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Chemical characterization and influence on the fitness of *Drosophila suzukii* of different yeast species and yeast-based attract-and-kill formulations

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Abbreviations

°C	degree Celsius
ACN	acetonitrile
Ala	alanine
ANOVA	analysis of variance
Arg	arginine
Asn	asparagine
Asp	aspartate
C. s.	*Clavispora santaluciae*
CAFE	capillry feeder
CDW	cell dry weight
CE	cholesterol ester
CE-MS	capillary electrophoresis-mass spectrometry
Cer	ceramide
CLSA	closed-loop-stripping analysis
CUDA	12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid
DG	diacyglycerol
DHS	dihydroshphingosine
DMNT	(E)-4,8-dimethylnona-1,3,7-triene
DSCD	*Drosophila suzukii* cornmeal diet
ESI	electrospray ionization source

FA	fatty acid
FAN	free amino nitrogen
FDR	false discovery rate
GC-MS	gas chromatography-mass spectrometry
Gln	glutamine
Glu	glutamate
GLVs	green leaf volatile
Gly	glycine
GP	phospholipids
h	hour
H.u.	*Hanseniaspora uvarum*
HILIC	hydrophilic interaction chromatography
His	histidine
HPAE-PAD	high performance anion exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography coupled with ultraviolet detector
HSD	honestly significant difference
I. t.	*Issatchenkia terricola*
ID-MS	isotope dilution-mass spectrometry
IEC	ion exchange chromatography

Ile	isoleucine
KOH	potassium hydroxide
LC	liquid chromatography
LCB	sphingoid long-chain base
LC-MS	liquid chromatography-mass spectrometry
Leu	leucine
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LRI	linear retention index
Lys	lysine
M. p.	*Metschnikowia pulcherrima*
m/z	mass to charge ratio
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MANOVA	multivariate analysis of variance
mbar	millibar
MEA	malt extract agar
MeOH	methanol
Met	methionine
MG	monoacyglycerol
MRM	multiple reaction monitoring
MS/MS	tandem mass spectrometry
MTBE	methyl tertiary butyl ether

MUFA	monounsaturated fatty acid
N	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
nd	not detected
ns	not significant
OD600	optical density at 600 nm
PAD	pulsed amperometric detection
PC	phosphatidylcholine
PCA	principal component analysis
PDA	potato dextrose agar
PDB	potato dextrose broth
PE	phophatidylethanolamine
PG	phosphatidylglycerol
Phe	phenylalanine
PHS	phytosphingosine
PI	phosphatidylinositol
PLS-DA	partial least squared discriminant analysis
Pro	proline
PS	phosphatidylserine
PUFA	poliunsaturated fatty acid
QC	quality control sample

RI	refractive index detection
rpm	round per minute
RSD	relative standard deviation
S	spinosad
S. c.	*Saccharomyces cerevisiae*
S. v.	*Saccharomycopsis vini*
SD	standard deviation
SE	steryl ester
Ser	serine
SFCA	surfactant-Free Cellulose Acetate
SIM	single reaction monitoring
SM	ceramide phosphocholine
SRM	selected reaction monitoring
T	timepoint
TG	triacyglycerol
Thr	threonine
TIC	total ion current
Try	tryptophan
Tyr	tyrosine
UHPLC – QTOFMS	liquid chromatography electrospray ionization time of flight mass spectrometry

UHPLC-QqQ	liquid chromatography electrospray ionization triple quadrupole mass spectrometry
UV	ultraviolet
UV-Vis	UV-Visible
v/v	volume/volume
Val	valine
VOC	volatile organic compound
YGM	yeast growth media
YMM	yeast minimal medium
$\chi 2$	Chi-squared

1 Introduction

1.1 The worldwide invasion of *Drosophila suzukii*

1.1.1 Origin and diffusion of the species

The vinegar fly *Drosophila suzukii* (Matsumura) (Diptera Drosophilidae), also known as the spotted wing drosophila, is an invasive pest native to South East Asia, which is causing serious economic damages worldwide in the agricultural sector (Calabria, Máca, Bächli, Serra, & Pascual, 2012; Cini et al., 2014; De Ros, Conci, Pantezzi, & Savini, 2015; Dos Santos et al., 2017; Hauser, 2011). Little is known about its exact geographical origin: the species was described by Matsumura in 1931 in Japan, where it was firstly recorded in 1916 and where few years later it was found to be widely distributed on cherries and grapes (Kanzawa, 1939). The fly was also recorded in the east of China (Peng, 1937), as well as in numerous other Asian countries including the Korean peninsula (Kang & Moon, 1968), Taiwan (Lin, Tseng, & Lee, 1977), and Myanmar (Toda, 1991). Due to its high capacity to disperse, coupled with the lack of efficient control strategies, *D. suzukii* quickly spread across USA and in Canada, Mexico, and Europe (Hauser 2011; Cini, Ioriatti, & Anfora, 2012). The first records in Europe refer to 2008 in Spain (Calabria et al., 2012). In Italy, the presence of *D. suzukii* was reported for the first time in 2009 in Trentino Alto-Adige, and the pest rapidly spread across the North East of Italy (Grassi, Palmieri, & Giongo, 2009; De Ros, Anfora, Grassi, & Ioriatti, 2013). Based on its rapid spread in numerous other European countries, all continental Europe seems to be at risk (Cini, Ioriatti, & Anfora, 2012).

1.1.2 Morphology and biology of the insect

D. suzukii are drosophilids. The body of the insect is approximately 2-3 mm long and the wingspan is about 5-6.5 mm (Kanzawa, 1939); they have red eyes, a yellow-brown thorax and black stripes on the abdomen (Cini, Ioriatti, & Anfora, 2012) (Figure 1). Males exhibit a dark spot on each wing (Figure 1B) and females possess a large and

serrated ovipositor (Kanzawa, 1939; J. C. Lee, Bruck, Dreves, et al., 2011; Walsh et al., 2011).

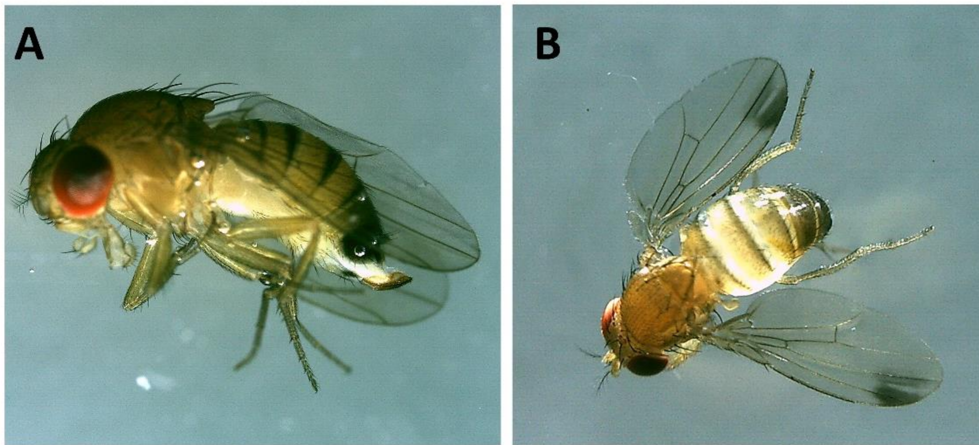

FIGURE 1: Example of *D. suzukii* adults: female (A) and male (B). Photos were taken by the Entomology Group of the Institute for Plant Health of Laimburg Research Centre.

The injuries that occur during infestations by *D. suzukii* are due to the direct damage caused by the larval feeding inside the fruit (Asplen et al., 2015), or to the indirect exposition of the fruit to pathogens after their oviposition (Cini, Ioriatti, & Anfora, 2012; Hamby, Hernández, Boundy-Mills, & Zalom, 2012; Ioriatti et al., 2015). Nonetheless, deteriorated fruits are more sensitive to attack by other drosophilids (Walsh et al., 2011). *D. suzukii* flies have short generation time (they reach maturity one or two days after emergence) and a high reproduction rate (Tochen et al., 2014). They undergo three stages of development before emerging as adults, consisting of: egg, larva, and pupa (Figure 2).

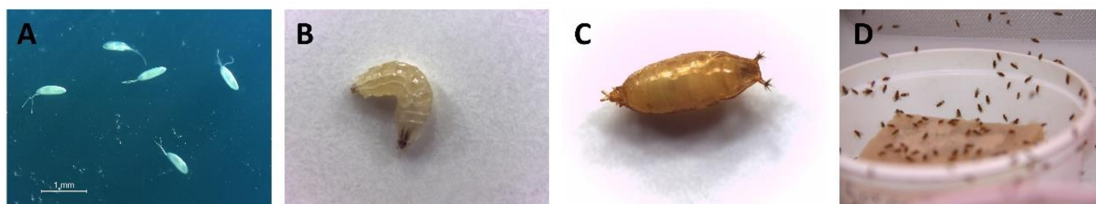

FIGURE 2: Life stages of *D. suzukii*: eggs (A), larva (B), pupa (C), and adult flies (D). Photos were taken by the Entomology Group of the Institute for Plant Health of Laimburg Research Centre.

In contrast with *Drosophila melanogaster*, females lay eggs in intact healthy ripe fruits (Burrack, Fernandez, Spivey, & Kraus, 2013; J. C. Lee, Bruck, Curry, et al., 2011;

Mitsui, Takahashi, & Kimura, 2006; Walsh et al., 2011). These characteristics, coupled with the wide host range including small and stone, wild and cultivated fruits (Bellamy, Sisterson, & Walse, 2013; De Ros, Anfora, Grassi, & Ioriatti, 2013; Elsensohn & Loeb, 2018; J. C. Lee et al., 2015; Mitsui, Takahashi, & Kimura, 2006), make this invasive insect pest a threat for numerous crops. Indeed, host plants suitable for the development of *D. suzukii*'s larvae include blueberry, strawberry, raspberry, blackberry, and cherry (De Ros, Anfora, Grassi, & Ioriatti, 2013), but there is also a risk for other fruits such as apricot, peach, nectarine, plum and some varieties of grape, including the typical South Tyrolean variety "Vernatsch". The host fruit preference for oviposition and suitability for larval development are influenced by numerous parameters: fruit characteristics, including sugar content, pH or firmness (Arnó, Solà, Riudavets, & Gabarra, 2016; Burrack et al., 2013; J. C. Lee et al., 2015; Little, Chapman, Moreau, & Hillier, 2016; Kinjo, Kunimi, Ban, & Nakai, 2013); fruit ripeness (Karageorgi et al., 2017); composition in proteins and carbohydrates (Hardin, Kraus, & Burrack, 2015); fruit species preferences (Abraham et al., 2015; Bellamy et al., 2013; Cloonan, Abraham, Angeli, Syed, & Rodriguez-Saona, 2018; J. C. Lee, Bruck, Curry, et al., 2011), and local abundance of hosts (Cini, Ioriatti, & Anfora, 2012). This species survives in a wide range of climatic conditions: though the optimal reproductive temperature occurs at ca. 23 °C, they can oviposit between 10 and 31 °C, and acclimated flies can survive up to 1°C (Ryan, Emiljanowicz, Wilkinson, Kornya, & Newman, 2016). The injury caused by *D. suzukii* is due to the feeding of larvae on fruits and to the physical damage of the fruit surface that leads to the development of microorganisms (bacteria, fungi, yeasts) including pathogens responsible for fruit deterioration (Cini, Ioriatti, & Anfora, 2012). To come into contact with the fruits, the flies detect signals like volatile compounds emitted by the fruits, by the plants and by fermentation products (Abraham et al., 2015; Bing, Gerlach, Loeb, & Buchon, 2018; Bolton, Piñero, Barrett, & Cha, 2019; Keesey, Knaden, & Hansson, 2015; Lasa, Toledo-Hernández, Rodríguez, & Williams, 2019; Liu et al., 2018; Piñero, Barrett, Bolton, & Follett, 2019; Revadi et al., 2015). The latter include numerous chemical compounds commonly produced by bacteria and yeasts that grow on ripe fruit and that insects associate with the presence of a sugar source (Madden et al., 2018).

1.1.3 Nutritional requirements and preferences of *Drosophila*

Only few relatively recent studies are focused on the nutritional needs of *D. suzukii* (Bellutti et al., 2018; Bing et al., 2018; Tochen, Walton, & Lee, 2016; Young, Buckiewicz, & Long, 2018). By contrast, studies concerning the nutritional requirements of *D. melanogaster* date back a long time and reveal that there are differences between species and diverse requirements between adults and larvae (Sang 1956; Sang 1959; Sang & King, 1961; Royes & Robertson, 1964). Sang & King (1961) stated that larvae of *D. melanogaster* need major nutrients for their development such as RNA, casein, cholesterol, and lecithin or choline. On the contrary, the absence of cholesterol or choline in the diet of adults did not influence fundamental biological processes such as oviposition; while the presence of fructose, which only slightly influenced the larval development, positively affected the egg production by adult flies (Sang & King, 1961). Ten amino acids (histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and arginine) are essential for *Drosophila* flies (Rudkin & Schultz, 1947). The lack of any of these compounds was shown to lead to a reduced egg production coupled with a lower viability of the eggs laid (Sang & King, 1961). The B vitamin complex, with the exception of vitamin B12, is essential for optimal larval growth (Sang 1956). The deficiency of potassium and magnesium in the diet resulted in a reduction of egg laying and higher mortality of adult flies (Sang & King, 1961). *D. melanogaster* is able to synthetize the necessary fatty acids for its survival, however, previous studies showed that the content and characteristics of dietary fatty acids influence the phospholipid composition of the cell membranes of the insect (Carvalho et al., 2012). On the contrary, the presence and the amount of specific phospholipid classes in various tissues of the insect's body is not influenced by the phospholipidic composition of their food (Carvalho et al., 2012).

Another aspect of *Drosophila*'s feeding is related to its preferences, behavior, and fitness as a response to a food source. Indeed, females of *D. melanogaster* fed with different yeasts, showed different egg laying behaviors (Anagnostou, Dorsch, & Rohlfs, 2010). Concerning specifically *D. suzukii*, recent works demonstrated that larvae reached a smaller adult body size after feeding on *H. uvarum*-based diets,

while exhibiting a strong attraction to the yeast *H. uvarum* in pairwise yeast feeding assays (Lewis & Hamby, 2019). This discrepancy between attractiveness and feeding benefits indicate that differences in the nutritional quality of a food source are not the only factor driving larval feeding choices in *D. suzukii*.

1.2 Interaction between yeasts and *D. suzukii* and its relevance for control strategies

1.2.1 The association between yeasts and *D. suzukii*

A complex interaction mechanism exists between yeasts and diverse *Drosophila* species. Yeasts represent a source of important nutrients for drosophilids (Becher et al., 2012) by providing the necessary proteins, amino acids, lipids, and vitamins to the fly (De Camargo & Phaff, 1957; Carvalho et al. 2012; Yamada, Deshpande, Bruce, Mak, & Ja, 2015; Steck et al. 2018; Bing, Gerlach, Loeb, & Buchon, 2018). Different species of yeast that colonize fruits constitute a source of nourishment for the larvae of *D. suzukii* and affect their development as well as the fecundity of adult females (Bellutti et al., 2018). It has been found that, as for other *Drosophila* species (Oakeshott, Vaeek, & Anderson, 1989), substrates colonized by yeasts are preferred for oviposition by *D. suzukii* over those where a high development of bacteria or molds occurs (Hamby, Hernández, Boundy-Mills, & Zalom, 2012).

Yeasts produce volatile organic compounds that attract *D. suzukii* (Hamby & Becher, 2016; Hamby, Hernández, Boundy-Mills, & Zalom, 2012; Lasa, Navarro-De-La-Fuente, Gschaedler-Mathis, Kirchmayr, & Williams, 2019; Scheidler, Liu, Hamby, Zalom, & Syed, 2015), with the strain, the species, and the growth medium playing a role in the attractiveness towards the fly (Lasa, Navarro-De-La-Fuente, Gschaedler-Mathis, Kirchmayr, & Williams, 2019). Additionally, they were shown to stimulate feeding in *D. suzukii* (Lewis & Hamby, 2019). Improving the knowledge related to the interaction between yeasts and *D. suzukii* could be helpful for the development of a control strategy, based on the exploitation of an interaction between insects and microorganisms already existing in nature.

1.2.2 The community of yeasts associated with *D. suzukii*

Numerous yeast's species are naturally associated to *D. suzukii* (Hamby, Hernández, Boundy-Mills, & Zalom, 2012; Bellutti et al., 2018; Lewis et al. 2019) (Figure 3).

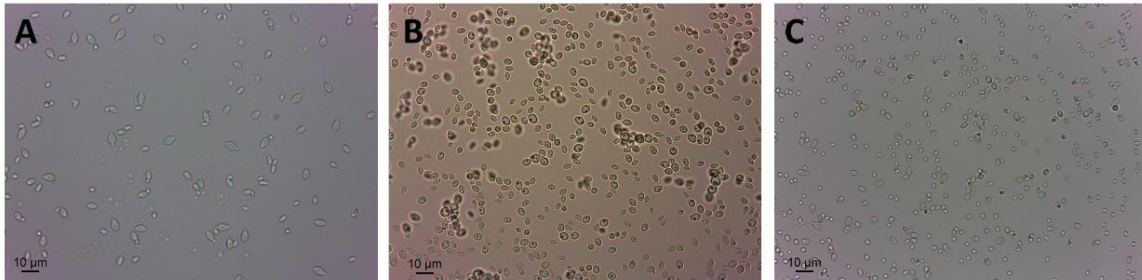

FIGURE 3: Examples of yeast species isolated from grapes infested by *D. suzukii* in South Tyrol. *Hanseniaspora uvarum* (A), *Metchenikovia pulcherrima* (B) and *Clavispora santaluciae* (C). Photos were taken by the Entomology Group of the Institute for Plant Health of Laimburg Research Centre.

Hanseniaspora uvarum was previously isolated both from larvae and adults of *D. suzukii* (Hamby, Hernández, Boundy-Mills, & Zalom, 2012; Knight, Basoalto, Yee, Hilton, & Kurtzman, 2016; Lewis, Koivunen, Swett, & Hamby, 2019), while other species, including *Metschnikowia pulcherrima*, *Issatchenkia terricola*, and *Pichia kluyveri*, were found on damaged fruit and on larvae of *D. suzukii* (Bellutti et al., 2018; Hamby, Hernández, Boundy-Mills, & Zalom, 2012; Lewis, Koivunen, Swett, & Hamby, 2019). The genus *Hanseniaspora* was reported as the predominant one among those isolated from infested fruits (Fountain et al. 2018; Hamby, Hernández, Boundy-Mills, & Zalom, 2012; Bellutti et al., 2018; Lewis, Koivunen, Swett, & Hamby, 2019) and the species *H. uvarum* as the most attractive one to *D. suzukii* adults compared to other yeasts (Scheidler, Liu, Hamby, Zalom, & Syed, 2015; Lewis & Hamby, 2019). Larvae prefer to feed on *H. uvarum* compared to other species as well (Lewis & Hamby, 2019; Bellutti et al., 2018). Trapping trials show that this species is also more attractive to *D. suzukii* adults compared to *Pichia kluyveri*, *Candida californica*, *Issatchenkia terricola*, *Saccharomyces cerevisiae*, and *Candida zemplinina* (Scheidler, Liu, Hamby, Zalom, & Syed, 2015).

1.2.3 Yeast-based control strategies against *D. suzukii*

Due to the biological characteristics of *D. suzukii*, the development of a control strategy against this pest is challenging (Cini, Ioriatti, & Anfora, 2012; Sial et al. 2019). The existing control of the pest in fruit cultivation is based on the chemical pest management. However, due to the low selectivity of most insecticides and to their residual presence on the surface of treated fruits (Haviland & Beers, 2012), new strategies should be adopted. Yeasts can be added to insecticides for the development of so called attract-and-kill formulations (Knight, Basoalto, Yee, Hilton, & Kurtzman, 2016; Mori et al., 2017). These control strategies are based on the use of attractant yeasts as adjuvants to improve the efficacy of baits (Figure 4) or to increase the ingestion of formulations based on the addition of insecticide to fermentation products and feeding attractants (Andreazza et al., 2017; Knight, Basoalto, Yee, Hilton, & Kurtzman, 2016; Noble et al., 2019; Roubos et al., 2019).

FIGURE 4: Example of a bait for *D. suzukii*. Photos were taken by the Entomology Group of the Institute for Plant Health of Laimburg Research Centre.

Thanks to their attractiveness and feeding stimulant effects towards *D. suzukii* flies, these microorganisms would allow to reduce the amount of insecticide necessary to be effective against infestations (Mori et al., 2017; Noble et al., 2019) and consequently reduce the environmental impact of chemicals used in the fight against *D. suzukii*. Therefore, the identification of the species that could increase the selectivity of the treatments and maximize the attractiveness and feeding stimulant effect of an attract-and-kill formulation is crucial. The limitation concerning the use of microorganisms for controlling the pest concerns the metabolic changes that occur during their growth and development, that lead to changes in the chemical

composition of the growth medium, resulting in the production or consumption of specific compounds. This can lead to nutritional competition mechanisms due to the consumption by microorganisms of potentially phagostimulant compounds for the fly. Additionally, modifications of the profile of VOCS of fermented products occur over time (Callejón, Margulies, Hirson, & Ebeler, 2012; Ye, Yue, & Yuan, 2014), with possible consequences for the attractiveness to *D. suzukii*. This indicates that not only the appropriate yeast species but also the growth medium and the growing conditions are relevant for the success of an attract-and-kill formulation. Additionally, considering the changes of the microbial metabolism that occur, it is essential to evaluate that the efficacy of the formulation is maintained over time.

1.3 Chemical characterization of yeasts

1.3.1 The importance of the characterization of yeasts for control strategies

An intriguing approach to understand the complex mechanism at the basis of the interaction between yeasts and *D. suzukii*, is based on the chemical characterization of the community of yeasts naturally occurring in association with *D. suzukii*. Indeed, the knowledge of the yeast metabolism and of the composition of yeast's cells and fermentation products concerning metabolites, lipids, and volatile organic compounds may be useful to improve existing control strategies against the pest. The identification of chemical compounds involved in the attractiveness of *D. suzukii* to specific yeast species coupled with the determination of metabolites able to induce desired feeding behaviors in the flies are important for the development of a successful attract-and-kill formulation.

1.3.2 Phagostimulant compounds towards *Drosophila* in fermentates

D. suzukii feeds on fruits and microorganisms growing on damaged fruits (Mitsui, Beppu, & Kimura, 2010; Tochen, Walton, & Lee, 2016; Walsh et al. 2011). As other *Drosophila* species, these insects need a sugar source and a correct proportion of protein and carbohydrates (Young, Buckiewicz, & Long, 2018). Vitamins, which are

also provided by symbiotic microorganisms (Becher et al., 2012; Douglas, 2017), are essential for the survival of *Drosophila* flies. Not only yeasts constitute a food source for *Drosophila*, but they also produce metabolites that, being perceived by adults or larvae, can induce repulsion against a food source or appetite stimulation. A yeast fermentate is commonly composed of water, yeast cells, nutrients present in the growth medium, a sugar source, vitamins, and minerals necessary for the survival and growth of yeasts. Nonetheless, all the nutrients available in a complex yeast growth medium plus metabolites produced by the microorganisms themselves constitute a suitable food source for *Drosophila* flies. Some of these compounds including carbohydrates, sugar alcohols, amino acids, organic acids, and lipids can induce specific feeding behavior in *Drosophila*.

1.3.2.1 Carbohydrates

Carbohydrates are polyhydroxy aldehydes or polyhydroxy ketones that occur either in an open chain form or in heterocyclic ring forms (Biermann & McGinnis, 2019). These biomolecules consist of atoms of carbon, hydrogen, and oxygen at different ratios, based on the empirical formula $C_m(H_2O)_n$. Besides being a primary source of energy for most living organisms, they are structural molecules, also involved in regulating physiological processes and in cellular communication systems, including enzyme-substrate specificity and molecular transport (Baker, 2020; Biermann & McGinnis, 2019). They are constituents of cell membranes (glycosphyngolipids) and of glycoproteins (Biermann & McGinnis, 2019).

Monosaccharides are the monomeric units that constitute carbohydrates. They can be divided into aldoses and ketoses based on the presence of an aldehyde or a ketone, respectively, when they are present in their acyclic form. When two or more monosaccharides are linked together through a glycosidic linkage, they constitute dimers, trimers and so on. Based on the degree of polymerization they can be distinguished between oligosaccharides (2 to 8 monosaccharide units) or polysaccharides (>8 monosaccharide units) (Biermann & McGinnis, 2019). Some polysaccharides, such as cellulose, are cell wall constituents, while others, like starch and glycogen, represent a storage form of glucose (Garg, Cowman, & Hales, 2008).

The metabolism of carbohydrates is essential for *Drosophila*, influencing growth, reproduction, and organismal maintenance (Mattila & Hietakangas, 2017). The various stages of development of *Drosophila* are associated to unique metabolic requirements. Indeed, though sugars are not needed for the maturation of larvae of *D. melanogaster* (Sang 1956), the life span of adult flies is much longer after the introduction of sugar to their diets (Hollingsworth & Burcombe, 1970). Among numerous carbohydrates, glucose, fructose, and sucrose were found to be the most effective in prolonging life of *Drosophila* (Kircher & Al-Azawi, 1985) and for a long time have been known to elicit a strong gustatory response by *Drosophila* flies (Falk, Bleiser-Avivi, & Atidia, 1976; Miyakawa, Fujishiro, Kijima, & Morita, 1980). Behavioral assays revealed that *Drosophila* flies extend proboscis to feed on glucose, trehalose, and glycerol (Slone, Daniels, & Amrein, 2007), and the availability of a suitable carbohydrate source was shown to increase the appetite of *D. suzukii* flies (Biolchini et al., 2017; Lebreton, Witzgall, Olsson, & Becher, 2014).

1.3.2.2 Sugar alcohols

Sugar alcohols, also known as polyols or polyhydric alcohols, are water-soluble carbohydrates derivatives obtained by the replacement of an aldehyde with a hydroxyl group (Shankar, Ahuja, & Sriram, 2013). They represent an energy and carbon storage source for the numerous organisms that produce them, including bacteria, yeasts, fungi, algae, lichens, and many plants (Awuchi, 2017).

These compounds are products of the metabolism of numerous microorganisms growing on the surface of fruits and influence physiological and feeding responses of *Drosophila* flies (Koseki, Koganezawa, Furuyama, Isono, & Shimada, 2004; Wisotsky, Medina, Freeman, & Dahanukar, 2011). The gene Gr64e confers responsiveness to glycerol in *Drosophila* (Kim et al., 2018; Wisotsky, Medina, Freeman, & Dahanukar, 2011). Studies focused on another sugar alcohol, erythritol, show its potential as a human-safe insecticide against *D. suzukii* due to its toxicity against the fly (Choi et al., 2017; Sampson, Werle, Stringer, & Adamczyk, 2017).

1.3.2.3 Amino acids

Amino acids are organic compounds that constitute the building blocks for the synthesis of proteins. Twenty proteinogenic amino acids are the units utilized by living cells for condensation into peptides and proteins (Barrett & Elmore, 1998). An α-amino acid is constituted by a backbone, that consists of an amine group (–NH2), a carboxyl group (–COOH), and a different side chain for each amino acid (R group) (Elliott & Elliott, 1997; Nelson & Cox, 2017). In aqueous neutral solution they are in their zwitterionic form. With the exception of glycine, which has no asymmetric carbon atom, all amino acids that constitute proteins are in L-configuration. Through a condensation reaction, two amino acids can be linked together generating a dipeptide after the formation of the peptide bond (-CO-NH-) (Elliott & Elliott, 1997).

Specific amino acids induce different appetitive larval behaviors in *Drosophila*, with no correlations with their dietary necessities nor with their chemical characteristics (Croset, Schleyer, Arguello, Gerber, & Benton, 2016). On the contrary, other research indicate that imbalances in the essential amino acid profile affects the larval food intake in *Drosophila* (Bjordal, Arquier, Kniazeff, Pin, & Le, 2014). The addition of essential amino acids to a dietary restriction diet of *Drosophila* leads to a higher fecundity and a lower lifespan (Grandison, Piper, & Partridge, 2009). Methionine, one of the essential amino acid for *Drosophila* flies, influences their fecundity and lifespan (Grandison, Piper, and Partridge 2009; Schutz 2008; Lee et al. 2014); while other three amino acids, which are not essential for the fly, namely glutamic acid, alanine, and aspartic acid, can stimulate food consumption by *Drosophila* (Yang et al., 2018).

1.3.2.4 Organic acids

Organic acids are acidic compounds resulting from hydrolysis, biochemical metabolic processes, and microbial activities (Vargas, 2017). They are intermediate and final products of fundamental cellular metabolic pathways like the glycolysis and the citric acid cycle (Elliott & Elliott, 1997).

The presence of organic acids affects the feeding behavior of *Drosophila*. These flies tend to reject too acidic food and to have adverse responses to carboxylic acids, including acetic acid and citric acid (Charlu, Wisotsky, Medina, & Dahanukar, 2013;

Liman, Zhang, & Montell, 2014; Revadi et al., 2015). Additionally, numerous receptors of *Drosophila* flies are involved in the taste of specific acidic compounds, enabling the insects to discriminate foods based on the presence/abundance of specific acidic compounds rather than just pH (Rimal et al., 2019). On the other hand, while fed *Drosophila* flies showed aversion to acetic acid, starved ones showed a strong positive appetitive response towards this compound (Devineni, Sun, Zhukovskaya, & Axel, 2019). Other studies revealed that supplementation of citric acid improves health span in *Drosophila* (Panchal & Tiwari, 2016). These findings reflect the complexity of the requirements as well as appetitive or aversive responses of *Drosophila* to diverse organic acids.

1.3.2.5 Lipids

Lipids are ubiquitous non-polar compounds involved in a high number of biological processes. They occur in all cell types and constitute a storage energy source; they are components of cell membranes, they contribute to cellular structure, and have a role as signaling and regulation molecules (Fahy, Cotter, Sud, & Subramaniam, 2011; Gurr, et al., 2016). The term "lipid" defines heterogeneous not chemically well-defined molecules that are not soluble in water but soluble in nonaqueous solvents like chloroform, alcohols, and hydrocarbons. Indeed, numerous different lipid classes exist such as esters of the glycerol: mono-, di-, and triacylglycerols (TG), the latter constituting natural fats and oils. Other lipids are represented by phospholipids and glycolipids, where the glycerol hydroxyl group is esterified with phosphorus or sugar, respectively. Some lipids are instead based on sphingosine, while others, like isoprenoids, steroids, and hopanoids, derive from the five-carbon hydrocarbon isoprene (Gurr et al., 2016).

Yeasts constitute a lipid source for *Drosophila* by providing TGs, steryl esters (SE) sphingolipids, unsaturated and saturated fatty acids (FA), phospholipids, and sterols to its diet (Ejsing et al., 2009; Kaneko, Hosohara, Tanaka, & Itoh, 1976; Koch, Schmidt, & Daum, 2014). Despite *Drosophila* is able to synthetize *de novo* the fatty acids needed for its survival (Carvalho et al., 2012), previous studies demonstrated that the gene Gr64e controls the behavioral and electrophysiological responses of *Drosophila* to fatty acids (Kim et al., 2018). Capillary feeding assays showed

that *Drosophila* flies prefer to feed on a fatty acid solution rather than water (Masek & Keene, 2013), meaning that also this compound class should be taken into account with reference to *Drosophila*'s nutritional preferences. Except for fatty acids, little is known about the influence of other lipid classes on the feeding behavior or preferences of *Drosophila*.

1.3.3 Liquid chromatography for the characterization of chemical compounds

Liquid chromatography (LC) is a widely used technique for the determination and quantification of a variety of chemical compounds in numerous application fields including biomedical, environmental, chemical, and pharmaceutical sectors. A chromatographic analysis is aimed at separating chemical compounds of interest in a complex matrix within a relatively short time. Due to the advances in this technique, nowadays it is possible to analyze a large number of compound classes of interest for industry and research. The separation of molecules is based on the iterative interaction of the analyte, the stationary phase, and the mobile phase. The stationary phase consists of a resin derivatized with polar, apolar, or functional groups, depending on the specific application. The solvents used, called mobile phases, allow the elution by removing the interaction of the analytes with the stationary phase.
The numerous applications of LC are related to the possibility to link it with numerous detection systems including UV-visible spectrometry, unit- and high-resolution mass-spectrometry, fluorescence spectrometry, or even a secondary chromatographic system (Hage & Carr, 2012).

1.3.3.1 Ion exchange chromatography for the analysis of carbohydrates, sugar alcohols, and organic acids

Ion exchange chromatography (IEC) is based on the ionic interaction between analyte, stationary phase, and mobile phase. This technique is commonly used for numerous applications, ranging from the analysis of drinking water, food and beverages, pollutants, and groundwater. The separation is based on the ion exchange between a mobile phase, consisting of the eluent, and a stationary solid phase, consisting of specific ion exchange resins. The IEC is based on chemical interactions

between ions in solution and the ionic functional groups of the stationary phase that interact with the analytes and with the mobile phase ions of opposite polarity (Sarzanini & Cavalli, 2002).

The chemical characterization of fermentation and culture media is of great interest in numerous application fields. Yeast culture media are usually chemically complex, containing sugars, organic acids, anions, amino acids, vitamins, proteins, and yeast extract. Such a complex matrix requires sensitive and selective analytical procedures to be correctly characterized. Anion exchange chromatography (AEC) has been used to determine carbohydrates, sugar alcohols, and organic acids present in fermentates. For these applications, the mobile phase consists of highly alkaline sodium hydroxide (NaOH) or potassium hydroxide (KOH) solutions, converting even neutral sugars to their oxyanions, which are separated on the AE column. Two main detectors are used: conductimetric or amperometric detectors. The conductivity relies of the electric conductivity of the ionic analytes, which is directly proportional to their mobility (Sarzanini & Cavalli, 2002). The conductivity detector requires a suppressor that allows to reduce the conductivity signal of the eluent. Carbohydrates and sugar alcohols are detected selectively via their specific redox potential. The redox reaction produces an electric current that is proportional to the concentration of the analyte (Eith, Schnepf, Kolb, & Seubert, 2007). This is also a reason why it is possible to selectively determine these compound classes in a complex mixture containing thousands of other apolar compounds and cations, which are not retained on the column. The IEC coupled with pulsed amperometric detection (PAD) is among the most frequently used approaches for the analysis of carbohydrates and sugar alcohols (Pereira da Costa & Conte-Junior, 2015), because of its higher selectivity compared to high performance liquid chromatography coupled with ultraviolet detector (HPLC-UV) and its higher sensitivity and selectivity compared to refractive index detection (RI) (Paredes, Maestre, Prats, & Todolí, 2006). This technique allows the detection of these compounds in complex matrices, since the peculiarity of carbohydrates and sugar alcohols to be oxidized and thus selectively determined through amperometric technologies (Hanko & Rohrer, 2000). Additionally, the wide range of linear response at increasing concentrations of compounds detected

through IEC, allows to avoid multiple dilutions required in case of analytes having high differences in their concentrations among samples (Hanko & Rohrer, 2000).

Numerous studies reported chromatographic applications for the determination of organic acids, including reversed-phase HPLC-UV (Cunha, Fernandes, & Ferreira, 2002; Destandau et al., 2005; Sturm, Koron, & Stampar, 2003) or mass spectrometry (Flores, Hellín, & Fenoll, 2012; Ross, Tu, Smith, & Dalluge, 2007). Nonetheless, these techniques have some limitations. The wavelength of 210 nm, usually used for the UV detection of organic acids, lacks in selectivity (Geng, Zhang, Wang, & Zhao, 2008), while high detection limits were reported using mass spectrometric approaches due to the high ionic strength (Ross, Tu, Smith, & Dalluge, 2007). Ion exchange chromatography was found to be suitable for the profiling of carboxylic acids in the presence of inorganic anions in complex matrices such as bacterial cell cultures, yeast fermentate (Geng, Zhang, Wang, & Zhao, 2008), wort, and fermented samples resulting from different phases of beer production (Boyles, Boyles, & Company, 1992), indicating that IEC represents a technique of choice for the analysis of these compounds.

1.3.3.2 Liquid chromatography electrospray ionization triple quadrupole mass spectrometry (UHPLC-QqQ) for the analysis of amino acids

Mass spectrometry is a powerful and sophisticated technique that allows the identification and the quantitative analysis of an extremely wide range of molecules belonging to different compound classes based on their mass to charge ratio (m/z).

Four steps are needed for a mass spectrometric analysis:

1) Ionization of the molecules

2) Acceleration of ions by an electric field

3) Separation of ions with different masses

4) Ion detection and mass determination

The electrospray ionization source (ESI) is a commonly used ion source in mass spectrometry coupled to a LC system. The sample is nebulized at atmospheric pressure in an electric field. The sample is solubilized in a volatile solvent and then

introduced at atmospheric pressure into the ESI source through a capillary to which a voltage is applied responsible for the ionization of the solution. A spray of charged droplets is formed at the outlet of the capillary. The solvent is evaporated under a nitrogen and heat flow until multi-charged ions of the gas-phase analyte are released. Positive or negative molecular ions can be produced from a sample depending on the polarity of the capillary voltage (ESI- or ESI+) and the solvent used. From the same molecule, multiple molecular ions are formed, each with a different number of charges, which are then sent to the analyzer under high vacuum conditions, thus obtaining a mass spectrum of ions of interest (Hage & Carr, 2012). ESI is considered a soft ionization technique, leading to intact molecular ions with limited fragmentation, which, however, can be tuned to the needs of the experimenter.

The different types of existing analyzers have the role of separating ions according to their m/z using a magnetic and/or electrical field. Quadrupole mass analyzers are commonly used in association with HPLC. Specifically, the triple quadrupole, consisting of three in-line quadrupoles, is a unit-resolution tandem mass spectrometry analyzer often used for targeted quantitative analyses. The first and third quadrupoles act as mass filters, while the central quadrupole is a collision cell. The quadrupole consists of four parallel metal rods through which the selected ions are filtered before reaching the collision cell, where they are fragmented (Fanali, Haddad, Poole, & Riekkola, 2017). Different scan modes can be performed: precursor ion scan, neutral loss scan, product ion scan, and selected reaction monitoring (De Hoffmann, 1996). In selected reaction monitoring (SRM) mode, the signal acquisition can be performed in single (SIM) and multiple reaction monitoring (MRM). In SRM mode the first quadrupole selects the m/z of the molecule of interest (precursor ion), this ion is fragmented in the collision cell, while the third quadrupole filters one fragment of the precursor ion selected in the first quadrupole. SIM mode implies that only a single ion transition is monitored, while MRM mode allows to multiple ion transitions to be monitored at the same time (Rossi & Sinz, 2001).

Numerous studies report applications of LC coupled with triple quadrupole mass spectrometry for the quantitative analysis of amino acids in fermentation products (Dalluge, Smith, Sanchez-Riera, McGuire, & Hobson, 2004; Preinerstorfer, Schiesel, Lämmerhofer, & Lindner, 2010; Qu et al., 2002). This technique offers high specificity

and sensitivity thanks to the possibility to confirm the identity of compounds not only based on their molecular weight, but also based on their fragmentation patterns (Preinerstorfer, Schiesel, Lämmerhofer, & Lindner, 2010). Additionally, it is optimal for quantitative analysis compared to high-resolution mass spectrometry, which is recommended for accurate qualitative analyses (Mozzi, Ortiz, Bleckwedel, De Vuyst, & Pescuma, 2013).

1.4 The emerging field of metabolomics

An innovative tool for high throughput studies of the chemical profile of microorganisms, cells and tissues is represented by metabolomics. This powerful analytical strategy exploits sophisticated chromatographic and detection technologies (gas chromatography-mass spectrometry [GC-MS], liquid chromatography-mass spectrometry [LC-MS], matrix assisted laser desorption ionization-time of flight [MALDI-TOF]) to obtain a relative quantification of diverse compound classes that constitute a complex matrix. It requires numerous steps, including sample collection and storage, protein precipitation, extraction, data acquisition, data processing, and data transformation (Weckwerth, 2007). Sample preparation procedures need to be specific for the type of sample to analyze, based on the composition of the cell membrane, and possibly the cell wall, and the resistance of the cell to physical treatments (Caudy, Mülleder, & Ralser, 2017). The choice of data acquisition techniques and methods should take into account the nature of the sample and the type of analysis to perform. Different suitable and specific techniques are recommended for targeted compound classes profiling, for comparative metabolomic studies, for *de novo* identification of unknown compounds, as well as for metabolic fingerprinting (Fiehn, 2002). Finally, one major challenge in metabolomics studies is represented by the data processing and interpretation steps. Firstly, data preprocessing is required (Shurubor, Paolucci, Krasnikov, Matson, & Kristal, 2005; van der Werf, Jellema, & Hankemeier, 2005): the negative effect of the instrumental noise should be minimized, and retention times aligned. Data generally need to be scaled, centered, and transformed in order reduce differences between the orders of magnitude of the concentrations of metabolites,

fold changes consequent to a variation between two conditions to compare, fluctuations due to biological variation, or technical variability due to analytical biases (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006). Afterwards, numerous software can be chosen for statistical and visual interpretation and representation of data. The numerous advantages of metabolomics include the possibility of a high throughput analysis of a wide range of different molecules, the exploration of unknown metabolites, and the ability to determine relative changes in metabolite abundances among samples and metabolic conditions. The main disadvantages are related to the necessity to take compromises in the method development, leading to a reduction of the accuracy of the analysis for some metabolites or compound classes (Weckwerth, 2007). A typical workflow used for metabolomics is reported in Figure 5.

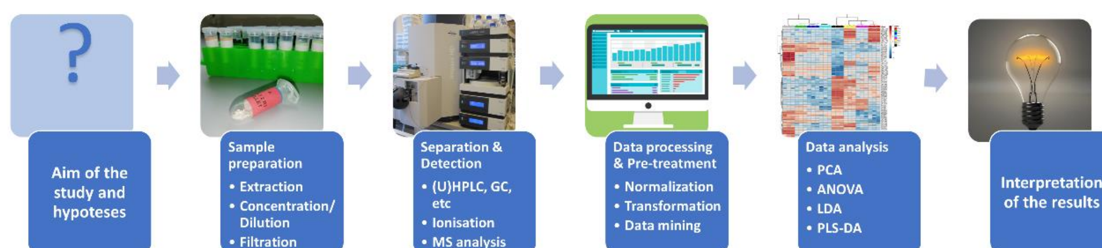

FIGURE 5: Metabolomic workflow.

1.4.1 Yeast metabolomics and lipidomics

Due to its genetic characteristics as well as its importance in the food industry and in biotechnology, the yeast has always been considered an excellent model for studying the metabolism of eukaryotic cells (Caudy, Mülleder, & Ralser, 2017). In a metabolomic approach for the analysis of yeasts, numerous variables should be considered when choosing an analytical technique, including the yeast strain and species, the growth conditions, and the turnover rate (Caudy, Mülleder, & Ralser, 2017). So far, diverse metabolic approaches have been used to study the eucaryotic metabolism through yeasts and fungus, such as targeted and untargeted metabolic profiling as well as metabolic flux analysis (Sailwal et al., 2020). For each application, it is fundamental to pay attention to numerous sample preparation steps and data acquisition as well as data processing procedures. In their review, Sailwal et al. (2020)

have recently summarized all stages of a yeast metabolomic workflow, as listed below:

- **Yeast cultivation and sample preparation:** This involves working under sterile and controlled conditions during the cultivation of the cells. A quick collection of an amount of the fermentate is required, as well as a quenching step aimed at stopping the biochemical activities of the micro-organisms. The subsequent extraction procedure should be specific based on the type of analysis to perform and should avoid the degradation of metabolites.
- **Analysis of the samples through MS-based metabolomics:** This step includes to choose techniques such as LC-MS, GC-MS, MALDI-TOF, isotope dilution-mass spectrometry (ID-MS), or capillary electrophoresis-mass spectrometry (CE-MS), based on the nature of the metabolites of interest.
- **Data processing and statistics:** It starts with the observation of raw data, followed by pre-processing, data transformation, and normalization. These stages are intended to reduce the instrumental noise, the technical variation, or the risk of wrong metabolite annotation. Finally, it is possible to proceed with statistical analyses, including univariate, multivariate, system-based, or predictive approaches.

