



Yeast species affects feeding and fitness of *Drosophila suzukii* adults

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Abstract

Yeasts play an important role in the life cycle and biology of the insect pest *Drosophila suzukii* (Matsumura), commonly known as the spotted wing drosophila (SWD). Adult and larvae of this species are known to feed and benefit from yeast in their diet. In addition, yeasts were found to be attractive to SWD and were repeatedly found to be associated with SWD. Among those, *Hanseniaspora uvarum* is the most commonly mentioned. The present study explores the chemical composition and the effects of three *H. uvarum* strains and five yeast species (*Saccharomyces cerevisiae*, *Candida* sp., *Issatchenkia terricola*, *Metschnikowia pulcherrima* and *Saccharomycopsis vini*) in the diet of SWD adults. The different yeast species used in this study influenced mortality, fecundity and ingestion by SWD females. *Hanseniaspora uvarum* and *S. vini* were preferably ingested and increased fecundity of SWD females. The intra- and extracellular concentrations of compounds, such as amino acids, carbohydrates, sugar alcohols and organic acids, produced or consumed by yeasts differed among the species. Knowledge of the interaction of different yeast species with SWD and specific differences in the profile of compounds of yeast can help to improve the development of control strategies against the insect pest by promoting the ingestion of attract-and-kill formulations based on the combinations of yeasts and an appropriate insecticide.

Keywords Fecundity · *Hanseniaspora uvarum* · Ingestion · Spotted wing drosophila · Yeast metabolites

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Key message

- Naturally occurring yeasts play an important role in SWD development.
- Mortality, fecundity and ingestion by SWD adults are influenced by the yeast species in their diet.
- The concentrations of nutritional compounds in the fermentation broth of different yeasts vary due to differences in the methods they produce or consume compounds.
- Chemical analysis of selected yeast cultures helps to understand the interaction between SWD and putative associated yeast species.

Introduction

Yeasts play an important role in the interaction with *Drosophila* flies. They constitute a food source and produce volatile compounds attractive to the insect. As previously shown in *Drosophila* flies, yeast communities are to some extent species specific (Chandler et al. 2012), and the density and

community structure of yeasts on damaged fruit is influenced by the flies (Stamps et al. 2012), which benefit from yeast in their diet (Becher et al. 2012).

Drosophila suzukii (Matsumura), also known as the spotted wing drosophila (SWD), is an invasive insect pest (Hauser 2011). The ability of SWD to lay eggs in healthy ripe fruits, their short generation time and high reproductivity (Tochen et al. 2014) make it an important pest for a wide range of small and stone fruits, both wild and cultivated (Bellamy et al. 2013; De Ros et al. 2013; Elsensohn and Loeb 2018; Lee et al. 2015; Mitsui et al. 2006). Oviposition and host fruit suitability for larval development are influenced by several factors, including fruit species preferences (Abraham et al. 2015; Bellamy et al. 2013; Cloonan et al. 2018; Lee et al. 2011); fruit characteristics, such as sugar content, pH or firmness (Arnó et al. 2016; Burrack et al. 2013; Lee et al. 2015); and protein and carbohydrate content (Hardin et al. 2015).

In their habitat, SWD flies feed on damaged fruits and other sugar sources, including the yeast flora naturally growing on sugar-rich reserves (Mitsui et al. 2010; Tochen et al. 2016; Walsh et al. 2011). In contrast to the common vinegar fly, *Drosophila melanogaster*, SWD females search for healthy intact soft-skinned fruits in suitable stages of ripeness to oviposit their eggs (Burrack et al. 2013; Lee et al. 2011; Mitsui et al. 2006). Searching for undamaged fruit or other food sources, the flies use signals, such as volatile compounds emitted by the fruits (Abraham et al. 2015; Liu et al. 2018; Revadi et al. 2015) and the leaves (Bolton et al. 2019; Keesey et al. 2015; Piñero et al. 2019) or fermenting products (Lasa et al. 2019b; Bing et al. 2018; Camargo and Phaff 1957; Steck et al. 2018). Damage to the fruit is caused by the SWD larvae and by microorganisms growing inside the infested fruit (Cini et al. 2012). The populations of yeasts and other microorganisms inside the damaged fruit are likely established through microorganisms present on adult SWD flies (Hamby and Becher 2016). *Hanseniaspora uvarum* was frequently isolated from *Drosophila* before (Chandler et al. 2012) as well as from SWD larvae and adults (Hamby et al. 2012; Knight et al. 2016; Lewis et al. 2019). It was also found that SWD larvae would rather feed on *H. uvarum* than on alternative yeasts, such as *S. cerevisiae* (Lewis and Hamby 2019). Several other yeast species, such as *Metschnikowia pulcherrima*, *Issatchenkia terricola* and *Pichia kluyveri*, have been isolated from SWD larvae and fruit damaged by SWD (Bellutti et al. 2018; Hamby et al. 2012; Lewis et al. 2019). In laboratory trapping assays, *H. uvarum* was the most attractive yeast for SWD adults compared to strains from *Pichia kluyveri*, *Candida californica*, *Issatchenkia terricola*, *Saccharomyces cerevisiae* and *Candida zemplinina* (Scheidler et al. 2015). Larvae of SWD benefit from yeast in their diet (Bellutti et al. 2018; Hardin et al. 2015; Lewis and Hamby 2019), but little is known about the

effect of naturally occurring yeast species in the diet of SWD adults and the influence of yeasts on substrate food quality.

The present study focuses on the influence of yeast species on the diet of SWD adults to provide additional insights into the biology of this insect and to clarify the association between SWD adults and certain yeast species. Since yeasts could be exploited for the development of attract-and-kill strategies against SWD (Knight et al. 2015; Mori et al. 2016), additional data concerning their effect on fecundity, feeding and mortality of SWD adults can be useful for the establishment of focused control strategies. For this purpose, the fecundity over a long lifetime period of SWD fed with different yeasts was observed. Cultures of yeasts isolated from feeding tunnels of SWD larvae in infested grape berries were grown on two different commercial yeast culture media and offered as food sources to SWD flies to understand the influence of individual yeast species on SWD flies. To characterize the quality of the various nutrient sources and to explain the findings of the insect trials, the content of carbohydrates, amino acids, sugar alcohols and organic acids in the yeast fermentation broths was measured.

Materials and methods

Yeast material

Table 1 lists the yeasts used in this study. The yeast cultures except for *Saccharomyces cerevisiae* were isolated from feeding tunnels of SWD larvae found in infested grapes in South Tyrol in 2009 (Bellutti et al. 2018). *Saccharomyces cerevisiae* strain S288c is a conventional laboratory strain.

