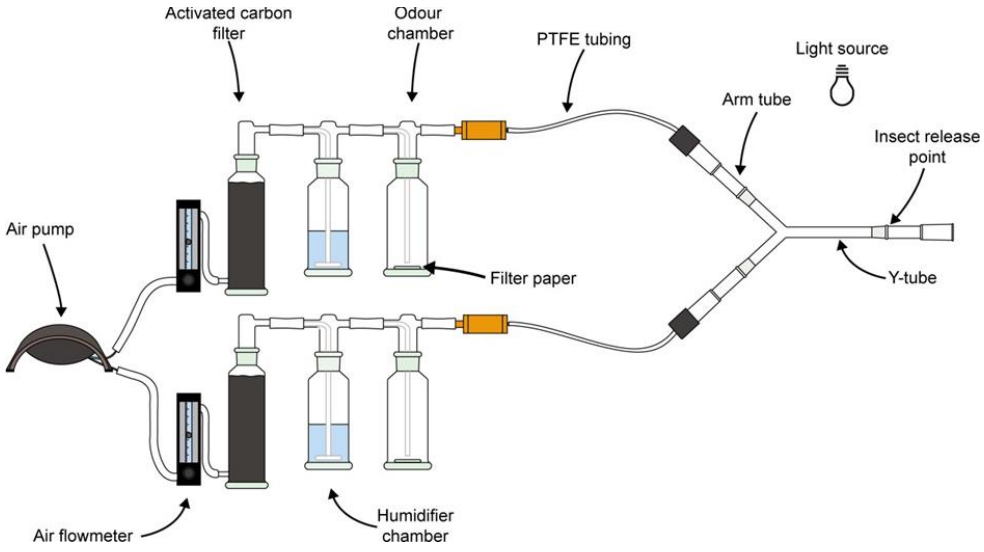


Diagram 5.1. Schematic representation of the two-choice Y-tube olfactometer used in the bioassays. The olfactometer consisted of a 20-cm-long main arm with 1.5 cm internal diameter and two 12-cm-long lateral arms with a 60° angle at the Y-junction. Details of the various parts and connections were as follows: Airflow was generated by an electric air pump containing two separate outlets. The airflow was controlled at 700 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 demineralised water, respectively. Next, the purified and humidified air passed through glass odour chambers containing the test odours. Finally, the air containing the test odours was directed into 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 simulate

the 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 (diagram is adopted from(Goelen, 2020)).

To test a given yeast medium, 150 µL of the cell-free 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 another filter paper was placed in the second chamber, on which 150 µL of blank YPD 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 renewed with cleaned glassware. At the end of the assay, all olfactometer parts were thoroughly cleaned with tap water, distilled water, acetone and finally pentane, after which the parts were placed overnight in an oven at 150°C. All bioassays were conducted at 23 ± 2°C, 60 ± 5% RH and performed between 09:00 and 16:00 h.

In the first set of experiments, parasitoid behaviour was evaluated by simultaneous application of two odours in different conditions, including (i) odour of the blank medium vs water, (ii) odour of the three yeast strains vs blank medium, (iii) odour of the ATF1 deletion mutant supplemented with acetate esters vs blank medium, and (iv) odour of the ATF1 deletion mutant supplemented with acetate esters vs the wild type strain or the ATF1 overexpression mutant. Experiments were performed with a 1000× diluted cell-free fermentation medium, as preliminary experiments revealed suboptimal responses with higher concentrations (data not shown). The supplemented samples of the ATF1 deletion mutant contained either ethyl acetate (99.5%, Acros Organics), isoamyl acetate (>95%, Sigma-Aldrich, Saint Louis, MO, USA) or phenylethyl acetate (98%, Sigma-Aldrich) at concentrations that matched the ones present in the diluted media from the wild type yeast or the overexpression mutant, or combinations of these compounds. For each test, 150 µL medium was loaded on a filter paper (37 mm; Macherey-Nagel, Düren, Germany) and subsequently put in one of the olfactometer odour chambers. In the second set of experiments, parasitoid response was evaluated by subjecting the parasitoids to two concentrations of ethyl acetate (0.1 ppm and 1 ppm), isoamyl acetate (0.001 ppm and 0.01 ppm) or phenylethyl acetate (0.001 ppm and 0.01 ppm) dissolved in diethyl ether vs diethyl ether. Again, 150 µL was loaded on a filter paper, and 30 s later the filters were put in the odour chambers of the olfactometer set-up.

5.2.4 Data analysis

Olfactometer bioassay

For each yeast mutant and wild type, parasitoid olfactory response was analysed using a Generalised Linear Mixed Model (GLMM) based on binomial distribution (the choice is binary: for either control side or treatment side) with a logit link function (logistic regression) using yeast treatment as a 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. The number of parasitoids choosing the control or treatment side in each cohort was entered as the response variable. To examine the preference of the investigated parasitoid for mVOCs produced by each of the tested yeast fermentation media, we

tested the null hypothesis (H0) that the parasitoid shows no preference for any olfactometer arm (i.e. 50:50 response) by testing H0: logit = 0, which equals a 50:50 distribution. In addition, 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 fermentation yeast media. The results were presented by calculating the Preference Index (PI) by dividing the difference between the number of parasitoids choosing the fermentation odours and the parasitoids choosing the control by the total number of responding insects. Additionally, a GLMM was used to determine whether different fermentation media had a significant influence on the olfactory response of *Trichopria*, by using the number of parasitoids in each cohort choosing either the control or the treatment side of the Y- shape as a dependent variable and source of fermentation as a fixed factor. The release of each cohort (n = 12) was again included in the model as a random factor.

Chemical analysis

Non-metric multidimensional scaling (NMDS) was performed on the fermentation media*volatile peak area matrix by using a Bray-Curtis distance matrix (Vegan package in R). Permutational multivariate analysis of variance (perMANOVA) was carried out on the strain*volatile peak area matrix to test for significant differences in the 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, univariate ANOVA followed by Tukey's HSD test were performed on the summed peak areas of the compounds belonging to the same chemical class, when strains were grouped according to the olfactory response. For the statistics in the boxplots and overview table (i.e. GC data), we performed a pairwise Tukey HSD test. All statistical analyses and evaluation of normality and homoscedasticity of the data were performed in R version 4.0.2 (Team, 2013).

5.3 Results

5.3.1 mVOC composition of different mutants and the wild type of *Saccharomyces cerevisiae*

To determine the chemical composition of mVOCs in cell-free fermentation media, the fermentation media of each biological replicate (n=4) of the deletion mutant KY3734, overexpression mutant KY3735, wild type Y182 and three blank controls were analysed in a Headspace Gas Chromatography system, coupled to a Flame Ionization Detector (HS-GC-FID). mVOC composition differed significantly between the tested groups, as presented in PCA Fig 1. Overall, the volatiles produced in the largest amounts belonged to higher alcohols and acetate esters. Figure 5.1 presents the principal component analysis, which separated all the tested samples into different groups.

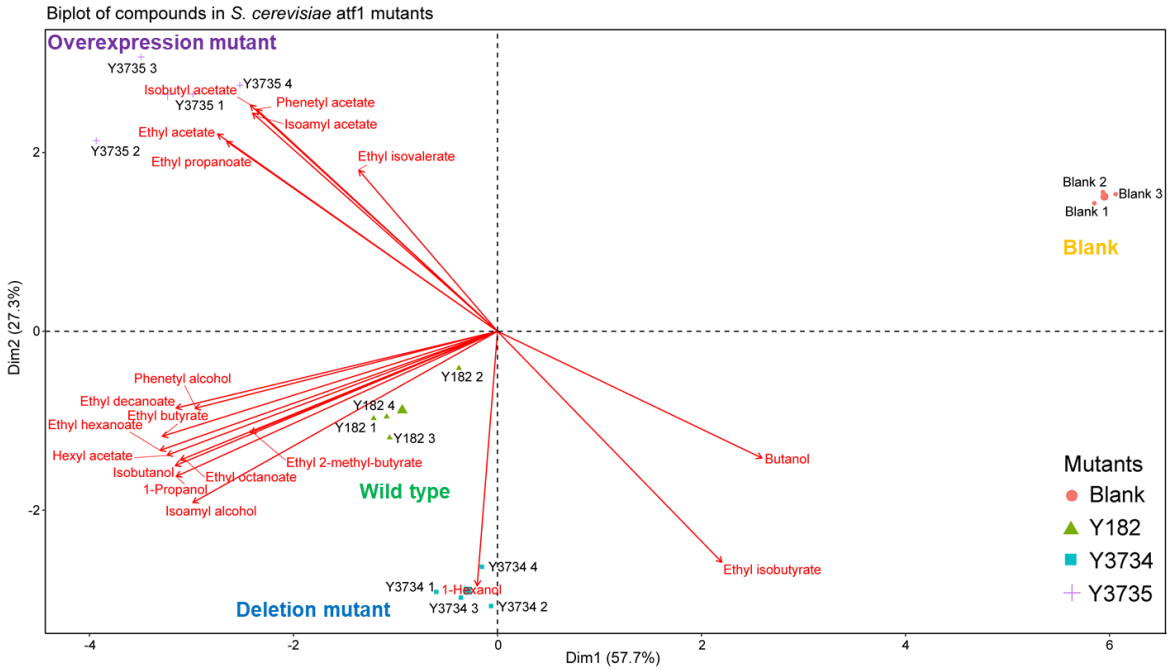


Figure 5.1. Principal component analysis of odour response profiles. The first two components are shown (total variance explained by the first two principal components Dim 1= 57.7% and Dim2= 27.3 %).

For a few compounds, their concentration was significantly higher in the wild type compared to the yeast deletion mutant KV3734 (i.e. ethyl acetate, isoamyl acetate, and butanol), indicating that these compounds were reduced after ATF⁻ double-deletion.

5.3.4 Differences in mVOC composition between *Saccharomyces cerevisiae* mutants and wild type strain Y182

Grouping the yeast lines (deletion mutant KY3734, overexpression mutant KY3735, and wild type Y182) based on the effect of their mVOC blends on the olfactory response of *Trichopria drosophilae* showed that the deletion mutant neutral to parasitoids produced significantly less amounts of 1-propanol (P= 0.01), butanol (P=0.01), ethyl acetate (P= 0.015), isobutyl acetate (P= 0.01), and isoamyl alcohol (P=0.00), compared to the attractive mutant wild type Y182 (Table 5.1). This analysis confirmed that acetate esters were considerably reduced or even completely abolished in the ATF⁻ deletion mutant strain, while no significant difference was observed in the concentration of other volatile compounds such as higher alcohols.

Table 1. Aroma Production by Wild type Y-182 and atf1⁻ Strain					
Compound (ppm)	**WT-Y182**	**SD**	**atf⁻**	**SD**	**p Value**
1-hexanol	0	0.00	0.03	0.00	0
1-propanol	48.65	1.69	55.08	2.63	0.01
4-vinylguaiacol	0	0.00	0.00	0.00	ND
Butanol	93.40	1.49	100.45	3.48	**0.01**
Ethyl 2-methyl butyrate	0.19	0.09	0.19	0.08	1.00
Ethyl acetate	28.05	0.85	15.73	1.23	**0.015**
Ethyl butyrate	0.22	0.03	0.23	0.01	0.96
Ethyl decanoate	0.11	0.01	0.12	0.01	0.84
Ethyl hexanoate	0.04	0.00	0.05	0.00	0.64
Ethyl Isobutyrate	0.05	0.00	0.05	0.00	0.98
Ethyl Isovalerate	0.06	0.10	0.00	0.00	1.00
Ethyl octanoate	0.10	0.00	0.10	0.01	0.97
Ethyl propanoate	0.30	0.01	0.00	0.00	**0**
Ethyl valerate	0	0.00	0.00	0.00	ND
Hexyl acetate	0.01	0.00	0.01	0.00	0.53
Isoamyl Alcohol	124.22	9.79	149.18	5.38	**0.00**
Isobutanol	79.65	2.98	79.65	3.04	1
Isobutyl acetate	0.16	0.02	0.03	0.00	**0.01**
Isoamyl Acetate	0.72	0.05	0.36	0.01	0.36
Octyl acetate	0	0.00	0.00	0.00	ND
Pentyl Acetate	0	0.00	0.00	0.00	ND
Phenetyl acetate	0.10	0.01	-0.01	0.01	0.260
Phenetyl alcohol	12.50	3.73	11.37	0.79	0.961
Propyl acetate	0	0.00	0.00	0.00	ND

Table 5.1. The concentration of aroma compounds (in parts per million) produced by the diploid wild type Y182 strain and the ATF⁻ double-deletion mutant during fermentation. Four fermentations were performed for each strain, and each was analysed in four technical replicates. The significant difference in acetate ester between the wild type and deletion mutant is shown in bold type. These results show that the only compounds significantly affected by ATF⁻ deletion were acetate esters. ND= not detected.

In contrast, the overexpression mutant KY3735 attractive to the parasitoid wasp produced significantly higher amounts of 1-propanol (P= 0.00), butanol (P=0.00), ethyl acetate (P= 0.00), ethyl isobutyrate (P= 0.00), isoamyl alcohol (P=0.00), and isoamyl acetate (P= 0.00), compared to the neutral deletion mutant KY3734 (Table 5.2). Chemical analysis revealed the concentration of aroma compounds in the fermentation of the wild type, deletion mutant and overexpression mutant, focusing on higher alcohols and acetate esters, and the main differences were expressed in the concentration and production of acetate esters.

Table 2. Aroma Production by atf1⁻ Strain and Overexpression mutant					
Compounds (ppm)	**atf1⁻**	**SD**	**OM**	**SD**	**p Value**
1-hexanol	0.03	0.00	0	0	0
1-propanol	55.08	2.63	47.86	1.55	**0.00**
4-vinylguaiacol	0	0	0	0	ND
butanol	100.5	3.48	91.60	1.83	**0.00**
Ethyl 2-methyl butyrat	0.19	0.08	0.18	0.09	1.00
Ethyl acetate	15.73	1.23	96.07	7.63	**0**
Ethyl butyrate	0.23	0.01	0.23	0.03	1.00
Ethyl decanoate	0.12	0.01	0.12	0.02	0.99
Ethyl hexanoate	0.05	0.00	0.04	0.00	0.77
Ethyl Isobutyrate	0.05	0.00	0	0	**0**
Ethyl Isovalerate	0	0	0.64	0.65	0.17
Ethyl octanoate	0.10	0.01	0.10	0.01	0.88
Ethyl propanoate	0	0	0.48	0.02	**0**
Ethyl valerate	0	0	0	0	ND
Hexyl acetate	0.01	0.00	0.01	0.00	0.35
Isoamyl Alcohol	149.2	5.38	116.5	6.26	**0.00**
Isobutanol	79.65	3.04	72.88	5.27	0.15
Isobutyl acetate	0.03	0.00	0.83	0.08	0
Isoamyl Acetate	0.36	0.01	2.96	0.49	**0.0000004**
Octyl acetate	0	0	0	0	ND
Pentyl Acetate	0	0	0	0	ND
Phenetyl acetate	-0.01	0.01	0.67	0.13	**0.0000005**
Phenetyl alcohol	11.37	0.79	12.57	3.95	0.96
Propyl acetate	0	0	0	0	ND

Table 5.2. The concentration of aroma compounds (in parts per million) produced by the ATF⁻ double-deletion mutant KY3734 and the ATF double-overexpression mutant KV3735 during fermentation. Four fermentations were performed for each strain, and each was analysed in four technical replicates. The significant difference in acetate ester between the deletion mutant KV3734 and overexpression mutant KV3735 is shown in bold type. These results show that the only compounds significantly affected by ATF⁻ deletion were acetate esters. ND = not detected.

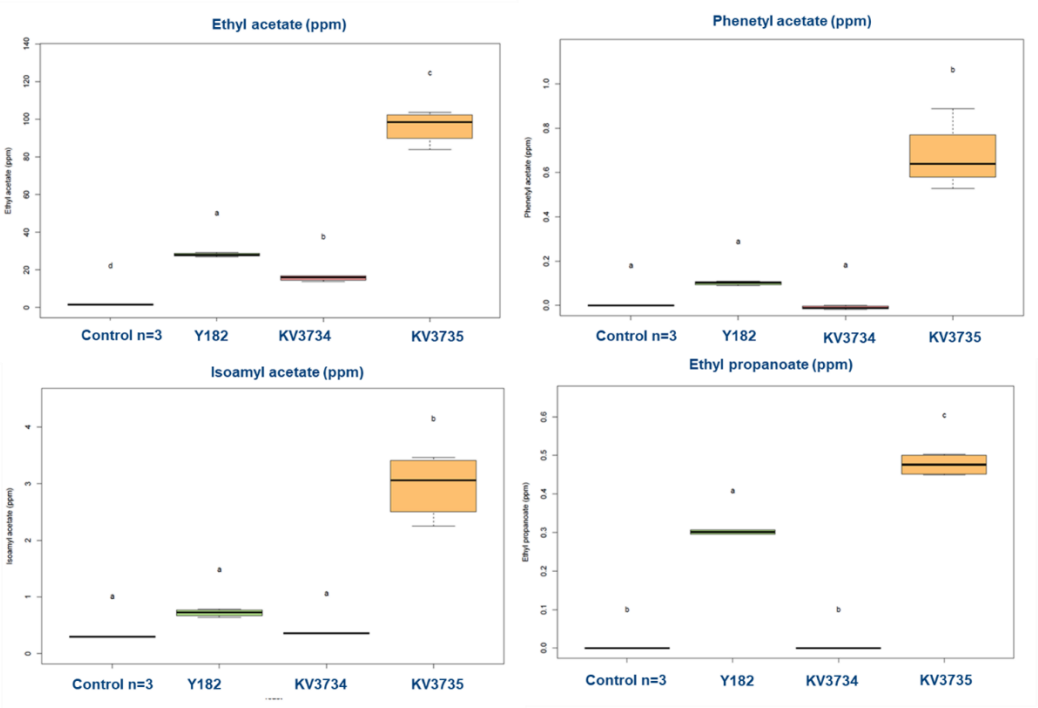


Figure 5.2. Mean concentration of (SE) peak area normalized to 2-heptanol (IS) of microbial volatile organic compounds (mVOCs) produced by different mutant strains of *Saccharomyces cerevisiae*. The values shown are the sum of the peak areas of corresponding compounds, as detected by MXT-5 equipped GC-MS, and results from three biological replicates for blank control (n=3) and four replicates for the tested mixture of mutants and wild type. Control = non-inoculated, yeast-free YPD medium; Y182=YPD inoculated with diploid wild type strain; KV3734 = YPDA inoculated with the ATF double-deletion mutant strain and KV3735 = YPD inoculated with ATF1 overexpressed mutant strain. All analysis was performed on cell-free YPD medium. Different letters indicate significant differences (P< 0.001) between the production of compounds emitted by different yeast mutants and the wild type based on a non-parametric pairwise Tukey HSD test.