Lipidomics is a branch of metabolomics that focuses on non-polar compounds and can be useful to understand the lipid composition of a sample as well as the biological role of lipids. Despite the complexity of the lipidome of eukaryotic cells, the numerous progresses in mass spectrometry enabled the exploration of diverse compound classes. Differences between the lipidic composition of diverse cellular organelles and compartments, as well as changes in the profile of lipids involved in signaling and regulation pathways, are reflected in the lipidome of a cell (van Meer, 2005). One of the issues in lipidomics is related to the complicated procedures of sample preparation, aimed at exploring a large number of molecular classes. Additionally, as for metabolomics, the computational and bioinformatic interpretation of data represents one of the main barriers for lipidomic studies (Wenk, 2005). The relatively simple lipidome of yeast, together with a wide

knowledge of the genes involved in its lipid metabolism constitute an advantage and a good starting point for lipidomic studies (Gaspar et al., 2007). Among the numerous researches and reviews on lipidomics (Casanovas et al., 2015; Ejsing et al., 2009; Li, Yang, Bai, & Liu, 2014; Watson, 2006; Wenk, 2005; Wolf & Quinn, 2008), only few are focused on comparative yeast lipidomics (Aloklah, Alhajali, & Yaziji, 2014; Augustyn, Ferreira, & Kock, 1991; Hein & Hayen, 2012; Kaneko, Hosohara, Tanaka, & Itoh, 1976; Viljoen, Kock, & Lategan, 1986), which is a branch of this discipline of great interest for the exploration of different species, strains, and growing conditions.

1.5 Motivation, aim of this thesis and working hypotheses

1.5.1 Motivation

During the last decade, the insect pest *D. suzukii* rapidly spread from Asia across USA and Europe, causing severe economic losses in agriculture. A relevant aspect concerning the diffusion and the damage due to *D. suzukii* infestations, is the lack of an efficient and sustainable control strategy. So far, repeated applications of insecticides are often necessary to obtain satisfactory results, with a consequent negative impact on the environment.

Sustainable management systems are challenging due to the lack of a detailed knowledge of the biology of the species, of its population dynamics, and of its interaction with other organisms populating infested fruit crops. This last point is highly interesting for research, since a successful control strategy against this pest could be based on the interaction between insects and microorganisms that naturally populate crops at risk. So far, numerous strategies were proposed to face the challenge of controlling infestations by *D. suzukii*, including mass trapping using vinegar containing baits and traps composed of attractive fruits, fruit juices, or fermentation products. Focusing only on the attractiveness, however, may not be sufficient. Although this aspect is of fundamental importance, there are other factors that affect the functionality of a bait, including the insect's feeding behavior and long-term effectiveness. An intriguing possibility is represented by the so-called attract-and-kill formulations. An attract-and-kill formulation consists of a product that contains: i) attractive substances luring the insect to the formulation; ii) feeding stimulants that induce the fly to feed on the formulation; iii) an insecticide, killing the insect due to the contact or the feeding on the formulation. For the success of such a strategy, a combination of ingredients capable to ensure sufficient selectivity, long-term effectiveness, and low environmental impact is required. Numerous studies report the improvement of baits after the addition of yeasts, indicating the key role of these microorganisms in the attractiveness towards *D. suzukii*. Yeasts represent a relevant portion of the community of microorganisms associated to *D. suzukii*. *Drosophila* flies associate the presence of yeasts to a sugar-rich source, and thus a

suitable food source for its development and egg-laying. Flies recognize volatile organic compounds produced by yeasts and react to them through attraction or repulsion behaviors. Besides being attractive to the fly, yeasts constitute a relevant food source in its diet. They produce vitamins that are essential for the survival of *Drosophila*, provide proteins, lipids, and secrete chemical compounds able to stimulate feeding or provoke food rejection. Concerning both attractiveness and feeding stimulation, these microorganisms can induce behavioral responses of opposite valence; therefore, it is crucial to identify the conditions that maximize the positive effects given by the presence of yeast in the bait. To reach this scope, it is fundamental to combine expertise in different scientific fields: behavioral and entomological studies are necessary for understanding the responses of the insect to yeast-based attract-and-kill formulations, and the exploration of the chemical composition of yeasts is required to understand the chemical mechanism behind the efficacy of the product, in order to optimize it.

Consequently, this thesis was embedded in the project Dromytal (ERDF 1021, CUP H32F16000420009), which aimed at broadening the knowledge about the interaction between yeasts and *D. suzukii*, in order to develop a prototype of an attract-and-kill formulation to be used in the field as an innovative and sustainable control strategy against this insect pest.

This thesis focuses on the chemical characterization of non-volatile compounds present in fermentates made using yeasts associated with *D. suzukii* and in a yeast-based attract-and-kill formulation. This chemical approach of characterizing yeast metabolites represents an innovation in the context of microorganism-insect interaction studies, which could help explaining the basis of this complex mechanism of association, from a chemistry perspective.

1.5.2 Aim of this thesis

The main objective of this study is the chemical characterization of yeasts associated with *D. suzukii* and yeast-based attract-and-kill formulations. The effect of the chemical composition of yeasts on the yeast-insect interaction and on the efficacy of yeast-based control strategies would be assessed.

For such a study, numerous steps are required. Firstly, the presence and the amounts in yeast fermentates of relevant compounds for the association mechanism between *D. suzukii* and yeasts should be investigated using advanced and reliable analytical techniques.

Secondly, a prototype of a yeast-based attract-and-kill formulation should be developed and its efficacy in controlled conditions over a defined time-period should be assessed.

Studies concerning entomological responses of *D. suzukii* after exposure to various yeasts and yeast-based treatments (performed by of the Entomology Group of the Institute for Plant Health of the Laimburg Research Centre – Urban Spitaler) and the profile of volatiles emitted by yeasts (performed by the Faculty of Science and Technology of the Free University of Bozen-Bolzano – Irene Castellan) were needed to interpret the results of this thesis and are therefore included in it.

1.5.3 Working hypotheses

Given the above-mentioned motivations and objectives, the following working hypotheses were established based on the three main experimental parts of this study.

1.5.3.1 Quantification of chemical compounds present in yeasts associated with *D. suzukii*

- Yeasts contain nutritionally relevant compounds for *D. suzukii*.
- Differences concerning the chemical composition of diverse dietary yeasts affect the feeding behavior of *D. suzukii*.

1.5.3.2 Development of a prototype of a yeast-based attract-and-kill formulation

- A formulation for an attract-and-kill approach against *D. suzukii* can be developed based on yeasts fermentates.
- The key compounds, which attract and feed *D. suzukii* in this formulation, can be determined and monitored over time.

1.5.3.3 Metabolomics of yeasts associated with *D. suzukii*

- A metabolomic approach allows to explore the presence in yeasts of potentially phagostimulant compounds towards *D. suzukii*.
- The analysis of the metabolome of different yeast species associated with *D. suzukii* enables to differentiate them based on their metabolic profile.

2 Materials and methods

2.1 Materials

2.1.1 Yeasts

The yeasts used for this study are reported in Table 1. With the exception of *Saccharomyces cerevisiae*, all yeasts were isolated from feeding tunnels of *D. suzukii* larvae found in infested grapes in South Tyrol in 2009 (Bellutti et al., 2018). *Saccharomyces cerevisiae* strain S288c is a conventional laboratory strain.

TABLE 1: List of the yeast strains used for the experiments.

Yeast species	Strain	Accession number*	Abbreviation
Saccharomyces cerevisiae	S288c		S.c. S288c
Hanseniaspora uvarum	LB-NB-1.21	KP298009	H.u. 1.21
Hanseniaspora uvarum	LB-NB-2.2	MK567898	H.u. 2.2
Hanseniaspora uvarum	LB-NB-3.4	MK567905	H.u. 3.4
Issatchenkia/Pichia terricola	LB-NB-2.1	MK567903	I.t. 2.1
Metschnikowia pulcherrima	LB-NB-3.2	KP298012	M.p. 3.2
Saccharomycopsis vini	LB-NB-1.33	KP298011	S.v. 1.33
Clavispora santaluciae	LB-NB-3.3	KP298013.1	C.s. 3.3

*The accession numbers were deposited in GenBank NCBI

2.1.2 Chemicals

2.1.2.1 Reagents

Formic acid (LC-MS grade) and methyl tertiary butyl ether (MTBE, HPLC grade) were obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (ACN, LC-MS grade),

isopropanol (LC-MS grade) and methanol (MeOH, LC-MS grade) were purchased from VWR International Srl (Milan, Italy). Toluene (HPLC grade), ammonium formate (LC-MS grade), ammonium acetate (≥98%), ethanol (≥99.8 %), NaOH (50 % w/w), dichloromethane (GC grade), and heptane (HPLC grade) were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). The ultrapure water was prepared with a Millli-Q-water purification system (EMD Millipore Corporation, Billerica, MA, USA).

2.1.2.2 Standards

Analytical standards of the compounds listed in Table 2 were used for the quantitative analyses of carbohydrates, sugar alcohols, amino acids, and organic acids. All standards were provided by Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

TABLE 2: List of the standards used for quantitative analyses. The purity of the standard was reported, when available.

Compound	Classification	Category	Purity (%)
L-Alanine	Amino acids	Analytical standard	≥99.0
L-Arginine monohydrochloride	Amino acids	Analytical standard	>98.0
L-Asparagine	Amino acids	Analytical standard	>98.0
L-Aspartic acid	Amino acids	Analytical standard	>99.0
L-Glutamic acid	Amino acids	Analytical standard	≥99.0
L-Glutamine	Amino acids	Analytical standard	≥99.5
Glycine	Amino acids	Analytical standard	≥99.0

L-Histidine	Amino acids	Analytical standard	>99.0
L-Isoleucine	Amino acids	Analytical standard	≥99.5
L-Leucine	Amino acids	Analytical standard	≥99.5
L-Lysine	Amino acids	Analytical standard	>98.0
L-Methionine	Amino acids	Analytical standard	≥99.5
L-Ornithine monohydrochloride	Amino acids	Analytical standard	≥99.0
L-Phenylalanine	Amino acids	Analytical standard	≥99.0
L-Proline	Amino acids	Analytical standard	>99.5
L-Serine	Amino acids	Analytical standard	≥99.5
L-Threonine	Amino acids	Analytical standard	≥99.5
L-Tryptophan	Amino acids	Analytical standard	≥99.5
L-Tyrosine	Amino acids	Analytical standard	≥99.0
L-Valine	Amino acids	Analytical standard	≥99.5
D-(+)-Glucose	Carbohydrates	Analytical standard	≥99.5
D-(+)-Trehalose dihydrate	Carbohydrates	Analytical standard	≥99.0
Glycerol	Sugar alcohols	Analytical standard	>99.0
D-Sorbitol	Sugar alcohols	Analytical standard	>98.0

D-(+)-Arabitol	Sugar alcohols	Analytical standard	≥99.0
Sodium acetate anhydrous	Organic acids	Analytical standard	>99.0
L-(+)-Lactic acid	Organic acids	Analytical standard	≥98.0
Succinic acid	Organic acids	Analytical standard	>99.0
L-(-)-malic acid	Organic acids	Analytical standard	>99.0
Fumaric acid	Organic acids	Analytical standard	>99.0
Citric acid	Organic acids	Analytical standard	>99.0
Ammonium formate	Organic acids	Analytical standard	>99.0
Pyruvic acid	Organic acids	Analytical standard	>99.0
α-ketoglutaric acid	Organic acids	Analytical standard	>99.0
DL-Isocitric acid, trisodium salt hydrate	Organic acids	Analytical standard	>99.0
Cis-aconitic acid	Organic acids	Analytical standard	>99.0
DL-Phenylalanine-3,3-d2	Amino acids	Internal standard	-
L-Lysine-4,4,5,5-d4 hydrochloride	Amino acids	Internal standard	-
L-Glutamic acid-2,3,3,4,4-d5	Amino acids	Internal standard	-
L-Alanine-2,3,3,3-d4	Amino acids	Internal standard	-

For lipidomics, the compounds listed in Table 3 were used as internal standards (IS). Analytical IS were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

TABLE 3: List of the IS used for lipidomics.

Compound	Classification

CE (22:1)	Cholesterol esters
Ceramide (d18:1/17:0)	Ceramides
Cholesterol d7	Cholesterol
CUDA (12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid)	Fatty acids and conjugates
DG (12:0/12:0/0:0)	Diacylglycerols
DG (18:1/2:0/0:0)	Diacylglycerols
FA (16:0)-d3	Fatty acids and conjugates
LPC (17:0)	Lysophosphatidylcholine
LPE (17:1)	Lysophosphatidylethanolamine
MG (17:0/0:0/0:0)	Monoacylglycerols
PC (12:0/13:0)	Phosphatidylchloline
PE (17:0/17:0)	Phophatidylethanolamine
PG (17:0/17:0)	Phosphatidylglycerols
SM (d18:1/17:0)	Ceramide phosphocholines
Sphingosine (d17:1)	Sphingoid long-chain bases
TG d5 (17:0/17:1/17:0)	Triacylglycerols

Analytical standards of volatile organic compounds (VOCs) were provided by Sigma Aldrich (Merck KGaA, Darmstadt, Germany) and are listed in Table 4.

TABLE 4: List of the analytical standards used for the identification of VOCs. The purity of the standard was reported, when available.

Compound	**Classification**	**Purity (%)**
Benzaldehyde	Aldehydes	≥99
2-phenylethanol	Alcohols	≥99
Octanoic acid	Acids	≥99
2-phenylethyl acetate	Esters	99
Methyl acetate	Esters	≥98
Methyl salicylate	Esters	≥99
Indole	Polycyclic heteroarenes	≥99
(*Z*)-3-hexenyl butyrate	Green leaf volatiles	≥98
Linalool	Terpenes	≥95
(E)-4,8-dimethylnona-1,3,7-triene	Terpenes	≥90
Beta-caryophyllene	Terpenes	≥80

Humulene	Terpenes	≥96.0
(*E,E*)-alpha-farnesene	Terpenes	Mixture of isomers

2.1.3 Yeast culture media and *D. suzukii* diet

Chloramphenicol yeast glucose broth (5 g/L yeast extract) was purchased from Merck KGaA (Darmstadt, Germany). Glucose, sodium chloride (NaCl) and glycerol were provided by Sigma-Aldrich (Merck, KGaA, Darmstadt, Germany). Potato dextrose agar (PDA, 4 g/L potato starch from infusion, 20 g/L dextrose, 15 g/L agar) and potato dextrose broth (PDB) were provided by Difco™ (Becton Dickinson, Le Pont de Claix, France). Malt extract agar (MEA) (30 g/L malt extract, 3 g/L peptone from soymeal and 15 g/L agar) was provided by Sigma-Aldrich (Merck, KGaA, Darmstadt, Germany). Yeast minimal medium (YMM) – yeast nitrogen base without amino acids – was provided by Sigma-Aldrich (Merck, KGaA, Darmstadt, Germany).
D. suzukii Cornmeal Diet (DSCD(a)) with dry deactivated yeast and dry baker's yeast were provided by Küchle GmbH & Co. KG (Günzburg, Germany).

2.1.4 Laboratory Equipment

2.1.4.1 Instruments

- Eppendorf Centrifuge 5810 R (Eppendorf, Hamburg, Germany)
- Speedvac - Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany)
- Freeze dryer (Labconco Corporation, Kansas City, MO, USA)
- Spectrophotometer - Cary 60 UV-Visible (UV–Vis) (Agilent Technologies, Santa Clara, CA, USA)
- Multichannel pipette - Eppendorf Researchplus (Eppendorf, Hamburg, Germany)
- Thermomixer (Eppendorf, Hamburg, Germany)
- pH meter - Crison GLP 21 (Hach, Düsseldorf, Germany)
- Fuchs Rosenthal counting chamber (Hecht Assistent®, Sondheim vor der Rhön, Germany)

- High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) Dionex ICS 5000 (Thermo Fisher, Waltham, MA, USA)
- Liquid chromatography electrospray ionization triple quadrupole mass spectrometry (UHPLC-QqQ) - Dionex UltiMate 3000 UHPLC - TSQ Quantiva (Thermo Fisher, Waltham, MA, USA)
- Liquid chromatography electrospray ionization time of flight mass spectrometry (UHPLC – QTOFMS) - Dionex UltiMate 3000 UHPLC (Thermo Fisher, Waltham, MA, USA) - Impact HD qTOF (Bruker, Billerica, MA, USA)
- GC-MS - GC 7890A coupled with a MS 5975C Network (Agilent Technologies, Santa Clara, CA, USA).
- Digital Oenological Distiller SUPE DE SV (Gibertini, Novate Milanese, Italy)
- Thermostatic bath (Gibertini, Novate Milanese, Italy)
- Electronic densimeter DMA 4500 (Anton Paar, Graz, Austria)
- Drying oven (WTB Binder, Tuttlingen, Germany)

2.1.4.2 Software

- Microsoft Office 16 (Microsoft Corporation, Redmond, WA, USA)
- R version 3.6.3 (R Development Core Team)
- SPSS (IBM SPSS statistic 24)
- MS-DIAL
- Metaboanalyst
- ChemRICH
- Enhanced MSD ChemStation E.02.02.1431 (GC-MS)
- Chromeleon 6.80 (IEC, UHPLC)
- TraceFinder 3.2 (QqQ)
- otofControl 3.4 (QTOFMS)
- Bruker Daltonics Hystar 3.2 (QTOFMS)
- Bruker Compass DataAnalysis 4.2 (QTOFMS)

2.2 Methods

2.2.1 Yeast cultivation

Yeasts were cultivated by the Entomology Group of the Institute for Plant Health of the Laimburg Research Centre.

2.2.1.1 Yeast cultivation on solid media

Yeast cultures used for oviposition assays on solid media were grown on Petri dishes (diameter 6 cm) on MEA or PDA. 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 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% 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.

2.2.1.2 Yeasts cultivated in liquid medium used for Capillary Feeder assay (CAFE assay) and quantification of targeted compounds

For long-term storage, purified yeast isolates were cultivated in chloramphenicol yeast glucose broth consisting of 5 g/L yeast extract (Merck, KGaA, Darmstadt, Germany), 20 g/L glucose (Sigma-Aldrich, Merck, KGaA, Darmstadt, Germany), 0.1 g/L chloramphenicol (Merck, KGaA, Darmstadt, Germany), and maintained frozen in 20% glycerol (Merck, KGaA, Darmstadt, Germany) at -80 °C.

Fermentates of the yeast species reported in Table 1 were used to feed *D. suzukii* adult flies. Yeasts were grown in 1 L PDB at 25 °C, on a rotary shaker at 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 for four days, 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 growth phase. The yeast fermentates were stored at –80 °C

until use for the CAFE assay and for the semi-field experiment, while for the chemical characterization of intra- and extracellular compounds, a quenching step was performed immediately after the 30 h growth.

Preliminary trials were performed using a medium (YMM) with a known composition. The same conditions used for yeasts grown in PDB were applied, though in this case the inoculum (1 mL) was prepared with a loop full of yeast cells cultivated on YMM + Agar (15 g /L). As mentioned above, after 30 h of growth in liquid medium (YMM) the fermentates were stored at –80 °C until use for the CAFE assay, while a quenching step was applied before the extraction of targeted intra- and extracellular compounds.

2.2.1.3 Yeasts cultivated in liquid medium used for lipidomics and for the yeast-based attract-and-kill formulation

A slightly different method was used for the second and third experiment: yeasts were grown in 220 mL PDB in the same conditions reported for the first experiment. Six replicates of the inoculum (0.1 mL) were prepared for each yeast used for the first experiment (Figure 6) and lipidomic of intracellular yeast metabolites was performed. One replicate of the H. u. 2.2 culture and one of *S. vini* were further stored at –80 °C and used for the evaluation of the persistence of a yeast-based attract-and-kill formulation in semi-field conditions.

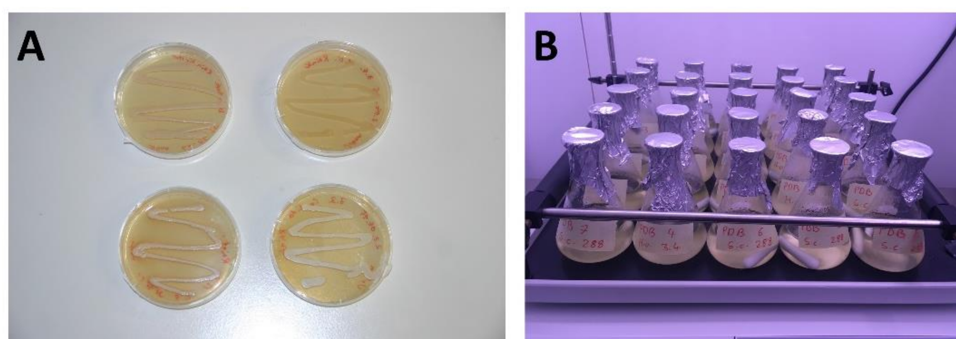

FIGURE 6: Cultivation of yeasts. Yeasts culture on solid medium (PDA) (A); yeast fermentates grown in PDB at 25 °C, on a rotary shaker in Erlenmeyer flasks (B).

2.2.2 Measurement of standard parameters of the yeast cultures

Number of cells per mL, optical density at 600 nm (OD600), pH, cell dry weight (CDW) of yeast pellet and alcohol content were measured after 30 h of growth. The values of the monitored parameters of yeasts grown in PDB are shown in Table 5, while results concerning yeasts grown in YMM used for preliminary trials are summarized in Table S1.

- **Number of cells/mL**

The number of cells was counted using a Fuchs Rosenthal counting chamber consisting of 16 squares of 1 mm^2 each. The cell number on five squares was counted to calculate the mean number of cells per mL.

- **OD600**

After detecting the absorbance at a wavelength of 600 nm of the blank sample (PDB or YMM), 1mL of fermentate was transferred in a cuvette and the OD at 600 nm was measured in triplicate using the Spectrophotometer - Cary 60 UV-Visible (UV–Vis).

- **pH**

The pH value was measured with a pH meter (Crison GLP 21), after calibration with proper buffer solutions (pH 4.01, pH 7.00 and pH 10.01).

- **CDW**

An amount of 30 mL of fermentate was transferred in a 50 mL centrifuge tube and centrifuged at 25 °C at 4000 rpm for 10 min. The supernatant was discarded, and the pellet dried in a drying oven for 24 h at 103 °C. Measurements were performed on five replicates for each yeast.

- **Alcohol content**

The alcohol content of fermentates was determined through distillation. An amount of 100 mL of sample was poured into a distillation flask, placed in a thermostatic bath

at 20 °C and 10 drops of antifoam were added before starting the distillation. After collecting about 90 mL of the distillate, the hydroalcoholic solution was placed in a thermostatic bath at 20 °C and made up to volume with water, transferred into a beaker and the relative density was measured with an electronic densimeter. The table of Reichard was then used to determine the alcohol content (vol %). Data concerning alcohol content were collected by Christof Sanoll (Wine and Beverages Laboratory, Institute for Agricultural Chemistry and Food Quality of the Laimburg Research Centre).

TABLE 5: Standard parameters of the yeast cultures monitored after 30 h growth in PDB.

Experiment	Yeast	Cells/mL	OD600	pH	CDW (mg/mL fermentate)	Alcohol (vol %)
CAFE assay and quantification of targeted intra- and extracellular compounds	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.s. 3.3	1.41×10^8	1.97	4.29	1.71	0.92
Lipidomics of intracellular yeast metabolites	S.c. S288c		1.98 ± 0.03		1.57 ± 0.18	
	H.u. 1.21		1.86 ± 0.02		1.42 ± 0.11	
	H.u. 2.2		1.83 ± 0.05		1.48 ± 0.15	
	H.u. 3.4		1.86 ± 0.04		1.59 ± 0.06	
	I.t. 2.1		1.90 ± 0.04		1.32 ± 0.17	
	M.p. 3.2		2.02 ± 0.05		1.94 ± 0.15	
	S.v. 1.33		1.78 ± 0.10		1.63 ± 0.13	
	C.s. 3.3		2.04 ± 0.07		1.77 ± 0.25	
Persistence of a yeast-based attract-and-kill formulation in semi-field conditions	H.u. 2.2	6.40×10^7	1.80	4.13	1.42	
	S.v. 1.33	n/a*	1.78	3.97	1.68	

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

2.2.3 Insect rearing

Rearing and all entomological assays were performed by the Entomology Group of the Institute for Plant Health of the Laimburg Research Centre in the laboratory under controlled conditions (22 ± 1 °C, 75 ± 3% relative humidity, photoperiod of L16:D8). The mass rearing was refreshed numerous times each year with pupae collected in South Tyrol, Italy, from various fruits in different fields. The larvae were reared on DSCD(a) with dry deactivated yeast and dry baker's yeast 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 used.

2.2.4 Cage design used for oviposition assays

Cages used for the oviposition trials after feeding *D. suzukii* with yeasts grown on solid medium were made of white polystyrene boxes (C.I.B. Srl, Sona, Italy), 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 Figure S1.

2.2.5 Oviposition assays

A number of 10 male and 10 female 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.s. 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 adult flies 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 *D. suzukii*. The three components were changed daily, except during weekends, the mortality of adult flies 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 PDA assay, seven yeasts (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.s. 3.3) were cultivated on PDA. The mortality of adult flies and the number of eggs laid on the three components were measured daily over 30 days. Three replicates of the PDA assay were performed.

2.2.6 Preparation of the attract-and-kill formulation

Three formulations were tested on potted grape plants, namely:

- PDB containing spinosad
- *H. uvarum* fermentate (H.u. 2.2) containing spinosad
- *S. vini* fermentate (S.v. 1.33) containing spinosad

An amount of 11.32 µL/L LaserTM (480 g/L spinosad, Corteva Agriscience™, Wilmington, DE, USA) were added to H.u. 2.2, S.v. 1.33or PDB with a resulting active ingredient of 5.43 mg spinosad per liter.

2.2.7 Grape plants cultivation and treatments in the greenhouse

Rooted grafted vines of the local variety “Edelvernatsch Lb 43” on rootstock SO4 were potted in 4-L pots filled with standard soil (SP ED63 T coarsely, Einheitserde®, Sinntal-Altengronau, Germany). The plants were grown for two months in a greenhouse (Figure 7) and treated one time a week for 20 min against powdery mildew with

vaporized sulfur using a sulfur burner. No sulfur treatments were performed during the experimental period.

FIGURE 7: Potted grape plants grown for two months in the greenhouse.

The temperature and the relative humidity in greenhouse were monitored over the experimental period (Figure 8).

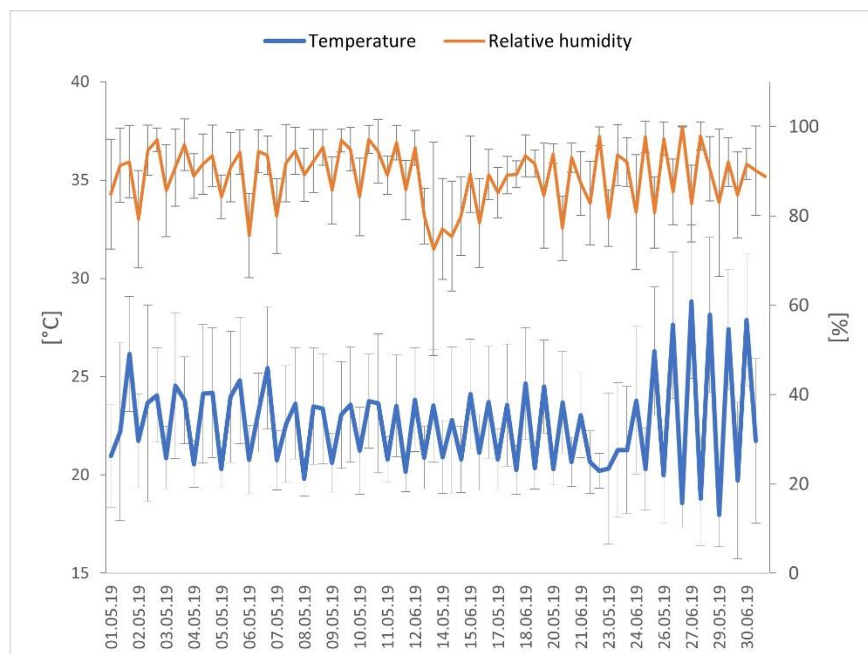

FIGURE 8: Mean daily temperature and relative humidity measured in the greenhouse over the experimental period. The y-scale on the left indicates the temperature values (°C) and the y-scale on the right the relative humidity (%). Standard devitation is reported for both variables.

Four different treatments were performed on grape leaves in greenhouse for further laboratory trials of mortality and oviposition and for the analyses of non-volatile compounds: i) insecticide-free PDB (PDB), ii) insecticide-containing PDB (PDB + S), iii) insecticide-containing *H. uvarum* fermentate (H. u. + S) and iiii) insecticide-containing *S. vini* fermentate (S. v. + S). The three treatments were applied to ten plants at the same time. Ten leaves per plant were treated with 10 drops of 10 µL each using a multichannel pipette. Leaves belonging to five plants were ripped off at timepoint one (T1) - one day after treatment -, while leaves belonging to other five plants were ripped off at timepoint two (T2) - one week after treatment. All treated leaves belonging to each of the five plants per timepoint were ripped off and used for further *D. suzukii* assays and chemical analyses. For mortality and oviposition assays and for the analyses of non-volatile compounds the same plants were used (five leaves for assays and five for chemical analyses). Single plants were considered as replicates.

Since the amount of 10 drops was not sufficient for the detection of VOCs, for volatiles collection a slightly different treatment was performed. Six plants were treated with *H. uvarum* (H. u.) and each plant was considered as a replicate. Five leaves belonging to one plant were treated with 500 µL per leaf using an airbrush (Hansa 681, Harder & Steenbeck, Norderstedt, Germany) to cover the upper surface. The volatile collections were performed one day before treatment (T0 - VOCs), as soon as the formulation dried on the leaves surface (ca. 30 min after treatment) (T1 - VOCs) and five days after treatment (T2 - VOCs). The VOCs emitted by six untreated plants were collected and considered as a control.

2.2.8 Ingestion and mortality assays

Entomological trials were performed by the Entomology Group of the Institute for Plant Health of the Laimburg Research Centre.

2.2.8.1 CAFE assay

The eight yeasts grown for 30 h in PDB or in YMM reported in Table 1 were used to feed *D. suzukii*. The daily ingestion by *D. suzukii* adults of different yeasts fermentates and PDB was measured with a modified CAFE assay (Ja et al., 2007). Females 48 ±

12 h after their emergence from the pupal stage were used in the CAFE assay. 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 fermentate, 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-µL glass capillary tube (Drummond Scientific Company, Broomall, PA, USA). The glass capillaries were held in place with a strip of parafilm wrapped around the capillary at 1 cm height. Every day, yeast fermentates were thawed at room temperature and mixed with a vortex mixer at 1800 rpm for 1 min. Ten microliters of yeast fermentate was offered through the capillary once a day. The daily consumption was measured in mm based on the liquid level and converted into µL. Inside the Eppendorf tube, an agar disk (diameter 8 mm) 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 *D. suzukii* 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 µL per day.

2.2.8.2 Mortality and oviposition assays (treated leaves in the greenhouse)

Leaves treated with the formulations PDB + S, H. u. + S and S. v. + S applied in the greenhouse were used for laboratory trials of mortality and oviposition of *D. suzukii* adult flies. Five leaves belonging to the same plant were cut and refreshed in an Erlenmeyer flask filled with tap water and closed with cotton to avoid the contact of the flies with water. The leaves were placed into the insect cage together with a small Petri dish (diameter 6 cm) with cotton soaked in 10 mL of a 5% sucrose solution and two ripe cherries for oviposition. After 24 h and after 48 h the mortality of males and females was assessed, the total number of eggs laid per cage counted and the cherries replaced. Single cages were used as replicates (n = 5). The insect cages were kept in climatic chambers at 22 ± 1 °C, with 65 ± 5% relative humidity and a

photoperiod of L16:D8. The mortality was calculated as the total mortality over 24 h and 48 h and the oviposition as eggs laid over the first 24 h and between 24 and 48 h. A picture of the cage is reported in Figure 9.

FIGURE 9: Cage used for the laboratory mortality and oviposition trials.

2.2.9 Preparation of standards and stock solutions

2.2.9.1 Quantitative analyses of targeted intra- and extracellular compounds

Stock solutions at a concentration of 1000 mg/L of DL-Phenylalanine-3,3-d2 and L-Alanine-2,3,3,3-d4 were prepared by diluting 10 mg of standard in 10 mL of a solution of MilliQ water and ACN in a ratio 1:1. The other two IS DL-Phenylalanine-3,3-d2 and L-Alanine-2,3,3,3-d4 were prepared by diluting 5 mg of standard in 10 mL of the same MilliQ water and ACN solution. A mixture of these four IS (amino acids IS mix solution) was prepared at a concentration of 10 mg/L in water:ACN (v:v/1:1).The mixture was spiked into each sample for routine quality control and quantification. For the other analytical standards, mother solutions were prepared at a concentration of 10000 mg/L in MilliQ water. Few drops of NaOH 1 M were added to promote the solubilization of some compounds.

2.2.9.2 Lipidomics

- **Preparation of IS mixture in MeOH and MTBE:**

A mixture of 14 lipid standards was prepared in MeOH and a solution of CE 22:1 in MTBE with the exact concentrations shown in Table 6. The mixtures were further sonicated for 5 minutes. Both the IS mixture in MeOH and the CE 22:1 in MTBE were stored in a sealed glass under nitrogen at -30 °C until use for lipid extraction.

TABLE 6: Final concentrations of internal standards in MeOH and MTBE mixtures.

Lipid Standard	Concentration [µg/mL]	Diluted in
PE (17:0/17:0)	1.7	MeOH
PG (17:0/17:0)	6.8	
LPC (17:0)	1.1	
Sphingosine (d17:1)	0.25	
Ceramide (d18:1/17:0)	0.56	
SM (d18:1/17:0)	0.45	
FA (16:0)-d3	56.3	
PC (12:0/13:0)	0.45	
Cholesterol d7	23.4	
TG d5 (17:0/17:1/17:0)	0.28	
DG (12:0/12:0/0:0)	2.3	
DG (18:1/2:0/0:0)	13.5	
MG (17:0/0:0/0:0)	4.5	
LPE (17:1)	0.56	
CE 22:1	75.0	MTBE

2.2.9.3 VOCs

Mother stock solutions of VOCs were prepared by diluting 1 mg of pure chemical standard in 1 mL of dichloromethane. Stock solutions were further diluted 1:1000 prior injection into the GC-MS.

2.2.10 Extraction of targeted intra- and extracellular compounds

For the extraction of intracellular compounds, the method of Boer et al. (Boer, Crutchfield, Bradley, Botstein, & Rabinowitz, 2010) was used, with further modifications. Ten milliliters of yeast fermentate were directly quenched in 20 mL of –80 °C methanol and centrifuged for 5 min at 4000 rpm in a –80 °C prechilled rotor in a centrifuge at –10 °C. After centrifugation, the supernatant was discarded, and 0.2 mL of amino acids IS mix solution (10 mg/L) plus 0.8 mL of –20 °C extraction solvent (ACN:MeOH:water=2:2:1) were added to the pellet. The sample was extracted for 15 min in an ultrasonic cold bath, controlling that the temperature did not exceed 10 °C. The suspension was centrifuged, and the supernatant set aside. The pellet was reextracted under the same conditions adding 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). An aliquot of the extract was transferred to a HPLC vial and directly analyzed for the quantification of amino acids. One part of the extract (0.2 mL) was dried using a speed vac 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.

For the analysis of extracellular metabolites, yeast fermentate and 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 (ACN:MeOH:water=2:2:1) after the addition of amino acids IS mix solution (10 mg/L), and the amount of amino acids was measured. Filtered yeast fermentate was diluted 1 to 10 with water for the analysis of carbohydrates and sugar alcohols. For PDB, after filtration a 1 to 100 dilution was necessary given the high amount of glucose. For organic acids, filtered yeast fermentates and PDB were diluted 1 to 5 with water before analysis.

2.2.11 Leaves sample preparation for analyses of non-volatile compounds

Non-volatile chemical compounds, including yeast metabolites present on the surface of treated leaves were analyzed. Each leaf was washed with 10 mL of MilliQ water, using a multichannel pipette and with the help of a funnel to collect the eluate (Figure 10).

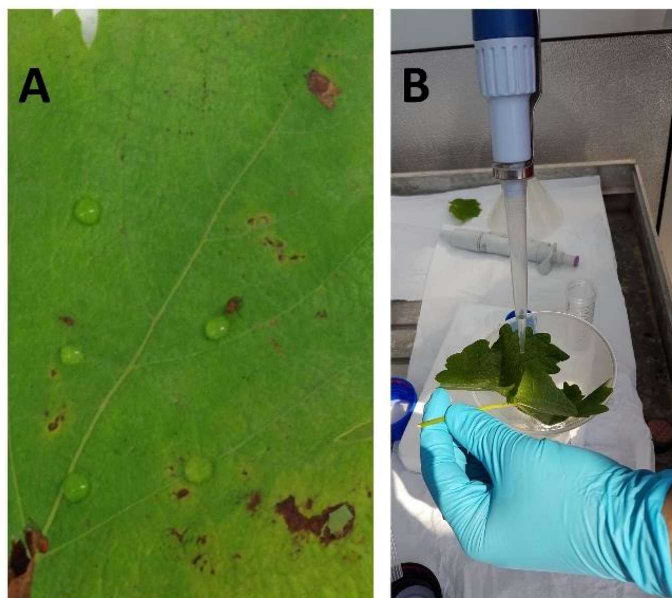

FIGURE 10: Detail of the leaf treatment and washing procedure. Drops of 10 µL volume on the surface of treated leaves (A); washing procedure (B).

The eluate from five leaves belonging to one plant were pooled, filtered (hydrophilic SFCA filters, 0.2 µm) and 1 mL was transferred in a HPLC vial for the analyses of carbohydrates and sugar alcohols. For organic acids, 500 µL of the filtered sample were freeze dried, resuspended in 100 µL of MilliQ water and transferred in a HPLC vial for further analyses. For amino acids, 500 µL of filtered sample were transferred in a HPLC vial containing 480 µL of acetonitrile and 20 µL of IS amino acids mix (50 mg/L) before analysis. The liquid formulations (H. u. + S; S. v. + S; PDB + S; PDB) were analyzed before their application on leaves (T0) after filtration and proper dilution.

2.2.12 Analytical methods for quantitative analyses of targeted compounds

The analytical methods reported below were used for the quantification of the intra- and extracellular amounts of amino acids, carbohydrates, sugar alcohols, and organic acids in fermentates made with eight yeasts and in culture media (PDB and YMM). The same methods were used for the analyses of the above-mentioned compounds eluted from the surface of leaves treated with the attract-and-kill formulations (PDB + S, S. v. + S, and H. u. + S) and with PDB.

Concerning the quantification of targeted intra- and extracellular polar compounds in eight yeasts and two media, three technical replicates were analyzed. Otherwise, for the analyses of the treated leaves with the yeast-based attract-and-kill formulations in semi-field conditions, each plant was considered as a replicate (n=5).

2.2.12.1 Amino acids

For the quantification of amino acids, an UHPLC-QqQ instrument in MRM mode was used. Separation procedures was based on previous studies (Paglia et al., 2012) with further modifications on a hydrophilic interaction chromatography (HILIC) column (Acquity BEH Amide 2.1×150 mm, 1.7 µm with ACQUITY UPLC BEH Amide VanGuard precolumn, 130 Å, 1.7 µm, 2.1 mm x 5 mm) at 45 °C. The low resolution between the peaks of the two isomers leucine and isoleucine did not enable the quantification of these two amino acids as separate compounds. In the results discussed, therefore, the sum of the contents of the two molecules is reported. The HILIC solvents used included: Solvent A (water with 0.1% formic acid) and Solvent B (ACN with 0.1% formic acid). Flow rate was 400 µL/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, amino acids IS mix solution was spiked into each sample. 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.

2.2.12.2 Carbohydrates, sugar alcohols, and organic acids

Samples were analyzed using HPAE-PAD for the quantification of carbohydrates and sugar alcohols and using a conductivity detector for organic acids. Separation of carbohydrates and sugar alcohols was achieved using a Dionex CarboPac PA10 analytical column (4×250 mm) and a Dionex CarboPac PA10 precolumn (4×50 mm) by isocratic elution with a 40 mM sodium hydroxide solution, and the column was regenerated using a 200 mM NaOH solution 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 PAD was used to monitor the eluted carbohydrates and sugar alcohols. Organic acids were analyzed using a Dionex Ion Pac ATC-HC trap (9×75 mm) before a Dionex AG11-HC precolumn (4×50 mm) and a Dionex Ion Pac AS11-HC column (4×250 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 a previous work (Geng, Zhang, Wang, & Zhao, 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.

2.2.13 Extraction of intracellular lipids

For the extraction of intracellular lipids, 10 mL of fermentate were quenched in 20 mL methanol at −80 ◦C and centrifuged at −10 ◦C for 5 min at 4000 rpm using a −80 ◦C prechilled rotor. The supernatant was discarded, and the pellet was freeze-dried. Ten mg of freeze-dried cell pellet was weighted, and intracellular lipids were extracted using the procedure of Showalter et al. (2018). A volume of 225 µL of −20 ◦C methanol containing the IS mixture in MeOH (see Preparation of standards and stock solutions) and 750 µL of −20 ◦C CE 22:1 in MTBE was added to the pellet. Samples were shaken for 6 min at 4 ◦C using a Thermomixer and 188 µL of milliQ water was added. Samples were vortexed, centrifuged, and 350 µL of the upper layer was collected, evaporated to dryness using a SpeedVac vacuum concentrator, and re-

suspended in MeOH:toluene (9:1, v/v) containing 50 ng/mL CUDA. Samples were vortexed, sonicated for 5 min, and centrifuged before analysis. Pooled samples were used as a quality control and extraction performed in blank Eppendorf-tubes were used as blanks.

2.2.13.1 Freeze-drying procedure

The freeze-drying procedure used for the yeast cell pellet was based on Schoug et al. (Schoug, Olsson, Carlfors, Schnürer, & Håkansson, 2006), with further modifications. Samples were introduced into the freeze-dryer after freezing them at -80 °C. Once the freeze-dryer reached -40 °C, the vacuum was lowered to 0.08 mbar. Then, a temperature ramp was applied as shown in Table 7.

TABLE 7: Freeze-drying conditions used for the yeast cell pellet.

begin temp [°C]	End-temp [°C]	Temp-ramp [°C/Min]	hold [h]	Vacuum [mbar]
-40	-10	0.25	30	0.08
-10	10	0.25	0.5	0.08
10	-4	0.50	0.1	0.08
-4	-4	0.00	Until collection	0.08

2.2.14 Analytical methods for lipidomics

Chromatographic and mass spectrometric conditions were based on Showalter et al. (2018). A Waters Acquity UPLC CSH C18 (100 mm length × 2.1 mm id; 1.7 µm particle size) column with a Waters Acquity VanGuard CSH C18 pre-column (5 mm × 2.1 mm id; 1.7 µm particle size) maintained at 65 ◦C was used for RPLC-QTOFMS analysis. In positive ion mode, solvent A was 60:40 v/v ACN:water with 10 mM ammonium formate and 0.1% formic acid and solvent B was 90:10 v/v isopropanol:ACN with 10 mM ammonium formate and 0.1% formic acid. In negative ion mode, solvent A was 60:40 v/v ACN:water with 10 mM ammonium acetate and solvent B was 90:10 v/v isopropanol:ACN with 10 mM ammonium acetate. The flow rate was set at 0.6 mL/min, with a 15 min gradient as reported in the paper. The instrument Impact HD

QTOF equipped with an Ultimate 3000 UHPLC was used for LC-MS analysis. Five µL of the re-suspended sample was injected in ESI positive ion mode, while the injection volume in negative ion mode was 10 µL. The mass spectrometric conditions were as follows: m/z range, 60–1700; capillary voltage, 3500 V; nebulizer gas (nitrogen), 2.4 bar; dry gas (nitrogen), 8 L/min in positive ion mode and 12 L/min in negative ion mode; dry temperature, 325 ◦C in positive ion mode and 200 ◦C in negative ion mode. For MS/MS, the collision energy was set at 20 eV in positive and in negative ion mode, and the spectra rate was 13 Hz with 4 precursor ions per cycle. Sodium formate was used as a calibrant for maintaining mass accuracy.

2.2.15 Analyses of VOCs in the greenhouse

The collection and analyses of VOCs were performed by the Faculty of Science and Technology of the Free University of Bozen-Bolzano.
The VOCs were collected via closed-loop-stripping analysis (CLSA) (Figure 11) and analyzed in gas chromatography–mass spectrometry.