Yeast cultivation on solid media

For long-term storage, purified isolates were cultivated in chloramphenicol yeast glucose broth (5 g/L yeast extract (Merck, Germany), 20 g/L glucose (Sigma-Aldrich, Germany), 0.1 g/L chloramphenicol (Merck, Germany) and maintained frozen in 20% glycerol at -80 °C. The yeast cultures for the SWD assays were grown on Petri dishes (diameter 6 cm) on malt extract agar (MEA) (30 g/L malt extract, 3 g/L peptone from soymeal and 15 g/L agar; Merck, Italy) or potato dextrose agar (PDA) (4 g/L potato starch (from infusion), 20 g/L dextrose, 15 g/L agar; Difco™ Becton–Dickinson, France). To inoculate the Petri dishes, a loop full of yeast cells cultivated on MEA or PDA was transferred in a 2-mL Eppendorf tube filled with 1 mL 0.9% NaCl (Merck, Italy) solution and vortexed for 10 s at 1800 rpm. Then, 0.1 mL yeast cell suspension was pipetted into the Petri dishes with culture medium and spread evenly across the surface. Petri dishes containing culture medium only were inoculated with 0.1 mL 0.9%

Table 1 Yeasts used in this study

Yeast species	Strain	Accession number*	Abbreviation
<i>Saccharomyces cerevisiae</i>	S288c		S.c. S288c
<i>Hanseniaspora uvarum</i>	LB-NB-1.21	KP298009	H.u. 1.21
<i>Hanseniaspora uvarum</i>	LB-NB-2.2	MK567898	H.u. 2.2
<i>Hanseniaspora uvarum</i>	LB-NB-3.4	MK567905	H.u. 3.4
<i>Issatchenkia/Pichia terricola</i>	LB-NB-2.1	MK567903	I.t. 2.1
<i>Metschnikowia pulcherrima</i>	LB-NB-3.2	KP298012	M.p. 3.2
<i>Saccharomycopsis vini</i>	LB-NB-1.33	KP298011	S.v. 1.33
<i>Candida</i> sp.	LB-NB-3.3	KP298013	C.sp. 3.3

*The accession numbers were deposited in GenBank NCBI

NaCl as yeast-free control. All Petri dishes including the yeast-free control were kept at 22 °C, offered to the flies 48 to 72 h after inoculation and checked for contaminations prior to use. Forty-eight hours after inoculation, the yeast colonies covered the whole Petri dishes containing culture media.

Yeast cultivation in liquid medium

Yeasts were grown in 1 L PDB (24 g/L Difco™ Potato Dextrose Broth) at 25 °C, 120 rpm for 30 h in a 2-L Erlenmeyer flask closed with cotton and aluminum foil. The inoculum (1 mL) was prepared with a loop full of yeast cells cultivated on PDA, which was transferred to a 2-mL Eppendorf tube filled with 1 mL PDB and vortexed for 10 s at 1800 rpm. Preliminary trials showed that after 30 h all yeast cultures reached the stationary phase. Number of cells per mL (Fuchs Rosenthal counting chamber), optical density at 600 nm (OD₆₀₀) (Cary 60 UV–Vis, Agilent), pH value (pH meter, Crison GLP 21), dry weight (DW) of yeast pellet (centrifugation of fermentation broth, removal of the supernatant, drying of the pellet at 103 °C) and alcohol content (distillation) were measured after 30 h of growth. The values are shown in Table 2. The yeast fermentation broths were stored at –80 °C until use.

Table 2 Cell concentration (cells/mL), optical density at 600 nm (OD₆₀₀), pH value, dry weight (DW) and alcohol content of yeast fermentation broth measured after 30 h of growth in potato dextrose broth (PDB)

Yeast	Cells/mL	OD ₆₀₀	PH	DW pellet (mg/mL fermentation broth)	Alcohol (vol %)
S.c. S288c	7.28 × 10 ⁷	1.94	4.13	1.30	1.01
H.u. 1.21	6.06 × 10 ⁷	1.71	4.40	0.72	0.78
H.u. 2.2	3.80 × 10 ⁷	1.59	4.24	0.55	0.93
H.u. 3.4	2.90 × 10 ⁷	1.58	4.31	0.57	0.78
I.t. 2.1	1.59 × 10 ⁷	1.96	4.25	1.47	0.71
M.p. 3.2	3.64 × 10 ⁷	1.84	4.25	1.66	0.87
S.v. 1.33	n/a*	1.81	3.97	1.63	0.54
C.sp. 3.3	1.41 × 10 ⁸	1.97	4.29	1.71	0.92

*Cell counting was not possible for the mycelial yeast *S. vini*

Insects

Rearing and all SWD assays were performed in the laboratory under controlled conditions (22 ± 1 °C, 75 ± 3% relative humidity, photoperiod of L16:D8). The mass rearing was refreshed on multiple occasions each year with pupae from various fruits from the field collected in South Tyrol, Italy. The larvae were reared on a *Drosophila suzukii* Cornmeal Diet (DSCD_(a)) with dry deactivated yeast and dry baker's yeast (Küchle GmbH & Co. KG, Günzburg, Germany) sprinkled on the surface (Bellutti et al. 2018). The rearing also contained 5% sucrose solution on cotton. Males and females that emerged from the pupal stage within 24 h were kept together in an insect cage with cotton soaked in 5% sucrose solution until they were used in the assays.

Oviposition assays

Cages used for the oviposition trials were made of white polystyrene boxes (CIB Verona, 18 cm long, 18 cm wide and 6 cm high) with three closable openings with screw plugs on the bottom of the box for changing the three components. The top was closed with a white mesh (mesh size 1 × 0.625 mm). The three components were (1) one Petri dish (diameter 6 cm) with culture medium or culture medium and yeasts culture (diets), (2) one Petri dish (diameter 6 cm)

with water agar (15 g/L Agar–agar, Merck, Italy) covered with 0.1 mL 5% sucrose solution (sterilized by autoclaving) and (3) a piece (6 by 6 cm) of folded paper towel soaked in 1 mL 5% sucrose solution. The components were placed in matching lids to easily replace them daily. The cage design is shown in “Electronic Supplementary Material (Fig. S1).”

At day 0 of the oviposition assays, 10 male and 10 female SWD flies of known age (36 ± 12 h after emergence from pupal stage) were placed in the cages. Each cage was considered as a replicate. Dead flies were removed and not replaced; therefore, the number of flies decreased over the experimental period. The sex of the dead flies was determined, and the mortality was recorded. Different diets were tested simultaneously, and the replicates started at different time points. The daily oviposition was calculated as the total number of eggs laid per female on the three components.

Three different methods were used in the oviposition assays. In the malt extract agar assay (MEA assay), three yeast species (M.p. 3.2, C.sp. 3.3 and H.u. 3.4) were tested on MEA, and a yeast-free MEA served as control. In the MEA assay, the mortality of SWD adults and the number of eggs laid on the three components were counted daily over 50 days. Seven replicates of the MEA assay were performed. The yeast growth media assay (YGM assay) assessed the yeast culture media MEA and PDA to evaluate their suitability as a nutrient medium for SWD. The three components were changed daily, except on weekends, and the mortality of SWD adults and the number of eggs laid on the three components were measured over 30 days. Five replicates of the YGM assay were performed. In the potato dextrose agar assay (PDA assay), seven yeasts (S.c. S288c, H.u. 1.21, H.u. 2.2, H.u. 3.4, I.t. 2.1, M.p. 3.2, S.v. 1.33 and C.sp. 3.3) were cultivated on PDA. The mortality of SWD adults and the number of eggs laid on the three components were measured daily over 30 days. Three replicates of the PDA assay were performed.

To evaluate whether the different yeasts or the yeast-free culture media impact oviposition, a linear mixed effect analysis was applied for all oviposition assays (Winter 2013). Yeasts or medium and day (without interaction term) were included in the model as fixed effects. The replicates, which started at different time points, were included in the model as random effects. The oviposition data entered the analyses as numbers of eggs laid per female and day. To avoid deviations from homoscedasticity or normality, squared root data transformation was performed for the dataset of the PDA assay and YGM assay, while a cubic root transformation was performed for the MEA assay. To find significant effects in the mean first occurrence of oviposition in the cages, a Kruskal–Wallis test was performed followed by a Wilcoxon rank sum test for pairwise comparison. The results were adjusted using FDR (false discovery rate) methodology. Survival curves were evaluated using the Kaplan–Meier

method followed by a log rank test. P values were adjusted using FDR methodology.