5.3.5 Olfactory response of *Trichopria drosophilae* to different mutants of *Saccharomyces cerevisiae*

In a Y-shape olfactometer, we tested *T. drosophilae* preference for different mutants of *Saccharomyces cerevisiae* and the wild type Y182 strain in 10% YPD broth, fermented for seven days. This experiment aimed to assess the olfactory response of the parasitoid to the volatile emissions of the mutants and wild type strain of ***Saccharomyces cerevisiae.*** The different mutants were tested against a blank medium (YPD broth without yeast cell) (Figure 5.3). We tested all four biological replicates. Preference index were negative for the **deletion mutant Y3734** in all three biological replicates, while in one case it was neutral but not statistically significant (PI= 0,09 (P=0.321); PI= -0,13 (P=0.477); PI= -0,46 (P=0.003); PI= -0,40 (P=0.015)). The PIs for **wild type Y182** were negative for all biological replicates (PI = -0, 70 (P=1e-o4); PI= -0, 14 (P=0.372); PI= -0, 43 (P=0.009); PI= -0, 30 (P=0.072)). The PIs for the overexpression mutant **Y3735** were (PI = 0,23; PI = -0,44; PI= -0,34 ; PI= -0,47). Overall, most of the tested strains had negative PI-values.

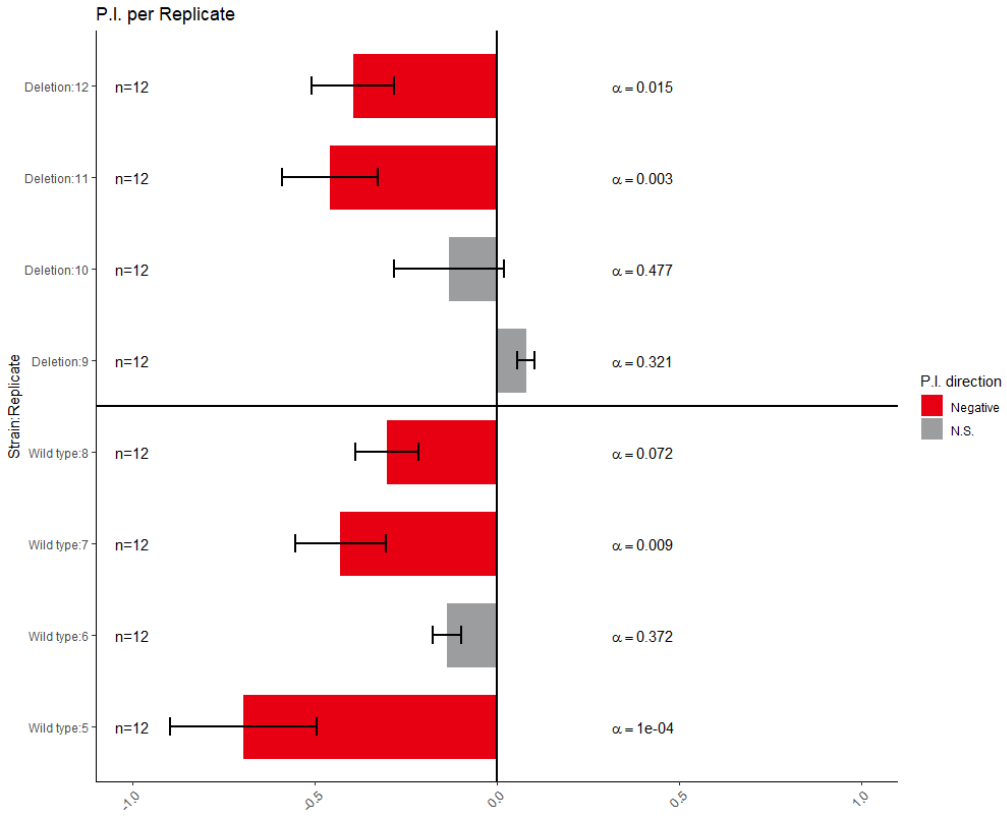


Figure 5.3. Olfactory response of naïve 24 h-old female adult *Trichopria drosphilae*, when given a choice between the odour of a tested yeast (deletion-null mutant Y3734; wild type Y182 strain;) grown in 10% YPD broth and fermented for 7 days, and the odour of the blank YPD broth medium in a Y-shape olfactometer. Insect response is expressed as a Preference Index (PI), which is calculated by dividing the difference between the number of parasitoids choosing the yeast odour and parasitoids choosing the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females). Non-responders were excluded from the statistical analysis. Red bars on the left of the 0 axis indicate the olfactory response to the deletion mutant with P values as follows (P=0.015; P=0.003; P=0.477; P= 0.321), grey bars in the upper part of the graph indicate a non-significant response to the deletion mutant (P=0.477; P= 0.321), the grey bar in the lower part of the graph indicates a non-significant response (P=0.372) to the wild type, when compared to theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). Overall, parasitoid responsiveness was higher than 80%.

5.3.6 Olfactory response of *Trichopria drosophilae* to different *Saccharomyces cerevisiae* mutants in samples diluted 1000 times

Since all the preference indexes were negative in the previous experiment with natural concentrations, we diluted the samples, first by ten, then by 100, and finally by 1000 to see whether concentration plays a role in changing the olfactory preferences of *Trichopria*. The results are only presented here for the 1000 time dilution concentration (Figure 5.4). In the concentration diluted 1000 times, overexpression mutant KY3735 had a positive PI= 0,68 (P <0.001), while wild type Y182 had a PI= 0.56 (P<0.001). Deletion mutant KY3734 had a negative PI= -0,02 (P=0.876).

We then wanted to restore the attractiveness of deletion mutant KY3734 by adding compounds known from previous studies to be the main mediators of insect behaviour. The acetate ester compounds ethyl

acetate (EA) and isoamyl acetate (IA) were added to the deletion mutant at a concentration normally present in the wild type, tested first, then in the concentration of overexpression mutant, both diluted 1000 times. The aim was to mimic the concentration of these compounds from the wild type and overexpression mutant. The preference index of the restored deletion mutant KY3734, which mimics the concentration of the EA+IA wild type, was positive, with a PI = 0.384615 (P=0.019), and the preference index of the deletion mutant with the addition of EA+IA mimicked the concentration of overexpression, with a PI= 0.5 (P=0.003).

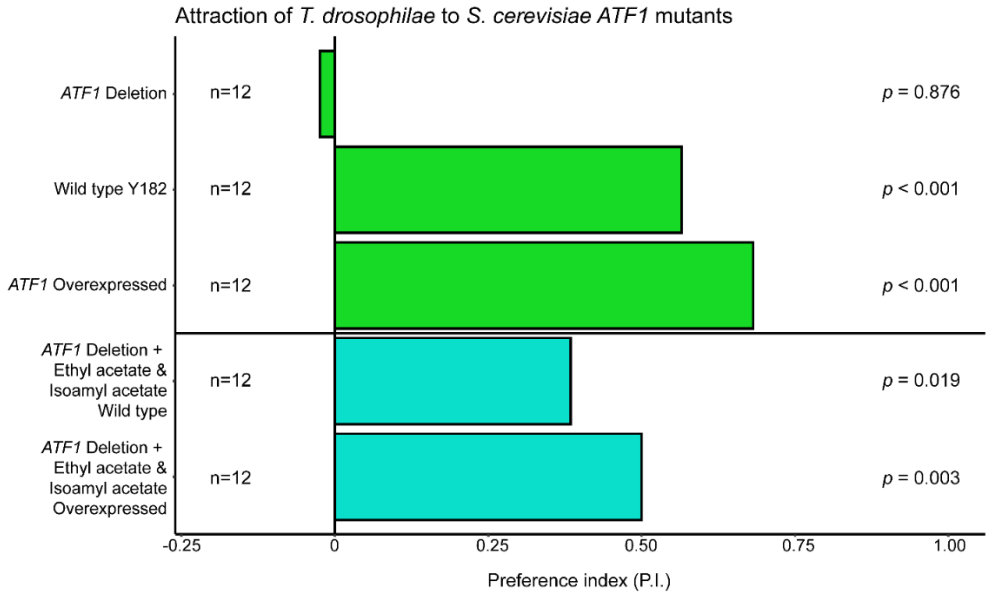


Figure 5.4. Olfactory response of naïve 24 h-old female adult *Trichopria drosphilae*, when given a choice between the odour of a tested yeast diluted 1000 times (deletion-null mutant KY3734; wild type Y182; overexpression mutant KY3735 grown in 10% YPD broth and fermented for seven days, and the odour of the blank YPD broth medium in a Y-shape olfactometer. Insect response is expressed as a **Preference Index (PI)**, which is calculated by dividing the difference between the number of parasitoids choosing yeast odours and parasitoids choosing the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females). Non-responders were excluded from the statistical analysis. The green bar on the left of the 0 axis indicates a non-significant olfactory response to the deletion mutant (P=0.876), the green bar in the middle indicates a significant attractive response (P<0.001) to the wild type Y182, and the last green bar indicates a significant attractive response (P<0.001) to the overexpression KY3735 mutant, when compared to theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). There is no significant difference between the attraction of the wild type and the overexpression yeasts media. Overall, parasitoid responsiveness was higher than 80%.

5.4 Discussion

This study assessed the olfactory responses of an important *Drosophila* endoparasitoid *Trichopria drosophilae* to microbial volatiles (mVOCs) produced by the common baker's yeast species *Saccharomyces cerevisiae*. The results proved our initial hypothesis about the attraction of this parasitoid to mVOCs produced by *S. cerevisiae* in a Y-shape olfactometer. Furthermore, we investigated whether the chemical compounds in mVOC blends differed between an attractive wild type, overexpression of ATF 1 gene and neutral delete mutants of AFT1 gene yeast strains. PCA of volatiles analysed in a Headspace Gas Chromatography system, coupled to a Flame Ionization Detector (HS-GC-FID) showed clear separation of the wild type, overexpression and delete mutant from each other. Additionally, we restored the impaired attraction in the deletion mutant by adding a mimicking concentration of important acetate esters present

in the wild type and the overexpressed mutant. Overall, these findings highlight the role of the same volatile cues across trophic levels.

Saccharomyces cerevisiae is a model yeast that has been used for different research purposes because of its ability to be easily genetically modified and quickly reproduced (Duarte, Herrgård, & Palsson, 2004). During fermentation, *S. cerevisiae* produces several volatile acetate esters and other complexes, namely volatile compounds (Verstrepen, Derdelinckx, et al., 2003). Furthermore, the physiological role of acetate esters produced in yeast *S. cerevisiae* has been examined in relation to fungal dispersal (Christiaens et al., 2014). Aromatic compounds have been shown to be produced in yeast as a result of an evolutionary mutualist relationship between yeast and insects (Becher, Hagman, et al., 2018; Christiaens et al., 2014). Likewise, some studies have shown the importance of acetate ester in the choice of meeting and oviposition sites (Becher et al., 2012; Mori et al., 2017). Yeast fermentation metabolic products are capable of changing nectar chemistry and in this way, of attracting insects for dispersal (Sobhy et al., 2018). Nectar yeast produced VOCs that are used as a learning cue by parasitoids (Sobhy et al., 2019). *S. cerevisiae* emits a broad spectrum of volatiles (Verstrepen, Van Laere, et al., 2003), but for *Trichopria,* we proved the importance of acetate esters.

In an evolutionary context, microorganisms and insects evolved together and adapted their metabolic and olfactory traits in a mutualistic relationship. Volatile cues emitted by yeasts that mediate *Drosophila* behaviour are a result of ecological coadaptation between the two (Becher, Hagman, et al., 2018; Scheidler et al., 2015). Evolutionary adaptation of yeast that produces volatiles to be attractive to *Drosophila* for dispersal has been shown in (Christiaens et al., 2014). Pathogenic bacteria enhance dispersal by disturbing Drosophila social communication (Ian W Keesey et al., 2017).

Insects develop special olfactory circuits and classes of numerous sensory neurons, expressed in olfactory receptors that are specialised for detection of diverse upcoming olfactory cues from the environment and respond to ether positive or harmful properties of the habitat milieu. Innovative genetic techniques allowed us to render the volatile production in the yeast and in this way, create ATF1 deletion KV3734 and overexpression KV3735 mutants. Our results show that *T. drosophilae* females responded differently to the mVOCs of all three tested yeast media. The overexpressed yeast medium and the wild type yeast medium showed significant attraction for *Trichopria*, while the deletion mutant was neutral when tested in a Y tube olfactometer.

To emphasise the importance of acetate ester produced by *Saccharomyces cerevisiae,* in our investigation we provided a deletion mutant with a mixture of important acetate esters in two different concentrations. By doing so, we restored the attraction to KV3734: KV3734 + EA + IA (Y182) => to mimic the wild type; andKV3734 + EA + IA (KV3735) => to mimic the overexpression mutant. This indicates the importance of these two acetate esters for *Trichopria* attraction. While these two additional different tests showed attractiveness, they did not show a statistical difference when compared with the wild type and overexpression mutant. Restoration of attraction in KV3734 showed the importance of two aromatic compound acetate esters: ethyl acetate and isoamyl acetate. Given this observation, our results seem to strongly support our initial hypothesis that the same mVOCs produced by *Saccharomyces cerevisiae* are attractive to host flies and its parasitoid *Trichopria drosophilae*. Adding important acetate esters to the deletion mutant in the concentration mimicking the overexpression and wild type concentration of these esters was not as attractive as the wild type and overexpression themselves. In the yeast fermentation volatile milieu, there are other compounds that could be important for the attraction or composition of background odour. Acetate esters emitted from yeast fermentation attract Drosophila, but fusel alcohols such as 2-phenyl-ethanol, 3-methyl-1-butanol, and ethanol also contribute to attractiveness (Becher, Hagman, et al., 2018)

Our finding that a parasite of *Drosophila* utilises *S. cerevisiae* volatiles for host habitat location and VOCs originating from host habitats as cues enabling searching behaviour, is in agreement with a previous study (M Dicke et al., 1984), where *Leptopilina heterotoma* (Thomson) (Hymenoptera: Eucoilidae), a

Drosophila sp. parasitoid, utilised VOCs from *S. cerevisiae*. Research on the importance of mVOCs and their ecological role has increased in the last decade (Davis et al., 2013). Recent studies with mVOCs from bacteria indicate their importance in trophic interactions and mediation of insect behaviour across trophic levels and between aphid parasitoids and their hyperparasitoids (Goelen et al., 2020). In contrast to our study, the study on bacterial volatiles used by natural enemies of aphids, *Aphidius colemani*, in the search for a host habitat, was also tested on its hyperparasitoid *Deudrocerus aphidum***,** but this study did not find a conspecific volatile common for both trophic levels.

In our experiments on the attractiveness of wild type and overexpression mutants, we did not find any statistical differences. These results were obtained once we diluted samples 1000 times in the blank medium. *Trichopria* was repelled by the original yeast medium concentration. This behaviour could be explained by the presence of some repellent compounds in intolerable amounts, or because significant biological compounds were so concentrated that they had a repellent effect on *Trichopria*. Therefore, our final dilution by 1000 gave us a strong attraction for wild and overexpressed mutants. At the same time, the lower dilution was attractive but not as significant as our final x1000 dilution. This might indicate that *T. drosophilae* requires a lower concentration of key compounds for attraction or that the concentration of some compounds in the original media mixture of *Saccharomyces cerevisiae* was too high and masked otherwise attractive compounds (Aartsma et al., 2017). Compound concentration affects insect behaviour, and this is also well known from other studies; it is known that the minimum volatile concentration eliciting a behavioural response may differ between parasitoids (Aartsma et al., 2017; Bruce & Pickett, 2011; Bruce et al., 2005). Therefore, the importance of volatile concentration for parasitoid attraction is not surprising.

The yeast VOC blends comprised typical microbial fermentation products, such as methylated, low molecular weight higher alcohols and acetate esters. However, some compounds, like ethyl octanoate, ethyl acetate, 1 propanol, isoamyl acetate, ethyl hexanoate, 1-hexanol, ethyl octanoate, and benzaldehyde, are also commonly reported as typical plant volatiles (Bruce & Pickett, 2011; Dudareva, Klempien, Muhlemann, & Kaplan, 2013; J.-E. Lee et al., 2009). Visualisation of differences in VOCs emitted by different mutants and the wild type using PCA, in which the loading vectors of most VOCs were clearly separated from each other, suggested distinct VOC profiles when we compared all the tested groups. Indeed, low VOCs released by the deletion mutant explain the poor olfactory responses of naïve females of *Trichopria*. A significant reduction in the production of acetate esters is the reason for the neutral response of *T. drosophilae* to the deletion mutant. Odour concentration is important, since the results were obtained once the tested mixtures were diluted 1000 times in blank cell media. In general, the tested mixture emitted the same set of volatile compounds, and most VOCs produced by *Saccharomyces cerevisiae* deletion and overexpression mutants and the wild type had differences in concentration and significantly different ratios in terms of the production of acetate esters. This indicates that the presence of acetate esters in the tested yeast mutants elicits a different response in *Trichopria,* depending on the concentration of the compounds and specific ratios, determined by the presence or absence of the ATF1 gene. More specifically, VOC compositions highly attractive to *T. drosophilae* had a higher concentration of acetate esters, in particular ethyl acetate with a concentration of around 100 ppm, while alcohol esters and acids that increase during malolactic fermentation with bacteria improved attraction to *Drosophila suzukii* in the open field (Chapter 3, (J.-E. Lee et al., 2009)). Ethyl acetate and isoamyl acetate in particular are attractive to *Drosophila suzukii* (Cha et al., 2012; Feng et al., 2018; Revadi et al., 2015). The attractiveness of phenethyl esters such as phenethyl acetate to SWD is recognised, not as a single compound but in combination with other phenethyl esters (Kleiber et al., 2014). A fraction of the volatile compounds in a natural blend ratio could induce behavioural responses, rather than the whole blend (Bruce & Pickett, 2011; Conchou et al., 2019; De Moraes, Lewis, Pare, Alborn, & Tumlinson, 1998; Piñero & Dorn, 2007). For *D. suzukii,* a smaller number of compounds in a specific concentration and ratio is responsible for attraction (Cha et al., 2017; Feng et al., 2018; Najar-Rodriguez, Galizia, Stierle, & Dorn, 2010). However, in the case of the oriental fruit moth *Cydia*

molesta (Tortricidae: Olethreutinae: Grapholitini), the ratio of constituents in a volatile mixture could be varied to a certain degree without reducing female attraction (Najar-Rodriguez et al., 2010).

Aliphatic and aromatic hydrocarbons such as 2-phenylethanol (aromatic alcohol) are common floral volatiles of most roses, together with phenylethyl acetate from various fermentation products, and are attractive for D. *melanogaster* (Zhu et al., 2003).

With our method of GC analysis, characterisation of aromatic compounds can be problematic because certain odours are highly volatile and may be lost during extraction or can co-elute with a solvent used for extraction during GC analysis. A trap with apple cider vinegar in the open field for SWD had a high number of two beneficial parasitoids (*L. boulardiand* and *L. heterotoma*) (Wang et al., 2016). This also indicates that similar volatile cues are used by other drosophila parasitoids. Apple cider vinegar as a SWD attractant is characterised by a high concentration of ethyl octanoate, phenethyl alcohol, ethyl acetate, ethyl acetate, hexyl acetate, and ethyl decanoate. While ethyl octanoate is described as close-range attractant in the lab and synergistically increases the attraction of other compounds attractive to SWD in the open field (Feng et al., 2018), in studies on sister Drosophila species in Chapter 4 ethyl octanoate did not show any activity. Ethyl acetate and phenethyl alcohol are synergistic for SWD in potentially attractive lures. Phenethyl alcohol is a component of rose aroma. All these volatiles described could also play a role in *Trichopria* attraction, and they were detected in the chemical analysis reported in this chapter.