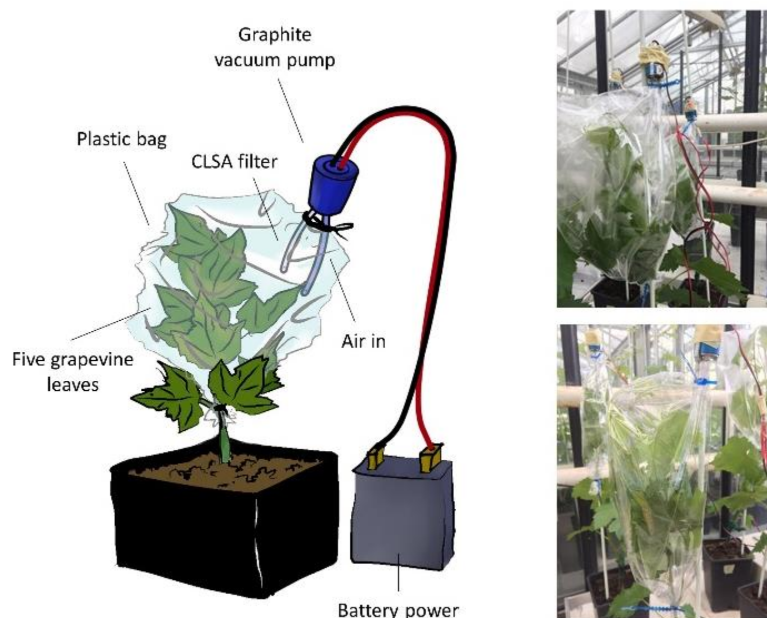

FIGURE 11: Scheme of the VOCs collection via CLSA.

To reduce variation in chemical profiles due to the plant circadian rhythm all collections were performed at a regular time between 12 pm and 3 pm. The treated

shoots were not covered during the five days between the first and the second collection. Untreated leaves were considered as a control. Plant materials were held in a VOC-bag (Cuki® oven bag, Cofresco). Charcoal filtered air was pushed in via an inlet port at a rate of 500 mL/min while air was sucked out via an outlet port at a rate of 400 mL/min, creating a positive pressure in the bag for three hours. The airflow was maintained using a 12 V graphite vacuum pump (Fürgut, Tannheim, Germany) using Teflon tubes and ferrule connections. The outlet air passed through an adsorbent trap (glass tube, 6.5 × 0.55 × 0.26 cm) loaded with 1.5 mg activated charcoal (CLSA filter LR-type; Brechbühler AG). The VOCs were eluted from the adsorbent traps with 100 µL GC-grade dichloromethane and stored at -80 °C. Adsorbents were cleaned after each collection using three rinses with approximately 50 µL of HPLC-grade heptane, HPLC-grade methanol then GC-grade dichloromethane and baked 10 min at 160 °C. Two µL of extract were injected on a non-polar HP-5MS column (30 m × 0.25 mm ID, 0.25 µm film thickness, 7890A, Agilent Technologies) in splitless mode when the inlet valve was at 280 °C. Helium was used as carrier gas at a flow rate of 1.2 mL/min and a velocity of 39.92 cm/s. The starting temperature of 50 °C was held for 1.5 min, followed by an increase of 7.5 °C/min until a temperature of 250 °C was reached and then held for 10 min.

VOCs emitted by the yeast fermentate was also collected via CLSA before their application on leaves (T0 - VOCs). A double airflow pump system was used: charcoal-filtered air was pushed at rate of 1 L/min into a 250-mL Pyrex glass bottle containing 100 mL of yeast sample; simultaneously, CLSA filters (1.5 mg activated charcoal, LR-type, Brechbühler AG, Schlieren, Switzerland) fitted into the plastic lid of the glass bottle were connected to the outflow pump using a short Teflon tube, drawing out air at a rate of 0.4 L/min. The CLSA filters were then eluted with 100 µL of GC-grade dichloromethane solvent in 1.1-mL GC glass vials (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and stored in a freezer at -80 °C until use for subsequent GC-MS analysis.

The chromatogram was recorded in the full scan mode m/z 20–400 amu, the electron ionization was set at 70 eV and the ion source temperature at 250 °C. Data acquisition and analysis were carried out using ChemStation software. A commercially available mixture of n-alkane standards (nC8-nC40, Sigma-Aldrich, Merck KGaA, Darmstadt,

Germany) was used to calculate the linear retention indices (LRI) (van den Dool & Dec. Kratz, 1963). Compounds were annotated initially by comparing their mass spectra with those in the databases NIST 14 (Gaithersburg, MD, USA) and Wiley7 (Wiley, Hoboken, NJ, USA). The identity of all compounds, with the exception of 1,8-cineole and trans-alpha-bergamotene, was confirmed by comparison with reference standards.

2.2.16 Data processing and statistics

For the oviposition assays, to evaluate whether the different yeasts or the yeast-free culture media influenced the oviposition, a linear mixed effect analysis was applied (Winter, 2013). Yeasts or medium and day (without interaction term) were included in the model as fixed effects. The replicates were considered as random effects. The oviposition data were included in 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. Survival curves were evaluated using the Kaplan–Meier method followed by a log rank test. P values were adjusted using FDR methodology.

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 comparisons. Survival was evaluated using the Kaplan–Meier method followed by a log rank test. P values were adjusted using FDR methodology.

For the identification of lipids full-scan and tandem mass spectrometry (MS/MS) analyses were performed. MS-DIAL was used for deconvolution, peak picking, alignment, and annotation (Tsugawa et al., 2015). LipidBlast was used as the library for compound identification with an identification score cut-off of 85% and a retention time tolerance of 0.1 min. The level of identification of lipids was two for all compounds based on Sumner et al. (2007). The nomenclature used was based on Züllig et al. (Züllig, Trötzmüller, & Köfeler, 2020): the bond type level was reported in the case of annotation based on a high-resolution full-scan (MS1), while double-bond positions were indicated in case of MS/MS-based annotation. Peak heights were submitted to Metaboanalyst. Values were normalized using class-based IS and

further by log transformation before statistical testing. To assess the quality of the data, pooled quality control samples (QCs) were distributed evenly in the analytical batches, relative standard deviation (RSD%) among the QCs of each IS was calculated and the clustering of QCs samples was visually inspected through principal component analysis (PCA). Pareto scaling was performed for PCA and partial least squared discriminant analysis (PLS-DA). An analysis of variance (ANOVA) with Tuckey honestly significant difference (HSD) post-hoc testing, Student t-tests, and pairwise multivariate analysis of variance (MANOVA) were done in SPSS. To evaluate significantly impacted lipid clusters between S.c. S288c and H.u. 3.4, a chemical similarity enrichment analysis (ChemRICH) was performed using the Kolmogorov–Smirnov-test for statistical analysis (Barupal & Fiehn, 2017). Lipidomics data have been deposited into the EMBL-EBI MetaboLights database (Haug et al., 2020) with the identifier MTBLS1955. The complete dataset can be accessed here: https://www.ebi.ac.uk/metabolights/MTBLS1955/descriptors.

For the greenhouse data, the mortality and oviposition data were analyzed performing a one-way ANOVA to highlight differences between treatments. The equality of error variance was verified with a Levene's test. Multiple comparisons were performed with Bonferroni's procedure. To evaluate the variation of the concentration of metabolites over time, a one-way ANOVA was performed using Tukey's post hoc test for pairwise comparison. Nonparametric tests (Wilcoxon statistic with Bonferroni's correction to adjust the significance level) were performed whenever at least one of the conditions to apply parametrical tests (normal distribution, variance homogeneity) was not satisfied. To evaluate significant differences between VOCs emitted by treated and non-treated leaves per each timepoint, peak areas were compared using one-way ANOVA followed by post hoc Tukey's test. All statistical analyses were performed using the software R or SPSS.

3 Results

3.1 Oviposition assays on solid media

In the MEA assay, flies were fed with three selected yeasts (M.p. 3.2, C.s. 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 *D. suzukii* adults (Figure S2). The different diets had a significant effect on the number of eggs laid over the test period of 50 days ($\chi^2_{(3)}$=963.19, p < 0.0001). Significantly fewer eggs were laid by females fed with MEA compared to all yeast cultures grown on MEA. Additionally, significant differences among the three yeast species were found, with H.u. 3.4 leading to the highest oviposition. The different diets had no effect on the survivorship of males ($\chi^2_{(3)}$=7.50, p=0.058) but influenced the survivorship of females ($\chi^2_{(3)}$=9,41, p=0.024). No significant differences were found among diets after adjusting for multiple comparisons (p<0.05).

In the YGM assay, two yeast growth media, MEA and PDA, were compared to evaluate their influence on oviposition and mortality of *D. suzukii* adults. Yeast growth medium was found to affect the fecundity of females: PDA led to significantly higher fecundity compared with MEA ($\chi^2_{(1)}$=45.04, p<0.0001) (Figure S3). The different culture media had no significant influence on the survivorship of males ($\chi^2_{(1)}$=2.20, p = 0.138) or females ($\chi^2_{(1)}$=1.147, p=0.284).

After verifying the positive effect of PDA on the fecundity in comparison to MEA, the number of yeasts to include in the PDA trial was widened: seven different yeasts (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.s. 3.3) and yeast-free PDA were tested over a period of 30 days. Significant differences concerning the egg laying curves were found after feeding flies with different diets ($\chi^2_{(7)}$=123.33, p < 0.0001) (Figure S4). The diets had no significant influences on the survival of males ($\chi^2_{(7)}$=4.16, p=0.760), but had a significant influence on the survival of females ($\chi^2_{(7)}$=16.59, p=0.020). More females survived after feeding with H.u. 1.21 compared to those fed with I.t. 2.1 (p=0.041). Data were acquired and elaborated by the Entomology Group of the Institute for Plant Health of the Laimburg Research Centre.

3.2 CAFE and mortality assays

To evaluate the acceptance of the different yeast-based diets by *D. suzukii* adult flies, the CAFE assay was performed by measuring the daily ingestion by *D. suzukii* females of fermentates of the yeast species included in the study and of liquid media (PDB or YMM) over a four-day period.

Considering yeasts grown in PDB and the medium itself, the ingested amount tended to increase over the test period. Except for day 1 ($\chi 2$ (8) = 13.18, p = 0.106), significant differences in the daily ingestion of the different yeast fermentates were observed: day 2 ($\chi 2$ (8) = 47.07, p < 0.001); day 3 ($\chi 2$ (8) = 19.47, p = 0.014; day 4 ($\chi 2$ (8) = 23.28, p = 0.003). The total ingestion over four days was calculated considering the females which survived the whole test period (n = 6 to 20). Significant differences were observed concerning the total ingestion over the experimental period ($\chi 2$ (8) = 34.98, p < 0.001). The diets also affected the survival of flies over four days ($\chi 2$ (8) = 78.967, p < 0.001). The three *H. uvarum* strains, S. v. 1.33, and PDB were associated to the highest ingestion rate coupled with the lowest mortality; while the mortality rate of *D. suzukii* females fed with C.s. 3.3 or S.c. S288c over the whole test period was 70%. Mean values and standard deviations (SD) of the daily ingested amounts and statistical differences are reported in Table 8 together with mortality rate after feeding with each yeast.

TABLE 8: Daily mortality and daily ingestion (mean ± SD) per female (n = 20) fed with yeast fermentates and PDB.

	Day 1		Day 2		Day 3		Day 4	
Yeast	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.s. 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

Besides the yeasts grown in PDB, the CAFE assay was performed also for yeasts grown in the same conditions in YMM. Results are summarized in Table S2. Data show that, starting from day 2, the ingested amounts were on average much lower than those reported for yeasts grown in PDB, indicating that the lack of some nutritional compounds in the growth medium was probably responsible for the lower feeding by the flies. Concerning mortality, a similar trend was observed as that found after feeding flies with yeasts cultivated in PDB, with maximum mortality rate for flies fed with S.c. S288c (100 %), followed by C.s. 3.3 (95 %), over the four-days period. In addition, higher mortality rates were observed after feeding flies with the other yeasts cultivated in YMM and the medium itself compared with those cultivated in PDB (Table S2).

Though the use of a chemically defined medium gives the advantage to evaluate the effects of single compounds on the nutritional behavior and mortality of *D. suzukii*, the higher cost together with the lower efficacy of YMM as feeding stimulant for *D. suzukii* compared to an undefined media such as PDB represent a disadvantage. The choice of a chemically undefined growth medium implies a more extensive chemical characterization.

Data were acquired and elaborated by the Entomology Group of the Institute for Plant Health of the Laimburg Research Centre.

3.3 Comparison of intra- and extracellular concentrations of targeted compounds

The intra- and extracellular concentrations of targeted compounds including carbohydrates, sugar alcohols, organic acids, and amino acids in the eight yeast fermentates cultivated in PDB or YMM and in the two media were assessed. Overall, 36 intracellular and 34 extracellular compounds were quantified.

In most of the cases, extracellular compounds made the largest contribution to the total amount of nutrients, as they were present in much higher concentrations than intracellular compounds. The culture medium (PDB) was found to contain the highest amounts of amino acids and carbohydrates.

Average amounts of intra- and extracellular compounds in yeasts grown in PDB and medium alone (PDB) are summarized in Table 9 and Table 10, respectively. Differences between samples were visualized through an heatmap (Figure 12). Figure 13 shows the average extracellular amount of total carbohydrates, organic acids, and sugar alcohols in PDB and fermentates.

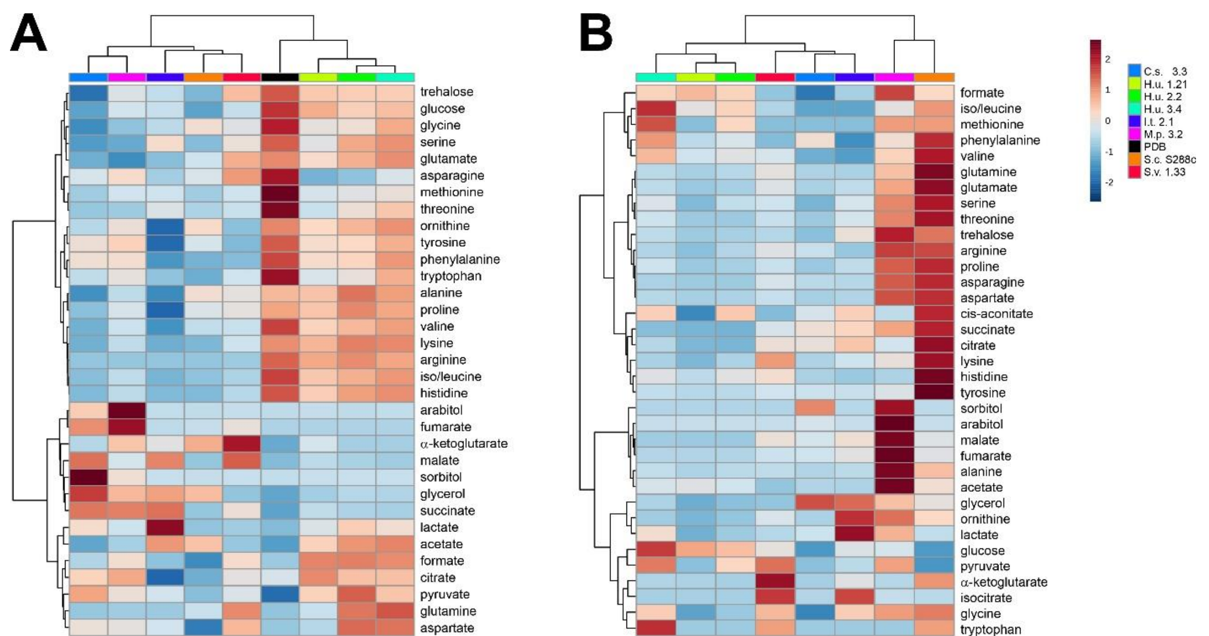

FIGURE 12: Heatmaps of extracellular (A) and intracellular (B) compounds detected in the eight selected yeasts and in PDB. Concentrations of single compounds are displayed using a color scale indicating differences concerning relative metabolite quantities between yeasts, ranging from red (higher amounts), to white (intermediate values), to blue (lower amounts). Both rows and columns are clustered using Euclidean distances and a Ward clustering algorithm. Mean values (n = 3) for each sample are shown.

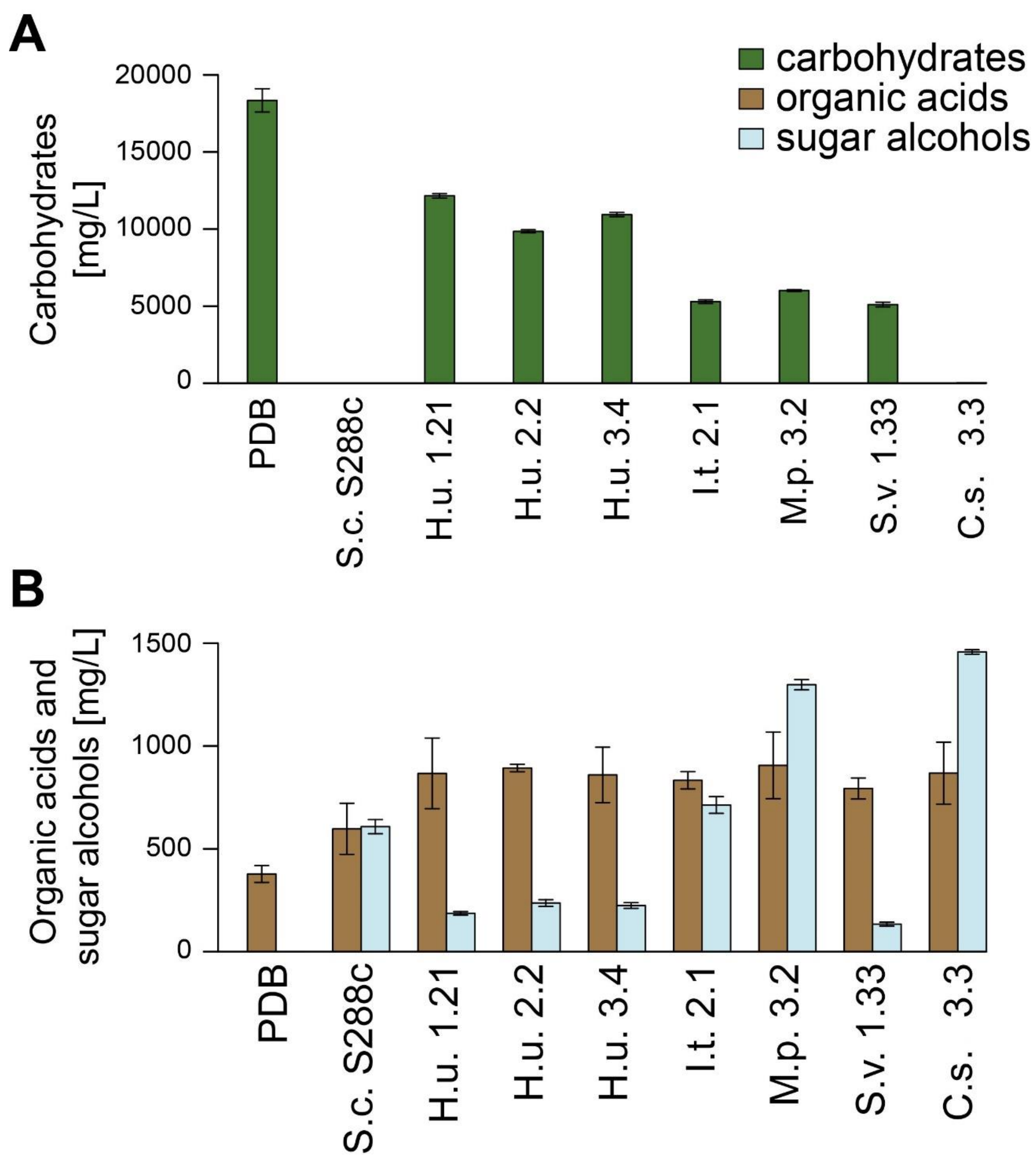

FIGURE 13: Extracellular amounts of targeted compounds in the selected yeasts and PDB. Total carbohydrates (A), organic acids and sugar alcohols (B). Values (mean ± SD) are reported as mg/L of fermentate or culture medium.

TABLE 9: Mean (n=3) concentration of intracellular compounds in yeasts cultivated in PDB (mg/L ± SD). The first column indicates the compound class. Abbreviations: n.d. = not detected.

Compound class	Compound	C.s. 3.3	H.u. 1.21	H.u. 2.2	H.u. 3.4	I.t. 2.1	M.p. 3.2	S.c. S288c	S.v. 1.33
amino acids	leucine/isoleucine	n.d.	0.0658 ± 0.0097	0.0854 ± 0.0083	0.1557 ± 0.0117	n.d.	0.0649 ± 0.0078	0.1145 ± 0.0046	0.0384 ± 0.0060
	alanine	0.1381 ± 0.0111	0.1340 ± 0.0152	0.1995 ± 0.0114	0.2667 ± 0.0176	0.2253 ± 0.0341	2.3971 ± 0.2318	1.0113 ± 0.0360	0.2535 ± 0.0203
	arginine	0.7680 ± 0.0516	0.3507 ± 0.0538	0.6266 ± 0.0412	0.5960 ± 0.054	0.4278 ± 0.1421	2.3676 ± 0.1891	2.3113 ± 0.4701	0.9680 ± 0.0850
	asparagine	0.0571 ± 0.0023	0.0381 ± 0.0002	0.0405 ± 0.0003	0.0499 ± 0.0006	0.0566 ± 0.0026	0.2143 ± 0.0153	0.2517 ± 0.0184	0.0827 ± 0.0047
	glutamine	0.0245 ± 0.0050	0.0066 ± 0.0011	0.0337 ± 0.0107	0.0432 ± 0.0028	0.0134 ± 0.0015	0.2462 ± 0.0236	0.5973 ± 0.0253	0.1327 ± 0.0321
	glycine	0.0831 ± 0.0079	0.1031 ± 0.0158	0.1243 ± 0.0129	0.1827 ± 0.0207	0.1831 ± 0.0372	0.2102 ± 0.0078	0.2198 ± 0.0084	0.1959 ± 0.0183
	histidine	0.2819 ± 0.0253	0.3342 ± 0.0687	0.4541 ± 0.0556	0.4411 ± 0.0735	0.2407 ± 0.0830	0.2995 ± 0.0309	1.2188 ± 0.2598	0.5295 ± 0.0655
	lysine	0.7266 ± 0.0670	0.3725 ± 0.0385	0.7339 ± 0.0162	0.6945 ± 0.0543	1.0037 ± 0.3388	1.2767 ± 0.1103	3.1881 ± 0.6038	2.1023 ± 0.2755
	methionine	0.0115 ± 0.0006	0.0126 ± 0.0002	0.0153 ± 0.0006	0.0236 ± 0.0016	0.0113 ± 0.0001	0.0191 ± 0.0010	0.0177 ± 0.0008	0.0118 ± 0.0004
	ornithine	0.0857 ± 0.0230	0.0483 ± 0.0029	0.0705 ± 0.0054	0.0783 ± 0.0041	0.3023 ± 0.1089	0.2567 ± 0.0245	0.1659 ± 0.0355	0.1230 ± 0.0364
	phenylalanine	0.0852 ± 0.0053	0.0529 ± 0.0048	0.0639 ± 0.0039	0.1245 ± 0.0088	n.d.	0.0822 ± 0.0099	0.1650 ± 0.0050	0.0335 ± 0.0016
	proline	0.0553 ± 0.0053	0.0516 ± 0.0124	0.0746 ± 0.0116	0.1226 ± 0.0062	0.1040 ± 0.0203	0.3837 ± 0.0335	0.4489 ± 0.0364	0.1157 ± 0.0149
	serine	0.0529 ± 0.0023	0.0560 ± 0.0039	0.0873 ± 0.0099	0.1032 ± 0.0024	0.1017 ± 0.0122	0.1784 ± 0.0092	0.2337 ± 0.0201	0.0994 ± 0.0052
	threonine	n.d.	n.d.	n.d.	0.0477 ± 0.0111	0.0594 ± 0.0214	0.1916 ± 0.0115	0.2833 ± 0.0104	0.0440 ± 0.0036
	tryptophan	n.d.	n.d.	n.d.	0.0246 ± 0.0043	n.d.	n.d.	0.0147 ± 0.0018	0.0165 ± 0.0031
	tyrosine	0.0525 ± 0.0034	0.0198 ± 0.0017	0.0230 ± 0.0033	0.0411 ± 0.0022	0.0469 ± 0.0071	0.0892 ± 0.0092	0.6207 ± 0.0265	0.0654 ± 0.0124
	valine	0.0183 ± 0.0009	0.0528 ± 0.0064	0.0678 ± 0.0056	0.1019 ± 0.0089	0.0063 ± 0.0012	0.0864 ± 0.0061	0.1649 ± 0.0038	0.0583 ± 0.0074
	aspartate	0.0905 ± 0.0010	0.0912 ± 0.0002	0.0946 ± 0.0058	0.1105 ± 0.0054	0.1027 ± 0.0074	0.3463 ± 0.0247	0.3808 ± 0.0188	0.1268 ± 0.0068
	glutamate	0.6875 ± 0.0699	0.4273 ± 0.0490	0.6500 ± 0.0477	1.0241 ± 0.02260	1.0843 ± 0.1398	3.5000 ± 0.2772	6.2366 ± 0.4563	1.6124 ± 0.2313
carbohydrates	trehalose	0.2139 ± 0.0230	1.8257 ± 0.1858	2.6117 ± 0.2374	4.6626 ± 0.1493	9.5215 ± 0.6653	28.6308 ± 3.7242	21.4927 ± 0.7573	3.8176 ± 0.3751
	glucose	0.3982 ± 0.0358	49.1578 ± 5.0886	43.0967 ± 3.6118	68.1497 ± 1.9933	27.1483 ± 3.3711	25.1602 ± 4.9079	0.6354 ± 0.0825	30.3727 ± 4.1546
sugar alcohols	arabitol	1.5096 ± 0.0891	0.0452 ± 0.0061	n.d.	0.0313 ± 0.0034	n.d.	23.6626 ± 3.0141	n.d.	n.d.
	glycerol	4.1882 ± 0.4306	0.5900 ± 0.0733	0.8984 ± 0.0922	1.3081 ± 0.0196	3.9049 ± 0.5227	2.8401 ± 0.4854	2.0838 ± 0.3196	0.9649 ± 0.2145
	sorbitol	2.3356 ± 0.1631	0.0150 ± 0.0018	0.0332 ± 0.0050	0.0391 ± 0.0004	0.1130 ± 0.0134	3.7914 ± 0.4602	0.1680 ± 0.0015	0.0334 ± 0.0020
organic acids	lactate	0.1339 ± 0.0124	0.0818 ± 0.0064	0.1050 ± 0.0405	0.1667 ± 0.0184	0.3147 ± 0.0111	0.2070 ± 0.0302	0.1283 ± 0.0248	0.1179 ± 0.0273
	acetate	0.1323 ± 0.0143	0.2124 ± 0.0527	0.1849 ± 0.0289	0.1985 ± 0.0399	0.1307 ± 0.0545	0.6455 ± 0.0610	0.2525 ± 0.1142	0.0914 ± 0.0086
	formate	0.0651 ± 0.0257	0.2056 ± 0.0403	0.1852 ± 0.0264	0.1858 ± 0.0226	0.1297 ± 0.0006	0.2612 ± 0.0464	0.1838 ± 0.0264	0.1246 ± 0.0263
	pyruvate	0.1617 ± 0.0064	0.1640 ± 0.0364	0.2647 ± 0.0272	0.3396 ± 0.0201	0.1964 ± 0.0124	0.3099 ± 0.0439	0.1247 ± 0.0405	0.3428 ± 0.0627
	succinate	0.8709 ± 0.0868	0.1698 ± 0.0298	0.1480 ± 0.0646	0.2419 ± 0.0203	1.0034 ± 0.0427	1.3193 ± 0.0733	1.9821 ± 0.0845	0.6666 ± 0.1141
	malate	0.3251 ± 0.0335	n.d.	n.d.	0.0183 ± 0.0014	0.5343 ± 0.0129	1.9778 ± 0.1049	0.3974 ± 0.0498	0.4965 ± 0.015
	α-ketoglutarate	0.0315 ± 0.0031	0.0378 ± 0.0004	0.0326 ± 0.0064	0.0481 ± 0.0036	0.1173 ± 0.0043	0.0537 ± 0.0035	0.2817 ± 0.0471	0.4720 ± 0.0687
	fumarate	0.0583 ± 0.0114	n.d.	n.d.	0.0114 ± 0.0020	0.1008 ± 0.0138	0.5896 ± 0.0373	0.0931 ± 0.0273	0.0496 ± 0.0093
	citrate	3.3222 ± 0.2697	1.4388 ± 0.1561	1.4007 ± 0.1598	2.3134 ± 0.0536	4.2895 ± 0.3730	2.8628 ± 0.2895	7.4893 ± 0.3883	3.441 ± 0.1765
	isocitrate	0.0463 ± 0.0099	n.d.	n.d.	n.d.	0.8463 ± 0.2183	0.1049 ± 0.0167	0.0634 ± 0.0192	0.8877 ± 0.1791
	cis-aconitate	0.2082 ± 0.0363	0.1115 ± 0.0193	0.2622 ± 0.0267	0.2600 ± 0.0316	0.2563 ± 0.0029	0.1920 ± 0.0180	0.3661 ± 0.0175	0.1522 ± 0.0345

TABLE 10: Mean (n=3) concentration of extracellular compounds in PDB and in yeasts cultivated in PDB (mg/L ± SD). The first column indicates the compound class. Abbreviations: n.d. = not detected.

Compound class	Compound	C.s. 3.3	H.u. 1.21	H.u. 2.2	H.u. 3.4	I.t. 2.1	M.p. 3.2	S.c. S288c	S.v. 1.33	PDB
amino acids	leucine/isoleucine	3.5633 ± 0.1305	46.6433 ± 2.7412	53.5233 ± 1.5431	60.6467 ± 0.4623	0.2500 ± 0.0200	18.6133 ± 0.8980	5.0167 ± 0.8822	13.8100 ± 0.3551	81.6300 ± 0.1992
	alanine	16.4800 ± 0.5981	66.7033 ± 3.4279	85.0167 ± 1.4778	75.7933 ± 1.0627	14.7433 ± 0.1815	41.7467 ± 0.8846	56.7267 ± 10.2393	52.2633 ± 0.6439	69.2733 ± 0.1193
	arginine	n.d.	24.3700 ± 0.6264	28.3067 ± 2.3612	24.9500 ± 1.9107	n.d.	0.3933 ± 0.0737	n.d.	1.5800 ± 0.0755	33.3633 ± 0.1193
	asparagine	5.8567 ± 0.4474	0.7100 ± 0.2982	1.8200 ± 0.2600	6.1200 ± 0.9528	2.9600 ± 0.0529	8.6933 ± 0.4043	6.3167 ± 1.2436	13.1867 ± 0.3667	20.4300 ± 0.8150
	glutamine	0.4433 ± 0.0666	2.3067 ± 0.0814	9.1167 ± 0.5856	10.2233 ± 1.8760	0.6600 ± 0.0436	0.7933 ± 0.0971	2.3533 ± 0.3313	8.3867 ± 0.2574	0.2433 ± 0.0153
	glycine	9.6433 ± 0.2532	26.7933 ± 3.8613	28.1933 ± 5.4881	35.9967 ± 3.1766	21.3133 ± 0.6586	16.66 ± 1.2742	28.4267 ± 0.4007	25.5200 ± 1.0658	49.7533 ± 1.3303
	histidine	n.d.	6.3667 ± 1.0970	8.4767 ± 1.1906	9.1233 ± 0.8864	n.d.	2.3333 ± 0.1626	n.d.	2.1200 ± 0.2800	11.2867 ± 0.1680
	lysine	0.7667 ± 0.1856	35.5100 ± 2.0814	45.2633 ± 1.7067	43.5267 ± 0.9872	n.d.	13.3100 ± 0.6745	1.9833 ± 0.0874	15.6567 ± 0.4466	42.7300 ± 0.2946
	methionine	n.d.	3.5300 ± 0.0800	4.3667 ± 0.2754	5.1700 ± 0.5719	3.0333 ± 0.0058	3.1367 ± 0.0208	n.d.	3.0267 ± 0.0058	22.8333 ± 0.8208
	ornithine	2.4833 ± 0.0681	3.7067 ± 0.1250	4.3100 ± 0.2498	4.8333 ± 0.0709	0.2300 ± 0.0100	3.3733 ± 0.3723	3.6167 ± 0.6134	1.8733 ± 0.0058	4.9700 ± 0.0361
	phenylalanine	24.3700 ± 0.4084	26.0633 ± 1.7654	27.8033 ± 0.2173	37.9800 ± 0.6560	0.1167 ± 0.0153	25.0633 ± 0.7404	5.2800 ± 1.0341	7.2533 ± 0.0513	48.9367 ± 0.5090
	proline	14.7467 ± 1.4880	28.3667 ± 2.0600	33.9767 ± 1.5799	31.3467 ± 3.8974	4.3833 ± 0.2558	21.31 ± 0.6478	21.7133 ± 3.6791	23.1700 ± 0.4850	31.7133 ± 1.8163
	serine	5.3200 ± 0.2972	17.3200 ± 0.9954	25.9800 ± 1.5853	28.1667 ± 3.6965	20.1767 ± 0.0757	6.7533 ± 0.3201	9.0567 ± 1.3203	18.7300 ± 0.5469	32.3200 ± 1.7655
	threonine	4.6933 ± 0.3083	11.6433 ± 1.8510	18.5733 ± 2.4914	25.1233 ± 1.6636	14.9767 ± 0.6506	5.7333 ± 0.3355	7.9133 ± 0.0702	11.5200 ± 0.7600	54.9367 ± 0.7489
	tryptophan	6.6133 ± 0.0751	7.7100 ± 0.5682	8.7167 ± 0.2774	12.2733 ± 0.8605	4.3867 ± 0.1097	8.5667 ± 0.2380	3.2267 ± 0.4737	7.2500 ± 0.3487	19.1933 ± 0.7051
	tyrosine	8.5900 ± 0.3208	9.0000 ± 0.6359	9.4000 ± 0.1825	11.1933 ± 1.3600	0.2800 ± 0.0200	9.7400 ± 0.5881	7.2167 ± 1.1104	4.2267 ± 0.3707	14.2100 ± 0.8229
	valine	4.1300 ± 0.1562	19.2433 ± 1.3248	22.4067 ± 0.5727	24.9867 ± 0.2454	0.4600 ± 0.0100	11.9167 ± 0.4528	11.0533 ± 2.0742	11.6567 ± 0.5835	32.9767 ± 0.7270
	aspartate	39.1200 ± 6.0014	31.0667 ± 5.0935	57.0367 ± 7.2433	56.0000 ± 8.6227	34.5367 ± 2.1946	37.9867 ± 8.9214	14.7400 ± 2.3390	47.6033 ± 1.3439	27.2867 ± 1.8329
	glutamate	111.5300 ± 2.6738	246.2033 ± 16.5216	297.4833 ± 15.4931	327.8967 ± 34.9017	129.6200 ± 6.5018	71.8067 ± 9.8043	191.7900 ± 37.0893	299.1133 ± 13.2533	332.5533 ± 17.9729
carbohydrates	trehalose	60.6803 ± 0.6565	98.7060 ± 1.4198	97.7203 ± 1.8767	97.6617 ± 2.3232	84.1860 ± 1.8882	88.2823 ± 0.7345	74.6680 ± 4.0534	100.7430 ± 3.6667	115.2000 ± 1.9058
	glucose	29.9790 ± 5.0226	12155.2800 ± 142.6690	9851.3657 ± 100.2664	10942.6907 ± 136.7896	5293.7787 ± 119.8561	6013.4423 ± 57.9379	12.4383 ± 1.3007	5108.0020 ± 149.9309	18345.0867 ± 757.065
sugar alcohols	arabitol	181.9763 ± 2.9928	10.3070 ± 0.0767	2.7673 ± 0.1005	4.9860 ± 0.0901	5.0977 ± 0.3900	618.0170 ± 4.8302	0.5543 ± 0.0384	11.0220 ± 0.3208	n.d.
	glycerol	977.4053 ± 15.3745	172.0347 ± 8.7023	228.7917 ± 16.6127	214.6057 ± 13.5399	704.0297 ± 40.2218	627.9663 ± 20.0297	604.5747 ± 34.3595	118.5923 ± 8.9651	n.d.
	sorbitol	298.0077 ± 3.0182	3.9583 ± 0.1238	4.6797 ± 0.0225	4.7410 ± 0.0733	4.2917 ± 0.1905	52.4883 ± 0.6394	2.9913 ± 0.2135	3.9733 ± 0.0807	n.d.
organic acids	lactate	29.4668 ± 4.5288	23.2312 ± 1.4746	33.7555 ± 3.7586	28.2907 ± 1.04700	68.0967 ± 6.4021	20.1942 ± 1.4938	10.3603 ± 1.1543	12.7693 ± 0.9217	8.2807 ± 1.2571
	acetate	18.6437 ± 2.6962	162.5765 ± 35.8497	218.9067 ± 7.8895	232.8330 ± 35.7900	217.0375 ± 18.4078	68.3542 ± 11.2463	178.5072 ± 33.7477	55.5647 ± 2.0899	8.4258 ± 1.6489
	formate	5.6022 ± 1.2805	11.1620 ± 3.1256	11.2643 ± 0.8372	10.9890 ± 1.6854	4.5608 ± 0.7896	7.9723 ± 2.7948	2.5680 ± 0.9419	8.2042 ± 0.5568	4.9780 ± 0.6194
	pyruvate	110.9747 ± 15.8522	91.0140 ± 20.1080	133.7740 ± 17.1413	96.9668 ± 14.1874	66.4042 ± 7.8807	79.0720 ± 20.2150	39.7067 ± 6.0231	69.7465 ± 3.7543	n.d.
	succinate	204.2367 ± 35.9040	77.2942 ± 15.0643	63.7690 ± 2.3458	59.6200 ± 12.0879	208.2798 ± 4.4573	199.0438 ± 112.8661	47.2017 ± 11.2726	115.1632 ± 11.0549	14.992 ± 1.6281
	malate	73.9590 ± 14.7883	17.5868 ± 4.1009	11.9617 ± 0.9828	9.6140 ± 3.1075	68.7633 ± 1.6292	25.5755 ± 4.8228	6.2248 ± 1.6940	79.4802 ± 2.1022	1.9830 ± 0.3221
	α-ketoglutarate	18.5113 ± 1.8576	27.3422 ± 6.1390	14.2277 ± 0.4739	14.3812 ± 3.6862	32.1228 ± 0.8242	50.0857 ± 6.5774	56.9713 ± 15.4894	96.3307 ± 3.3204	n.d.
	fumarate	13.2502 ± 2.3609	n.d.	n.d.	n.d.	1.2917 ± 0.1465	22.4068 ± 3.1181	1.7298 ± 0.1971	4.9858 ± 0.931	n.d.
	citrate	399.0823 ± 76.0174	468.0665 ± 96.0087	416.7702 ± 16.4653	417.9923 ± 69.9615	171.2793 ± 6.9942	441.2817 ± 118.1483	256.6802 ± 56.8957	359.5375 ± 27.8559	343.7190 ± 37.5547
	isocitrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	cis-aconitate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

With its average glucose extracellular concentrations of 18.34 g/L, compared to values ranging from 0.01 g/L in S.c. S288c to 12.15 g/L in H.u. 1.21, PDB was found to be much richer in glucose compared to the fermentates. An extracellular amount of total amino acids of 0.93 g/L was found on PDB, while in fermentates values ranged from 0.25 g/L in I.t. 2.1 to 0.83 g/L in H.u. 3.4, indicating that both for glucose and amino acids a consumption by yeasts generally occurred. The yeasts C.s. 3.3 and S.c. S288c consumed almost all the available glucose within 30 h of growth. All ten amino acids (histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and arginine) essential for *Drosophila* flies (Sang & King, 1961) were found in PDB. The three *H. uvarum* strains consumed less amino acids than other yeasts. Some amino acids were lacking in the extracellular environment of three yeast fermentates: a deficiency of arginine, histidine and lysine was found in I.t. 2.1, while arginine, histidine, and methionine were lacking in S.c. S288c and C.s. 3.3. These amino acids were present intracellularly, however, very low concentrations of methionine were detected. In all samples, glutamic acid exhibited the greatest extracellular concentration among amino acids. As shown in Figure 13, sugar alcohols and organic acids were instead secreted by yeasts. The intracellular concentrations of most compounds were much lower in all yeast samples compared to the corresponding extracellular amounts (Table 9, 10). Two of the organic acids, cis-aconitic acid, and isocitric acid, could only be detected inside the cells and not in the extracellular environment.

To exclude the possible toxic effects of alcohol produced during fermentation, the alcohol content was measured in fermentates made with yeasts grown in PDB. 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 (Table 5). The LD50 of *Drosophila* flies is greater than 1 vol % (Merçot, Defaye, Capy, Pla, & David, 1994; Chakir, Peridy, Capy, Pla, & David, 1993), therefore it is improbable that the alcohol content found in the samples would influence the results.

The amounts of the same intra- and extracellular compounds found in PDB and fermentates made with yeasts grown in PDB were analyzed in YMM and fermentates made with yeasts grown in YMM, as reported in Table S3 (intracellular) and Table S4 (extracellular). Data show an evident contamination of the growth medium with

amino acids. Specifically, a large amount of methionine (109.2 mg/L) and tryptophan (27.2 mg/L) were found, while asparagine (2.1 mg/L), glycine (3.7 mg/L), histidine (7.7 g/L), proline (1.8 g/L), and serine (2.1 g/L) were present at low concentrations. The source of contamination could not be identified; therefore, it was not possible to understand if it was a laboratory-internal cross-contamination or if the supplied product had a defect. This issue affected the CAFE assay results, making the data unreliable. The presence of amino acids in the medium certainly influenced the growth of yeasts and, consequently, the feeding behaviour of *D. suzukii* fed with fermentates. The finding of such a problem using laboratory conditions and therefore a lower risk of contamination compared to a potential field application of a prototype yeast-based formulation, confirms that the use of a non-chemically defined and cost-effective growth medium represents an advantage for a future prospect of using a yeast-based product for commercial purposes.

To evaluate whether the contamination problem was due to the presence of the amino acids that could be detected or rather to other amino acids and small peptides, the free amino nitrogen was measured in all samples analyzed using the system NOPA, a rapid 2-reagent method used in association with a spectrophotometer. Results showed a good agreement with data concerning PDB and fermentates made with yeasts grown in PDB, while in YMM and in fermentates made with yeasts grown in YMM a large amount of amino nitrogen was found, which was probably due to the presence of small peptides that were not detected with LC-MS. In Table S5 the procedures used to calculate the amino nitrogen based on the amounts of single amino acids are reported as well as the protocol used for the analysis of free amino nitrogen. Results concerning the amount of amino nitrogen calculated and measured are summarized in Table S6. Figure S5 shows the correlation between data calculated based on the sum of single amino acids and those measured with the spectrophotometer.

Concerning glucose consumption by yeasts, as observed for fermentates made with yeasts grown in PDB, C.s. 3.3 and S.c. S288c consumed almost all the available glucose within 30 h. As reported for yeasts grown in PDB, yeast metabolism resulted in the production of sugar alcohols and organic acids. In this case, trehalose was found to be a product of yeast metabolism, since it was lacking in YMM and present in

fermentates. Citric acid, which was the most abundant organic acid in PDB and fermentates, was absent in the minimal medium as well as in the extracellular environment of fementation broths. It was only present, together with isocitric acid, in trace amounts inside the cells. As for yeast grown in PDB, cis-aconitic acid was only present inside yeast cells.

3.4 Relationship between extracellular compounds, ingestion, and mortality

Since numerous differences among the chemical profiles of the various yeast species were observed, it would not be possible to find a simple correlation between the different concentrations of a single compound between yeasts and entomological or behavioral responses such as higher/lower ingestion or mortality rates. However, summarizing the results of the CAFE assay of yeasts grown in PDB and extracellular concentrations of glucose, total amino acids, and glycerol, as shown in Figure 14, it is possible to point out some relationships.

Indeed, lower ingestion was generally linked to a corresponding higher mortality rate. Likewise, lower concentrations of amino acids and glucose corresponded to higher amounts of glycerol, indicating that the metabolism of yeasts results in the production of compounds like sugar alcohols. Crossing entomological and chemical data, a link between the availability of extracellular glucose and amino acids and a higher ingestion by *D. suzukii* flies coupled with a lower mortality was observed. On the contrary, higher glycerol concentrations corresponded to higher mortality. Deficiency in glucose was found to be associated with the highest mortality rates.

