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Two Gut-Associated Yeasts in a Tephritid Fruit Fly have Contrasting Effects on Adult Attraction and Larval Survival

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Abstract Yeast-insect interactions have been well characterized in drosophilid flies, but not in tephritid fruit flies, which include many highly polyphagous pest species that attack ripening fruits. Using the Queensland fruit fly (*Bactrocera tryoni*) as our model tephritid species, we identified yeast species present in the gut of wild-collected larvae and found two genera, *Hanseniaspora* and *Pichia,* were the dominant isolates. In behavioural trials using adult female *B. tryoni*, a fruit-agar substrate inoculated with *Pichia kluyveri* resulted in odour emissions that increased the attraction of flies, whereas inoculation with *Hanseniaspora uvarum*, produced odours that strongly deterred flies, and both yeasts led to decreased oviposition. Larval development trials showed that the fruit-agar substrate inoculated with the 'deterrent odour' yeast species, *H. uvarum,* resulted in significantly faster larval development and a greater number of adult flies, compared to a substrate inoculated with the 'attractive odour' yeast species, *P. kluyveri,* and a yeast free control substrate. GC-MS analysis of volatiles emitted by *H. uvarum* and *P. kluyveri* inoculated substrates revealed significant quantitative differences in ethyl-, isoamyl-, isobutyl-, and phenethyl- acetates, which may be responsible for the yeast-specific olfactory responses of adult flies. We discuss how our seemingly counterintuitive finding that female *B. tryoni* flies avoid a beneficial yeast fits well with our understanding of female choice of oviposition sites, and how the contrasting behavioural effects of *H. uvarum* and *P. kluyveri* raises interesting questions regarding the role of yeast-specific volatiles as cues to insect vectors. A better understanding of yeast-tephritid interactions could assist in the future management of tephritid fruit fly pests through the formulation of new "attract and kill" lures, and the development of probiotics for mass rearing of insects in sterile insect control programs.

Keywords Microbial · Insect · *Hanseniaspora* · *Pichia* · *Bactrocera* · Olfaction · Microbiome · Phytobiome · Odours · Volatiles

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Introduction

The role of yeasts in multitrophic microbe-insect-plant interactions is a rapidly expanding field of research that is shedding new light on the behavioural ecology of herbivorous insects (Vega and Dowd 2005; Gibson and Hunter 2010). Many yeast species utilise insect dispersal for colonising new environments, and have evolved symbiotic relationships with their insect vectors (Christiaens et al. 2014). Volatile organic compounds (VOCs) emitted by yeasts are thought to act as olfactory signals to attract insect vectors (Davis et al. 2013), and in return for transport to a suitable substrate, yeasts provide the adult insect (Starmer and Aberdeen 1990) or developing larvae (Rohlfs and Kürschner 2010) with nutrition that promotes its survival and reproduction.

Symbiotic relationships between insects and their associated yeasts received considerable attention in *Drosophila* (Chandler et al. 2012). In *Drosophila melanogaster* and

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Drosophila suzukii, yeasts promote adult oviposition and reproductive output, and provide nutrients that benefit larval development (Anagnostou et al. 2010; Stamps et al. 2012; Yamada et al. 2015; Mori et al. 2016). Volatiles emitted by yeasts take a precedent over host fruit volatiles in these two species (Becher et al. 2012; Scheidler et al. 2015), and species-specific preferences for different yeasts (Starmer and Fogleman 1986; Scheidler et al. 2015) may have played a role in the evolution of niche separation between these two species (Barker et al. 1988; Buser et al. 2014). In the wake of the rapid spread of the spotted wing drosophila, *D. suzukii*, an understanding of the role yeasts play in attracting these flies has formed the basis of current and emerging "attract and kill" pest management strategies, which use odour attractants to monitor and mass trap this highly destructive pest of ripening fruits (Hamby and Becher 2016).

Far less is known about the role of yeasts in the ecology of tephritid fruit flies. Tephritids include many species of highly polyphagous pests that continue to cripple horticultural economies around the globe (Clarke et al. 2011; Sarles et al. 2015). Some of the most problematic species are in the genera *Ceratitis* (e.g. Medfly, *C. capitata*), *Bactrocera* (e.g. Oriental fruit fly, *B. dorsalis*), *Anastrepha* (e.g. Mexican fruit fly, *A. ludens*), and *Rhagoletis* (e.g. apple maggot fly *R. pomonella*), which contain species that attack ripening fruits and have a major impact on market access (Clarke et al. 2005; Malacrida et al. 2007; Sarles et al. 2015). Recent studies on tephritids have identified species of yeast in the larval alimentary canal (Deutscher et al. 2016), although unlike drosophilids, there are no studies investigating whether these yeasts provide the larvae with any benefit. Standard yeast supplements, however, are essential ingredients in culture media for rearing these insects for study, and mass release in sterile insect programs (Pérez-Staples et al. 2009); and yeast-based "protein baits" are used in adult tephritid trapping programs worldwide (Balagawi et al. 2014; Navarro-Llopis et al. 2015; Varikou et al. 2016).

Our study aimed to address the knowledge gap that exists in understanding yeast-insect interactions in tephritids. We used the Queensland fruit fly, *Bactrocera tryoni*, a major pest in fruit orchards across eastern Australia (Clarke et al. 2011), as our model system. We began with the molecular identification of yeasts found in the gut of wild *B. tryoni* larvae collected from three host fruits; peaches (*Prunus persica*), cherry plums (*Prunus cerasifera*) and strawberry guavas (*Psidium cattleyanum*); identifying two predominant species, *Hanseniaspora uvarum* and *Pichia kluyveri*. The influence of the two yeasts on adult attraction and oviposition preference was investigated using two different behavioural assays, and GC-MS analysis was conducted to identify yeast volatile emissions. Larval survival in the presence of yeasts was tested by exposing *B. tryoni eggs* to orange-agar substrates inoculated with one of the two yeasts, recording development time and survival to pupation and adulthood.

