Project title:	**The use of highly attractive yeast strains for controlling *Drosophila suzukii* (spotted wing drosophila).**
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Project leaders:	Michelle Fountain (NIAB EMR) Matthew Goddard (University of Lincoln)
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Location of project:	NIAB EMR and University of Lincoln
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[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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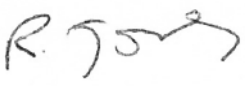
Signature  Date 24 Sep 2018

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CONTENTS

GROWER SUMMARY

Headline

- A number of yeasts have proved to be attractive to *D. suzukii* (SWD) in laboratory choice tests.

Background and expected deliverables

Since being identified in the UK in 2012 *Drosophila suzukii* – spotted wing drosophila (SWD) has started to cause commercial damage in soft and stone fruit plantations, resulting in yield losses and increasing expenditure on control methods. *D. suzukii* is currently controlled by plant protection products, crop hygiene measures and insect exclusion mesh. With more stringent measures being imposed on the use of plant protection products, often resulting in the withdrawal of particular products, combined with the threat of insecticide resistance from a limited number of active ingredients, new control methods need to be developed and optimised.

Complex interactions take place between fruit, microbial organisms and *Drosophila* species and understanding these is important in developing strategies for the control of *D. suzukii*. Yeasts are an essential source of nutrients for *Drosophila*. They are not only important for oviposition but also larval development. Some yeast species, most notably *Hanseniaspora uvarum,* are attractive to *D. suzukii* and have the potential to produce highly attractive and selective baits. Potentially, yeasts can be deployed in two distinct ways for controlling *D. suzukii*.

The first is precision monitoring, which traps numerous adult *D. suzukii* and is widely available and easily implemented, but is labour-intensive. To date this method has not been demonstrated to reduce crop damage. Trapping is recommended for the monitoring and detection of *D. suzukii* and lure-and-kill strategies and it could be used in integrated pest management of *D. suzukii*. However, more attractive and selective baits are needed to reduce the capture rates of non-target species. This would also make detecting *D. suzukii* females easier as they can be mistakenly identified for other *Drosophila* species without the aid of a microscope.

The second is the attract-and-kill technique, which combines yeasts with plant protection products to attract flies to the control agent. This technique offers potential within IPM programmes. This system may achieve a reduction of the volume of synthetic plant protection products applied whilst simultaneously increasing the targeted exposure of *D. suzukii.* It could also reduce the exposure to non-target species to plant protection products and reduce residues in fruit. A study of the literature and results from AHDB project SF 145 have

demonstrated that combining plant protection products with the yeast species *H. uvarum,* increases mortality and reduces egg laying compared to plant protection products alone.

Yeast has been widely used as an attractant in SWD monitoring traps. Dried baker's yeast has been typically used in fermentation-based baits. Since 2012, there has been a focus on the potential use of the yeast species *H. uvarum* in control strategies for SWD. Although, *H. uvarum* is known to be attractive to *D. suzukii*, not many other yeast species have been tested for attractiveness. This project will not only test the attractiveness of yeast species from an existing culture collection but also yeasts that will be isolated from ripening fruit (strawberries, raspberries, blueberries and cherries). Unlike the majority of *Drosophila* species *D. suzukii* oviposit in ripening fruit, so yeast from ripening fruit may not only be attractive but selectively attractive to *D. suzukii.* In nature microbes on the surface of fruit are complex and, currently, only single yeasts have been tested for attractiveness. This project will also test the attractiveness of combinations of yeasts.

The main aim of this project is to identify highly attractive yeast species alone and in combination and then utilise these in the control of *D. suzukii.* Additionally, this project will characterise microbial communities on ripening fruit and investigate identified yeast for attraction to *D. suzukii* as well as its potential use in control strategies.

Summary of the project and main conclusions

Three candidate yeast species that are attractive to *D. suzukii* have been identified; *Hanseniaspora uvarum*, yeast coded 218 and 190*. Drosophila simulans* was shown to be indifferent to all three of the yeasts while *D. melanogaster* was indifferent to two of the three. Both are common non-target species often captured in *D. suzukii* monitoring traps. Additionally, multiple strains of *H. uvarum* are also attractive to *D. suzukii*, a yeast species that in the context of *D. suzukii* has received a lot of attention in the literature and is known to be attractive to *D. suzukii*. This highlights the potential for yeast to produce attractive and selective baits for *D. suzukii.* This work will continue for a further two years.

Financial benefits

Drosophila suzukii is an economically damaging pest that causes yield losses in both soft and stone fruit crops. This project has the potential to improve *D. suzukii* control. The attractive yeast species and strains identified by this project could potentially be exploited in the monitoring and control of *D. suzukii* in IPM strategies to more effectively combat this pest.

Action points for growers

- At this early stage of this three-year project, there are currently no action points for growers.

SCIENCE SECTION

Introduction

Drosophila suzukii (Matsumra) is a highly polyphagous pest that is endemic to Southeast Asia and has recently spread around the globe. In 2008 *D. suzukii* was first detected on the American mainland in California (Bolda *et al.,* 2010). *Drosophila suzukii* then spread quickly to many other countries and is now also present in most northern temperate regions (Rota-Stabelli *et al.,* 2013; Asplen *et al., 2015*). It was first detected in the UK in 2012 (Harris and Shaw, 2014). It is likely that *D. suzukii's* global spread is due to human-aided transportation (Hauser, 2011; Calabria *et al.,* 2012). Since its recent spread around the globe, *D. suzukii* has caused massive economic losses. Total losses attributed to *D. suzukii* in 2008 in three states in the USA in strawberries, blueberries, raspberries, blackberries and cherries totalled $511.3 million (Bolda *et al.,* 2010). In Europe, *D. suzukii* causes substantial losses, with 80% losses to strawberry crops in a region in the south of France and between 60-80% losses of strawberry crops in Italy in 2010 and 90% losses of late-harvested cherries and 90-100% of blueberry crops being affected in some regions (Grassi *et al.,* 2011). Unlike most *Drosophila* species, *D. suzukii* oviposits in ripening fruit; this ability derives from female *D. suzukii* having a morphologically modified ovipositor (Atallah *et al.,* 2014). Oviposition can lead to mechanical damage in the form of puncture wounds in the surface of fruit. Once the larvae hatch they cause additional mechanical damage by feeding, resulting in unmarketable fruit (Goodhue *et al.,* 2011). The open wounds inflicted on the fruit allow secondary infection by bacteria and yeast species (Loriatti *et al.,* 2017). In addition to this, the damage inflicted on fruit by *D. suzukii* allows oviposition entry by other insects that lack the ability to oviposit in undamaged ripe fruit (Walsh *et al.,* 2011).

Microbes associated with fruit

Microbes are essential components of agricultural and natural ecosystems. Fruits provide conditions that support microbial growth with good water availability and access to sugars and other nutrients (Berg *et al.,* 2016). Microbes inhabiting the surface of plants form complex communities whilst fulfilling a variety of roles, some beneficial, some neutral and some pathogenic to plants. Several factors have an effect on fruit microbial communities; geographic location (Gayevskiy and Goddard, 2012; Taylor *et al.,* 2014; Bokulich *et al.,* 2014), plant organ, fruit ripening stage (Barata *et al.,* 2012), farming practices (Martins *et al.,* 2014), fruit species (Leff and Fierer, 2013) and even fruit variety (Cordero-Bueso *et al.,* 2011; Gayevskiy and Goddard 2012). Some studies reported little variation between fungal communities and geographic region in some fruit species but variation in others (Vepštaitė-

Monstavičė *et al.,* 2018). Niche generally explains more variance in fungal communities than geographic location (Morrison-Whittle and Goddard, 2015).

