The Diversity and Function of Indigenous Yeasts in the Okanagan Region of British Columbia.

by

Sarah Marie A Lyons

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The following individuals certify that they have read, and recommend to the College of Graduate Studies for acceptance, a thesis/dissertation entitled:

The Diversity and Function of Indigenous Yeasts in the Okanagan Region of British Columbia.

submitted by Sarah Marie A. Lyons in partial fulfillment of the requirements of

the degree of Master of Science.

Dr. Daniel M Durall, Irving K. Barber Faculty of Science

Supervisor

Dr. Wesley Zandberg, Irving K. Barber Faculty of Science

Supervisor

Dr. Michael Deyholos, Irving K. Barber Faculty of Science

Supervisory Committee Member

Dr. Vivien Measeday, Wine Research Centre, Faculty of Land and Food Systems

Supervisory Committee Member

Dr. Thu Thuy Dang, Irving K. Barber Faculty of Science

University Examiner

Abstract

Consumer preference in the wine industry is encouraging wine makers to produce more unique wines than ever before. One way in which to add such distinctiveness to a wine is to utilize the microbial diversity present in each region. This can be done by spontaneous fermentations; however, this carries risks of spoilage or stuck fermentations and lacks the consistency of inoculated fermentations. More research is needed to understand the diversity of yeasts in vineyards and how some indigenous strains could be utilized by wineries in a more controlled fashion. My thesis aims to better characterize the microbial diversity in vineyards and how they respond to environmental perturbations as well as to characterize the fermentative properties of unique strains isolated from fermentations in the Okanagan region.

To accomplish this, I first investigated the fungal communities on grapes in 3 vineyards in the Okanagan region after environmental disturbances. Experimental vines were exposed to either simulated forest fire smoke, one of three agricultural sprays, or both smoke and sprays. Amplicon based Next Generation Sequencing was then used to profile the fungal communities on the grapes before exposure, two weeks after exposure, and again at harvest. We revealed that fungal communities are robust to these disturbances and no differences were found after exposure between the experimental vines and the control vines.

In the second research chapter of my thesis, I performed controlled fermentations of chardonnay juice using unique strains isolated from wineries in the Okanagan region. These strains were found to be abnormally competitive in previously conducted winery fermentations, some even out-competing commercially sourced yeast strains. Others were found in relatively high abundance in wineries for multiple years in a row. By analyzing the fermentation kinetics

and the production of volatile compounds using GC-MS, we found that many of these strains had comparable fermentation kinetics to commercial strains at both 12 °C and 15 °C, and produced unique compound profiles that could be used by local wineries to produce more complex and regionally specific wines.

Lay Summary

The final flavour of a wine is determined not only by grape variety and fermentation techniques, but also by the environment in which the grapes were grown. The unique consortia of yeast and bacteria that are found living in vineyards are part of what makes wine from a certain region unique. Some of these yeast in the vineyard enter the fermentation and can even take over fermentations in which commercial yeast strains were added. Winemakers would like to know more about what flavours these indigenous yeast strains produce so that they can use them in a more controlled deliberate manner. Changing environmental conditions may be altering these microbial communities. I found that indigenous yeast communities in vineyards were robust to environmental perturbations. I also found that some of these indigenous yeasts produce unique flavours and are able to compete with commercial yeast strains.

Preface

Chapter 2 is original research, based on an experimental design, which was developed by Dr. Wesley Zandberg to test the efficacy of 3 potential prophylactic sprays against the uptake of volatile phenolic compounds into grapes from forest fire smoke. I modified the original experimental design with the guidance from my supervisors, Dr. Daniel Durall and Dr. Wesley Zandberg, as well as from Dr. Sydney C Morgan. I assited with the application of smoke in vineyards and conducted all sampling from the field with the assistance of Stephanie McCann and Samantha Sanderson who contributed to sample collection and processing before DNA extraction. Library preparation for Illumina sequencing was performed by me under the guidance of Dr. Sydney C Morgan. Illumina sequencing was performed at IBEST core facility at the University of Idaho by Dan New and technicians under his supervision. I analyzed Illumina sequencing data with the guidance from Dr. Sydney C Morgan. I also conducted all statistical analyses and data visualization.

I designed the fermentation experiments described in chapter 3 under the guidance of my supervisors Dr. Daniel Durall and Dr. Wesley Zandberg. The experimental set up and sample collection was performed by me with the assistance of Stephanie McCann and Samantha Sanderson. I performed the Gas Chromatograph Mass Spectrometer (GC-MS) sampling, optimization, and data acquisition at the Supra Research and Development facility (Kelowna, BC). The resulting chemical profiles, data processing, statistical analysis, and data visualization were all performed by me. A version of chapter 3 has been submitted for publication to the OENO One Journal.