Infochemicals such as the aromatic compounds acetate esters mediate organism behaviour from the first to the second trophic level, as well as on the third trophic level. Searching behaviour of natural enemies is important on tritrophic levels. Plant volatiles mediate the searching behaviour of insects, especially at a longer distance. Long distance volatiles are not emitted just by plants, but also by microorganisms present on plants and in the surroundings. The use of innovative genetic modified techniques allowed us to understand ecological interactions and investigate the importance of specific volatiles regulated by the alcohol acetyl transferase gene, the AFT1 gene. From our findings, acetates esters as long-distance volatiles mediate the interaction between organisms on trophic levels. Overall our trials with different dilutions and concentrations clarified the relative importance of specific concentrations of volatile cues.

Our studies add to knowledge on the importance of microbial chemical information, specifically in relation to *Trichopria drosophilae* searching for a host organism. However, it is so far unclear what the other ecological roles of acetate esters produced by *Saccharomyces cerevisiae* are. It is unclear why *Saccharomyces cerevisiae* produces acetate esters that act as semiochemicals across trophic levels. Furthermore, it also opens the question of why yeast produces volatiles that will eventually attract insects for its dispersal and consequently its parasitoids.

5.5 Conclusion

Very little is known about the ecological role and bio function of volatiles emitted by microorganisms and especially their function on other trophic levels. Our study expands our knowledge about the ecological role and biological function of mVOCs in the foraging behaviour of parasitoids. We clearly showed the attraction of the *Drosophila* generalist endopupal parasitoid *Trichopria drosophilae* to aromatic volatiles, acetate esters produced by the baker's yeast *Saccharomyces cerevisiae*. We further confirmed this result with a deletion yeast mutant, and by restoring the phenotype with the addition of the missing volatiles. Our study highlights the importance of using molecular approaches to understand ecological interactions and render organisms in such a way as to explain the physiological and biological activity of certain genes, chemical compounds etc. in laboratory behavioural assays. Yeast produces volatiles that attract drosophila for dispersal (Christiaens et al., 2014). As regards *Leptopilina heterotoma,* chemical signals from a host habitat emitted by yeast are important biological cues for searching by its host Drosophila (M Dicke et al., 1984). Furthermore, these findings may be utilised to better understand the behaviour of

parasitoids and their attraction in the open field, and consequently lead to improvement of biological pest control.

While it is true that we restored attraction in the deletion mutant and proved significant attraction of *T. drosophilae* towards acetate ester, it could be argued that this attraction may be due to limited spatial circulation of volatiles without the presence of other background odours, as takes place in natural conditions. Cues derived from microbes are likely to function in combination with with plant-derived cues, and acetate esters could be an important component of the complex chemical environment for trophic levels. This and similar findings need to be confirmed in more natural conditions, such as semi-field and open field conditions. Additionally, future studies should focus on the technological transfer of the knowledge obtained in this study and similar studies, and the possibilities of boosting biocontrol for crops.

In the context of biological control of *Drosophila suzukii,* Chapter 5 provides new information linking the interaction of the two species. This knowledge could be directly utilised in a SWD management system.

Chapter 6

General conclusion and perspectives

6.1 Main results of this study

Oenoccocus oeni strains were shown to be the most attractive of the different LAB species and strains tested in field and laboratory conditions for *D. suzukii*

With the growth in the world population, increasing global demand for food for human and animal consumption is a huge challenge for sustainable crop production. Natural enemies (parasitoids and predators) control 99% of agricultural crop pests (DeBach & Rosen, 1991), yet crop and food production is severely affected, with a reduction of 25-50% as a result of insects, weeds and pathogens (Pimentel et al., 1991). Direct harmful effects of pesticides are reported in more than 20000 human victims yearly (Forget, 1991). Among the challenges arising from climate change and the transformation of agroecosystems, there is the problem of invasive insect species, which heavily affect agricultural production. Invasive insects can establish themselves in new areas, where their development can progress due to a suitable climate and lack of natural enemies. Farmers have few options to mitigate attack by these insects. Current control tactics using pesticides need to be replaced with more sustainable methods to counter invasive insect species. Monitoring is the first step in any management programme to determine the characteristics of a pest. For this purpose, there are no efficient lures, traps, baits registered up to date. Spotted-wing Drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), native to Eastern Asia, is an invasive alien species in Europe and the Americas, where it is a severe pest of horticultural crops, including soft fruit and wine grapes. The conventional approach to controlling SWD infestations involves the use of insecticides, but the frequency of application for population management is undesirable.

Consequently, alternative strategies are urgently needed. Effective and improved trapping is important as an early risk detection tool. We studied bacterial impact on enhanced attractiveness in a commercial bait (Droskidrink®) under field and laboratory conditions in **Chapter 2**. Initially, one species (*Oenococcus oeni*) of lactic acid bacteria was found to release chemical compounds proving attractive to SWD. This bacterial culture increased the attractiveness of the Droskidrink bait, which resulted in higher capturing of SWD in traps. Therefore, our findings suggest the use of the bacterial culture inside homemade SWF traps. Using this kind of bait can minimise the risk of pest outbreaks in fruit orchards in both domestic and wild environments. Our pest management approach is farmer-friendly in every way, and suitable for the food sector. Currently, monitoring of *Drosophila suzukii* is based on the use of traps baited with attractants. The current food baits have not provided entirely efficient monitoring, and full control of SWD is challenging, even with the use of insecticide control for SWD (Diepenbrock, Rosensteel, Hardin, Sial, & Burrack, 2016). The use of bacteria as bio-catalysers to produce bioactive volatiles to *D. suzukii* may improve attraction of the flies. We therefore carried out work to enhance the attractiveness of Droskidrink® food bait to *D. suzukii* by utilising lactic acid bacteria species. Different food baits based on the use of Droskidrink® were assessed for their appeal to flies in a Droso-Trap® in a commercial vineyard. *Oenococcus oeni*, *Pediococcus* spp., and *Lactobacillus* spp. were used in some of the baits. Additionally, the performance of the most attractive species, *O. oeni*, inoculated into Droskidrink® was assessed in laboratory tests. The response of female flies to the volatiles emitted by Droskidrink® inoculated with *O. oeni* strains was recorded using electroantennography (EAG). Preliminary field assessment of food baits established that *O. oeni* was the most attractive species. Laboratory tests identified the three strongest *O. oeni* strains. Volatiles extracted from the headspace of baits inoculated with *O. oeni* elicited electroantennographic (EAG) responses from *D. suzukii* antennae. Droskidrink® inoculated with *O. oeni* is a highly attractive bait for *D. suzukii* monitoring. Further research was carried out to characterise the chemical profile of liquid baits inoculated with selected bacteria, to provide optimal growth conditions for inoculated bacteria in the open field and to set up new efficient traps in **Chapter 3**.

The results showed that the attractiveness of DD increased by up to two-fold with the addition of commercially available *O. oeni* when combined with an innovative trap design

We approached the control of invasive spotted-wing drosophila *Drosophila suzukii* using a baiting system that manipulates insect behaviour without the use of toxic or non-sustainable chemicals. The results of our work can be reutilised for monitoring and mass trapping of this devastating invasive species. In an innovative smart-design trap system, we used odours that attract flies and decrease damage in open field scenarios. Our trapping system can efficiently detect the first spring arrival of *D. suzukii* in agricultural fields and therefore represents a good early monitoring tool. We conducted three years of laboratory and open-field trials in different berry crops. As a source of odour attraction, we used a mixture of wine, apple cider vinegar, and different commercially available wine fermentation bacteria. The research described in this chapter aimed to improve Droskidrink® (DD), a commercially available attractant for SWD. We focused on the chemical and behavioural effects of adding the bacterium *Oenococcus oeni* (Garvie) to Droskidrink® and used a new trap design to enhance the effects of attractive lures. The new trap design is a delta trap modified by creating a hole in the centre of the traps at the bottom, into which a 30-mL cup is inserted with two cotton balls and 15 mL of the attractant mixture. The results of our work showed an unexpected set of chemical compounds that are products of bacterial fermentation. We demonstrated that the microbial volatile compounds produced by *O. Oeni* are responsible for the increase in the attractiveness of the bait and could later be utilised for the development of a better trapping system. Our results showed that the attractiveness of DD was increased by up to two-fold with the addition of commercially available *O. oeni* when combined with an innovative trap design. The new trap-bait combination increased the number of males and especially females captured at low population densities.

Volatile organic compounds showing repellent activity were revealed and these could be utilised for untargeted insect capture in the design of more selective innovative trapping concepts for plant protection from *Drosophila suzukii*

In preliminary field trials conducted in a New Zealand orchard where SWD has not yet arrived, the most abundant species in lures for different Drosophila was *D. simulans*. Therefore, we chose this *Drosophila* species as the main subject of our research. Chemical compounds in commercial lures were tested with gas-chromatography following two different timelines. In the first week of lure exposure in semi-field conditions, the first tests took place to identify repellent volatile organic compounds. The second tests followed in the third week, aiming to find attractive volatile compounds in the lure. Extraction of volatiles from existing lures from PFR for *Drosophila* took place with direct headspace VOC collection analysis. We tested the chemical compounds in different lures with gas-chromatography mass spectrometry. The neurophysiology of *Drosophila simulans* on different VOCs was assessed by means of gas chromatography coupled with electro-antennographic detection (GC-EAD) and GC-sensillogram recordings. This experiment led to partial identification of the compounds that are electro-physiologically active in *Drosophila simulans*. In the second part of the work, we created a blend of VOCs, selected according to their biological activity in our electrophysiological studies and a review of the literature. Different VOC blends were tested with pitfall behavioural experiments (in laboratory conditions - multi-choice), but time restrictions did not allow testing in semi-field conditions as had been planned. The compounds showing repellence activity were benzaldehyde, eugenol, ethanol, ethyl isovalerate, phenethyl acetate, isoamyl lactate, 1-octen-3-ol, ethyl caproate, limonene, p-cymene, and valeric acid. The use of compounds with a repellent effect for untargeted insect capture would allow the design of more selective innovative trapping concepts for plant protection technologies. The final goal of this chapter is to improve the attractiveness of baits for *D. suzukii* and develop new trapping systems for early detection and management of this pest. The

conditions and substrates required to maximise trapping efficiency will be selected based on the potential future use of baits and VOCs under field conditions, based on the knowledge obtained from experiments conducted in **Chapters 3** and **4**.

The olfactory response of *Trichopria drosophilae* showed significant attractiveness towards x1000 diluted wild type and overexpression strains of *Saccharomyces cerevisiae*

Mutualism between microbes and flying insects is a well-established evolutionary system in which microbes produce volatile organic compounds that attract their insect vectors, and insects benefit from a rich protein source in return. Although studies of microbes and insect mutualism have led to understanding of the importance of chemical signalling between these two trophic levels, there has not been sufficient investigation of the ecological role of these same chemical cues on higher trophic levels. Furthermore, studies across trophic levels are needed to understand the importance of specific volatile organic compounds and the way they mediate the behaviour of parasitoids and predators. We therefore focused our attention on investigating the importance of *Saccharomyces cerevisiae* produced volatiles for the drosophila generalist endoparasitoid *Trichopria drosophilae*. We used a null mutant of *Saccharomyces cerevisiae* with the AFT1 gene knocked out, an ATF1 overexpression mutant and the wild type Y182 of *S. cerevisiae*. Strains were grown in 10% YPD broth and fermented for seven days. Volatile profiles were determined with gas chromatography and the use of a mass spectra detector. Parasitoid behaviour was tested in Y-tube olfactometer. We showed that *Saccharomyces cerevisiae* olfactory cues mediate *T. drosophilae* attraction. These findings could be utilised in integrated pest management, where biological control of insect pests is a crucial component.

6.2 Perspectives

Overall, this PhD thesis provides a better understanding of the behaviour of *Drosophila suzukii* and the effects of mVOCs on its attraction. Further, this PhD study has provided new insight into chemical communication between bacteria, *drosophila suzukii* and the parasitoid *Trichopria drosophilae*. The knowledge obtained allows improved IPM control, using microbe-based insect semiochemicals and an innovative trap design to lure and suppress a decreasing number of SWD in the crop, and leads to increased understanding of the effects of key VOCs that govern behaviour across trophic levels. However, additional research is required to improve and simplify the trapping system and mVOC application, together with other essential cues for SWD. A number of potential routes deriving from previous studies and our own studies are outlined below, to suggest ideas for further investigation.

6.2.1 Limitation and challenges of insect semiochemicals in IPM for *Drosophila suzukii*

The chemical environment inhabited by insects is quite complex and dynamic, and depends on many factors. The overall picture of interaction between organisms in a given ecological system should be carefully examined. Ecological system interaction can be compared to a symphony orchestra (Van Der Putten, 2017), where each player has a unique role. Ecological and scientific observation should focus on the role of each main player in an ecosystem; host plants, the microbiome of the plant surroundings and soil, insect herbivores, sap-sucking feeders, various dipterous pests, and their friends and foes. Semiochemicals and infochemicals convey different biological and ecological information to organisms in a certain environment. Inner and intraspecific relations between organisms are arranged on different trophic levels. Plants are the first trophic level, while herbivores, sap-sacking insects, and fruit flies (e.g. *Drosophila suzukii*), for instance, are the second trophic level. Parasitoids, predators of the second trophic level, belong to third trophic level, whereas the enemies of parasitoids and predators represent the next

trophic level (Marcel Dicke & Baldwin, 2010; Harvey, Gols, Vet, & Kruidhof, 2012; Vet & Dicke, 1992). Differing conditions and the environment influence the way semiochemicals convey information and its function and efficacy.

Selection pressure of the intricate environment and convoluted interactions among different trophic levels force insects to adapt to diverse, dynamic changes. Infochemicals, a semiochemical subcategory, convey chemical information, and are the primary mode of interaction between trophic levels. Insects have evolved sensory systems and nervous systems to locate suitable host plants or hosts (Galizia & Sachse, 2010). The function and ability of infochemicals to convey information are affected not just by abiotic environmental conditions, but also by biotic interaction between all the participants in a given niche (Aartsma et al., 2017). Complex odour bouquets from a plant, the plant epiphytic microbiome, within trophic levels can convey information that is positive for insects and their parasitoids, negative for both or negative to just one trophic level (Vet & Dicke, 1992). Insects are well-designed to detect chemical cues through spatial and temporal resolution of the natural environment. Understanding chemical ecology and the evolution of plant-insect interaction in the context of a multitrophic perspective is a challenging task. More than half a century has passed since the discovery of the sex pheromone Bombykol released by the female silkworm moth and chemical ecology research. Yet, there is still no knowledge of fully understood interaction mediated by chemical cues for any natural enemy or invasive insect species (Vet & Dicke, 1992). Obtaining more knowledge from multitrophic interaction in the natural environment and revealing the evolutionary mechanism and patterns will provide information to be used in biological control.

Primary and secondary metabolites in plants change according to many factors—herbivore attack changes plant VOC emission (Kaplan, 2012). Whether individual compounds or compound mixture are relevant for the attraction of insects depends on ways of perceiving olfactory cues, which have evolved differently in different species (Meiners, 2015). Background odours may indicate the presence of a host and help in habitat searching.

Insects have developed a sensitive olfactory system to locate a suitable habitat or host (Bruce & Pickett, 2011). Insects have a spatial-temporal resolution of the synchronous firing of ORNs tuned to several VOCs, and the olfactory system can detect relevant odour cues in a complex environment, despite background noise. The insect olfactory system has extremely high sensitivity and specificity for certain volatiles (Hansson, 1999) and specific ORNs tuned to specific compounds (Hansson & Anton, 2000). This highly specialised olfactory system enables the insect to function in the midst of a chaotic mixture of diverse odours in the environment. It is now known that most insects recognise plants by detecting blends of ubiquitous compounds (Bruce et al., 2005; Najar-Rodriguez et al., 2010). However, many insects respond to typical compounds that are attractive across different taxonomic groups. The question is whether some volatile compounds are redundant, independently of the emitting source.

A decade of research on *D. suzukii* has revealed that a combination of volatiles is more attractive and gives better behavioural responses than a single tested compound. The suggestion is that outside the context of blends, single compounds are perceived as non-host cues. A combination of volatiles is essential for insects in general. Quantitative and qualitative differences in the blend and composition can have a significant effect on insect behavioural responses.

Lately, more studies on *D. suzukii* have focused on so-called redundancy. Redundancy means that not all the components in a blend are essential for attraction, and that different orders of magnitude in the ratio between compounds may be perceived in the same way without changing insect behaviour (Cha et al., 2014b; Feng et al., 2018; Najar-Rodriguez et al., 2010). Furthermore, compounds that are part of a blend in different concentrations, for instance in high doses, may act as a repellent. Blends and volatile plumes allow insects not only to distinguish host plant species from non-host species, but also to recognise unsuitable habitats. The composition of volatile blends helps with host recognition and learning ability for future response. Plant or fruit odour is perceived differently according to the insect's different physiological

stages, and the different phenological status of plants (De Moraes et al., 1998). *D. suzukii* is a real example of this.

Insect responses and behaviour mediated by mVOCs represent learned behaviour (Sobhy et al., 2019) and a genetically conserved trait (Becher, Hagman, et al., 2018).

6.2.2 Potential application of the research results to improve IPM for *Drosophila suzukii*

Agriculture must be prepared to deal with the problem of invasive insect species, following increasing shifting of ecological niches resulting from climate change. *Dropshila suzukii* is one of the invasive species causing the most substantial economic losses to farmers and soft fruit production. In the context of IPM and its practical application, a holistic approach is required in dealing with each invasive species. Invasive species can be combated, and their number brought down to low economic thresholds, when different strategies are applied simultaneously.

This study has resulted in a new trap design, as demonstrated in **Chapter 3.** This could be scaled up and deal more efficiently with *Dropshia suzukii* than other currently available traps. It is particularly suitable and easy to use for big farms, because there is no need to apply homemade liquid attractant. One major challenge with traps for invasive insects is still selectivity and the attraction of non-target organisms inside the traps. In **Chapter 5,** we found that crucial acetate esters present in the Droskidrink® wine-vinegar solution are also emitted as infochemicals from *Saccharomyces cerevisiae* yeast and in laboratory conditions are highly attractive to one of most effective *Drosophila* spp. parasitoids, *Trichopria drosophilae*. These findings show why *T. drosophilae* is also found as a non-target species in traps for *Dropshila suzukii*.

A slow-release dispenser could be an alternative and lasting solution. The technology behind designing a dispenser to be placed inside the delta trap should be considered. Volatiles selectively attractive to SWD could aid in the monitoring and early discovery of SWD in the field after overwinter time. Narrowing down the number of effective, attractive compounds should lead to greater efficiency and selectivity of the trap.

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Appendix

Appendix to Chapter 1: No supplementary files for Chapter

Appendix to Chapter 2:

Table A 2.1: Field assessment of different lactic acid bacteria species and strains. Number of SWD caught per trap per day for each of the treatments for three successive trials (95% confidence limits).