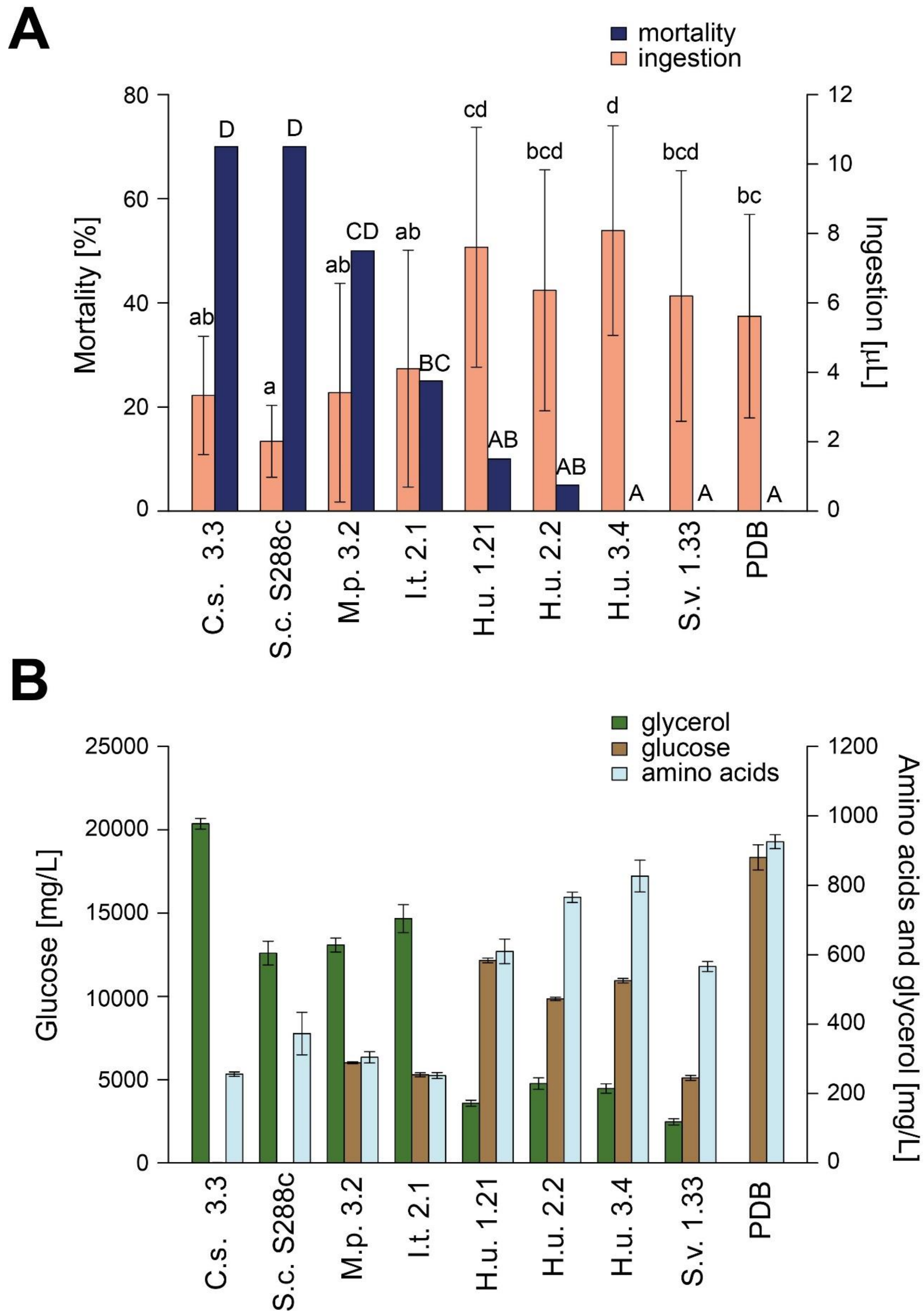

FIGURE 14: Relationship between CAFE assay results and chemical data. Total ingestion (mean ± SD) and mortality (%) of *D. suzukii* females over the four-day experimental period in the CAFE assay (A). Extracellular amount of glucose, glycerol and total amino acids in the eight selected yeasts and PDB (B). Values (mean ± SD) are reported as mg/L of fermentate or culture medium.

3.5 Laboratory assessment of mortality and oviposition by *D. suzukii* after treatment of potted plants in the greenhouse with the yeast-based attract-and-kill formulation

The mortality and the oviposition of *D. suzukii* were measured in the laboratory after exposure of the flies to treated leaves one day after treatment (T1) and one week after treatment (T2). Data concerning the treatment with *S. vini* (S. v. + S) are treated in a separate section since they only refer to entomological assay and to the analysis of non-volatile compounds, while the VOCs characterization is missing. Results concerning the formulation based on *S. vini* (S. v. + S) are reported in the appendix.

Concerning treatments with PDB, PDB + S, and H. u. + S, it was observed that after exposure of the flies to leaves one day after treatment, the mortality and the oviposition were significantly affected by the different treatments (Figure 15). Flies were exposed to treated leaves for 48 h. Already after 24 h a significant effect of the treatment on the mortality of *D. suzukii* flies was found ($F_{2, 7.673} = 35.481$, $p < 0.001$), which was confirmed after 48 h ($F_{2, 12} = 122.00$, $p < 0.001$). Specifically, after exposure to leaves treated with H. u. + S, significantly more flies died compared with PDB or PDB + S ($p < 0.05$), with a 5-fold higher mortality within the first 24 h and 4.3-fold higher mortality after 48 h of exposure to H. u. + S compared to PDB. There was no statistical difference, instead, between PDB and PDB + S ($p < 0.05$). The exposure of flies to leaves to which different treatments were applied did not significantly affect the oviposition after 24 h of exposure ($F_{2, 12} = 0.158$, $p = 0.855$). A significant effect of the treatment became visible between 24h and 48 h of exposure ($F_{2, 12} = 9.621$, $p = 0.003$). Since oviposition was evaluated as the number of eggs laid per cage, these values depend on the number of females that survived and that were able to lay eggs. A higher oviposition was observed between flies exposed to leaves treated with insecticide-free PDB compared to those exposed to PDB + S or H. u. + S ($p = 0.05$), while the two treatments H. u. + S and PDB + S were found to have a similar effect ($p < 0.05$).

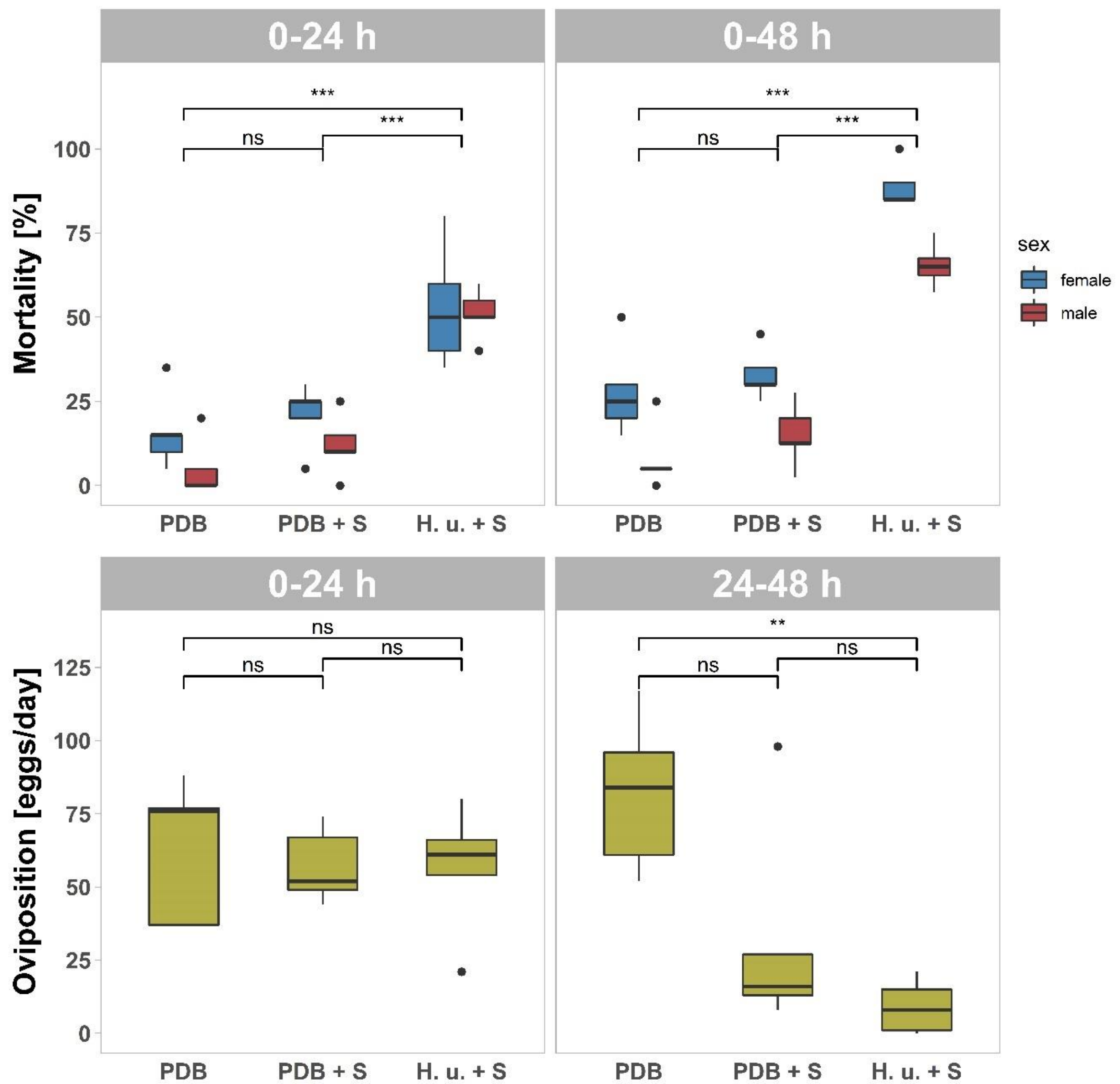

FIGURE 15: Results of mortality and oviposition trials one day after treatment. Mortality and oviposition (n = 5) of *D. suzukii* adults after 24 h and 48 h of exposure to leaves treated with potato dextrose broth (PDB), PDB plus spinosad (PDB + S), or *H. uvarum* plus spinosad (H. u. + S) one day after treatment (T1). Oviposition was measured once after 24 h and a second time between 24 and 48 h of exposure. Asterisks indicate significant differences between treatments ($p < 0.05$). Not significant differences are indicated (ns). Outliers are represented by dots.

One week after treatment (T2), *D. suzukii* flies were exposed to treated leaves using the same methodology used for T1. As shown in figure 16, there was still a significant effect of the different treatments on the mortality after 24 h ($F_{2, 6.190} = 694.376$, $p < 0.001$) and 48 h ($F_{2, 7.151} = 131.912$, $p < 0.001$), with no significant difference in the mortality comparing flies exposed to PDB and PDB + S ($p < 0.05$) and significantly

higher mortality of flies exposed to H. u. + S in comparison with the other treatments ($p < 0.05$). As observed at T1, there were no differences in the number of eggs laid within the first 24 h ($F_{2, 12} = 3.239$, $p = 0.075$), while a significant effect of the various treatments on the oviposition was observed after 48 h of exposure ($F_{2, 12} = 26.609$, $p < 0.001$). Lower oviposition was observed after exposure to leaves treated with H. u. + S compared to the treatments with PDB or PDB + S ($p < 0.05$).

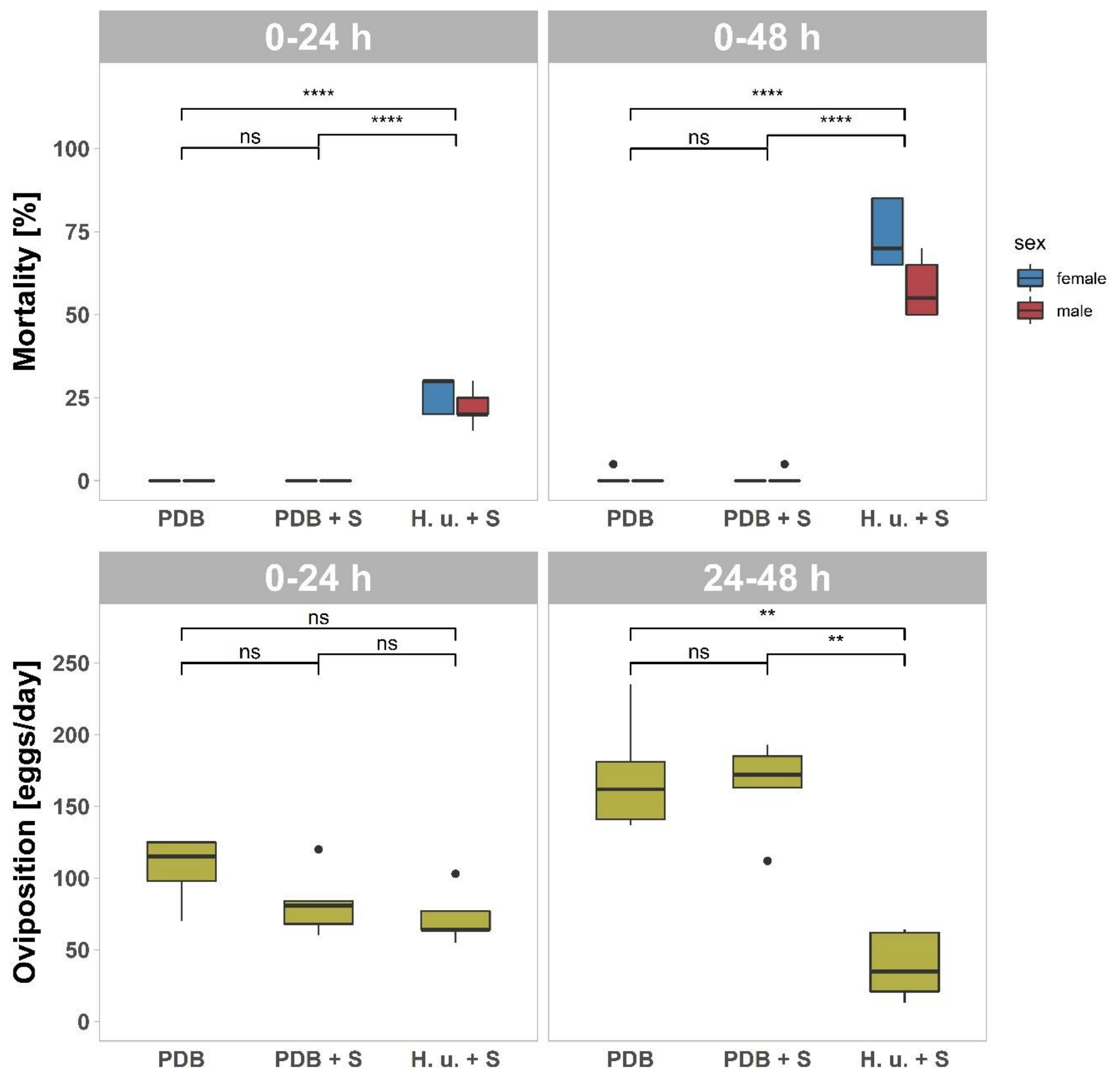

FIGURE 16: Results of mortality and oviposition trials one week after treatment. Mortality and oviposition (n = 5) of *D. suzukii* adults after 24 h and 48 h of exposure to leaves treated with potato dextrose broth (PDB), PDB plus spinosad (PDB + S), or *H. uvarum* plus spinosad (H. u. + S) one week after treatment (T2). Oviposition was measured once after 24 h and a second time between 24 and 48 h of exposure. Asterisks indicate significant differences between

treatments ($p < 0.05$). Not significant differences are indicated (ns). Outliers are represented by dots.

Figure S6 and S7 summarize all data concerning mortality and oviposition of flies exposed to leaves treated with the various formulation tested, including *S. vini* plus spinosad. Though no statistical results are reported, Figure S6 shows the efficacy of S. v. + S was comparable to that of H. u. + S one day after treatment, concerning the reduction of the oviposition due to the increase of the mortality rate of *D. suzukii* flies. However, its efficacy one week after treatment was much lower compared to H. u. + S (Figure S7). Data were acquired and elaborated by the Entomology Group of the Institute for Plant Health of the Laimburg Research Centre.

3.6 Determination of targeted chemical non-volatile compounds on the surface of treated leaves in the greenhouse

Targeted chemical non-volatile compounds, including carbohydrates, sugar alcohols, amino acids and organic acids present on the dried surface of treated leaves were measured. Additionally, the content of the same compounds in culture and fermentates was analyzed (T0) to obtain a starting level of their concentration before their application on leaves. For the reasons mentioned above, data concerning S. v. + S are reported in a separate section. The concentrations and the evolution of the chemical composition of the surface of leaves treated with insecticide-free PDB and with PDB + S over time were very similar to each other.
Overall, two carbohydrates (glucose and trehalose) and two sugar alcohols (glycerol and arabitol) were found (Figure 17). The concentration of the carbohydrates and sugar alcohols (except for glycerol in PDB + S) present on the leaves treated with PDB + S and H. u. + S significantly decreased over time ($p < 0.05$). Though a large amount of glucose was consumed by yeasts within 30 h of growth prior to application of the formulation on leaves, a certain amount of carbohydrates and sugar alcohols was still present on the surface of leaves one week after treatment.

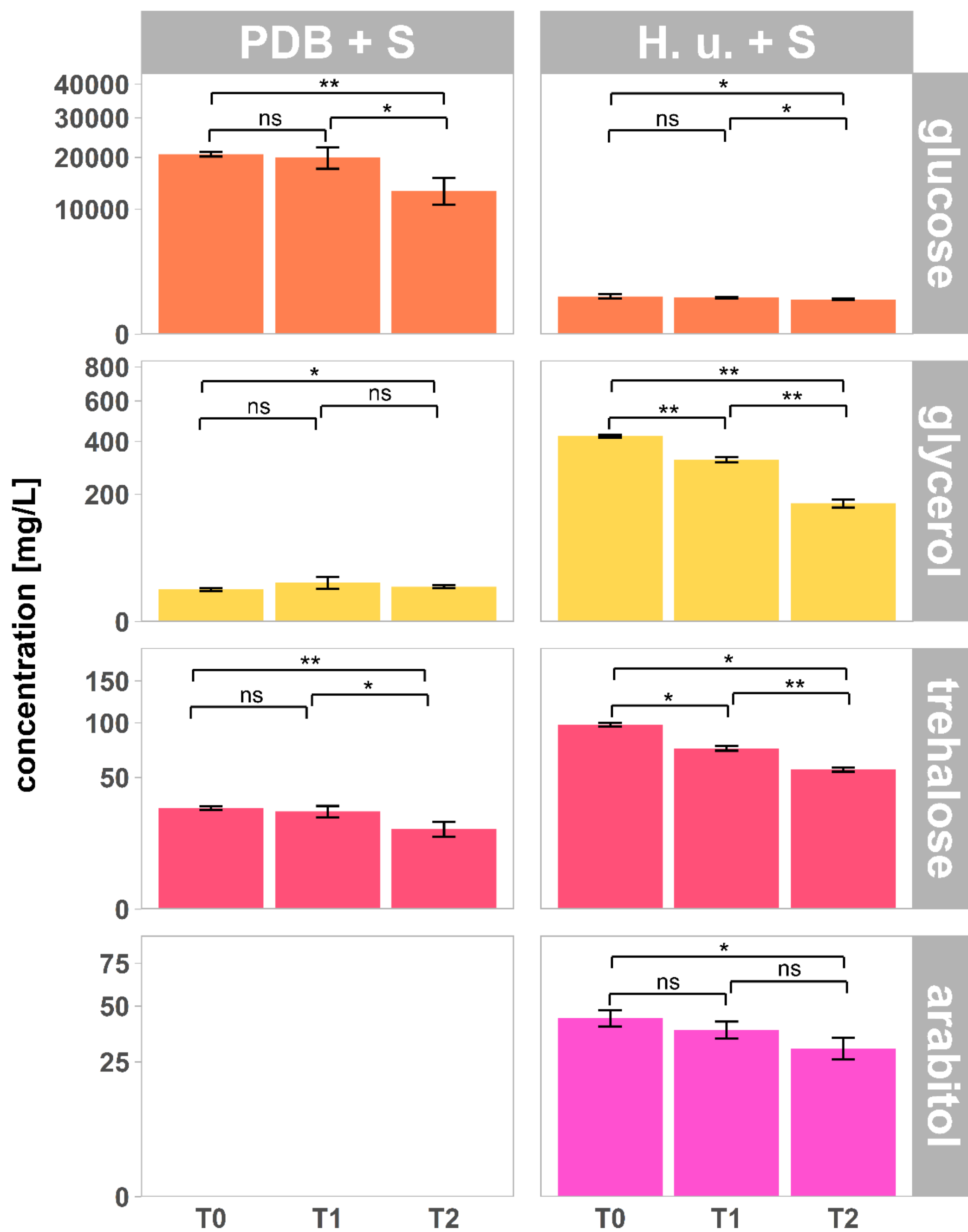

FIGURE 17: Amounts of carbohydrates and sugar alcohols in culture medium/fermentate and on the leaves surface. Concentration of glucose and sugar alcohols (mean ± SD; n = 5) in PDB + S (T0), in the fermentate H. u. + S (T0) and on the surface of leaves treated with PDB + S and H. u. + S collected one day (T1) and one week (T2) after treatment. Arabitol was not detected at any of the timepoints in PDB + S. Asterisks indicate significant differences between timepoints for each treatment ($p < 0.05$). Not significant differences are indicated (ns).

The concentration of 17 amino acids was determined (Figure 18 and Table 11), with PDB + S reporting higher concentrations compared to H. u. + S, indicating amino

acids consumption by yeasts. The concentration of most of the amino acids significantly decreased over time, with few exceptions: no changes were observed at the three timepoints for tyrosine in H. u. + S (*p* = 0.879), as well as glycine (*p* = 0.078), serine (*p* = 0.293), threonine (*p* = 0.085), and tyrosine (*p* = 0.486) in PDB + S. Values of glutamine were variable among replicates (Table 11). Glutamic acid was the most abundant amino acid among all samples analyzed.

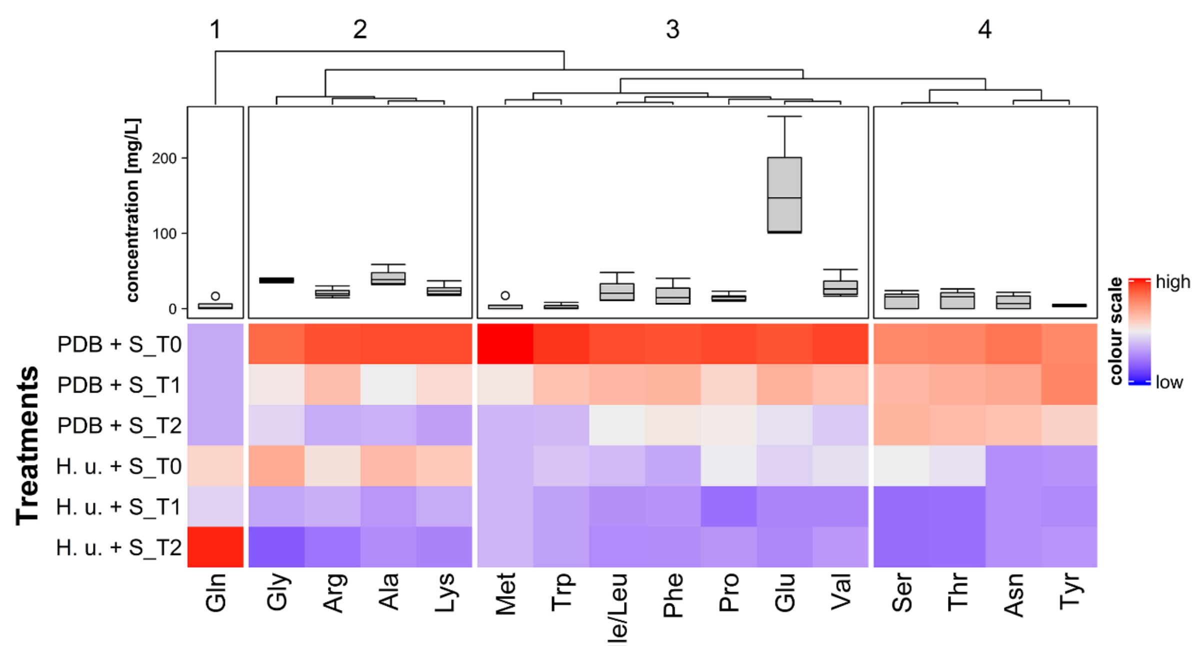

FIGURE 18: Heatmap of the amino acids in culture medium/fermentate and on the leaves surface. Relative concentrations of amino acids in PDB + S and H. u. + S fermentate (T0) and on the surface of leaves treated with PDB + S and H. u. + S collected one day (T1) and one week (T2) after treatment. Three letter codes were used to indicate amino acids. Boxplots show the concentration of amino acids over all six treatments. Clustering of the amino acids was performed using Ward method with Euclidian distance, and the split was based on the k-means algorithm (k = 4).

TABLE 11: Concentrations of amino acids (mg/L of formulation) in culture medium/fermentate and on the leaves surface. Amounts (mean ± SD; n = 5) of single amino acids in PDB + S and

H. u. + S fermentate (T0) and on the surface of leaves treated with PDB + S and H. u. + S collected one day (T1) and one week (T2) after treatment. Concentrations that could not be measured are indicated (nd).

Compound (mg/L)	**HU+S**			**PDB+S**		
Timepoint	*T0*	*T1*	*T2*	*T0*	*T1*	*T2*
(Iso)leucine	16.97 ± 0.64	11.55 ± 0.73	10.77 ± 0.57	48.06 ± 2.03	32.93 ± 4.52	23.95 ± 4.39
Alanine	47.65 ± 1.83	33.17 ± 2.43	32.11 ± 3.68	58.67 ± 3.88	41.53 ± 7.56	35.58 ± 5.93
Arginine	21.79 ± 0.67	17.67 ± 0.93	14.48 ± 0.65	30.15 ± 1.3	24.07 ± 3.7	17.46 ± 4.54
Asparagine	nd	nd	nd	21.71 ± 4.04	16.69 ± 5.69	13.75 ± 2.38
Glutamate	142.92 ± 6.37	100.57 ± 6.59	102.33 ± 14.47	255.2 ± 14.8	200.53 ± 52.26	151.29 ± 21.37
Glutamine	6.2 ± 1.71	2.45 ± 0.91	16.65 ± 8.49	nd	nd	nd
Glycine	39.13 ± 0.97	36.07 ± 1.54	34.56 ± 2.41	40.51 ± 2.54	37.66 ± 2.44	36.96 ± 2.07
Lysine	27.87 ± 0.65	20.24 ± 0.99	17.5 ± 0.87	37.05 ± 1.64	26.52 ± 3	19.38 ± 2.88
Methionine	nd	nd	nd	17.46 ± 1.94	4.41 ± 1.86	nd
Phenylalanine	9.38 ± 0.32	6.85 ± 0.31	6.08 ± 0.27	40.23 ± 1.57	27.17 ± 3.62	19.67 ± 3.24
Proline	15.3 ± 2.44	10.16 ± 2.15	11.79 ± 1.53	23.17 ± 1.45	16.81 ± 3.59	15.71 ± 3.03
Serine	12.36 ± 0.53	nd	nd	23.95 ± 3.47	18.73 ± 4.83	19.14 ± 7.57
Threonine	11.79 ± 1.65	nd	nd	26.15 ± 1.31	21.13 ± 4.88	19.67 ± 5.61
Tryptophan	1.1 ± 0.23	nd	nd	8.32 ± 0.78	4.04 ± 1.14	0.73 ± 0.53
Tyrosine	2.98 ± 0.31	2.9 ± 0.44	3.02 ± 0.39	5.55 ± 0.3	5.59 ± 1.64	4.53 ± 2.07
Valine	27.38 ± 2.85	16.56 ± 2.45	18.97 ± 6.53	52.02 ± 5.08	36.52 ± 6.46	25.01 ± 6.47

Seven organic acids could be detected (Figure 19). As expected, the concentrations at T0 of succinate, acetate, pyruvate, and malate, which are normally produced and secreted by yeasts, were much higher in H. u. + S compared to PDB + S. The amounts of two other compounds, citrate, and formate, at T0 were instead similar between the two, indicating that these organic acids were already present in the growth medium rather than a result of the yeast metabolism. As observed in the previous analyses, citrate was the most abundant organic acid. A decreasing trend of the concentrations of most of the organic acids was observed, with the exception of malate in H. u. + S (p = 0.264), and formate (p = 0.170) and acetate (p = 0.403) in PDB + S, the concentrations of which did not change over time.

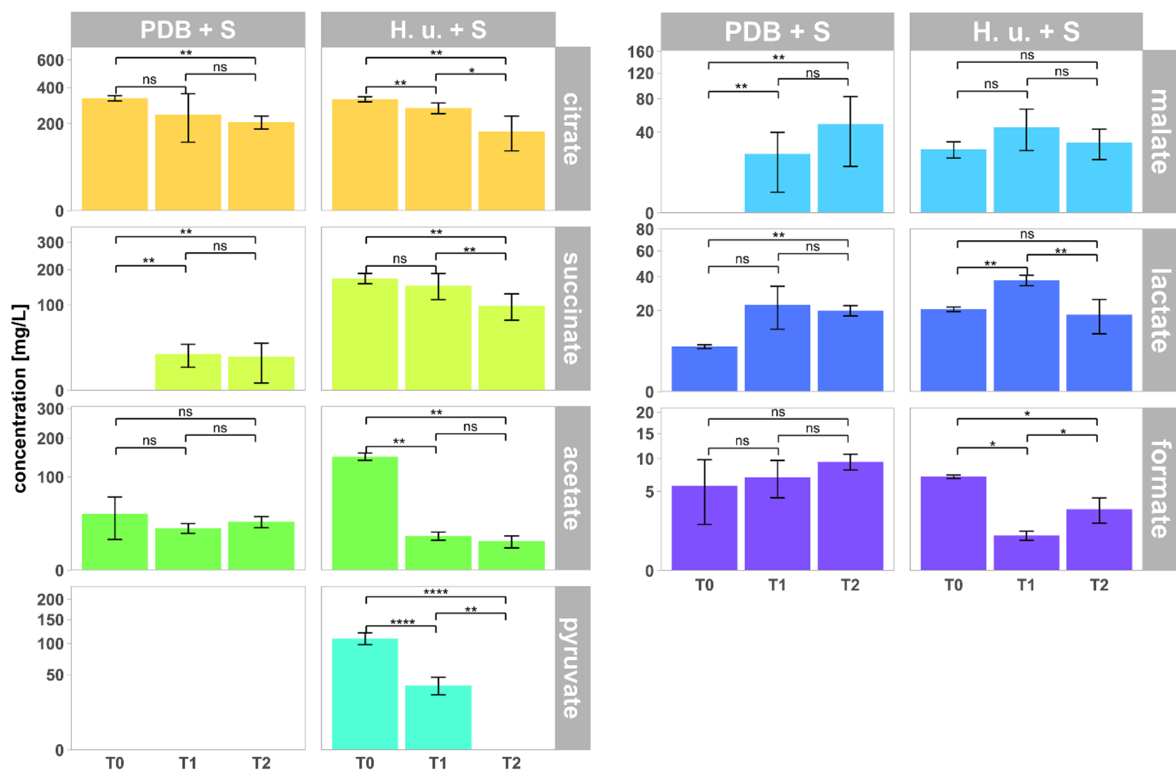

FIGURE 19: Amounts of organic acids in culture medium/fermentate and on the leaves surface. Concentration of organic acids (mean ± SD; n = 5) in PDB + S (T0), in the fermentate H. u. + S (T0) and on the surface of leaves treated with PDB + S and H. u. + S collected one day (T1) and one week (T2) after treatment. Pyruvate was not detected at any of the timepoints in PDB + S. Succinate and malate were not detected at T0 in PDB + S. Asterisks indicate significant differences between timepoints for each treatment ($p < 0.05$). Not significant differences are indicated (ns).

Results referred to S. v. + S are summarized in the appendix. Figure S8 shows the trends of the concentrations of each amino acid at T0, T1, and T2. Generally, as observed for H. u. + S, values tended to decrease over time. Glutamine showed a very high variability among replicates and glutamic acid was again found to be the most abundant amino acid. Concerning organic acids (Figure S9), as observed for H. u. + S, the concentrations of formate and acetate rapidly decreased over time. Glucose was found to be much more abundant in the fermentate of S. v. + S compared to H. u. + S, and its decrease over time was not relevant (Figure S10). On the contrary, sugar alcohols were less concentrated in this fermentate compared to H. u. + S (Figure S10). Surprisingly, the concentration of glucose in the fermentate collected before application was much higher than the amount found in H. u. + S. Since the culture

conditions were very similar to those used for the first trial (yeasts used for the CAFE assay and for the quantification of targeted non-volatile compounds), it is important to note that the yeast growth can easily change. In fact, the first quantitative analysis showed a higher amount of glucose in *H. uvarum* fermentate compared to *S. vini*, while these data show an opposite trend.

3.7 Determination of VOCs emitted by the surface of treated leaves in the greenhouse

The headspace collection of volatiles emitted by *H. uvarum* fermentate as well as those emitted by plants treated with *H. uvarum* and untreated plants was performed. A list of the collected volatiles with their average intensities is provided in Table 12, while chromatograms are reported in Figure 20. Two main compounds were released by yeast fermentate, benzaldehyde and 2-phenylethanol. Beside these two compounds, grapevine plants treated with *H. uvarum* as well as untreated plants, released numerous other compounds, including (*Z*)-3-hexenyl butyrate, beta-caryophyllene, trans-α-bergamotene, (*E,E*)-alpha-farnesene, other VOCs known to be emitted by grapes, such as germacrene D (May, Lange, & Wüst, 2013), or by grapevines, like humulene (Chalal et al., 2015) and by other plants, like 1,8-cineole (Erland, Rheault, & Mahmoud, 2015). The compound 1,8-cineole was the most abundant compound released by the non-treated grapevine leaves. After treatment with *H. uvarum*, the profile of volatiles changed: the concentrations of benzaldehyde and 2-phenylethanol significantly decreased over time, while some VOCs that were not detected in grapevine leaves were released by the plant just after the treatment with *H. uvarum*, including octanoic acid, 2-phenylethyl acetate, methyl salicylate, and (*E*)-4,8-dimethylnona-1,3,7-triene, and were still present five days after treatment, albeit in lower amounts. On the contrary, the concentration of indole, linalool (unidentified isomer), and (*E,E*)-alpha-farnesene significantly increased after treatment and five days later. The latter was the most abundant VOC released by the treated grapevine leaves five days after application. Significant differences between non-treated and treated leaves were observed for most of the terpenes detected. Overall, one aldehyde, one alcohol, one acetate, one acid, one green leaf volatile, two aromatic compounds, and eight terpenes were detected. Data were acquired and elaborated by the Faculty of Science and Technology of the Free University of Bozen-Bolzano.

TABLE 12: Total Ion Chromatogram (TIC) peak area of volatile organic compounds (VOCs) from yeast-treated and non-treated grapevine leaves collected by Closed Loop Stripping Analysis (CLSA). Average amounts (n=6) of VOCs measured in TIC† are indicated. The following abbreviations are used: Linear Retention Index (LRI), Green Leaf Volatiles (GLVs), (E)-4,8-dimethylnona-1,3,7-triene (DMNT), not detected (nd). Significant differences ($p < 0.05$) are reported using different letters and asterisks (≥ 0.05 = ns; < 0.05 = *; ≤ 0.01 = **; ≤ 0.001 = ***).

No	**Compound**	**LRI$^{\forall}$ on HP-5MS**	**Reference LRI**	**Grapevine Leaves (T0)**	**Grapevine Leaves + *H. uvarum* (T1)**	**Grapevine Leaves + *H. uvarum* (T2)**	**Significance (ANOVA *p* Value, df = 2,15)**
ALDEHYDES 1	Benzaldehyde $^{\alpha}$	961	965	0.33 ± 0.28 ^{a}	2.85 ± 1.35 ^{b}	1.20 ± 1.07 ^{ab}	F = 8.039, 0.004 **
ALCOHOLS 2	2-phenylethanol $^{\alpha}$	1114	1116	0.18 ± 0.20 ^{a}	1.09 ± 0.45 ^{b}	0.48 ± 0.36 ^{a}	F = 8.648, 0.004 **
ACIDS 3	octanoic acid $^{\alpha}$	1171	1175	nd ^{a}	0.70 ± 0.30 ^{b}	0.53 ± 0.39 ^{b}	F = 8.431, 0.003 **
ACETATES 4	2-phenylethyl acetate $^{\alpha}$	1258	1265	nd ^{a}	1.48 ± 0.60 ^{b}	0.96 ± 0.63 ^{b}	F = 11.27, 0.001 **
AROMATICS							
5	methyl salicylate $^{\alpha}$	1193	1190	nd ^{a}	12.73 ± 9.00 ^{b}	2.25 ± 2.17 ^{a}	F = 14.45, *p* < 0.001 ***
6	Indole $^{\alpha}$	1291	1288	nd ^{a}	0.57 ± 0.24 ^{ab}	1.82 ± 1.85 ^{b}	F = 3.75, 0.047 *
GLVs 7	(*Z*)-3-hexenyl butyrate $^{\alpha}$	1188	1180	0.06 ± 0.08	2.63 ± 2.39	3.87 ± 4.99	F = 1.846, ns
TERPENES							
8	1,8-cineole	1030	1030	7.80 ± 4.37	24.95 ± 14.86	15.98 ± 12.13	F = 2.851, ns
9	Linalool $^{\alpha}$	1103	1101	nd ^{a}	1.53 ± 0.88 ^{ab}	2.79 ± 1.54 ^{b}	F = 9.299, 0.002 **
10	DMNT $^{\alpha}$	1117	1105	nd ^{a}	9.07 ± 4.70 ^{b}	7.51 ± 5.32 ^{b}	F = 6.996, 0.007 **
11	beta-caryophyllene $^{\alpha}$	1418	1418	0.20 ± 0.15 ^{a}	8.90 ± 3.83 ^{b}	5.80 ± 5.39 ^{ab}	F = 6.659, 0.008 **
12	Humulene $^{\alpha}$	1452	1440	0.29 ± 0.11 ^{a}	5.11 ± 1.94 ^{b}	3.49 ± 2.66 ^{b}	F = 8.334, 0.004 **
13	germacrene D $^{\alpha}$	1480	1480	0.80 ± 0.46 ^{a}	4.61 ± 2.08 ^{b}	3.35 ± 2.41 ^{b}	F = 5.478, 0.016 *
14	trans-alpha-bergamotene	1495	1496	0.15 ± 0.22	1.99 ± 0.62	3.63 ± 3.66	F = 3.284, ns
15	(*E,E*)-alpha-farnesene $^{\alpha}$	1508	1500	0.13 ± 0.11 ^{a}	25.02 ± 13.40 ^{a}	136.25 ± 115.62 ^{b}	F = 5.816, 0.013 *

$^{\forall}$ Linear Retention Indices as calculated from experimental retention times; † The amount in TIC of each compound in the yeast cultures is the mean peak area of six replicates; mean ± standard deviation, divided by 10^6 (in TIC/ 3 h) of volatile compounds calculated from six replicates. $^{\alpha}$ VOCs confirmed by comparison with reference standards.

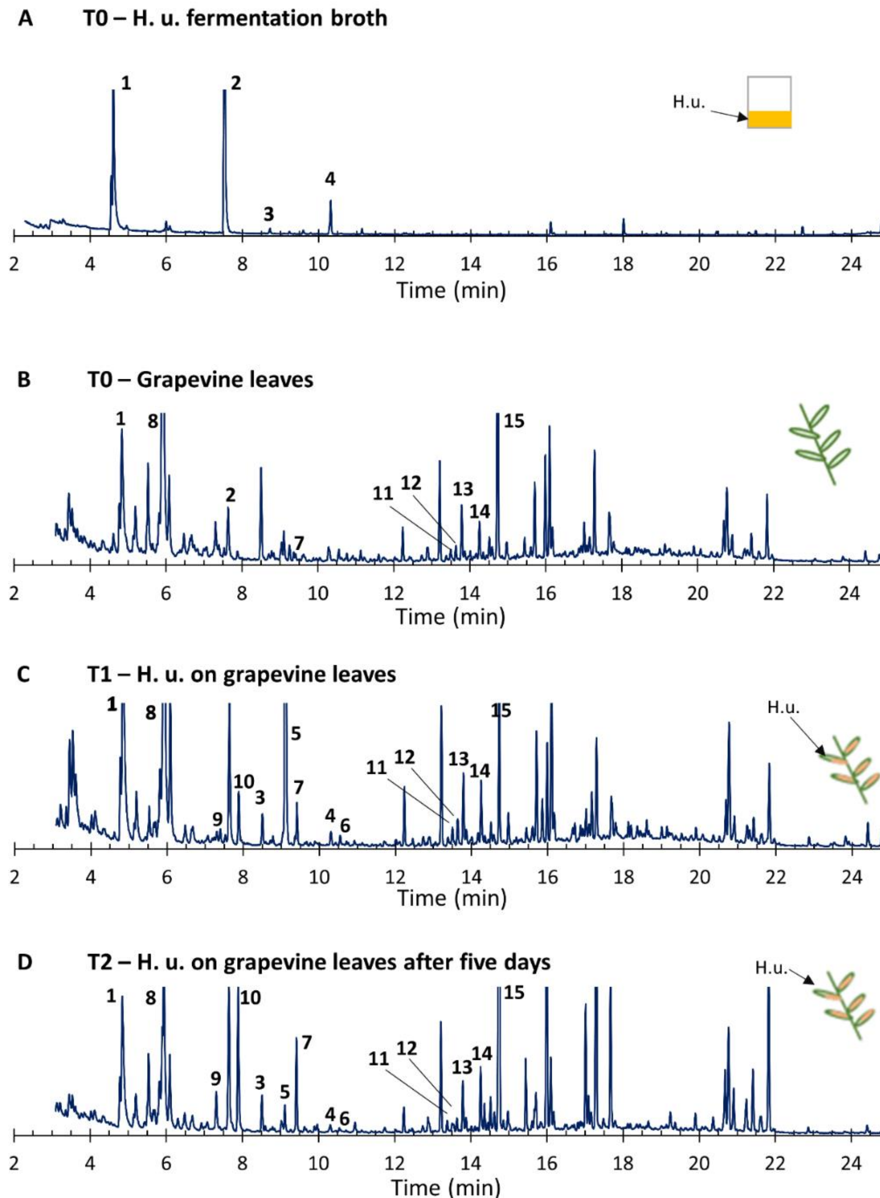

FIGURE 20: Chromatograms of headspace collection of yeast fermentate and leaves. H. u. fermentate (A), grapevine leaves (B), grapevine leaves with H. u. 30 min after application (C), grapevine leaves with H. u. 5 days after application (D). All headspaces were collected with close loop stripping analysis (CLSA) for 3 h. Chemicals identified from H. u. are reported: 1. benzaldehyde; 2. 2-phenylethanol; 3. octanoic acid; 4. 2-phenylethyl acetate; 5. methyl salicylate; 6. indole; 7. (*Z*)-3-hexenyl butyrate; 8. 1,8-cineole; 9. linalool; 10. (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT); 11. beta-caryophyllene; 12. humulene; 13. germacrene D; 14. trans-alpha-bergamotene; 15. (*E,E*)- alpha–farnesene. All graphs are on the same scale showing the total ion chromatogram peaks in time.

3.8 Analysis of the lipid profile of yeasts

3.8.1 Differences in the global lipid profile between yeast strains

A LC-QTOF based approach was performed in order to annotate/identify a large number of lipids belonging to diverse compound classes, present in the intracellular environment of the eight yeasts under investigation.

Overall, 171 compounds including phospholipids (GP), sterols, FA, Cer, sphingoid long-chain bases (LCB), MG, DG, and TG were annotated (Table S7). Prior to data analysis and statistics, peak heights were normalized using class-based IS, log transformation, and pareto scaling. Data distribution before and after pre-processing was inspected through PCA (Figure 21).