Ingestion assay

The daily ingestion of different yeasts (S.c. S288c, H.u. 1.21, H.u. 2.2, H.u. 3.4, I.t. 2.1, M.p. 3.2, S.v. 1.33 and C.sp. 3.3) grown in liquid medium (PDB) and yeast-less PDB were measured with a modified Capillary Feeder assay (CAFE assay) (Ja et al. 2007). The females were used in the CAFE assay 48 ± 12 h after their emergence from the pupal stage. Males and females hatched together, and the chosen females were only given water for 5 h before they entered the CAFE assay. For each tested fermentation broth, 20 females were kept individually in one Eppendorf tube (2-mL safe-lock tubes) with the lid positioned downward. Single flies were considered as replicates. Dead flies were not replaced; therefore, the number of flies decreased during the experiments due to the observed mortality. For air circulation, the Eppendorf tubes had three holes (diameter 1 mm) on the sides (at the 1.5-mL mark) and one hole at the bottom for insertion of a 10- μ L glass capillary tube (Drummond Scientific Company, USA). The glass capillaries were held in place with a strip of parafilm wrapped around the capillary at 1 cm height. Every day, yeast fermentation broths were thawed at room temperature and mixed with a vortex mixer at 1800 rpm for 1 min. Ten microliters of yeast fermentation broth was offered through the capillary once a day. The daily consumption was measured in mm based on the liquid level and converted into μ L. Inside the Eppendorf tube, an agar disk (diameter 8 mm, 15 g/L Agar–agar, Merck, Italy) placed in the lid provided an additional water source. The capillaries and agar disks were changed every 24 h, and ingestion and mortality were observed every 24 h over 4 days for single flies. For each solution, three Eppendorf tubes without SWD females were used daily to measure the evaporation rate. The daily evaporation was subtracted from the experimental readings. The mean evaporation was 1.6 ± 0.6 μ L per day.

To identify significant effects in the daily consumption in the CAFE assay, a Kruskal–Wallis test was performed followed by a Wilcoxon rank sum test for pairwise comparison. The results were adjusted using FDR methodology. Survival was evaluated using the Kaplan–Meier method followed by a log rank test. P values were adjusted using FDR methodology.

Chemical analysis

For the analysis of metabolites, liquid chromatography mass spectrometry grade (LC–MS grade) solvents and reagents were used. Analytical standards of carbohydrates, organic acids, sugar alcohols and amino acids that were quantified

in PDB and in fermentation broth samples (“Electronic Supplementary Material (Table S1)”) and isotope-labeled internal standards (IS) (DL-Phenylalanine-3,3-d₂; L-Lysine-4,4,5,5-d₄ hydrochloride; L-Glutamic acid-2,3,3,4,4-d₅; and L-Alanine-2,3,3,3-d₄) were purchased from Sigma-Aldrich (Germany).

Sample preparation: intracellular compounds

Samples were prepared as described by Boer et al. (2010), with further modifications. Ten milliliters of yeast fermentation broth was directly quenched in 20 mL of -80°C methanol and centrifuged for 5 min at 4000 rpm in a -80°C pre-chilled rotor in a centrifuge at -10°C . After centrifugation, the supernatant was discarded, and 0.2 mL of 10 mg/L IS mix solution (DL-Phenylalanine-3,3-d₂; L-Lysine-4,4,5,5-d₄; L-Glutamic acid-2,3,3,4,4-d₅; and L-Alanine-2,3,3,3-d₄) plus 0.8 mL of -20°C extraction solvent (acetonitrile:methanol:water = 2:2:1) were added to the pellet. Then, the sample was extracted for 15 min in an ultrasonic cold bath, and the temperature did not exceed 10°C . The suspension was centrifuged, and the supernatant set aside. The pellet was re-extracted under the same conditions with 1 mL of extraction solvent for 15 min in ultrasonic bath. The suspension was again centrifuged, and the supernatants were pooled (total extraction volume 2 mL, IS final concentration 1 mg/L).

An aliquot of the extract was transferred to a high-performance liquid chromatography (HPLC) vial and directly analyzed for amino acid determination. One part of the extract (0.2 mL) was dried using a speed vac (Eppendorf Concentrator 5301, Eppendorf, Italy) and resuspended in 0.2 mL of milliQ water for the quantification of carbohydrates and sugar alcohols. Another part of the extract (0.5 mL) was dried using a speed vac and resuspended in 0.15 mL of milliQ water for the determination of organic acids.

Sample preparation: extracellular compounds and culture broth

For the analysis of extracellular metabolites, yeast fermentation broth and medium (PDB) were filtered using hydrophilic Surfactant-Free Cellulose Acetate (SFCA) filters (0.2 μm). One part of the filtered sample was diluted 1 to 10 with extraction solvent (acetonitrile:methanol:water = 2:2:1) after the addition of IS mix solution (final IS concentration 1 mg/L), and the amount of amino acids was measured.

Filtered yeast fermentation broth was diluted 1 to 10 with water for the analysis of carbohydrates and sugar alcohols. For filtered PDB, a 1 to 100 dilution was necessary given the high amount of glucose. For organic acids, filtered yeast fermentation and PDB were diluted 1 to 5 with water before analysis.

Analytical methods

The same yeast cultures used for the CAFE assay were used for the chemical characterization (Table 2). Analyses were performed in triplicates.

For the determination of amino acids, samples were analyzed using liquid chromatography electrospray ionization triple quadrupole mass spectrometry (UHPLC-QqQ, Dionex UltiMate 3000 UHPLC TSQ Quantiva, Thermo Fisher, US) in multiple reaction monitoring (MRM) mode. Separation procedures followed Paglia et al. (2012) with further modifications on a hydrophilic interaction chromatography (HILIC) column (Acquity BEH Amide 2.1 \times 150 mm, 1.7 μm with ACQUITY UPLC BEH Amide VanGuard precolumn, 130 \AA , 1.7 μm , 2.1 mm \times 5 mm) at 45°C . The HILIC solvents used included Solvent A (water with 0.1% formic acid (FA)) and Solvent B (acetonitrile (ACN) with 0.1% FA). Flow rate was 400 $\mu\text{L}/\text{min}$. The gradient was as follows: $t=0$, 99% B; $t=0.1$, 99% B; $t=7$ min, 30% B; $t=7.1$ min, 99% B; $t=10$ min, 99% B. The autosampler temperature was 4°C , and injection volume was 2 μL . For routine quality control and quantification, IS of four metabolites (DL-Phenylalanine-3,3-d₂; L-Lysine-4,4,5,5-d₄ hydrochloride; L-Glutamic acid-2,3,3,4,4-d₅; and L-Alanine-2,3,3,3-d₄) were spiked into the samples. Values are reported as mg/L and the amount was calculated with a calibration curve based on the ion ratio between each analyte and the relative IS used. The spray voltage was set at 3200 V when operating in positive ion mode and 3500 V in negative ion mode. Vaporizer temperature and ion transfer tube temperature were set at 275°C and 325°C , respectively.