Yeasts, which are single-celled fungi, colonise various surfaces of plants and are more abundant and have greater community diversity on fruits compared to blossoms (Vadkertiová *et al.,* 2012). A small number of abundant taxa dominate yeast communities on fruit (Hamby *et al.,* 2012). *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Metschnikowia pulcherrima* and *Pichia kluyveri* were regularly isolated from various fruit surfaces; including apples, plums and pears using culture-based methods (Vadkertiová *et al.,* 2012). Apples are dominated by the fungal phylum Ascomycota and Basidiomycota (Vepštaitė-Monstavičė *et al.,* 2018). Analogous to bacterial populations on apples, little difference was detected between geographic location and fungal population on apples (Vepštaitė-Monstavičė *et al.,* 2018). Yeast communities change over time on nectarines (Janisiewicz *et al.,* 2010), plums (Janisiewicz *et al.,* 2014) and grapes (Martins *et al.,* 2014). Strawberries harbour a wide variety of fungal taxa (Abdelfattah *et al.,* 2016). Similar to apples, the diversity of fungal communities on strawberries differs significantly with fruit maturity and between different parts of the plant including, flowers and leaves (Abdelfattah *et al.,* 2016).

Yeasts associated with Drosophila.

Drosophila are saprotrophic and thus dependent on microbes for nutrition. Microbes are required for the development of both *D. suzukii* and *D. melanogaster* (Meigen) (Bing *et al.,* 2018). *Drosophila* are attracted to yeasts (Dobzhansky *et al.,* 1956) and yeast is used in fermentation baits to catch *Drosophila*. Female flies prefer to oviposit on yeast-colonised substrates (Oakeshott *et al.,* 1989), resulting in an increased development rate of larvae (Meshrif *et al.,* 2016; Bellutti *et al.,* 2018). The same yeast lineages are often present in *Drosophila* irrespective of species, geographical location or diet (Chandler *et al.,* 2012). In *D. melanogaster* survival rate and development time vary with yeast species availability (Meshrif *et al.,* 2016). Giving *D. melanogaster* larvae access to either *Pichia toletana* or *M. pulcherrima* decreased development time whilst simultaneously increasing survival rates. *Pichia toletana* was more beneficial than *M. pulcherrima*; in addition, choice tests revealed that *P. toletana* was more attractive to *D. melanogaster* larvae (Meshrif *et al.,* 2016). Both larvae and adult *D. melanogaster* engage in niche construction, manipulating yeast communities to differing degrees (Stamps *et al.,* 2012). Yeasts are immobile and therefore rely, in part, on insects to transport them to new habitats. *Drosophila* adults may vector yeast, potentially inoculating yeast onto new fruit surfaces (Gilbert, 1980; Starmer *et al.,* 1987; Stamps *et al.,* 2012; Buser

et al., 2014) with a significant effect on communities (Stamps *et al.,* 2012) and *Drosophila* have been shown to vector yeast in the laboratory (Christiaens *et al.,* 2014; Buser *et al.,* 2014). There are however limited studies demonstrating this in nature, but a strain of *Saccharomyces cerevisiae* that is attractive to *D. simulans* has been shown to be dispersed more frequently than an unattractive strain of *S. cerevisiae* in a vineyard setting (Buser *et al.,* 2014). Additionally, flies had higher fecundity when associated with more attractive yeasts (Buser *et al.,* 2014) and thus these studies provide some evidence to support the existence of a mutualism between yeast and *Drosophila* as both gain fitness advantages by interacting (Christiaens *et al.,* 2014; Buser *et al.,* 2014), but there is not enough evidence to assume that this mutualism extends to hold generally between yeast and *Drosophila* (Günther and Goddard, 2018). In addition, larvae have considerable effects on yeast species composition, reducing yeast diversity on fruits (Stamps *et al.,* 2012). Larvae and adults from four different *Drosophila* species had a preference for certain yeast species when given the choice; this preference differed between larvae and adults of the same species (Cooper, 1960). Stamps *et al.* (2012) also demonstrated that *D. melanogaster* larvae consistently encouraged the development of yeast communities comprised of three yeast species; *Candia cailfornica*, *Candidia zemplina* and *P. kluyveri* (Stamps *et al.,* 2012).

Yeast species vary in attractiveness to *D. melanogaster* with *Saccharomyces* species generally being more attractive than non-*Saccharomyces* species (Palanca *et al.,* 2013). Additionally, yeasts isolated from fruit were more attractive than non-fruit isolated yeast (Palanca *et al.,* 2013). *Saccharomyces cerevisiae* strains also vary in attractiveness to both *D. melanogaster* (Palanca *et al.,* 2013; Schiabor *et al.,* 2014) and *D. simulans* (Sturtevant) (Buser *et al.,* 2014). Only single yeasts have been tested for attractiveness to *Drosophila* species (e.g. Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015; *Scheidler et al., 2015*). It is not known whether there is a synergistic or additive effect of combinations of different yeasts, as occurs in nature, on attractiveness. In reality, microbial communities on fruit are much more complex (e.g. Taylor *et al.,* 2014). Yeast combinations warrant further investigation and may have the potential to produce both attractive and selective baits.

D. suzukii and yeast.

There are complex interactions between fruit, microbes and *Drosophila* species and understanding these interactions is important in the control of *D. suzukii* (Hamby and Becher, 2016). Yeast have been utilised for trapping *Drosophila* for a long time (e.g. Da Cunha *et al.,* 1951). Yeast can be exploited in baits to attract *D. suzukii,* with baits incorporating yeast capturing more *D. suzukii* than baits like apple cider vinegar (Iglesias *et al.,* 2014). *D. suzukii*

is associated with the yeast species *H. uvarum*; one study found, from the total 28 yeast species identified, *H. uvarum* was the dominant yeast species isolated from both *D. suzukii* larvae and adults, followed by *Issatchenkia terricola* and *P. kluyveri* (Hamby *et al.,* 2012). Non-Saccharomyces yeasts from the genera *Hanseniaspora*, *Pichia* and *Candida* have also been identified from the guts of adult winter morph *D. suzukii* (Fountain *et al.,* 2018). Hamby et al. (2012) affirmed that as the abundance of *H. uvarum* was higher in *D. suzukii* compared to other *Drosophila* species (Heed *et al.,* 1976) this could mean that there was a specific association between *D. suzukii* and *H. uvarum* making it an ideal candidate for a yeast-based bait (Hamby *et al.,* 2012). If this is the case, it could be fundamentally important in the creation of selective and attractive bait for *D. suzukii.* In addition, *H. uvarum* was the most attractive to *D. suzukii* when tested against multiple yeast species (Scheidler *et al.,* 2015). However, *H. uvarum* is common in the environment and on fruits. *Hanseniaspora uvarum* has been isolated from cherries, raspberries (Hamby *et al.,* 2012), apples, plum, pears (Vadkertiová *et al.,* 2012) and grapes (Barata *et al.,* 2012 and Gayevisky and Goddard, 2012). *H. uvarum* is also attractive to other *Drosophila* species, including *D. melanogaster* (Scheidler *et al.,* 2015; Palanca *et al.,* 2013; Hoang *et al.,* 2015), making it unlikely to be a species-specific relationship. However, as *H. uvarum* is attractive to *D. suzukii* (Scheidler *et al.,* 2015) it is still an important candidate yeast which warrants future investigation into its attractiveness to *D. suzukii*.