Trial	Treat	Male	Female	Total	%female
1	A	20.2 (15.0,27.2)	15.9 (11.2,22.6)	36.1 (27.8,47.0)	44.0 (35.1,53.3)
	B	11.0 (7.4,16.5)	12.0 (8.0,17.9)	23.0 (16.6,31.9)	52.0 (40.4,63.3)
	C	7.8 (4.8,12.6)	7.7 (4.7,12.7)	15.5 (10.4,23.1)	49.7 (35.9,63.5)
	D	11.0 (7.4,16.5)	9.9 (6.4,15.4)	21.0 (14.9,29.6)	47.3 (35.5,59.4)
	E	12.7 (8.7,18.5)	11.2 (7.4,17.0)	23.9 (17.3,33.0)	46.8 (35.8,58.2)
	F	9.3 (6.0,14.5)	10.2 (6.6,15.8)	19.5 (13.7,27.9)	52.2 (39.7,64.4)
2	A	13.1 (8.0,21.6)	9.0 (5.6,14.3)	22.1 (13.7,35.6)	40.6 (36.3,45.0)
	B	11.7 (6.9,19.8)	7.6 (4.6,12.6)	19.3 (11.6,32.2)	39.3 (34.8,44.1)
	C	3.9 (1.6,9.8)	4.5 (2.3,8.7)	8.4 (3.9,18.2)	53.3 (46.1,60.4)
	D	6.7 (3.4,13.5)	5.3 (2.9,9.7)	12.0 (6.3,23.0)	44.0 (38.2,50.0)
	E	11.4 (6.7,19.5)	6.8 (4.0,11.7)	18.3 (10.8,30.9)	37.3 (32.7,42.1)
	F	9.5 (5.3,17.0)	6.4 (3.7,11.1)	15.9 (9.0,27.8)	40.2 (35.2,45.4)
	G	1.4 (0.3,6.4)	0.9 (0.2,3.9)	2.2 (0.5,10.0)	38.3 (25.9,52.5)
3	A	40.6 (25.4,64.8)	38.0 (26.1,55.3)	78.6 (51.7,119.5)	48.3 (44.6,52.1)
	B	30.7 (17.9,52.6)	21.4 (13.0,35.3)	52.1 (31.1,87.2)	41.0 (36.6,45.6)
	C	22.0 (11.7,41.6)	15.0 (8.2,27.2)	37.0 (20.1,68.2)	40.4 (35.2,45.9)
	D	25.5 (14.1,46.0)	19.0 (11.2,32.4)	44.5 (25.5,77.7)	42.8 (37.9,47.8)
	E	33.8 (20.2,56.4)	20.5 (12.3,34.2)	54.2 (32.7,89.8)	37.8 (33.5,42.2)
	F	22.9 (12.3,42.7)	15.0 (8.2,27.2)	37.8 (20.7,69.2)	39.5 (34.4,44.9)
	G	13.1 (5.7,29.8)	10.0 (4.8,20.8)	23.0 (10.6,49.9)	43.2 (36.5,50.2)
	H	3.2 (0.6,16.9)	2.3 (0.5,10.6)	5.5 (1.1,26.8)	42.4 (29.3,56.7)
	I	8.5 (3.1,23.5)	8.0 (3.5,18.1)	16.5 (6.6,41.1)	48.6 (40.5,56.7)
	L	5.5 (1.6,19.7)	3.9 (1.2,12.7)	9.5 (2.8,31.7)	41.5 (31.3,52.4)

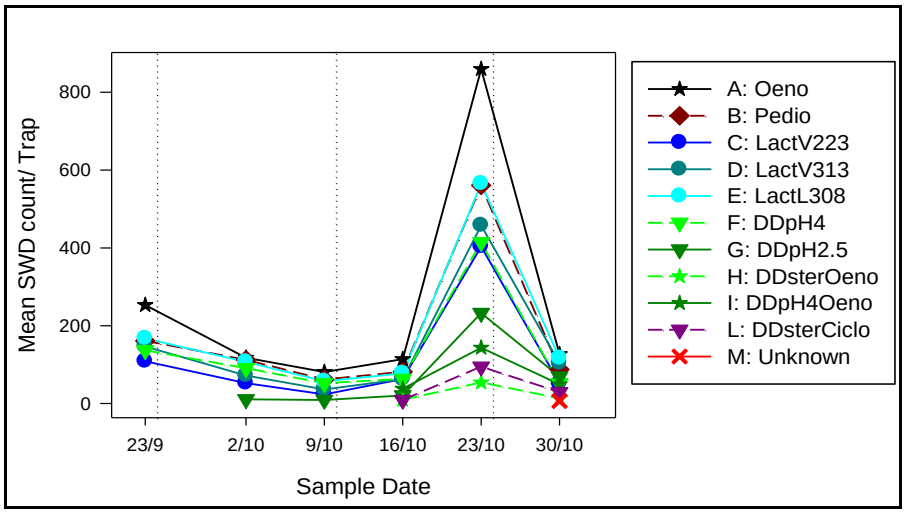


Figure A 2.1: Mean number of *D. suzukii* catches in different baited traps, in three trials conducted for comparing the attractiveness of Droskidrink food baits inoculated with different lactic acid bacteria in a commercial vineyard.

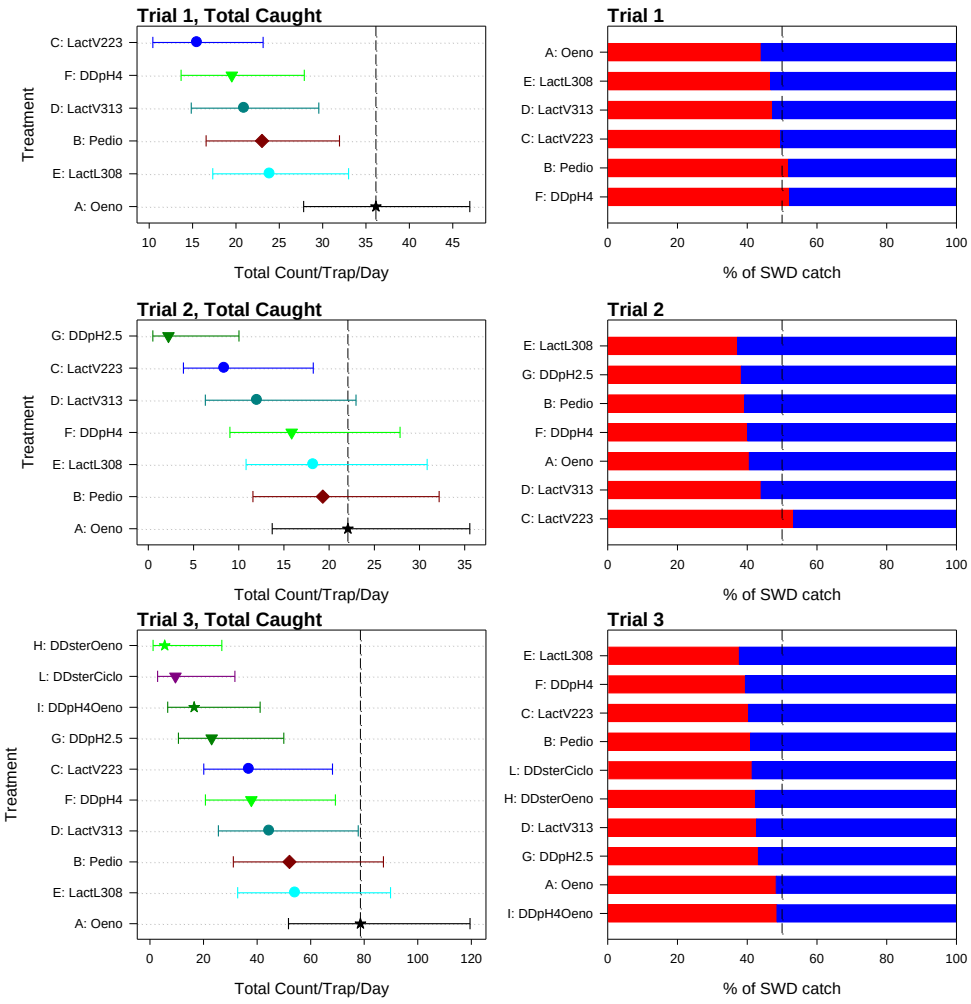


Figure A 2.2: Left: Mean total SWD caught per trap per day for the three trials (including the final assessment, trial 3), with treatment in increasing order of the mean. Error bars are 95% confidence limits, and vertcal dshed line is at the mean for treatment A. Right: percentage of the total SWD that were female or male, for each of the three trials. Treatments are in increasing order of the % female. Dashed vertical line is at 50%.

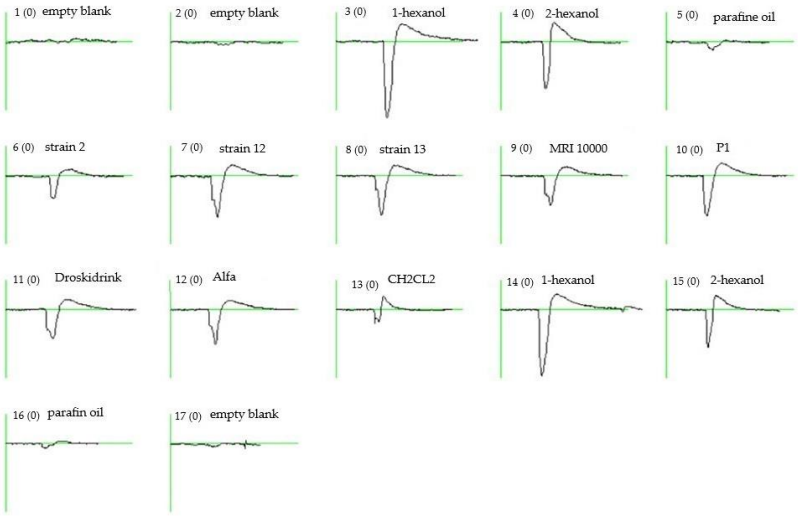


Figure A 2.3: Electroantennogram plots recorded from SWD antenna, in response to volatile collection of O. oeni strains in Droskidrink® Bait. Control stimuli: empty blank, paraffin oil, and dichloromethane solvent. Reference compounds: 1-hexanol, 2-hexanal. Baits: commercial DD, O. oeni reference strain (MRI 10000), strain 2, strain 12, and strain 13.

Table A 2.2: Assessment of *O. oeni* strains' growth in Droskidrink® bait. Differences in the mean absorbance of bacterial growth in MRSm medium considering the main DD limiting parameters for *O. oeni* growth (pH 4.0), and ethanol (4%), acetic acid (45 g/L) at temperature (15 °C).

	pH (4.0)	Acetic acid (45 g/l)	Ethanol (4%)	Temperature (15°C)
Strain 1	0.075	-0.005	-0.125	-0.044
Strain 2	0.091	0.025	-0.011	-0.018
Strain 3	-0.002	0.003	0.042	0.017
Strain 4	0.092	-0.020	0.022	-0.052
Strain 5	0.144	0.029	0.019	0.099
Strain 6	0.130	0.032	-0.067	-0.067
Strain 7	0.068	-0.008	-0.014	0.040
Strain 8	0.046	-0.029	-0.083	-0.032
Strain 9	-0.008	0.007	0.119	-0.026
Strain 10	0.083	-0.005	0.130	-0.058
Strain 11	0.081	0.017	0.187	0.003
Strain 12	0.117	-0.001	0.164	-0.009
Strain 13	-0.008	-0.003	0.205	-0.014
Strain 14	0.079	0.000	0.164	-0.022
Control	-0.990	-0.043	-0.755	-1.403

Table A 2.3: Electroantennography responses of SWD females to volatile collection of *O. oeni* strains in Droskidrink® Bait. Mean responses (mV) of mated female antennae elicited by commercial DD and DD inoculated with different O. oeni strain 2, strain 12 and strain 13. Control stimuli: empty blank, paraffin oil, and dichloromethane solvent. Reference compounds: 1-hexanol, 2-hexanal. Baits: commercial DD, O. oeni reference strain (MRI 10000), strain 2, strain 12, and strain 13. The standard deviation of the means is reported.

Samples	Mean	SDV
Empty blank	0.15	0.06
1-Hexanol	5.80	1.35
2-Hexanal	3.51	1.14
Paraffin oil	0.79	0.37
Dichloromethane	1.33	0.76
O. oeni strain 2	3.71	0.36
O. oeni strain 12	3.46	0.35
O. oeni strain 13	3.52	0.30
MRI 10000	3.30	0.34
Droskidrink	3.19	0.02

Appendix to Chapter 3:

Picture A 3.1. Trap types: C- Red cup with a white lid; DIB- Delta trap with an insulated bottle; DC- Delta trap with a cup without a lid, with two cotton balls.

In the field trials, we wanted to determine which combination of O. oeni strain and trap performed best. In order to create an optimum environment for colonization and reproduction of the bacteria, the pH of the liquid lure was raised to 3.8 using potassium hydroxide (KOH) (monohydrate granular AR [ARS]). Eight treatments were tested in Trial 1.

Trial 1: Treatment A; Droskidrink (DD) natural pH 2.6 in cup shaped trap., B; DD with ajusted pH up to 3.8 in cup shaped trap., C; Che-Landolt solution (Pherocon SWD dispenser, Trécé Inc., Adair, OK, USA) in cup shape trap., D; DD with pH 3.8 with addition of O.oeni strain Alpha in cup shaped trap., E; DD with pH 3.8 with addition of O.oeni strain Beta in cup shaped trap., F; DD with pH 3.8 with addition of O. oeni strain Beta, citric acid in cup shaped trap., G; DD with pH 3.8 with addition of O. oeni strain Beta in DIB trap design., H; DD with pH 3.8, addition of *O. oeni* strain Beta in delta trap with uninsulated bottle (DUB) trap design. All treatments had 200 ml of liquid tested solution.

Trial 2: In addition, the amount of bacterial inoculum used for the preparation of the treatments was reduced from 0.5 g to 0.2 g L^{-1}. The fermentation of DD by and O. oeni was conducted in the laboratory for one week prior to the field trials under a controlled temperature of 22 ± 2°C. Eight treatments were tested. Treatment A; DD with pH 2.6 in cup shaped trap., B; DD with ajusted pH up to 3.8 in cup shaped trap., C; Che-Landolt solution (Pherocon SWD dispenser, Trécé Inc., Adair, OK, USA) in cup shape trap., D; DD with pH 3.8 with addition of *O.oeni* strain Alpha in cup shaped trap., E; DD with pH 3.8 with addition of *O. oeni* strain Beta, citric acid in cup shaped trap., G; DD with pH 3.8, addition of O. oeni strain Beta in delta trap with a cup (DC a) without lead and 20 ml amount of liquid in the cup., H; DD with pH 3.8, addition of *O. oeni* strain Beta in delta trap with DCb trap design with 15 ml amount of liquid on two cotton balls. Treatments from A to F had 200 ml of tested baits, while treatment G had just 20 ml and treatment H 15 ml amount of liquid.

Trial 3: Trial 3 was the last phase of the fieldwork and involved the assessment and development of a different trap as well as the improvement of traps used in previous trials. This trial used six treatments. Treatment A; DD with natural pH 2.6 in cup shaped trap., B; DD with ajusted pH up to 3.8 in cup shaped trap., C; Che-Landolt solution (Pherocon SWD dispenser, Trécé Inc., Adair, OK, USA) in cup shape trap., D; DD with pH 3.8 with addition of O.oeni strain Alpha in DC b shaped trap 15 ml of liquid on two cotton balls., E; DD with pH 3.8, addition of *O. oeni* strain Beta in delta trap with DC b trap design with 15 ml amount of liquid on two cotton balls., F; DD with pH 3.8, addition of O. oeni strain Beta in delta trap with a cup DC b without lead and 15 ml amount of liquid in the cup and citric acid.

Table A 3.1: Trapping experiments with fermentation in open field

Mean counts of *Drosophila suzukii* per day per trap, and % female capture (95% confidence limits)

Trial	Treatment	Male	Female	Total	%Female
Trial 1					
	A: Cw	39.0 (31.5,48.3)	14.5 (11.6,18.1)	53.5 (43.2,66.2)	27.1 (25.5,28.7)
	B: Kw	23.8 (18.1,31.3)	11.2 (8.7,14.4)	35.0 (26.9,45.5)	32.0 (29.9,34.1)
	C: ChLn	0.2 (0.0,3.8)	0.2 (0.0,1.4)	0.4 (0.0,4.8)	41.5 (22.3,63.6)
	D: ⊚⊚Kn	6.6 (3.9,11.1)	3.5 (2.2,5.5)	10.1 (6.2,16.4)	34.5 (30.5,38.6)
	E: ⊚5Kn	16.8 (12.1,23.2)	7.9 (5.8,10.7)	24.6 (18.0,33.7)	32.0 (29.5,34.6)
	F: ⊚⊚KnCA	13.0 (9.0,18.8)	6.4 (4.5,8.9)	19.3 (13.6,27.6)	32.9 (30.1,35.9)
	G: ⊚5K⊚IBn	0.7 (0.2,3.5)	0.8 (0.3,2.1)	1.5 (0.4,5.4)	52.0 (41.3,62.6)
	H: ⊚⊚K⊚UBn	0.7 (0.1,3.4)	0.6 (0.2,1.8)	1.3 (0.3,5.1)	48.0 (36.6,59.6)
Trial 2					
	A: Cw	23.1 (15.1,35.3)	14.9 (10.0,22.2)	37.9 (25.2,57.1)	39.2 (35.9,42.5)
	B: Kw	30.0 (20.7,43.6)	18.8 (13.1,26.8)	48.8 (34.0,70.0)	38.4 (35.6,41.4)
	C: ChLw	0.4 (0.0,9.5)	0.2 (0.0,5.8)	0.7 (0.0,14.7)	33.8 (14.7,60.1)
	D: ⊚2Kw	16.7 (10.1,27.4)	11.4 (7.2,18.0)	28.0 (17.4,45.1)	40.6 (36.8,44.5)
	E: ⊚2Kw	32.1 (22.4,45.9)	20.5 (14.6,28.9)	52.6 (37.2,74.5)	39.1 (36.3,41.9)
	F: ⊚2KwCA	22.0 (14.3,34.0)	14.8 (10.0,22.1)	36.9 (24.3,55.8)	40.3 (37.0,43.7)
	G: ⊚5K⊚Cw	6.4 (2.9,14.4)	7.2 (4.0,12.8)	13.6 (6.9,27.0)	52.7 (47.0,58.3)
	H: ⊚2K⊚Cw	9.9 (5.1,18.9)	10.6 (6.6,17.0)	20.4 (11.7,35.7)	51.7 (47.1,56.3)
Trial 3					
	A: Cn	2.8 (1.8,4.2)	4.2 (2.8,6.3)	6.9 (4.6,10.4)	60.2 (56.1,64.2)
	B: Kn	3.9 (2.7,5.5)	4.4 (3.0,6.6)	8.3 (5.7,12.0)	53.4 (49.7,57.2)
	C: ChLn	0.0 (0.0,*)	0.0 (0.0,*)	0.0 (0.0,*)	100.0 (*,100.0)
	D: ⊚2K⊚n	6.1 (4.6,8.1)	9.6 (7.4,12.6)	15.7 (12.0,20.7)	61.2 (58.5,63.8)
	E: ⊚2K⊚n	10.7 (8.6,13.3)	18.1 (14.9,22.0)	28.8 (23.6,35.2)	62.8 (60.8,64.7)
	F: ⊚ 2K⊚nCA	7.9 (6.1,10.1)	12.7 (10.0,16.0)	20.5 (16.2,26.0)	61.7 (59.3,64.0)

Figure A 3.1: Trapping experiments with fermentation in open field,

For each trial, for each treatment: Mean *Drosophila suzukii* catches per trap at each assessment (a, d, g); Dot plots of mean total catch trap^{-1} day^{-1} with 95% confidence limits, sorted by the means, with dotted vertical line at the mean for treatment A (control), (b, e, h), and percentage of the total catch that was female or male (c, f, i). Note that for h, the upper confidence limit for a mean of 0 is not shown as it is difficult to obtain. Treatment codes are as in Table 1: Traps are C cup with a lid; ⊚ delta trap; IB: insulated bottle; UB uninsulated bottle. Delta traps without a bottle have a cup without lid with two cotton balls. Liquid components are: K KOH added; ⊚ Alpha; ⊚ Beta; 2 rate 0.2; 5 rate 0.5; CA citric acid added. n: no Liquid Replacement; w: Liquid replaced weekly.