Materials and Methods

Isolation of Yeasts from *B. tryoni* Larval gut

B. tryoni larvae were collected from ripe, infested, cherry plums (*Prunus cerasifera),* peaches (*Prunus persica*), and strawberry guavas (*Psidium cattleyanum*) picked from trees in orchards around Victoria, Australia. Fruits were transported to the laboratory in sterile ziplock bags, where larvae were extracted and identified morphologically using a taxonomic key (White and Elson-Harris 1992). In addition, larvae allowed to develop in fruits to pupation stage were used to confirm species identification. A random sample of extracted larvae were individually surface sterilized in 5% NaClO for 30 s, followed by a rinsing in 70% ethanol solution, and then placed in 1 mL MilliQ water in a 1.5 mL Eppendorf tube. Absence of fungal cells on surface sterilized larvae was confirmed by streaking 1 μL of the MilliQ water containing larva on Sabouraud Dextrose Agar (SDA) (40 g/L Dextrose, 10 g/L Peptone, 15 g/L agar, 34 μg /mL chloramphenicol). Surface sterilized larvae were then transferred to individual 1.5 mL Eppendorf tubes containing 50 μL MilliQ water and crushed using a sterile pestle to release gut contents. The crushed larvae were then streaked on SDA agar. Yeast-like colonies were re-streaked up to four times on fresh agar plates to obtain pure isolates. Identified yeasts were stored at 4 °c on individual SDA plates between experiments and restreaked every 2 weeks, or placed in 30% glycerol at −80 °c for longer term storage.

Molecular Identification of Yeasts

A single colony from the pure isolate was selected for DNA extraction as per Löoke et al. 2011. The internal transcribed spacer (ITS) region was amplified using ITS1/ITS4 primers (White et al. 1990). The D1/D2 region of 28S rDNA was also amplified using NL1/NL4 primers to confirm species assignment for isolates used in behavioral assays (Kurtzman and Robnett 1997). Reactions were set up using OneTaq DNA Polymerase (New England BioLabs, Ipswich, Massachusetts, USA). PCR products of expected length were visualized on agarose gel, then purified and sequenced in both directions by Macrogen Inc., South Korea (http://www.macrogen.com) using the same primers. Consensus sequences were manually assembled and edited according to chromatograms in Bioedit (Hall 1999). Nucleotide sequences were deposited on GenBank with accession numbers KY977691-KY977703. Tentative species assignments were made based on BLASTn alignments against the NCBI GenBank database.

Phylogenetic Analysis

Isolates with identical ITS nucleotide sequences were pooled, leaving 13 genotypically distinct strain sequences, which were

then aligned with 58 additional taxonomically related type sequences obtained from the NCBI Genbank Database. Sequences were aligned using MUSCLE (Edgar 2004) then excess sequence trimmed using trimAl (Capella-Gutiérrez et al. 2009) with automated settings. The models function in MEGA 6 (Tamura et al. 2013) was used to select the appropriate model of nucleotide sequence evolution. Kimura two-parameter model with discrete gamma distribution was selected to best describe the substitution pattern among 24 different nucleotide substitution models. The evolutionary history was inferred using the Maximum Composite Likelihood method. All positions containing gaps were eliminated leaving 177 positions in the final dataset. Confidence at each node was assessed by 1000 bootstrap replicates. The *Filobasidium magnum* ITS sequence was used as an outgroup.

Adult fly Responses to Yeast Odours

Olfactory Responses A three-choice odour-trap assay was conducted to test the attraction of mature, mated and young, virgin female flies to orange-agar media infected with *Hanseniaspora uvarum, Pichia kluyveri* or a sterile control. Fly traps were assembled from 70 mL screw-capped sterile pots (TechnoPlas, South Australia) containing 15 mL of orange-agar (preservative free orange juice, 15 g/L agar). A 1.5 mL Eppendorf tube, cut 5 mm from the narrow end, was slotted through a puncture hole in the lid. The lid surrounding the trap entry was sealed with *Parafilm* to prevent odours escaping. Fly traps were inoculated with 250 μL of a high cell density (OD 600 ≥ 9) suspension of the treatment yeasts *Hanseniaspora uvarum* (HU) or *Pichia kluyveri* (PK) (on SDA growth media) or SDA media only for the control. Traps were incubated overnight at 25 °C then placed in mesh cages (30 cm × 30 cm ×30 cm) in a triangular formation with equal distance (18 cm) between each treatment. 30 female flies were released into the cage at 9:30 AM and left for 8 h, rotating the trap position hourly to control for positional bias. Trap catches were counted at the end of the 8 h period. Trials using mature and virgin females were conducted at the same time but in separate cages. Mature females were 12 days old had been given ad libitum access to protein (hydrolysed brewer's yeast), sugar and water. Virgin females were 5 days old and provided with only sugar and water to prevent sexual maturation (Meats and Leighton 2004). All flies were starved of sugar and protein for 12 h before commencing the experiment. Twenty replicates were carried out for each treatment, for mated and virgin female flies (5 replicates of mated and virgin flies run in parallel over 4 days).

Oviposition Responses A three-choice assay was conducted with mated female flies, using oviposition pots inoculated with either HU, PK or a sterile control. Oviposition pots were assembled from 30 mL plastic pots, pierced with 8 holes equidistantly around the side of the pot to allow females to insert their ovipositor but not the whole abdomen. Sterile filter paper was inoculated with a high cell density (OD 600 ≥ =9) suspension of yeast grown in autoclaved orange juice then placed around the inside surface of the oviposition pots. Pots were arranged in 30 × 30 × 30 cm cages as per the olfactory trap experiment. 10 mated females (16 day old), reared as described previously, were released into each test cage at 9:30 AM and left for 8 h, with the position of each treatment pots rotated hourly. At the end of the trial, eggs laid on the filter paper lining and inside the pot were counted. Twelve replicates were carried out for each treatment.

Larval Development on Yeasts

Freshly laid *B. tryoni* eggs were collected on filter paper, then washed with 0.4% NaClO followed by autoclaved deionized water. 20 surface sterilized eggs were placed on an orange juice-agar medium (preservative free orange juice, 15 g/L agar, autoclaved) in deep petri dishes (50 mm × 24 mm). A small section (5 mm × 5 mm) at the edge of the petri dish was inoculated with 1 μL of pure *Hanseniaspora uvarum*, *Pichia kluyveri* cells using a sterile loop or left sterile as a control, forming three treatment groups (HU, PK, Control). Petri dishes were sealed with parafilm with small holes pierced with a sterile entomological pin to allow gas exchange and decrease humidity, then placed in an incubator set at 23 °C, 60% external RH, 16:8 h light:dark. After 7 days, the Petri dish lids were removed to allow late instar larvae to emerge from the media. Although this increased the chances of contamination, this step was necessary to enable larval pupation. Treatments were checked daily for larvae at the "popping" stage, which occurs prior to pupation (larvae attempt to propel themselves out of the substrate) (Drew 1987). At this stage, larvae were transferred to vermiculite to complete pupation. Time until pupation, number of pupae, and emerging adults were measured. Twenty replicates were carried out for each treatment.