Drosophila suzukii control utilising yeast

Currently *D. suzukii* is controlled through the use of plant protection products, crop hygiene measures and insect exclusion mesh. With more stringent measures being increasingly implemented on the use of plant protection products, often resulting in the withdrawal of particular products combined with the threat of insecticide resistance from a limited number of active ingredients, new control methods need to be developed and improved.

There are two potential approaches for yeast in *D. suzukii* control. Firstly, mass trapping is a relatively cheap method of control which is widely available and easily implementable, but this method can be labour-intensive (Lee *et al.,* 2011). Trapping is essential for the monitoring and detection of *D. suzukii*, and greater incidence of *D. suzukii* have been recorded in spring when fruit infestation levels were low and vice versa in early July. This suggests lures may be less attractive than ripening fruit (Beers *et al.,* 2011) highlighting the need to develop more attractive and selective baits. Currently, baits based on fermentation products are often used to trap *D. suzukii*. Lure-and-kill strategies using traps are very important candidates in the IPM management of *D. suzukii*. However, for mass trapping more attractive and selective

baits are needed to reduce the capture rates of non-target species; this would also make detecting *D. suzukii* females easier as they can be mistakenly identified for other *Drosophila* species without the aid of a microscope. Therefore, reducing the numbers of other *Drosophila* species in traps with a bait that is selective to *D. suzukii* would make it easier to process trap captures (Hamby *et al.,* 2012). Secondly, one promising avenue of research is attract-and-kill strategies which combine yeast with pesticides thus attracting flies to kill them (Hamby and Becher, 2016; Mori *et al.,* 2016). This may enable a reduction of the amount of synthetic pesticide input generally whilst simultaneously increasing the targeted exposure of *D. suzukii.* This importantly means it may reduce the exposure to non-target species to pesticides and reduce residues on fruit. *Drosophila suzukii* females lay fewer eggs and mortality increases when they are exposed to *H. uvarum* combined with the Spinosad pesticide (Mori *et al.,* 2017) demonstrating the potential for utilising yeast combined with pesticides for control.

Aims

In this project I began to test the following null hypotheses:

H1-There is no significant difference between the attractiveness of different yeast species to different *Drosophila* species *(D. melanogaster*, *D. simulans* and *D. suzukii)*.

H2-There is no significant difference between the attractiveness of different *H. uvarum* strains to *D. suzukii*.

H3-There is no synergistic or additive effect of combining yeast species on the attractiveness to either *D. melanogaster*, *D. simulans* or *D. suzukii*.

H4-There are no significant differences in the yeast communities on ripening fruit, regardless of fruit type or stage of ripening.

H5-There is no significant difference between highly attractive yeasts or yeast blends to *D. suzukii* in comparison to currently available commercial baits.

The aims addressed by this report were;

1. Test attractiveness and repulsiveness of individual yeast species to *D. melanogaster*, *D. simulans* and *D. suzukii* in the laboratory.
2. Characterise yeast communities on ripening fruit, strawberries, raspberries, blueberries and cherries. Yeast associated with ripening fruit will then be tested for their attraction, both individually and in combinations, to *D. suzukii.*

Materials and methods

Drosophila maintenance

Three *Drosophila* species; *D. melanogaster*, *D. simulans* and *D. suzukii,* were used in these experiments. *D. melanogaster* originated from stocks provided by Prof. R. Newcomb (Plant and Food Research, New Zealand). *D. simulans* were from wild populations collected in the field from Kumeu River Winery, New Zealand by Dr C. Buser. *D. suzukii* cultures were derived from an Italian strain, which was established at NIAB EMR in 2013.

Summer morph *D. suzukii* populations were housed in BugDorm cages (32.5 x 32.5 x 32.5 cm) (MegaView Science Co., Ltd.) situated in a temperature-controlled room, which provided a constant temperature of 25°C and a 16:8 h light: dark photoperiod (Shaw *et al.,* 2018). Damp blue absorbent paper was placed on the base and roof of the cages to provide humidity (96%) inside the cages. *D. melanogaster* and *D. simulans* were maintained under the same temperature and light conditions as *D. suzukii* but cultured in standard *Drosophila* tubes (35 mL) (Gosselin FLY35-02, Fisher scientific). All *Drosophila* were fed on Drosophila Quick Mix Medium blue (Blades Biological Ltd) sprinkled with dried baker's yeast. *D. suzukii* were also cultured on media comprising of 100% distilled H_20, 1% agar, 9% sugar, 9% pre-cooked ground maize, 2% baker's yeast, 5% malt 1% soy flour, 0.3% propionic acid and 0.3% Methylparaben (methyl 4-hydroxybenzoate) pre-dissolved in 70% ethanol (as per Shaw *et al.,* 2018). Additionally, *D. suzukii* were also regularly provided with frozen raspberries (Shaw *et al.,* 2018).

Yeast cultures

Yeast species were cultured from the Goddard culture collection, University of Lincoln, stored on glycerol at -80°C, originating from different locations including New Zealand, UK and USA (Table 1). Yeast species were grown on YPD (yeast extract 1%, peptone 2%, dextrose 2%) agar plates at 30°C for 24 hours; if required, the growth period was extended for a further 24 hours.

Table1: Origin, source and strain of yeast species used in choice tests.

Yeast Strain	Origin	Source	Reference
EC-1118	France	Commercial wine yeast	Lallemand Inc.
162	New Zealand	Chardonnay juice	Anfang *et al.,* 2009.
218	New Zealand	Pinot noir ferment	Goddard culture collection
150	New Zealand	Beehive	Anfang *et al.,* 2009.
164	New Zealand	Chardonnay ferment	
212	New Zealand	Syrah fruit	Gayevskiy *et al.,* 2012.
198	New Zealand	Sauvignon Blanc ferment	Goddard culture collection
190	New Zealand	Sauvignon Blanc ferment	Goddard culture collection
166	New Zealand	Sauvignon Blanc ferment	Goddard culture collection
98-3	United Kingdom	*D. subobscura*	Goddard culture collection
44-1	United Kingdom	*D. subobscura*	
201	New Zealand	Chardonnay fruit	Gayevskiy *et al.,* 2012.
206	New Zealand	Chardonnay fruit	Goddard culture collection
209	New Zealand	Chardonnay fruit	
11-382	United States	*D. suzukii*	Phaff Yeast culture collection, UC-Davis
44-9	United Kingdom	*D. subobscura*	Goddard culture collection
28-1	United Kingdom	*Drosophila* sp.	
28-5	United Kingdom	*Drosophila* sp.	
28-9	United Kingdom	*Drosophila* sp.	