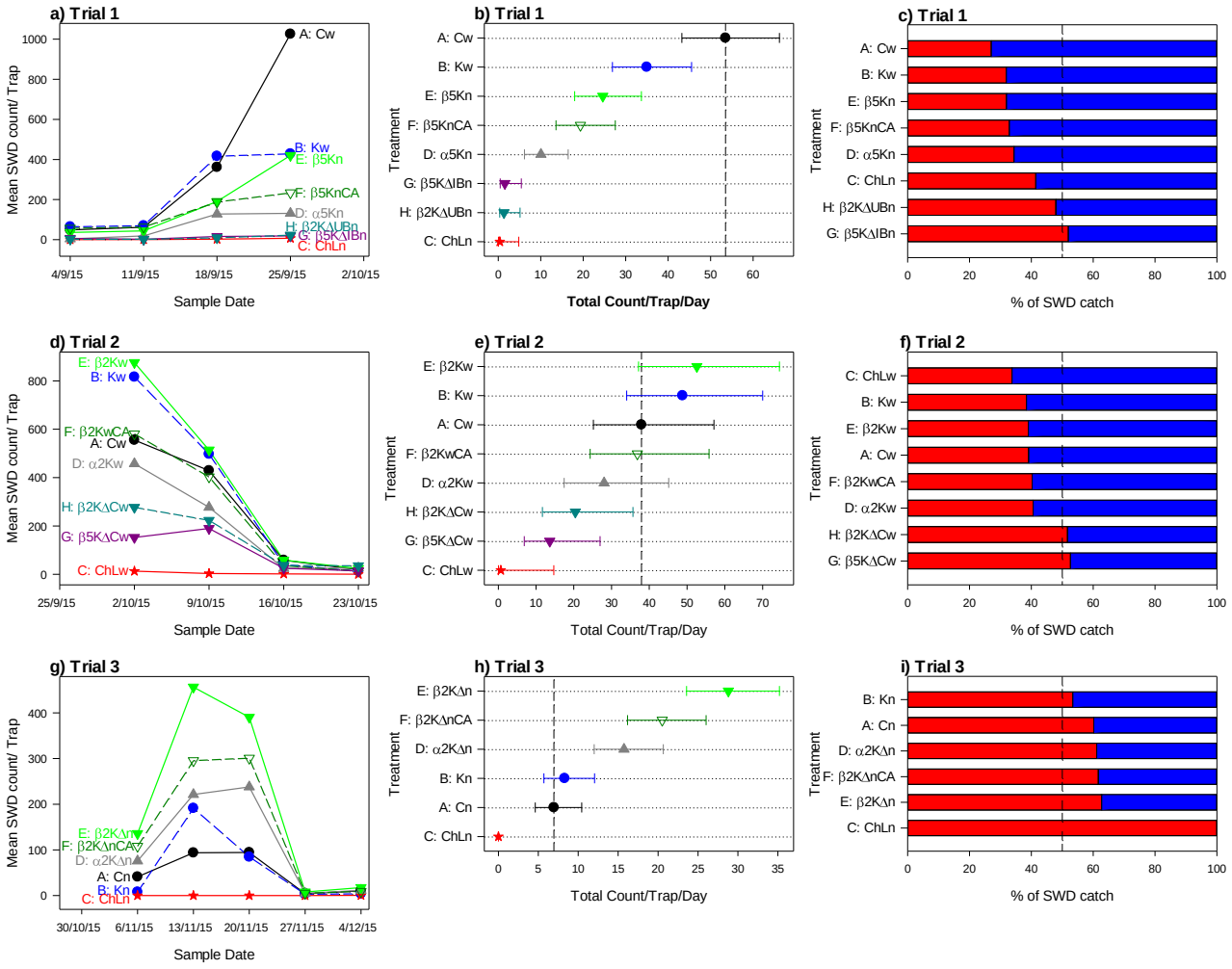
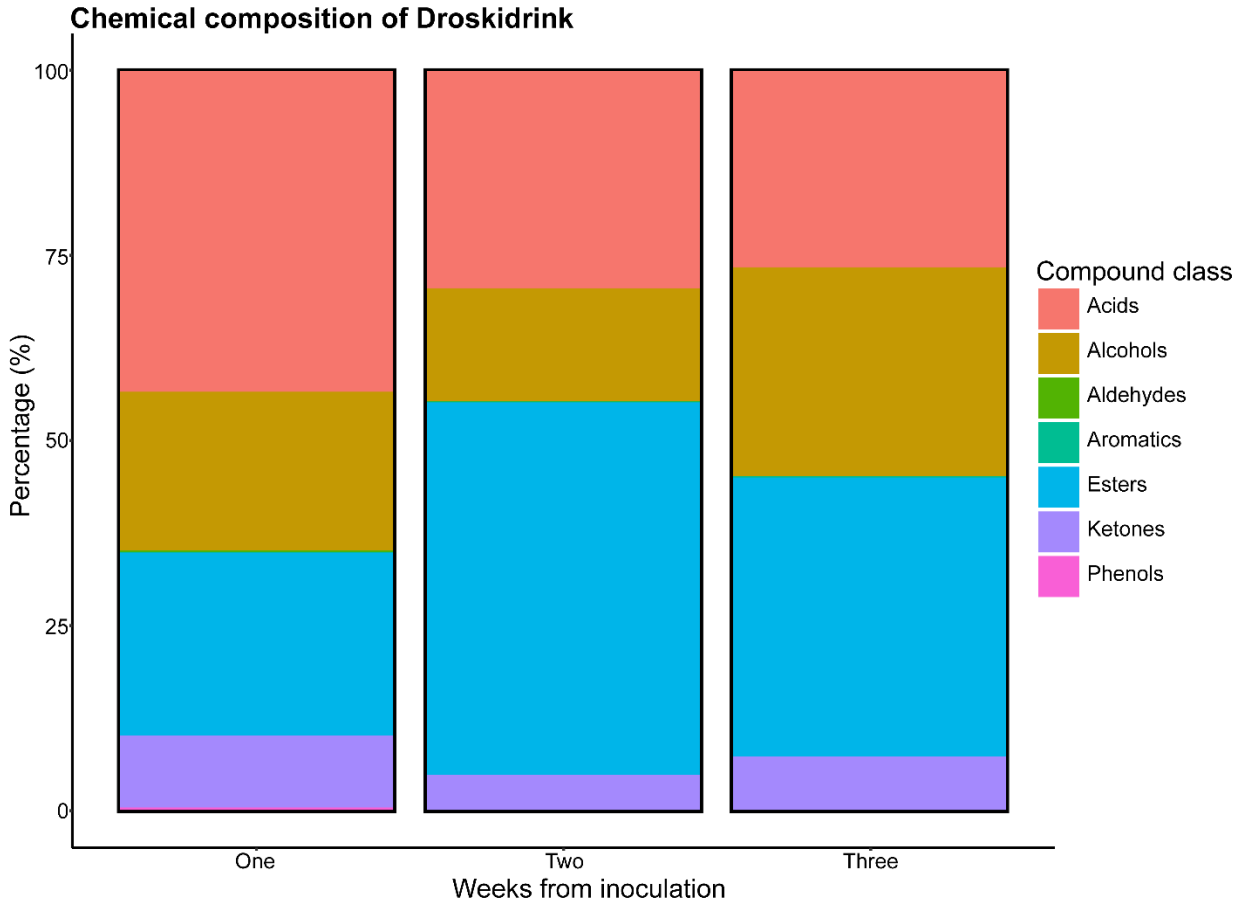
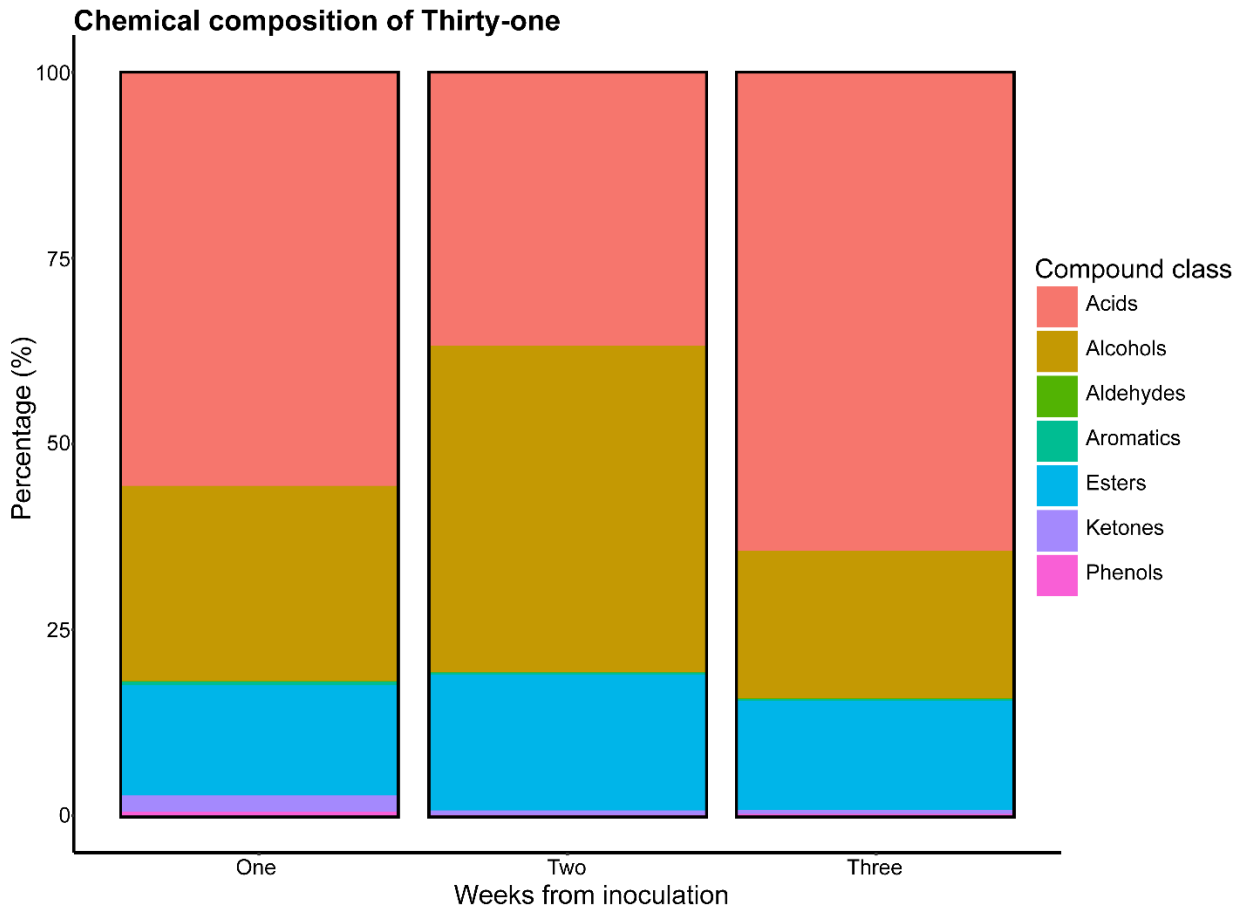


Figure A 3.2. a, b, c. Aligned analyses of the percentages were calculated by dividing the peak area of that compound over the total peak area of the compounds found, a) Droskidrink; b) Oenococcus oeni Strain 31; c) O. oeni strain PN4.



a


Chemical composition of Thirty-one
100
75
50
25
0
Percentage (%)
One
Two
Three
Weeks from inoculation
Compound class
Acids
Alcohols
Aldehydes
Aromatics
Esters
Ketones
Phenols

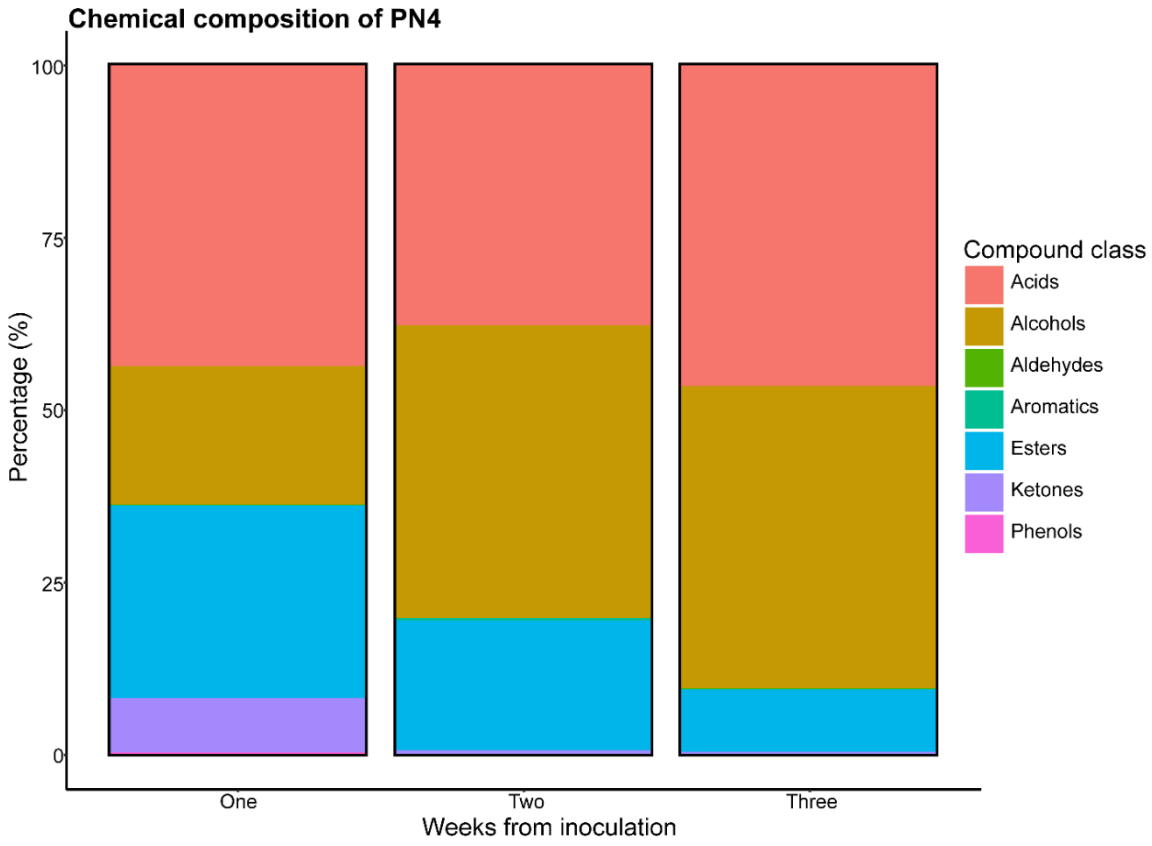
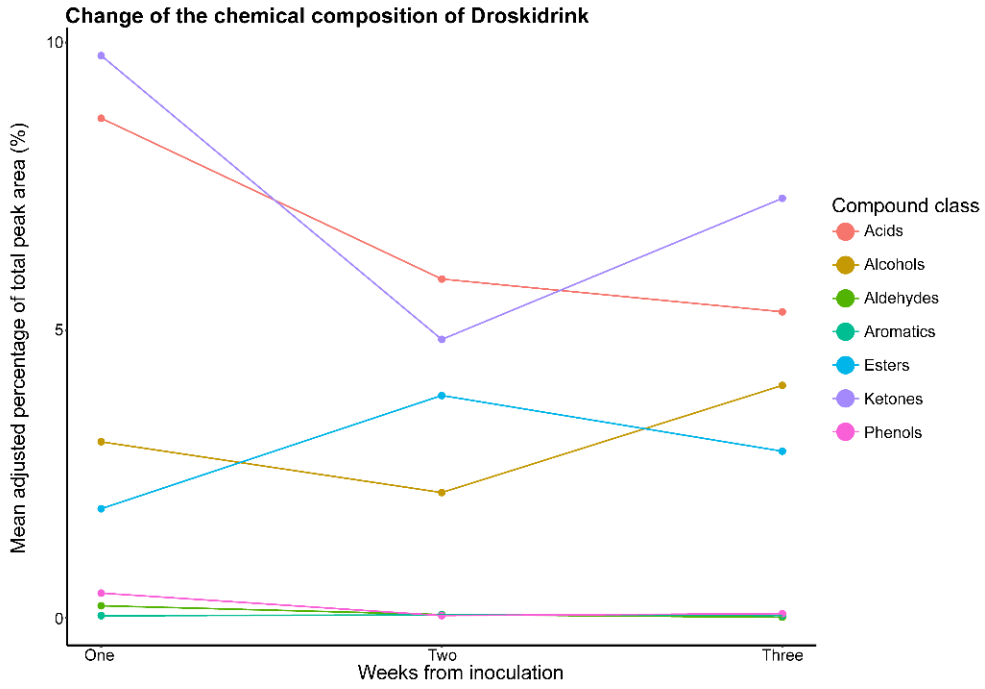
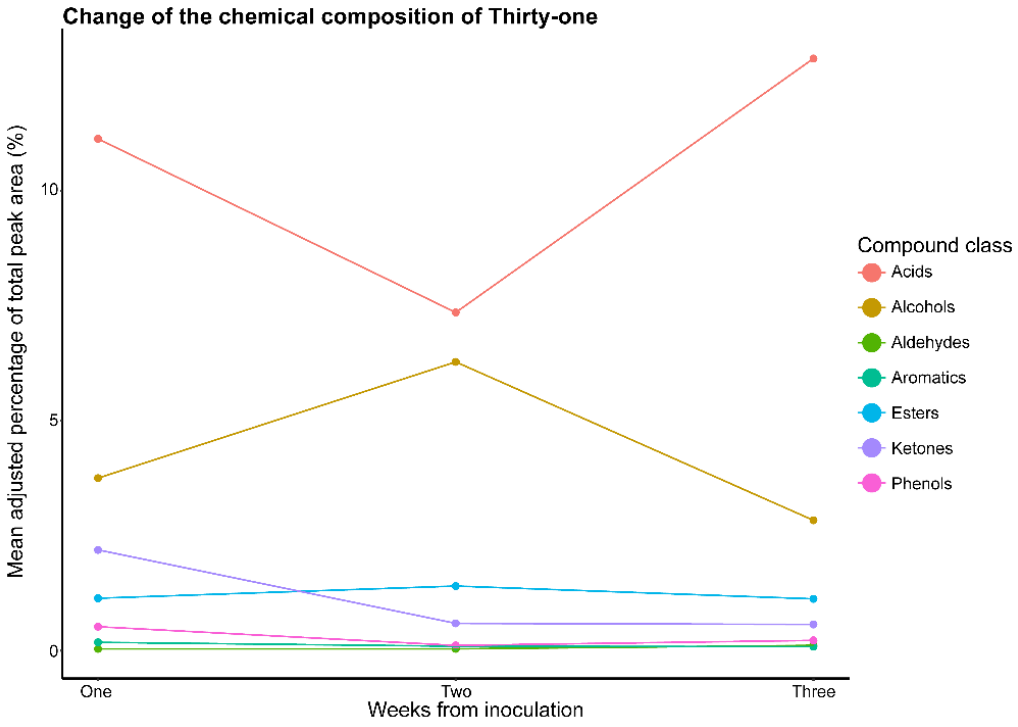

