Original Article

**Hippophae rhamnoides** berry related *Pichia kudriavzevii* yeast volatiles modify behaviour of *Rhagoletis batava* flies

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**HIGHLIGHTS**

- *Pichia kudriavzevii* yeasts were isolated from ripe *Hippophae rhamnoides* berries.
- Thirty-five yeast volatiles were identified from the headspace of *P. kudriavzevii*.
- Esters and alcohols contributed by 32% and 66% to the total blend amount.
- Ten of those volatiles elicited antenna responses of *Rhagoletis batava* flies.
- Mixture of synthetic olfactory active compounds attracted *R. batava* males and females.

**GRAPHICAL ABSTRACT**

**ABSTRACT**

Olfactory cues have a large impact on insect behaviour and fitness consequently showing potential in pest management. Yeast released volatiles are used by insects as olfactory cues for finding feeding and oviposition sites. The yeast strain SB-16-15 was isolated from spontaneous fermentation of *Hippophae rhamnoides* berries and identified as *Pichia kudriavzevii*. Thirty-nine volatiles were sampled from the headspace of *P. kudriavzevii* yeasts by solid phase micro extraction and identified by gas chromatography and mass spectrometry techniques. Ten of those volatiles elicited antennal responses of *Rhagoletis batava* flies, one of the most serious pest of *H. rhamnoides* berries. In the two-choice experiments, *R. batava* flies preferred the mixture composed of nine synthetic compounds analogous to electroanntenographic active volatiles released by the yeasts compare to the solvent control. Female flies were significantly attracted to the mixture at the concentration 0.1 µL mL⁻¹ and showed no preference to the mixture at the...
Gas chromatography-mass spectrometry

Gas chromatography–electroantennogram detection

Concentration 1 μL mL⁻¹ versus control while males reacted positively to the synthetic blend at the concentration 1 μL mL⁻¹. Herein, for the first time, behaviour modifying effect of *H. rhamnoides* berry related yeast volatiles was shown suggesting these semiochemicals have potential in use for monitoring *R. batava* flies.

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### Introduction

Sea buckthorn, *Hippophae rhamnoides* L. (Rosales: Elaeagnaceae) is a hardy, deciduous shrub native to Europe and Asia [1]. In Europe, *H. rhamnoides* is naturally distributed on river banks and coastal dunes along the Baltic Coast of Finland, Poland, Germany as well as along the Gulf of Bothnia in Sweden [1,2]. Due to drought resistance, rapid development of an extensive root system, capability of holding the soil on fragile slopes, ability to fix nitrogen due to association with nitrogen-fixing symbiotic Actinomycetes and conserve other essential nutrients, *H. rhamnoides* is an ideal plant for soil erosion control and land reclamation [3,4]. Sea buckthorn berries are among the most nutritious fruits known with vitamin C contents of 360 mg 100 g⁻¹ of berries for the European subspecies *H. rhamnoides* [5] to 2500 mg 100 g⁻¹ of berries for the Chinese subspecies *H. r. sinensis* [6]. Phytochemicals from berries and essential oil produced from berry pulp and seeds diminish inflammation, have antibacterial effect, relieve pain, promote regeneration of tissues, and have anticancer and radio-protective activity [1.7–11].

Sea buckthorn fly, *Rhagoletis batava* Hering (Diptera: Tephritidae), is the most harmful insect pest of sea buckthorn berries. In the years of mass development of *R. batava* flies without applying pest control means, damage reach 100% [12]. As far as we know *R. batava* damage sea buckthorn berries only, being a highly specialized pest. *R. batava* species was described in the Netherlands [13] and was known only in a few European countries causing no economically important damage to *H. rhamnoides*, however for a long time, this species has been a serious pest of sea-buckthorn in Western Siberia and Altai Region [12,14]. In 1970, Kolomiec has described the Siberian population of sea-buckthorn flies as a new subspecies, *Rhagoletis batava obscuriosa* Kol. On the basis of range expansion data of *R. batava* and notably increased damage level of *H. rhamnoides* berries in the new distribution areas of the flies, Stalàžis and Balalaikins [15] have postulated that the more aggressive *R. b. obscuriosa* flies from Siberia is spreading through the eastern part of Russian Federation to Central and Western Europe. There are number of environment-friendly pest control strategies that could potentially be used to keep *R. batava* population below an economically significant level including natural limiting factors like parasites or entomopathogenic fungi; sterile insect technique; or methods based on application of semiochemicals, for example lure and kill, push-pull techniques and etc. [16–18]. Moreover, semiochemicals are widely used in monitoring programs including efficiency control of pest density regulating methods [16]. Unfortunately, up to our knowledge, no semiochemicals have been identified for *R. batava* species.