Ferment Preparation

A preculture was prepared using YPD media. Individual yeast species were transferred from YPD agar plates using a sterile inoculation loop to 15 ml falcon tube containing 3 ml of YPD media. This preculture was incubated at 30°C and 180rpm for 24 hours; the optical density was ascertained after inoculation and at 24 hours using a spectrophotometer (Jenway 6705). The ferments were then created by inoculating sterile strawberry juice with approximately 10^5 per ml yeast cells (Buser *et al.,* 2014, Günther *et al.,* 2015). Strawberry juice was cold-sterilised by filtration, where juice was passaged through a 0.2 µL filter system (Corning 1L Filter system 0.2 µL). Prior to being subjected to filtration, strawberry juice was pre-filtered through two layers of muslin to help reduce clogging of the filter which could potentially reduce

efficiency of the filtration process. The brix and optical density (OD) of the yeast ferments were determined at 48 hours. The brix was ascertained using a refractometer (HI 96801, Hanna instruments) and the OD was again measured using a spectrophotometer. One ferment was used per yeast, so as to reduce variation.

Choice tests

To ascertain the attractiveness of yeast species, two-way choice test experiments were carried out. Choice tests using T-maze, or similar variants, are often utilised apparatus to determine preference to various olfactory stimuli in *Drosophila* (e.g. Begg and Hogben 1946; Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015).

Two-way choice tests using multiple T-maze apparatus were employed to ascertain the attractiveness of yeasts (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al., in press*). Situated at each side of the T-Maze apparatus was 10 ml of 1:1000 dilution of either a yeast ferment or sterile fruit juice control. Experiments were run for 30 minutes in the absence of light to ensure that any choice made was done solely on olfactory cues (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015). T-maze position was randomised in regard to their orientation, in addition to treatments being reversed, to account for any directional bias in the room.

Flies were anaesthetised using CO_2 for a maximum of 10 minutes to separate out the number of females required and then starved for 24 ± 1 hours prior to the experiment start. For *D. suzukii* flies were starved for 17 ± 1 hours then anaesthetised and sexed. Flies were again anaesthetised briefly using CO_2 before being inserted in the centre of the T-Maze apparatus (Figure 1). Between 60-80 mated adult females between 3-12 days old of either *D. melanogaster*, *D. simulans* and *D. suzukii* were added to each T-maze. Each replicate run consisted of one replicate per yeast for all treatments; this was repeated six times. Thus, ensuring that each cohort of flies, bred from the original populations, was subjected to all treatments. Ensuring that the physiological state of each cohort of *Drosophila* was similar to reduce variation between yeasts tested.

After each replicate run, T-mazes were placed in a freezer to euthanise the flies prior to counting. To determine attractiveness of each yeast species an Attraction Index (AI) was calculated (AI = the total number of flies in the arm corresponding to yeast ferments subtracted from the number of flies in the arm corresponding to the sterile strawberry juice, divided by the total number of flies making a choice) (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015). After completion of the experiment the sliding doors of the T-maze were closed (Figure 1). Any flies that left the central compartment, passed either sliding door thus

occupying either arm, were deemed to have made a choice (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015). The data was analysed using the binomial distribution to determine whether the choices made by the flies differed significantly from a random distribution (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015).

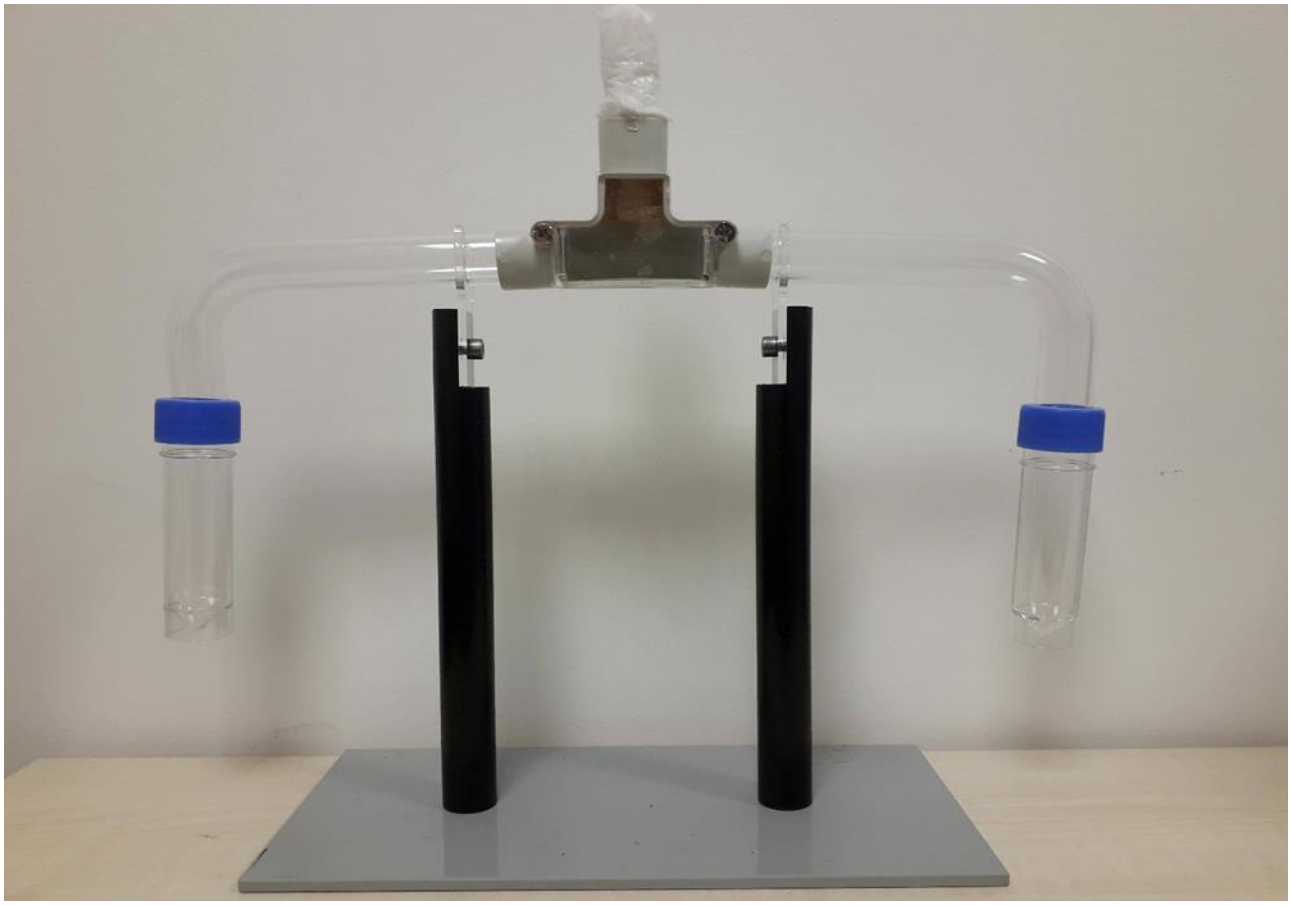

Figure 1: Set up of T-maze apparatus. 10 ml of 1:1000 dilution of yeast ferment or sterile fruit juice for a control was placed in the vials attached to the T-maze arms, a piece of circular mesh was placed in between the top of the vial and the end of the T-maze arms. This prevented the *Drosophila* individuals interacting with the ferments/fruit juice, whilst simultaneously allowing diffusion of odours from the different treatment throughout the T-maze. For all experiments using *D. suzukii* damp blue absorbent paper was included in the centre of the T-maze to increase humidity. *Drosophila* individuals were placed in the T-maze after being anaesthetised. A cotton wool plug prevented any flies escaping. The doors of the T-maze were opened in the absence of light, so as to prevent the flies making a choice based on visual cues.