Chemical composition of PN4
100
75
50
25
0
Percentage (%)
One
Two
Three
Weeks from inoculation
Compound class
Acids
Alcohols
Aldehydes
Aromatics
Esters
Ketones
Phenols

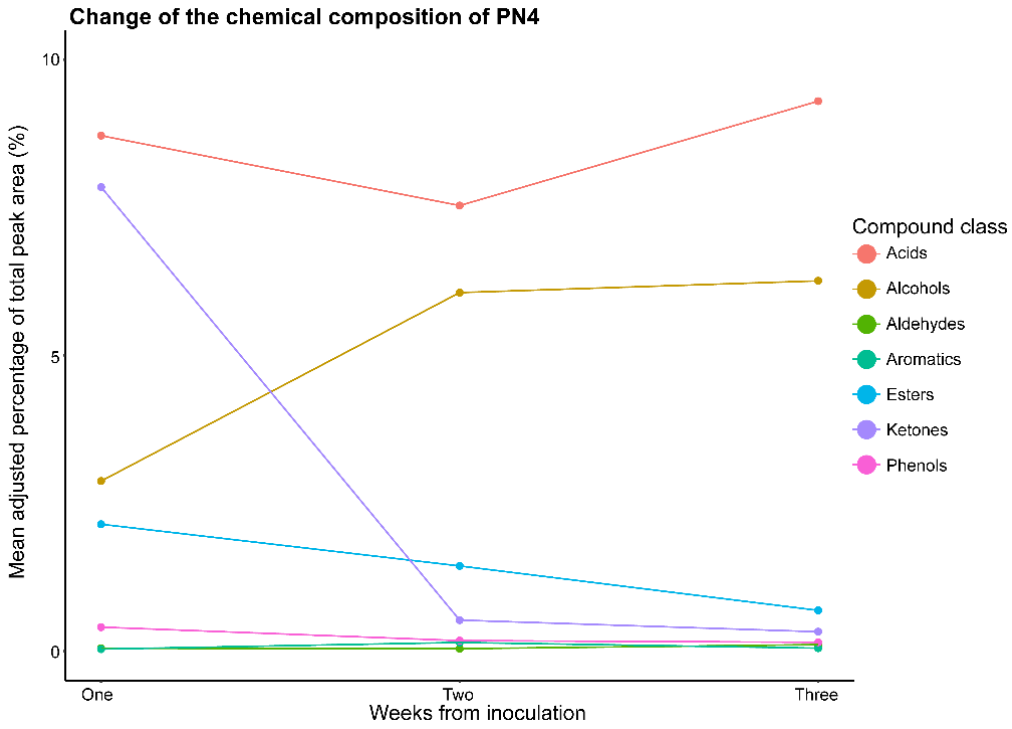
Figure A 3.3 a,b,c. Comparison with main chemical groups present in Droskidrink a, strain 31, strain PN4, and their change under influence of *Oenococcus oeni* and fermentation time. Compound belonging to same chemical group and their total peak area were compared between samples.



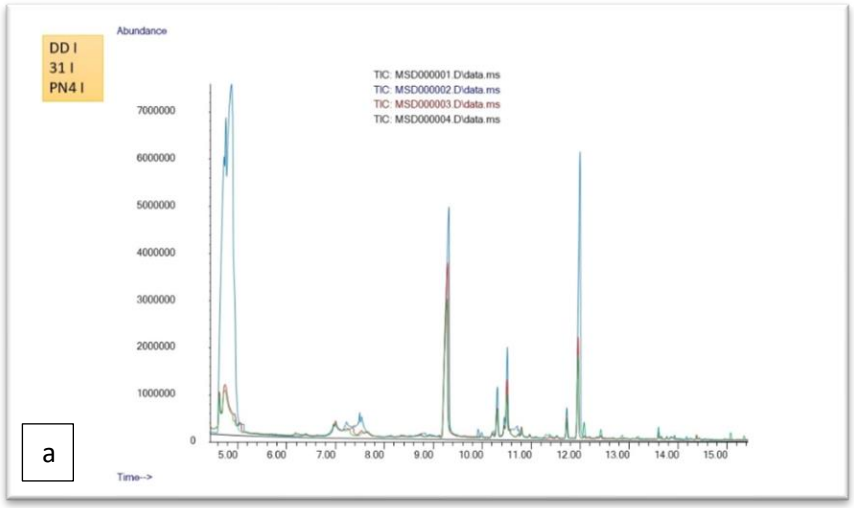
a

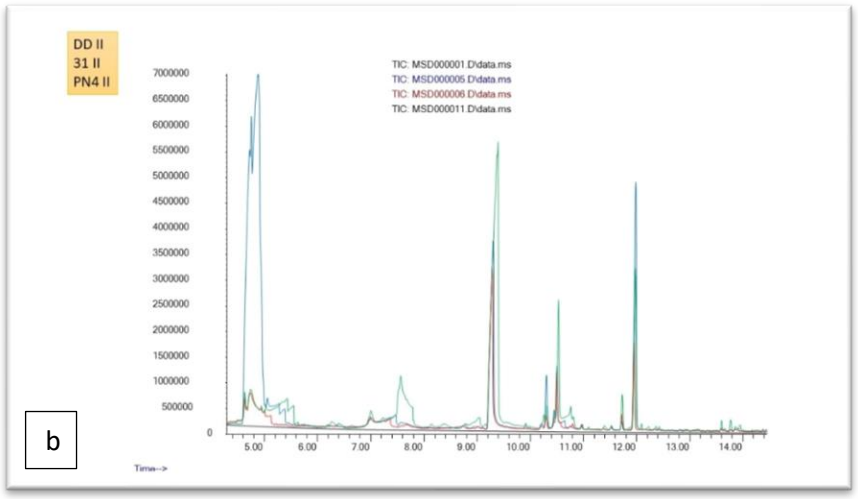


b


Change of the chemical composition of PN4
Mean adjusted percentage of total peak area (%)
10
5
0
One
Two
Three
Weeks from inoculation
Compound class
Acids
Alcohols
Aldehydes
Aromatics
Esters
Ketones
Phenols

c


DD I
31 I
PN4 I
Abundance
TIC: MSD000001.D\data.ms
TIC: MSD000002.D\data.ms
TIC: MSD000003.D\data.ms
TIC: MSD000004.D\data.ms
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
14.00
15.00
Time-->
a


DD II
31 II
PN4 II
TIC: MSD000001.D\data.ms
TIC: MSD000005.D\data.ms
TIC: MSD000006.D\data.ms
TIC: MSD000011.D\data.ms
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
14.00
Time-->
b

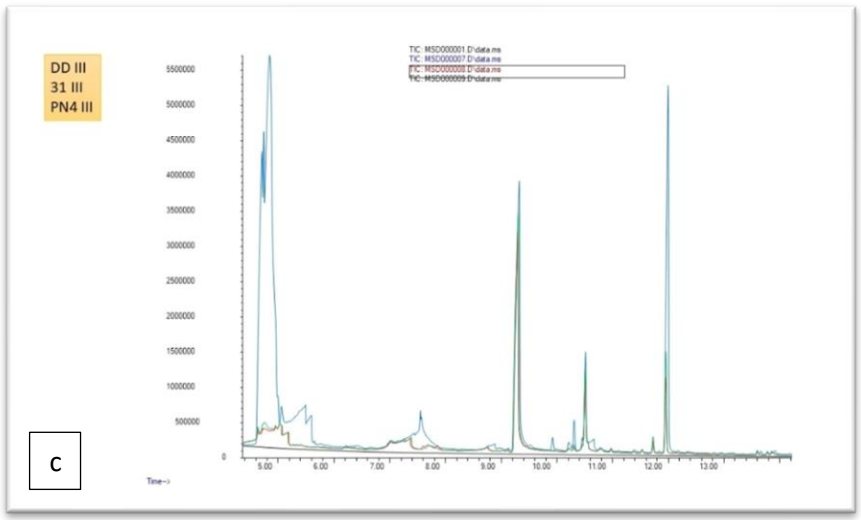
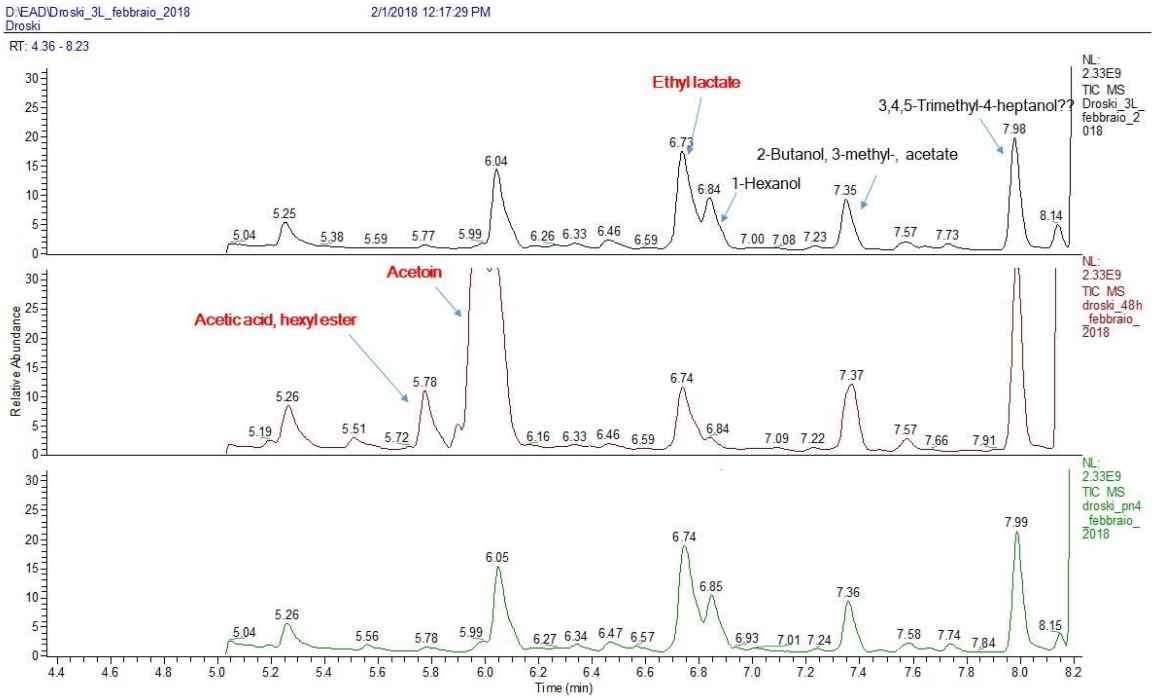

DD III
31 III
PN4 III
TIC: MSD000001.D\data.ms
TIC: MSD000007.D\data.ms
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
Time-->
c

Figure A 3.4. a, b, c. Aligned different CG-MS data chromatographs showing how different strains of lactic acid bacteria changing the volatile profile of the sample.

A) 1-week old headspace extracts. DD I: 1 week old Droskidrink; 31 I- with the addition of *Oenococcus oeni* strain 31; PN4 I- droskidrink with the addition of *O. oeni* strain PN4. B) 2-week old headspace extracts. DD II: 2 week old DD; 31 II: DD with the addition of *O. oeni* strain 31 2 week old; PN4 II: DD with the addition of *O. oeni* strain PN4 2 week old. C) 3-week old headspace extracts. DD III: 3-week old DD; 31 III: DD with the addition of *O. oeni* strain 31 3-week old, PN4 III: DD with the addition of *O. oeni* strain PN4 3-week old.



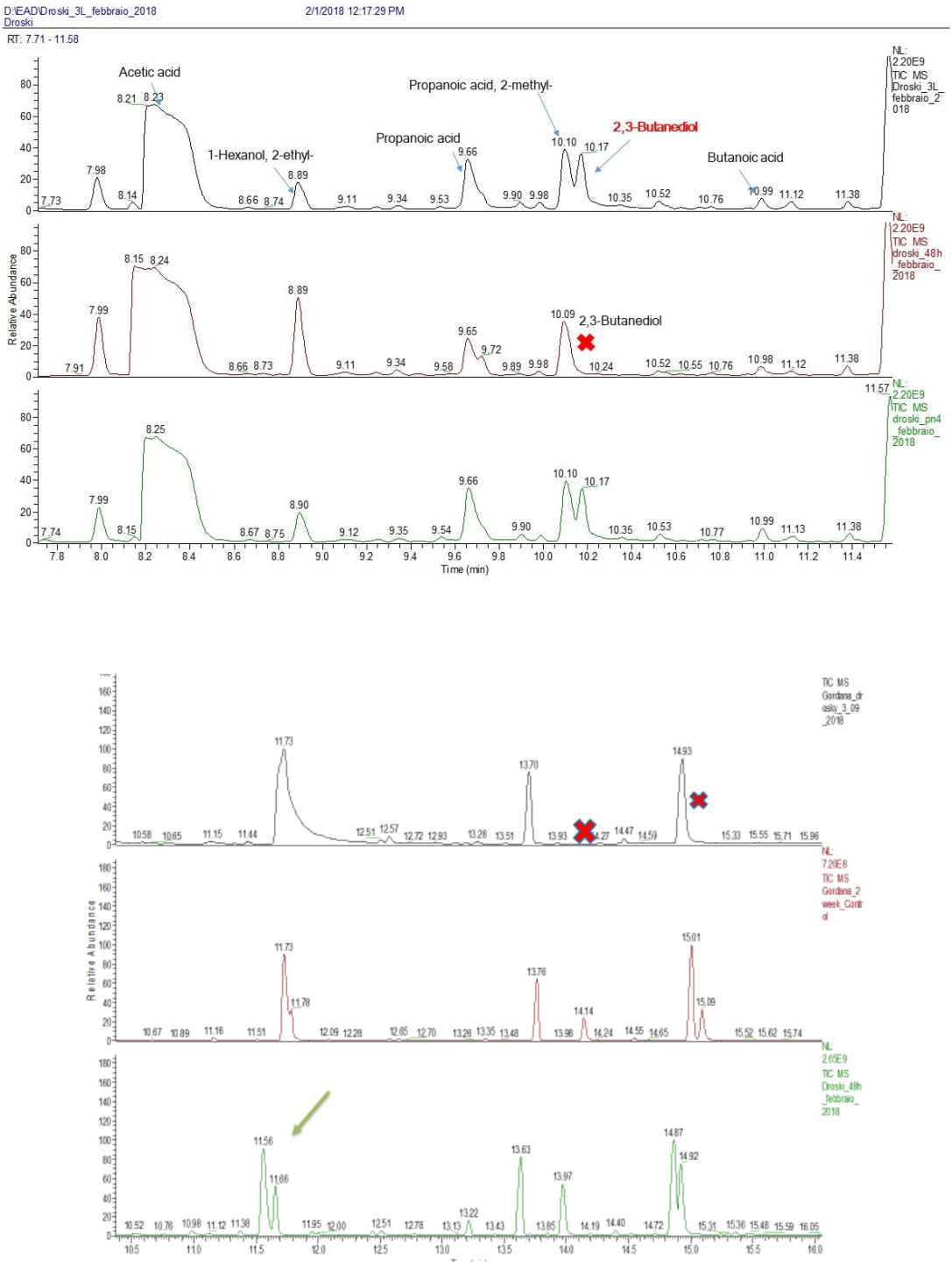
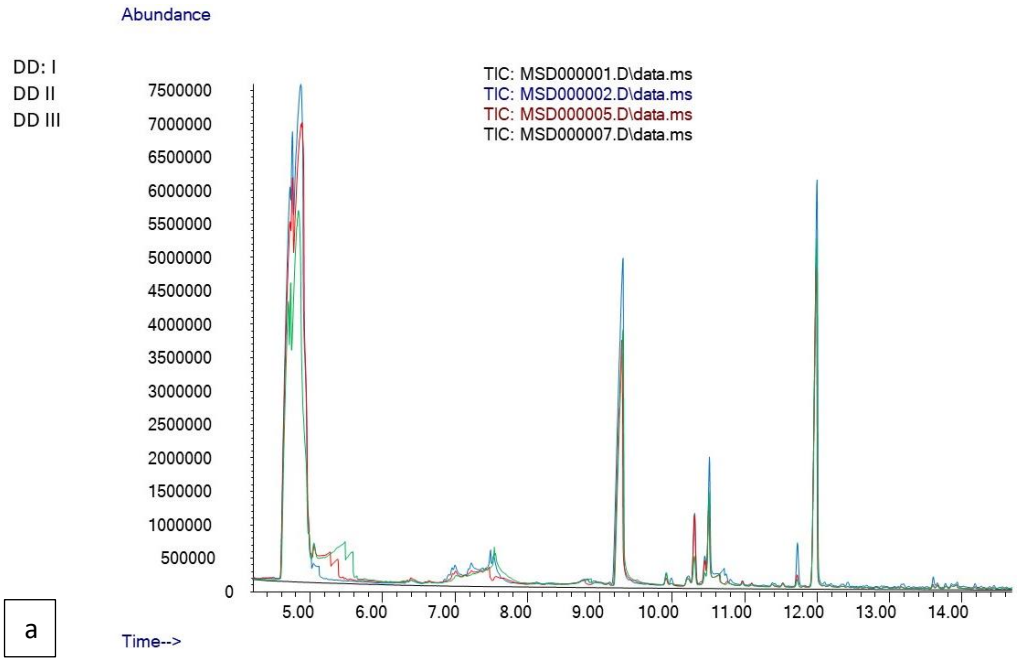


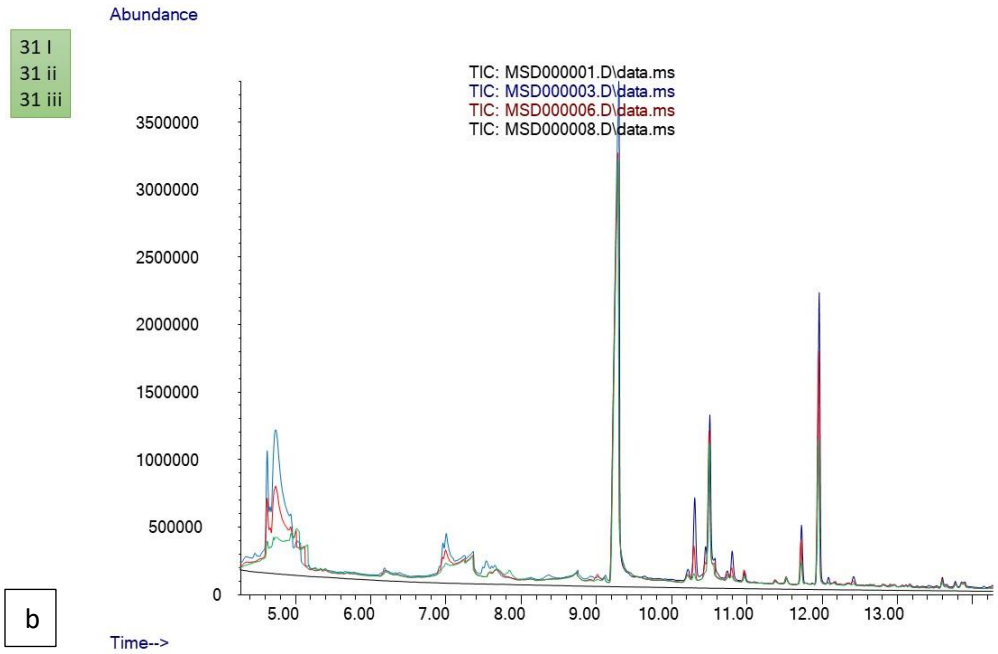
Figure A 3.5. GC-MS analyses from polar ZB-wax collum, with the addition of *O. Oeni* strain Pn4 and strain 31, visible VOCs profile changes in samples inoculated by two different *O. oeni* sub-populations.