The goal of this study was to identify volatiles released by yeasts colonizing *H. rhamnoides* berries that could have behaviour modulating effect on *R. batava* flies. The aims have been formulated to determine whether: (i) some yeast species populating *H. rhamnoides* berries could be isolated and cultivated under laboratory conditions; (ii) some volatiles produce by yeast elicit electroantennographic responses in *R. batava* females and males; (iii) a blend comprised of synthetic compounds analogous to electroantennographic active volatiles have a behaviour modifying effect on *R. batava* flies under laboratory conditions.

### Material and methods

#### Insects

Sea buckthorn flies used in laboratory studies, were collected in May 2018 as puparia in soil under sea buckthorn shrubs with damaged berries located in organic sea buckthorn plantation (global positioning system (GPS) coordinates: 55°15'12.179''N, 25°26'23.049''E) in Stacjava village, Moleštai district, Lithuania. Each puparium was separately placed in 14 mL glass vial containing wet 3 cm² filter paper inside and corked by foam stoppers. Vials were placed in a climate chamber “Fitotron” (Weiss Gallenkamp, UK) under 20–24 °C, 16L:8D (light:dark) photoperiod and 65–75% relative humidity. Two times a week 2–3 drops of water were added on a filter paper to maintain high humidity inside a vial. Emerged adults were kept in the same vials in walk-in climate room under 18–20 °C, natural day light photoperiod, 50–60% relative humidity and fed on 10% sugar solution in water.

#### Yeast isolation and identification

*H. rhamnoides* berries were sampled from the private farm located in the Vilnius region of Lithuania (GPS coordinates: 54°75'20.0''N, 25°27'99.6''E) in the mid-September 2016. Sea buckthorn berries were aseptically collected by using scissors cleaned with 70% ethanol, placed into sterile bags, transported to the laboratory and processed within 2 h after harvesting. Thirty grams of berries were placed into sterile tubes with 5% dextrose solution and kept for 15 days at a 18 °C temperature. Serial dilutions were made in a Ringer (Merck authorized distributor Biotecha UAB, Vilnius, Lithuania) solution, plated on yeast extract-peptone-dextrose (YPD)-agar plates (1% yeast extract, 1% peptone, 2% dextrose, 2% agar) [19] containing 50 μg mL⁻¹ chloramphenicol and incubated for 2–3 days at 25 °C. Selected colonies were applied for morphological analysis and molecular identification.

DNA was isolated from fresh yeast culture (24 h) by using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer’s instructions. For identification of yeast, the regions between the 18S rRNA and 28S rRNA genes containing two non-coding internal transcribed spacers (ITS-A and ITS-B) separated by the 5.8S rRNA gene were polymerase chain reaction (PCR)-amplified using ITS1 (5′-TCCGTAAGTGAACCTGCGG-3′) and ITS4 (5′-TCTCCGGCTATTGAGATATGC-3′) primers [20,21] and sequenced at Base Clear (Leiden, Netherlands). The obtained sequences were compared with those found in the Nucleotide Similarity Search database (NCBI BLAST+), FASTA network service at the European Bioinformatics Institute (EMBL-EBI, Cambridge, UK).

#### Chemicals

The compounds used in behaviour tests namely 3-methylbut-1-yl propionate (≥99% chemical purity), 3-methylbutan-1-ol (≥99% chemical purity), ethyl octanoate (≥99% chemical purity), 2-phenylethyl acetate (≥99% chemical purity) and 2-phenyl ethanol (≥99% chemical purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA); ethyl propionate (99% chemical purity), 3-
methylbut-1-yl acetate (99% chemical purity), ethyl hexanoate (99% chemical purity) were purchased from Alfa Aesar (Ward Hill, MA, USA); ethyl acetate (≥99% chemical purity), hexane as a solvent (≥99% chemical purity) was obtained from Carl Roth (Karsruhe, Germany).

D (+)-Glucose monohydrate (obtained from Merck Millipore, Darmstadt, Germany), yeast extract, peptone and agar (purchased from Liofilchem, Rosedegli Abruzzi, Italy) were used for YPD-agar medium preparation and yeast cultivation. To avoid bacterial contamination, YPD medium was supplemented with chloramphenicol (purchased from Applichem, Darmstadt, Germany).