Samples were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) for the quantification of carbohydrates and sugar alcohols and using a conductivity detector for organic acids (Dionex ICS 5000; Thermo Fisher, USA). Separation of carbohydrates and sugar alcohols was performed using a Dionex CarboPac PA10 analytical column (4 \times 250 mm) and a Dionex CarboPac PA10 precolumn (4 \times 50 mm). Separation was achieved by isocratic elution with a 40 mM sodium hydroxide solution (50% w/w; Sigma-Aldrich, Germany), and the column was regenerated using a 200 mM NaOH solution (50% w/w; Sigma-Aldrich, Germany) for 10 min. The flow rate was set at 1.2 mL/min, the column temperature at 30°C and injection volume was 20 μL . The total run time was 30 min, and a pulsed amperometric detector was used to monitor the eluted carbohydrates and sugar alcohols. Organic acids were analyzed using a Dionex Ion Pac ATC-HC trap (9 \times 75 mm) before a Dionex AG11-HC precolumn (4 \times 50 mm) and a Dionex Ion Pac AS11-HC column (4 \times 250 mm) coupled with a conductimetric detector. The column temperature was set at 30°C , and the injection volume was 25 μL . Chromatographic conditions were based on

previous studies (Geng et al. 2008). For analysis of carbohydrates, sugar alcohol and organic acid quantitation of each compound was calculated based on the calibration curves of corresponding analytical standards.

Statistical analyses

All statistical analyses were performed with R (R Core Team 2019). Visual inspections of residual plots were performed for all models. Significance of growing medium or yeasts were obtained with likelihood ratio tests of the full model with the effect of the growing medium against the model without that effect.

Post hoc tests in linear mixed models were calculated by computing estimated marginal means (EMMs) for specified factors or factor combinations in the models (Searle et al. 1980), and P values were corrected for multiple comparisons using the Tukey method. The following R packages were used: lme4 (Bates et al. 2015) to fit the mixed models, lmerTest (Kuznetsova et al. 2017) for approximating degrees of freedom for the t and F tests of the models and emmeans (Lenth 2019) to perform the post hoc tests in the linear mixed models. The R package survival (Therneau and Grambsch 2000) was used to fit the survival data for the Kaplan–Meier method, and survminer (Kassambara and Kosinski 2018) was used to calculate the pairwise comparisons between group levels. Heatmaps of intra- and extracellular metabolites were generated using MetaboAnalyst 4.0 (Chong et al. 2019).

Results

Oviposition assay

The distribution of the eggs among the three components was considered as the result of the different surface structures. Only the Petri dish with water agar and the culture media without yeast exhibited a fruit-skin-like surface that allowed an easy insertion of the eggs with the serrated ovipositor of the female. In the MEA and PDA assays over all cages containing yeast cultures grown on MEA or PDA ($n = 21 + 21$), on average (\pm SD) $15.3 \pm 15.4\%$ of the eggs were laid into the yeast culture (1), $65.5 \pm 22.0\%$ into the water agar (2) and $19.2 \pm 13.4\%$ into the piece of folded paper (3). It was assumed that the different surfaces of the yeast colonies depending on the yeast species had no effect on the total oviposition given that most eggs were laid in the water agar in all yeast-containing cages. In the MEA and YGM assays overall cages containing a yeast-free MEA control ($n = 7 + 5$), on average (\pm SD) $32.9 \pm 13.2\%$ of the eggs were laid into the MEA (1), $32.3 \pm 16.0\%$ into the water agar (2) and $34.8 \pm 17.5\%$ into the paper towel

(3). In the PDA and YGM assays containing a yeast-free PDA control ($n = 7 + 5$), on average (\pm SD) $70.5 \pm 15.1\%$ of the eggs were laid into the PDA (1), $21.2 \pm 16.7\%$ into the water agar (2) and $8.3 \pm 8.8\%$ into the paper towel (3).

Yeasts grown on MEA

In the MEA assay, SWD flies were fed with three selected yeasts (M.p. 3.2, C.sp. 3.3 and H.u. 3.4) grown on solid medium (MEA) and yeast-free MEA to evaluate the effect of the yeasts on fecundity and mortality of SWD adults. The average first occurrence of oviposition in the cages (\pm SD) was 4.64 ± 1.83 days after the flies entered the assay. No influence of the diets on the first occurrence of oviposition was detected ($\chi^2_{(3)} = 1.63, p = 0.653$). The average number of eggs laid per fly increased during the first 30 days, whereas only a slight increase was observed until the end of the test period, i.e., day 50 (Fig. 1). The different diets had a significant effect on the number of eggs laid over the test period (50 days) ($\chi^2_{(3)} = 963.19, p < 0.0001$). Significantly fewer eggs were laid by SWD females fed with MEA compared to all yeast cultures grown on MEA. Additionally, significant differences among the three yeast species were found, and H.u. 3.4 led to the highest oviposition.

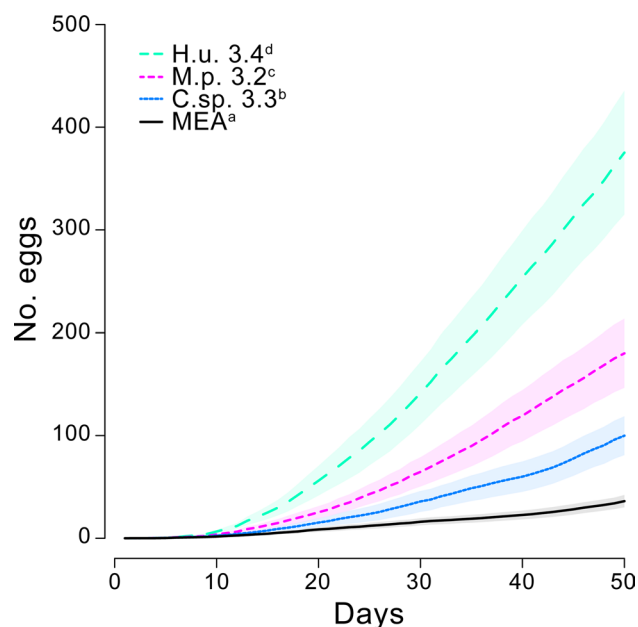


Fig. 1 Cumulative number (mean \pm SE; $n = 7$) of eggs per SWD female laid in the MEA assay over a period of 50 days depending on the yeast species present on malt extract agar: without yeast (MEA), *Hanseniaspora uvarum* (H.u. 3.4), *Metschnikowia pulcherrima* (M.p. 3.2) and *Candida* sp. (C.sp. 3.3). Significant differences between total oviposition rates are indicated by sample names followed by different letters ($p < 0.05$) in the figure legend

Over all treatments, significantly more males (88.93%) than females (80.71%) survived the test period of 50 days ($\chi^2_{(1)}=7.29, p=0.007$). The different diets had no effect on the survivorship of SWD males ($\chi^2_{(3)}=7.50, p=0.058$) but influenced the survivorship of SWD females ($\chi^2_{(3)}=9.41, p=0.024$). No significant differences were found among diets after adjusting for multiple comparisons ($p < 0.05$).

Yeast growth media without yeast

In the YGM assay, two yeast growth media, MEA and PDA, were compared to assess differences in their suitability as a nutrient medium for SWD and to evaluate their influence on oviposition and mortality of SWD adults. Yeast growth media used as SWD food affected female fecundity, and PDA led to significantly increased fecundity compared with MEA ($\chi^2_{(1)}=45.04, p < 0.0001$) (Fig. 2). As also observed in the MEA assay, the egg laying started at a low level after an average (\pm SD) of 1.4 ± 0.55 days on PDA and 3.2 ± 1.30 days on MEA ($\chi^2_{(1)}=5.26, p=0.022$).