Table A.3.2. . b. Compounds that get smaller in intensity or higher, on the polar column, in the second and third week old extracts

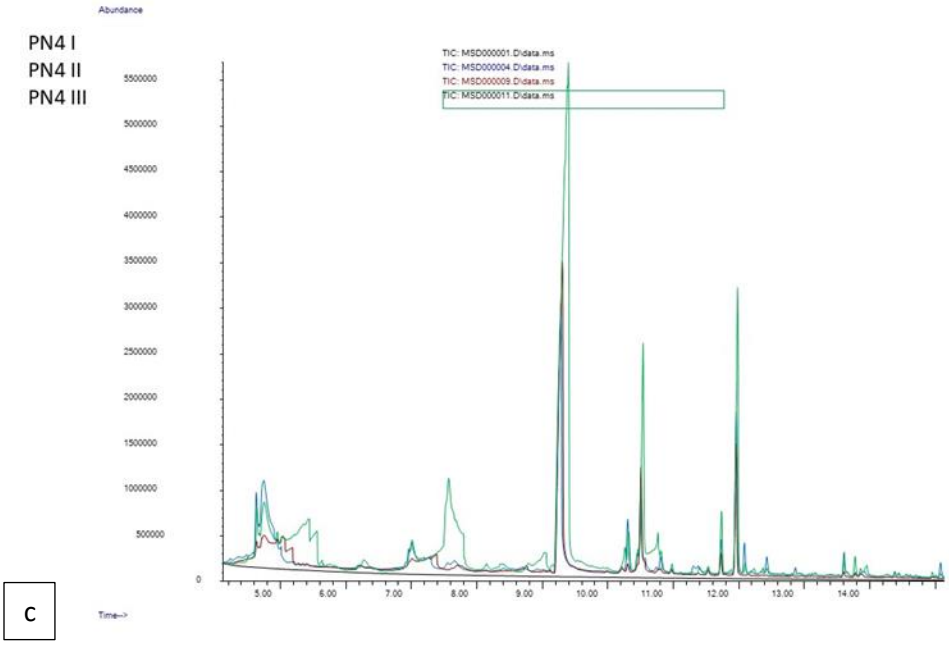
Second week old extracts					Third week old extracts				
comound name	RT	CAS	Pn4	31	comound name	RT	CAS	Pn4	31
1 Butanol-2- methyl	4.94	137-32-6	>	>	1-butanol-3 methyl	4.84	123-51-3	>	>
Propanoic acid, 2 hyd	6.87	97-64-3	>	>	Propanoic acid, 2 hydroxy	6.78	97-64-3	>	>
1- hexanol 3- ethyl	9.02	104-76-7	>	>	Propanoic acid, 2 methyl	10.19	79-31-2	>	>
Propanoic acid, 2 met	10.27	79-31-2	>	>	un known	12.57		>	>
Hexanoic acid	14.14	142-62-1	>	>	Benzene acid, ethyl ester	13.28	101-97-3	>	>
Benzyl alcohol	14.45	100-51-6	>	>	Phenylethyl alcohol	14.93	60-12-8	>	>
Octanoic acid	16.84	124-07-2	>	>	Triacetin	16.92	102-76-1	>	>
Sorbic acid	17.92	110-44-1	>	>	Phenol, 2,4- bis (1,1 dimethyletl	19.65	96-76-4	>	>



a



b



c

Figure A 3. 6 a, b, c. The changing in volatile profile between the same mixtures over time

Table A 3. 3. a. Compounds that get smaller in intensity or higher, on the polar column, in the first week old extracts

First week old extracts				
comound name	RT	CAS	Pn4	31
acetic acid, hexyl ester	5.78	142-92-7	<	<
1 hexanal	6.84	111-27-3	>	>
Ethyl lactate	6.73	97-64-3	>	>
Acetic acid	8.23	64-19-7	similar	similar
Propanoic acid	9.66	79-09-4	>	>
Propanoic acid, 2 methyl	10.1	79-31-2	>	>
Butanoic acid	10.99	107-92-6	>	>
Propanediol diacetate	12.51	628-66-0	>	>
ß- phenethyl acetate	13.63	103-45-7	<	<
Hexanoic acid	13.98	142-62-1	<	<
Benzyl alcohol	14.4	100-51-6	>	>
Phenylethanol	14.87	60-12-8	similar	similar
Octanoic acid	16.69	124-07-2	<	<
Triacetin	16.85	102-76-1	<	>
Sorbic acid	17.76	110-44-1	<	<
Phenol 4-ethyl	18.08	123-07-9	<	<
Decanoic acid	19.16	334-48-5	<	<
Phenol, 2,4 bis (1,1 dimethy	19.57	96-76-4	<	<
Benzoic acid	20.97	65-85	<	<

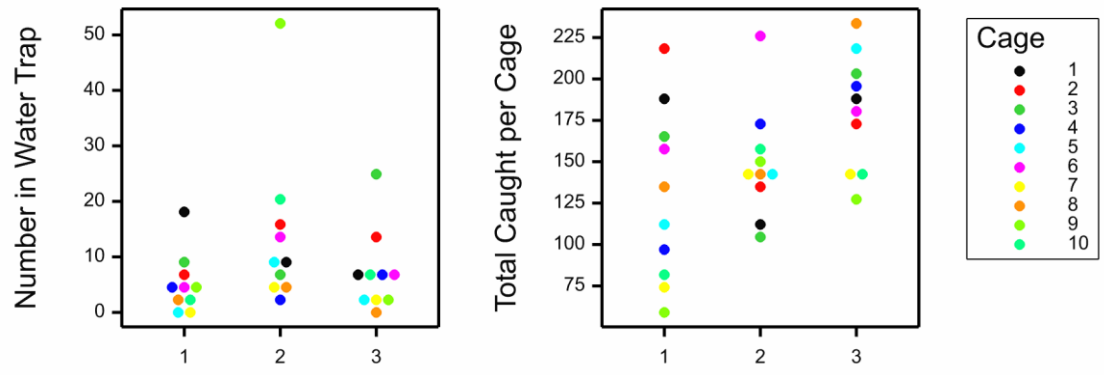


Figure A 3.7 : First Trial. , The number of flies caught in each water trap, and the total flies caught per cage, for each run (x-axis).

Table A 3.4: Percentage of insects out of those that were caught, that were on the traps with each of the each ten lures; and percentage of all insects caught on traps of each type that were female. (95% confidence limits). For Water, an estimate over all three runs is shown, as well as estimates for each of the three runs.

	Week	**Water**	**DD**	**31**	**PN4**
Male	Water	6.5 (4.3,9.9)	5.9 (2.5,13.4)	10.1 (5.7,17.4)	3.7 (1.5,9.0)
	I		43.0 (32.9,53.8)	28.7 (21.0,37.9)	25.7 (18.7,34.3)
	II		29.8 (21.0,40.5)	29.5 (21.7,38.7)	35.0 (27.0,43.9)
	III		21.3 (13.8,31.3)	31.7 (23.6,41.0)	35.5 (27.5,44.5)
Female	Water	5.0 (3.5,7.1)	2.7 (1.1,6.4)	7.8 (4.7,12.7)	4.6 (2.6,8.1)
	I		39.4 (32.5,46.7)	33.8 (27.3,41.0)	25.0 (20.0,30.8)
	II		35.0 (28.3,42.2)	30.8 (24.5,37.9)	37.4 (31.6,43.6)
	III		22.9 (17.4,29.7)	27.6 (21.6,34.6)	33.0 (27.4,39.1)
Total	Water	5.8 (4.1,8.2)	4.3 (1.9,9.1)	9.1 (5.5,14.6)	4.2 (2.1,8.0)
	I		41.1 (33.3,49.4)	31.0 (24.4,38.5)	25.4 (19.8,31.9)
	II		32.5 (25.2,40.6)	30.1 (23.5,37.5)	36.2 (29.8,43.1)
	III		22.1 (16.0,29.7)	29.9 (23.3,37.3)	34.3 (28.0,41.1)
%Female	Water	42.1 (33.3,51.4)	32.7 (16.9,53.7)	38.5 (26.8,51.7)	55.3 (38.0,71.3)
	I		48.9 (42.3,55.4)	48.9 (41.9,56.0)	49.2 (42.2,56.3)
	II		55.0 (47.6,62.2)	46.0 (38.9,53.2)	51.7 (45.8,57.5)
	III		53.0 (44.0,61.8)	41.5 (34.7,48.8)	48.2 (42.1,54.2)

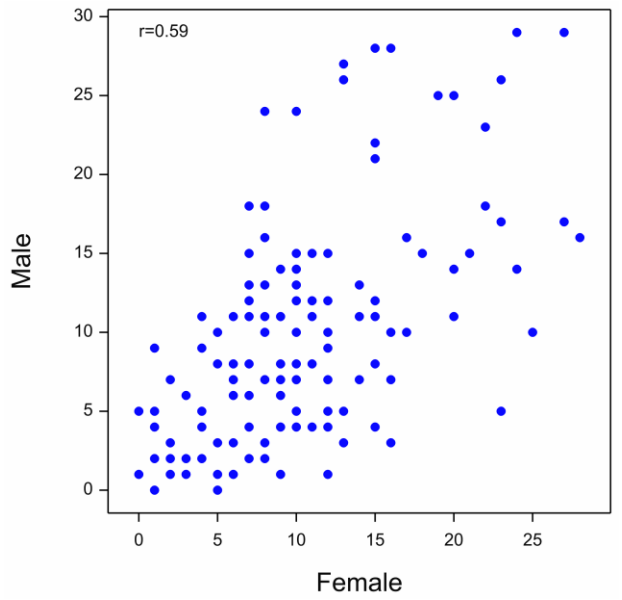


Figure A 3.8 : Second Trial. Relationship between the number of males per trap and the number of females.

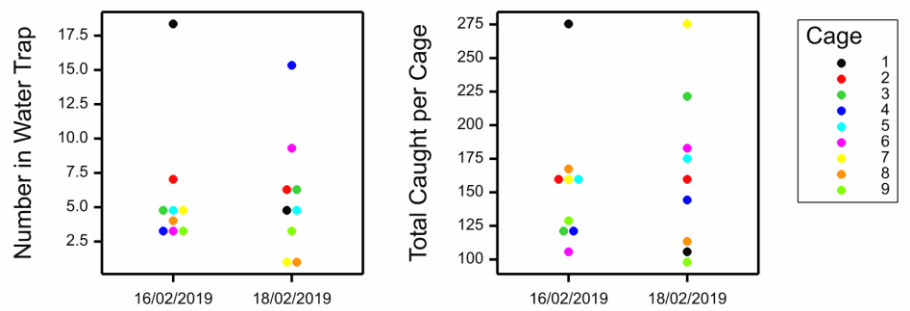


Figure A 3.9: Second Trial. , The number of flies, caught in each water trap, and the total flies caught per cage, for each date (x-axis).

Table A 3.5: Percentage of insects out of those that were caught, that were on the traps with each of the each ten lures; and percentage of all insects caught on traps of each type that were female. (95% confidence limits). For Water, an estimate over all three runs is shown, as well as estimates for each of the three runs.

Var	Week	Water	DD	31	PN4
Male	Water	4.3 (3.0,6.1)			
	DD		14.9 (12.4,17.7)		
	I			10.0 (8.0,12.5)	11.7 (9.5,14.3)
	II			12.2 (9.9,14.8)	14.7 (12.2,17.6)
	III			17.8 (15.1,20.8)	14.6 (12.1,17.4)
Female	Water	3.0 (2.0,4.6)			
	DD		14.3 (11.9,17.0)		
	I			9.5 (7.6,11.9)	12.1 (9.9,14.7)

	II			13.3 (11.0,16.0)	16.0 (13.5,18.8)
	III			15.8 (13.3,18.6)	16.0 (13.5,18.9)
Total	Water	3.6 (2.6,5.0)			
	DD		14.6 (12.5,16.9)		
	I			9.8 (8.1,11.8)	11.9 (10.0,14.0)
	II			12.8 (10.8,15.0)	15.3 (13.3,17.7)
	III			16.7 (14.6,19.2)	15.3 (13.2,17.7)
%Female	Water	42.9 (30.6,56.0)			
	DD		50.2 (43.7,56.7)		
	I			50.0 (42.1,57.9)	52.2 (44.9,59.3)
	II			53.5 (46.5,60.4)	53.3 (46.9,59.6)
	III			48.2 (42.2,54.3)	53.6 (47.2,59.9)

Appendix to Chapter 4:

Table A 4.1. Tested compounds on GC-MS polar column

Suffix	Compound	Synonym	CAS	Kovats NP	Kovats P	Mix
2	Propanone	Acetone	67-64-1	486	819	d_polar
2	Propanol	Isopropyl alcohol	67-63-0	486	927	d_polar
	Propyl acetate		109-60-4	708	973	d_polar
3	Methyl-1-butanol	Isoamyl alcohol	123-51-3	736	1209	d_polar
	Methyl lactate		2155-30-8	751	1330	d_polar
	Isobutyl acetate		110-19-0	771	1012	d_polar
2	Methylpropanoic acid	Isobutyric acid	79-31-2	772	1570	d_polar
2	Methylbutanoic acid	2-Methylbutyric acid	116-53-0	861	1662	d_polar
E2	Hexenol		928-95-0	862	1405	d_polar
2	Methylbutyl acetate		624-41-9	880	1125	d_polar
	Ethanol		64-17-5	427	932	e_polar
	Acetic acid		64-19-7	610	1449	e_polar
	Ethyl acetate	Acetidin	141-78-6	612	888	e_polar
	Methyl butyrate		623-42-7	722	982	e_polar
	Hexanal		66-25-1	800	1083	e_polar
	Butyric acid	Butanoic acid	107-92-6	805	1625	e_polar
E2	Hexenal		6728-26-3	854	1216	e_polar
3	Methylbutanoic acid	Isovaleric acid	503-74-2	863	1666	e_polar
	Hexanol		111-27-3	868	1355	e_polar
2	Heptanone		110-43-0	891	1182	e_polar
2	butanone,		78-93-3	598	907	f_polar
	Propionic acid		79-09-4	700	1535	f_polar
	Ethyl propionate		105-37-3	709	953	f_polar
2	methyl butanol		137-32-6	739	1208	f_polar
2	butyl acetate,	Sec butyl acetate	105-46-4	760	987	f_polar
Z3	Hexen-1-ol		928-96-1	857	1382	f_polar
2	Furylmethanol	Furfuryl alcohol	98-00-0	859	1660	f_polar
	Isoamyl acetate	Isopentyl acetate	123-92-2	876	1122	f_polar
	Acetoin		513-86-0	713	1284	separate

Appendix to Chapter 5: No supplementary files for Chapter

Appendix to Chapter 6: No supplementary files for Chapter

Chemical compound explanation:

Acetic acid

Acetic acid is a simple monocarboxylic acid containing two carbons. It has a role as a protic solvent, a food acidity regulator, an antimicrobial food preservative and a Daphnia magna metabolite. It is a conjugate acid of an acetate. Acetic acid, glacial appears as a clear colorless liquid with a strong odor of vinegar. Flash point 104°F. Density 8.8 lb / gal. Corrosive to metals and tissue. Used to make other chemicals, as a food additive, and in petroleum production. Acetic acid is a product of the oxidation of ethanol and of the destructive distillation of wood. It is used locally, occasionally internally, as a counterirritant and also as a reagent. (Stedman, 26th ed) Acetic acid otic (for the ear) is an antibiotic that treats infections caused by bacteria or fungus.

Acetoin

Acetoin is a methyl ketone that is butan-2-one substituted by a hydroxy group at position 3. It has a role as a metabolite. It is a methyl ketone and a secondary alpha-hydroxy ketone. Hence sinks in water. Used to make other chemicals. Acetoin is a product of fermentation. It is a component of the butanediol cycle in microorganisms. Pubchem; '3-Hydroxybutanone, also known as acetoin or acetyl methyl carbinol, is a chemical compound composed of carbon, hydrogen and oxygen. Its formula is C4H8O2. It is a colourless or pale yellow to green yellow liquid with a pleasant buttery odour. It is used as a food flavoring and a fragrance. **Acetoin**, also known as 3-hydroxybutanone or acetyl methyl carbinol, is an organic compound with the formula $CH_3CH(OH)C(O)CH_3$. It is a colorless liquid with a pleasant, buttery odor. It is chiral. The form produced by bacteria is (R)-**acetoin.** Acetoin is a neutral, four-carbon molecule used as an external energy store by a number of fermentive bacteria.

Isoamyl alcohol-3-Methyl-1-butanol

Isoamylol is an primary alcohol that is butan-1-ol in which a hydrogen at position 3 has been replaced by a methyl group. It has a role as a xenobiotic metabolite, a Saccharomyces cerevisiae metabolite and an antifungal agent. It is a primary alcohol, a volatile organic compound and an alkyl alcohol. It derives from a hydride of an isopentane. Isoamyl alcohol is a colorless liquid with a mild, choking alcohol odor. Less dense than water, soluble in water. Hence floats on water. Produces an irritating vapor. Isopentanol or Isoamyl alcohol is one of several isomers of amyl alcohol. It is a by-product of gut microbial fermentation (PMID: 17452087). It can be produced by 3-methylbutanal reductase (EC 1. 1. 1. 265) from 3 methylbutanal. Isoamyl alcohol is the major higher chain alcohol in alcoholic beverages and is present in cider, mead, beer, wine, and spirits to varying degrees, being obtained by the fermentation of starches.

