Screening for microbial volatiles related to *Cydia pomonella* infestation in walnut fruits (*Juglans regia*)

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Screening for microbial volatiles related to *Cydia pomonella* infestation in walnut fruits (*Juglans regia*)

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Abstract

Cydia pomonella is a pest of several trees belonging to the family of *Rosaceae* but also of *Juglans regia.* Screening for volatiles attracting *Cydia pomonella* to *Rosaceae spp.* have been ongoing for years and has led to the discovery of pear ester as a potent host plant attractant. The origin of pear ester is suggested to be microbial, and yeasts from the genus of *Metschnikowia* has been shown to emit this volatile. In this pilot study we aim at identifying possible host plant cues, attracting *Cydia pomonella* to *Juglans regia.*

We sample volatiles from *Cydia pomonella* infested walnuts and from related larval frass, in addition we sample volatiles from microbes isolated from both infested nuts and frass, in the search for pear ester and a number of other key volatile compounds known from headspaces of *Metschnikowia spp.* with the ability to induce antennal response in *Cydia pomonella*. The selected key compounds are nonanal, decanal, 2-nonanone, 3-methylbutyl ethanoate, 2-phenylethanol, sulcatone, 3-methylbutyl propanoate and ethyl hexanoate.

Volatile collections are done with both dynamic- and static sampling methods and the samples are analysed using gas chromatography coupled with mass spectrometry. These methods proved sufficient in detecting all key compounds but pear ester, in the microbial- and frass headspaces. Continued sampling of microbial volatiles is needed, and based on this study, the recommended procedure going forward is dynamic headspace sampling. Other methods like community level metagenomics and DNA sequencing of individual microbes should also be considered, as well as adapting the inoculation time, temperature, and media to match the optimal conditions for pear ester emittance from *Metschnikowia spp.*

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Abbreviations

CAR/PDMS	carboxen/polydimethyl-siloxane
DB-wax	Polyethylene glycol coated, polar, column, produced by Agilent J&W
DHS	Dynamic headspace sampling
DVB	Divinylbenzene
FID	Flame ionization detector
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
GC-MSD	Gas chromatography - single quadrupole mass spectrometry
GC/Q-TOF-MS	Gas chromatography coupled with Quadrupole Time-Of-Flight Mass Spectrometry
HES	High efficiency ion source
HP-5	(5%-phenyl)-methylpolysiloxane coated, non-polar, column, produced by Agilent J&W
RI	Retention index
SHS	Static headspace sampling
SPME	Solid phase microextraction
SSR	Single sensillum recordings
t_i	Retention time
TOF	Time of flight (detector)
VOC	Volatile organic compound
YPD	Yeast extract peptone dextrose

Glossary

Chromatogram

The product of chromatography. A plot showing the separation of a mixture into its components. The plot contains a vertical axis depicting counts (%) and a horizontal axis depicting time from injection (min). Each peak in a chromatogram represents a single compound.

Flame ionization detector

Detection of ions formed from combusting a volatile compound with a hydrogen flame, creating an ion current, which is proportional to the concentration of the given volatile in the analyzed sample (Flame Ionization Detector (FID), Most Popular GC Detector | Agilent, n.d.).

Gas chromatography

A method for separating compounds in a mixture. A mixture/sample is injected and vaporized and is then carried by the mobile phase (an inert gas, often helium) through a column which is lined with a stationary phase (Libretexts 2023). The interaction between the individual compounds in the mixture and the lining with the stationary phase, separates the compounds, and leads to differing retention times for each component in the sample.

Headspace

"*The volume above a liquid or solid in a closed container*" ("Headspace" 2024).

High efficiency ion source

The first part of mass spectrometry, is ionizing the compounds, prior separated using gas chromatography. The ionization is done with an electron beam, with an increased efficiency due to
multiple reflections, increasing the electron flight path (Kernan 2016). This results in an enhanced chance for ionization, as the electron has a higher probability of "striking" the compounds.

Kairomone

Interspecific communication with semiochemicals, benefitting the receiver and harming the emitter.

Mass spectrometry

A method to sort ions based on their mass to charge ratio (Brown & Beynon 2024). The results are presented as a mass spectrum.

Mass spectrum

A plot displaying data obtained from mass spectrometry. Vertical axis is the relative abundance (%) and the horizontal axis is the mass to charge ratio (m/z) (Brown & Beynon 2024). The plot is like a "fingerprint" of a given compound, displaying the abundance of its ionized components.

Odorant receptor / Olfactory receptor

Receptors with the ability to detect odorants, which prompt the sense of smell.

Oviposition

To deposit eggs.

Pheromone

Intraspecific communication with semiochemicals.

Receptor clade

A grouping of receptor with a common origin.

Retention index

A system-independent constant, calculated from retention times, making it possible to compare results from different GC-MS instruments, obtained using similar analytical methods.

Retention time

The amount of time a compound spends in the column of the GC-MS instrument.

Semiochemical

A chemical emitted by an organism that induces behavioral changes in another organism, of same or different species.

Single sensillum recordings

"SSR is a form of extracellular electrophysiology, where action potentials generated by OSNs (olfactory sensory neurons) within single sensilla on the insect antenna can be measured by an electrode in contact with the extracellular receptor" (Olsson & Hansson 2013).

Single quadrupole mass spectrometry

The quadrupole works as a mass filter, where ions are separated based on their mass to charge ratio, ending with a detection phase, where the quantity of ions with similar mass to charge is determined. From this data a mass spectrum is created (Principles and Operation of Single Quadrupole Mass Spec | Danaher Life Sciences, n.d.).

Time-of-Flight Mass Spectrometry

TOF-MS is additional to single quadrupole mass spectrometry, increasing the sensitive of the MS equipment, providing more precise mass spectra ("Time-of-Flight Mass Spectrometry," 2011).

TOF technology is based on measuring the time it takes for an ion with a known kinetic energy, to travel a certain distance, and with this information, it is possible to calculate the mass of the ion.

1. Introduction

1.1 Background

Volatile organic compounds (VOCs) are involved in the communication between plants and insects (Krieger & Breer 1999). These chemical cues emitted by plants may guide the insect to appropriate sites for oviposition and food. Insects are able to recognize and differentiate between VOCs due to highly sensitive and specific tuned olfactory receptors on the insect antennae. Identifying volatile chemicals that induce changes in insect behavior (semiochemicals), are central for future sustainable plant protection strategies, creating possibilities for mating disruption, mass trapping and identification of trap crops (Gonzales et al. 2016; Bengtsson et al. 2012).