Yeast Volatiles Analysis

Yeast media was as prepared as before, using 70 mL sterile pots containing 15 mL of orange-agar inoculated with 250 μL of 72 h yeast cultures, and incubated overnight at 25 °C. Sterile orange-agar media was used as a control. Volatiles were sampled from two treatment pots placed in 3 L glass flasks. Air purified through a 500 mL gas wash bottle filled with activated charcoal (8–20 mesh, Supelco, USA) was drawn from one end of the flask at 500 mL min^{-1}. Volatiles were trapped on the opposite side of the flask using 100 mg of a Porapak Q adsorbent (80 mesh, Supelco, USA) packed between two silanized glass wool plugs in a glass Pasteur pipette (4 mm inner diameter). Collections were run for 8 h, and volatiles were eluted from the adsorbent with 300 μL of

dichloromethane (≥ 99.9% residue analysis, Sigma Aldrich, Australia). Next, 300 ng of two internal standards was introduced in the samples, in the form of 5 μl of a mixture of *n*-octane (≥ 99%, puriss.; Sigma Aldrich, Australia) and nonyl acetate (≥ 97% FCC grade, Sigma Aldrich, Australia) prepared in dichloromethane. Quantification of analytes was achieved by comparing peak areas of compounds eluting between 5 and 10 min with the peak area of *n*-octane (early eluent) while peak areas of compounds eluting after 10 min were compared to that of nonyl acetate (late eluent)

Samples were analysed by GC-MS on an Agilent 7890A Gas Chromatograph coupled with an Agilent 7000 Tandem triple quadrupole mass spectrometer equipped with a non-polar column DB-5MS (30 m × 0.25 mm × 0.25 μm). Helium was used as carrier gas. An Autosampler (GC Sampler 120, Agilent Technologies) was used to inject 2 μL of the samples. Injection was performed in pulsed splitless mode at 250 °C. Initial oven temperature was set at 30 °C held for a minute before increasing at 10 °C/min to 200 °C, and then to 250 °C at 20 °C/min, maintained for 3 min. Ionization was performed in EI mode (70 eV) and scan range was set between 35 and 350 amu. Compounds were identified by comparison of their mass spectra with a NIST14 mass spectral library, and confirmed using Kovats indices calculated on non-polar (DB-5MS) and polar columns (Supelcowax 10, Supelco, USA).

Data Processing and Statistical Analysis

Behavioural Experiments Trap catches and egg counts from the two 3-choice trials were analysed as proportional data. As data did not conform to a normal distribution (Kologorov-Smirnov test) following arcsin transformation, we used the non-parametric Kruskal-Wallis test (KW), and a Dunn's test for post hoc analysis (with Šidák correction on alpha value). The difference between virgin and adult fly catches in traps was analysed with a Mann-Whitney U test. *Larval development trials.* Larval trials were analysed using KW tests. All data analysis was carried out using Rstudio software (Rstudio Team 2016). *Volatile analysis.* Due to the extensive number and diversity of compounds detected, we considered only the most consistently found volatiles that accounted for at least 1% of the chemical profile in any of the treatments. Quantities of shortlisted compounds were used as variables for ordination using non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix (excluding limonene, essentially emanating from the orange-agar). An ANOSIM test using the same type of matrix was used to test for dissimilarities between treatments. Pairwise comparisons were made using sequential Bonferroni corrections. SIMPER analysis was subsequently performed to determine which compounds were responsible for differences in chemical profiles. Volatiles data were analysed using PAST version 2.17c.

Results

Yeast Diversity in *B. tryoni* Larval gut

A total of 91 unique yeast isolations were made from 48 individual *B. tryoni* larvae. The isolates formed 13 genotypically distinct strains and were tentatively assigned to 12 described species or genera based on BLASTn sequence similarity and phylogenetic analysis (Fig. 1, Supplemental Fig. 1 (full tree)). Two strains genotypically distinct in the ITS region were both assigned as *Pichia kluyveri*. The vast majority of yeast isolates belonged to the budding yeasts (phylum Ascomycota, sub-phylum Saccharomycotina). The most frequently isolated yeasts belonged to the genus *Pichia*, including *Pichia kluyveri*, *Pichia kudriavzevii* and *Pichia terricola.* The second most frequently isolated yeasts belonged to the genus *Hanseniaspora* and included *Hanseniaspora uvarum*, *Hanseniaspora opuntiae/meyeri* and *Hanseniaspora guilliermondii*. A number of other ascomycetous yeast species were isolated at lower frequency and included *Wickerhamomyces* sp., *Starmerella bacillaris*, *Kluyveromyces* sp., *Torulaspora* sp. and *Satumispora diversa*. The only basidiomycetous yeast to be isolated belonged to the genus Naganishia (sub-phylum Agarimycotina) but exact species assignment was not possible solely based on ITS sequence. Any isolate that could not be assigned to a recognized species with at least 99% sequence identity or a bootstrap support cut-off of at least 70 was assigned to genus only. Due to low nucleotide sequence variation in the ITS region between species of the *Hanseniaspora* genus (Cadez et al. 2003), species assignments for the three *Hanseniaspora* strains isolated in this study are to be considered tentative. Larvae in different fruits varied in abundance of yeast species (Supplemental Table 1), however a number of *Pichia* and *Hanseniaspora* yeasts consistently appeared. Because of their frequency in sampled *B. tryoni* larvae, the yeasts *Pichia kluyveri* and *Hanseniaspora uvarum* were investigated further for their effects on larval development and adult behaviour.