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Abbreviations

Aberviation	Full Terminology
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
GCMS	Gas chromatography mass spectrometry
HPLC	High pressure liquid chromatography
ITS	Interal transcribed spacer
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PERMANOVA	Permutational analysis of variance
PCA	Principle component analysis
PCR	Polymerase chain reaction
PCoA	Principle coordinate analysis
RNA	Ribonucleic acid
TIC	Total ion current
VBNC	Viable but not culturable
VOC	Volatile organic compound
VPC	Volatile phenolic compound
YPD	Yeast peptone dextrose

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There are many people that I would like to thank for their mentorship and support during my time here. First and foremost, I want to thank my supervisors Dr. Daniel Durall and Dr. Wesley Zandberg who have given me this opportunity. Your faith in my abilities has drastically changed the course of my life for which I will be forever grateful. I would also like to thank the members of my committee, Dr. Vivien Measeday and Dr. Michael Deyholos whose guidance and input has kept my research on track. I would like to extend a special thank-you to Dr. Sydney Morgan who has not only been an invaluable mentor, but also a wonderful and supportive friend.

There are three more people without whom I couldn't have made it through this degree. Cailan, I thought I was renting a room but instead you gave me a home. Thank you for teaching me so much about bikes, cars, and climbing. Your companionship, enthusiasm for everything, and supernatural ability to fix anything have made you the most incredible roommate I will ever have. Lisa, I don’t even know where to start, thank you for always being such an amazing listener with plenty of wine and brilliant advice. Your friendship has been an unexpected treasure that I will always be grateful for. Bruno, I couldn’t dream of a better adventure partner. Thank you for all the climbing, skiing, and biking days. You always brought out the best in me and I will cherish those memories forever.

Dedication

For my mother, who taught me to never stop climbing.

Chapter 1: Introduction

In the course of this research I have explored the diversity and function of indigenous vineyard yeasts in the Okanagan region. This chapter provides an introduction to the dynamics of yeast populations in the vineyard throughout grape ripening and what is currently known about the response of these communities to the ecological disturbances of agricultural sprays and forest fire smoke exposure. I also outline the grape ripening process and how flavors are developed during growth. I then discuss how indigenous and commercially purchased yeasts behave during fermentation with respect to how they affect the production of organoleptic compounds under different temperatures. Additionally, I discuss what is known about how temperature can affect the fermentation dynamics and organoleptic compound production. Finally, I introduce the aims and predictions of my research.

1.1 Succession of Fungi on Grapes During Ripening

Wine flavour characteristics begin to take shape while the grapes are ripening on the vine and are defined by more than just the grape varietal. While grapes are growing, complex microbial community assemblages of fungi and bacteria live on the surface of the grapes, leaves, bark, and in the soil (Vitulo *et al.*, 2019). The microbial assemblages growing in vineyards have been implicated in the health and pathogen resistance of the vines; they have also been shown to affect the final aroma/flavor profile of wines resulting in unique *terroir* qualities of wines (Gilbert *et al.*, 2013; Bokulich *et al.*, 2016; Liu *et al.*, 2017). Microbial populations in the vineyard, either on the plant or in the soil, confer pathogen resistance and allow for the availability of essential nutrients to vines, which subsequently promotes their growth. These communities are affected by many factors such as grape cultivar, temperature, humidity, sunlight, interannual climate variation, vineyard management practices, and the ecosystems surrounding the vineyard (Miura *et al.*, 2019). These assemblages can be so sensitive to different

conditions that even microclimates within the same vineyard can host different species, which may lead to biogeographic regionalization (Setati *et al.*, 2012). Yeast species have also been shown to fluctuate across regions from year to year (Barata, Malfeito-Ferreira and Loureiro, 2012). Yeast species and filamentous fungi representing over 100 different genera have been identified on grapes in vineyards showing impressive diversity (Barata, Malfeito-Ferreira and Loureiro, 2012; Rousseaux *et al.*, 2014; Morgan, du Toit and Setati, 2017).