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is a pest of primarily apples (*Malus spp.*), pears (*Pyrus spp.*), quinces (*Cydonia spp.*) belonging to the family of *Rosaceae*, and of walnuts (*Juglans spp.*) belonging to *Juglandaceae* (Walker III et al. 2016). Codling moth larvae feeds on the fruits of the host plants, causing economically significant losses worldwide. Studies on host plant volatiles has identified the kairomone pear ester (ethyl (2E, 4Z)-2,4-decadienoate) as an attractant, guiding female moths towards oviposition and feeding sites and male moths towards habitat and potential mates (Light et al. 2001; Trona et al. 2013; Erdei et al. 2023; Wan et al. 2019). The codling moth detects pear ester with the odorant receptor (CpomOR3) placed within the lepidopteran pheromone receptor clade, found on both male and female antennas (Bengtsson et al. 2014; Wan et al. 2019).

Pear ester was first discovered in the headspace of Bartlett pears and has since been detected from quinces and apples (Jennings 1961; Lopéz et al. 2022; Berger, Drawert 1984). Pear ester was also detected in the headspace from *Metschnikowia spp.* fermentations, supporting the thesis of Witzgall et al. (2012) suggesting microbial origin of codling moth host plant attractants (Gonzales 2017; Witzgall et al. 2012). A strong connection between *Metschnikowia spp.*, and codling moths was also uncovered in the study by Witzgall et al. (2012), where *Metschnikowia spp.* inoculated apples attracted more female codling moths laying more eggs, compared to the control study on sterile apples. Larvae feeding on the yeast-inoculated apples had accelerated development and a decreased mortality rate, and the larvae frass functioning as an inoculant, helped sustain *Metschnikowia spp*. colonies in the apple galleries. These findings promote studying the interaction between insects-microbes-plants.

Assuming that microbes facilitate communication between host plants and the codling moth, they may also help bridge the gap between VOC profiles of the different host plant families *Rosaceae* and *Juglandaceae*. To date, pear ester has not been detected from walnuts, ripe or unripe. Tests with pear-ester baited traps in walnut orchards were effective in catching codling moths, implicating that walnut-feeding codling moths too are sensitive to pear ester (Light et al. 2001).

Pear ester is not the only VOC coupling *Metschnikowia spp.* and *C. pomonella*. Ljunggren et al. (2019) identified multiple compounds from the headspace of *Metschnikowia spp.* fermentations, which have also shown to induce antennal response in *Cydia pomonella* (Lindblom 2012). This pertains following compounds: nonanal, decanal, 2-nonanone, 3-methylbutyl ethanoate, 2-phenylethanol, sulcatone, 3-methylbutyl propanoate and ethyl hexanoate. Furthermore, nonanal and decanal act synergistically with pear ester in trapping codling moths in baited traps, suggesting a multifaceted host plant cue (El-Sayed et al. 2012).

1.2 Methods for sampling of volatile organic compounds

Static headspace sampling (SHS) with solid phase microextraction (SPME) is done in an enclosed space without air exchange, inserting an adsorbent-coated fiber (Tholl et al. 2006). The SPME fiber equilibrates with volatiles in the gaseous phase sample. After end sampling time, the fiber is retracted, and ready for analysis using gas chromatography coupled with mass spectrometry (GC-MS).

Dynamic headspace sampling (DHS) needs a constant airflow through the sample container, carrying the volatile compounds towards an adsorbent filled volatile trap (Tholl et al. 2006). Incoming air is purified using active charcoal to minimize contamination. The trapped volatiles on the adsorbent filter are eluted using a solvent, creating a storable sample, to be analyzed using GC-MS.

1.3 Research aims

This study is part of the initiating research into microbial volatiles, related to *Cydia pomonella* infestation in walnut fruits. The objective of this study is to identify selected volatile compounds through headspace samplings of infested walnuts, codling moth frass, and from microbes isolated from both substrates.
Nine compounds, known from the headspace of *Metschnikowia spp.* with the ability to induce antennal response in the codling moth, have been selected for this study. Of these volatiles, pear ester is of the greatest interest, as this kairomone is a known host plant cue, guiding the codling moth towards host plants from the family of *Rosaceae*.

Parallel with this, we wish to evaluate the two methods used: Static- and dynamic headspace sampling, to make recommendations for continued research going forward.

1.4 Hypothesis and research questions

In this study, we assume that pear ester is fully or partially produced by microbes living on the exterior or interior of the host plants, and that these microbes function as a host plant bridge between *Juglandaceae spp.* and *Rosaceae spp.* **We hypothesize that pear ester is emitted from the walnuts (*Juglans regia*) microbiome and should be detectable in the headspace of *Cydia pomonella* infested walnut kernels and in larvae frass retreated from same infested fruits.**

Research questions:

- Is pear ester detectable in the headspaces of:
  1) microbes isolated from *Cydia pomonella* infested walnuts and *C. pomonella* frass?
  2) *C. pomonella* infested walnuts?
  3) *C. pomonella* frass acquired from infested walnuts?
- Is it possible to detect and identify other known volatiles registered by *C. pomonella* antennae?
- Which method is most suitable for headspace collection, and what are the methodological adjustments to consider?

2. Material and methods

2.1 Microbial isolation and cultivation

Healthy and infected walnuts are acquired from SENURA - Station d'expérimentation nucicole, Rhône-Alpes, 385A route de St Marcellin, 38160 CHATTE, France.

We isolate microbes from codling moth frass/infested walnut kernels and healthy kernels on yeast extract peptone dextrose (YPD) agar. Based on morphological comparison, candidate colonies from infected kernels or frass, which are not present on the healthy kernels, are isolated on separate agar plates. In this study, five out of 33 colonies are selected for detailed analysis. The selection is based on a subjective comparison of the microbial colors and consistencies, aiming to select a diverse range of microbes, from various frass and walnut samples.

The five candidate microbes are grown in 75ml liquid YPD broth in conical flasks for 24 hours in a 28°C incubater, to achieve higher density. The flasks have been autoclaved prior to use. We prepare three replicates per microbe for SHS and 1 replicate per microbe for DHS. Additionally, one replicate per microbe is grown for 72hrs followed by SHS.

Two replicates of *C. pomonella* frass obtained from the infested walnuts are grown in 75ml YPD broth for 32hrs and 39hr respectively.

2.2 Sampling of volatile organic compounds

2.2.1 Static headspace sampling with SPME

The SPME fibers are activated/cleaned prior to sampling using the inlet port of the GC-MS (Agilent 5973) heating the fiber till 250°C for five minutes. The fiber coating consists of 50 µm divinylbenzene (DVB) and 30 µm carboxen/polydimethyl-siloxane (CAR/PDMS).