Adult fly Responses to Yeast Odours

Olfactory Responses (Trap Test) Mated adult female *B. tryoni* responded well to the traps presented in the 3-choice experiment (mean response = 59 ± 4% flies), and showed significantly different responses to odours emitted from the different inoculation treatments ($\chi^2_{(2)} = 30.5$, $P < 0.001$, KW). Traps containing orange-agar media inoculated with *Pichia kluyver*i were the most attractive, catching significantly more flies than those inoculated with HU (PK = 60 ± 5%, HU = 7 ± 1%, $P < 0.05$) and the sterile control (Control = 33 ± 6%, $P < 0.05$)(Fig. 2a). Traps containing *H. uvarum* appeared to have a deterrent effect, catching significantly fewer flies than the control ($P < 0.05$). Virgin flies

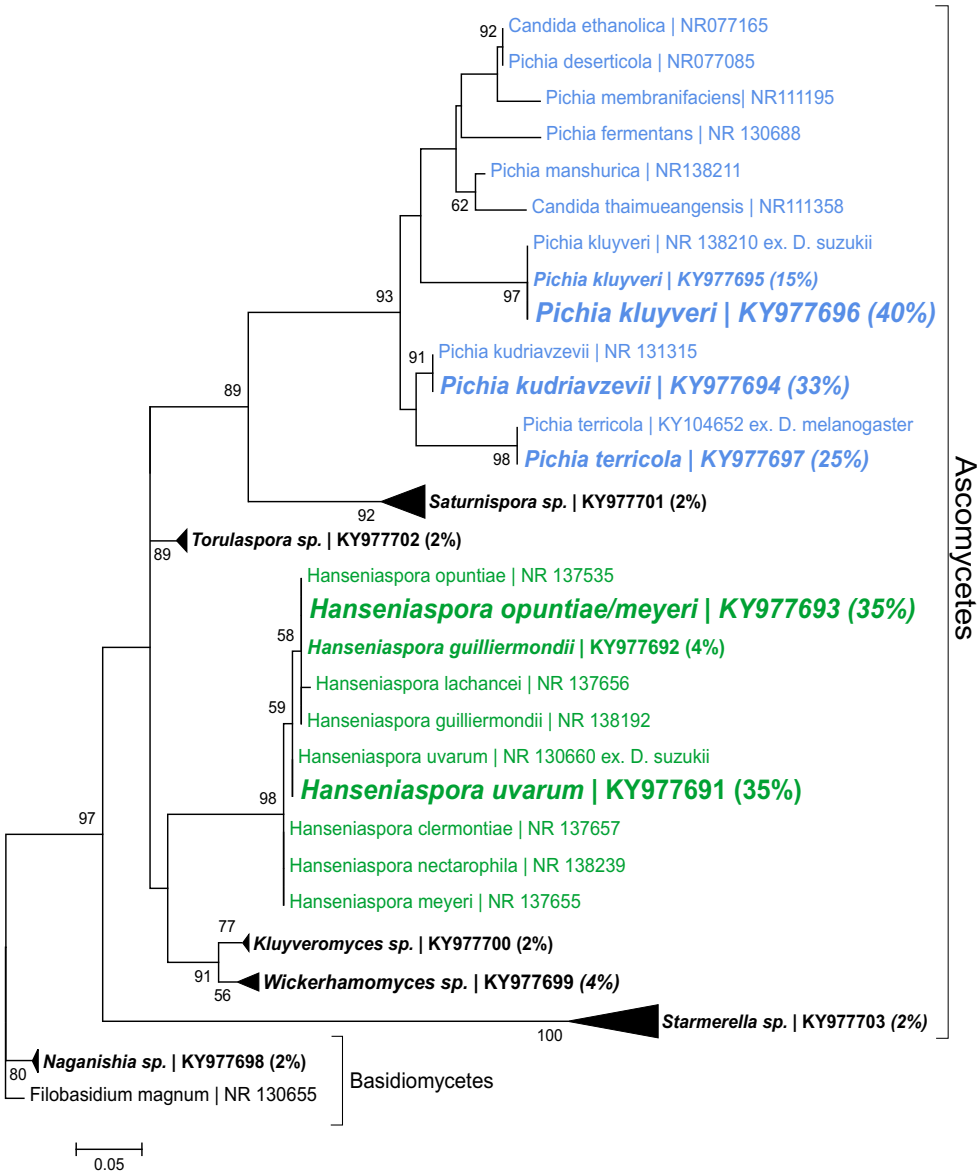


Fig. 1 Maximum likelihood tree of the ITS region of rRNA for 13 genotypically distinct yeast strains isolated from *Bactrocera tryoni* larval gut in this study. Clades of irregularly isolated species have been collapsed (see Supplementary information for full tree). Tentative species ID is given as the top match on a BLASTn search. Percentage of larvae ($N = 48$) carrying each strain is listed after the name. Yeast species that have been previously isolated from other insect species are indicated with 'ex.'. Branch lengths are proportional to number of substitutions per site. Alignment length is 177 positions. Bootstrap values of ≥ 50% are indicated (1000 replicates). Full tree presented in Supplemental Fig. 1

responded very poorly to all treatments, with traps catching significantly fewer flies than in mated experiments (means: virgin = 9 ± 2%, mated = 59 ± 4%, Z = −5.39, $P < 0.05$, Mann-Whitney). Catch numbers of virgin flies were deemed too low for further statistical analysis.

Oviposition Responses Mated adult females *B. tryoni* responded well to the oviposition pots (mean eggs laid per trial = 198.25 ± 21.3), revealing significantly different preferences for laying on different yeast inoculation treatments ($\chi^2_{(2)} = 24.4$, $P < 0.001$, KW). Mated females showed a significant preference for ovipositing on the control egg laying pot (orange-juice alone) over pots containing *P. kluyveri* grown in orange juice (Control = 166.3 ± 18.6, PK = 11.4 ± 1.6, $P < 0.05$) or *H. uvarum* grown in orange juice (mean = 20.5 ± 4, $P < 0.05$) (Fig. 2b).