Ethyl butyrate

Ethyl butyrate is a butyrate ester resulting from the formal condensation of the hydroxy group of ethanol with the carboxy group of butyric acid. It has a role as a plant metabolite. It derives from an ethanol. Ethyl butyrate appears as a clear colorless liquid with a pineapple-like odor. Flash point 78°F. Less dense than water and insoluble in water. Vapors heavier than air. Ethyl butyrate is found in apple. Ethyl butyrate is present in many fruits e. g. apple, apricot, banana, plum, tangerine etc. Ethyl butyrate is a flavouring ingredient. Ethyl butyrate, also known as ethyl butanoate, or butyric ether, is an ester with the chemical formula CH3CH2CH2COOCH2CH3, with one oxygen having a double bond. It is soluble in propylene glycol, paraffin oil and kerosene. It can be synthesized by reacting ethanol and butyric acid. This is a condensation reaction, meaning water is produced in the reaction as a byproduct.

Ethyl lactate

Ethyl 2-hydroxypropanoate is the ethyl ester obtained of 2-hydroxypropanoic acid. It has a role as a metabolite. It is a secondary alcohol, a lactate ester and an ethyl ester. It derives from a 2-hydroxypropanoic acid. Ethyl lactate appears as a clear colorless liquid with a mild odor. Flash point 115°F. Denser than water and soluble in water. Vapors heavier than air. Ethyl lactate is found in alcoholic beverages. Ethyl lactate is present in cabbage, peas, vinegar, bread,

roasted chicken, butter, blackberry, pineapple, raspberry and various wines and spirits. Ethyl lactate is a flavouring agent. Ethyl lactate, also known as lactic acid ethyl ester, is a monobasic ester formed from lactic acid and ethanol, commonly used as a solvent. This compound is considered biodegradable and can be used as a water-rinsable degreaser. Ethyl lactate is found naturally in small quantities in a wide variety of foods including wine, chicken, and various fruits. The odor of ethyl lactate when dilute is mild, buttery, creamy, with hints of fruit and coconut.

Butanoic acid

Butyric acid is a straight-chain saturated fatty acid that is butane in which one of the terminal methyl groups has been oxidised to a carboxy group. It is a straight-chain saturated fatty acid and a fatty acid 4:0. It is a conjugate acid of a butyrate. Butyric acid appears as a colorless liquid with a penetrating and unpleasant odor. Flash point 170°F. Corrosive to metals and tissue. Density 8.0 lb /gal. A four carbon acid, $CH_3CH_2CH_2COOH$, with an unpleasant odor that occurs in butter and animal fat as the glycerol ester.

Isoamyl acetate

Isoamyl acetate is the acetate ester of isoamylol. It has a role as a metabolite and a *Saccharomyces cerevisiae* metabolite. It derives from an isoamylol. Isopentyl acetate is found in apple. Isopentyl acetate is present in many fruit aromas, especially banana. Isopentyl acetate is used in banana flavouring. Isoamyl acetate has a strong odor (similar to Juicy Fruit or a pear drop) which is also described as similar to both banana and pear. Banana oil is a term that is applied either to pure isoamyl acetate or to flavorings that are mixtures of isoamyl acetate, amyl acetate, nitrocellulose and other flavors. Pear oil commonly refers to a solution of isoamyl acetate in ethanol that is used as an artificial flavor. Isoamyl acetate, also known as isopentyl acetate, is an organic compound that is the ester formed from isoamyl alcohol and acetic acid. It is a clear colorless liquid that is only slightly soluble in water, but very soluble in most organic solvents.

Acetoin acetate

Acetoin acetate is found in alcoholic beverages. Acetoin acetate is present in pineapple, paw paw, arctic bramble, red wine, cocoa and roast chicken. Acetoin acetate is a flavouring ingredient.

1-Hexanol

Hexan-1-ol is a primary alcohol that is hexane substituted by a hydroxy group at position 1. It has a role as a plant metabolite. Hexanols appears as a clear colorless liquid mixture of isomeric six-carbon alcohols with similar chemical properties. Vapors are heavier than air. Used to make pharmaceuticals and as a solvent. 1-Hexanol is an organic alcohol with a six carbon chain and a condensed structural formula of $CH_3(CH_2)_5OH$. This colorless liquid is slightly soluble in water, but miscible with ether and ethanol. Two additional straight chain isomers of 1-hexanol exist, 2-hexanol and 3-hexanol, both of which differ by the location of the hydroxyl group. Many isomeric alcohols have the formula $C_6H_{13}OH$. 1-hexanol is believed to be a component of the odour of freshly mown grass. It is used in the perfume industry.

Ethyl 3-hydroxybutyrate- Grape butyrate

Ethyl 3-hydroxybutyrate is the fatty acid ethyl ester of 3-hydroxybutyric acid. It has a role as a metabolite. It derives from a 3-hydroxybutyric acid.

Benzaldehyde

Benzaldehyde is an arenecarbaldehyde that consists of benzene bearing a single formyl substituent; the simplest aromatic aldehyde and parent of the class of benzaldehydes. It has a role as a flavouring agent, a fragrance, an odorant receptor agonist, a plant metabolite. Benzaldehyde appears as a clear colorless to yellow liquid with a bitter almond odor. Flash point near 145°F. More denser than water and insoluble in water. Hence sinks in water. Vapors are heavier than air. The primary hazard is to the environment. Immediate steps should be taken to limit spread to the environment. Easily penetrates the soil to contaminate groundwater and nearby waterways. Used in flavoring and perfume making. Benzaldehyde is an aromatic aldehyde used in cosmetics as a denaturant, a flavoring agent, and as a

fragrance. Currently used in only seven cosmetic products, its highest reported concentration of use was 0. 5% in perfumes. Benzaldehyde is a generally regarded as safe (GRAS) food additive in the United States and is accepted as a flavoring substance in the European Union. Because Benzaldehyde rapidly metabolizes to Benzoic Acid in the skin, the available dermal irritation and sensitization data demonstrating no adverse reactions to Benzoic Acid were considered supportive of the safety of Benzaldehyde. It has characteristic odour on burnt sugar, almond and woody.

Ethyl hexanoate

Ethyl hexanoate is a fatty acid ethyl ester obtained by the formal condensation of hexanoic acid with ethanol. It has a role as a metabolite. It is a fatty acid ethyl ester and a hexanoate ester. Ethyl hexanoate is found in alcoholic beverages. Ethyl hexanoate is found in many fruits, clove bud, corn oil, Camembert cheese, milk, fruit brandies, sparkling wine and Bourbon vanilla. Ethyl hexanoate is used in perfumes and fruit flavours.

Isovaleric acid

Isovaleric acid is a C5, branched-chain saturated fatty acid. It has a role as a plant metabolite and a mammalian metabolite. It is a short-chain fatty acid, a methylbutyric acid and a branched-chain saturated fatty acid. It is a conjugate acid of an isovalerate. Isopentanoic acid is a colorless liquid with a penetrating odor. It is slightly soluble in water. It is corrosive to metals and to tissue. Isovaleric acid, is a natural fatty acid found in a wide variety of plants and essential oils. Isovaleric acid is clear colorless liquid that is sparingly soluble in water, but well soluble in most common organic solvents.

3-Hexenoic acid, ethyl ester

Ethyl 3-hexenoate is a fatty acid ethyl ester of 3-hexenoic acid. It has a role as a metabolite. It derives from a 3-hexenoic acid.

Nonanal

Nonanal a plant metabolite. It is a saturated fatty aldehyde and a n-alkanal. It derives from a nonanoic acid. Nonanal is a satruated fatty aldehyde formally arising from reduction of the carboxy group of nonanoic acid. Nonanal is a clear brown liquid characterized by a rose-orange odor. Insoluble in water. Found in at least 20 essential oils, including rose and citrus oils and several species of pine oil. Nonanal belongs to the family of Medium-chain Aldehydes. These are An aldehyde with a chain length containing between 6 and 12 carbon atoms.

1-Hexanol, 2-ethyl

2-ethylhexan-1-ol is a primary alcohol that is hexan-1-ol substituted by an ethyl group at position 2. It has a role as a volatile oil component and a plant metabolite. 2-ethyl hexanol appears as a dark brown liquid with an aromatic odor. Insoluble in water and less dense than water. Flash point between 140 - 175°F. Contact may irritate skin, eyes and mucous membranes. May be toxic by ingestion, inhalation and skin absorption. xi-2-Ethyl-1-hexanol is found in alcoholic beverages. xi-2-Ethyl-1-hexanol occurs in corn, olive oil, tobacco, tea, rice, tamarind, grapes, blueberries etc.

Hexanoic acid

Hexanoic acid, also known as caproic acid, is the carboxylic acid derived from hexane with the chemical formula CH 3 4COOH. It is a colorless oily liquid with an odor that is fatty, cheesy, waxy, and like that of goats or other barnyard animals. Hexanoic acid is a C6, straight-chain saturated fatty acid. It has a role as a human metabolite and a plant metabolite. It is a straight-chain saturated fatty acid and a medium-chain fatty acid. It is a conjugate acid of a hexanoate. Caproic acid appears as a white crystalline solid or colorless to light yellow solution with an unpleasant odor. Insoluble to slightly soluble in water and less dense than water. Contact may severely irritate skin, eyes and mucous membranes. May be toxic by ingestion, inhalation and skin absorption. Used to make perfumes. Caproic acid is a colourless oily liquid that smells like cheese. It is a fatty acid found naturally in various animal fats and oils. It is safe for human dietary consumption up to levels of 1g/kg.

1- Octanol

Octan-1-ol is an octanol carrying the hydroxy group at position 1. It has a role as a plant metabolite. It is an octanol and a primary alcohol. Octanol appears as a clear colorless liquid with a penetrating aromatic odor. Insoluble in water and floats on water. Vapors heavier than air. Vapors may irritate the eyes, nose, and respiratory system.

Ethyl butyrate- Butyric acid ethyl ester

Ethyl butyrate is a butyrate ester resulting from the formal condensation of the hydroxy group of ethanol with the carboxy group of butyric acid. It has a role as a plant metabolite. It derives from an ethanol. Ethyl butyrate appears as a clear colorless liquid with a pineapple-like odor. Flash point 78°F. Less dense than water and insoluble in water. Vapors heavier than air. Ethyl butyrate is found in apple. Ethyl butyrate is present in many fruits e. g. apple, apricot, banana, plum, tangerine etc. Ethyl butyrate is a flavouring ingredient. Ethyl butyrate, also known as ethyl butanoate, or butyric ether, is an ester with the chemical formula CH3CH2CH2COOCH2CH3, with one oxygen having a double bond. It is soluble in propylene glycol, paraffin oil and kerosene. It can be synthesized by reacting ethanol and butyric acid. This is a condensation reaction, meaning water is produced in the reaction as a byproduct.

Acetic acid, hexyl ester

Hexyl acetate is the acetate ester of hexan-1-ol. It has a role as a metabolite. It derives from a hexan-1-ol. Hexyl acetate appears as a colorless liquid with a mild sweet odor. Flash point 113°F. A moderate fire risk. Inhalation may cause adverse effects. Insoluble in water and very soluble in alcohols and ethers. When heated to high temperatures emits acrid smoke and fumes. Used as a solvent and as a propellant in aerosols. Hexyl acetate is found in alcoholic beverages. Hexyl acetate is used in fruit essences, and fruit aroma concentrates. Hexyl acetate is present in wines, black tea, soya bean and other food.

Sorbic Acid

Sorbic acid is a hexadienoic acid with double bonds at C-2 and C-4; it has four geometrical isomers, of which the trans,trans-form is naturally occurring. It is a hexadienoic acid, a polyunsaturated fatty acid, a medium-chain fatty acid and an alpha,beta-unsaturated monocarboxylic acid. It is a conjugate acid of a sorbate. Sorbic acid appears as white powder or crystals. Melting point 134.5°C. Slightly acidic and astringent taste with a faint odor. (2E, 4E)-2, 4-Hexadienoic acid is a preservative for many foodstuffs. Generally used as K salt or (less frequently) as Ca salt. (2E, 4E)-2, 4-Hexadienoic acid is an antimicrobial agent against a wide variety of microorganisms, especially yeasts and moulds. (2E, 4E)-2, 4-Hexadienoic acid is a preservative action more efficient in acidic foods. Typical usage levels 500-2000 pp.

Phenylmethyl acetate- Acetic acid, phenylmethyl ester

Benzyl acetate is the acetate ester of benzyl alcohol. It has a role as a metabolite. It is an acetate ester and a benzyl ester. Benzyl acetate is a colorless liquid with an odor of pears. (USCG, 1999)

Benzyl acetate is found in alcoholic beverages. Benzyl acetate occurs in jasmine, apple, cherry, guava fruit and peel, wine grape, white wine, tea, plum, cooked rice, Bourbon vanilla, naranjila fruit (Solanum quitoense), Chinese cabbage and quince. Benzyl acetate is a flavouring agent Benzyl acetate is an organic compound with the molecular formula C9H10O2. It is the ester formed by condensation of benzyl alcohol and acetic acid. It is one of many compounds that is attractive to males of various species of orchid bees, who apparently gather the chemical to synthesize pheromones; it is commonly used as bait to attract and collect these bees for study.

Phenylethyl Alcohol

2-phenylethanol is a primary alcohol that is ethanol substituted by a phenyl group at position 2. It has a role as a fragrance, a Saccharomyces cerevisiae metabolite, a plant metabolite, an Aspergillus metabolite and a plant growth retardant. It is a primary alcohol and a member of benzenes. An antimicrobial, antiseptic, and disinfectant that is used also as an aromatic essence and preservative in pharmaceutics and perfumery. 2-Phenylethanol is found in almond. 2-Phenylethanol is a flavouring ingredient.

Benzyl alcohol

Benzyl alcohol is an aromatic alcohol with the formula $C_6H_5CH_2OH$. The benzyl group is often abbreviated "Bn", thus benzyl alcohol is denoted as BnOH. Benzyl alcohol is a colorless liquid with a mild pleasant aromatic odor. It is a useful solvent due to its polarity, low toxicity, and low vapor pressure. Benzyl alcohol is an aromatic alcohol that consists of benzene bearing a single hydroxymethyl substituent. It has a role as a solvent, a metabolite, an antioxidant and a fragrance. Benzyl alcohol appears as a clear colorless liquid with a pleasant odor. Slightly denser than water. Flash point 194°F. Boiling point 401°F. Contact may irritate skin, eyes, and mucous membranes. May be slightly toxic by ingestion. Used to make other chemicals.

Phenethyl acetate

Phenethyl acetate is the ester resulting from the condensation of acetic acid and phenethyl alcohol. Like many esters, it is found in a range of fruits and biological products. It is a colorless liquid with a rose and honey scent and a raspberry-like taste.

Phenethyl acetate is the acetate ester of 2-phenylethanol. It has a role as a metabolite and a *Saccharomyces cerevisiae* metabolite. It derives from a 2-phenylethanol. 2-Phenylethyl acetate is found in apple. 2-Phenylethyl acetate is a flavouring ingredient.

Ethyl phenylacetate- Benzeneacetic acid, ethyl ester

Ethyl phenylacetate is found in alcoholic beverages. Odoriferous constituent of many plants. Ethyl phenylacetate is present in apple, grapefruit, guava fruit, papaya, melon, pineapple, wheat bread, crispbread, wines, fruit brandies, shoyu, bael (Aegle marmelos), sake, and ceriman (Monstera deliciosa). Ethyl phenylacetate is a flavouring ingredien.

Euginol

Eugenol is an allyl chain-substituted guaiacol, a member of the allylbenzene class of chemical compounds. It is a colorless to pale yellow, aromatic oily liquid extracted from certain essential oils especially from clove oil, nutmeg, cinnamon, basil and bay leaf.

Eugenol is a phenylpropanoid formally derived from guaiacol with an allyl chain substituted para to the hydroxy group. It has a role as an allergen, a plant metabolite, a human blood serum metabolite and a sensitiser. It is a phenylpropanoid, a monomethoxybenzene and a member of phenols. It derives from a guaiacol. Eugenol is a naturally occurring phenolic molecule found in several plants such as cinnamon, clove, and bay leaves.

Triacetin

Triacetin is a triglyceride obtained by acetylation of the three hydroxy groups of glycerol. It has fungistatic properties (based on release of acetic acid) and has been used in the topical treatment of minor dermatophyte infections. It has a role as a plant metabolite, a solvent, a fuel additive, an adjuvant, a food additive carrier, a food emulsifier, a food humectant and an antifungal drug. It derives from an acetic acid. Triacetin is found in fruits. Triacetin is a flavouring agent, adjuvant; formulation aid, humectant, solvent and vehicle. Triacetin is present in papaya (Carica papaya. A triglyceride that is used as an antifungal agent.

Diethyl succinate- Butanedioic acid, diethyl ester

Diethyl succinate is a flavour ingredient. Utilised by bactrocera halforiae (Diptera, tephritidae dacinae, Dacini)

Benzothiazole

Benzothiazole is an organic heterobicyclic compound that is a fusion product between benzene and thiazole. The parent of the class of benzothiazoles. It has a role as a plant metabolite, a xenobiotic and an environmental contaminant. Benzothiazole is found in common persimmon. Benzothiazole is isolated from cranberries Benzothiazole is a colorless, slightly viscous liquid with a melting point of 2 ¬∞C, and a boiling point of 227-228 ¬∞C. The density of benzothiazole is 1. 238 g/ml (25 ¬∞C). It is a heterocyclic organic compound. Benzothiazole has no household use. It is used in industry and research.

Ethyl caprylate- ethyl octanoate

Ethyl octanoate is a fatty acid ethyl ester resulting from the formal condensation of octanoic acid with ethanol. It has a role as a metabolite. It is a fatty acid ethyl ester and an octanoate ester. Ethyl octanoate is found in alcoholic beverages. Ethyl octanoate is used in many fruit flavourings. Ethyl octanoate is a constituent of plant oils. Also present in Swiss cheese, Camembert cheese, wheat bread, port wine, plum brandy, sparkling wine, apple, apricot, banana, cherry, orange, grapefruit, plum and other fruits.

Methyl salicylate

Methyl salicylate appears as colorless yellowish or reddish liquid with odor of wintergreen. Methyl salicylate (oil of wintergreen or wintergreen oil) is an organic ester naturally produced by many species of plants, particularly wintergreens. Methyl salicylate is a benzoate ester that is the methyl ester of salicylic acid. It has a role as a flavouring agent, a metabolite and an insect attractant. It is a benzoate ester and a member of salicylates. It derives from a salicylic acid.The compound was first extracted and isolated from plant species Gaultheria procumbens in 1843. It can be manufactured synthetically and it used as a fragrance, in foods, beverages, and liniments. It forms a colorless to yellow or reddish liquid and exhibits a characteristic odor and taste of wintergreen. For acute joint and muscular pain, methyl salicylate is used as a rubefacient and analgesic in deep heating liniments. It is used as a flavoring agent in chewing gums and mints in small concentrations and added as antiseptic in mouthwash solutions.

Dimethylacetophenone

Prodox 146

2,4-di-tert-butylphenol is a member of the class of phenols carrying two tert-butyl substituents at positions 2 and 4. It has a role as a bacterial metabolite, an antioxidant and a marine metabolite. It is an alkylbenzene and a member of phenols.

I am turning into Goblin

at least it looks like that

Into some strange creature

that I cannot long recognise

in the mirror of my soul

PhD thesis writing, it is not

a process, after all, it is an

transformation that happens

or not