A halved infested walnut and a halved healthy walnut, placed in separate 50ml glass tube, are sampled for 2h using a single SPME fiber. The same applies to the prepared frass samples and two blank YPD broths.

The microbial liquid cultures are sampled for 5h inserting two SPME fibers.

a. b. c. d.

Figure 1. Volatile collection procedure from microbial colonies. a) Isolated microbe from codling moth frass/infected kernel – grown on YPD agar. b) Transfer microbe using an inoculation loop to 75 ml liquid YPD broth. c) Static headspace volatile sampling with two SPME fibers for each microbial sample. d) Injection of respective SPME fiber in GC-MS equipped with either a non-polar HP-5 or polar DB-wax capillary column.

2.2.2 Dynamic headspace sampling

One replicate per microbe and a blank YPD broth are sampled using DHS. Cleaning of the volatile trap (50-mm Super Q trap, 80/100 mesh) is done prior to each sampling using 3ml methanol followed by 3ml redistilled hexane and the glassware is cleaned at 375°C for 8h.

The inoculated broth is transferred to a 2-L glass container with an air in- and outlet. Incoming air is filtered by charcoal, and outgoing air passes through the adsorbent trap. Sampling is done for approximately 24h, whereafter the adsorbed volatiles are extracted from the trap using 300 µl redistilled hexane. Sample volumes are reduced, and stored at -19°C, until analysis.

2.3 GC-MS analysis

All microbial volatile samples acquired through both DHS and SHS, are analyzed on both a 7890 Gas Chromatograph coupled with 7250 Quadrupole Time-Of-Flight Mass Spectrometry (GC/Q-TOF-MS) (Agilent technologies), equipped with a non-polar HP-5 capillary column and a 7890B Gas Chromatograph coupled with 5977 Single Quadrupole Mass Spectrometry (GC-MSD) (Agilent technologies), equipped with a polar DB-wax capillary column. The frass- and walnut volatile samples are only analyzed on the GC/Q-TOF-MS. Inlet-temperature is 225°C followed by a temperature ramp starting at 40°C for 2 minutes, increasing at 5°C/min until 270°C (holding for 10 minutes). When injecting a liquid, a solvent delay is added, corresponding to 6 minutes on the GC-MSD and 10 minutes on the GC/G-TOF-MS.

We analyze a synthetic pear ester, to use as a reference during chromatogram analysis. 2µl of a 1ng/µl solution is injected in splitless mode on both GC-MS instruments. We also analyze a linear alkane series. 2µl of a 5ng/µl solution containing linear alkanes from seven to thirty carbon length are weekly injected on splitless mode on both GC-MS instruments. The linear alkane series help us to verify the functionality of the equipment and to calculate Kováts retention indices to tentatively identify the volatile components of the walnut-, codling moth frass-, and microbial samples.

2.4 Calculation of Kovat´s retention index

The equation for Kováts retention index:

$$RI_i = 100\left(\frac{t_i - t_x}{t_{x+1} - t_x} + x\right)$$

RI_i is the retention index calculated for peak i.

t_i is the retention time for peak i.

t_x is the retention time for alkane C_x, representing nearest alkane peak before peak i.

t_{x+1} is the retention time for alkane C_{x+1}, representing nearest alkane peak after peak i.

x is carbon length of the alkane.

Calculating the retention indices (RI) are part of tentatively identifying the volatile compounds. The compounds are initially recognized based on their mass spectra, after which their calculated RIs are compared to that found in online libraries (NIST webbook, Pubchem).

3. Results

3.1 Pear ester

Pear ester is not detected in the headspace of the included microbes, frass samples nor walnut samples. In figure 2, a section of the chromatograms from all microbial samples analyzed on GC/Q-TOF-MS, with the chromatogram of a synthetic pear ester for reference, can be seen. The peaks appearing at a similar retention time as that of pear ester, are identified as 3-phenylfuran, based on their mass-spectra.

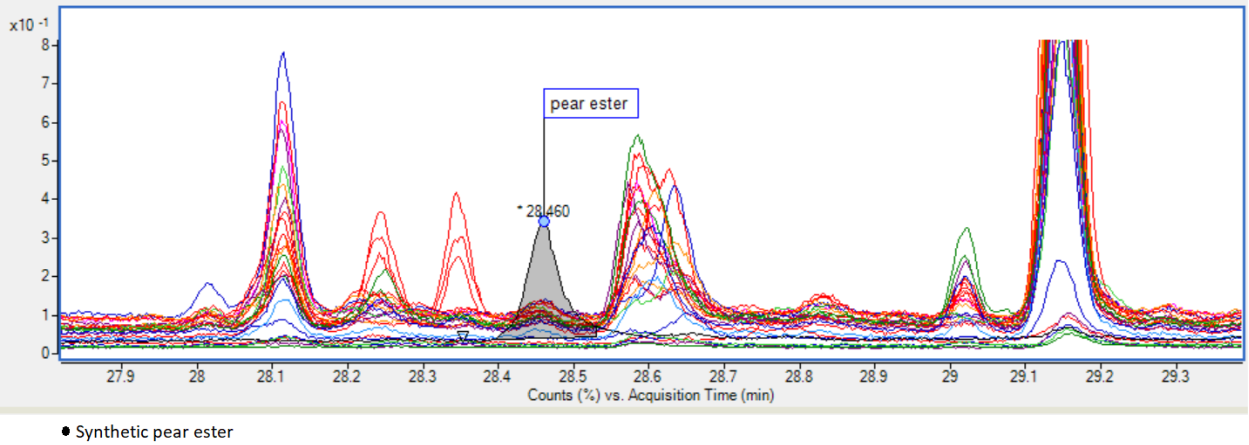

Figure 2. Section of the chromatograms from static and dynamic samplings of microbial headspaces, with the chromatogram of a synthetic pear ester for reference. Analysis completed on GC/Q-TOF-MS equipped with a HP-5 column. The peaks from the microbial samples with similar retention time as pear ester are tentatively identified as 3-phenylfuran.

3.2 Key volatile compounds

3.2.1 Microbial samples

The eight selected VOCs are all conceivably identified in the microbial headspaces. For a tentative identification, the experimental retention indices must be within ±15 compared to retention indices from NIST online library, on both polar and non-polar columns. In the identification table below (see table 1) calculated retention indices are noted in case the given compound is detected in the microbial headspace using either SHS or DHS. The relative abundancies of the key compounds vary between the microbial samples. Figure 3 shows the relative abundancies represented by the relative peak areas in each chromatogram. The data stems from both DHS and SHS analyzed on the GC/Q-TOF-MS.