Overall, 84% of the females and 92% of the males survived until day 30 ($\chi^2_{(1)}=3.06, p=0.80$). The observed mortality was 88% for males and 80% for females on PDA and 96% for males and 88% for females on MEA. The different culture media showed no significant influence on the

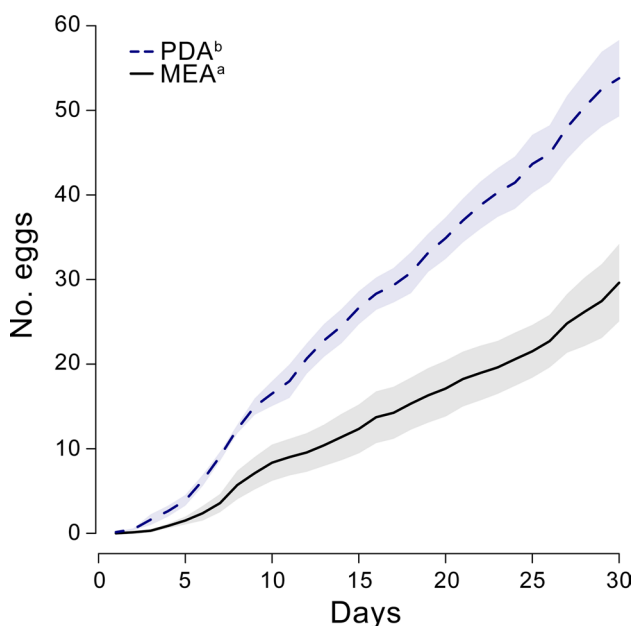


Fig. 2 Cumulative number (mean \pm SE; $n=5$) of eggs per SWD female laid in the YGM assay over a period of 30 days on malt extract agar (MEA) or on potato dextrose agar (PDA). Significant differences between the diets are indicated by sample names followed by different letters ($p < 0.05$) in the figure legend

survivorship of SWD males ($\chi^2_{(1)}=2.20, p=0.138$) or females ($\chi^2_{(1)}=1.147, p=0.284$).

Yeasts grown on PDA

After verifying the promoting effect of PDA on fecundity in comparison to MEA, the PDA assay tested eight different yeasts (S.c. S288c, H.u. 1.21, H.u. 2.2, H.u. 3.4, I.t. 2.1, M.p. 3.2, S.v. 1.33 and C.sp. 3.3) and yeast-free PDA over a period of 30 days. The first eggs were laid on average (\pm SD) 2 ± 0.93 days after the flies entered the assay. The effect of the diets on the first occurrence of oviposition was not significant ($\chi^2_{(7)}=6.065, p=0.532$). Significant differences in the egg laying curves were found between the different diets ($\chi^2_{(7)}=123.33, p < 0.0001$) (Fig. 3). Greater than eighty percent of females (82.08%) and males (87.08%) survived until day 30 ($\chi^2_{(1)}=1.810, p=0.532$). The observed survival on the yeast grown on PDA ranged from 93.3% for males and 96.7% for females on H.u. 1.21 to 76.7% for males and 73.3% for females on H.u. 2.2. The different diets had no significant influences on the survival of SWD males ($\chi^2_{(7)}=4.16, p=0.760$), but a significant influence on females ($\chi^2_{(7)}=16.59, p=0.020$) was noted. Significant more females survived on H.u. 1.21 compared to I.t. 2.1 ($p=0.041$).

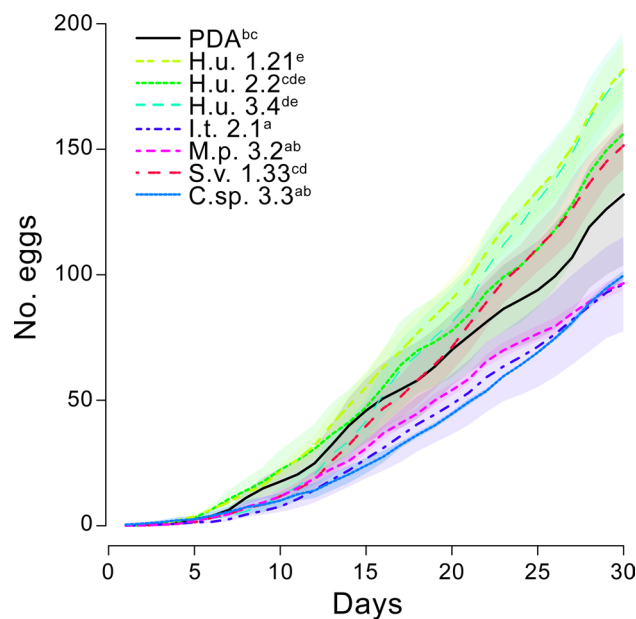


Fig. 3 Cumulative number (mean \pm SE, $n=3$) of eggs per SWD female laid in the PDA assay over a period of 30 days depending on the yeast species present on potato dextrose agar without yeast (PDA) and one of three *Hanseniaspora uvarum* strains (H.u. 1.21, H.u. 2.2, H.u. 3.4), *Issatchenkia terricola* (I.t. 2.1), *Metschnikowia pulcherrima* (M.p. 3.2), *Saccharomyces vini* (S.v. 1.33) and *Candida* sp. (C.sp. 3.3). Significant differences between diets are indicated by sample names followed by different letters ($p < 0.05$) in the figure legend

Ingestion assay

To evaluate the acceptance of the different yeast substrates by SWD females, the CAFE assay was performed to measure the daily ingestion over a four-day period. The ingested amount of yeast fermentation broths grown in PDB and yeast-free PDB showed an increasing trend over the four-day test period. Comparing the daily ingested amount of different yeast broths, except for day 1 ($\chi^2_{(8)} = 13.18$, $p = 0.106$), significant differences were found at day 2 ($\chi^2_{(8)} = 47.07$, $p < 0.001$), day 3 ($\chi^2_{(8)} = 19.47$, $p = 0.014$) and day 4 ($\chi^2_{(8)} = 23.28$, $p = 0.003$). At day 4, pairwise comparison showed no significant differences (Table 3). The mortality rates for the single days are also shown in Table 3.

Profile of intra- and extracellular compounds

Comparison of intra- and extracellular concentrations

Overall, 36 intracellular and 34 extracellular compounds were quantified in PDB and in the eight yeast fermentation broth samples listed in Table 2. Average values of intra- and extracellular compounds are summarized in “Electronic Supplementary Material (Table S2 and Table S3),” while heatmaps of the extracellular and intracellular concentrations of the compounds analyzed are reported in Fig. 4. Generally, extracellular compounds made the largest contribution to the total amount of nutrients, as they were present in much higher concentrations than intracellular compounds. Regarding amino acids and carbohydrates, the highest amount of these nutrients was found in the medium itself.

While 18.34 g/L of glucose was found in PDB, extracellular concentrations in fermentation broth samples ranged from 0.01 g/L in S.c. S288c to 12.15 g/L in H.u. 1.21. The total extracellular amount of amino acids was 0.93 g/L in PDB, ranging from 0.25 g/L in I.t. 2.1 to 0.83 g/L in H.u. 3.4 in fermentation broth samples. Consumption of amino acids and carbohydrates by yeasts was observed since their concentrations decreased compared to those found in PDB with few exceptions. In addition, secretion of sugar alcohols and organic acids by yeasts was detected (Fig. 4a). Although clear differences in the profile of intracellular compounds were identified among yeasts (Fig. 4b), their concentrations were very low compared to extracellular compounds. This is not surprising since amino acids constitute building blocks of proteins, and glucose is clearly involved in sugar metabolism. Two of the organic acids, cis-aconitic acid and isocitric acid, could only be detected inside the cells and not in the extracellular environment.