Identification of yeast

Restriction Fragment Length Polymorphism (RFLP) which exploits homogenous DNA sequences using restriction enzymes that cut DNA at certain sequence of bases (Williams, 1989) was used to identify yeast isolates. The flanks of the internal transcribed rDNA spacer (ITS) regions are conserved across yeasts but the internal area is variable and thus may be exploited for identification. As well as absolute length, different restriction enzymes can be used to create fragmentation patterns; for example, restriction enzymes like CfoI, HaeIII and HinfI can be used to create a unique profile for many different species (Esteve-Zarzoso *et al.,* 1999). Here I used this method to create a library of fragmentation patterns to aid in the identification of any yeast isolated.

DNA extraction

Single colonies of yeast species, grown on YPD agar at 30°C for 24hours, were added to 10 µL of Zymolyase buffer and mixed 10 µL of 0.5U Zymolyase (an enzyme that attacks yeast cell walls) and then incubated for 30 minutes at 37°C after which they were placed at -80°C for 15 minutes and then heated for 10 minutes at 95°C. 80 µl of sterile water was added, then spun down and supernatant removed and stored at -80°C. Prior to storage the DNA was assessed using both a nanodrop and fluorescent assay.

Polymerase Chain Reaction (PCR)

PCR reactions were set up using 25 µl GoTaq master mix 5 µl of ITS1 and ITS4 primer mix, 16 µl autoclave water per reaction and 4 µl extracted DNA. Each reaction included a negative control (4µl autoclave water) and a positive control (*S. cerevisiae* DNA). The PCR programme used was as follows; 95°C for 2 mins, then 30 cycles of 95°C for 30 seconds 54°C for 30 seconds 72°C for 1.30 mins followed by a final extension time at 72°C for 10 mins. The PCR product was electrophoresed on a 1% agarose gel containing 10 µl sybr safe dye per 100 ml TAG buffer. The gel was run for 25 mins at 120V, after which the size of the PCR product was ascertained.

RFLP

PCR products were digested using *CfoI, HaeIII* and *HinfI* restriction endonuclease, (Esteve-Zarzoso *et al.,* 1999). Additionally, *Hanseniaspora* species were also digested using *DdeI*. For *HinfI*, 10 µL of PCR product was digested with 4 µL of *HinfII* (10,000 U/ml), 2 µL 10X CutSmart® Buffer and 4 µL water. For *HaeIII* 10 µL of the PCR product was digested with 0.4 µL of *HaeIII* (10,000 U/ml), 2 µL 10X CutSmart® Buffer, 7.6 µL water. For *CfoI* 10 µL of the

PCR product was digested with 0.5 µL CfoI (10U/µl), 2 µL 10x buffer B, 0.2 µL BSA and 7.3 µL water. For *DdeI* 10 µL of the PCR product was digested with 0.5 µL DdeI (10U/ul), 2 µL 10x buffer 0.2 µL BSA and 7.3 µL water. All these reactions were incubated at 37°C for 1 hour and then heated at 80°C for 20 mins to deactivate the enzyme. The restriction digest product was electrophoresed on 3% agarose gel (1g normal agarose + 2g ultra-pure agarose dissolved in 100ml TAE buffer). The number and size of fragments was recorded for each enzyme.

Microbes on fruit

Fruit samples were collected from four different ripening stages of strawberries, raspberries, blueberries and cherries (Figure 2). Samples were collected from three different locations in Kent. Four stages from all fruit cherry, blueberry, raspberry and strawberry were collected and processed (Figure 2). Ten fruit, 20 for blueberries, were aseptically removed from plants with as much of the stalks or calyx of the fruit being removed as possible without damaging the fruit epicarp. Six replicates of fruit species at each stage were used. After collection, the fruit was surface-washed with autoclaved water to remove microbes. The water used to wash the fruit was collected in 50 ml falcon tubes and centrifuged for 30 mins at 4,500rpm, whereupon the supernatant was discarded leaving approximately 2 ml in the tube. The pellet was then re-suspended in the remaining water and separated. 1 ml was added to 2 ml safe-lock tube and centrifuged for 30 mins at 13,000rpm whereupon the supernatant was discarded and the pellet frozen at -80°C for DNA extraction. Approximately 1 ml was mixed with equal parts 30% glycerol to create a 15% solution and frozen at -80°C for cultural-based analysis. 100 µL was added to 500 µL of YPD media and incubated at 30°C for 3 days. Cells were collected by centrifuging for 10 mins at 13,000, the supernatant was removed and the collected cells were re-suspended in 15% glycerol and stored at -80°C.

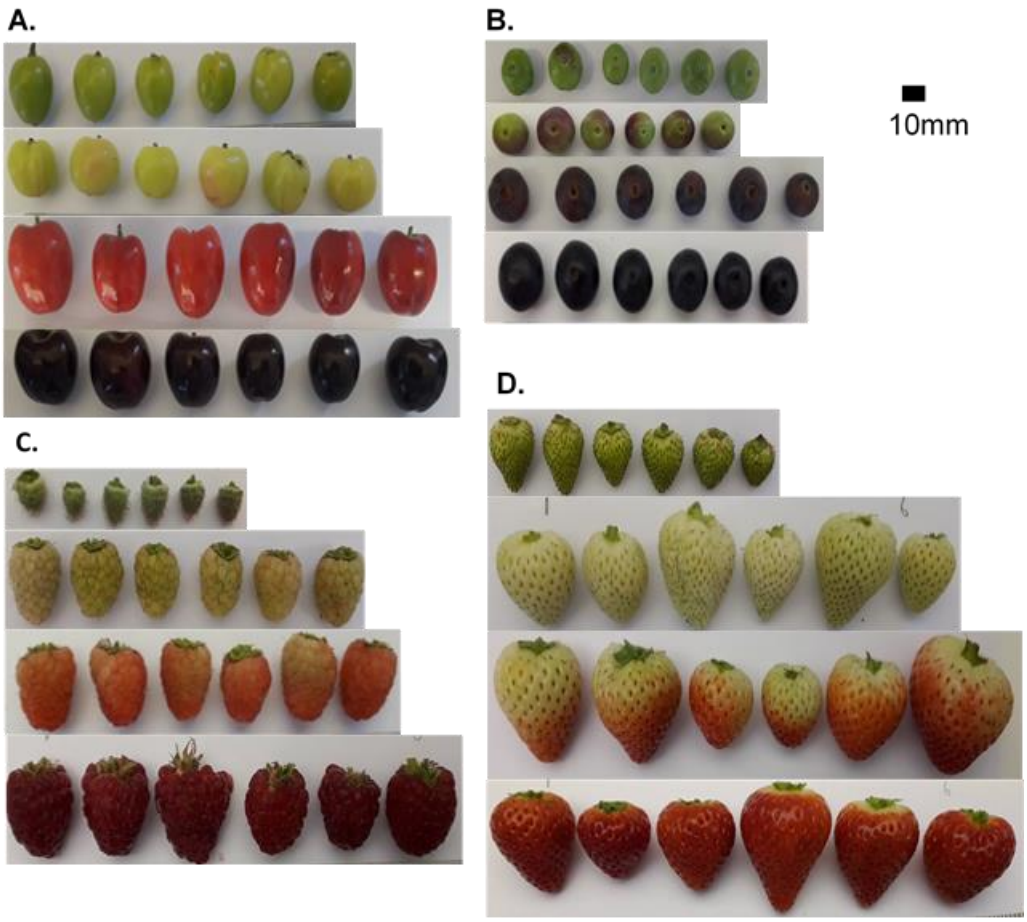

Figure 2: Four stages of fruit sampled and processed. A. green, white/pink, red and harvest (top to bottom) of cherry. B. green, green/purple, purple and harvest (top to bottom) of blueberry. C. green, white, pink and harvest (top to bottom) of raspberry, D. green, white and red/white (pink) and harvest (top to bottom) of strawberry.