3.2.2 Walnut- and frass samples

Static headspace sampling with SPME of infested and healthy walnuts, do not reveal any of the eight key compounds. The volatile samples from frass inoculated YPD broth potentially contain five out of the eight key compounds. In table 2, experimental retention indices are calculated and noted for tentatively identified compounds in the frass samples. Important to notice, that the frass volatile samples have only been analyzed on the GC/Q-TOF-MS with a non-polar column.

Table 1. Identification table from DHS and SHS of the selected microbes. Calculated retention indices are noted in case the given compound is tentatively identified. Orange coloring indicates compounds was detected on the GC/Q-TOF-MS with a HP-5 column, green coloring indicates detection on the GC-MSD with a DB-wax column and purple coloring symbolizes detection on both instruments.

Sample identifier	Microbe	Replicate	Method	Inoculation time	Sampling time	GCMS Column	nonanal	decanal	2-nonanone	3-methylbutyl ethanoate	2-phenylethanol	sulcatone	3-methylbutyl propanoate	ethyl hexanoate
SHS_BLANK	YPD broth	1	SHS	0h	5h	HP-5	-	1208	-	-	-	989	-	-
						DB-WAX	1378	-	-	-	-	1319	-	-
DHS_BLANK_174	YPD broth	1	DHS	24h	24h	HP-5	1106	-	-	-	-	-	-	-
						DB-WAX	-	-	-	-	-	-	-	-
SHS_microbe1_1	microbe 1	1	SHS	24h	5h	HP-5	-	-	1094	877	1118	989	970	-
						DB-WAX	-	-	-	1110	1873	1317	-	-
SHS_microbe1_2	microbe 1	2	SHS	24h	5h	HP-5	-	-	1094	877	1118	989	970	-
						DB-WAX	-	-	-	1110	1873	1317	-	-
SHS_microbe1_3	microbe 1	3	SHS	24h	5h	HP-5	-	1208	1094	877	1118	989	-	-
						DB-WAX	-	-	-	1110	1873	1317	-	-
SHS_microbe1_72h	microbe 1	1	SHS	72h	5h	HP-5	-	1208	-	-	1118	989	-	-
						DB-WAX	1377	1482	-	1110	1873	1317	-	-
DHS_microbe1_169	microbe 1	1	DHS	24h	24h	HP-5	-	1208	1094	877	1118	989	970	999
						DB-WAX	-	-	1371	1110	1873	1317	1176	-
SHS_microbe3_1	microbe 3	1	SHS	24h	5h	HP-5	-	1208	1094	877	1118	989	-	-
						DB-WAX	-	-	-	1110	1873	1318	-	-
SHS_microbe3_2	microbe 3	2	SHS	24h	5h	HP-5	-	1208	-	877	1118	989	-	-
						DB-WAX	-	-	-	1110	1873	1318	-	-
SHS_microbe3_3	microbe 3	3	SHS	24h	5h	HP-5	-	1208	-	877	1118	989	-	-
						DB-WAX	-	-	-	-	1873	1318	-	-
SHS_microbe3_72h	microbe 3	1	SHS	72h	5h	HP-5	-	-	-	-	1118	989	-	-
						DB-WAX	1377	-	-	1110	1873	1318	-	-
DHS_microbe3_170	microbe 3	1	DHS	24h	24h	HP-5	-	-	-	877	1118	989	-	-
						DB-WAX	-	-	-	1110	-	1318	-	-
SHS_microbe20_1	microbe 20	1	SHS	24h	5h	HP-5	-	1208	1094	877	1118	989	-	-
						DB-WAX	1377	1482	-	1110	1872	1317	-	-
SHS_microbe20_2	microbe 20	2	SHS	24h	5h	HP-5	-	1208	1094	877	1118	989	-	-
						DB-WAX	1377	-	-	1110	1872	1317	-	-
SHS_microbe20_3	microbe 20	3	SHS	24h	5h	HP-5	-	-	1094	877	1118	989	-	-
						DB-WAX	-	-	-	1110	1872	1317	-	-
SHS_microbe20_72h	microbe 20	1	SHS	72h	5h	HP-5	-	1208	-	-	1118	989	-	-
						DB-WAX	1377	1482	-	1110	1872	1317	-	-
DHS_microbe20_176	microbe 20	1	DHS	24h	24h	HP-5	1106	-	-	877	1118	989	970	-
						DB-WAX	-	-	-	1110	1872	1317	-	-
SHS_microbe22_1	microbe 22	1	SHS	24h	5h	HP-5	1106	1208	-	-	1118	989	-	-
						DB-WAX	1377	1482	-	-	1872	1316	-	-
SHS_microbe22_2	microbe 22	2	SHS	24h	5h	HP-5	1106	1208	-	-	-	989	-	-
						DB-WAX	1377	1482	-	-	1872	1316	-	-
SHS_microbe22_3	microbe 22	3	SHS	24h	5h	HP-5	-	1208	-	-	1118	989	-	-
						DB-WAX	1377	-	-	-	1872	1316	-	-
SHS_microbe22_72h	microbe 22	1	SHS	72h	5h	HP-5	-	1208	-	-	1118	989	-	-
						DB-WAX	1377	1482	-	-	1872	1316	-	-
SHS_microbe22_72h_16h	microbe 22	1	SHS	72h	16h	HP-5	-	1208	-	877	1118	989	-	-
						DB-WAX	1377	1482	-	1110	1872	1316	-	-
DHS_microbe22_175	microbe 22	1	DHS	24h	24h	HP-5	1106	-	-	-	-	989	-	-
						DB-WAX	-	-	-	-	1872	1316	-	-
SHS_microbe 28_1	microbe 28	1	SHS	24h	5h	HP-5	-	1208	-	877	1118	989	-	-
						DB-WAX	-	-	-	1109	1872	1317	-	-
SHS_microbe28_2	microbe 28	2	SHS	24h	5h	HP-5	-	1208	-	877	1118	989	-	-
						DB-WAX	1377	1482	-	-	1872	1317	-	-
SHS_microbe28_3	microbe 28	3	SHS	24h	5h	HP-5	-	1208	-	-	1118	989	-	-
						DB-WAX	1377	-	-	-	1872	1317	-	-
SHS_microbe28_72h	microbe 28	1	SHS	72h	5h	HP-5	-	-	-	-	1118	989	-	-
						DB-WAX	1377	-	-	1109	1872	1317	-	-
DHS_microbe28_172	microbe 28	1	DHS	24h	24h	HP-5	1106	1208	-	877	-	989	-	-
						DB-WAX	1377	1482	-	1109	1872	1317	-	-