Effect of Yeasts on Larval Development

Inoculated yeasts grew prolifically on the orange agar substrate and formed a lawn across surface of the diet, providing developing larvae ad libitum access to yeast for feeding (Fig. 3). Yeast inoculation had a significant influence on larval development time ($\chi^2_{(2)} = 78.1$, $P < 0.001$, KW) (Fig. 2c). Larvae on *H. uvarum* inoculated media reached pupation significantly faster than those with *P. kluyveri* (HU = 14.4 ± 0.2 days, PK = 23 ± 0.7 days, $P < 0.05$) or the sterile control media (Control = 24 ± 0.3 days, $P < 0.05$). Yeast inoculation also significantly influenced the total number of larvae successfully reaching pupation ($\chi^2_{(2)} = 40.4$, $P < 0.001$, KW) and emerging as adults ($\chi^2_{(2)} = 39.5$, $P < 0.001$, KW) (Fig. 2d). Significantly more larvae pupated from the *H. uvarum* inoculated media compared to media inoculated with *P. kluyveri* (HU = 7.3 ± 0.6, PK = 1.55 ± 0.23, $P < 0.05$) or the sterile control media

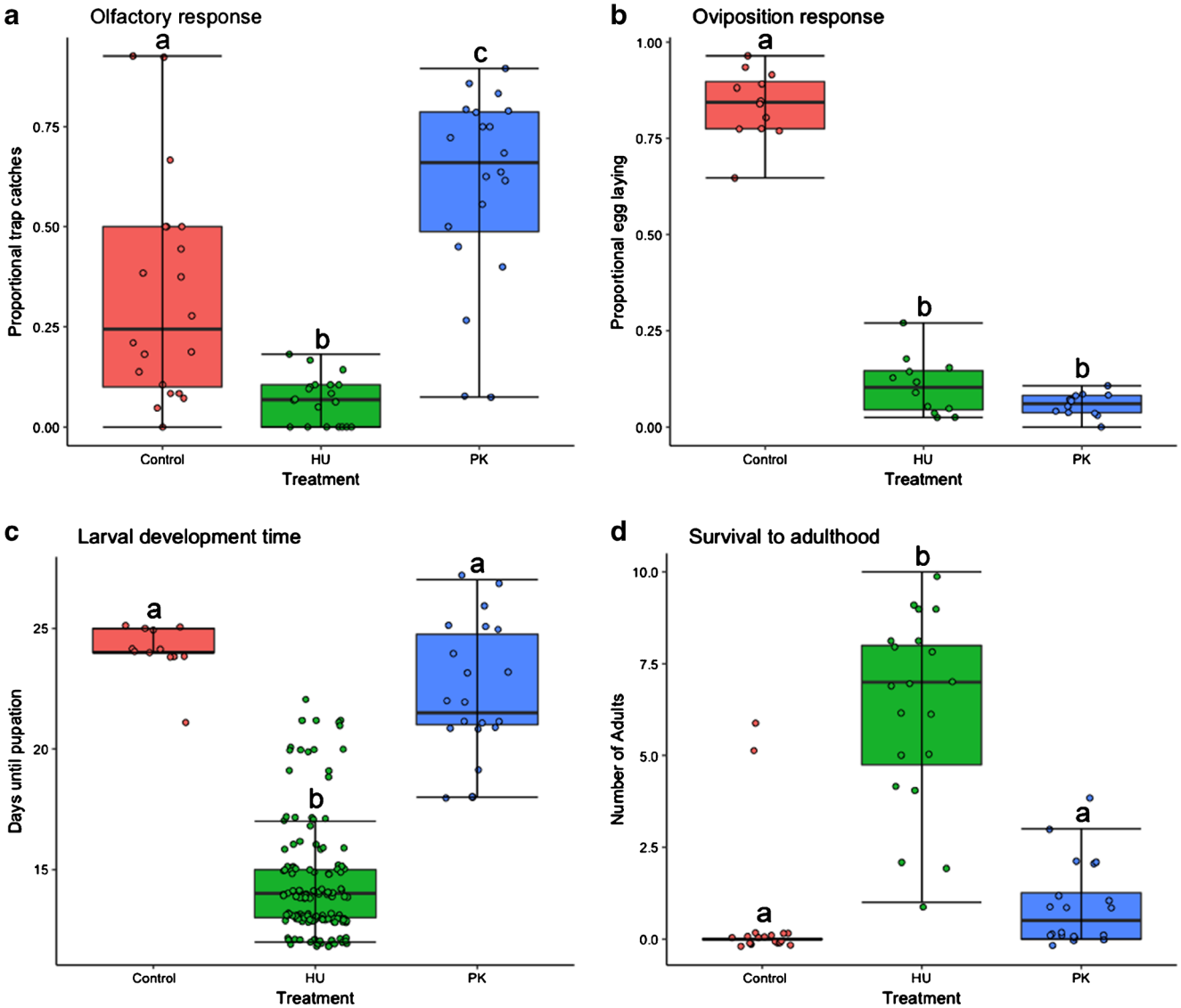


Fig. 2 Olfactory and oviposition responses, and larval survival: **a** preference of mated *Bactrocera tryoni* mated females to yeasts inoculated on orange-agar or a sterile control ($N = 20$). **b** Proportions of eggs laid by mated *B. tryoni* females on egg laying pots inoculated with orange juice and treatment yeasts or an orange juice control ($N = 20$). **c** Time until larval pupation from 20 eggs placed on orange juice agar inoculated with a treatment yeast or left sterile as control ($N = 20$). **d** Number of adults emerged. Different letters indicate statistical significance ($P < 0.05$)

(Control = 0.6 ± 0.4, $P < 0.05$). In addition, significantly more adults emerged from treatments inoculated with *H. uvarum* compared to treatments inoculated with *P. kluyveri* (HU = 6.3 ± 0.6, PK = 0.9 ± 0.3, $P < 0.05$), or sterile control (Control = 0.55 ± 0.4, $P < 0.05$). There were no significant differences in larval development time, number of pupae, or adult emergence between *P. kluyveri* inoculated media and the sterile control. This may have been due to two replicates in the control treatment that became contaminated (by yeast-like colonies) upon removal of all Petri dish lids at 7 days (which was necessary to allow late instar larvae to emerge from the diet and pupate). Only in these two replicates of the control did larvae successfully reach pupation and adulthood (see outliers in Fig. 2d).