**Sampling of yeast-produced volatiles**

The solid phase micro-extraction (SPME) technique [22] was used to sample the headspace of P. kudriavzevii yeast strain SB-16-15. The yeasts were cultivated in polystyrene Petri dishes (Ø 55 mm x 14 mm) poured with 14 mL of YPD-agar for 2 days at 25 °C. As control, YPD-agar plates were used for sampling background odours. Before each collection period, the routine purification of SPME fibres coated with polydimethylsiloxane-divinylbenzene polymer (DVB/PDMS, 65 μm coating layer thickness, Supelco, Pennsylvania, USA), was conducted at 240 °C for about 10 min in a GC injector. Afterwards, the needle of SPME syringe was pierced through a small hole made in a wall of a Petri dish just above the yeast culture; the fibre was pushed out from the needle and exposed to the headspace for 60 min at room temperature. After sampling was finished, the fibre was transferred to the injection port of gas chromatograph and volatiles were thermally desorbed from the fibre during 2 min.

**Gas chromatography–electroantennogram detection and identification of volatiles**

Coupled gas chromatography-electroantennogram detection (GC-EAD) was performed using a Clarus 500 gas chromatograph (PerkinElmer, Waltham, MA, USA) and the Synthech electroantennogram detection system (Hilversum, Netherlands) [23]. The GC injector and the detector temperatures were set at 240 °C. The oven temperature was maintained isothermally at 40 °C for 1 min, after which it was raised to 200 °C at a rate of 5 °C min⁻¹, then increased to 240 °C at a rate of 10 °C min⁻¹, and then maintained isothermally for 11 min. Hydrogen at the flow rate 1.5 mL min⁻¹ was used as a carrier gas. Five antennae from the flies of each sex were used for the GC-EAD analysis. Relative amounts of the compounds were determined as areas under chromatographic peaks.

The identification of volatiles was carried out using a Shimadzu gas chromatograph GC-2010 coupled with Shimadzu mass selective detector MS-QP 2010 Plus (Kyoto, Japan) [23–25]. The GC was equipped with Restek Stabil-Wax column (30 m x 0.25 mm x 0.25 μm, Bellefonte, PA, USA) and operated under the same conditions as described in the GC-EAD experiments, except that helium was used as the carrier gas at the flow rate 1.5 mL min⁻¹. The yeast-derived volatile compounds were identified by comparison of their mass spectral data and their retention indexes with the corresponding data available from NIST version 2.0 mass spectra search programme (National Institute of Standards and Technology, USA) and with those of synthetic standards for the compounds indicated in the Table 1 using the software GCMSsolution version 2.71 (Shimadzu, Kyoto, Japan).

**Behaviour tests**

A Y-tube olfactometer (14.5 cm main tube, 10 cm arms, 130° branching angle, 0.9 cm inner diameter) was used to test preference of flies to synthetic samples versus control [26]. Four 18 W tube type lamps (T8/840, Colorlux plus, NARVA, Germany) covered with white, mat, plastic shield (65 cm length, 42 cm width) at a distance of 23 cm were placed in front of the Y tube of the olfactometer. Positive phototaxis is characteristic for sea buckthorn flies and the light stimulated the insects to move towards the light source. The Y tube was held at a 10° angle upward from horizontal on a holder. The arms of the olfactometer were connected to separate glass tubes that contained the stimulus versus control. Clean air was pushed at a rate of 0.5 L min⁻¹ through each arm using a clean air delivery system CADS-4CPP (Sigma Scientific LLC, Micanopy, FL, USA).

Bioassays were conducted to test attraction of the blend comprised of ethyl acetate, ethyl propionate, 3-methylbutyl acetate, 3-methylbutyl propionate, 3-methylbutanol, ethyl hexanoate, ethyl octanoate, phenylethyl acetate and 2-phenyl ethanol at the ratio 42:2:30:1:8:1:4:5:7 against a control. This ratio was selected based on relative amounts of EAD-active headspace volatiles released by P. kudriavzevii. The blend was tested at 1 μL mL⁻¹ and 0.1 μL mL⁻¹ concentrations by dispensing 10 μL of the prepared solution onto a filter paper strip (5 x 40 mm). After 0.5 min of solvent evaporation, the filter paper strip was placed in the glass tube connected to one arm of the olfactometer. The same size filter paper was treated with 10 μL of hexane and after solvent evaporation was placed in the other arm serving as control. The olfactometer was dismantled and the glassware was cleaned with hexane after each test, soaked overnight in distilled water, and dried for 2 h in an oven by rising the oven temperature from 100 to 200 °C. Silicone parts of the Y-tube olfactometer were cleaned with hexane, soaked overnight in distilled water, and air dried or replaced between the tests.