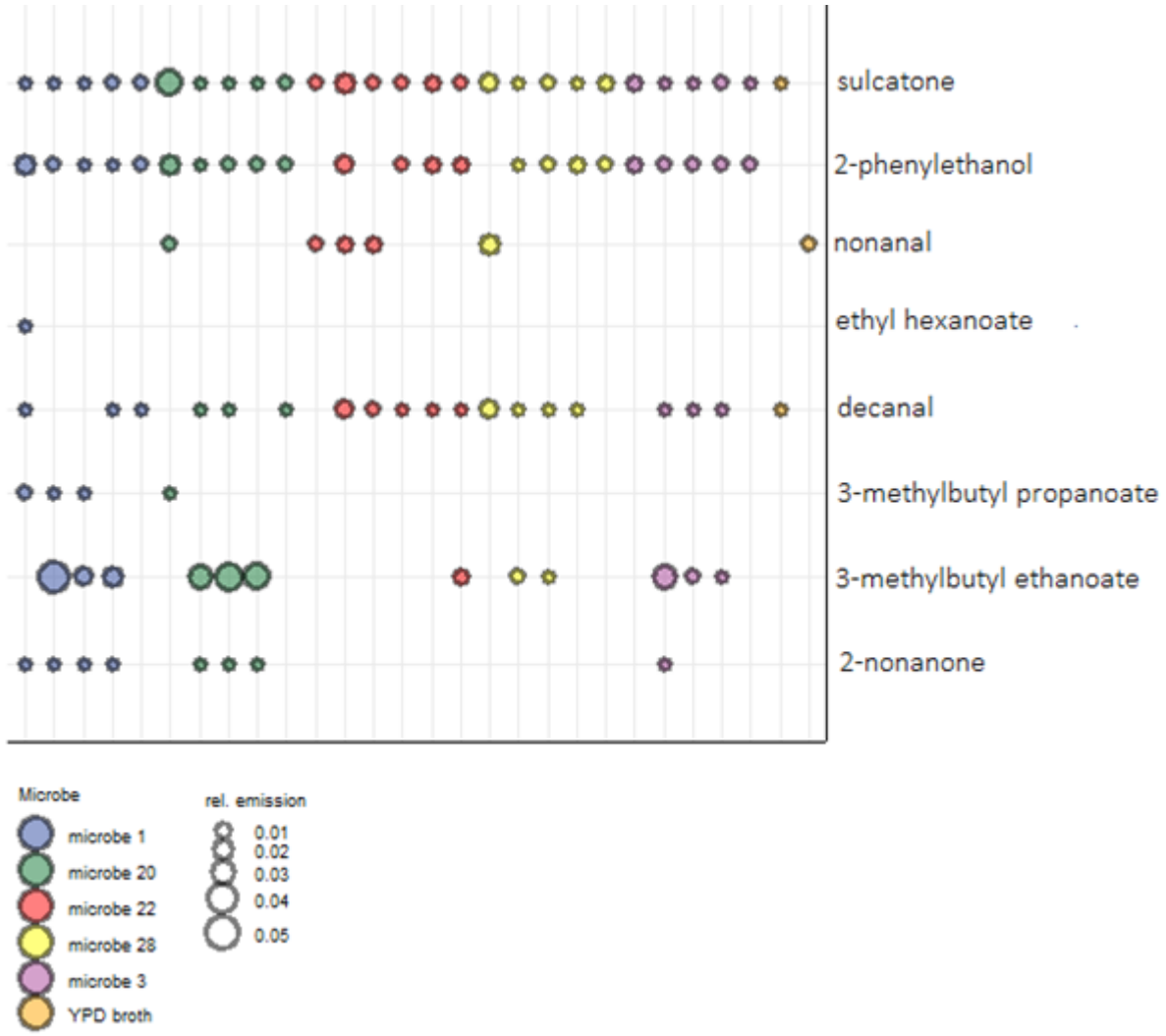

Figure 3: Relative abundance of selected key compounds in the microbial headspaces, represented by their relative peak area. The area of the tentative identified peak is divided by the total peak area in the given chromatogram. Data stems from both dynamic and static headspace sample, analyzed on the GC/Q-TOF-MS equipped with a HP-5 column.

Table 2. Identification table from static headspace sampling of walnuts and codling moth frass. Calculated retention indices are noted in case the given compound is tentatively identified. Sampling time is 2h, after which the SPME fiber is analyzed on the GC/Q-TOF-MS with a HP-5 column.

Sample identifier	Sample	Replicate	Innoculation time	nonanal	decanal	2-nonanone	3-methylbutyl acetate	phenylethyl alcohol	sulcatone	3-methylbutyl propanoate	ethyl hexanoate
Healthy nut_1	Halfed healthy nut	1	-	-	-	-	-	-	-	-	-
Infested nut_1	Halfed infested nut	1	-	-	-	-	-	-	-	-	-
Control broth_1	YPD broth	1	-	-	-	-	876	-	-	-	-
Frass in broth_1	Frass in YPD broth	1	39h	-	-	1094	875	1121.1	-	-	999
Control broth_2	YPD broth	2	-	-	-	-	874	1121.1	-	-	-
Frass in broth_2	Frass in YPD broth	2	32h	-	-	-	874	1121.1	988	-	1000

3.4 Methodological differences

The methodological differences reveal themselves during analysis of the chromatograms. For instance, there is a clear difference in the chromatograms from DHS and SHS, when comparing the numbers of peaks. Figure 4 shows a section of the chromatograms from DHS and SHS of microbe 28. The total number of peaks in the chromatograms from SHS ranges from 200-286, and from DHS the total number of peaks is 110.