Yeast Volatile Analysis The main constituents of the orange agar medium headspace were *β*-myrcene, limonene, 4,5-dimethyl-nonane, linalool, terpinen-4-ol, dodecane, α-terpineol, carvone and perilla aldehyde. These compounds were found in all treatments, although the emission rates of some of the volatiles differed in the presence of yeasts (e.g. limonene, carvone and perilla aldehyde). Other compounds such as ethyl acetate, ethyl propionate, isobutyl acetate, hexanol, isoamyl acetate, hexyl acetate, phenethyl alcohol and phenethyl acetate were present only in small quantities in the medium, but were markedly more abundant when the medium was inoculated with yeasts (Fig. 3a and Table 1). Differences among the headspace odours of the three treatments (HU, PK, Control)

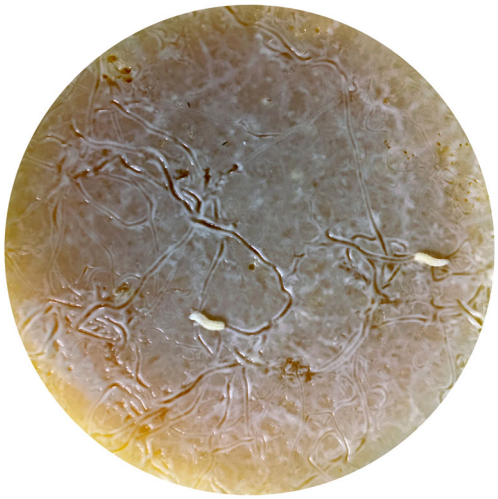


Fig. 3 Eight day old *Bactrocera tryoni* larvae feeding on orange-agar substrate inoculated with *Hanseniaspora uvarum*. Two larvae feed on the surface, with tracks across the lawn of (white) yeast clearly visible (the remainder of the larvae are concealed within the substrate)

were confirmed using multivariate analysis. Significant differences were found in the chemical profiles of the three treatments (one-way ANOSIM; Bray-Curtis distance; $R = 0.89$ $P < 0.001$,), and between all treatment groups (orange-agar control vs *H. uvarum*, $P = 0.0048$; orange-agar control vs *P. kluyveri*, $P = 0.006$ and *H. uvarum* vs *P. kluyveri*, $P = 0.0045$) (Fig. 3b). There were similarities in the array of volatiles produced by the two yeast species, but the quantities and ratios of these volatiles differed noticeably (see Fig. 4a and c). SIMPER analysis revealed that 90% of the dissimilarities between the two yeasts resided in the emissions of five compounds; namely isoamyl acetate (~60%), phenethyl acetate (~17%), ethyl acetate (~5%), isobutyl acetate (~4%) and 2-methyl-1-butanol (~3%).

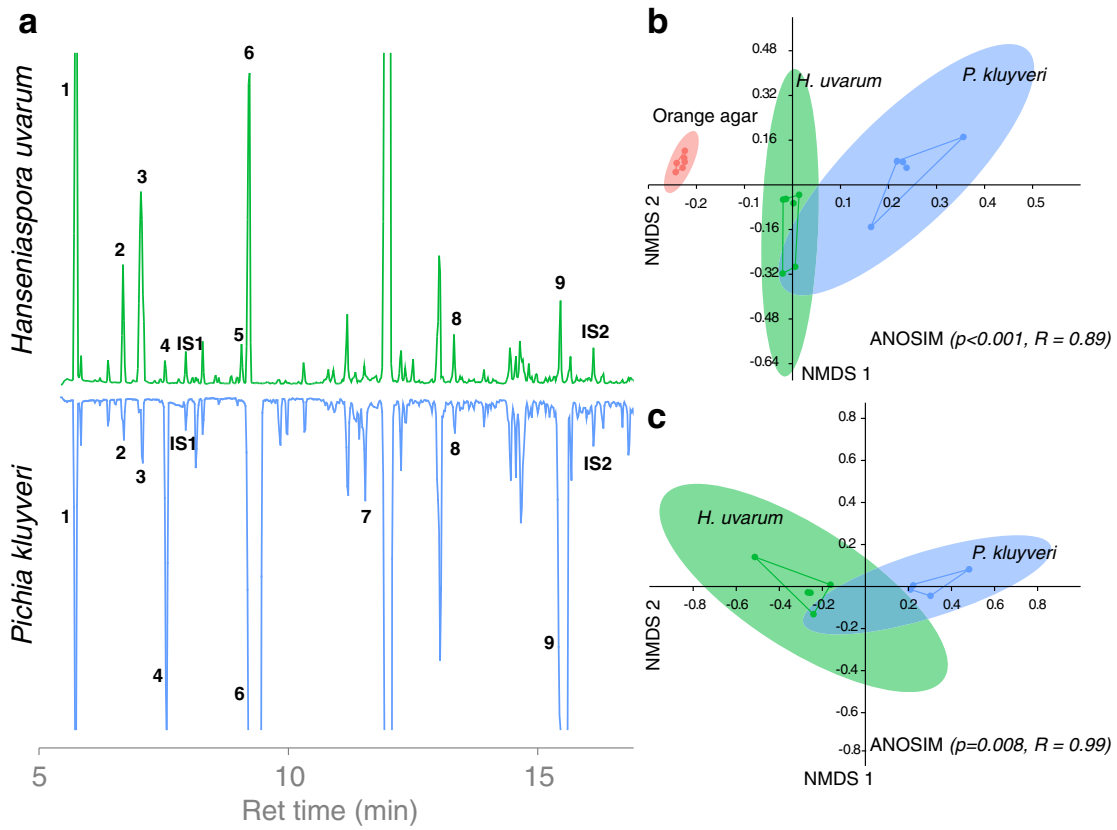
Discussion

The cohort of yeasts we isolated from the gut of Queensland fruit fly, *B. tryoni* larvae represent a similar diversity to those isolated from larvae of frugivorous *Drosophila* species (Lachance et al. 1995; Hamby et al. 2012). *Hanseniaspora* and *Pichia* were the most prevalent yeast genera, and are common fruit-associated yeasts (Molnárová et al. 2014). *H. uvarum* and *P. kluyveri*, which were the two most frequently isolated species in this study, are considered to be mutualistic yeasts in *Drosophila* (Becher et al. 2012; Hamby et al. 2012; Hamby and Becher 2016). Adult female *D. melanogaster* and *D. suzukii* are attracted towards volatiles produced by these and other yeasts (Scheidler et al. 2015), which when inoculated into the fruit can provide the developing fruit fly larvae with nutritional benefits (Hamby and Becher 2016). The yeast benefits in this mutualistic relationship through utilizing the fly as a vector as transport from

Table 1 Headspace volatiles from an orange-agar medium (control) and orange-agar innoculated with *Hanseniaspora uvarum* or *Pichia kluyveri*. Yeast cultures were grown for 24 h at 25°C