Male and female adults between 2 and 5-day-old were used in the experiment. Flies were allowed feeding on the sucrose solution until used in olfactometer bioassays. Single fly was released into the Y olfactometer at the end of the main tube. Pre-choice duration, i.e. the time within which a fly must have reached the branch point, was 15 min. A fly was considered to have made a choice when the fly reached the distal end of the glass tube containing the stimulus or a solvent control irrespectively whether the fly switched arms or not before reaching the odour source. If the fly did not choose an arm within 15 min, it was considered as not making choice. The positions of the two Y-tube arms were reversed after every five tests. All insects were observed individually and used in a bioassay only once. Out of 106 flies tested 11 specimens (all of them were females) failed to make a choice (10.4%). The tests were carried out at 25 ± 2 °C, 60% RH, between 10.00 and 17.00 h local time.

**Statistical analysis**

Nonparametric Mann-Whitney U test (Statistica 6.0, StatSoft, Inc., Tulsa, OK, USA) was applied to evaluate differences of volatile amounts between yeast and control samples. Generalised linear mixed model (GLMM), logistic regression (glm) with binomial distribution of dependent variable (fly choice: 0, 1) was used to determine whether the effects of independent variables such as the treatment (factorial: control and stimulus), and sex (factorial: male and female) were significant. Afterwards, we evaluated the effect of treatment on fly choice in each fly sex (male or female) and for each stimulus concentration separately. The effect of the stimulus concentration has only been evaluated in the female flies. In all models, experimental replication was treated as a random variable. Statistical evaluations were carried out with program R, version 3.5.1 and RStudio version 1.1.463 (R Core Team, 2017; RStudio Team, 2018).
Pichia kudriavzevii yeast produced volatiles and their electroantennographic activity to Rhagoletis batava flies.