Results

In the first year, at least some work has been completed to contribute to testing H1, H2 and H4; results are presented here.

H1-There is no significant difference between the attractiveness of different yeast species to different *Drosophila* species *(D. melanogaster*, *D. simulans* and *D. suzukii)*.

Two-way choice tests

In total, 12 different yeast species were tested for attractiveness to *D. suzukii*, *D. melanogaster* and *D. simulans* with two-way choice tests experiments; n=6 for all samples will be carried out. Choice test are ongoing, currently all replicates for *D. suzukii* have been carried out but only 5 for *D. melanogaster* and four for *D. simulans* (Table 1).

Drosophila suzukii had no preference for either side in the sterile fruit juice control; suggesting that there was no bias (AI=0.08, P=0.26). Of the 12 species tested against *D. suzukii,* three species were significantly attractive including *H. uvarum* (AI=0.25, P=0.0067), yeasts coded 162 (AI=0.28, P=0.0012) and 190 (AI=0.36, P<0.001), nine indifferent and none repulsive (Figure 3A).

Drosophila melanogaster had no significant preference for either side in the sterile fruit juice control; suggesting that there was no bias (AI=0.18, P=0.093). Of the 12 species tested against *D. melanogaster*, six were significantly attractive including yeasts EC-1118. (AI=0.30, P<0.001), 162 (AI=0.31, P<0.001), 150 (AI=0.20, P=0.0034), 212 (AI=0.19, P=0.0012), 190 (AI=0.088, P=0.0051) 166 (AI=0.36, P<0.001), six indifferent and no repulsive (Figure 3B).

Drosophila simulans also had no preference for either side of the apparatus in the sterile fruit juice control assays (AI=0.03, P=0.58). Two of the yeast species were significantly attractive to *D. simulans,* 164 (AI=0.14, P=0.023) and 212 (AI=0.21, P<0.001), ten species were indifferent, and no species were significantly repulsive (Figure 3C). It is worth noting that choice tests are ongoing with only five replications of each treatment having been carried out thus far for *D. melanogaster* and four for *D. simulans* to date. Therefore, this data should be treated with caution.

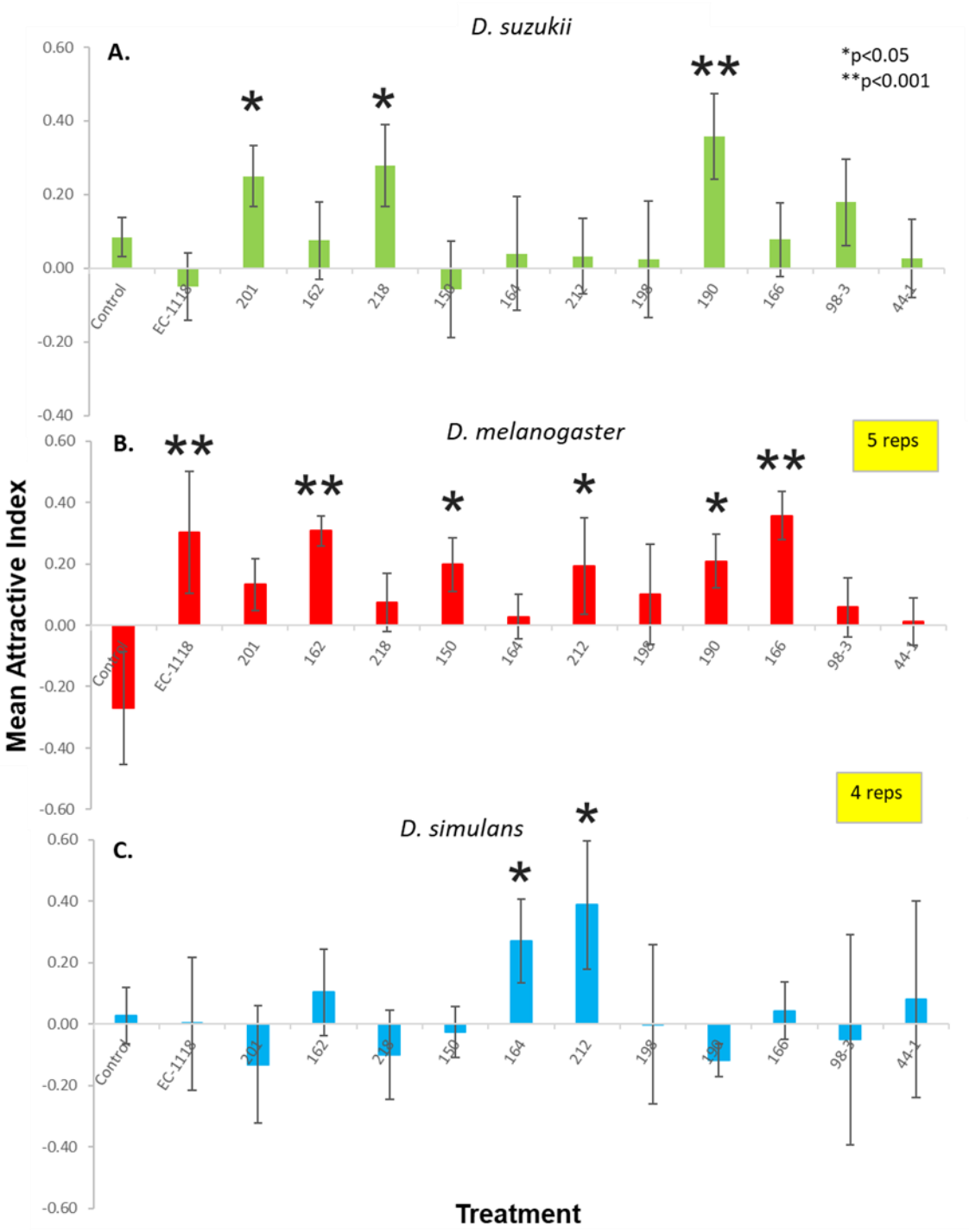

Figure 3: A. shows the attraction of various yeast species fermented in strawberry juice to *D. suzukii* (N=6). B. shows the attraction of the same yeast to *D. melanogaster.* C. shows the attraction of the same yeast to *D. simulans*. Attraction is shown as an attraction index, worked out as the number of flies choosing yeast divided by the total number of flies making a choice. Asterisks above or below bars represent any significant attraction. *denotes a p value less than 0.05 and **denotes a P value less than 0.001. Mean AI plotted ± Standard Error.