			Orange-agar control ($n = 6$)		Orange-agar + *H. uvarum* ($n = 6$)		Orange-agar + *P. kluyreri* ($n = 6$)	
Ret time (min)	Compounds	KI*	Amount (ng.hr.$^{-1}$)	SE	Amount (ng.hr.$^{-1}$)	SE	Amount (ng.hr.$^{-1}$)	SE
5.72	Ethyl acetate	666	1.6	1.2	1048.4	147.9	799.9	166.4
6.68	Ethyl propionate	724	0.2	0.2	170.3	43.6	36.8	4.3
7.07	2-Methyl-1-butanol	747	–	–	536.4	117.3	203.8	49.2
7.55	Isobutyl acetate	776	0.4	0.4	43.6	7.4	606.2	146.1
9.10	1-Hexanol	869	8.9	6.6	84.8	29.5	12.3	5.8
9.25	Isoamyl acetate	878	50.3	28.0	897.9	263.7	9564.4	2509.3
11.19	*β*-Myrcene	992	205.3	61.2	182.6	79.6	169.3	41.6
11.54	Hexyl acetate	1013	2.1	2.1	44.9	16.7	118.2	38.2
12.07	Limonene	1044	9743.1	2736.9	6445.4	3540.3	5743.1	1686.7
12.26	4,5-dimethyl-nonane	1056	88.8	20.8	53.9	19.4	56.9	17.1
12.99	Linalool	1100	598.6	94.5	439.7	152.9	432.1	86.2
13.34	Phenethyl alcohol	1122	0.7	0.7	107.2	24.6	156.5	59.9
14.46	Terpinen-4-ol	1193	127.4	24.5	99.0	30.1	94.9	15.7
14.56	Dodecane	1200	128.6	47.4	42.1	10.6	57.2	14.9
14.66	*α*-Terpineol	1207	179.3	18.3	99.9	30.7	183.0	53.3
15.39	Carvone	1256	100.0	10.3	45.2	28.0	20.0	4.0
15.46	Phenethyl acetate	1261	18.9	18.9	125.3	40.5	2762.2	995.4
15.90	Perilla aldehyde	1290	30.8	13.9	–	–	0.8	0.8

*Retention indices calculated on a non-polar column (DB-5MS)



1. Ethyl acetate; **2.** Ethyl propionate; **3.** 2-Methyl-butanol; **4.** Isobutyl acetate; **5.** 1-Hexanol; **6.** Isoamyl acetate; **7.** Hexyl acetate; **8.** 2-Phenylethyl alcohol; **9.** 2-Phenylethyl acetate; **IS1.** n-Octane (internal standard 1); **IS2.** Nonyl acetate (internal standard 2).

Fig. 4 Yeast headspace volatile analysis. **a** Chromatograms of the headspace of *H. uvarum* (green) and *P. kluyveri* (*blue*) inoculated into orange-agar medium. Peaks annotated with numbers indicate the most noticeable differences between the two yeast volatiles profiles. **b** Two-dimensional plot representing the volatile profile of orange-agar alone (red), orange-agar + *H. uvarum* (green) and orange-agar + *P. kluyveri* (*blue*) using non-metric multidimensional scaling based on a Bray-Curtis dissimilarity matrix. Ellipses represent a 95% confidence interval around different classes. **c** Similar plot only representing the two yeast volatiles profiles in orange-agar

one fruit to another (Christiaens et al. 2014). A second *Hanseniaspora* isolate closely related to *H. opuntiae* and *H. meyeri* was also consistently isolated from *B. tryoni* larvae in our study, but could not be unambiguously assigned to either species and was therefore not analysed further. Work is currently ongoing to develop new molecular markers in tephritid-associated yeasts with higher phylogenetic resolution to enable more precise species assignment.

Whilst the presence of the same gut-associated yeast species in *B. tryoni* as in drosophilid fruit flies might suggest a similar mutualistic relationship between insect and yeast, our bioassays revealed striking differences in the olfactory behavior of adult flies, and also in larval survival. This might point to fundamental differences in the behavioural- and chemical- ecology of yeast-insect-plant interactions between these fruit fly families. In our olfactory trap assay, female *B. tryoni* flies were presented with a three-way choice of odours from orange-agar substrate inoculated with either *H. uvarum, P. kluyver*i, or sterile orange-agar media. Significantly fewer mated female flies were caught in the *H. uvarum* traps compared to the orange-agar sterile control, and significantly more flies were caught in *P. kluyveri* traps compared to the control: thus *H. uvarum* emitted a deterrent odour, whilst *P. kluyveri* emitted an attractant odour. Oviposition choice tests exposing the same three substrates directly to the flies, demonstrated that both yeasts had a strong deterrent effect, receiving significantly fewer eggs compared to the orange-agar control.

Our study on Queensland fruit fly larvae suggests that the presence of *H. uvarum* is highly beneficial to larval fitness. Larval development and survival to adulthood were significantly higher when larvae were exposed to orange-agar substrate inoculated with *H. uvarum* compared to sterile orange-agar (where, excluding two contaminated plates, survival was negligible). Development on *H. uvarum* inoculated substrate was around one and a half times faster than *P. kluyveri* inoculated substrate (averaging 14 days from egg to pupae) and survival to adulthood seven times higher. It was particularly notable that larval development and survival to adulthood on

orange agar inoculated with *P. kluyveri* was not significantly different from the sterile control. The different influences of these two yeasts may arise from the presence or absence of essential key nutrients (Gibson and Hunter 2010) or production of extracellular enzymes that help break down components of the fruit (Molnárová et al. 2014), and may relate to the stage of decomposition during which these yeasts predominate (*P. kluyveri* being at a more advanced stage than *H. uvarum*). Another explanation may relate to the production of bioactive extracellular metabolites that may either protect the larva from biotic stressors such as fungal and bacterial pathogens from the local environment (Witzgall et al. 2012), or prove harmful to the insect (Haidani et al. 2008). Survival of larvae to adulthood on *H. uvarum* was comparable to that found when *B. tryoni* larvae develop on ripe nectarines (Masry et al. 2016). Fresh orange juice was chosen as the base for our fruit-agar media, as citrus is considered an inferior host for Queensland fruit fly (Muthuthantri and Clarke 2012) and might therefore highlight any beneficial influence of the yeast. Other fruit substrates that provide better nutrition for *B. tryoni*, or whole fruits instead of artificial media, may yield greater total survival in the presence or absence of yeasts.