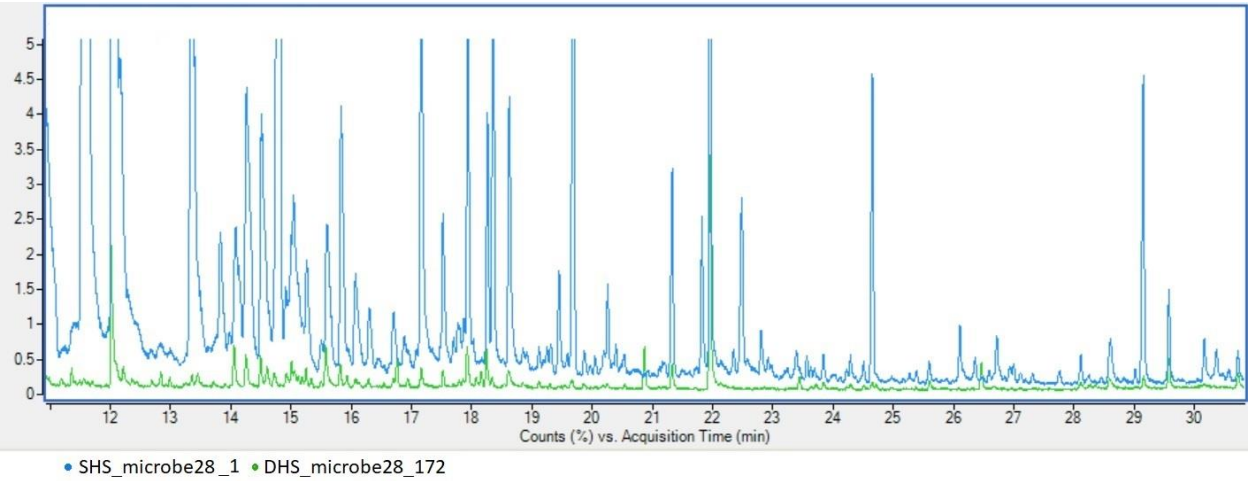

Figure 4. Section of chromatograms from SHS and DHS of microbe 28. Analysis completed on GC/Q-TOF-MS equipped with a HP-5 column. SHS_microbe28_1 and DHS_microbe28_172 are the sample identifiers, see table 1 for further information.

Another methodological difference is visible when comparing the chromatograms from a blank YPD broth with that of the microbial samples. The chromatograms from SHS of the five microbes differ marginally from that of the YPD broth, concerning both peak size and number (see figure 5). The opposite is true concerning the chromatograms from DHS of microbes depicting greater variation from that of the blank YPD broth (see figure 6).

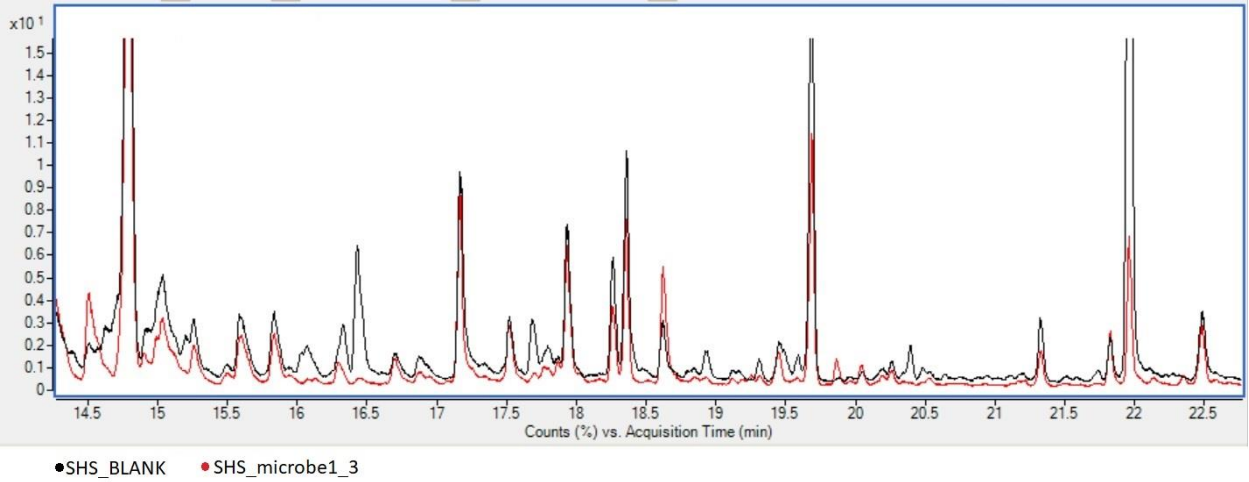

Figure 5. Section of chromatograms from static headspace sampling of blank YPD broth and microbe 1. Analysis completed on GC/Q-TOF-MS. SHS_BLANK and SHS_microbe1_3 are the sample identifiers, see table 1 for further information.

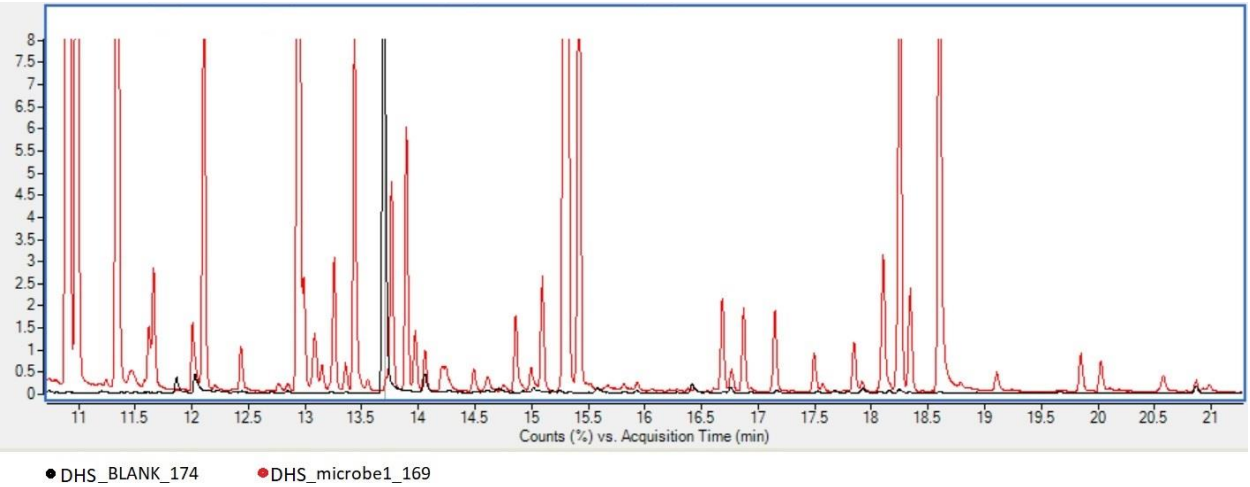

Figure 6. Section of chromatograms from dynamic headspace sampling of a blank YPD broth and microbe 1. Analysis completed on GC/Q-TOF-MS. DHS_BLANK_174 and DHS_microbe1_169 are the sample identifiers, see table 1 for further information.

The relative abundancies of key compounds also vary between the methods and between the different inoculation times. Figure 7 displays the relative abundance of the feasibly identified compounds in different cultures of microbe 1. Data stems from both DHS and SHS. The relative abundance is represented by the relative area of a given peak.