Carbohydrate consumption and secretion of fermentation products

The extracellular concentrations of carbohydrates, sugar alcohols and organic acids in fermentation broths and PDB are shown in Fig. 5. The results show the consumption of carbohydrates and the secretion of metabolism and fermentation products, such as sugar alcohols and organic acids. The yeasts C.sp. 3.3 and S.c. S288c consumed almost all the available glucose within 30 h of growth.

Amino acid profile

The intra- and extracellular concentrations of amino acids found in fermentation broths and PDB are shown

Table 3 Mean daily ingestion per SWD female ($\mu\text{L} \pm \text{SD}$) of yeast fermentation broth or PDB and daily mortality (% from the initial number ($n = 20$)) in the CAFE assay

Yeast	Day 1		Day 2		Day 3		Day 4	
	Ingestion (μL)	Mortality (%)	Ingestion (μL)	Mortality (%)	Ingestion (μL)	Mortality (%)	Ingestion (μL)	Mortality (%)
PDB	0.27 ± 0.36^a	0	1.13 ± 1.19^{bc}	0	2.00 ± 1.48^{ab}	0	2.22 ± 1.09^a	0
S.c. S288c	0.64 ± 0.95^a	0	1.20 ± 1.32^{bc}	10	1.47 ± 1.27^{ab}	10	0.80 ± 0.79^a	50
H.u. 1.21	0.57 ± 0.56^a	0	1.60 ± 1.27^{cd}	0	1.94 ± 1.84^{ab}	0	3.33 ± 1.67^a	10
H.u. 2.2	0.71 ± 0.69^a	0	1.22 ± 1.30^{bc}	0	1.72 ± 1.16^{ab}	0	2.73 ± 1.59^a	5
H.u. 3.4	0.70 ± 1.01^a	0	2.25 ± 1.14^d	0	2.35 ± 1.49^b	0	2.78 ± 1.73^a	0
I.t. 2.1	0.43 ± 0.45^a	0	0.58 ± 0.66^{ab}	0	1.17 ± 1.19^{ab}	5	1.77 ± 1.53^a	20
M.p. 3.2	0.29 ± 0.42^a	0	0.29 ± 0.39^a	0	1.39 ± 1.66^{ab}	5	1.39 ± 1.49^a	45
S.v. 1.33	0.77 ± 0.75^a	0	0.91 ± 0.60^{bc}	0	1.44 ± 1.48^{ab}	0	3.11 ± 2.07^a	0
C.sp. 3.3	0.22 ± 0.28^a	0	0.63 ± 0.71^{ab}	0	0.81 ± 1.01^a	10	1.80 ± 0.80^a	60

Single females were considered as replicates. Due to mortality, the number of replicates decreases and varies over the test period. Significant differences between ingestion within the days are indicated by different letters ($p < 0.05$)

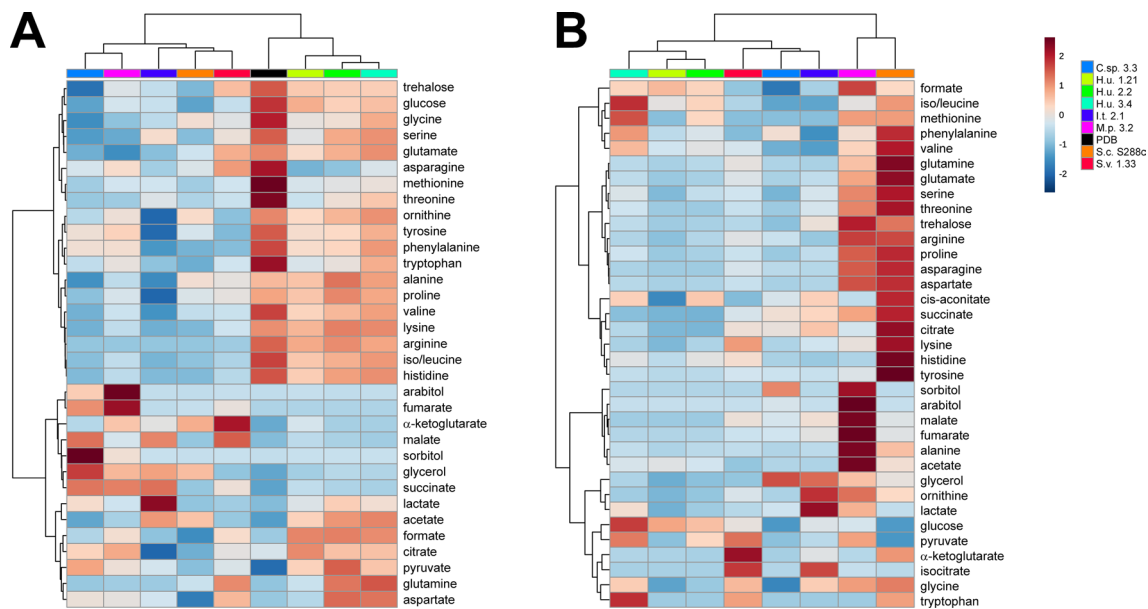


Fig. 4 Heatmaps of extracellular (a) and intracellular (b) compounds detected in the eight selected yeasts and in PDB. Concentrations of single compounds are displayed using a color scale ranging from red (higher amounts) to blue (lower amounts) as shown in the legend.

Both rows and columns are clustered using Euclidean distances and a Ward clustering algorithm. Average values ($n=3$) for each sample are shown

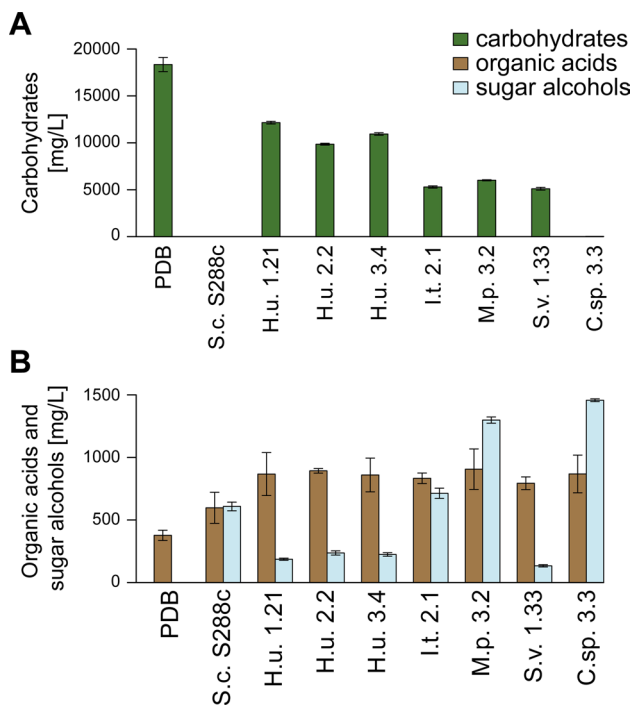


Fig. 5 Extracellular amount of total carbohydrates (a), organic acids and sugar alcohols (b) in the eight selected yeasts and in PDB. Values (mean \pm SD) are reported as mg/L of fermentation or culture broth

in “Electronic Supplementary Material (Table S2 and Table S3).” Ten amino acids (histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan,

valine and arginine) are essential for *Drosophila* flies (Sang and King 1961). Regarding extracellular concentrations, PDB was rich in essential amino acids. The three *H. uvarum* strains consumed less amino acids than other yeasts. Some amino acids were lacking in three yeast fermentation broths. Specifically, a deficiency in extracellular concentrations of arginine, histidine and lysine was found in l.t. 2.1, while arginine, histidine and methionine were lacking in S.c. S288c and C.sp. 3.3. These deficient amino acids were present intracellularly, but methionine was present at low concentrations. In all samples, glutamic acid exhibited the greatest extracellular concentration among amino acids.