That a beneficial yeast species living in the gut of *B. tryoni* larvae should deter female adults flies when present in fruits, might appear somewhat counterintuitive in terms of an evolutionary or ecological explanation—particularly in light of the work on *Drosophila,* where both yeast species (and *H. uvarum* in particular) are highly attractive to adult flies (Scheidler et al. 2015; Mori et al. 2016). We suggest, however, that our results fit well with our understanding of the behavior of these tephritid fruit flies. As with many pest tephritids around the globe, the Queensland fruit fly prefers ripening fruits that are hanging on the tree, and ovipositing females will actively avoid laying into fruits that are already infested with larvae (Fitt 1984). Larval infestation may decrease fruit quality through depletion of resources, escalated fruit drop, or larval competition (Bateman 1972; Duyck et al. 2006), providing selection pressure that would favour the recognition of infestation-associated cues by ovipositing adult fruit flies. Given that *H. uvarum* is frequently associated with *B. tryoni* larvae, and emits a distinct odour, the olfactory system of female flies may have evolved to recognize specific *H. uvarum* volatiles as a predictable cue for decreased fruit quality. In our oviposition trials using the same orange-agar media, significantly fewer eggs were laid in the presence of either *H. uvarum* or *P. kluyveri* compared to sterile controls. This differs from *Drosophila* studies (Barker et al. 1988; Becher et al. 2012; Mori et al. 2016) and codling moth (Witzgall et al. 2012) where yeast volatiles were found to promote oviposition, and further supports the hypothesis that female avoidance of the yeast relates to oviposition in larval infested (or decomposing) fruits.

The underlying olfactory physiology and behavioural ecology behind the contrasting responses to *H. uvarum* odour and *P. kluyveri* odour in the trap assay needs further elucidation. Whilst we have presented an explanation for why *H. uvarum* odours might elicit a deterrent response, the attractiveness of *P. kluyveri* implies a different underlying ecological explanation (especially as this yeast had no benefit to larval survival). Yeast headspace analysis on inoculated orange-agar revealed that a similar suite of volatiles were produced by both species (ethyl acetate, ethyl propionate, isobutyl acetate, hexanol, isoamyl acetate, hexyl acetate, phenethyl alcohol and phenethyl acetate), but with significant quantitative differences in the emission of particular volatile components. The concentrations of isoamyl acetate and phenethyl acetate alone (respectively 10- and 15-fold higher in *P. kluyveri* compared to *H. uvarum*), accounted for 77% of the dissimilarity between both yeast species. Our results partially concur with an earlier study in which these two compounds were found to predominate the headspace of the same yeast species inoculated on potato dextrose agar (Scheidler et al. 2015). However, the authors found that isoamyl acetate was twice as abundant in *H. uvarum* than *P. kluyveri*. This difference may be the consequence of headspace sampling methods employed (i.e. solid-phase microextraction, SPME, used in their study as opposed to dynamic sampling in the present work); or the growth media used for culturing the yeasts. We also found that the yeasts appeared to alter the concentrations of constituents emanating from the orange-agar substrate. For instance, the concentrations of limonene were reduced by up to 40% in *P. kluyveri* and 30% in *H. uvarum* inoculated orange-agar compared to the sterile substrate (Table 1). Limonene biotransformation by microbes (including yeasts) has been previously described by Duetz et al. (2003). Although some potential degradation products such as carvone, and α-terpineol were found in our collections, their concentrations did not increase in the headspace of yeast-inoculated orange-agar, providing little support for a hypothetic biotransformation of limonene. Nevertheless, the apparent decrease of some minor and potentially toxic terpenoids, such as *ß*-myrcene, linalool, terpinen-4-ol and perilla aldehyde, remains intriguing and raises the question as to whether yeasts also play a substrate detoxification role during larval development. Other compounds such ethyl acetate, ethyl propionate (previously found to be highly attractive to *B. tryoni* (Cunningham et al. 2016)) and 2-methyl-1-butanol, accounted for more than 10% of the dissimilarity between the two-yeast species, and were all more abundant in *H. uvarum*. In contrast, isobutyl acetate and hexyl acetate appeared to be produced in higher amounts by *P. kluyveri*. Overall, our results demonstrate that not only is *B. tryoni* behaviour influenced by yeast-produced volatiles, but quantitative variations of these compounds may provide crucial olfactory information mediating attraction and oviposition preferences. Future work including electrophysiological studies (GC-EAD and GC-SSR) to confirm olfactory detection conducted in parallel with behavioural studies using synthetic blends will allow us to further investigate volatile-driven behavioural discrimination in fruit flies.

Dispersal of yeasts by insects is a common phenomenon either through gut contents (Reuter et al. 2007; Coluccio et al. 2008) or adhesion to the body of the insect (Christiaens et al. 2014), and the question remains as to how *B. tryoni* adult females obtain *H. uvarum* if they are deterred by the odour as mated females. Our results showed very low attraction to any fruit or yeast odours in newly-emerged unmated adult females, however adult flies may still be attracted to the yeast at some point in the immature stage or soon after mating, as has been shown in *D. suzukii* (Mori et al. 2016). A previous study found no evidence of yeast cells within crop contents of *B. tryoni* adults, when were examined under the microscope (Vijaysegaran et al. 1997), but further studies may be needed here to confirm whether adult flies carry these yeasts internally, or whether the vectoring of yeasts is on external body parts only.

The use of fungal volatiles as attractants for insect pests shows great potential as part of integrated pest management strategies (Hamby and Becher 2016; Holighaus and Rohlfs 2016; Mori et al. 2016). Further investigation of the volatile compounds produced by attractive yeasts such as *P. kluyveri* could aid in lure development for attract and kill approaches. Attractant yeasts could also be used for novel methods of biocontrol by increasing the effectiveness of entomopathogenic viruses or by RNAi expression (Murphy et al. 2016). The sterile insect technique (SIT) is an important focus for the control of Queensland fruit fly and other tephritids (Hendrichs et al. 1995; Dominiak et al. 2003; Dyck et al. 2005). An important requirement for the mass-rearing of flies in SIT is a high-quality diet (Parker 2005), and the selection of suitable species of live yeasts (associated with the target fruit fly species in nature) could improve the quality of artificial diets. We recommend further work focusing on *H. uvarum* as a potential probiotic to supplement diets of the Queensland fruit fly and other pest tephritids around the world.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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