| No | Compound | RI | CAS No | Group | ID | Amount | EAD |
|----|----------|----|--------|-------|----|--------|-----|
| 1  | Ethyl acetate | 898 | 141-78-6 | E | L, RI | 0.06 ± 0.02 | 11.17 ± 6.09* |
| 2  | Ethanol | 902 | 64-17-5 | OH | L, RI | 0.10 ± 0.02 | 11.50 ± 2.04^* |
| 3  | Ethyl propionate | 915 | 110-35-7 | E | RC | 0.01 ± 0.00 | 1.07 ± 0.00%^* |
| 4  | 2-Methylprop-1-yl acetate | 985 | 110-19-0 | E | RC | – | 0.41 ± 0.16 |
| 5  | Ethyl butanoate | 1013 | 105-54-4 | E | RC | – | 0.14 ± 0.03 |
| 6  | Ethyl 2-methylbutanoate | 1033 | 7452-79-1 | E | RC | – | 0.07 ± 0.01 |
| 7  | But-1-yl acetate | 1063 | 123-86-4 | E | L, RI | 0.10 ± 0.02 | 0.02 ± 0.01^* |
| 8  | 2-Methylpropan-1-ol | 1095 | 78-83-1 | OH | L, RI | – | 1.07 ± 0.02 |
| 9  | Unknown | 1099 | | | | | |
| 10 | 3-Methylbut-1-yl acetate | 1105 | 123-92-2 | E | RC | 0.10 ± 0.07- | 9.04 ± 2.35^* |
| 11 | 3-Methylbutyl 1-propionate | 1176 | 105-68-0 | E | RC | – | 0.22 ± 0.01 |
| 12 | 2-Methylbutan-1-ol | 1207 | 137-32-6 | OH | RC | – | 4.17 ± 0.21 |
| 13 | 3-Methylbutan-1-ol | 1213 | 123-51-3 | OH | RC | 0.21 ± 0.11 | 20.11 ± 1.49^* |
| 14 | Ethyl hexanoate | 1224 | 123-66-0 | E | RC | – | 0.26 ± 0.07 |
| 15 | Styrene | 1238 | 108-42-5 | AR | – | – | 0.14 ± 0.06 |
| 16 | 3-Methylbutyl 2-methylbutanoate | 1258 | 27625-35-0 | E | RC | 0.01 ± 0.001 | 0.04 ± 0.001^* |
| 17 | 3-Methylbutyl 3-methylbutanoate | 1287 | 659-70-1 | E | RC | 0.06 ± 0.03 | 0.02 ± 0.02 ns |
| 18 | 2,5-Dimethyl pyrazine | 1316 | 136-32-0 | O | L, RI | 0.09 ± 0.05 | 0.05 ± 0.01 ns |
| 19 | Heptyl acetate | 1382 | 112-06-1 | E | L, RI | – | 0.01 ± 0.001 |
| 20 | Ethyl octanoate | 1430 | 106-32-1 | E | RC | – | 0.45 ± 0.09 |
| 21 | Acetic acid | 1499 | 64-19-7 | AC | L, RI | – | 0.03 ± 0.001 |
| 22 | 3-Methylbut-1-yl hexanoate | 1456 | 2198-61-0 | E | RC | – | 0.03 ± 0.01 |
| 23 | 2-Ethylhexanol-1-ol | 1488 | 1074-76-0 | OH | RC | 0.12 ± 0.09 | 0.04 ± 0.01 ns |
| 24 | 2-Methylpropionic acid | 1562 | 79-31-2 | AC | L, RI | – | 0.20 ± 0.05 |
| 25 | Unknown | 1620 | | | | | |
| 26 | Butanoic acid | 164 | 107-92-6 | AC | L, RI | – | 0.58 ± 0.14 |
| 27 | Unknown | 1055 | | | | | |
| 28 | 3-Methylbutanoic acid | 1703 | 503-74-2 | AC | L, RI | – | 0.1 ± 0.02 |
| 29 | Ethyl 2-phenylethanoate | 1752 | 101-97-3 | E | L, RI | – | 0.01 ± 0.001 |
| 30 | Methoxy-phenyl oxime | 1767 | 100-42-5 | AR | – | – | 0.03 ± 0.01 |
| 31 | 2-Phenylethyl acetate | 1795 | 105-45-7 | E | RC | – | 1.71 ± 1.1 |
| 32 | 3-Methylbutyl decanoate | 1839 | 2306-91-4 | E | L, RI | – | 0.12 ± 0.01 |
| 33 | 2-Phenylethyl acetate | 1858 | 1207-32-6 | OH | L, RI | – | 0.13 ± 0.02 |
| 34 | 2-Phenyl ethanol | 1894 | 123-92-2 | OH | L, RI | – | 0.05 ± 0.01 |
| 35 | Phenyl-2-propynyl acetate | 1982 | 140-26-1 | E | L, RI | – | 0.01 ± 0.002 |
| 36 | Octanoic acid | 2044 | 124-07-2 | AC | L, RI | – | 0.15 ± 0.01 |
| 37 | Hexadecanone | 2119 | 18787-63-8 | K | L, RI | – | 0.03 ± 0.01 |
| 38 | 2-Hexadecanone | 2224 | 2292-51-2 | K | L, RI | – | 0.05 ± 0.02 |

No. is a number of compound as indicated in the Fig. 1; a superscript following compound name indicates the reference reporting that the compound was identified from a volatile blend released by P. kudriavzevii yeast; RI – retention index (polar DB-Wax fused silica capillary column 30 m × 0.25 mm i.d., 0.25 μm film thickness); CAS No – chemical abstract service number; Group – group of chemical compound; ID – identification; EAD – electroantennographic detection; AR – aromatic; AC – acid; E – ester; OH – alcohol, K - ketone; O – other compound; L – NIST and MassFinder3 libraries; RC – reference compound; all values in the columns headed Amount are the absolute amounts expressed as areas under the chromatographic peaks and have to be read as numbers times 100,000; ns – not-significant according to nonparametric Mann-Whitney U test; values in the columns headed EAD represent number of antennae which responded to the compound and values in the brackets indicates how many time the compound was tested; NR – no response. **P < 0.001. ^*P < 0.01. *P < 0.05.