Relationships between extracellular compounds, ingestion and mortality

Various yeasts and PDB have different chemical profiles. Therefore, it is not possible to explain the results of this study based on linear correlations among single compounds and ingestion or mortality. However, some relationships were noted among ingestion, mortality and the chemical profile of different samples. The results of the CAFE assay and extracellular concentrations of glucose, total amino acids and glycerol are summarized in Fig. 6. Data on the consumption of females only, which survived the four-day experimental period, were included in the calculation of the total ingestion over four days ($n=6$ to 20). Significant differences were identified for the total ingestion over the four-day period ($\chi^2_{(8)} = 34.98$,

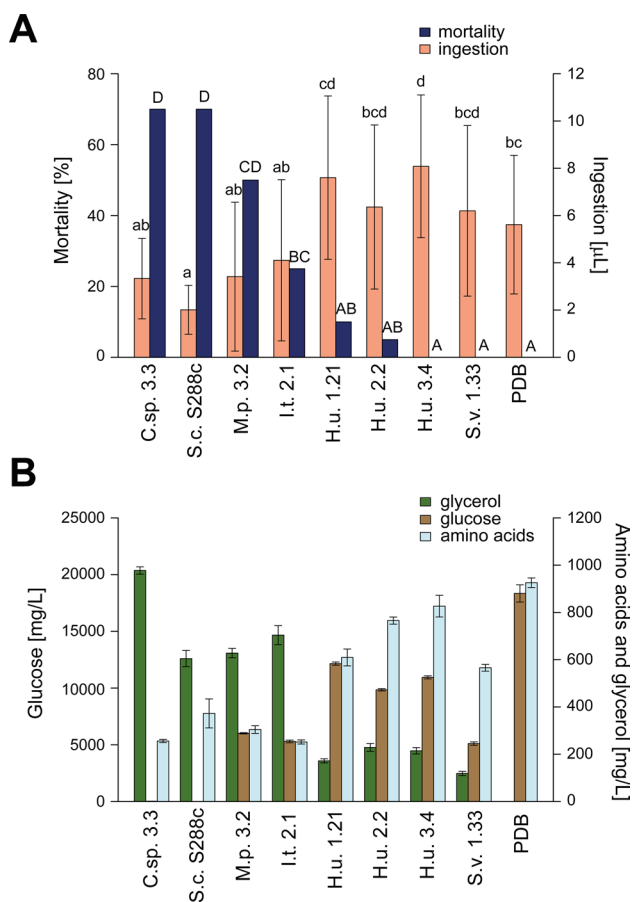


Fig. 6 Total ingestion per SWD female (mean \pm SD) and mortality (%) of SWD females in the CAFE assay (a). Single females were considered as replicates ($n=20$). Data on the ingestion only of females that survived the four-day experimental period were included in the calculation of the total ingestion over four days (resulting $n=6$ to 20). Significant differences between diets are indicated by different letters ($p<0.05$). Extracellular levels of glucose, glycerol and total amino acids in the eight selected yeasts and PDB (b). Values (mean \pm SD) are reported as mg/L of fermentation or culture broth

$p<0.001$). Different diets also significantly influenced the mortality over four days ($\chi^2_{(8)}=78.967, p<0.001$). The lowest mortality and the highest ingestion rate were observed for the three *H. uvarum* strains, S.v. 1.33 and PDB. Overall, a link between lower ingestion coupled with higher mortality could be observed (Fig. 6a), and an opposite relationship was observed between glucose and total amino acids versus glycerol concentration (Fig. 6b). Coherence between the supply of glucose and amino acids and increased ingestion and lower mortality as well as a link between increased glycerol levels and increased mortality are observed.

Discussion

The present study investigates the effect of different yeast species on the life history traits of SWD adults. The culture media PDA showed a positive effect on the fecundity of SWD compared with MEA, and an even increased fecundity rate was observed after inoculation of PDA with *H. uvarum* and S.v. 1.33, demonstrating generally positive effects of these two yeasts on SWD fecundity. Furthermore, SWD females feeding on *H. uvarum* or S.v. 1.33 ingested an increased amount of yeast broth compared with the other four yeast species tested, and a lower mortality of SWD females was observed. Relevant and phagostimulant compounds for SWD, such as carbohydrates and amino acids, were detected in PDB and in fermentation broths. Two yeasts, S.c. S288c and C.sp. 3.3, consume almost all available glucose within 30 h growth, reducing the amount available for SWD flies. *Hanseniaspora uvarum* tend to consume generally less nutrients within 30 h compared to the other species.

A trend toward different egg laying behaviors of *D. melanogaster* fed with different yeasts was previously reported by Anagnostou et al. (2010). The results of this study confirm their hypothesis for SWD and show that H.u. 3.4 cultivated on MEA leads to higher fecundity compared to C.sp. 3.3 and M.p. 3.2. Finding food sources rich in *H. uvarum* might offer an advantage for SWD females. A negative effect of *M. pulcherrima* on *Drosophila* larvae was reported by Anagnostou et al. (2010). On MEA without yeast, fecundity was very low, and all three yeasts exhibited increased oviposition. This study found that egg laying increases slowly over 50 days. A similar oviposition curve with a slow increase in egg laying was observed by Jaramillo et al. (2015).

Lasa et al. (2019a) showed that different growth media influence the attractiveness of yeasts to SWD. A significant difference in the fecundity was observed between yeast growing media with higher oviposition on PDA compared to MEA. In the fecundity assay on PDA, the three *H. uvarum* strains and S.v. 1.33 had positive influences on fecundity, while I.t. 2.1, M.p. 3.2 and C.sp. 3.3 had negative effects. A CAFE assay with the PDB growth medium was performed that included the model organism *Saccharomyces cerevisiae* strain S288c. The three *H. uvarum* strains and S.v. 1.33 led to a higher ingestion and lower mortality of SWD females compared to other yeasts. Interestingly, *S. vini* has rarely been mentioned as being associated with SWD (Bellutti et al. 2018). The results show that *S. vini* potentially represents a yeast with positive effects for SWD, recommending it for further studies. *Hanseniaspora uvarum* is attractive for SWD adults (Scheidler et al. 2015) and larvae (Lewis and Hamby 2019). Based on the

results of this study, SWD adult females also benefit from *H. uvarum* in their diet. Lewis and Hamby (2019) showed that larvae reared on *H. uvarum* reached a smaller adult body size compared to adults reared on diets prepared with *S. cerevisiae*, *P. kluyveri* or *I. terricola*. The reason may be different dietary requirements of larvae and adults.

To exclude the possible toxic effects of alcohol produced during the fermentation process, the alcohol content was measured in fermentation broths. Values ranged from a minimum of 0.54 vol % in S.v. 1.33 to a maximum of 1.01 vol % in S.c. S288c. The LD50 of *Drosophila* flies is generally greater than 1 vol % (Merçot et al. 1994; Chakir et al. 1993), and an influence on the results is therefore not likely.