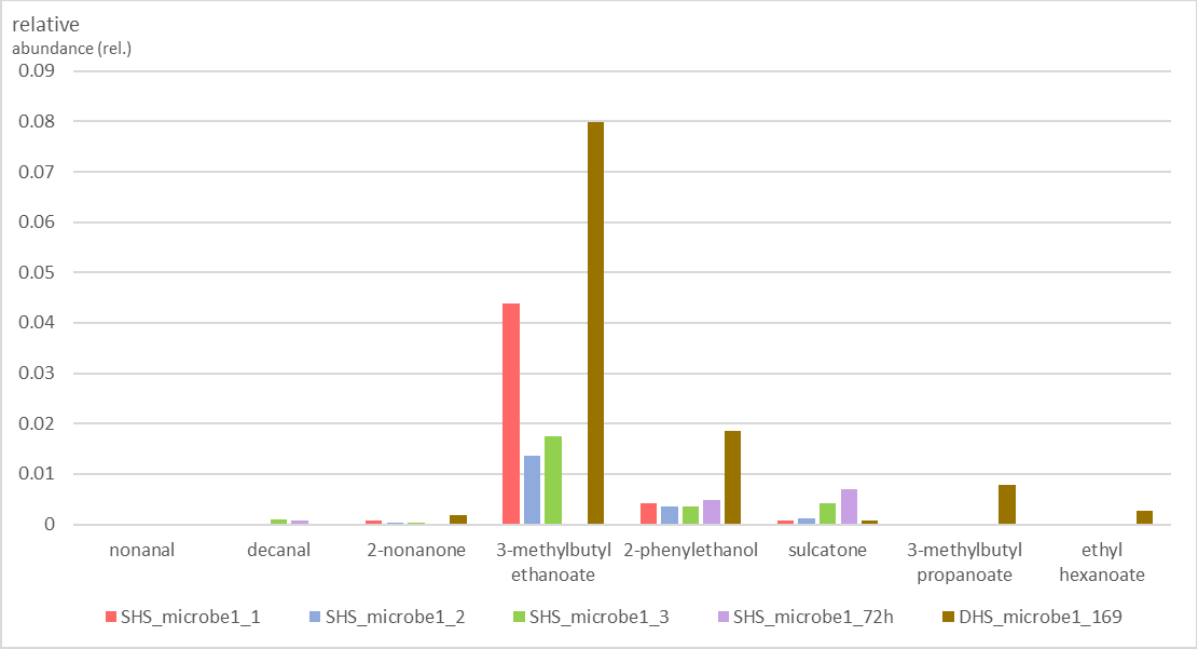

Figure 7. Relative abundance of selected key volatile compounds in replicated cultures of microbe 1, sampled using either SHS or DHS. Inoculation time for all analytes were 24h, except for Microbe1_72h which was grown for 72h. The abundance is represented by the relative peak area, calculated from dividing the area of the given peak by the total peak area in the given chromatogram. Samples are analyzed on GC/Q-TOF-MS. SHS_microbe1_1, SHS_microbe1_2, SHS_microbe1_3, SHS_microbe1_72 and DHS_microbe1_169 are the sample identifiers, see table 1 for further information.

4. Discussion

4.1 Pear ester

Pear ester is not detected in the headspace of the walnut, nor codling moth frass, nor microbial samples. This may be due to several factors. The samples may simply not emit pear ester, or they do emit pear ester, but in quantities too low for the GC-MS to detect it. Using single sensillum recordings (SSR) coupled with gas chromatography increases the sensitivity of the analysis and may therefore reveal pear ester in minute concentrations, if not from the five microbial headspaces, then perhaps from the headspaces of *C. pomonella* frass or infested walnuts. GC-SSR is a method that makes use of *Drosophila melanogaster* bred to express CpomOR3, the *C. pomonella* odorant receptor tuned to pear ester (Gonzales 2017). Coupling of the single sensilla response with flame ionization detector (FID) may confirm the presence of pear ester in the given sample.

We should also assess the medium, temperature and inoculation time used in this study, when preparing the microbial inoculated broths. Ljunggren et al. (2019) grew *Metschnikowia saccharicola* in 100 ml of liquid minimal medium for 24h in a 25°C incubator, followed by successful detection of pear ester in the sample headspace. Further tests with *M. saccharicola* with differing media, temperature and inoculation times should determine how to promote a growth curve and metabolism resulting in pear ester emittance. Adapting our procedure to such data, would increase the probability of pear ester emittance from our microbial samples, given that pear ester is part of their microbial metabolism.

In this study, we examine the headspace of only five microbes, which is too few to represent the total volatile profile of the walnut microbiome. Pardatscher and Schweigkofler (2009) isolated 3.880 microbial cultures from twig, leaf, and fruit of *Juglans regia*, of which 96.4% belonged to fungi and 3.6% to bacteria. These numbers emphasize the diversity and abundance of the phytomicrobiome, while stressing how small a fraction of the microbiome, we have studied. Continued and extended research of walnut-associated, microbial volatiles is needed. Another approach in the search for pear ester would be DNA sequencing of individual microbial isolates and a community level metagenomics, in an attempt to identify microorganism known to emit pear ester, such as *Metschnikowia spp.* This method was not feasible in this study due to time limitation but should be part of future research.

4.2 Selected volatile compounds

The eight selected key compounds are detected in the headspaces of the five microbes (see table 1). The finding of these compounds known to induce antennal response in the codling, supports the theory that microbes are an essential part of the interaction between host plant and codling moth, and that microbes may be part of producing the host plant cues.

3-Methylbutyl ethanoate has the highest relative abundance in all microbial samples with a 24h inoculation time, except microbe 22 (figure 3). 3-Methylbutyl ethanoate has a relatively high vapor pressure and is more volatile than the other VOCs we investigate, which may explain its abundance (*Isopentyl Acetate Mixture of Isomers, Reagent Grade, 98 123-92-2,* n.d.). In the 72h inoculated samples the dominance of this VOC is less pronounced, indicating that 3-methylbutyl ethanoate may only be produced in the early phase of microbial growth (see figure 7). The 72h inoculation time was introduced to test its potential effect on microbial metabolism concerning pear ester emittance from the microbial samples, but it had no effect in this regard. The fact that microbe 22 do not emit 3-methylbutyl ethanoate suggests that we have succeeded in selecting different microbes, at least regarding this microbe.