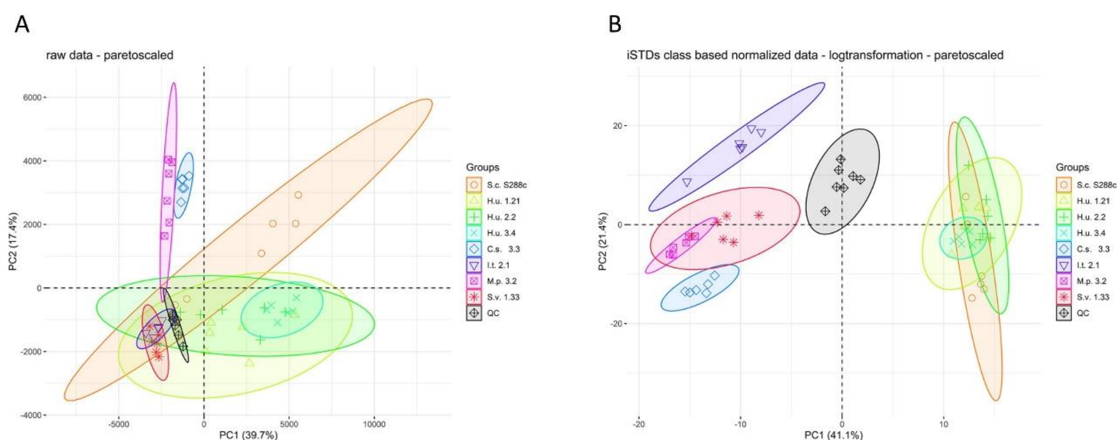

Figure 21: Two-dimensional score plots of the PCAs performed including the entire dataset of six biological replicated for each of the eight yeasts under investigation and seven pooled quality control samples (QCs). PCA with pareto scaled raw data (A). PCA after data normalization using class-based IS, log transformation and pareto scaling (B).

Data pre-processing was necessary to reduce the spread of biological replicates in order to better distinguish the yeast species and strains based on their lipid profiles. After pre-processing, QCs were in the middle of the PCA score plot, as desirable (Figure 21B). The PCA showed that some yeast species could be sorted into discrete clusters and the three strains of *H. uvarum* clustered together. Also, the yeast S. c. S288c was found to cluster together with the three *H. uvarum* strains, indicating similarities concerning their lipid profiles.

An ANOVA revealed that significant differences exist between yeasts concerning all annotated compounds, with two exceptions: FA(18:0) and DG(16:0_18:1).

The PCA was calculated a second time, after excluding pooled quality control samples (QCs) to emphasize the differences between yeasts. Score plot, scree plot and box plots of the scores of the first five principal components are shown in Figure 22.

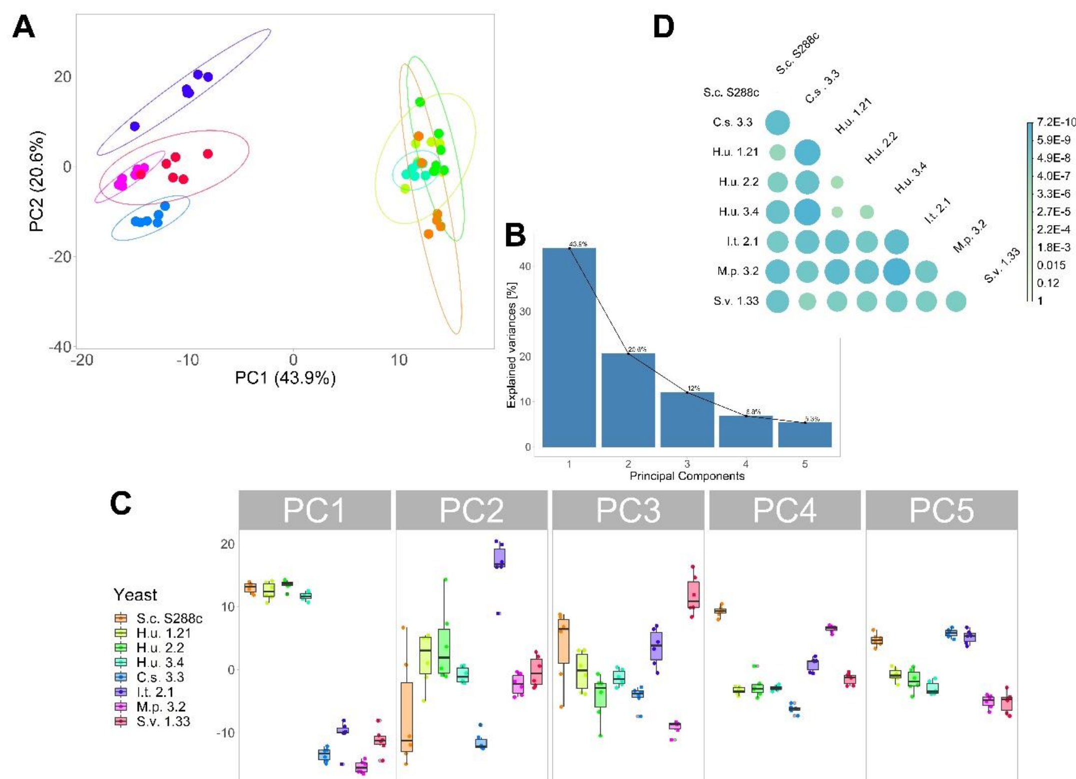

FIGURE 22: Results of the PCA and of the MANOVA. Elaborations were performed including the entire dataset of six biological replicated for each of the eight yeasts under investigation. Score plot (A), scree plot (B) and box plots (C) of the first five principal components resulting from the PCA. Heat map of the pairwise MANOVA between yeast species (D). Result of the MANOVA tests between yeasts based on the first five principal components of the PCA is reported. Differences among species in the yeast lipid chemistry are highlighted using a colour scale and bigger dots for highly significant differences.

The first two principal components explained 64.5% of the variation in the lipid profile, with principal component one accounting for 43.9% and principal component two for 20.6% of the total variation. GPs, DGs, and TGs containing PUFAs negatively influenced the first principal component as well as unsaturated free FAs and ergosterol. Principal component two was strongly influenced in a positive direction

by most of the FAs, including PUFAs, and negatively by most of the GPs. The statistical significance of the differences among the overall yeast lipidome profiles was determined by pairwise MANOVA of the first five principal components. Principal components four and five were included for the calculation of the MANOVA since they were found to be more informative for the discrimination between *H. uvarum* strains and *S. cerevisiae* compared to the first three principal components (Figure 22C). Except for H.u. 2.2 and H.u. 1.21 (p = 0.011), H.u. 1.21 and H.u. 3.4 (0.026), all the species were found to be significantly different from each other (p = 0.005), as shown in the heat map of the MANOVA results (Figure 22D).

In order to emphasize the differences between species and identify the compounds most responsible for discrimination, a partial least squares discriminant analysis (PLS-DA) was performed. This statistical method was not used for predictive purposes, but rather to identify the compounds most responsible for differentiation between yeast species. The 15 most important features for the first principal component identified by PLS-DA and corresponding VIP scores are reported (Figure 23). The top positive contributors include phosphatidylinositole PI (34:1), TG (54:3), PC (30:2), and PI (34:2), indicating GPs and TGs as highly responsible for the discrimination between species.

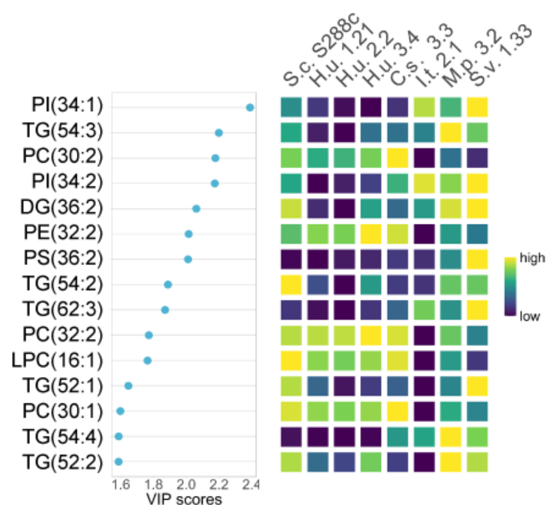

FIGURE 23: Most important features and corresponding VIP scores for the first principal component of the PLS-DA. Elaborations were performed including the entire dataset of six biological replicated for each of the eight yeasts under investigation.

Besides providing an overview of similarities and differences between species, a hierarchical clustering dendrogram allows to evaluate hierarchical relationships and distance between the species and strains under investigation. Spearman distance and Ward clustering algorithm were used to generate the dendrogram shown in Figure 24. Results of the hierarchical clustering confirm similarities and differences observed in the PCA and showed that the biological variability is lower in comparison to the differences among species. Two main clusters were observed: cluster one (C.s. 3.3, M.p. 3.2, S.v. 1.33, and I.t. 2.1) and cluster two (three *H. uvarum* strains and S.c. S288c). The three strains belonging to *H. uvarum* clustered together, indicating a strong similarity between them.

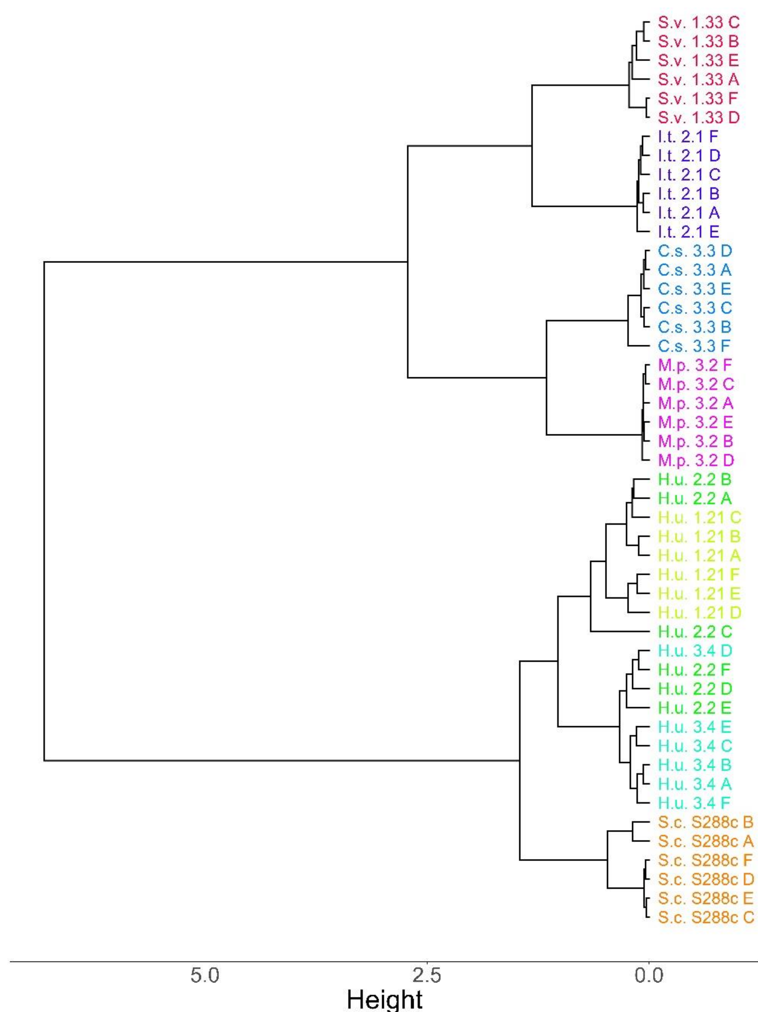

FIGURE 24: Hierarchical clustering dendrogram of the eight yeasts under investigation. The dendrogram was obtained using Spearman distance and Ward clustering algorithm including all the annotated metabolites for each of the six biological replicates (A to F) per each one of the eight yeasts under investigation.

3.8.2 Differences in the lipid profile of single compound classes

3.8.2.1 GPs, DGs, and TGs

Numerous classes of phospholipids were found in yeasts, including phosphatidylserine (PS), PC, PE, PG, phosphatidylinositol (PI), LPE, and LPC (Figure 25A). These compounds were found to be helpful for discrimination between yeast species in comparative studies (Hein & Hayen, 2012). The same study showed that *S. cerevisiae* is poor in GPs containing fatty acids with more than two double bonds. According to the abovementioned works, the GP pattern was found to be informative for the discrimination between yeasts, with the two species, C.s. 3.3 and M.p. 3.2, being richer in GPs compared to the others and S.c. S288c being poor in GPs containing PUFAs. Odd-numbered fatty acids in the acyl chains of PCs and LPCs were annotated in the three species C.s 3.3, M.p 3.2, and S.v. 1.33, with C.s 3.3 containing the highest concentrations.

The relative amounts of DGs and TGs in the various species were visualized through heatmaps (Figure 25B and 26). A similar DG and TG pattern between the three *H. uvarum* strains was observed. As for GPs, a lack of PUFAs in the acyl chains of DGs and TGs was observed in *S. cerevisiae*, as previously reported (Aloklah et al., 2014), and in the three *H. uvarum* strains, in accordance with previous studies (Augustyn, Ferreira, & Kock, 1991). A compound class important for the discrimination between the two similar species *H. uvarum* and *S. cerevisiae* was that of TGs (Figure 22C). S.v. 1.33 was generally richer in higher concentrations and a larger variety of DGs and TGs compared to the other species. Odd-numbered fatty acids in the acyl chains of TGs were annotated in the three species C.s 3.3, M.p 3.2, and S.v. 1.33, with S.v. 1.33 containing the highest concentrations in TGs containing odd-numbered fatty acids.

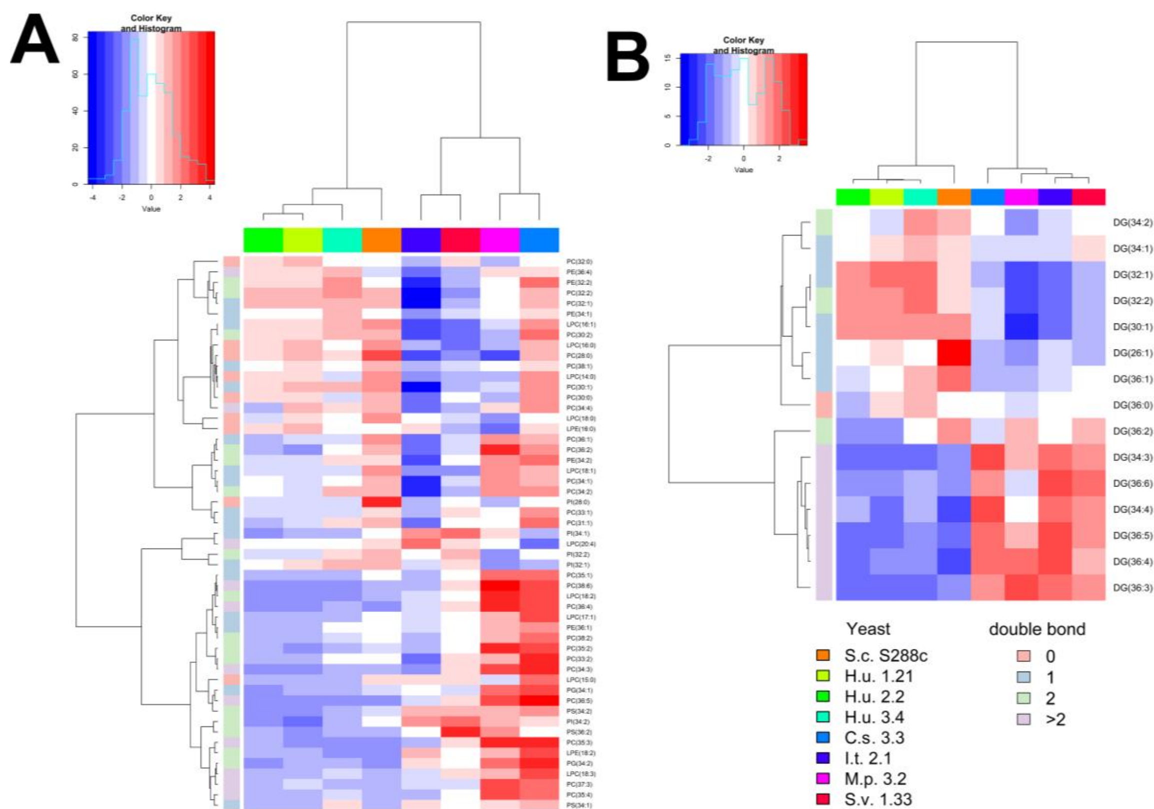

FIGURE 25: Heat maps of GPs (A) and DGs (B) in the yeast species and strains included in the study. Intensities of single compounds are displayed using a color scale ranging from red (higher values) to blue (lower values) as shown in the legend. Both rows and columns are clustered using Spearman distance and Ward clustering algorithm. Average values (n=6) for each yeast are shown.

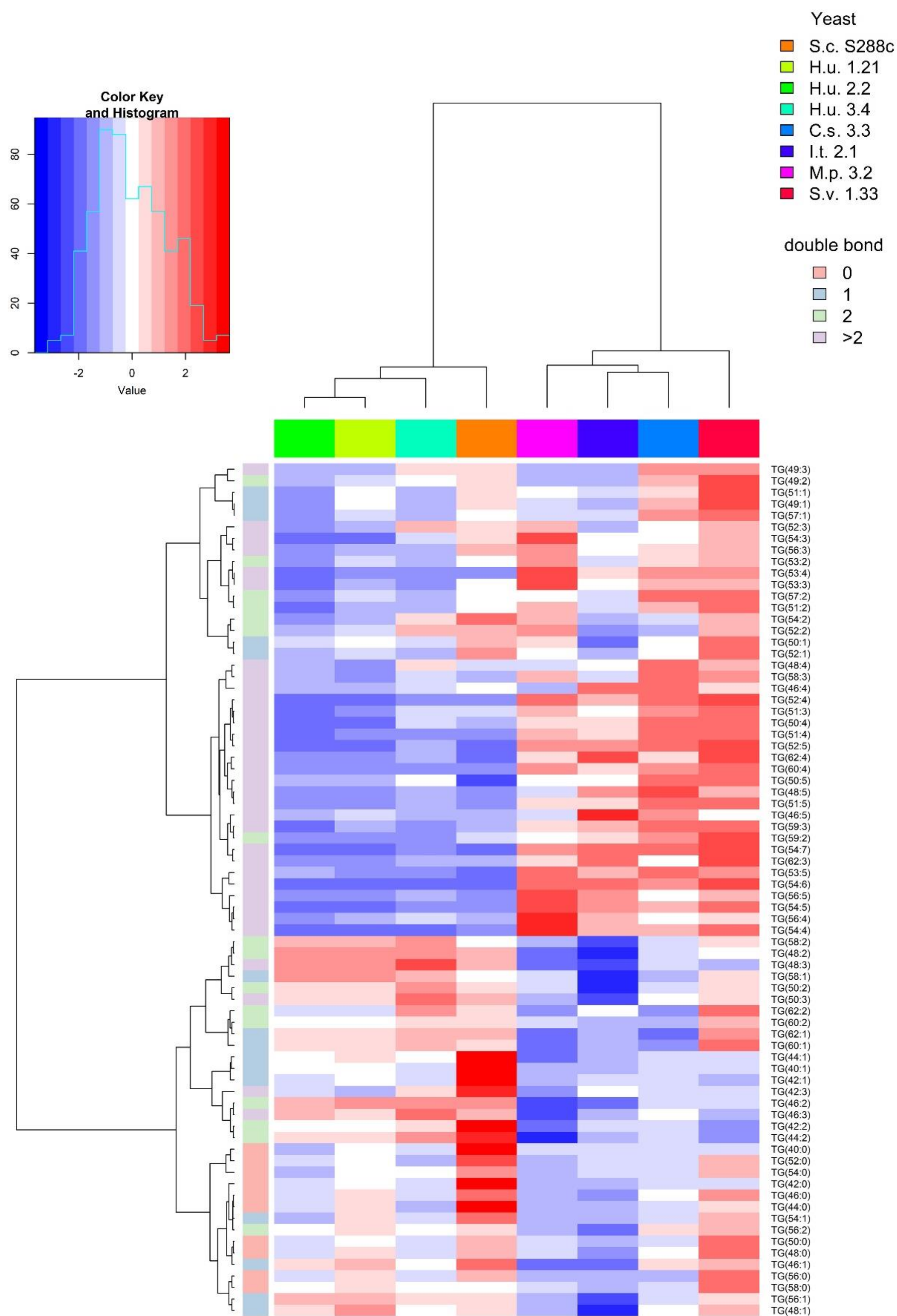

FIGURE 26: Heat map of the TGs in the yeast species and strains included in the study. Intensities of single compounds are displayed using a color scale ranging from red (higher values) to blue (lower values) as shown in the legend. Both rows and columns are clustered using Spearman distance and Ward clustering algorithm. Average values (n=6) for each yeast are shown.

3.8.2.2 FA Ceramides, LCB, and sterols

More than 20 different FAs were found in the analyzed samples. PUFAs were lacking or present at low amounts in *S. cerevisiae* and the three *H. uvarum* strains (Figure 27), S.v. 1.33 was the richest species in oleic acid.

Cer and LCB are components of sphingolipids; four Cer and two LCB, phytosphingosine (PHS) and dihydroshphingosine (DHS), were annotated. Among sterols, only ergosterol was found, with higher relative amounts in S.v. 1.33, I.t. 3.2, and M. p. 3.2 and the lowest amount in H.u. 3.4 (Figure 27).

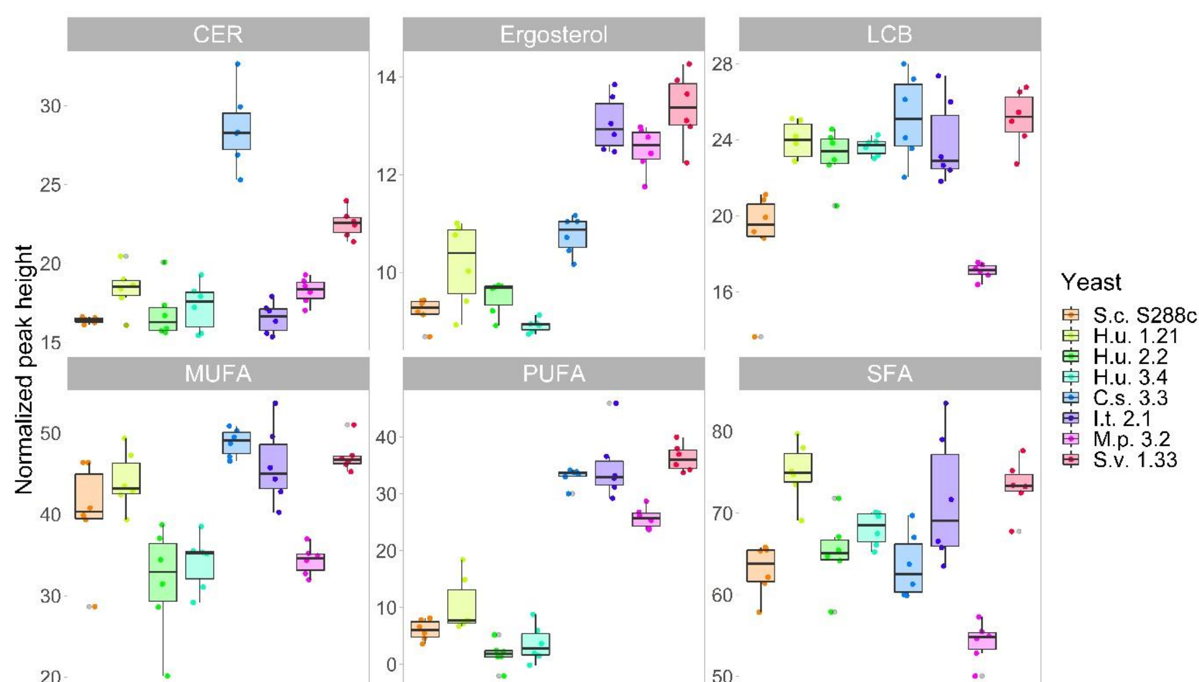

FIGURE 27: Boxplot of Cer, Ergosterol, LCB, and FA in the yeasts included in the study. Distribution of total ceramides (Cer), ergosterol, long-chain base (LCB), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) in the six biological replicates for each yeast.

3.8.3 Chemical enrichment analysis for the comparison between yeasts

The lipid profile of the laboratory *S. cerevisiae* strain was compared to that of the naturally occurring yeast *H. uvarum* through a chemical enrichment analysis (ChemRICH). This statistical analysis is based on similarities and diversities between

groups of metabolites. Results of the analysis are visualized in a two-dimensional impact plot representing the significantly altered clusters of lipids (Figure 28).

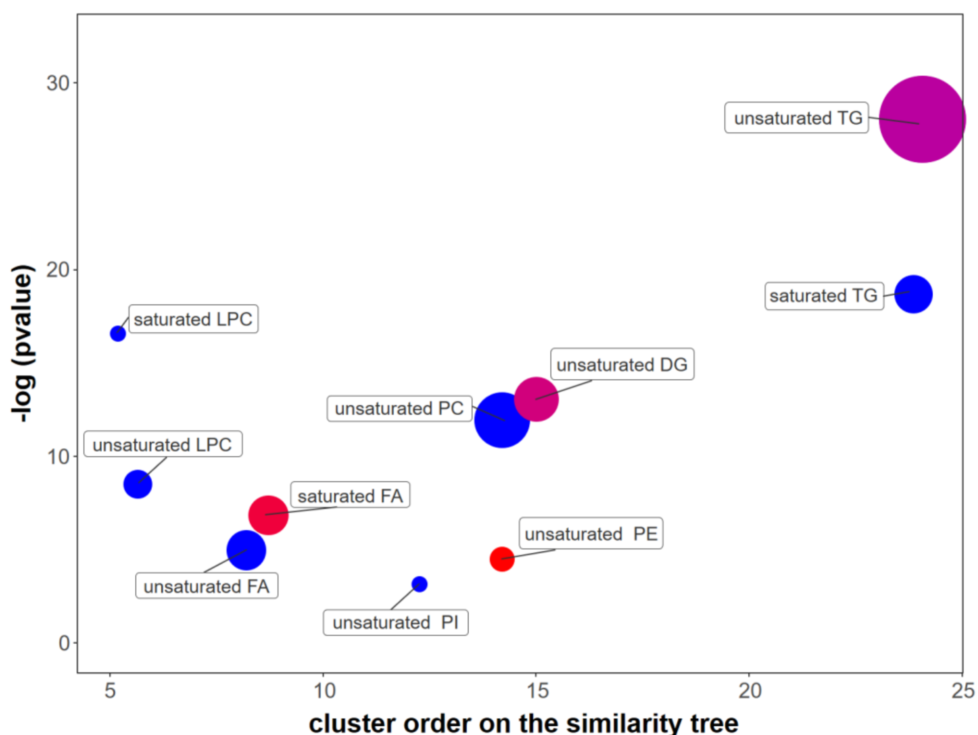

FIGURE 28: ChemRICH results plot shows the comparison between H.u. 3.4 and S.c. S288c. Each node represents a significantly altered cluster of lipids. The most significantly impacted lipid clusters are at the top of the y-axis. Node sizes account the number of lipids in the clusters, while the node color scale represents the proportion of lipids having higher (red) or lower (blue) concentrations in H.u. 3.4 compared to S.c. S288c. The purple node indicates that in the clusters of unsaturated DG and TG there is a number of compounds that are more concentrated in H.u. 3.4 and other compounds that are more abundant in S.c. S288c. Only significantly impacted clusters are shown (p = 0.05).

All the saturated TG, unsaturated PC, LPC, unsaturated FA, and unsaturated PI were at lower concentrations in H.u. 3.4 compared to S.c. S288c (blue nodes), while saturated FA and unsaturated PE were present at higher concentrations in H.u. 3.4 (red nodes). The highest number of significantly impacted compounds was recorded for unsaturated TG. This compound class, together with unsaturated DG are characterized by a similar number of lipids having higher or lower concentrations in either of the two yeasts (purple nodes). Enrichment results are reported in Table 13.

TABLE 13: Results of the ChemRICH analysis of H.u. 3.4 versus S.c. S288c.

Cluster name	Cluster size	*p* value	FDR	Key compound	Altered metabolites	Increased	Decreased	Increased ratio	Altered Ratio
unsaturated TG	64	6.5E-13	7.8E-12	TG(48:3)	25	10	15	0.4	0.4
saturated TG	10	7.5E-9	4.5E-8	TG(46:0)	8	0	8	0	0.8
saturated LPC	4	6.2E-8	2.5E-7	LPC(18:0)	4	0	4	0	1
unsaturated DG	14	2.1E-6	6.3E-6	DG(34:4)	9	5	4	0.6	0.6
unsaturated PC	23	6.5E-6	1.6E-5	PC(34:4)	4	0	4	0	0.2
unsaturated LPC	6	2E-4	3.9E-4	LPC(18:1)	4	0	4	0	0.7
saturated FA	11	1.1E-3	1.8E-3	FA(28:0)	6	5	1	0.8	0.5
unsaturated FA	11	6.8E-3	0.01	FA(20:1)	3	0	3	0	0.3
unsaturated PE	5	0.011	0.015	PE(32:2)	3	3	0	1	0.6
unsaturated PI	4	0.042	0.05	PI(34:1)	2	0	2	0	0.5
unsaturated PS	3	0.053	0.056	PS(34:1)	2	1	1	0.5	0.7
saturated PC	3	0.056	0.056	PC(28:0)	2	0	2	0	0.7

4 Discussion

In this study, the chemical basis was established for the functional explanation of a yeast fermentation-based prototype of an attract-and-kill formulation towards the fruit fly *D. suzukii,* which is a common pest in vineyards and cherry production. It could be demonstrated that specific yeasts produce compounds, which attract the fly and are components of its nutrition. This yeast-based attraction and feeding mechanism can be effectively exploited to reduce the amount of insecticide needed on the plants, thus increasing its efficacy over time. Specific compounds could be identified, which promote these effects.

For the three respective chapters of this study the following theses can be formulated, which are explained and discussed in the next paragraphs:

Quantification of chemical compounds present in yeasts associated with *D. suzukii*

- Yeast fermentates contain nutritionally relevant compounds for *D. suzukii*, like amino acids, carbohydrates, sugar alcohols, and organic acids.
- The feeding behavior of *D. suzukii* fed with diverse yeast species and strains can be referred to differences concerning the chemical composition of yeast fermentates in feeding stimulants and essential nutrients for the fly.
- Consumption or production of metabolites by yeasts during their growth can positively or negatively affect the availability of nutrients for *D. suzukii* fed with various yeast fermentates.

Development of a prototype of a yeast-based attract-and-kill formulation

- A fermentate based on *H. uvarum* cultivated in PDB with the addition of spinosad was found to be promising for its use as a prototype of an attract-and-kill formulation.
- Chemical compounds of relevance for the diet of *D. suzukii* are present on the surface of leaves treated with the formulation prototype developed.
- The long-lasting efficacy of the formulation prototype against *D. suzukii* relates to the time course of chemical changes upon treatment.

Metabolomics of yeasts associated with *D. suzukii*

- A lipidomic approach allows to find a large number of potentially feeding stimulant non-polar compounds towards *D. suzukii.*
- The analysis of the lipid profiles of yeasts associated with *D. suzukii* is useful for species discrimination based on their lipidome, and for highlighting fine similarities and diversities between species and strains.

4.1 Chemical characterization of targeted polar compounds in fermentates and yeast growth media

The results of the entomological trials reported showed that different dietary yeasts influence the feeding behavior and survival of *D. suzukii* adults. Experiments performed using yeasts grown on solid medium MEA showed that the presence of yeast in the diet of *D. suzukii* increases its ability to produce eggs, not affecting the viability of the insects. Using PDA as a growth medium for yeasts, *D. suzukii* were found to be more fecund than those fed on MEA, again, with no effect on viability. Based on these results, the dataset was expanded to eight different yeasts grown in PDA: a laboratory strain of *S. cerevisiae* and five yeast species isolated from feeding tunnels of *D. suzukii* larvae in infested grapes (S. *vini*, *I. terricola*, *M. pulcherrima*, *C. santaluciae*, and three strains of *H. uvarum*). A positive effect on the oviposition by *D. suzukii* females after feeding on the three *H. uvarum* strains, *S. vini* and, to a lesser extent, PDA, compared to *M. pulcherrima*, *I. terricola* and *C. santaluciae* was observed. This trend toward different egg laying behaviors of *D. suzukii* fed with different yeasts, already suggested by Anagnostou et al. (Anagnostou, Dorsch, & Rohlfs, 2010) for *D. melanogaster*, indicates the importance of the presence of appropriate nutrients and specific yeasts in *D. suzukii* diet to promote their fitness. The eight yeasts under investigation were also found to affect the feeding preferences of *D. suzukii* when offered as a food source as fermentates made with yeasts grown in liquid medium PDB. Also in this case, the three *H. uvarum* strains, *S. vini*, and PDB positively affected the fitness of the insect, leading to a lower mortality and to the ingestion of a higher amount of fermentate or culture medium compared to the other four yeast species tested. Besides PDB, a chemically defined minimal

medium (YMM) was tested using the same conditions reported for yeasts grown in PDB, however, in this case, ingested amounts were much lower, and mortality of flies was higher.

Based on these observations, it was deemed appropriate to explore the differences in the chemical composition of the yeasts used in the aforementioned feeding and survival assays.

The choice of the compounds to characterize for such a study was based the interest in finding essential nutrients for the survival of *D. suzukii* and potentially phagostimulant compounds. Therefore, the extracellular and intracellular concentrations of carbohydrates, sugar alcohols, organic acids, and amino acids were measured in yeast fermentates cultivated in PDB and in YMM liquid media. 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 & Daignan-Fornier, 2012). For this reason, it was found necessary to also examine the yeast growth media.

The undefined medium PDB was found to contain numerous nutrients necessary for the survival and development of *D. suzukii*, such as carbohydrates and amino acids (Markow & O'Grady, 2008; Tochen, Walton, & Lee, 2016). This explains its effectiveness as a food source as observed in the feeding assay, even without the addition of yeast. Generally, nutrient consumption or secretion of products of the yeast metabolism and fermentation was observed when comparing the medium with the fermentates. Considering yeasts grown in PDB, *H. uvarum* tended to consume less nutrients within 30 h compared to the other species. Two species, *S. cerevisiae* and *C. santaluciae*, consumed most of the available glucose within 30 h growth in both media (YMM and PDB), reducing the amount of an appropriate carbon source for the feeding of *D. suzukii* flies. The low amount of glucose in these two yeast fermentates could be a possible explanation for the low ingestion and high mortality observed in the feeding assay. The supply of a suitable energy source, such as carbohydrates, increases the appetite of *D. suzukii* flies (Biolchini et al., 2017), and dietary glucose was reported to modulate appetite in *Drosophila* flies (Lebreton, Witzgall, Olsson, & Becher, 2014). Therefore, it is supposed that the availability of glucose in the diet of *D. suzukii* can contribute to the feeding stimulation. The

knowledge of the nutritional requirements and behavior of *D. suzukii* adults can be exploited for management strategies against this pest (Mori et al., 2017; Tochen, Walton, & Lee, 2016). Cowles et al. (2015) demonstrated how the addition of carbohydrates (sucrose) to insecticides enhanced lethality in field tests by increasing the food intake by *D. suzukii* flies.

Concerning carbohydrates, beside glucose, trehalose was measured. No differences among yeasts grown in PDB and with the medium itself concerning their extracellular trehalose concentration were found. On the contrary, trehalose was lacking in YMM, while yeasts grown in YMM produced and secreted this compound. *Drosophila* flies possess gustatory receptors for trehalose (Isono et al., 2005), and this compound was found to elicit a response of sugar neurons (Dahanukar, Lei, Kwon, & Carlson, 2007). Behavioral assays based on the proboscis extension reflex demonstrated that *Drosophila* flies extend proboscis to feed on glucose, trehalose, and glycerol (Slone, Daniels, & Amrein, 2007). These findings indicate that also trehalose can contribute to the promotion of feeding by *D. suzukii*.

Yeast metabolites, such as sugar alcohols and organic acids, are secreted in the extracellular environment (Kayingo, Kilian, & Prior, 2001; Ljungdahl & Daignan-Fornier, 2012). Some of them, like acetic acid, may affect the attractiveness towards *Drosophila* (Erasmus, Cliff, & Van Vuuren, 2004; Hamby & Becher, 2016; Vilela-Moura et al., 2011). It was demonstrated that *Drosophila* rejects overly acidic food and shows adverse responses to carboxylic acids, including acetic acid and citric acid (Charlu, Wisotsky, Medina, & Dahanukar, 2013; Liman, Zhang, & Montell, 2014; Revadi et al., 2015). In addition, sweet perceiving neurons were found to be inhibited by acid taste. However, an increase in sugar concentration allowed to overcome food rejection (Charlu, Wisotsky, Medina, & Dahanukar, 2013). Therefore, an appropriate combination of sugar and acid concentrations is crucial to favor the acceptance of a food source by *Drosophila* flies. More focused investigations will be needed to understand the role of single organic acids present in the yeast-base diets tested and in YMM and PDB on the feeding behavior of *D. suzukii*. Since numerous organic acids were detected, showing variable concentrations among yeasts, the experimental setup performed would not allow to correctly evaluate the response of *D. suzukii* to single carboxylic acids. Nonetheless, because of their peculiarity of eliciting specific

feeding behaviors in *Drosophila*, these compounds should be taken into account for further investigations.

Concerning sugar alcohols, glycerol was the most abundant compound secreted by all yeasts. *Drosophila* flies are able to detect glycerol, since the gene Gr64e confers responsiveness to this compound (Kim et al., 2018; Wisotsky, Medina, Freeman, & Dahanukar, 2011). Additionally, glycerol was reported to influence physiological and behavioral feeding responses in *Drosophila* flies (Koseki, Koganezawa, Furuyama, Isono, & Shimada, 2004; Wisotsky, Medina, Freeman, & Dahanukar, 2011). Although glycerol was found to have a minimal effect on the survival of *Drosophila* (Díaz-Fleischer et al., 2019), some nonnutritive sugar alcohol sweeteners, such as erythritol, show potential as a human-safe insecticide against *D. suzukii* given their potential toxicity (Choi et al., 2017; Sampson, Werle, Stringer, & Adamczyk, 2017).

Different amino acids induce diverse appetitive larval responses in *Drosophila*. Croset et al. (Croset, Schleyer, Arguello, Gerber, & Benton, 2016) observed that no correlations exist between specific feeding behaviors of *Drosophila* and the availability of essential/nonessential amino acids in its diet. In contrast, other studies demonstrated that an imbalance in the concentration of essential amino acids affects the larval food intake by *Drosophila* (Bjordal, Arquier, Kniazeff, Pin, & Le, 2014). Grandison et al. (Grandison, Piper, & Partridge, 2009) observed how the addition of essential amino acids to a restricted diet of *Drosophila* led to a higher fecundity and a lower lifespan. The present study found the fermentates from the three *H. uvarum* strains (grown in PDB), together with PDB, richer in essential amino acids compared other yeast species. The yeast *I. terricola* consumed all available arginine, histidine, and lysine from PDB, whereas a deficiency of extracellular arginine, histidine, and methionine was found in *S. cerevisiae* and *C. santaluciae* grown in PDB. The lack or low concentrations of numerous essential and non-essential amino acids in YMM and in fermentates of yeasts grown in YMM, reflected in a lower ingestion of this yeast-based diets and YMM by *D. suzukii*, highlight the necessity of a diet with a balanced amino acid composition.

The minimal medium used was supposed to be amino acid-free, however a contamination problem occurred, that biased the results concerning YMM. Indeed, numerous amino acids were found in YMM, some of which were present at high

concentrations (methionine, tryptophan). However, numerous essential amino acids were missing, and this dietary restrictions for *D. suzukii* fed with YMM or with yeasts grown in YMM were reflected in a lower ingestion of yeast-free medium and fermentates by the flies, indicating the importance of a rich and balanced diet for the feeding stimulation of adult flies.

Although yeasts can compensate the lack of specific amino acids by providing proteins to *Drosophila* (Bing, Gerlach, Loeb, & Buchon, 2018; De Camargo and Phaff 1957; Steck et al. 2018; Yamada, Deshpande, Bruce, Mak, & Ja, 2015; Phaff, Miller, and Shifrine 1956), the lack of specific free amino acids observed in this study seemed to negatively affect the fitness of *D. suzukii*. Methionine is known to influence the fecundity and the lifespan of *Drosophila* (Grandison, Piper, & Partridge, 2009; B. C. Lee et al., 2014; Schutz, 2008; Troen et al., 2007). This amino acid was consumed by *C. santaluciae* and *S. cerevisiae*, the two yeasts associated with the highest mortality rate of *D. suzukii*. On the contrary, this was the amino acid present at the highest concentration in YMM and highly consumed by yeasts grown in this medium. Glutamic acid was the most abundant amino acid in all yeast fermentates grown in PDB and in PDB itself. Although this amino acid is not essential in the *Drosophila* diet, previous studies demonstrated that three amino acid compounds, namely glutamic acid, alanine, and aspartic acid, stimulate food consumption in *Drosophila* (Yang et al., 2018).

Summarizing, the diets based on PDB and on the fermentates of the three *H. uvarum* strains grown in PDB were found to be richer in glucose and in essential amino acids for *Drosophila* compared to the other yeasts under investigation. *H. uvarum* was already reported to be attractive for *D. suzukii* adults (Scheidler, Liu, Hamby, Zalom, & Syed, 2015) and larvae (Lewis & Hamby, 2019). These findings indicate that, besides being attractive to the fly, this yeast species may be preferred over other species in the insect’s diet because of the lower competition with *D. suzukii* for nutrient utilization, in growing conditions analogous to those reported in this study. Concerning fermentates made with yeasts grown in PDB, the two yeast species associated to the highest mortality and lower ingestion by the flies, namely *C. santaluciae* and *S. cerevisiae*, were lacking in glucose and some essential amino acids. These results indicate that the supply of amino acids and glucose seem to be related

to the feeding stimulation and the reduction of mortality of *D. suzukii*. Organic acids and sugar alcohols as products of the yeast metabolism are able to affect feeding behavior of *Drosophila* flies. Specifically, a connection was found between increasing glycerol content in the extracellular environment and increasing mortality of *D. suzukii* fed with yeast fermentates grown in PDB. Taking into account bibliographic data demonstrating a low toxicity of glycerol towards *Drosophila* (Díaz-Fleischer et al., 2019), this result would not seem to indicate that the abundance of glycerol is the direct cause of the high mortality, but rather that its high concentration indicates a flourishing metabolic activity by yeasts, with consequent consumption of nutrients necessary for the survival of the insect.

Data concerning YMM revealed that this medium is less indicated for the growth of yeasts intended for use as a dietary supplement for *D. suzukii*, since the lack of numerous nutrients leads to competitive phenomena between yeasts and insects for the consumption of essential nutrients. Also, the characterization of YMM revealed the presence of FAN and single free amino acids, index of contamination. On one hand, the contamination of YMM made it impossible to objectively evaluate results, on the other hand it revealed how a multidisciplinary approach that combined chemical data with entomological studies represents a successful strategy for avoiding a wrong interpretation of biological data.

4.2 Efficacy and persistence of the yeast-based attract-and-kill formulation against *D. suzukii* in the greenhouse

Due to the biological and ecological characteristics of *D. suzukii*, the development of an effective control strategy that allows to avoid severe economic losses is extremely challenging (Cini, Ioriatti, and Anfora 2012; Sial et al. 2019; De Ros, Anfora, Grassi, & Ioriatti, 2013). Nowadays, insecticides or exclusion netting can be used to control the pest (Beers, Van Steenwyk, Shearer, Coates, & Grant, 2011; Leach, Van Timmeren, & Isaacs, 2016; Sial et al., 2019). Alternatively, fermentation products including acetic acid and yeast volatiles were already utilized for control strategies (Cha et al., 2013; Iglesias, Nyoike, & Liburd, 2014; Lasa, Toledo-Hernández, Rodríguez, & Williams, 2019), and yeasts were used as adjuvants in insecticide sprays to increase the efficacy

of attract-and-kill strategies by promoting the ingestion by *D. suzukii* of formulations based on attractant yeasts and an insecticide (Knight, Basoalto, Yee, Hilton, & Kurtzman, 2016; Noble et al., 2019; Roubos et al., 2019).