Based on the availability of appropriate carbon, ammonium and nitrogen sources as well as the presence of specific amino acids in the extracellular environment, yeasts regulate their metabolism and growth (Ljungdahl and Daignan-Fornier 2012). Studies about the use of yeasts in control strategies are based on different methods, such as washing the cells from Petri dishes with different sugar-containing liquids (Noble et al. 2019), revitalizing active dry yeast with sugar solution (Knight et al. 2015; Roubos et al. 2019) and often adjusting the cell number (Mori et al. 2016). Since nutrients present in the substrate influence yeast metabolism and therefore the availability of nutrients for the SWD in a yeast-based diet, a microbiological cost-effective commercial medium (PDB) was chosen, and both intra- and extracellular compounds were analyzed separately.

With few exceptions, the concentrations of extracellular compounds were considerably increased compared with the concentration of intracellular compounds. Overall nutrient consumption or secretion of products of yeast metabolism and fermentation was observed comparing PDB and fermentation broths. The fact that PDB is rich in the nutrients necessary for the development of SWD, such as carbohydrates and amino acids (Markow and O'Grady 2008; Tochen et al. 2016), explains its effectiveness as a food source as demonstrated in the results of the CAFE assay, even without the addition of yeast. The supply of a suitable energy source, such as carbohydrates, increases the appetite of SWD flies (Biolchini et al. 2017), and dietary glucose modulates appetite in *Drosophila* flies (Lebreton et al. 2014). Therefore, the availability of glucose in the diet of SWD is associated with the ingestion. Increased understanding of adult nutrient requirements and nutritional behavior can improve management of SWD (Mori et al. 2016; Tochen et al. 2016). Cowles et al. (2015) demonstrated how the addition of carbohydrates (sucrose) to insecticides targeting SWD enhanced lethality in field tests by increasing the food intake by SWD flies. The yeasts C.sp. 3.3 and S.c. S288c consume almost all available carbohydrates (glucose). The low ingestion of these two yeast fermentation broths in the CAFE assay could

be due to the reduced amount of carbohydrates available for SWD compared to other yeasts and PDB. As a result of yeast metabolism, compounds, such as sugar alcohols and organic acids, are produced and secreted by microorganisms (Kayingo et al. 2001; Ljungdahl and Daignan-Fornier 2012). Some of these compounds, such as acetic acid, may affect the production of aromatic compounds, influencing the attractiveness to *Drosophila* (Erasmus et al. 2004; Vilela-Moura et al. 2011, Hamby and Becher, 2016). Ten amino acids (histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and arginine) are essential for *Drosophila* flies (Sang and King 1961). Previous studies on *Drosophila* demonstrated how different amino acids can produce different appetitive larval responses, and no correlations with the essential/nonessential dietary requirements for amino acids or their chemical properties were observed (Crosset et al. 2016). In contrast, other studies demonstrated that essential amino acid imbalances influence the larval food intake in *Drosophila* (Bjordal et al. 2014). Grandison et al. (2009) also associated the addition of essential amino acids to a dietary restriction diet of *Drosophila* with increased fecundity and decreased lifespan. On the other hand, little is known about the effect of essential and non-essential amino acids on the diet of SWD. In the present study, *H. uvarum* strains contain generally increased abundance of essential amino acids compared with other yeasts, while some yeasts consume specific essential amino acids. The yeast I.t. 2.1 consumes arginine, histidine and lysine, whereas a deficiency in extracellular arginine, histidine and methionine was found in S.c. S288c and C.sp. 3.3. Although yeasts are known to be a protein source in the diet of *Drosophila* (Bing et al. 2018; Camargo and Phaff 1957; Phaff et al. 1956; Steck et al. 2018; Yamada et al. 2015), a high mortality rate was found after four days in SWD fed with C.sp. 3.3 or S.c. S288c, which suggests that the lack of specific free essential amino acids can influence the fitness of the insect. Methionine, an essential amino acid known for its influence on fecundity and lifespan of *Drosophila* (Grandison et al. 2009; Lee et al. 2014; Schütz 2008), was consumed by the two yeasts species (C.sp. 3.3 and S.c. 288) associated with a high mortality in SWD. Glutamic acid was the most abundant amino acid in both PDB and fermentation broths. Although this amino acid is not essential in the *Drosophila* diet, previous studies demonstrated that three amino acidic compounds, glutamic acid, alanine and aspartic acid stimulate food consumption in *Drosophila* (Yang et al. 2018). *Hanseniaspora uvarum* consumed a smaller amount of important nutrients, such as carbohydrates and amino acids, from the culture medium. This finding could explain why increased ingestion by SWD fed with *H. uvarum* was observed. The highest mortality in the CAFE assay was found in SWD females fed

C.sp. 3.3 or S.c. S288c, and yeasts consumed most of the glucose and specific amino acids. Overall, amino acids and the glucose supply seem to be related to the promotion of ingestion and reduction of the mortality. On the other hand, glycerol appears to be associated with a lower survival rate in SWD. This compound also influences physiological and behavioral feeding responses in *Drosophila* (Koseki et al. 2004; Wisotsky et al. 2011). In addition, some nonnutritive sugar alcohol sweeteners, such as erythritol, show potential as a human-safe insecticide against SWD given their possible toxicity (Choi et al. 2017; Sampson et al. 2017). However, glycerol had a minimal effect on fly mortality (Díaz-Fleischer et al. 2019). Although not all compounds that were assessed were related with SWD, these data provide insight in the composition of naturally occurring yeasts grown in an artificial growth medium.

The biology of SWD makes it difficult to develop an effective control strategy (Cini et al. 2012; Sial et al. 2019) to avoid severe economic losses (De Ros et al. 2013). As a control strategy against SWD, suitable yeasts can be used as adjuvants in insecticide sprays to increase ingestion in attract-and-kill formulations based on the association of attractant yeasts and an insecticide (Andreazza et al. 2017; Knight et al. 2015; Noble et al. 2019; Roubos et al. 2019). To date, insecticides were registered against SWD, and technical strategies, such as exclusion netting, were applied to control this pest (Beers et al. 2011; Leach et al. 2016, Sial et al. 2019). Monitoring and control strategies based on acetic bacteria and yeast volatiles were developed (Lasa et al. 2019b; Cha et al. 2013; Iglesias et al. 2014). For example, *H. uvarum* is known to be attractive for SWD flies (Scheidler et al. 2015) and can increase ingestion compared to growth medium without yeast (Mori et al. 2016). Normally, insects are attracted to specific volatile compounds associated with a food source (Davis et al. 2013). Given its attractiveness, yeast could prove more suitable than odorless adjuvants, such as sucrose, which has already shown to increase the effectiveness of insecticides against SWD (Cowles et al. 2015). For example, *H. uvarum* is attractive for SWD flies (Scheidler et al. 2015) and increases ingestion compared to growth medium without yeast (Mori et al. 2016). This finding should be considered in view of development of attract-and-kill control strategies.

The findings of the present work are useful for understanding the differences in the fecundity, ingestion and mortality of SWD adults among yeast strains, providing relevant information for the development of attract-and-kill control strategies against SWD. Additionally, the chemical characterization of yeast-based food provides insights concerning potentially phagostimulant components that may be exploited for pest management, indicating that not only the aromatic compounds but also nonvolatile metabolites of the yeast play a role in the association between yeast and SWD.

Author contributions

US and SS performed the entomological assays. FB and DE performed the chemical analyses. US, SS, IC, EHK and SA designed the entomological research. FB, DE, PR and RFV designed the chemical research. FB and US analyzed the yeast broths and wrote the manuscript. ND contributed to statistical analyses. All authors reviewed and approved the final version of the manuscript and contributed to the interpretation of the data.

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Compliance with ethical standards

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

Ethical standard Ethical standards of institutional, national and international guidelines were considered and followed.

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