Results and discussion

Pichia kudriavzevii yeast and their odours

Based on Next generation sequencing (NGS) results, the vast majority of sea buckthorn-associated fungal microorganisms and yeasts were described as unidentifiable (87.9%) and at the species level they were assigned to uncultured fungi [21]. Application of fermentation-based enrichment and cultivation techniques revealed that about 68% of the cultured yeast population obtained from the tested sample of H. rhamnoides berries, collected in early autumn of 2016, was composed of P. kudriavzevii yeast. The sequence identity match of isolated yeast strain SB-16-15 was 100% compare to that of Pichia kudriavzevii culture B-WHX-12-19 in GenBank (Fig. 1). To our knowledge, there are no data published dealing with direct isolation and analysis of cultivable yeasts associated with H. rhamnoides berries. P. kudriavzevii yeast have been found in significant amounts on the berries of various grape cultivars [27–29] on apple, pear and plum fruits [30] as well as on cherry fruits [31].

Gas chromatographic – mass spectrometric analyses revealed 39 compounds 35 of which were exclusively present or occur in significantly large amounts in the headspace samples obtained from P. kudriavzevii yeast compare to those of blank samples. The yeast released volatiles were dominated by 19 esters followed by 6 alcohols and 5 volatile fatty acids as well as by 3 ketones, 3 unknown compounds and 2 other type substances. Quantitatively, esters and alcohols contributed by 32% and 66% to the total blend amount, respectively. Five compounds, namely, ethyl acetate, ethanoic acid, 3-methylbut-1-yl acetate, 3-methylbutan-1-ol and 2-phenyl ethanol were released at the largest quantity and contributed to 85% of total blend amount (Table 1).

The same compounds released at the large quantities by kudriavzevii yeast cultivated in malt extract broth medium were reported by Wu et al [32], except 3-methylbut-1-yl acetate comprising 3% of total content while in our analysis the ester...
contributed by 18% to the total composition of the blend. Taking into consideration biosynthetic origin of the compounds [22] produced by *P. kudriavzevii*, the volatiles derived from the carbohydrate catabolism and fermentation pathway have been detected, for example, ethanol, acetic acid and ethyl acetate; from the fatty acids biosynthesis and degradation pathway, for example, octanoic acid, ethyl hexanoate and ethyl octanoate; and from amino acid synthesis and degradation pathway like 2-methylbutan-1-ol, 3-methylbutanoic acid and 2-phenyl ethanol, while volatiles produced by terpene biosynthetic pathway have not been detected.

Electroantennographic activity of yeast-produced volatiles

GC–EAD analyses of *P. kudriavzevii* headspace collections (n = 10) showed that antennae of *R. batava* flies were able to detect 10 volatile compounds in total (Fig. 2). There were no qualitative differences detected in EAD responses between females and males. Chemical structure and EAD activity comparison showed that all ten EAD active compounds elicited antenna responses of both females and males in nearly all replicates tested, except two aromatic compounds, namely 2-phenylethyl acetate and 2-phenyl ethanol which showed activity in two of five male and in three of five female antennae recordings, respectively. Moreover, the compounds 3-methylbut-1-yl acetate and 3-methylbutan-1-ol bearing a methyl group at the carbon 3, showed clear EAD activity, while the presence of methyl moiety closer to a functional group, i.e. at the carbon 2 in the structurally similar compounds, i.e. 2-methylbut-1-yl acetate and 2-methylbutan-1-ol, obliterated EAD activity.

As olfaction is essential sensory modality in insects used to find suitable food sources, oviposition sites and mates [33], ten volatiles present in the headspace of *P. kudriavzevii* yeasts which elicited...
EAD responses of *R. batava* flies, indicate that yeasts may have ability to modulate searching behaviour of flies towards feeding and oviposition sites. The EAD responses of flies to yeasts’ volatiles are the best studied in the family Drosophilidae [34–37] showing that yeast species complexes specific for drosophilid hosts and for host plant berries’ development stages could be resolved solely based on volatile constituents [38,39] which provides base for observing preference for certain host-yeast complex affecting performance of flies. Preference for certain host-yeast complexes leads to specialisation and niche separation which in turn have impact on a sensory specialization for odours enhancing suitable habitat detection [34,36,38]. The olfactory-chemosensory part of associations between Tephritidae flies including those of the genus *Rhagoletis* and yeasts has received far less attention despite that *Rhagoletis* flies are considered direct pests of economic importance.