H. uvarum was already reported for its attractiveness towards *D. suzukii* (Scheidler, Liu, Hamby, Zalom, & Syed, 2015). Odorless components found in yeast fermentates, such as sucrose, were already reported to increase the effectiveness of insecticides against *D. suzukii* (Cowles et al., 2015). Therefore, it is expected that the presence of attractive yeast volatiles coupled with the presence of sugars could improve the efficacy of a yeast-based bait. The exploration of the chemical composition of yeasts used for attract-and-kill formulations against *D. suzukii* seems to be highly relevant for the improvement of yeast-based control strategies against this pest.

After verifying that specific yeast fermentates are rich in chemical compounds that can stimulate feeding by *D. suzukii* in the conditions reported in this study, in this experimental part these microorganisms were used for the development of a prototype of an attract-and-kill formulation, the efficacy and persistence of which were assessed through a semi-field experiment.

Based on the findings mentioned above, the species *H. uvarum* and *S. vini* were selected for this purpose and cultivated in PDB, in the previously reported conditions. Spinosad, an insecticide allowed for integrated pest management, was added to fermentates to obtain the yeast-based attract-and-kill formulation. The semi-field experiment performed aimed at evaluating the efficacy and the persistence over a one-week period of the attract-and-kill liquid formulation after treatment of potted grape plants in a greenhouse.

The efficacy of the formulation was assessed through laboratory assays based on indirectly measuring the ingestion of the formulation by the fly through the evaluation of the mortality rate of *D. suzukii* flies after exposure to leaves treated with the attract-and-kill formulation and on the measurement of the oviposition. The addition of both yeasts under investigation to the insecticide resulted in an increase in the mortality rate and a reduction of egg-laying by *D. suzukii* flies exposed to treated leaves compared to what was observed for untreated leaves or leaves treated with PDB with the addition of spinosad (PDB + S). The treatment based on *S. vini* plus spinosad (S. v. + S) was found to be as effective as that with *H. uvarum*

plus spinosad (H. u. + S) one day after treatment, however its lower efficacy one week after treatment represents a limitation of the use of this microorganism for control strategies, in the conditions adopted for this study. The low efficacy of the treatment with PDB + S was surprising, since in the previous experiment, PDB was found to be rich in relevant nutrients for the feeding and survival of *D. suzukii*, including sugars, which were already described as feeding stimulants for *D. suzukii* in baits in combination with insecticides (Cowles et al., 2015; Knight, Basoalto, Yee, Hilton, & Kurtzman, 2016). These findings suggest that yeast metabolites and VOCs are crucial for a satisfactory result. Indeed, under the given experimental conditions, the flies were able to feed on the sucrose solution avoiding the contact with treated leaves. This means that the presence of attractive fermentation products in yeast fermentate attracted flies to find the insecticidal bait on the leaves. At the same time, the presence of nutrients and yeast metabolites stimulated the feeding by *D. suzukii* flies, leading to a higher mortality compared to leaves treated with PDB + S. These results strongly suggest that emitted volatiles together with feeding-stimulants contained in the yeast fermentate enhanced the efficacy of the bait.

Since the hypothesis was that the observed mortality of *D. suzukii* is related to attractiveness or feeding stimulation towards specific constituents of the formulation, potential phagostimulants were analyzed, in order to determine whether changes in their profiles were correlated with the efficacy of the formulation. Numerous potentially feeding stimulants were found in fermentate as well as in PDB within the first experiment reported. The same compound classes were explored for this trial: the concentrations of carbohydrates, sugar alcohols, amino acids, and organic acids on the surfaces of treated leaves that were offered to *D. suzukii* flies during the laboratory trials were measured. Additionally, non-volatile compounds present on the surface of leaves were collected and their evolution over time was assessed.

As already observed for the previous experiment, yeasts consumed glucose and produced sugar alcohols. Unlike the previous experiment, also the content of trehalose was higher in yeast fermentate compared to PDB. Generally, a reduction of the concentrations of carbohydrates and sugar alcohols over time was observed in all samples with few exceptions. This reduction in the sugar's concentration can be

attributed to biodegradation processes by epiphytic microorganisms populating the surface of leaves. Indeed, trehalose represents a carbon source for bacteria (Argüelles, 2000) and numerous lactic acid bacteria that populate the grape surface use diverse sugars and sugar alcohols as substrate for their growth (Zaunmüller & Unden, 2009). A different environmental fate can be supposed for glycerol: this compound undergoes rapid biodegradation and has a photodegradation half-life of 6.8 h (Wernke, 2014). This explains its reduced concentration after exposure to light observed in H. u. + S.

Carbohydrates act as feeding stimulants towards *D. suzukii* (Biolchini et al., 2017). *Drosophila* flies also possess gustatory receptors for trehalose (Isono et al., 2005), and this compound was reported to elicit a response of sugar neurons (Dahanukar, Lei, Kwon, & Carlson, 2007). Behavioral assays demonstrated that *Drosophila* flies extend proboscis to feed on glucose, trehalose, and glycerol (Slone, Daniels, & Amrein, 2007) and that the gene Gr64e confers responsiveness to glycerol in *Drosophila* (Kim et al., 2018; Wisotsky, Medina, Freeman, & Dahanukar, 2011). All these previous findings indicate that not only glucose possesses phagostimulant properties towards *D. suzukii* but also trehalose and glycerol can influence the feeding behavior of the insect. Therefore, the lowering of the concentration of glucose after 30 h of yeast growth compared to PDB may not necessarily be a limiting factor for the feeding acceptance of the attract-and-kill formulation by *D. suzukii*, since this reduced concentration of glucose is a result of the production of other potentially stimulant compounds resulting from its metabolism.

Though the concentration of carbohydrates and sugar alcohols significantly decreased over time, a certain amount of these compounds was still available one week after treatment, indicating that their presence may still be reason for feeding stimulation of *D. suzukii*, and, consequently, high mortality. On the other hand, mortality also decreased over time, indicating a potential relationship between a reduction in the concentrations of compounds capable of stimulating ingestion by *D. suzukii* and reduced efficacy of the formulation.

In a similar way to what was observed for carbohydrates and sugar alcohols, the concentration of amino acids tended to decrease over time, with few exceptions. This indicates that also for these compounds probably biodegradation occurs. According

to the results reported for the first experiment, glutamic acid was the most abundant amino acid present in PDB, as well as in yeast fermentates. As already mentioned, this compound can stimulate feeding in *Drosophila* flies (Yang et al., 2018) and therefore, it is expected that its presence on the surface of leaves up to seven days after treatment may be one of the factors associated to the ingestion of the formulation by the flies. Although after treatment with fermentates a reduction over time of some amino acids reported to be essential for *Drosophila* was observed, the formulation was still effective against *D. suzukii* up to one week after treatment. This suggests either that the low amount of these compounds does not influence the feeding acceptance of a food source by *D. suzukii*, or that their lack is compensated by the biosynthesis of macromolecules by yeasts. However, since the experimental design does not allow to evaluate the effect of each compound separately, only speculations can be made concerning the effect of each amino acid on the efficacy of the formulation. It is presumably a combination of numerous factors including the presence of specific volatile and non-volatile compounds as well as the contact toxicity of spinosad coupled with the exposure time and toxicity due to the feeding that determine the effectiveness of the formulation.

The evolution of the profile of organic acids over time was found to be different from that of sugars and amino acids. The concentrations of some of these compounds, such as lactate and malate, on the surface of leaves treated with fermentates of both yeasts tended to increase over time, compared to that observed at T0, indicating that it is possible that some other microorganisms grew on the surface of treated leaves and produced carboxylic acids. The concentration of other organic acids decreased over time (acetate, pyruvate, citrate, formate), however there are different explanations for this phenomenon, including biodegradation, photodegradation (pyruvate), and evaporation (formate and acetate). As expected, based on its chemical characteristics, after drying on the leaf surface, acetate was probably present as a vapor in the ambient atmosphere (Wernke, 2014), since its concentration was significantly reduced between T0 and T1 in H. u. + S. As for acetate, the reduction of the concentration of formate on the surface of leaves to which the treatment with H. u. + S was applied, is probably due to the high volatility of this compound (Salthammer, 2016). The rapid degradation of pyruvate could be

explained as a result of the photolysis in presence of sunlight (Grosjean, 1983). For the success of an attract-and-kill formulation it is worthy to evaluate the profile of carboxylic acids since these compounds can affect the feeding behavior of *Drosophila*: flies reject too acidic food and showed adverse responses to some carboxylic acids, such as acetic acid and citric acid (Charlu, Wisotsky, Medina, & Dahanukar, 2013; Liman, Zhang, & Montell, 2014; Revadi et al., 2015). Without a sufficient amount of sugar able to overcome food rejection, sweet perceiving neurons of *Drosophila* are inhibited by acid taste (Charlu, Wisotsky, Medina, & Dahanukar, 2013). Therefore, an appropriate composition in sugars and acids is crucial for the acceptance of a food source by *Drosophila* flies.

One last observation comparing fermentates of *S. vini* and *H. uvarum* concerns the amount of glucose. The fermentate of *S. vini* was found to be richer in glucose compared to that of *H. uvarum*. This result was surprising since the chemical characterization of fermentates performed during the first experiment revealed an opposite trend, though the growth conditions of the yeast cultures were very similar. This critical issue should be considered in the interest of developing a product for commercial purposes, since it is important to use controlled and standardized growth conditions, in order to reduce the variability of the characteristics of the yeast cultures.

Data concerning the profile of volatiles emitted by treated and untreated leaves were collected via CLSA. Since the aim of this study was to develop a methodology that could allow to collect volatiles emitted by treated plants *in situ*, to reduce potential interfering effects due to the presence of spinosad, insecticide was not added to fermentate and culture medium, and a higher amount of formulation was applied on the surface of each leaf. Only two treatments were considered: PDB and *H. uvarum*. Despite these differences in the experimental design, these data provide study material and support to the entomological assays and the characterization of non-volatile compounds.

An interesting aspect is that numerous VOCs were induced in the plant after the treatment with *H. uvarum*, and changes in the VOCs profile over time were observed. This indicates that not only VOCs emitted by yeasts may be relevant for the attractiveness, but also those produced by the plants as a response to the yeast-

based treatment should be considered for the development of a successful attract-and-kill formulation. Concerning the persistence of the VOCs over the time-period monitored, as for non-volatile compounds, changes in their profile were observed. The concentrations tended to decrease in most of the cases, while for some compounds (indole, linalool and (*E,E*)-alpha-farnesene) a significant increase in their concentration over time was observed.

Taken together, these results indicate that the presence of yeasts in the attract-and-kill bait is necessary to allow the fly to find it. The complex chemical composition and the abundance of nutrients in the fermentates, while promoting ingestion of the formulation by *D. suzukii*, could lead to the development of epiphytic microorganisms on the surface of treated leaves, which consume the nutrients in the formulation and produce other metabolites that could affect its efficacy against *D. suzukii* over time. Besides biodegradation phenomena, degradation of certain compounds due to the presence of light, air, or high temperatures can affect the stability of the formulation over time.

4.3 Lipidomics of different yeasts associated to *D. suzukii*: comparison between species and relationship with the fitness of the insect

The first part of this study consisted of a detailed examination of the composition in polar compounds chosen based on literature evidence demonstrating their presence in yeast and their importance for the nutrition of *D. suzukii*. Nevertheless, non-polar compounds like lipids represent an important source of nutrients for most of the living beings, including insects.

Besides producing polar metabolites with a nutritional relevance in the diet of *D. suzukii*, yeasts represent a source of lipids for these flies. The growth media selected for previous trials (PDB and YMM) are poor in lipids, since they do not contain oily substances, fat, or yeast extract. Therefore, it is supposed that the major source of lipids for *D. suzukii* fed with fermentates made with the yeasts under investigation is represented by yeast cells.

For the chemical characterization of the lipid composition of the eight yeasts selected for the first experiments, a semi-quantitative lipidomic approach was proposed instead of quantifying metabolites as for the previous experiment. This approach allows to compare the profile of a large number of lipids and to explore numerous compound classes. Another aspect was taken into account for this study: the biological variability. Indeed, after noticing some differences in the content of polar metabolites, as well as in the growth of the cultures of the same yeast prepared on two occasions for the two experiments illustrated above using the same procedure, it was decided to consider more biological replicates for a lipidomic investigation, both to have a more reliable data and to evaluate the biological variability. Six cultures of each yeast were grown in the same conditions, and samples were collected at the same time upon reaching the stationary growth phase; intracellular lipids were extracted, and samples were analyzed using UHPLC – QTOFMS.

Overall, 171 lipids were annotated. The yeast species could be distinguished from each other based on their lipid profile, while the three *H. uvarum* strains were found to be very similar to each other. It was previously reported that the FA profiles of different species belonging to the genus *Hanseniaspora* was very similar (Augustyn, Ferreira, & Kock, 1991). The findings of this research confirm this result and extend it to the other lipid classes investigated. Biological replicates were found to be similar in most of the cases, confirming the reproducibility of the procedure used for yeasts cultivation. The yeast species *C. santaluciae*, *M. pulcherrima*, *S. vini*, and *I. terricola* clustered together as well as the three *H. uvarum* strains and *S. cerevisiae*, indicating similarities in the profile of lipids of these two groups of species. Though for classification studies it will be recommended to use taxonomically defined strains, analogies and differences seemed to be not related with taxonomic relationships: *S. cerevisiae* and *I. terricola* belong to the family *Saccharomycetaceae* (Crous, Gams, Stalpers, Robert, & Stegehuis, 2004; Robert, Stegehuis, & Stalpers, 2005; Robert et al., 2013), and the other species belong to different families from each other. These considerations should only be considered at a general level as classification studies generally involve the use of chemically defined growth media (Hein & Hayen, 2012), which allow to standardize growth conditions and to influence as little as possible the metabolic responses of yeasts. Indeed, changes in the lipid metabolism can rapidly

occur (Gaspar et al., 2007) and are affected by the growth conditions and components of the growth medium (Tehlivets, Scheuringer, & Kohlwein, 2007; Tuller, Nemec, Hrastnik, & Daum, 1999), which can presumably differentially affect the metabolic responses of the various species.

A characteristic which was responsible for the clear difference between the three *H. uvarum* strains and *S. cerevisiae* compared to the other yeast species, was the deficiency in these two yeast species of PUFAs with more than two double bonds among free FA as well as in the acyl chains of GPs, DGs, and TGs. This is in accordance with previous studies that showed that *S. cerevisiae* is unable to produce PUFAs with more than two double bonds, in contrast with other species (Yazawa, Iwahashi, Kamisaka, Kimura, & Uemura, 2009). Though the species *H. uvarum* and *S. cerevisiae* were found to have a similar lipid profile, TGs were the compound class that highlighted the differences between these two species.

A peculiarity of *S. vini* is that this yeast contained the highest concentrations of a larger variety of DGs and TGs compared to the other yeasts.

Interestingly, odd-numbered fatty acids in the acyl chains of TGs, PCs and LPCs were found in *C. santaluciae*, *M. pulcherrima*, and *S. vini*, with the latter containing the highest amounts in TGs containing odd-numbered fatty acids. *C. santaluciae* was found to have the highest amounts of PCs and LPCs with this peculiarity. This characteristic may be of interest for studies focused on species discrimination based on the lipidome, since microbial lipids mainly contain even-numbered FAs (Kondo et al., 2014; Řezanka & Sigler, 2009).

An interesting compound class concerning the interaction between yeasts and *Drosophila* is represented by fatty acids. *Drosophila* is able to perceive these compounds, since it was shown that the gene Gr64e controls behavioral responses of *Drosophila* to FA (Kim et al., 2018) and capillary feeding trials showed that *Drosophila* flies prefer a fatty acid solution rather than water (Masek & Keene, 2013). Oleic acid, palmitoleic acid, palmitic acid and stearic acid are reported as the major FA of *S. cerevisiae* (Klug & Daum, 2014; Tuller, Nemec, Hrastnik, & Daum, 1999; Viljoen, Kock, & Lategan, 1986). Other works indicate that the *Saccharomycetaceae* family is characterized by a higher concentration of oleic acid compared to the *Saccharomycodaceae*, the family of *H. uvarum*, and the *Metschnikowiaceae*, the

family of *M. pulcherrima* (Viljoen, Kock, & Lategan, 1986). In this study, the concentrations of oleic acid (named FA 18:1) were similar between these three species, while *S. vini* was the richest species in this compound. This is an intriguing result since this compound was one of the fatty acids under investigation for its relevance for the fatty acid sensing mechanism in *Drosophila* flies (Kim et al., 2018; Masek & Keene, 2013). Among FA, also compounds with chains longer than 20 carbon atoms were annotated (very-long-chain fatty acids). This is a notable data that enriches the knowledge about the lipid composition of some yeast species concerning these compounds, which are poorly characterized because of the high variability of their concentrations as well as the complexity to detect and identify them without employing sophisticated LC-MS techniques (Řezanka & Sigler, 2009).

After determining the main similarities and differences among species, the next step was to compare results concerning the lipidome of yeasts with the results of the feeding assays performed within the first experimental part. Diets based on fermentates of the three *H. uvarum* strains and of *S. vini* lead to higher ingestion and lower mortality compared to the other species. These results were found to be related with a higher content of amino acids in these two yeasts, a lower amount of glycerol, and the availability of glucose, the deficiency of which in *S. cerevisiae* and *C. santaluciae* based-diets seemed to be linked with a high mortality of *D. suzukii* flies.

Similarly, the global profile of the lipid compounds was evaluated in relationship with the results of the feeding assay. The PCA showed that similarities and differences in the lipidome of different yeasts apparently do not match with *D. suzukii* feeding preferences or survival. Indeed, the lipid profile was similar between the two species *S. cerevisiae* and *H. uvarum*, which were shown to elicit opposite responses towards *D. suzukii* based on the feeding assay.

Previous studies were taken into account to evaluate the similarities and differences in the profile of lipids in relationship with *D. suzukii*. Similarities concerning the lipid profile of *M. pulcherrima* and *C. santaluciae* were observed. However, previous studies showed that these two species had different effects on the survival of *D. suzukii* larvae, since *C. santaluciae*, together with the three *H. uvarum* strains, positively affected the survival of *D. suzukii* larvae compared to *M. pulcherrima*

(Bellutti et al., 2018). It was demonstrated that larvae of *D. suzukii* prefer to feed on *H. uvarum* compared to *S. cerevisiae* and *I. terricola* (Lewis & Hamby, 2019).

These findings may apparently indicate that lipids do not play a role in the interaction between yeasts and *D. suzukii* concerning the behavioral responses of the fly to yeast-based diets. Or, on the other hand, they might indicate that considering the lipid profile in its entireness can be misleading. The lack of a match between *D. suzukii* feeding preferences or survival after feeding with yeast-based diets and similarities in the lipid profiles of yeasts that induced a similar response by the insect does not necessarily indicate that lipids do not have an influence on *D. suzukii*. Rather, that the information concerning specific compounds or compound classes is hidden behind global similarities and diversities in the lipid profile. To overcome this problem, a second approach was proposed: S*. cerevisiae* and *H. uvarum,* two species that showed to elicit opposite responses towards *D. suzukii* based on the feeding trials, were compared using a chemical enrichment analysis. This approach is commonly used for evaluation of changes in the chemical profile that occur after causing metabolic perturbations (Barupal & Fiehn, 2017). Some examples include the comparison of healthy and unhealthy subjects or treated and control samples. It represents an innovative tool for improving biological and biochemical interpretation of metabolomic data. These two species were chosen for two reasons. The first reason is that they were more than once reported to induce different responses in *Drosophila* flies, both concerning feeding behavior and attractiveness (Scheidler, Liu, Hamby, Zalom, & Syed, 2015). The second motivation refers to the fact that in this way it was possible to compare *H. uvarum*, a species isolated from grapes infested by *D. suzukii* and frequently reported in association with the pest (Fountain et al., 2018; Hamby, Hernández, Boundy-Mills, & Zalom, 2012), with a commonly used laboratory strain. Through the ChemRICH analysis it was possible to emphasized diversities between the two species that were not evident comparing the global lipid profile. Significantly higher or lower amounts in either of the two species under investigation were observed concerning compounds belonging to the lipid classes TG, DG, FA, and GP. This approach may be highly interesting for the identification of the main compounds and compound classes responsible for the development of control strategies against this pest. For example, *H. uvarum* was richer in saturated FA

compared to *S. cerevisiae*. Specifically, FA (28:0) was identified as a key compound based on the high statistical significance of the difference between the two yeasts. This compound class and especially this key compound may be singularly tested in further studies including quantitative chemical analysis, feeding trials, and behavioral or survival assays, to help explaining the complex yeast-insect interaction and the different responses of *D. suzukii* to diverse yeast species.

This example was reported to indicate the power of using a chemical enrichment analysis for the identification of key compounds that are responsible for differences between two conditions and its potential application for the identification of bioactive compounds towards *D. suzukii*.

5 Summary

Drosophila suzukii is an invasive insect pest causing severe economic losses to the cultivation of stone and soft fruits all over the world. Current control strategies are not sufficient. Recent studies focused on insect-associated microorganisms showed the potential to exploit this interaction mechanism for control strategies. Attractant volatiles and phagostimulant compounds produced by microorganisms that colonize ripe and overripe fruits increase the efficacy of baits or formulations. Yeasts represent a relevant percentage of the microorganisms on plants and fruit, providing vitamins, lipids, and proteins to *D. suzukii*. However, diverse yeast species were reported to elicit different responses in the feeding behavior or on the attractiveness towards *D. suzukii*, indicating that the selection of an appropriate species or strain is crucial for the success of a yeast-based control strategy. Additionally, it is important to verify the effectiveness of the strategy in short- and long-term studies.

In the present study, the effect of different yeasts on the fitness of *D. suzukii* was explored. The primary objective was to analyze the mechanism of interaction between eight yeasts and *D. suzukii*. The next step was to use the most promising yeasts for the development of a prototype attract-and-kill formulation based on fermentate containing the last-generation insecticide spinosad and to evaluate its efficacy and persistence over time.

Five yeasts isolated from infested grapes (*S. vini*, *I. terricola*, *M. pulcherrima*, *C. santaluciae* and three strains of *H. uvarum*) and a laboratory strain of *S. cerevisiae* were used to feed *D. suzukii* adults. The amount ingested and the mortality rate of flies fed with yeast fermentates and yeast growth media were measured, and the fermentates and culture media were chemically characterized using LC-QqQ and IEC for quantitative analyses of carbohydrates, sugar alcohols, amino acids, and organic acids. The various yeast-based diets differently affected the feeding behavior and the survival of *D. suzukii* flies. The lack of specific nutrients in some of the fermentates led to a lower feeding stimulation and a higher mortality of the flies compared to those containing a higher amount of essential nutrients and feeding stimulant compounds. Diets based on fermentates of the three *H. uvarum* strains, *S. vini* cultivated in PDB, and the medium itself (PDB) resulted in a higher ingestion by *D.*

suzukii flies, coupled with a very low mortality, compared to the other yeasts. On the contrary, *C. santaluciae* and *S. cerevisiae* cultivated in PDB lead to a very high mortality of *D. suzukii* flies and low ingestion. The chemical characterization of the fermentates and medium, allowed to quantify numerous nutrients relevant in the diet of *Drosophila*. Besides carbohydrates and amino acids, compounds resulting from yeast metabolism like organic acids and sugar alcohols were measured. PDB contained a higher amount of nutrients (carbohydrates and amino acids) compared to the fermentates, indicating consumption of such compounds by yeasts during their growth. On the contrary, most of the organic acids and all sugar alcohols were more concentrated in fermentates rather than in PDB. Extracellular concentrations of all compounds were much higher compared to the corresponding intracellular ones, indicating that some compounds are secreted by yeasts, while some other are consumed and used for metabolic processes and for the biosynthesis of macromolecules. Combining entomological results with chemical data, some interesting relationships were observed. When offered as food for *D. suzukii* flies, the fermentates of *C. santaluciae* and *S. cerevisiae* cultivated in PDB were poor in glucose, since it was already consumed by yeasts. The high mortality and low ingestion by *D. suzukii* fed with fermentates of these two yeasts may be a result of the lack of a suitable sugar source for their survival. This apparently simple connection found, reveals that competition mechanisms between yeasts and *D. suzukii* for a nutritional source occur. These phenomena should be taken into account for the optimization of a control strategy based on yeast-insect associations. This consideration may be extended to other nutrients including specific amino acids which were lacking in the abovementioned yeasts. The presence and the concentrations of sugar alcohols and organic acids may influence the feeding behavior of the fly, since *Drosophila* possesses gustative receptors for glycerol and tend to reject too acidic food. Based on the experimental design it was however not possible to address feeding preferences and mortality to single compounds, since each fermentate and the medium itself consist of a complex mixture of constituents, the presence, abundance, and countless combinations of which may influence the acceptance of the food source by the insect.

The second step of this study was to use *H. uvarum* and *S. vini*, the two most promising yeasts based on the previous results, for a prototype attract-and-kill formulation based on fermentate with the addition of the insecticide spinosad. This formulation was applied on potted grape plants in a greenhouse. Non-volatile and volatile compounds present on the surface of treated leaves were characterized, and the effect of the formulation on the survival and oviposition by *D. suzukii* was evaluated through laboratory trials. The same non-volatile compounds determined during the first experiment were quantified using the same analytical techniques, whereas VOCs were analyzed after CLSA collection. Results showed that the yeast-based formulation had a strong effect on the survival of the flies, leading to a much higher mortality compared to that observed after exposure of the flies to PDB with spinosad, without yeasts. At the same time, due to the high mortality, a reduction of the oviposition was observed. Though the previous experiment showed that the ingested amount of PDB was comparable to *H. uvarum* and *S. vini* fermentate, the results of this study revealed that the presence of yeasts is necessary for the fly to find the food source. Indeed, in the first experiment flies could not choose another food source and could move only in a limited space. On the contrary, the cage design of the second experiment allowed flies to move in a larger space and to access to an alternative sugar source, suggesting that the high mortality observed after exposure to leaves treated with fermentate plus spinosad was related to the attractants emitted by yeasts that enabled the fly to find the formulation. Phagostimulant compounds induced the fly to feed on treated leaves, leading to the ingestion of the insecticide. The formulation was still effective one week after treatment, with a mortality rate higher than 50 %. The concentration of most of the non-volatile compounds tended to decrease over time, however, numerous nutrients were still present on the surface of treated leaves up to one week after treatment. Similarly, numerous VOCs tended to decrease over time, however, some VOCs emitted by the plant seem to be induced by the treatment with the formulation, indicating that not only yeast volatiles but also VOCs emitted by the plant may play a role in the attractiveness towards *D. suzukii*.

Finally, the profile of lipids produced by yeasts was explored. So far, only polar compounds were considered, nevertheless yeasts are also a lipid source for

Drosophila. Additionally, *Drosophila* flies possess gustatory receptors to fatty acids, indicating the potential feeding stimulant capacity of these compounds. A high throughput semi-quantitative approach based on high resolution mass spectrometry (LC-QTOF) was performed for the determination of intracellular lipids in the eight yeasts selected for the first experiment cultivated in PDB. Since the biological variability had not been yet evaluated, six cultures per each yeast were grown under the same conditions and samples were collected at the same time. Results highlighted similarities and diversities among species and strains. The biological variation was low compared to the differences between species. The three *H. uvarum* strains had a similar lipid profile. More than 170 lipids were annotated. A chemical enrichment analysis was performed for a pairwise comparison between two yeasts (*H. uvarum* and *S. cerevisiae*), which allowed to highlight similarities and fine differences between compound classes and to identify key compounds that could be considered for further entomological studies.

Taken together, the studies of the present thesis provide extensive insights into the association between yeasts and *D. suzukii* and its importance for control strategies. The exploration of the effects of different yeast species on the fitness of the insect, as well as their chemical characterization provide a considerable progress of knowledge of the biology of the insect and of the metabolism of microorganisms associated to *D. suzukii*. Finally, the evaluation of the efficacy and persistence of a prototype yeast-based attract-and-kill formulation lays the foundations for a future development of a formulation to be applied in the field.

6 Zusammenfassung

Drosophila suzukii ist ein invasives Schadinsekt, das im Anbau von Stein- und Beerenfrüchten auf der ganzen Welt schwere wirtschaftliche Verluste verursacht. Die derzeit bekannten Bekämpfungsstrategien sind nicht ausreichend, um das Problem in den Griff zu bekommen. Neue Studien, die sich mit insektenassoziierten Mikroorganismen beschäftigten, zeigten das Potenzial, diesen Interaktionsmechanismus für Bekämpfungsstrategien auszunutzen. Lockende flüchtige Stoffe und phagostimulierende Verbindungen, die von Mikroorganismen produziert werden, die reife und überreife Früchte besiedeln, erhöhen die Wirksamkeit von Ködern oder Formulierungen. Hefen stellen einen relevanten Prozentsatz der mit *D. suzukii* assoziierten Mikroorganismen dar. Sie liefern dem Insekt Vitamine, Lipide und Proteine. Es wurde auch berichtet, dass verschiedene Hefespezies eine unterschiedliche Attraktivität auf *D. suzukii* haben und unterschiedliche Reaktionen im Fressverhalten hervorrufen, weshalb die sorgfältige Auswahl einer geeigneten Spezies oder eines Stammes für den Erfolg einer hefebasierten Bekämpfungsstrategie ausschlaggebend ist. Zusätzlich ist es wichtig, die Effektivität der Strategie kurz- und langfristig zu verifizieren.

In der vorliegenden Studie wurde der Einfluss verschiedener Hefen auf die Entwicklung und biologische Fitness von *D. suzukii* untersucht. Das primäre Ziel war es, den Mechanismus der Interaktion zwischen acht Hefen und *D. suzukii* zu analysieren. Der folgende eher anwendungsorientierte Schritt bestand darin, die vielversprechendsten Hefen für die Entwicklung eines Prototyps eines Attract-und-Kill-Verfahrens auf der Basis einer Hefekultur zu verwenden, die ein Insektizid der neuen Generation enthält, und dessen Wirksamkeit und Persistenz über die Zeit zu bewerten.

Fünf aus befallenen Trauben isolierte Hefen (*S. vini*, *I. terricola*, *M. pulcherrima*, *C. santaluciae* und drei Stämme von *H. uvarum*) und ein Laborstamm von *S. cerevisiae* wurden als Futter für adulte *D. suzukii* verwendet. Die aufgenommene Menge und die Mortalitätsrate von Fliegen, die mit Hefekulturen und Wachstumsmedia gefüttert wurden, wurden gemessen. Diese Hefekulturen und Wachstumsmedia wurden mittels LC-QqQ und IEC für quantitative Analysen von Kohlenhydraten,

Zuckeralkoholen, Aminosäuren und organischen Säuren chemisch charakterisiert. Die verschiedenen hefebasierten Diäten beeinflussten das Fressverhalten und das Überleben von *D. suzukii* Fliegen unterschiedlich. Der Mangel an spezifischen Nährstoffen in einigen der Hefekulturen führte zu einer geringeren Fütterungsstimulation und einer höheren Sterblichkeit der Fliegen im Vergleich zu jenen, die eine höhere Menge an essentiellen Nährstoffen und fütterungsstimulierenden Verbindungen enthielten. Diäten auf Basis von Hefekulturen der drei *H. uvarum*-Stämme, *S. vini* und kultiviert in PDB und dem Medium (PDB) wurden von *D. suzukii*-Fliegen in größeren Mengen aufgenommen im Vergleich zu den anderen Hefen und waren mit einer sehr geringen Mortalität verbunden. Im Gegensatz dazu führten *C. santaluciae* und *S. cerevisiae* kultiviert in PDB zu einer sehr hohen Mortalität der *D. suzukii* Fliegen und einer geringen Ingestion. Die chemische Charakterisierung der Hefekulturen und des PDB erlaubte die Quantifizierung zahlreicher Nährstoffe, die in der Ernährung von *Drosophila* relevant sind, darunter Kohlenhydrate und Aminosäuren. Die Hefen produzierten organische Säuren und Zuckeralkohole. PDB enthielt eine höhere Menge an Nährstoffen (Kohlenhydrate und Aminosäuren) im Vergleich zu den Hefekulturen, was auf den Verbrauch solcher Verbindungen durch die Hefen während ihres Wachstums hinweist. Im Gegensatz dazu waren die meisten organischen Säuren und alle Zuckeralkohole in den Hefekulturen höher konzentriert als in PDB. Die extrazellulären Konzentrationen aller Verbindungen waren viel höher als die entsprechenden Intrazellulären, was darauf hindeutet, dass einige Verbindungen von den Hefen abgesondert werden, während andere verbraucht und für metabolische Prozesse und für die Biosynthese von Makromolekülen verwendet werden. Bei der Gegenüberstellung der entomologischen Ergebnisse mit den chemischen Daten wurden einige interessante Zusammenhänge beobachtet. Die Hefekulturen von *C. santaluciae* und *S. cerevisiae* kultiviert in PDB waren extrem arm an Glukose als sie den *D. suzukii* Fliegen angeboten wurden. Die Glukose war zu diesem Zeitpunkt bereits von Hefen verbraucht worden. Die hohe Sterblichkeit und die geringe Ingestion von *D. suzukii*, die mit Hefekulturen dieser beiden Hefen gefüttert wurden, könnte auf das Fehlen einer geeigneten Zuckerquelle für ihr Überleben zurückzuführen sein. Dieser scheinbar einfache Zusammenhang zeigt, dass

Konkurrenzmechanismen zwischen Hefen und *D. suzukii* um eine Nahrungsquelle auftreten und für die Wirksamkeit einer Bekämpfungsstrategie, die auf Hefe-Insekten-Assoziationen basiert, berücksichtigt werden sollten. Diese Überlegung lässt sich auf andere Nährstoffe ausdehnen, einschließlich spezifischer Aminosäuren, die bei den oben genannten Hefen fehlten. Das Vorhandensein und die Konzentrationen von Zuckeralkoholen und organischen Säuren können das Fressverhalten der Fliege beeinflussen, da *Drosophila* gustative Rezeptoren für Glycerin besitzt und dazu neigt, zu saure Nahrung abzulehnen. Aufgrund des Versuchsaufbaus ist es jedoch nicht möglich, Fütterungspräferenzen und Mortalität auf einzelne Verbindungen zurückzuführen, da jede Hefekultur und das Medium selbst aus einem komplexen Gemisch von Bestandteilen bestehen, deren Vorkommen, Konzentration und unzählige Kombinationen die Akzeptanz der Nahrungsquelle durch das Insekt beeinflussen können.

In einem zweiten Schritt dieser Studie wurde mit *H. uvarum* und S. *vini* gearbeitet. Vorherige Ergebnisse haben gezeigt, dass diese zwei Hefen die vielversprechendsten für einen Prototyp eines Attract-und-Kill-Verfahrens auf Basis von Hefekulturen mit dem Zusatz des Insektizids Spinosad handelt. Diese Formulierung wurde in einem Gewächshaus auf die Blätter von getopften Traubenpflanzen ausgebracht. Nichtflüchtige und flüchtige Verbindungen, die auf der Oberfläche der behandelten Blätter vorhanden waren, wurden charakterisiert und ihre Wirkung auf das Überleben und die Eiablage von *D. suzukii* durch Laborversuche bewertet. Die gleichen nichtflüchtigen Verbindungen, die im ersten Versuch bestimmt wurden, wurden quantifiziert, während die VOCs nach der CLSA-Sammlung analysiert wurden. Die Ergebnisse zeigten, dass die Formulierung auf Hefebasis eine starke Auswirkung auf das Überleben der Fliegen hatte. Bei der Formulierung mit Hefe wurde eine viel höhere Sterblichkeit beobachtet als bei einer Exposition der Fliegen mit PDB und Spinosad ohne Hefen. Gleichzeitig wurde aufgrund der hohen Sterblichkeit eine Reduzierung der Eiablage beobachtet. Obwohl das vorherige Experiment zeigte, dass die aufgenommene Menge an PDB mit den *H. uvarum* and *S. vini* Hefekulturen vergleichbar war, zeigten die Ergebnisse dieser Studie, dass die Anwesenheit von Hefen für die Fliege notwendig ist, um die Nahrungsquelle zu finden. In der Tat konnten die Fliegen im ersten Experiment keine andere Nahrungsquelle wählen und

sich nur in einem begrenzten Raum bewegen. Im Gegensatz dazu erlaubte das Käfigdesign des zweiten Experiments den Fliegen, sich in einem größeren Raum zu bewegen und Zugang zu einer alternativen Zuckerquelle zu erhalten. Dies deutet darauf hin, dass die hohe Mortalität, nach der Exposition mit Blättern, die mit Hefekulture plus Spinosad behandelt wurden, mit den von den Hefen emittierten Lockstoffen zusammenhängt, die es der Fliege ermöglichen, die Formulierung zu finden. Die phagostimulierenden Verbindungen veranlassen die Fliege, an den behandelten Blättern zu fressen, was zur Aufnahme des Insektizids führt. Die Formulierung war noch eine Woche nach der Behandlung wirksam, mit einer Mortalitätsrate höher als 50 %. Die Konzentration der meisten nichtflüchtigen Verbindungen nahm mit der Zeit tendenziell ab, jedoch waren zahlreiche Nährstoffe noch bis zu einer Woche nach der Behandlung auf der Oberfläche der behandelten Blätter vorhanden. In ähnlicher Weise tendierten zahlreiche VOCs dazu, im Laufe der Zeit abzunehmen. Einige von der Pflanze emittierte VOCs scheinen jedoch durch die Behandlung mit der Formulierung induziert zu werden, was darauf hindeutet, dass nicht nur flüchtige Stoffe aus Hefen, sondern auch VOCs, die von der Pflanze unter bestimmten Bedingungen emittiert werden, eine Rolle bei der Attraktivität gegenüber *D. suzukii* spielen können.

Abschließend wurde das Profil der von den Hefen produzierten Lipide, sogenannte apolare Verbindungen, erforscht. In den bisherigen Versuchen wurden nur polare Verbindungen betrachtet, jedoch stellen Hefen auch eine Lipidquelle für *Drosophila* dar. Zusätzlich besitzen *Drosophila*-Fliegen Geschmacksrezeptoren für Fettsäuren, was auf die potenzielle phagostimulierende Kapazität dieser Verbindungen hinweist. Für die Bestimmung der intrazellulären Lipide in den acht für das erste Experiment ausgewählten Hefen kultiviert in PDB, wurde ein semiquantitativer Ansatz mit hohem Durchsatz auf der Basis hochauflösender Massenspektrometrie (LC-QTOF) durchgeführt. Da die biologische Variabilität noch nicht ausgewertet worden war, wurden sechs Kulturen pro Hefe unter den gleichen Bedingungen gezüchtet und die Proben zur gleichen Zeit gesammelt. Die Ergebnisse zeigten Gemeinsamkeiten und Unterschiede zwischen den Arten und Stämmen auf. Die biologische Variation war im Vergleich zu den Unterschieden zwischen den Spezies gering. Die drei *H. uvarum*-Stämme hatten ein ähnliches Profil. Mehr als 170 Lipide wurden annotiert. Zusätzlich

wurde eine chemische Anreicherungsanalyse für einen paarweisen Vergleich zwischen zwei Hefen (*H. uvarum* und *S. cerevisiae*) durchgeführt, die es erlaubte, Ähnlichkeiten und feine Unterschiede zwischen Verbindungsklassen hervorzuheben und Schlüsselverbindungen zu identifizieren, die für weitere entomologische Studien in Betracht gezogen werden könnten.

Zusammengenommen bieten die Studien der vorliegenden Arbeit umfassende Einblicke in die Assoziation zwischen Hefen und *D. suzukii* und deren Bedeutung für Kontrollstrategien. Die Erforschung der Auswirkungen verschiedener Hefearten auf die biologische Fitness des Insekts sowie ihre chemische Charakterisierung liefern einen erheblichen Erkenntnisfortschritt über die Biologie des Insekts und über den Stoffwechsel der mit *D. suzukii* assoziierten Mikroorganismen. Schließlich legt die Bewertung der Wirksamkeit und Persistenz eines Prototyps eines hefebasierten attract-and-kill-Verfahrens die Grundlage für die zukünftige Entwicklung einer Formulierung, die im Feld eingesetzt werden soll.

7 Outlook

The studies of the present thesis provide extensive insights into the association between yeasts and *D. suzukii*. The research focuses on the chemical characterization of different yeast species and strains and on the study of their effect on the fitness of *D. suzukii* adults. Additionally, the study aims at developing and evaluating the efficacy of a yeast-based attract-and-kill formulation against this insect pest. Besides expanding the knowledge about the chemical composition of yeasts naturally occurring in association with *D. suzukii* and their effect on the feeding behavior of the fly, this work represents a starting point for the development of an innovative and sustainable control strategy, based on the association between yeast and *D. suzukii*, a mechanism whose chemical basis has been investigated in this thesis. This opens new perspectives for further studies concerning the complex biology of *D. suzukii* and the possibility to exploit interaction mechanisms existing in nature for controlling infestations.

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9 Appendix

TABLE S1: Standard parameters of the yeast cultures monitored after 30 h growth in YMM.

Yeast	Cells/mL	OD600	pH	CDW (mg/mL fermentate)
S.c. S288c	$5.50x10^{7}$	1.966	2.53	1.37
H.u. 1.21	$1.91x10^{7}$	1.515	2.88	0.62
H.u. 2.2	$3.90x10^{7}$	1.784	2.72	0.84
H.u. 3.4	$6.00x10^{6}$	1.458	2.76	0.79
C.s. 3.3	$2.25x10^{8}$	2.003	2.48	1.67
S.v. 1.23	n/a*	1.462	2.57	1.42
I.t. 2.1	$4.39x10^{7}$	1.788	2.71	1.12
M.p. 3.2	$1.44x10^{7}$	1.610	2.50	1.44

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

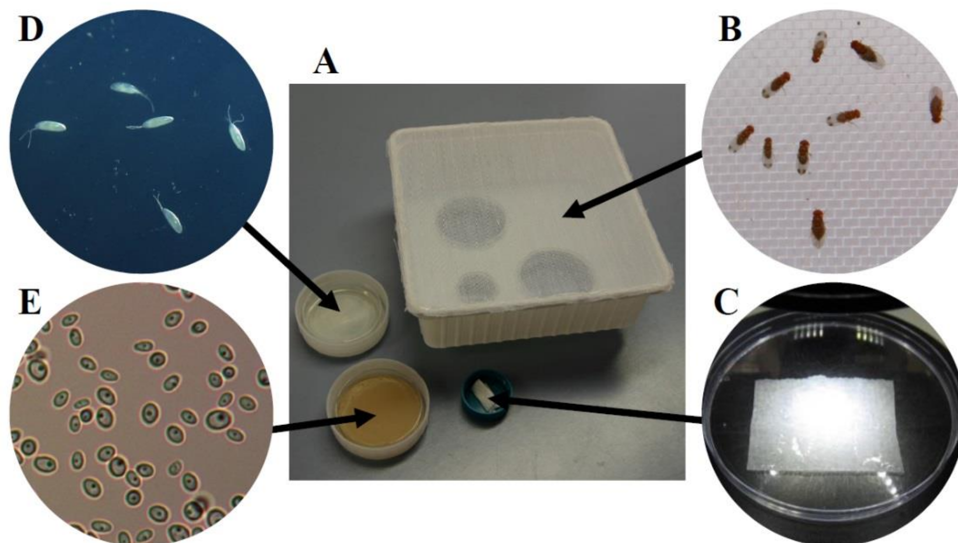

FIGURE S1: Cage design for the oviposition assays. (A) Experimental cages with three closeable openings with screw plugs on the bottom for changing the three components (Petri dish with agar and sucrose solution, Petri dish with yeast culture and a piece of paper towel with sucrose solution). (B) *D. suzukii* flies inside the cage sitting on the white mesh on the top of

the box, (C) paper towel as sugar source, (D) eggs laid in water agar, (E) yeast cells of *M. pulcherrima.* The paper towel was placed in the lid of a centrifuge tube and the petri dishes in the lids of two plastic cups. The lids match the three openings with screw plugs in the bottom.