Only five replicates of *D. melanogaster* and four of *D. simulans* have been carried out so far.

Yeast species varied in their attractiveness to all three *Drosophila* species (Figure 3), thus hypothesis 1 was rejected. The only consistent trend of attraction of yeast species across the three fly species in regards to consistent attraction where one yeast species exhibits the same attraction, repulsion or indifference across all species was with the yeast species 193, 98-3 and 44-1 all indifferent to the three fly species.

H2-There is no significant difference between the attractiveness of different *H. uvarum* strains to *D. suzukii*.

The attractiveness of eight different strains of *H. uvarum* was tested to *D. suzukii*. *D. suzukii* had no significant preference for either side in the sterile fruit juice control (AI=-0.15, P=0.18). Of the *H. uvarum* strains tested for attractiveness to *D. suzukii*, six were shown to be significantly attractive to *D. suzukii H. uvarum* (201) (AI=0.50, P=0.0020), *H. uvarum* (206) AI=0.15, P=0.013), *H. uvarum* (209) AI=0.21, P=0.0023), *H. uvarum* (11-382) AI=0.21, P=0.024), *H. uvarum* (28-1) AI=0.18, P=0.036) and *H. uvarum* (28-5) (AI=0.20, P=0.025) and two were shown to be indifferent and non-repulsive (Figure 4).

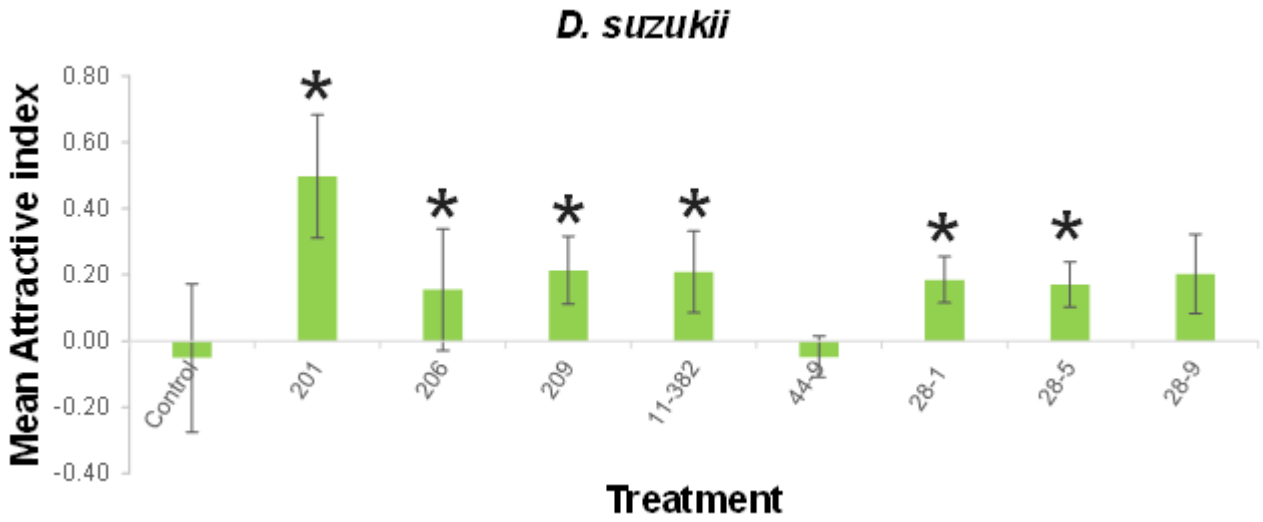

Figure 4: Shows the attraction of various *Hanseniaspora uvarum* strains (N=6) fermented in strawberry juice to *D. suzukii.* Attraction is shown as an attraction index, worked out as the number of flies choosing yeast divided by the total number of flies making a choice. Asterisks above or below bars represent any significant attraction or repulsion. *denotes a p value less than 0.05. Mean AI plotted ± Standard Error.

Hanseniaspora uvarum strains varied in their attractiveness to *D. suzukii*, with six being significantly attractive and two being indifferent (Figure 4), therefore H2 is rejected. All four of the New Zealand stains of *H. uvarum* tested were attractive, whereas only two out of four of the UK strains were attractive (Figure 4).

H4-There are no significant differences in the yeast communities on ripening fruit, regardless of fruit type or stage of ripening.

RFLP

RFLP experiments carried out to date have been to test and develop methods. Twenty yeast species and three strains of *H. uvarum* were digested separately with three different restriction enzymes (see Figure 5 for example); four in the case of *Hanseniaspora* species, to create a fragmentations patterns library to aid identification of yeast isolated from environmental samples (Table 3). In order to test this assay, this library and values from the literature (Esteve-Zarzoso *et al*., 1999) were used to identify yeast species isolated from three flies; two *D. subobscura* and one unidentified *Drosophila* caught in an apple orchard. Eleven *H. uvarum* isolates were identified, four *Kodamaea* species, three tentatively identified as an *Issatchenkia* species and one tentatively identified as a *Saccharomyces* species (Table 4).

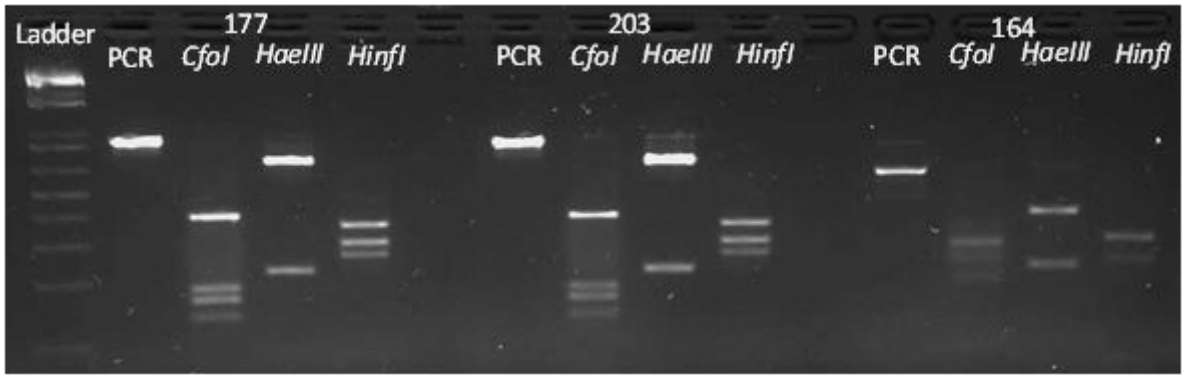

Figure 5: Example of A RFLP gel showing the pcr product and fragmentation pattern of three yeast *Candida glabrata* (177), *Candida stellata* (203) and *Candida zemplinina* (164), each digested with three restriction endonucleases (*CfoI, HaeIII* and *HinfI*). See Table 3 for sizes of fragmentation pattern. Ladder is 1 KB plus DNA ladder (Invitrogen) (size=100bp-1,200bp).

Table 3: Library of yeast. Created by using RFLP to build up fragmentation patterns for known species of yeasts.