**Behavioural responses of *Rhagoletis batava* flies to the blend of electroantennographic active compounds**

Statistical data evaluation by GLMM revealed that the independent variable treatment (control and stimulus) significantly influenced the fly choice ($\chi^2 = 11.24$, $P < 0.001$; $\beta$ estimate control $= 0.39$; OR $\pm$ SE $= 0.61 \pm 0.21$; $\beta$ estimate treatment $= 0.59$; OR $\pm$ SE $= 0.41 \pm 0.21$). However, the independent variable sex (male and female) had no significant effect on fly choice ($\chi^2 = 0.03$, $P = 0.85$; $\beta$ estimate female $= 0.24$; OR $\pm$ SE $= 0.76 \pm 0.56$; $\beta$ estimate male $= 0.06$; OR $\pm$ SE $= 0.94 \pm 0.32$). In the two-choice experiments, *R. batava* males preferred the mixture composed of nine synthetic compounds which were analogous to EAD active volatiles released by *P. kudriavzevii* yeasts compared to the control ($\chi^2 = 10.563$; $P = 0.001$; $\beta$ estimate stimulus $= 0.773$; OR $\pm$ SE $= 0.23 \pm 0.35$), whereas female flies showed no preference to the nine component mixture presented at 1 $\mu$L mL$^{-1}$ ($\chi^2 = 0.095$; $P = 0.758$; $\beta$ estimate stimulus $= 0.182$; OR $\pm$ SE $= 0.82 \pm 0.59$) but were attracted to the mixture at the concentration 0.1 $\mu$L mL$^{-1}$ ($\chi^2 = 4.972$; $P = 0.026$; $\beta$ estimate stimulus $= 0.559$; OR $\pm$ SE $= 0.44 \pm 0.36$) (Fig. 3).

Paleontology and molecular evolutionary biology data suggest that yeasts and insects have coexisted for 300–400 Ma years [40,41] long before the origin of angiosperms, which evolved 125–150 Ma years [42]. The long lasting coexisting between yeasts and insect resulted in the widespread and diverse nature of the association ranging from pathogenic and parasitic to mutualistic interactions, wherein the latter relationships are based on yeasts as a food for the insects and the insects as a vector for the yeasts [43–45]. Yeasts produce variety of odours [22] and substantially contribute to volatile chemical profile of a habitat affecting insect search for resources [46]. Behaviour modifying effect of volatiles released by yeasts have been observed in different insect orders [45,47–50], while behavioural activity of berry and fruit populating yeast odours was mostly reported in dipterans with major focus on drosophilid flies mainly from the genus *Drosophila* [34,38,45,46]. Scarce information is available for flies from the family Tephritidae [51–53].

Our results showed that both males and females positively responded to the mixture of nine EAD active yeast volatiles in the two-choice experiment under laboratory conditions. Notably, females were attracted to the lower concentration of the blend compare with males most probably due to differences in response to olfactory cues eliciting search for oviposition site compare to that mediating food source finding. *P. kudriavzevii* yeasts were characterised as well-fermenting yeasts [54,55] and were more abundant on damaged than on intact grape berries [27]. We assume that *P. kudriavzevii* yeast produced odours could indicate to flies easier access to berry interior and higher nutrient quality due to presence of yeasts for adult feeding and less suitability for larval development.

Olfour-mediated interactions between yeasts and insect are complex and diverse ranging from attractive to repellent depending on whether mutualistic or harmful yeasts inhabited a substrate. There is growing experimental evidence that yeast volatiles can be successfully used in integrated pest management programmes [56–58]. Our present work revealed that the blend composed of nine EAD active yeast volatiles attracted *R. batava* flies under laboratory conditions providing background for further optimisation of the blend and development of semiochemical based trap for monitoring and control of this pest in *H. rhamnoides* orchards.

**Conclusions**

*P. kudriavzevii* yeast inhabiting ripe *H. rhamnoides* berries have been successfully isolated and cultivated. Thirty-nine yeast associated volatiles were sampled and 35 identified from the headspace of *P. kudriavzevii*. Ten of those volatiles elicited antenna responses of *R. batava* flies. Nine component mixture comprised of synthetic compounds analogous to EAD active volatiles at the concentration 1 $\mu$L mL$^{-1}$ significantly attracted males while the females showed significant preference for the mixture at the lower 0.1 $\mu$L mL$^{-1}$ concentration. The behaviour modifying effect of the mixture indicates an application potential of EAD active volatiles in pest management programs of *R. batava* flies.

**Compliance with Ethics requirements**

This article does not contain any studies with human or animal subjects.

**Declaration of Competing Interest**

Authors declare no conflict of interest.

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