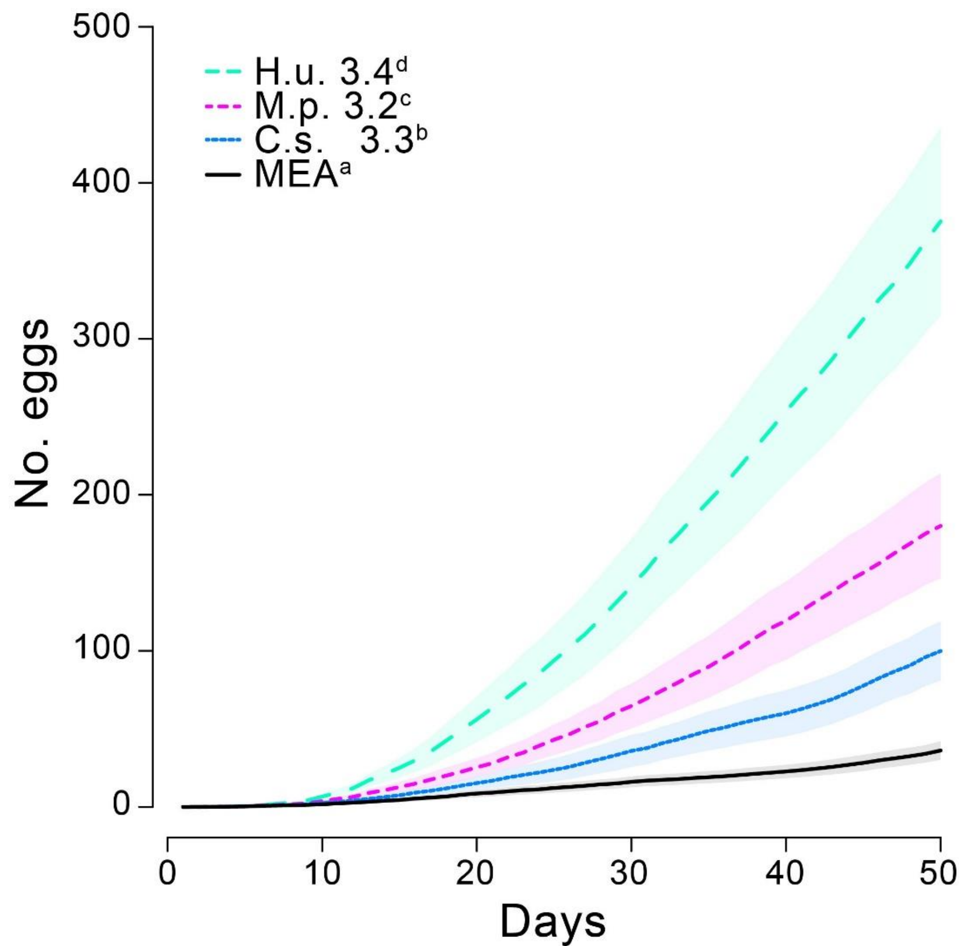

FIGURE S2: Oviposition by *D. suzukii* females fed with yeasts and MEA. Cumulative number (mean ± SE, n = 7) of eggs per female laid within the MEA assay over a period of 50 days. Significant differences ($p < 0.05$) between oviposition rate after feeding with the various diets are indicated using different letters.

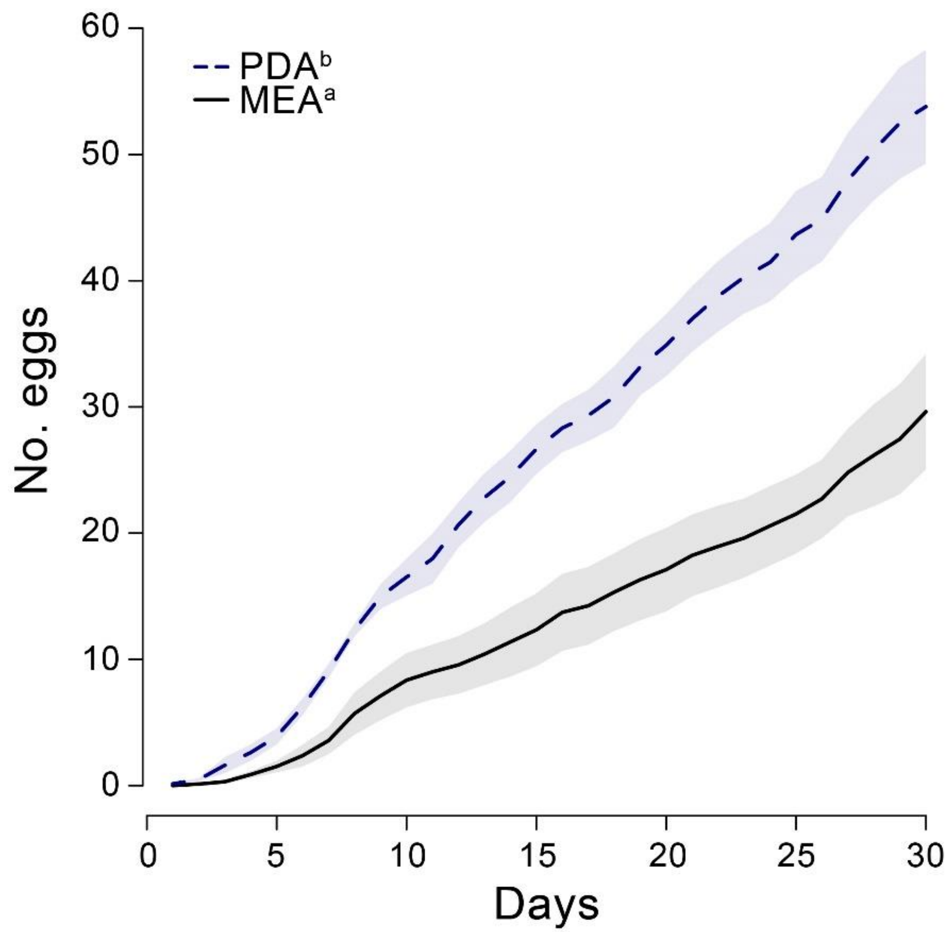

FIGURE S3: Oviposition by *D. suzukii* females fed with MEA and PDA. Cumulative number (mean ± SE, n = 5) of eggs per female laid within the MEA assay over a period of 50 days. Significant differences ($p < 0.05$) between oviposition rate after feeding with the two media are indicated using different letters.

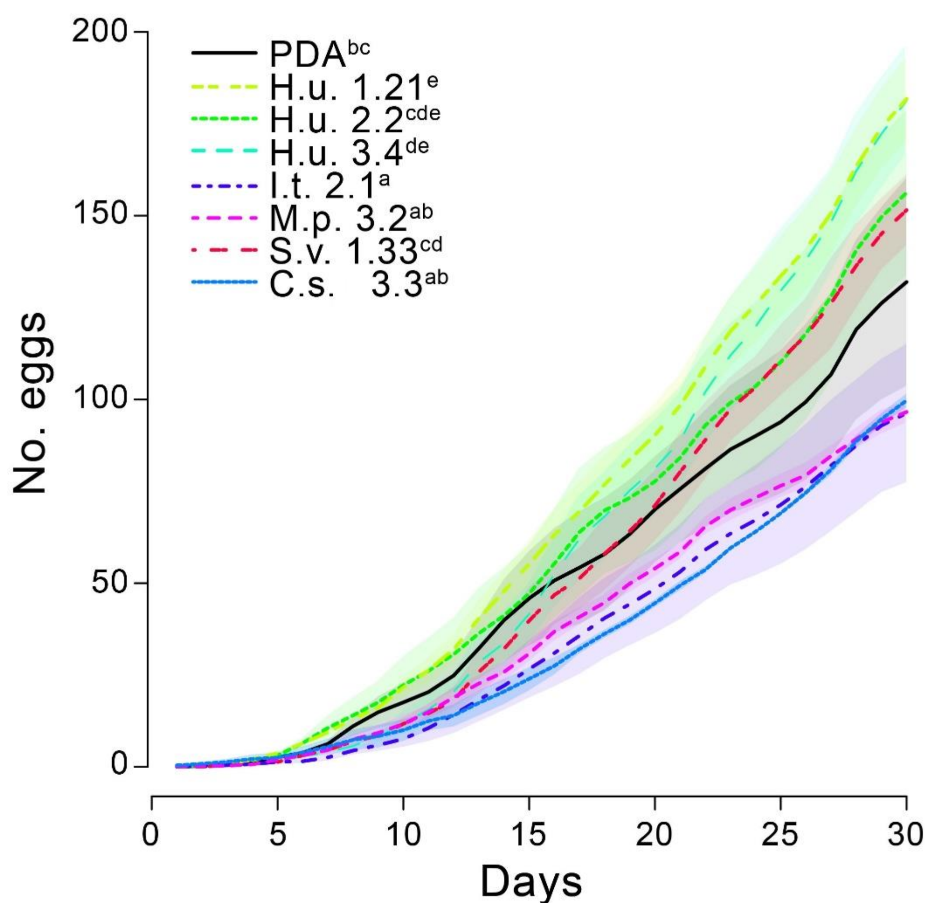

FIGURE S4: Oviposition by *D. suzukii* females fed with yeasts and PDA. Cumulative number (mean ± SE, n = 3) of eggs per female laid within the PDA assay over a period of 30 days. Significant differences ($p < 0.05$) between oviposition rate after feeding with the various diets are indicated using different letters.

TABLE S2: Daily mortality and daily ingestion (mean ± SD) per female (n = 20) fed with yeast fermentates and YMM.

	Day 1		Day 2		Day 3		Day 4	
Yeast	Ingestion (µL)	Mortality (%)	Ingestion (µL)	Mortality (%)	Ingestion (µL)	Mortality (%)	Ingestion (µL)	Mortality (%)
YMM	0.19 ± 0.33	0	0.46 ± 0.48	0	0.67 ± 0.58	5	1.66 ± 1.16	20
S.c. S288c	0.70 ± 0.12	0	0.13 ± 0.20	0	0.44 ± 0.61	40	n/a	60
H.u. 1.21	0.34 ± 0.84	0	0.27 ± 0.44	0	0.93 ± 0.89	0	1.59 ± 1.34	25
H.u. 2.2	0.00 ± 0.00	0	0.18 ± 0.27	0	0.72 ± 0.72	5	1.55 ± 1.34	40
H.u. 3.4	0.28 ± 0.31	0	0.29 ± 0.40	5	0.57 ± 0.69	5	1.88 ± 1.19	30
I.t. 2.1	0.12 ± 0.17	0	0.55 ± 0.34	0	0.66 ± 0.56	15	1.13 ± 1.10	25
M.p. 3.2	0.24 ± 0.29	0	0.05 ± 0.15	0	0.52 ± 0.47	15	0.14 ± 0.37	50
S.v. 1.33	0.03 ± 0.10	0	0.43 ± 0.42	0	0.76 ± 1.26	0	1.80 ± 1.27	15
C.s. 3.3	0.38 ± 0.25	0	0.40 ± 0.27	0	0.54 ± 0.52	25	0.46	70

TABLE S3: Mean (n=3) concentration of intracellular compounds in yeasts cultivated in YMM (mg/L ± SD). The first column indicates the compound class. Abbreviations: n.d. = not detected.

Compound class	Compound	C.s. 3.3	H.u. 1.21	H.u. 2.2	H.u. 3.4	I.t. 2.1	M.p. 3.2	S.c. S288c	S.v. 1.33
amino acids	leucine/isoleucine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0736 ± 0.0076	0.0117 ± 0.0030
	alanine	0.0634 ± 0.0049	0.0149 ± 0.0054	0.1102 ± 0.0151	0.1353 ± 0.0123	0.1885 ± 0.0145	0.7299 ± 0.0699	0.6392 ± 0.1320	1.9633 ± 0.4342
	arginine	0.2350 ± 0.0231	0.1609 ± 0.0250	0.2847 ± 0.0142	0.5917 ± 0.0254	0.3335 ± 0.0195	1.0299 ± 0.0615	2.1996 ± 0.0918	4.1857 ± 0.1734
	asparagine	0.0409 ± 0.0006	0.0415 ± 0.0013	0.0555 ± 0.0034	0.0633 ± 0.0022	0.0506 ± 0.0026	0.1105 ± 0.0137	0.3254 ± 0.0512	0.2986 ± 0.0323
	glutamine	0.0368 ± 0.0064	n.d.	0.7076 ± 0.0977	1.0101 ± 0.051	0.1909 ± 0.059	0.4585 ± 0.1379	1.7562 ± 0.5578	4.7615 ± 0.2829
	glycine	n.d.	n.d.	0.0263 ± 0.0028	0.0231 ± 0.0065	0.0129 ± 0.0043	0.0657 ± 0.0138	0.1239 ± 0.0298	0.7609 ± 0.1798
	histidine	0.0412 ± 0.0036	0.0229 ± 0.0049	0.0562 ± 0.0030	0.059 ± 0.004	0.0833 ± 0.005	0.0375 ± 0.0020	0.3213 ± 0.0087	0.3831 ± 0.0164
	lysine	0.0803 ± 0.0065	0.1800 ± 0.0256	0.3269 ± 0.0074	0.3454 ± 0.0183	0.2717 ± 0.0143	0.1731 ± 0.0140	2.1824 ± 0.0286	3.9174 ± 0.0787
	methionine	0.0372 ± 0.0023	0.0116 ± 0.0002	0.0313 ± 0.0018	0.0129 ± 0.0002	0.3326 ± 0.0988	0.6252 ± 0.1048	0.0688 ± 0.0123	2.4657 ± 0.4386
	ornithine	0.0353 ± 0.0014	0.0340 ± 0.0014	0.0482 ± 0.0013	0.0667 ± 0.0033	0.1113 ± 0.0051	0.0735 ± 0.0057	0.3359 ± 0.0095	0.2355 ± 0.0016
	phenylalanine	0.0023 ± 0.0002	n.d.	0.0035 ± 0.0004	0.0056 ± 0.0003	n.d.	0.0101 ± 0.0009	0.1301 ± 0.0129	0.1009 ± 0.0206
	proline	n.d.	n.d.	n.d.	n.d.	n.d.	0.0611 ± 0.0128	n.d.	0.1925 ± 0.047
	serine	0.0319 ± 0.0008	0.0351 ± 0.0021	0.0524 ± 0.0045	0.058 ± 0.0033	0.0433 ± 0.0034	0.1353 ± 0.0197	0.1720 ± 0.0323	0.5093 ± 0.0752
	threonine	n.d.	n.d.	0.0300 ± 0.0088	0.0628 ± 0.0178	0.0272 ± 0.0034	0.2533 ± 0.0798	0.5247 ± 0.1768	2.0134 ± 0.2441
	tryptophan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1295 ± 0.0177	2.3173 ± 0.1932
	tyrosine	0.0121 ± 0.0010	0.0127 ± 0.0021	0.0204 ± 0.0016	0.0161 ± 0.0007	0.0120 ± 0.0010	0.0145 ± 0.0017	0.4957 ± 0.0527	0.9728 ± 0.1239
	valine	0.0063 ± 0.0006	0.0133 ± 0.0039	0.0533 ± 0.0079	0.0835 ± 0.0036	0.0337 ± 0.0118	0.0970 ± 0.0291	0.4676 ± 0.1552	0.4215 ± 0.0647
	aspartate	0.0553 ± 0.0006	0.1196 ± 0.0011	0.1811 ± 0.0146	0.2473 ± 0.0286	0.1529 ± 0.0287	0.3479 ± 0.0928	0.5159 ± 0.0153	0.5283 ± 0.0276
	glutamate	0.0965 ± 0.0194	0.1819 ± 0.0390	0.5974 ± 0.0843	0.8529 ± 0.1069	0.6059 ± 0.0451	1.9617 ± 0.1917	3.5646 ± 0.3962	5.3102 ± 0.6502
carbohydrates	trehalose	0.0260 ± 0.0073	33.7923 ± 3.2995	49.9486 ± 2.4721	34.8014 ± 1.5837	18.1624 ± 0.1937	20.6474 ± 0.8967	10.2109 ± 0.3895	21.7390 ± 0.9418
	glucose	0.1344 ± 0.0245	64.4446 ± 4.0755	37.3445 ± 1.4954	55.5617 ± 9.6172	105.5552 ± 19.3374	37.7063 ± 7.1411	0.1234 ± 0.0113	111.8438 ± 1.4574
sugar alcohols	arabitol	1.1650 ± 0.0996	0.4593 ± 0.0603	0.7091 ± 0.0905	1.0100 ± 0.0588	2.2882 ± 0.0367	22.3870 ± 1.5629	n.d.	4.0723 ± 0.6130
	glycerol	4.3775 ± 0.4774	2.5156 ± 0.2921	3.7974 ± 0.2216	3.3686 ± 0.5765	1.6412 ± 0.3344	3.9603 ± 0.6785	8.7148 ± 0.6870	3.3882 ± 0.1851
	sorbitol	0.8801 ± 0.1005	0.0318 ± 0.0029	0.0747 ± 0.0147	0.0644 ± 0.0071	0.2695 ± 0.0557	3.5295 ± 0.2411	0.1019 ± 0.0187	0.5163 ± 0.0795
organic acids	lactate	0.0373 ± 0.0029	0.0703 ± 0.0136	0.0961 ± 0.0075	0.1166 ± 0.0269	0.2562 ± 0.0586	0.1598 ± 0.0190	0.4179 ± 0.0382	0.1944 ± 0.0035
	acetate	0.0211 ± 0.0048	0.1360 ± 0.0430	0.0869 ± 0.0139	0.0790 ± 0.0180	0.1522 ± 0.0263	0.1865 ± 0.0034	n.d.	0.1543 ± 0.0082
	formate	0.0072 ± 0.0013	0.0783 ± 0.0072	0.0671 ± 0.0021	0.0469 ± 0.0098	0.0567 ± 0.0100	0.0860 ± 0.0350	0.0382 ± 0.0112	0.0729 ± 0.0235
	pyruvate	0.0145 ± 0.0023	0.1751 ± 0.0299	0.2423 ± 0.0139	0.3468 ± 0.118	0.1259 ± 0.0172	0.2098 ± 0.0282	0.1788 ± 0.0205	0.2231 ± 0.0279
	succinate	0.2170 ± 0.0260	0.2501 ± 0.0110	0.4838 ± 0.0149	0.3692 ± 0.0653	0.3558 ± 0.0925	0.4808 ± 0.0771	0.1641 ± 0.0185	1.7527 ± 0.0937
	malate	0.0712 ± 0.0124	0.1994 ± 0.0375	0.3948 ± 0.0137	0.2645 ± 0.0317	0.3043 ± 0.0822	0.2865 ± 0.0539	0.2154 ± 0.0436	1.8669 ± 0.8480
	α-ketoglutarate	n.d.	0.0090 ± 0.0040	0.0282 ± 0.0013	0.0304 ± 0.0035	0.0339 ± 0.0074	0.1021 ± 0.0270	0.0448 ± 0.0042	0.2234 ± 0.0147
	fumarate	n.d.	n.d.	0.0431 ± 0.0042	0.0212 ± 0.0036	n.d.	0.1402 ± 0.0246	0.0219 ± 0.0012	0.3067 ± 0.0240
	citrate	n.d.	0.4189 ± 0.0724	0.6925 ± 0.0218	0.3982 ± 0.0513	0.2342 ± 0.0263	0.1592 ± 0.0292	0.1675 ± 0.0148	2.3894 ± 0.7494
	isocitrate	n.d.	n.d.	n.d.	n.d.	0.0654 ± 0.0098	n.d.	0.0356 ± 0.0070	1.1050 ± 0.2036
	cis-aconitate	0.0450 ± 0.0131	0.1496 ± 0.0028	0.2271 ± 0.0112	0.3041 ± 0.0161	0.1124 ± 0.0056	0.1799 ± 0.0233	0.2849 ± 0.0044	0.4893 ± 0.0554

TABLE S4: Mean (n=3) concentration of extracellular compounds in YMM and yeasts cultivated in YMM (mg/L ± SD). The first column indicates the compound class. Abbreviations: n.d. = not detected.

Compound class	Compound	C.s. 3.3	H.u. 1.21	H.u. 2.2	H.u. 3.4	I.t. 2.1	M.p. 3.2	S.c. S288c	S.v. 1.33	YMM
amino acids	leucine/isoleucine	n.d.	0.2033 ± 0.0404	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	alanine	0.1700 ± 0.020	n.d.	0.4400 ± 0.0100	n.d.	1.2900 ± 0.1418	0.0767 ± 0.0115	0.8000 ± 0.1253	1.0000 ± 0.1493	n.d.
	arginine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	asparagine	n.d.	0.6550 ± 0.3444	n.d.	0.9200 ± 0.1127	0.4767 ± 0.1328	0.5800 ± 0.1127	0.8000 ± 0.1300	n.d.	2.0833 ± 0.0231
	glutamine	0.2733 ± 0.0208	0.4667 ± 0.2376	1.9133 ± 0.1686	0.4833 ± 0.0751	0.7367 ± 0.1401	0.2533 ± 0.0231	1.1800 ± 0.2381	0.5833 ± 0.0351	n.d.
	glycine	n.d.	3.6200 ± 0.4757	3.1233 ± 0.1858	3.0500 ± 0.3219	4.2333 ± 0.7123	2.8967 ± 0.0306	4.7600 ± 0.4854	3.5700 ± 0.3378	3.7133 ± 0.5108
	histidine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.6833 ± 0.0611
	lysine	n.d.	n.d.	0.2133 ± 0.0681	n.d.	n.d.	n.d.	0.2233 ± 0.0404	0.2633 ± 0.1380	n.d.
	methionine	3.4533 ± 0.1464	n.d.	3.8033 ± 0.1793	n.d.	5.5800 ± 0.3897	3.5867 ± 0.0603	4.7533 ± 0.2237	3.2267 ± 0.0306	109.2000 ± 17.7203
	ornithine	n.d.	n.d.	n.d.	0.2433 ± 0.0153	n.d.	n.d.	0.2233 ± 0.0153	n.d.	n.d.
	phenylalanine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0767 ± 0.0153	n.d.	n.d.
	proline	1.855 ± 0.9445	5.9100 ± 1.5459	2.5933 ± 0.8113	4.0533 ± 0.6431	4.5667 ± 0.6710	2.9367 ± 0.1002	2.3767 ± 0.6361	2.4133 ± 0.6962	1.8200 ± 0.9339
	serine	2.4833 ± 0.4239	3.3000 ± 0.6265	2.7400 ± 0.1559	3.0633 ± 0.4899	2.9300 ± 0.4543	2.7867 ± 0.1815	2.7933 ± 0.3281	2.5100 ± 0.2663	2.0867 ± 0.0874
	threonine	1.7467 ± 0.0252	1.9767 ± 0.1250	1.9767 ± 0.1401	1.9833 ± 0.1955	3.9467 ± 0.4259	1.8400 ± 0.0954	2.0200 ± 0.0819	2.2333 ± 0.1721	n.d.
	tryptophan	0.2800 ± 0.0100	0.4033 ± 0.0416	0.3433 ± 0.0569	0.3700 ± 0.0557	2.1700 ± 0.3764	n.d.	3.0600 ± 0.8067	0.5933 ± 0.0321	27.1967 ± 4.6641
	tyrosine	0.1300 ± 0.0173	0.2167 ± 0.0404	0.1600 ± 0.0173	0.1567 ± 0.0115	0.1633 ± 0.0153	0.1333 ± 0.0058	0.2533 ± 0.0451	0.1600 ± 0.0265	n.d.
	valine	n.d.	0.7100 ± 0.1300	0.7833 ± 0.1514	0.6900 ± 0.0656	0.7600 ± 0.0794	0.6633 ± 0.0321	n.d.	0.8333 ± 0.0503	n.d.
	aspartate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	glutamate	1.5533 ± 0.2554	n.d.	n.d.	n.d.	n.d.	n.d.	2.4733 ± 0.1250	2.7167 ± 0.3953	n.d.
carbohydrates	trehalose	n.d.	17.3037 ± 0.3165	21.4953 ± 0.2971	13.7247 ± 0.0989	1.3860 ± 0.0778	5.4330 ± 0.1610	0.9850 ± 0.0719	6.7423 ± 0.1952	n.d.
	glucose	18.1527 ± 2.8789	11118.5547 ± 156.1750	6320.73 ± 23.8988	8996.1163 ± 67.2635	11919.695 ± 104.3465	5638.7267 ± 126.4182	1.3613 ± 0.2561	8840.0227 ± 145.5773	20535.5900 ± 742.2307
sugar alcohols	arabitol	105.4040 ± 1.1484	49.4457 ± 1.1940	53.7247 ± 0.3957	53.8873 ± 0.9228	12.4260 ± 0.1124	564.1557 ± 8.0433	n.d.	12.5310 ± 0.1570	n.d.
	glycerol	769.8673 ± 13.0734	442.7233 ± 13.9867	620.5487 ± 6.3764	528.8123 ± 9.4062	144.5223 ± 8.8352	465.6443 ± 14.3506	884.0333 ± 52.4647	257.2773 ± 13.5146	n.d.
	sorbitol	95.1377 ± 1.9137	1.9837 ± 0.0581	3.4767 ± 0.0289	2.0387 ± 0.1037	2.4677 ± 0.0883	50.7953 ± 1.0530	2.1133 ± 0.0314	3.5443 ± 0.2604	n.d.
organic acids	lactate	19.6260 ± 3.2949	15.2585 ± 7.7733	19.4653 ± 3.1567	25.4013 ± 2.3787	40.0103 ± 3.5694	12.3062 ± 2.6812	52.6435 ± 3.3960	12.0647 ± 0.9594	n.d.
	acetate	48.5022 ± 4.8462	136.3233 ± 69.7161	120.2737 ± 20.6451	106.7273 ± 21.1351	135.8755 ± 11.6822	42.1145 ± 8.303	197.5570 ± 11.7501	20.2232 ± 1.3034	n.d.
	formate	n.d.	n.d.	n.d.	n.d.	3.5537 ± 0.8121	5.1493 ± 2.0087	n.d.	7.9853 ± 2.0200	12.1155 ± 2.0809
	pyruvate	54.0095 ± 8.9967	64.5525 ± 34.0087	72.1868 ± 10.616	84.5752 ± 8.4351	21.6690 ± 2.3972	50.6428 ± 9.2687	20.8448 ± 3.2755	10.6088 ± 1.6099	n.d.
	succinate	59.6808 ± 6.6770	63.5568 ± 29.532	84.2870 ± 8.5965	75.1570 ± 10.1797	47.5182 ± 3.6683	73.5915 ± 11.919	10.7598 ± 0.3971	153.6698 ± 12.8328	n.d.
	malate	21.2020 ± 1.4122	39.8413 ± 18.4361	51.1950 ± 5.1862	40.7217 ± 5.9239	42.1712 ± 3.0151	32.3705 ± 5.7519	3.3738 ± 0.7391	82.5220 ± 9.0624	n.d.
	α-ketoglutarate	n.d.	n.d.	n.d.	n.d.	n.d.	26.4428 ± 5.0276	4.7148 ± 1.3157	14.1270 ± 3.6500	n.d.
	fumarate	n.d.	n.d.	n.d.	n.d.	n.d.	20.3043 ± 4.1570	n.d.	5.9835 ± 1.2598	n.d.
	citrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	isocitrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	cis-aconitate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

TABLE S5: Basic principle and calculation of free amino nitrogen based on single amino acids.

Calculation of Nitrogen from free amino acids	**Formula:**	$$\frac{\text{mg/L amino acid}_n \times \text{MW Nitrogen}}{\text{MW amino acid}_n}$$ This formula is applied to each free amino acid measured in LC-MS. The total amount of nitrogen is calculated by summing the contributions given by the single amino acids.
Measurement of free amino nitrogen (FAN)	**Principle:**	Primary amino groups (NH_2) are derived by a reaction of o-Phthaldialdehyde (OPA) and N-acetyl cysteine (NAC) to form isoindoles, that form a chromogenic complex proportional to the concentration of alpha-amino nitrogen in the sample. The reaction is measured using a spectrophotometer at a wavelength of 340 nm wavelength using a side wavelength of 700 nm. Proline does not contain any primary amino group and therefore is not measured using this procedure.

TABLE S6: Amount of nitrogen present in PDB and in fermentates made with yeasts grown in PDB calculated based on single amino acids and measured as free amino nitrogen (FAN). Data concerning free amino nitrogen were collected by Christof Sanoll (Wine and Beverages Laboratory, Institute for Agricultural Chemistry and Food Quality of the Laimburg Research Centre).

Sample	N from free amino acids (mg/L)	FAN (mg/L)	Difference (%)
PDB	94.3	106.5	11.5
C.s. 3.3	25.5	30.0	15.0
H.u. 1.21	63.5	77.0	17.5
H.u. 2.2	79.9	92.0	13.1
H.u. 3.4	86.4	95.0	9.0
I.t. 2.1	27.8	35.0	20.6
M.p. 3.2	32.1	41.0	21.7
S.c. S288c	40.7	51.0	20.2
S.v. 1.23	59.1	64.0	7.7

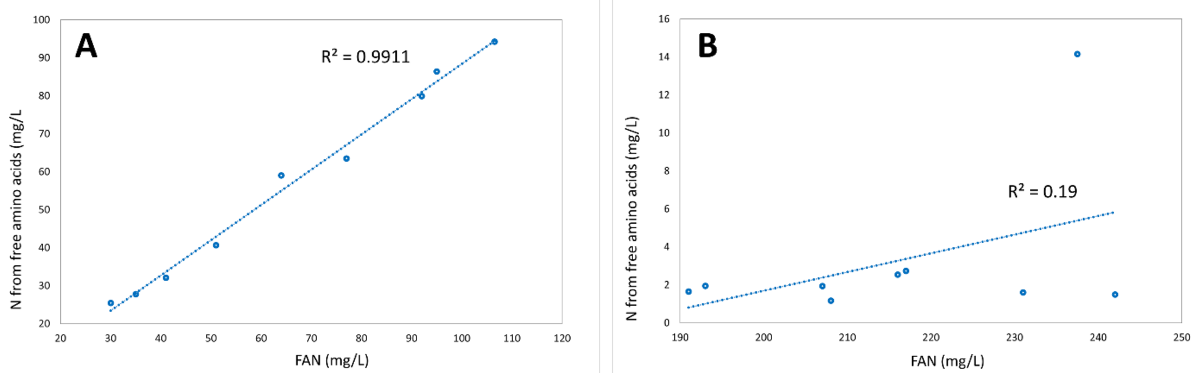

FIGURE S5: Linear regression between free amino nitrogen (FAN) and total nitrogen calculated based on single amino acids. (A) Correlation between the amino nitrogen amount found and calculated in PDB and fermentates of yeasts grown in PDB. (B) Correlation between the amino nitrogen amount found and calculated in YMM and fermentates of yeasts grown in YMM. Correlation coefficients (R^2) are reported for both curves.

TABLE S7: List of the lipids annotated (n = 171) and relative intensities in yeasts. Values are reported as peak height normalized using class-based IS.

Compound	C. s. 3.3	H. u. 1.21	H. u. 2.2	H. u. 3.4	I. t. 2.1	M. p. 3.2	S. c. S288c	S. v. 1.33
Cer(d34:0)	2646.22 ± 2557.24	79.76 ± 57.74	79.7 ± 29.14	91.33 ± 49.98	65.88 ± 54.22	74.83 ± 25.23	104.54 ± 45.02	73.93 ± 27.9
Cer(d34:1)	1133.78 ± 311.57	58.31 ± 21.75	46.32 ± 18.25	46.66 ± 11.02	23.56 ± 4.25	27.97 ± 5.23	41.55 ± 19.61	138.7 ± 23.25
Cer(d36:1)	232.17 ± 202.11	136.82 ± 100.19	60.69 ± 79.87	80.34 ± 99.96	101.95 ± 77.11	162.89 ± 32.98	24.32 ± 7.63	646.15 ± 156.37
FA(10:0)	0.47 ± 0.24	0.5 ± 0.15	0.41 ± 0.16	0.35 ± 0.12	0.79 ± 0.38	0.39 ± 0.13	2.25 ± 1.72	0.38 ± 0.13
FA(13:0)	3.69 ± 0.88	11.13 ± 10.11	3.16 ± 0.68	4.22 ± 1.59	5.42 ± 1.46	2.82 ± 0.4	4.03 ± 1.45	2.93 ± 0.72
FA(14:0)	140.58 ± 32.12	171.14 ± 76.84	48.42 ± 19.65	55.49 ± 12.79	67.28 ± 15.97	58.66 ± 12.13	84.4 ± 35.38	235.95 ± 49.49
FA(14:1)	104.27 ± 36.38	48.93 ± 31.9	10.29 ± 7.83	10.04 ± 6.73	3.23 ± 0.69	6.37 ± 1.56	59.7 ± 40.58	38.41 ± 23.91
FA(15:0)	37.66 ± 14.81	91.68 ± 99.48	14.14 ± 2.51	26.13 ± 13.51	54.45 ± 24.51	14.84 ± 2.32	22 ± 5.23	120.36 ± 28.37
FA(15:1)	11.59 ± 5.71	26.5 ± 34.41	1.29 ± 0.66	5.73 ± 4.92	3.13 ± 1.3	1.74 ± 0.54	6.91 ± 4.96	7.55 ± 1.62
FA(16:0)	2985.34 ± 631.57	6535.45 ± 3572.46	2752.12 ± 1295.31	2620.35 ± 463.86	6759.27 ± 4450.23	2061.68 ± 413.85	2255.91 ± 582.56	5869.1 ± 1078.86
FA(16:1)	6649.41 ± 2280.94	8284.53 ± 5813.02	4603.55 ± 3623.76	1572.06 ± 715.55	2302.57 ± 1372.47	419.11 ± 136.3	5361.99 ± 4264.39	6792.35 ± 3165.82
FA(18:0)	853.08 ± 109.23	1821.27 ± 2489.24	841.91 ± 136.41	805.79 ± 52.99	1958.3 ± 1962.94	1609.86 ± 1447.22	896.09 ± 235.77	2843.92 ± 2897.96
FA(18:1)	6356.07 ± 1164.39	3833.19 ± 2102.34	1760.55 ± 1135.14	1618.67 ± 212.12	17242.31 ± 14019.01	3336.42 ± 1445.63	4477.75 ± 3232.53	21110.6 ± 3447.3
FA(18:2)	11059.97 ± 2784.92	39.16 ± 36.7	8.6 ± 4.36	9.01 ± 3.09	14628.48 ± 18668.5	1731.89 ± 726.34	12.52 ± 5.03	15081.72 ± 3838.62
FA(18:3)	2039.91 ± 644.66	8.69 ± 3.41	3.58 ± 2.86	1.67 ± 0.69	2675.57 ± 3559.56	133.51 ± 43.39	1.62 ± 0.54	4003.05 ± 2419.87
FA(20:0)	166.05 ± 43.1	355.9 ± 106.49	217.85 ± 75.26	265.84 ± 36	214.84 ± 168.35	96.41 ± 12.23	174.69 ± 76.35	272.37 ± 50.4
FA(20:1)	125.01 ± 48.24	60.18 ± 20.43	27.63 ± 14.27	29.63 ± 14.43	988.88 ± 711.77	150 ± 45.2	74.33 ± 36.2	187.47 ± 41.49
FA(20:2)	27.62 ± 3.97	14.67 ± 19.73	0.72 ± 0.22	3.08 ± 3.48	138.65 ± 200.78	37.48 ± 11.36	2.89 ± 1.49	60.99 ± 25.07
FA(20:3)	16.27 ± 3.33	1.78 ± 2.12	0.23 ± 0.05	0.68 ± 0.64	56.71 ± 90.52	7.9 ± 2.84	1.45 ± 0.26	35.4 ± 22.01
FA(22:0)	70.41 ± 29.66	151.16 ± 26.06	85.1 ± 29.32	104.25 ± 16.08	95.32 ± 68.76	31.4 ± 3.23	49.08 ± 23.57	119.6 ± 25.43
FA(22:1)	8.48 ± 1.48	8.45 ± 3.56	3.72 ± 1.12	6.15 ± 1.38	48.16 ± 33.85	4.04 ± 1.08	6.37 ± 1.29	7.93 ± 2.34

FA(24:0)	386.45 ± 393.3	155.61 ± 37.56	88.97 ± 33.84	96.89 ± 15.72	113.66 ± 74.31	52.28 ± 7.33	44.13 ± 11	248.39 ± 71.18
FA(24:1)	13.42 ± 6.93	5.24 ± 3.6	1.25 ± 0.41	1.88 ± 0.63	10.07 ± 5.3	2.98 ± 0.44	1.3 ± 0.47	3.75 ± 0.78
FA(26:0)	102.31 ± 157.33	634.1 ± 178.75	536.91 ± 252.95	447.98 ± 48.01	740.45 ± 586.4	19.24 ± 3.45	235.12 ± 159.71	430.87 ± 111.57
FA(28:0)	5.79 ± 4.76	35.88 ± 10.34	45.42 ± 19.69	77.22 ± 10.64	77.68 ± 77.09	3.79 ± 1.8	8.33 ± 3.02	13.02 ± 3.34
Phytosphingosine (PHS)	10371.88 ± 3221.02	6381.53 ± 2449.31	6665.55 ± 3440.41	9567.98 ± 845.68	12741.85 ± 16091.96	1137.51 ± 100.14	1665.06 ± 967.12	7211.92 ± 2400.34
Dihydrosphingosine (DHS)	11477.8 ± 18435.53	3066.63 ± 1715.22	1835.55 ± 1140.2	1436 ± 456.74	2583.11 ± 2156.55	127.7 ± 35.6	500.78 ± 269.4	6946.31 ± 5824.5
Ergosterol	1791.86 ± 454.44	1320.67 ± 671.23	736.64 ± 158.06	484.39 ± 46.13	9050.32 ± 3686.77	6106.15 ± 1767.02	598.5 ± 105.48	11664.23 ± 5461.3
LPC(14:0)	229.13 ± 38.81	87.8 ± 59	85.12 ± 58.56	26.3 ± 9.56	7.92 ± 6.81	17.64 ± 4.57	227.84 ± 137.93	18.09 ± 9.05
LPC(15:0)	49.85 ± 12.69	2.13 ± 1.23	2.51 ± 1.22	1.87 ± 0.67	13.15 ± 13.33	3.5 ± 1.04	12.94 ± 7.3	7.61 ± 2.53
LPC(16:0)	2320.4 ± 525	2772.53 ± 1828	1831.91 ± 1303.31	888.46 ± 469.4	308.49 ± 310.1	322.86 ± 89.07	4022.59 ± 2010.44	126.17 ± 78.75
LPC(16:1)	4818.95 ± 1346.94	1789.86 ± 581.92	1845.42 ± 1417.06	2074.06 ± 867.84	20.17 ± 19.2	324.54 ± 62.17	5826.23 ± 3561.1	55.38 ± 31.52
LPC(17:1)	82.38 ± 25.64	1.66 ± 0.85	1.33 ± 0.57	1.89 ± 0.12	3.98 ± 3.26	11.58 ± 2.74	6.18 ± 3.03	3.97 ± 1.95
LPC(18:0)	131.03 ± 47.43	210.06 ± 123.54	121.64 ± 105.25	98.04 ± 28.02	159.21 ± 92.37	35.64 ± 13.58	715.35 ± 354.09	71.88 ± 29.84
LPC(18:1)	394.47 ± 122.55	60.16 ± 15.62	61.36 ± 36.97	66.51 ± 18.85	21.5 ± 15.83	636.86 ± 146.73	701.44 ± 352.84	23.93 ± 13.16
LPC(18:2)	1791.8 ± 589.3	2.79 ± 0.72	2.64 ± 0.51	3.24 ± 1.12	20.51 ± 24.79	4167.16 ± 1772.36	4.89 ± 1.73	28.79 ± 22.53
LPC(18:3)	425.38 ± 150.21	6.3 ± 5.72	3.92 ± 2.19	6.8 ± 4.65	12.65 ± 12.71	60.74 ± 15.12	4.11 ± 2.13	26.26 ± 22.32
LPC(20:4)	1.52 ± 3.24	2.97 ± 1.03	2.3 ± 0.49	2.31 ± 0.36	106.56 ± 208.8	4.18 ± 3.4	4.17 ± 1.31	4.39 ± 1.44
LPE(16:0)	1368.85 ± 209.83	3461.32 ± 2134.54	2860.24 ± 2510.25	1249.31 ± 497.7	2085.46 ± 1732.56	140.21 ± 36.41	1278.8 ± 604.09	389.68 ± 155.5
LPE(18:2)	2567.55 ± 608.18	33.51 ± 23.43	33.2 ± 24.36	13.12 ± 3.87	289.53 ± 163.97	380.07 ± 160.55	15.21 ± 5.77	111.4 ± 66.38
PI(28:0)	20.08 ± 12.19	11.34 ± 11.57	9.22 ± 3.83	9.65 ± 5.32	6.37 ± 3.4	6.86 ± 3.13	727.52 ± 405.36	16.58 ± 3.19
PI(32:1)	307.34 ± 227.09	1447.76 ± 610.69	1135.91 ± 766.77	1706.43 ± 313.12	605.81 ± 593.86	178.27 ± 59.21	1982.94 ± 1047.82	1524.94 ± 678.86
PI(32:2)	192.02 ± 140.71	128.72 ± 60.13	197.13 ± 164.82	302.39 ± 66.64	231.51 ± 210.7	49.92 ± 24.31	496.85 ± 293.29	538.46 ± 228
PI(34:1)	333.66 ± 177.67	389.62 ± 227.28	257.95 ± 255.37	215.06 ± 39.17	5139.05 ± 2983.23	1712.82 ± 311.17	1157.23 ± 316.28	9218.9 ± 5524.01
PI(34:2)	1030.97 ± 656.5	45.93 ± 22.88	73.17 ± 55.84	129.25 ± 41.47	3512.76 ± 1219.55	1659.98 ± 263.01	830.28 ± 463.05	5159.86 ± 1854.29
PC(28:0)	2013.41 ± 3327.11	1052.75 ± 928.73	1003.68 ± 944.05	426.95 ± 172.93	2.18 ± 0.45	2.25 ± 0.23	15679.61 ± 12483.01	74.33 ± 135.91
PC(14:0_16:0)	5831.19 ± 6869.38	1792.02 ± 1297.52	2115.86 ± 1652.82	670.14 ± 343.29	82.58 ± 58.36	250.79 ± 79.87	5514.63 ± 4339.09	1460.21 ± 1722.85