None of the selected VOCs are detected from the infested walnut, but from the frass samples, five of the eight compounds appear to be present in the inoculum headspaces (table 2). For conclusive identification of volatiles from infested walnuts and codling moth frass, additional analysis on a polar column is needed, along with calculation of associated retention indices. Technical replicates will also help confirm the compound identification.

4.3 Volatile sampling methods

The two methods (SHS and DHS) appear to be equally effective, concerning their ability to trap/absorb the selected key compound, as both methods lead to the detection of eight out of nine compounds, pear ester being the exception in both cases (see table 1 and 2).

Static headspace sampling with SPME is a relatively fast technique, with a simple set-up and a simple procedure for cleaning of both glassware and SPME fibers. This method allows for repeated sampling within a short time frame. The disadvantages of this method include the accumulation of heat and humidity in the sample container, which may decrease the efficiency of volatile extraction (Tholl et al. 2006). Additionally, saturation of the SPME fiber is a risk. Our results from SPME only show small differences between microbial headspaces and the headspace of a blank YPD broth (see figure 5). It appears that the blank YPD broth close to saturates the SPME fibers so that the volatile background potentially covers the small amounts of volatiles emitted from the microbes, making them complicated to identify. Future tests with SPME sampling of different inoculated growing media, e.g. minimal medium, should test the degree of saturation.

The chromatograms from our SPME samples are highly contaminated with siloxane-compounds from the fiber coating, resulting in excessive peaks in the chromatograms, which complicates the analysis of the chromatograms and requires extra time. The chromatograms from SHS contain an average of 230 peaks, compared to the chromatograms from DHS containing an average of 113 peaks. An additional challenge with SPME sampling, is not being able to re-analyze the sample. If such a need arises, a biological replication is the only option.

The SPME fibers we use, are coated with 3 different types of adsorbents; divinylbenzene (DVB), carboxen (CAR) and polydimethyl-siloxane (PDMS), which combined function as a bipolar adsorbent. Merck, the company that produces the fibers, recommends this coating for sampling of volatiles and semi-volatiles from C3-C20, with the molecular weight range from 40-275 g/mol, which matches the weight-range of our selected key compounds (Supelco. Analytical Products, n.d.). Despite the well-considered choice of fiber

coating, there is still a risk that individual compounds are not adsorbed by the coating and therefore overlooked in the analysis of the volatile samples.

Certain compounds are only detected on GC-MS instruments with either polar or non-polar column, while dual detection, with matching retention indices, are needed for tentative identification of stated compound. The GC/Q-TOF-MS is equipped with a high efficiency ion source (HES), which increases the efficiency of the ionization of the analytes, and a highly sensitive Q-TOF MS, which provides more precise mass spectra, than the GC-MSD equipment. This most probably explain why volatiles only are detected on the GC/Q-TOF-MS. However, nonanal in the headspaces of the microbial samples, are only identified on a polar column, except for sample SHS_microbe22_1 and SHS_microbe22_2 (see table 1). This is possibly connected with the functionality of the SPME fibers used. SPME fiber A is always run on the GC/Q-TOF-MS and SPME fiber B is always run on the GC-MSD. It appears that the SPME fiber A is unable to adsorb nonanal, for unknown reasons. This theory is strengthened by the fact that sample SHS_microbe22_1 and SHS_microbe22_2 are, as an exception, sampled using a different SPME fiber (fiber 4).

Dynamic headspace collection with a Super-Q trap, enable extraction of the trapped volatiles with a solvent, resulting in a storable solution. The chromatograms from DHS have distinct peaks, and are relatively less contaminated, compared to the chromatograms from SPME samplings. In the bar chart of figure 7 we see the different relative peak sizes from SHS and DHS of microbe 1, and for most of the key compounds, the relative peak area is greatest in the chromatogram from DHS of microbe 1. Having distinct peaks makes analysis of the chromatograms simpler.

Disadvantages of DHS includes the long sampling time (24h) and relatively long preparation time needed for cleaning equipment and setting it up, as well as time spend on extraction and concentration of the sample afterwards. The use of only one solvent also introduces the risk of the trapped volatiles not being soluble to the same extent in the select solvent. In this study we use hexane as a solvent, which proves sufficient in extracting our selected key compounds, except for pear ester, which is either not emitted, trapped, or extracted, although the former is most likely. The solvent also introduces the risk of contamination, which is why re-distilled hexane is recommended for ensuring a pure solvent. Another disadvantage of using a solvent, is the need for a solvent delay when analyzing the sample using GC-MS. Very volatile compound with a retention time lower than the solvent delay, will therefore not be detectable. The retention time for our key compounds is longer than the solvent delay, so this was not a problem in this study.

For continued analysis of volatiles from the microbiome of infected walnuts (*J. regia*) we recommend proceeding with dynamic headspace sampling. Since this study is a part of the basic research on the interaction between walnuts-microbes-*Cydia pomonella* it is likely that the ability to store the volatile samples, might prove advantageous if or when other analysis is needed. This recommendation is based on the given conditions that the selected key compounds are the same as in this study, as they have proven soluble in hexane, and all have retention times that are longer than the solvent delay.

5. Conclusion

Pear ester is not detected from the five microbes isolated from infested walnut kernels and codling moth larval frass. It is neither detected from the infested walnut nor from the frass samples. Continued study of volatiles from the microbiome of *Juglans regia* fruit is needed, to conclude whether pear ester is part of the host cue, guiding *Cydia pomonella* towards *Juglans regia,* as it is for multiple host plant in the family of *Rosaceae*. It should be considered to expand the research methods in use, to include methods for identification of microbes known to emit pear ester, such as yeasts belonging to the genus of *Metschnikowia.* Also, adapting the inoculation time, temperature, and media to match the optimal conditions for pear ester emittance from *Metschnikowia saccharicola*, will increase the possibility for emittance of pear ester from our microbial samples, given pear ester is part of their metabolism.

The key volatile compounds nonanal, decanal, 2-nonanone, 3-methylbutyl ethanoate, 2-phenylethanol, sulcatone, 3-methylbutyl propanoate and ethyl hexanoate, are all detectable in the five microbial headspaces using both static and dynamic headspace sampling, followed by GC-MS analysis. These compounds are selected for their documented antennal response in *Cydia pomonella* and are all known from the headspace of *Metschnikowia ssp.* (Lindblom 2012; Ljunggren et al. 2019).

Both static and dynamic headspace sampling prove sufficient in adsorbing the key compounds, and both methods are therefore appropriate for continued headspace sampling. That said, we recommend continuing with dynamic headspace sampling due to the listed advantages of this method, which especially applies to the storability of the samples.

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