		Restriction Enzyme			
Yeast Species	**ITS**	***Cfo I***	***Hae III***	***Hinfl***	***Dde I***
Metscnikowia pulcherrima	397	237, 90	413	208, 224	
Pichia kluyveri		169, 108, 80, 62	527	319	
Candida stellata	917	429, 194, 175, 143	763,235	399, 335, 277	
Pichia guilliermondi	837	300, 273, 105	384, 176, 95	317	
Pichia pijperi		279, 247, 200, 92	507, 234	388, 308	
Candida apicola	506	252, 195	465	287, 129	
Hanseniaspora uvarum (201)	755	321, 156	760	357, 207	257, 190, 78
Hanseniaspora uvarum (206)	735	316,150	772	258,208	83,158,259,369
Hanseniaspora uvarum (11-382)					267,192,89,
Zygosaccharomyces rouxii	556	232	384, 232, 139	377, 270, 149	
Torulaspora delbrueckii	837	342, 240, 159	864	472, 399	
Candida glabrata	917	421, 188, 167, 136	748, 270	386, 328, 270	
Saccharomyces cerevisiae	837	361, 334, 183	305, 245, 182	352, 170	
Candida railenensis	668	256, 90	448, 181, 87	386, 350	
Hanseniaspora occidentalis	533	222, 157	564, 458	287, 241	458, 121
Pichia sporocuriosa		107, 86	347, 151	261	
Saccharomyces uvarum	805	368, 172	531, 230, 151	390, 163	
Issatchenkia orientalis Kudryavtsev	597	381, 193, 62, 51	496, 92	319, 194	
Candida oleophila	683	404, 308, 97	453, 185, 90	333	
Candida zemplinina	669	328, 204, 217	447, 245	345, 261	
Kodamaea sp.	502	519	528	231	
Issatchenkia sp.		268, 200, 174, 139	425, 214, 129	425, 253, 144	

Table 4: RFLP fragmentation patterns for yeast isolated from *Drosophila.*

Yeast	ITS	Restriction Enzyme Cfo I	Hae III	HinfI	Dde I	Potential Yeast Species
fly 44-1	473	108, 68	355, 98	201, 151, 79		
fly 44-8	609	122, 71		220, 171, 100		*Issatchenkia* sp.?
fly 44-6	609			448, 120		
fly 44-4	851	295, 134	604,250	237, 212, 170, 111		*Saccharomyces sp.?*
fly 44-5	820			389, 178	260, 187	
fly 44-9	737	313, 164	786	362,217,	268, 181	
fly 28-1	834	362,151	898	406, 248	273, 181	
fly 28-2	829			405, 187	274, 197	
fly 28-3	837			374, 175	260, 186	
fly 28-4	837			386, 175	265, 197	*H. uvarum*
fly 28-5	837	340,151	896	375, 205	271, 183	
fly 28-6	816			399, 188	275, 196	
fly 28-7	865			410, 192	285, 210	
fly 28-8	902			412, 197	291, 231	
fly 28-9	896	360,167	930	398, 289	288, 193	
fly 98-3	587	616	639	308		
fly 98-5	616	571		237		*Kodamaea* sp.
fly 98-6	607	571		240		
fly 98-9	631	659	667	317		
fly 44-6	521		456,352			*?*
fly 44-7	837			220, 195	249, 167	*?*

Fruit samples

All four stages of each fruit (cherry, blueberry raspberry and strawberry) have been collected and processed for storage (Figure 2).

Discussion

The attractiveness of yeast species to different *Drosophila* was shown to vary (Figure 3); this is in line with previous studies (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015). *Drosophila suzukii* is significantly attracted to three yeast species; *H. uvarum*, 218 and 190 (Figure 3A). Scheidler *et al.* (2015) found that *H. uvarum* and *S. cerevisiae* were both significantly attractive to *D. suzukii.* In contrast, we have reported that *D. suzukii* is indifferent to *S. cerevisiae* (EC-1118). We assayed the attractiveness of yeast species fermented in strawberry juice whereas Scheidler *et al.* (2015) used culture media, Potato Dextrose Broth, which could account for the differences. Also, different strains of *S. cerevisiae* were used which could also account for the difference in attractiveness (Scheidler *et al.*, 2015). Different *S. cerevisiae* genotypes fermented in grape juice vary in their attractiveness to both *D. simulans* (Buser *et al.,* 2015) and *D. melanogaster* (Palancar *et al.,* 2013). In addition to the attractiveness of yeast species varying to *D. melanogaster* and *D. simulans*, the attractiveness of *H. uvarum* strains seems to vary, with six out of eight strains proving to be significantly attractive to *D. suzukii* (Figure 4). *D. melanogaster* was generally more attracted to the yeast tested (six attractive) when compared to *D. simulans* (two attractive) and *D. suzukii* (three attractive) (Figure 3).

Currently, existing baits used for attracting *D. suzukii* are both unselective and uncooperative with ripening fruit (Mori *et al.,* 2016). This makes sorting trap captures time-consuming and difficult as *Drosophila* are often hard to identify; *D. suzukii* females can be mistakenly identified for other *Drosophila* species without the aid of a microscope. As yeast species are differentially attractive to *Drosophila* species (Palanca *et al.,* 2013; Scheidler *et al.,* 2015; Figure 3) yeast-based baits could potentially produce a highly attractive and selective bait, potentially reducing non-target trap captures making it easier to detect *D. suzukii* and sort through trap captures.

Using RFLP we have created a library of fragmentation patterns for various yeast species (Table 3). This, along with existing databases (e.g. Esteve-Zarzoso *et al.,* 1999) was then

used to identify yeast species isolated for *Drosophila*. Eleven *H. uvarum* isolates have been identified (Table 4). These isolates produced a fragmentation pattern of two fragments two, one, two, two produced by *Cfo I*, *Hae III*, *Hinf I* and *Dde I* respectively. Four *Kodamaea* species were also identified by their distinctive pattern of one fragment for each restriction enzyme with *Cfo I* and *Hae III* not digesting and *Hinf I* producing one visible fragment half the size of the ITS pcr product (actual two band the same size). Two other genera were tentatively identified; three *Issatchenkia* species and a *Saccharomyces* species (Table 4).

Conclusions

Yeast vary in their attractiveness to *Drosophila* species (Figure 3). Three candidate yeast species that are attractive to *D. suzukii*: *H. uvarum*, 218 and 190 have been identified (Figure 3); all three of which are indifferent to *D. simulans* (Figure 3C) and two of which are indifferent to *D. melanogaster* (Figure 3B). Both are common non-target species often captured in *D. suzukii* monitoring traps. Additionally, multiple strains of *H. uvarum* are also attractive to *D. suzukii* (Figure 4), a yeast species that in the context of *D. suzukii* has received a lot of attention in the literature and is known to be attractive to *D. suzukii*, both in the literature e.g. Hamby *et al.,* 2012; Scheidler *et al.*, 2015; Mori *et al.,* 2017) and project SF 145. This highlights the potential for yeast to produce attractive and selective baits for *D. suzukii.*

Knowledge and Technology Transfer

I will be presenting a poster at the PGR symposium at University of Lincoln on the 10th October 2018 I will also be presenting a talk at the AHDB/NIAB EMR Association soft fruit day on the 21st November 2018 and presenting a poster at the AHDB Studentship Conference 26-27th November 2018. I also plan to attend ABB Crop Protection in Southern Britain 12-13th November 2018 and the ABB Advances in IPM 2018: Making it Work for the Farmer 12-13 December 2018 conferences. I will submit abstracts to present posters at both.

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