PC(16:0_14:1)	57333.33 ± 25593.88	17055 ± 14786.19	26990.25 ± 21668.04	20791.67 ± 14027.49	23.04 ± 17.74	2298.33 ± 977.37	58168.33 ± 47122.29	948.67 ± 801.42
PC(15:1_15:1)	37025.16 ± 11068.77	3827.14 ± 4672.25	4232.7 ± 3855.62	7787.18 ± 1690.54	37.97 ± 16.06	454.06 ± 187.4	15054.66 ± 14418.81	88.97 ± 55.95
PC(15:0_16:1)	5128.35 ± 1209.42	261.83 ± 358.3	221.8 ± 224.22	570.21 ± 189.73	46.61 ± 43.96	277.88 ± 94.74	1746.24 ± 1409.08	413.97 ± 253.67
PC(32:0)	1126.51 ± 1439.09	2221.75 ± 1605.01	1917.83 ± 1968.51	765.63 ± 229.21	280.45 ± 303.36	235.42 ± 70.95	888.67 ± 684.4	1392.5 ± 1326.55
PC(16:0_16:1)	223438.64 ± 53994.76	254123.99 ± 217454.98	274488.1 ± 180163.31	346725.56 ± 81057.75	299.63 ± 345.12	69531.61 ± 21208.01	352853.1 ± 258586.94	11602.31 ± 10734.37
PC(16:1_16:1)	479078.52 ± 81301.18	370596.94 ± 265567.35	376735.87 ± 211050.14	728125.29 ± 71222.45	268.33 ± 309.32	86598.53 ± 30832.85	439262.83 ± 320186.89	9639.02 ± 7300.76
PC(33:1)	2047.71 ± 800.71	232.05 ± 160.82	187.02 ± 38.66	289.94 ± 89.17	123.56 ± 60.98	581.39 ± 419.1	907.53 ± 708.17	774.24 ± 469.88
PC(15:0_18:2)	27447.58 ± 7387.86	171.76 ± 222.18	196.74 ± 167.76	501.58 ± 115.69	58.14 ± 56.16	2081.22 ± 637.97	1016.52 ± 965.29	953.21 ± 691.02
PC(16:0_18:1)	172595.72 ± 57000.19	30655.78 ± 30100.93	35355.64 ± 26593.64	37936.59 ± 14233.21	1188.08 ± 1515.67	315418.33 ± 58800.57	212331.85 ± 155773.56	21593.9 ± 15832.86
PC(16:0_18:2)	398603.43 ± 85183.09	48896.92 ± 58833.2	58039.48 ± 45302.97	150499.18 ± 26797.82	1490.09 ± 2249.57	292688.4 ± 64677.74	275304.35 ± 212802.05	17424.2 ± 13976.71
PC(14:0_20:3)	617947.92 ± 112394.85	368.59 ± 405.74	342.22 ± 238.78	755.36 ± 230.22	357.08 ± 392.08	230302.38 ± 58236.74	900.43 ± 742.18	6427.79 ± 5079.88
PC(34:4)	1892.57 ± 332.47	984.97 ± 532.4	406.55 ± 486.88	441.3 ± 85.36	14.79 ± 9.83	463.63 ± 155.85	669.06 ± 187.58	67.12 ± 49.88
PC(35:1)	587.68 ± 194.63	18.34 ± 8.56	21.24 ± 6.34	23.8 ± 6.77	29.06 ± 10.66	743.61 ± 253.41	113.85 ± 86.26	88.12 ± 16.7
PC(35:2)	5594.7 ± 1579.89	22.48 ± 10.94	28.8 ± 12.23	34.97 ± 10.23	36.21 ± 42.45	10004.75 ± 2913.21	135.9 ± 106.47	132.42 ± 102.8
PC(35:3)	22448.9 ± 5996.12	7.56 ± 3.52	11.32 ± 3.49	9.49 ± 6.79	26.6 ± 25.92	15434.93 ± 4680.71	7.79 ± 4.66	273.77 ± 279.69
PC(35:4)	7275.06 ± 1443.64	169.62 ± 139.61	148.22 ± 55.69	243.04 ± 106.96	183.5 ± 125.91	14183.75 ± 4979.64	125.4 ± 94.67	943.71 ± 893.7
PC(18:0_18:1)	9515.79 ± 9963.61	990.99 ± 890.94	895.66 ± 593.36	1319.35 ± 370.49	179.34 ± 189.67	15865.53 ± 5213.23	23640.17 ± 18674.99	1546.45 ± 1062.72
PC(18:1_18:1)	31150.44 ± 23606.55	483.67 ± 485.16	845.91 ± 653.61	2088.3 ± 470.01	176.11 ± 235.79	219502.92 ± 56514.43	20715.35 ± 17071.19	1911.37 ± 1416.21
PC(36:4)	9676.21 ± 2983.38	15.12 ± 7.21	19.65 ± 5.28	29.36 ± 10.41	70.25 ± 72.92	16332.79 ± 4239.58	22.28 ± 10.74	611.79 ± 548.04
PC(36:5)	139184.15 ± 34367.17	44.3 ± 15.95	41.78 ± 7.62	36.96 ± 8.1	208.5 ± 245.09	47910.04 ± 13605.43	53.25 ± 17.05	2334.85 ± 2410.38
PC(37:3)	199.78 ± 37.59	10.52 ± 5.5	8.11 ± 3.19	8.42 ± 3.99	11.7 ± 6.77	312.16 ± 81.21	7.71 ± 5.02	13.19 ± 4.55
PC(38:1)	105.82 ± 105.82	84.35 ± 66.35	60.18 ± 35.59	49.55 ± 6.82	25.33 ± 8.17	54.89 ± 33.76	182.41 ± 134.45	28.92 ± 11.89
PC(38:2)	345.16 ± 368.13	17.38 ± 10.93	17.65 ± 10.74	25.77 ± 9.47	19.42 ± 15.8	144.07 ± 138.77	31.91 ± 18.76	36.01 ± 13.81
PC(18:2_20:4)	96959.96 ± 26766.47	58.7 ± 27.42	53.08 ± 28.63	81.09 ± 18.29	219.11 ± 327.89	395792.34 ± 57316.19	167.63 ± 115.01	4013.87 ± 3493.89
PE(32:2)	18448.84 ± 4924.76	3770.42 ± 3981.73	5052.66 ± 4528.53	10872.55 ± 3363.44	25.08 ± 19.5	987.09 ± 325.91	2512.4 ± 1850.65	342.81 ± 227.38
PE(34:1)	3993.03 ± 1105.42	2821.89 ± 1673.12	3195.29 ± 2098.89	6028.98 ± 1043.18	787.78 ± 932.69	2574.58 ± 488.11	3075.67 ± 1986.73	1432.61 ± 475.32

PE(16:0_18:2)	29132.78 ± 6503.15	1451.06 ± 1510.66	1954.15 ± 1752.69	4280.38 ± 1587.24	320.09 ± 551.8	14139.78 ± 4018.49	5655.77 ± 4083.73	2679.73 ± 1329.47
PE(36:1)	269.67 ± 196.42	15.34 ± 12.45	14.5 ± 9.13	36.81 ± 14.61	36.54 ± 22.71	107.79 ± 31.15	64.9 ± 42.96	59.75 ± 30.97
PE(18:0_18:4)	133.95 ± 41.04	163.6 ± 24.5	200.74 ± 96.52	188.95 ± 18.71	18.35 ± 8.75	135.83 ± 19.21	76.95 ± 50.16	43.21 ± 11.03
PG(34:1)	1092.5 ± 249.8	15.43 ± 8.72	16.14 ± 17.08	19.17 ± 13.03	53.78 ± 12.91	562.47 ± 89.72	27.41 ± 11.09	38.75 ± 15.52
PG(34:2)	3050.24 ± 573.79	8.44 ± 3.07	11.42 ± 12.76	9.47 ± 5.95	80.6 ± 39.97	662.3 ± 101.1	7.79 ± 2.09	52.72 ± 12.86
PS(16:0_18:1))	2409.5 ± 824.48	394.08 ± 200.23	617.19 ± 446.53	1097.63 ± 350.15	1488.58 ± 799.98	1439.73 ± 214.1	289.07 ± 111.25	988.38 ± 458.2
PS(16:1_18:1)	5839.47 ± 1759.03	144.49 ± 62.54	178.9 ± 94.57	372.62 ± 96.04	4883.72 ± 2620.41	2737.8 ± 375.35	725.72 ± 306.73	3387.15 ± 1025.69
PS(36:2)	28.35 ± 14.53	3.88 ± 1.43	7.96 ± 6.4	6.43 ± 2.46	27.8 ± 17.18	128.62 ± 18.1	5.48 ± 1.51	1675.01 ± 790.39
MG(20:0)	389.31 ± 151.06	364.14 ± 228.99	249.39 ± 234.83	644.87 ± 118.97	245.26 ± 255.2	182.01 ± 38.72	304.86 ± 203.19	480.39 ± 155.8
DG(10:0_16:1)	0.3 ± 0.17	1.63 ± 1.04	1.07 ± 0.57	1.03 ± 0.19	0.48 ± 0.21	0.19 ± 0.05	63.85 ± 43.75	0.26 ± 0.1
DG(14:0_16:1)	15.14 ± 3.67	165.95 ± 57.84	227.19 ± 124.38	151.18 ± 57.58	3.62 ± 2.59	1.18 ± 0.32	187.85 ± 136.48	9.44 ± 3.76
DG(16:0_16:1)	123.25 ± 15.33	2686.48 ± 1043.31	2522.62 ± 1247.16	2603.04 ± 417.55	41.74 ± 33.72	22.9 ± 7.87	1113.8 ± 832.98	128.83 ± 53.06
DG(16:1_16:1)	195.62 ± 49.71	1893.17 ± 905.07	2302.49 ± 1447.82	3609.26 ± 307.98	29.77 ± 22.46	12.82 ± 4.99	1271.02 ± 937.41	86.49 ± 29.55
DG(16:0_18:1)	349.38 ± 35.82	689.2 ± 401.09	585.59 ± 308.4	814.34 ± 149.24	345.73 ± 185.97	317.37 ± 78.82	1052.49 ± 748.82	693.66 ± 216.13
DG(16:0_18:2)	709.63 ± 72.64	665.78 ± 382.15	891.41 ± 698.94	2000.62 ± 289.29	666.87 ± 328.74	302.8 ± 69.77	2151.69 ± 1507.34	858.35 ± 271.47
DG(16:1_18:2)	639.4 ± 142.4	4.3 ± 1.16	5.42 ± 0.67	5.35 ± 0.64	325.51 ± 183.9	156.81 ± 36.13	8.57 ± 3.04	221.38 ± 87.88
DG(16:1_18:3)	132.05 ± 35.83	0.85 ± 0.53	1.62 ± 0.9	1.64 ± 0.34	63.64 ± 44.57	7.04 ± 1.8	0.26 ± 0.09	42.84 ± 11.78
DG(18:0_18:0)	414.49 ± 118.58	510.36 ± 57.2	309.36 ± 182.4	591.39 ± 45.65	457.17 ± 143.15	317.01 ± 56.94	396.54 ± 173.82	365.65 ± 75.83
DG(18:0_18:1)	16.95 ± 7.2	48.54 ± 27.9	34.78 ± 18.97	75.26 ± 15.52	30.47 ± 16.05	17.78 ± 4.84	359.58 ± 253.72	33.47 ± 8.61
DG(18:1_18:1)	32.1 ± 18.13	13.5 ± 8.89	14.89 ± 11.41	45.88 ± 11.11	60.02 ± 28.94	125.85 ± 40.28	271.07 ± 197.24	148.52 ± 67.84
DG(18:1_18:2)	104.59 ± 18.44	1.16 ± 0.62	0.92 ± 0.27	1.08 ± 0.14	272.2 ± 148.18	295.92 ± 76.37	1.74 ± 0.8	139.19 ± 51.93
DG(18:2_18:2)	112.16 ± 22.03	1.6 ± 0.62	1.2 ± 0.56	1.9 ± 0.37	334.91 ± 187.23	109.59 ± 24.84	0.73 ± 0.15	58.88 ± 21.84
DG(18:2_18:3)	108.63 ± 28.95	2 ± 2.75	1 ± 0.31	2.29 ± 1.86	272.85 ± 146.53	25.99 ± 4.75	0.99 ± 0.25	87.83 ± 33.3
DG(18:3_18:3)	8.97 ± 3.58	0.26 ± 0.09	0.4 ± 0.17	0.63 ± 0.17	28.74 ± 19.4	0.7 ± 0.05	0.33 ± 0.13	12.91 ± 6.43

TG(12:0_12:0_16:0)	50.58 ± 10.83	76.4 ± 49.29	40.98 ± 11.31	50.94 ± 13.91	55.87 ± 19.52	45.79 ± 13.55	3649.18 ± 2910.6	55.6 ± 8.17
TG(12:0_12:0_16:1)	77.9 ± 48.65	211.35 ± 163.31	122.3 ± 76.23	62.59 ± 19.95	44.7 ± 11.46	70.19 ± 45.43	10589.44 ± 7757.87	71.84 ± 42.44
TG(14:0_14:0_14:0)	109.66 ± 30.76	216.09 ± 99.29	172.45 ± 66.3	163.95 ± 33.85	85.26 ± 25.2	77.58 ± 12.97	9856.08 ± 7571.14	167.74 ± 53.52
TG(42:1)	12.43 ± 2.3	18.82 ± 5.91	12.96 ± 5.32	11.98 ± 5.27	11.11 ± 6.09	5.81 ± 1.52	412.17 ± 133.12	6.64 ± 2.55
TG(12:0_14:1_16:1)	91.51 ± 34.65	176.68 ± 106.81	228.68 ± 127.87	400.82 ± 84	115.77 ± 66	18.57 ± 5.29	19900.19 ± 14593.06	39.37 ± 10.51
TG(12:1_14:1_16:1)	28.23 ± 9.63	16.47 ± 5.64	29.51 ± 12.4	57.86 ± 16.6	49.26 ± 31.89	9.47 ± 2.48	907.15 ± 681.8	20.14 ± 6.15
TG(14:0_16:0_14:0)	156.34 ± 101	302.81 ± 198.76	107.09 ± 63.06	90.54 ± 19.7	60.62 ± 13.35	73.28 ± 14.37	6890.68 ± 4833.32	388.48 ± 148.3
TG(12:0_16:0_16:1)	468.47 ± 173.73	1388.56 ± 1173.16	938.55 ± 655.76	680.47 ± 181.13	203.72 ± 94.53	58.01 ± 14.1	40422.89 ± 29125.05	438.89 ± 137.29
TG(12:0_16:1_16:1)	430.17 ± 120.64	1696.82 ± 1484.97	2353.91 ± 1820.39	4051.65 ± 1218.25	294.64 ± 148.96	30.92 ± 9.73	31445 ± 23002.2	180.58 ± 60.16
TG(14:0_16:0_16:0)	310.03 ± 147.91	456.07 ± 297.71	176.04 ± 101.16	162.32 ± 45.03	90.14 ± 30.14	145.72 ± 30.04	1995.85 ± 1221.46	896.6 ± 316.23
TG(14:0_14:0_18:1)	2231.08 ± 565.21	3973.82 ± 2101.54	2938.62 ± 2009.87	1441.1 ± 337	226.8 ± 100.69	309.58 ± 85.42	17263.79 ± 11159.32	3905.45 ± 1696.16
TG(14:0_16:1_16:1)	5684.19 ± 1721.52	36988.68 ± 21145.05	39252.16 ± 25882.31	50950.58 ± 17679.37	832.89 ± 394.75	388.21 ± 177.32	72078.15 ± 51021.64	4771.49 ± 1726.67
TG(12:0_16:1_18:2)	1719.31 ± 535.08	3421.59 ± 2350.52	6781.3 ± 6576.26	14929.71 ± 5289.06	518.19 ± 247.61	138.7 ± 51.57	9475.84 ± 6770.63	611.21 ± 171.74
TG(46:4)	313.61 ± 119.71	20.26 ± 7.7	24.58 ± 10.82	30.39 ± 5.21	269.07 ± 155.44	23.97 ± 6.03	52.88 ± 32.62	69.42 ± 20.83
TG(46:5)	58.65 ± 17.9	6.58 ± 3.35	4.49 ± 2.8	4.46 ± 1.31	207.1 ± 152.49	6.85 ± 2.23	4.63 ± 0.56	12.55 ± 4.94
TG(48:0)	239.26 ± 99.69	524.97 ± 373.46	235.82 ± 249.87	155.35 ± 33.12	84.34 ± 29.99	170.67 ± 16.37	611.78 ± 350.49	1116.25 ± 293.03
TG(16:0_16:0_16:1)	8511.57 ± 2786.97	28415.45 ± 15998.39	16678.75 ± 12803.14	8431.18 ± 2190.43	452.46 ± 221.82	3203.17 ± 912.12	18994.01 ± 11288.7	21120.33 ± 8294.48
TG(16:0_16:1_16:1)	14637.21 ± 4815.79	119885.12 ± 48989.08	122913.77 ± 65036.39	140719.02 ± 24103.47	845.45 ± 479.73	2810.67 ± 994.79	67886.58 ± 41467.96	21778.79 ± 9961.88
TG(16:1_16:1_16:1)	9783.19 ± 3397.25	104706.41 ± 62169.26	138714.23 ± 110402.34	310805.07 ± 65753.76	918.92 ± 490.28	1237.38 ± 425.56	77256.23 ± 54380.15	6963.57 ± 3581.86
TG(14:0_14:1_20:3)	3452.47 ± 1227.36	150.89 ± 89.42	283.24 ± 243.8	783.97 ± 346.67	675.61 ± 364.3	371.51 ± 130.02	384.36 ± 263.52	1315.63 ± 370.92
TG(48:5)	598.07 ± 208.1	15.42 ± 4.51	15.34 ± 4.22	23.87 ± 4.58	258.6 ± 141.26	37.78 ± 15.8	12.58 ± 3.06	162.32 ± 35.71
TG(16:0_16:0_17:1)	542.5 ± 252.27	350.49 ± 355.68	71.19 ± 20.01	120.8 ± 58.05	114 ± 34.15	154.88 ± 37.34	364.81 ± 175.82	3270.63 ± 1946.79
TG(16:1_16:1_17:0)	1201.99 ± 394.62	481.92 ± 416	233.23 ± 150.98	443.25 ± 113.35	196.73 ± 103.21	209.59 ± 42.35	1234.17 ± 841.59	4091.89 ± 2334.46
TG(14:0_17:1_18:2)	1122.36 ± 465.56	189.08 ± 129.06	241.37 ± 203.91	712.78 ± 292.04	179.64 ± 103.55	131.2 ± 29.12	680.75 ± 483.02	1660.29 ± 1000.54
TG(50:0)	66.71 ± 32.28	128.28 ± 107.84	78.27 ± 61.28	57.1 ± 16.22	37.51 ± 13.7	57.33 ± 11.64	279.9 ± 206.05	549.25 ± 240.79
TG(50:1)	7213.84 ± 4479.38	10635.58 ± 9884.04	5668.69 ± 5400.44	4937.19 ± 1800.05	970.78 ± 471.43	12534.52 ± 3054.93	20747.85 ± 13756.27	41657.98 ± 15771.69

TG(16:1_16:1_18:0)	24301.02 ± 7248.69	51297.96 ± 24429.31	52054.64 ± 33017.59	89935.8 ± 19110.19	3171.83 ± 1933.22	20554.4 ± 4673.46	69521.91 ± 39991.01	55596.84 ± 17903.77
TG(16:1_16:1_18:1)	27651.95 ± 8146.48	37507.16 ± 15766.07	50103.6 ± 41220.3	144219.07 ± 36867.2	3893.51 ± 2513.5	12776.32 ± 4020.67	73573.15 ± 45301.39	38612.01 ± 17849.03
TG(16:1_16:1_18:2)	13587.79 ± 4376.9	202.91 ± 114.32	238.38 ± 195.74	699.26 ± 266.16	2092.42 ± 1206.09	2720.48 ± 816.66	499.34 ± 352.22	11065.69 ± 4372.72
TG(16:1_16:1_18:3)	3934.85 ± 1359.82	201.09 ± 123.62	232.06 ± 163.72	513.63 ± 187.84	663.38 ± 389.88	470.35 ± 122.03	47.56 ± 29.23	2821.44 ± 784.56
TG(17:0_17:0_17:1)	90.41 ± 51.68	80.9 ± 76.75	22.43 ± 6	30.01 ± 12.44	48.14 ± 14.6	63.02 ± 21.09	102.13 ± 53.31	525.16 ± 311.93
TG(17:0_17:1_17:1)	685.94 ± 277.93	148.97 ± 131.42	56.09 ± 25.54	123.68 ± 33.62	188.92 ± 108.17	569.04 ± 174.75	451.89 ± 323.03	1641.95 ± 1027.33
TG(17:1_17:1_17:1)	1456.06 ± 442.3	96.75 ± 78.18	60.19 ± 46.73	236.97 ± 98.66	345.46 ± 286.59	824.74 ± 235.79	297.46 ± 221.26	2535.56 ± 1478.47
TG(16:1_17:2_18:1)	1274.44 ± 497.04	24.54 ± 7.89	14.01 ± 4.41	20.04 ± 9.02	278.49 ± 216.42	418.01 ± 122.55	16.41 ± 5.02	1541.94 ± 955.62
TG(51:5)	510.85 ± 234.33	12.27 ± 1.2	11.46 ± 2.64	16.54 ± 5.44	111.83 ± 72.67	98.81 ± 42.7	9.83 ± 3.69	456.44 ± 286.89
TG(52:0)	45.8 ± 15.35	64.52 ± 23.4	39.54 ± 14.02	36.19 ± 10.48	40.37 ± 8.9	34.08 ± 12.61	573.47 ± 424.74	177.93 ± 51.11
TG(16:0_18:0_18:1)	1363.65 ± 352.75	1335.82 ± 921.01	616.89 ± 441.31	749.77 ± 243.98	537.5 ± 256.3	1517.97 ± 777.66	9683.9 ± 7967.15	8023.4 ± 3857.04
TG(14:0_18:2_20:0)	5100.86 ± 1696.63	7541.32 ± 6014.27	6337.23 ± 5187.86	20925.47 ± 8563.22	3122.74 ± 2021.12	31172.81 ± 7466.72	36337.98 ± 23616.96	24469.5 ± 10193.07
TG(14:0_18:2_20:1)	11742.54 ± 3525.08	5346.93 ± 2781.68	7130.83 ± 6951.55	29983.16 ± 9897.34	6442.58 ± 5658.72	35167.79 ± 6523.36	23258.73 ± 13191.39	37917.48 ± 15772.85
TG(16:1_18:1_18:2)	12703.38 ± 3887.01	82.49 ± 37.54	76.14 ± 32.62	204.3 ± 87.69	6974.84 ± 6732.15	18434.9 ± 5607.83	215.85 ± 136.21	35038.78 ± 18641.31
TG(16:1_18:2_18:2)	9572.29 ± 3136.02	77.41 ± 36.6	69.2 ± 37.28	213.22 ± 87.36	4481.8 ± 3765.1	4508.18 ± 1410.21	51.5 ± 28.54	14251.63 ± 8121.67
TG(53:2)	83.33 ± 29.48	35.94 ± 22.14	19.94 ± 8.06	27.14 ± 10.26	49.09 ± 40.58	135 ± 59.54	54.59 ± 36.2	119.62 ± 55.89
TG(17:1_18:1_18:1)	354.89 ± 113.61	54.09 ± 47.82	22.71 ± 7.41	38.01 ± 11.8	179.62 ± 167.04	1195.98 ± 293.06	135.69 ± 86.11	393.28 ± 241.8
TG(53:4)	380.31 ± 110.23	17.84 ± 13.59	7.75 ± 4.67	12.36 ± 6.1	163.6 ± 169.78	1006.01 ± 289.51	13.69 ± 4.87	317.22 ± 205.69
TG (53:5)	432.75 ± 145.65	10.93 ± 3.61	14.56 ± 7.81	8.25 ± 2.78	192.11 ± 211.7	571.96 ± 179.97	5.75 ± 1.36	264.4 ± 186.39
TG(54:0)	191.9 ± 78.4	238.03 ± 46.67	162.02 ± 59.21	294.11 ± 48.96	198.09 ± 40.88	133.44 ± 24.72	1132.81 ± 819.38	464.37 ± 99.83
TG(18:0_18:0_18:1)	200.78 ± 50.55	436.99 ± 251.65	175.87 ± 122.03	199.42 ± 44.17	143.8 ± 46	138.71 ± 55.86	1905.39 ± 1892.02	750.64 ± 274.68
TG(14:0_18:2_22:0)	406.2 ± 105.78	442.1 ± 288.87	262.91 ± 197.84	1055.1 ± 396.18	367.07 ± 179.27	1670.93 ± 795.53	6453.37 ± 4821.32	1805.74 ± 821.89
TG(18:1_18:1_18:1)	947.31 ± 289.77	181.25 ± 65.4	168.73 ± 134	789.41 ± 360.43	1303.56 ± 839.59	12180.09 ± 3470.34	2992.54 ± 1940.62	4306.57 ± 2307.63
TG(18:0_18:1_18:3)	1572.9 ± 559.44	43.8 ± 11.45	46.92 ± 11.98	47.66 ± 11.8	2418.42 ± 2229.12	18968.22 ± 4609.43	61.66 ± 35.2	6463.94 ± 3726.96
TG(54:5)	570.95 ± 200.86	9.95 ± 3.5	9.81 ± 3.06	12.88 ± 2.61	999.99 ± 1321.14	3828.18 ± 1271.53	12.49 ± 2.47	2009.76 ± 1419.52
TG(54:6)	1989.04 ± 1081.48	13.99 ± 3.61	11.89 ± 2.36	11.12 ± 4.03	4433.67 ± 5740.45	3768.9 ± 1296.72	14.16 ± 11.65	7998.82 ± 7170.17

TG(54:7)	1531.75 ± 725.5	11.02 ± 4.97	9.28 ± 3.57	13.59 ± 4.99	2897.01 ± 3400.97	763.67 ± 194.53	11.13 ± 3.46	5470.61 ± 7326.33
TG(56:0)	31.96 ± 20.1	75.42 ± 21.71	42.45 ± 21.59	35.92 ± 14.03	27.08 ± 8.8	29.4 ± 4.46	153.03 ± 85.7	280.68 ± 74.97
TG(18:0_18:1_20:0)	247.08 ± 52.18	1314.34 ± 908.16	1121.23 ± 894.54	673.77 ± 287.34	38.97 ± 18.71	150.01 ± 58.57	936.34 ± 666.98	759.49 ± 296.49
TG(18:0_18:2_20:0)	237.1 ± 72.5	345.31 ± 222.9	189.65 ± 124.61	233.4 ± 90.59	46.65 ± 19.5	93.42 ± 39.31	451.99 ± 339.71	421.31 ± 124.78
TG(56:3)	69.88 ± 22.89	16.47 ± 4.12	11.46 ± 3.4	18.12 ± 5.4	55.24 ± 43.75	130.48 ± 50.97	109.82 ± 72.76	81.64 ± 38.21
TG(56:4)	24.49 ± 14.07	6.77 ± 3.31	4 ± 2.78	9.59 ± 2.44	76.19 ± 76.05	338.21 ± 107.05	7.48 ± 2.73	46.06 ± 42.62
TG(56:5)	27.34 ± 13.75	6.26 ± 3.34	5.38 ± 2.61	9.56 ± 5.47	122.69 ± 125.7	275.74 ± 74.59	4.1 ± 1.49	69.45 ± 47.46
TG(57:1)	125.32 ± 24.19	35.52 ± 19.3	15.42 ± 4.67	25.13 ± 13.09	29.35 ± 9.62	30.35 ± 11.11	52.4 ± 37.86	315.19 ± 187.59
TG(57:2)	177.4 ± 96.43	18.9 ± 13.69	7.28 ± 3.14	12.15 ± 5.1	20.66 ± 8.71	29.94 ± 11.22	36.65 ± 27.01	194.09 ± 112.52
TG(58:0)	23.12 ± 7.91	86.72 ± 58.28	89.94 ± 102.15	44.42 ± 41.67	19.78 ± 9.71	24 ± 4.02	43.01 ± 29.63	246.32 ± 79.07
TG(18:1_20:0_20:0)	444.11 ± 109.96	6832.04 ± 3949.26	6443.71 ± 4812.37	4778.9 ± 1676.62	114.72 ± 78.38	732.83 ± 366.3	2052.48 ± 1416.9	3009.36 ± 935.06
TG(14:0_22:0_22:2)	750.51 ± 177.99	4515.1 ± 2759.81	4525.24 ± 3478.49	6986.51 ± 2366.72	150.7 ± 55.97	705.69 ± 278.51	2037.22 ± 1476.23	2823.16 ± 936.22
TG(58:3)	292.21 ± 103.24	15.05 ± 5.48	24.61 ± 19.12	48.64 ± 33.6	41.23 ± 17.59	143.3 ± 83.14	34.16 ± 29.12	259.55 ± 75.73
TG(59:2)	364.34 ± 168.84	22.45 ± 8.11	19.63 ± 7.18	21.22 ± 8.38	194.61 ± 132.35	97.51 ± 33.82	66.01 ± 44.84	1097.31 ± 564.02
TG(59:3)	205.09 ± 86.31	7.92 ± 4.57	3.91 ± 1.85	6.14 ± 2.43	60.76 ± 32.37	42.89 ± 21.82	7.85 ± 2.61	155.97 ± 81.11
TG(18:1_21:0_21:0)	183.61 ± 49.66	2055.97 ± 1264.39	2112.86 ± 1511.08	2432.99 ± 823.98	403.36 ± 231.62	156.18 ± 66.8	1884.99 ± 1280.24	5378.55 ± 1638.39
TG(60:2)	5.86 ± 2.44	9.5 ± 3.11	12.44 ± 6.34	17.56 ± 11.26	8.55 ± 7.2	8.34 ± 2.88	12.91 ± 4.37	19.44 ± 3.55
TG(60:4)	131.44 ± 56.13	7.38 ± 3.77	8.59 ± 6.61	8 ± 2.97	63.44 ± 20.46	137.96 ± 63.6	7.21 ± 3.78	220.72 ± 65.59
TG(62:1)	19.13 ± 9.78	169.42 ± 104.89	201.12 ± 144.06	357.39 ± 120.16	56.11 ± 42.55	13.66 ± 5.19	410.66 ± 273.1	555.37 ± 163.32
TG(18:0_22:1_22:1)	48.93 ± 23.33	153.74 ± 73.44	225.14 ± 180.53	738.35 ± 210.1	210.75 ± 148.62	60.78 ± 31.41	489.53 ± 336.98	975.89 ± 306.85
TG(62:3)	46.3 ± 26.08	9.62 ± 4.38	8.89 ± 3.61	15.72 ± 4	376.23 ± 343.47	70.67 ± 33	18.85 ± 11.11	646.37 ± 216.8
TG(62:4)	41.01 ± 22.59	4.93 ± 1.1	6.42 ± 3.98	8.17 ± 2.37	484.2 ± 431.1	40.05 ± 20.67	3.67 ± 3.31	613.23 ± 255.21

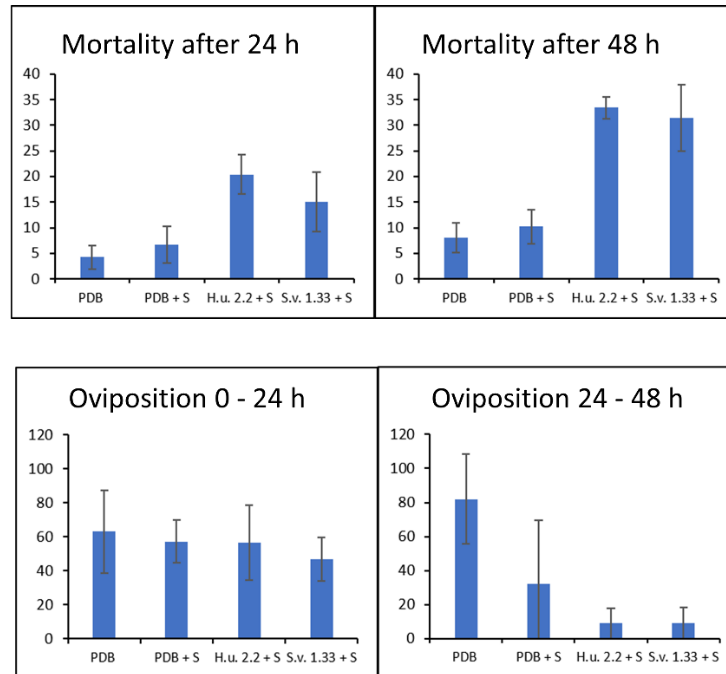

FIGURE S6: Results of mortality and oviposition trials with *H.uvarum* and *S. vini* one day after treatment. Mortality and oviposition (n = 5) of *D. suzukii* adults after 24 h and 48 h of exposure to leaves treated with potato dextrose broth (PDB), PDB plus spinosad (PDB + S), *H. uvarum* plus spinosad (H. u. 2.2 + S) and *S. vini* (S. v. 1.33 + S) one day after treatment (T1). Oviposition was measured once after 24 h and a second time between 24 and 48 h of exposure.

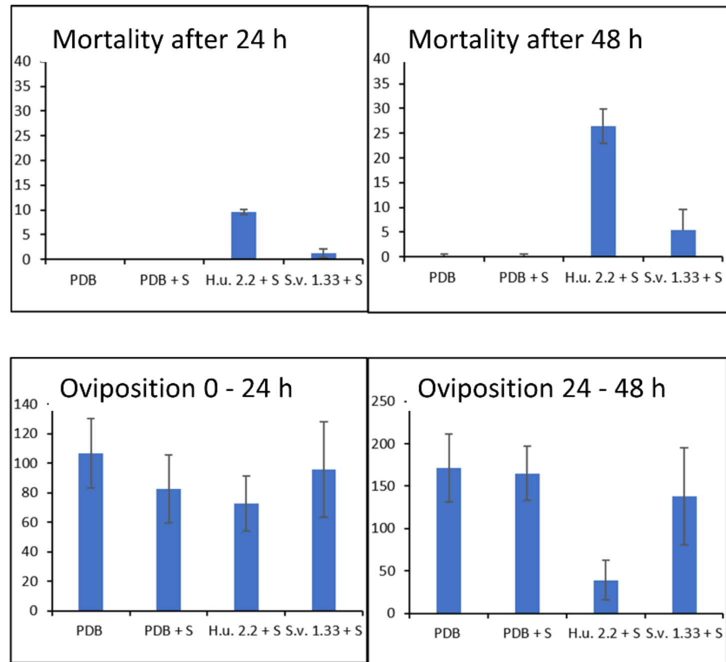

FIGURE S7: Results of mortality and oviposition trials with *H. uvarum* and *S. vini* one week after treatment. Mortality and oviposition (n = 5) of *D. suzukii* adults after 24 h and 48 h of exposure to leaves treated with potato dextrose broth (PDB), PDB plus spinosad (PDB + S), *H. uvarum* plus spinosad (H. u. 2.2 + S) and *S. vini* (S. v. 1.33 + S) one week after treatment (T2). Oviposition was measured once after 24 h and a second time between 24 and 48 h of exposure.

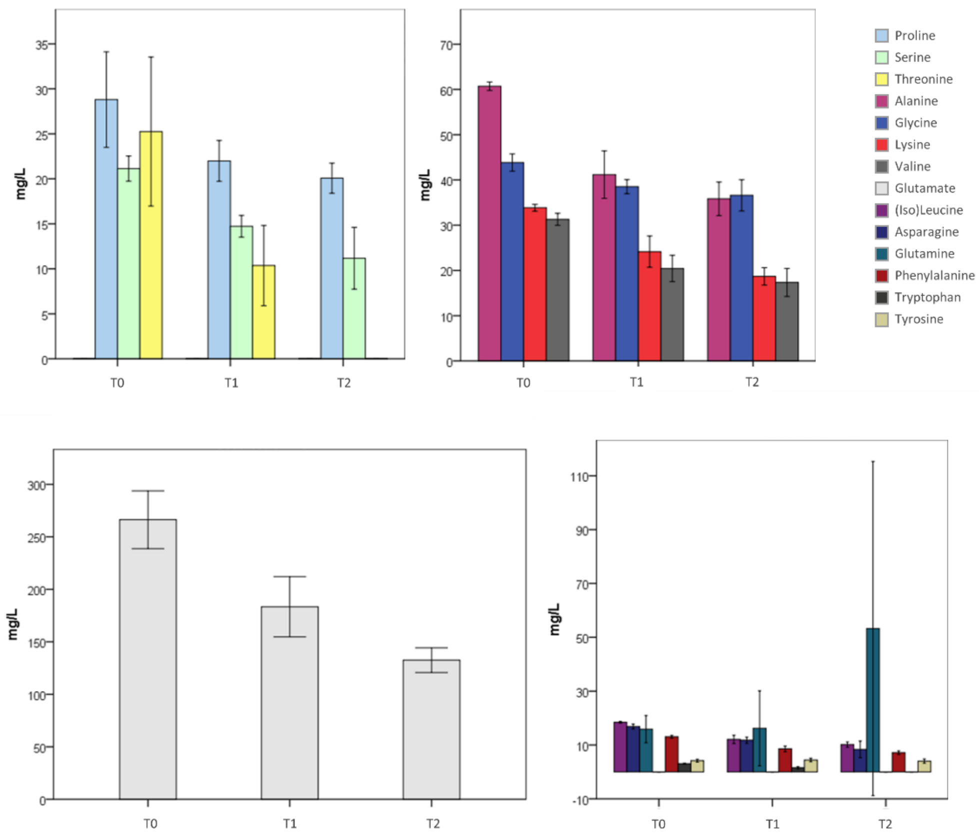

FIGURE S8: Amounts of amino acids in *S. vini* fermentate and on the surface of treated leaves. Concentrations (mean ± SD; n = 5) in the fermentate (T0) and on the surface of leaves collected one day (T1) and one week (T2) after treatment are reported.

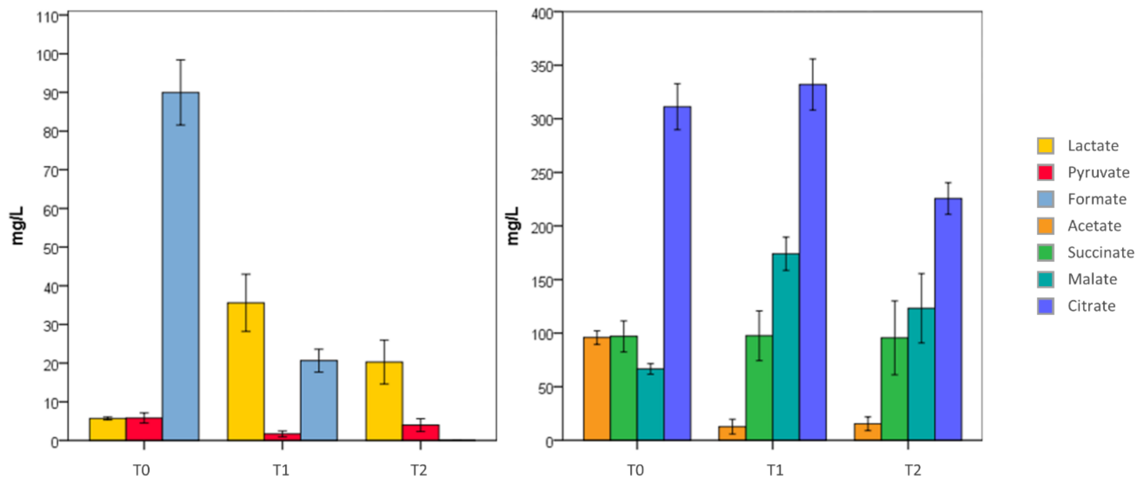

FIGURE S9: Amounts of organic acids in *S. vini* fermentate and on the surface of treated leaves. Concentrations (mean ± SD; n = 5) in the fermentate (T0) and on the surface of leaves collected one day (T1) and one week (T2) after treatment are reported.

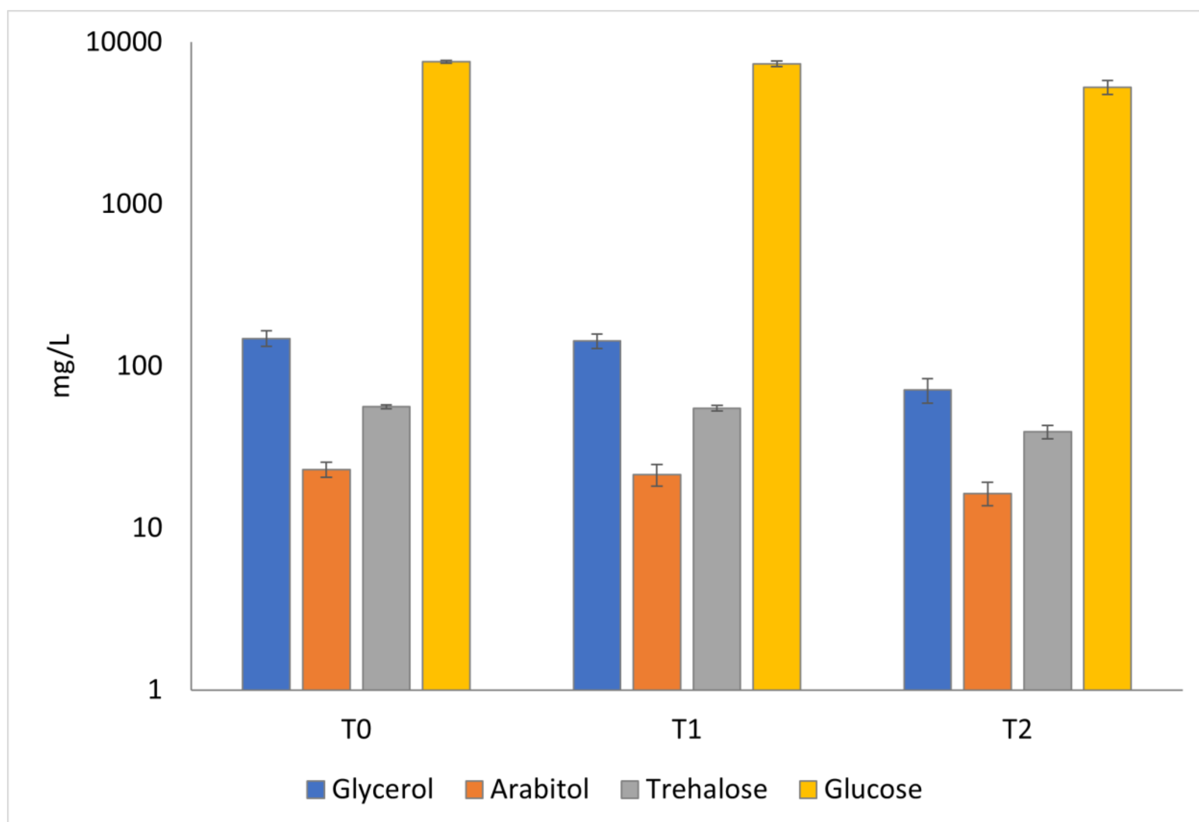

FIGURE S10: Amounts of carbohydrates and sugar alcohols in *S. vini* fermentate and on the surface of treated leaves. Concentrations (mean ± SD; n = 5) in the fermentate (T0) and on the surface of leaves collected one day (T1) and one week (T2) after treatment are reported.

10 List of publications

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First authorship publications in peer-reviewed journals derived from this thesis:

Bianchi, F.; Spitaler, U.; Castellan, I.; Cossu, C.S.; Brigadoi, T.; Duménil, C.; Angeli, S.; Robatscher, P.; Vogel, R.F.; Schmidt, S.; Eisenstecken, D. Persistence of a Yeast-Based (*Hanseniaspora uvarum*) Attract-and-Kill Formulation against *Drosophila suzukii* on Grape Leaves. Insects 2020, 11, 810; doi:10.3390/insects11110810.

Bianchi, F.; Spitaler, U.; Robatscher, P.; Vogel, R.F.; Schmidt, S.; Eisenstecken, D. Comparative Lipidomics of Different Yeast Species Associated to *Drosophila suzukii*. Metabolites 2020, 10, 352; doi:10.3390/metabo10090352.

Further participation in peer-reviewed journals derived from the collaboration with the Entomology group:

Spitaler, U.; Bianchi, F.; Eisenstecken, D.; Castellan, I.; Angeli, S.; Dordevic, N.; Robatscher, P.; Vogel, R.F.; Koschier, E.H.; Schmidt, S. Yeast species affects feeding and fitness of *Drosophila suzukii* adults. Journal of Pest Science 2020, 93, 1295–1309; doi:10.1007/s10340-020-01266-y.

Poster presentations:

Bianchi, F.; Spitaler, U.; Schmidt, S; Robatscher, P.; Eisenstecken, D. (2019). An untargeted lipidomics MS-based approach to identify bioactive compounds of different yeast species associated to *Drosophila suzukii*. 23.06.19-27.06.19, Metabolomics 2019, The Hague, Netherlands.

Bianchi, F.; Spitaler, U.; Schmidt, S; Robatscher, P.; Eisenstecken, D. (2019). MS-based approach to identify bioactive compounds of different yeast species associated to *Drosophila suzukii*. 27.05.19, 7th MS-J Day, Bozen, Italy.

Bianchi, F.; Spitaler, U.; Castellan, I.; Schmidt, S; Angeli, S.; Robatscher, P.; Eisenstecken, D. (2018). A metabolomic approach to evaluate the attractant and phagostimulant mechanism of different yeast species to *Drosophila suzukii*. 09.09.18-12.09.18, MOVISS: Metabolomic Bio and Data 2018, Vorau, Austria.

Oral presentations:

Bianchi, F. (10.09.2018). A metabolomic approach to evaluate the attractant and phagostimulant mechanism of different yeast species to *Drosophila suzukii*, MOVISS: Metabolomic Bio and Data 2018, Vorau, Austria.

Bianchi, F. (21.10.2019). Efficacy and persistency of a yeast-based "attract and kill" formulation against *Drosophila suzukii* on potted grapevine plants, Freising-Weihenstephan.

Participation to conference proceedings:

Spitaler, U.; Bianchi, F.; Castellan, I.; Rehermann, G.; Eisenstecken, D.; Becher, P. G.; Angeli, S.; Schmidt, S. (2019). An innovative management approach for spotted wing drosophila (*Drosophila suzukii*) using an environmentally friendly attract and kill formulation. In: Branco M.; Franco J. C.; Gross J. et al. (eds.).. IOBC-WPRS, Darmstadt, Deutschland, pp. 160-165. 20.01.19-25.01.19, Pheromones and Other Semiochemicals in Integrated Production and Integrated Protection of Fruit Crops, Lisbon, Portugal.

Student theses and contributions to this thesis:

Co-supervision of the Bachelor Thesis of Timothy Brigadoi at the University Claudiana (Bozen): " Misura di efficacia e persistenza di una formulazione a base di lievito e Spinosad contro la *Drosophila suzukii*: analisi dei costituenti chimici mediante cromatografia ionica e spettrometria di massa (Untersuchung der Effizienz und der Persistenz einer Formulierung auf Basis von Hefen und Spinosad gegen die Kirschessigfliege (*Drosophila suzukii*): Analysen der chemischen Inhaltsstoffe mittels Ionenchromatografie und Massenspektrometrie). The resulting raw data obtained in the experiments were partially incorporated into this thesis.

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