Throughout the growing season, the resident yeast populations change dynamically with environmental conditions and grape ripening progression (Bokulich *et al.*, 2013). Early in the growing season at berry-set, there is little available sugar. At this early stage, filamentous and dimorphic (producing both hyphae and yeast) fungi, mainly belonging to the phylum Ascomycota, are more abundant than strict yeast species. Examples of genera found during these early stages of the growing season include *Aureobasidium*, *Aspergillus, Alternaria, Penicillium*, *Cladosporium*, *Lewia, Davidiella, Erysiphe,* and *Botrytis* (Bokulich *et al.*, 2014; Morgan *et al.*, 2017, De Filippis *et al.*, 2017; Kecskeméti *et al.*, 2016). The dimorphic fungus *Aureobasidium pullulans* is the most dominant species found at this stage (Barata, Malfeito-Ferreira and Loureiro, 2012).

As grapes mature, they increase in size, sugar content, water content, elasticity, as well as decrease in acidity (Padilla *et al.*, 2016). This allows more growth of yeast populations showing increases in colony forming units (CFUs) from around 10^1-10^3 CFU/g at berry-set to more than 10^2-10^6 CFU/g at harvest (Setati *et al.*, 2012). The dominant species present on the grapes also changes. Most fungal species present on ripe berries are non-fermentative basidiomycetous yeasts such as *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp., *Filobasidium* spp., and *Aureobasidium pullulans* (Prakitchaiwattana, Fleet and Heard, 2004; Barata *et al.*, 2008).

There are also non-fermentative, or weakly fermentative, ascomycetous yeasts present including *Candida* spp., *Pichia* spp., and *Metschnikowia* spp. Fermentative Ascomycetous yeasts such as *Hanseniaspora* spp. can be dominant on ripe vineyard grapes, whereas *Zygoascus hellenicus* are present but only in low concentrations unless the skin of the berry has ruptured; in this case, they have access to more juice and can increase in abundance up to 10-fold (Nisiotou and Nychas, 2007; Barata, Malfeito-Ferreira and Loureiro, 2012). Even on ripe berries, strongly fermentative yeasts such as *Sacchaormyces cerevisiae* are present only in surprisingly low concentrations, around 10-100 CFU/g of berries (Garijo *et al.*, 2011).

1.2 Production of Secondary Metabolites and Precursors to Organoleptic Compounds Inside of Grapes

Flavour active compounds, or their pre-cursors that will be later transformed by fermentation are produced in the grapes as they mature and are affected by a complex interplay of vine genotype, environment, and vineyard management practices. These compounds are found either in the outer skin, called the 'exocarp', or in the inner flesh, called the 'mesocarp'. After berry-set, the mesocarp and exocarp undergo a period of rapid growth. The mesocarp begins to accumulate organic acids, especially malate (sour apple flavor) and tartrates (tart acidic flavors). In the exocarp, terpenes begin to form that will later become key aromatic compounds in the wine. Terpenes also develop and concentrate in the exocarp. Terpenes are a diverse group of compounds adding complexity to wines, more than 50 different terpenes have been identified in wine grapes (Marais, 2017). Following mesocarp and exocarp growth, a lag phase of berry growth occurs in which seeds start to mature.

The next phase of berry ripening is called, '*verasion*'. During which, the metabolic activity in the grape changes, glycolysis slows down and sugars begin to accumulate in the mesocarp. The grapes begin using malate as a source of carbon, and volatile organic compounds

(VOC's) are biosynthesized. Red grape varietals accumulate anthocyanins in the exocarp giving them a rich, red hue. Other compounds including polyphenolics, terpenoids, and esters are produced and stored as non-volatiles by being conjugated to glycosides or amino acids (Lund and Bohlmann, 2006). These conjugated compounds will later be liberated by glycosidase and peptidase enzymes during fermentation allowing them to be perceived by the consumer. The harvesting of the grapes is timed to balance acidity and sweetness, generally around 12-14 weeks after berry-set. Grapes are then crushed and can be processed in a variety of ways to achieve maceration prior to fermentation, depending on the style of the wine being produced. During the maceration process, phenolic compounds in the grapes such as tannins and anthocyanins are leached from the grape skins, seeds and stems into the must.

1.3 Response to Environmental Disturbances

Environmental disturbances can affect both the health of the grapes as well as the resident microbial populations. These disturbances can be in the form of vineyard management practices such as pruning and application of agricultural sprays or in the form of environment changes like alterations in weather patterns and smoke exposure from forest fires. Vineyard management practices will alter the soil and can increase or decrease bacterial and fungal diversity (Hernandez and Menéndez, 2019). Practices that can alter microbial populations in the soil include light exposure, cover crop use, tillage, compost application, and conventional, organic, or biodynamic systems. After an environmental disturbance, there is evidence to suggest that the grapes are re-colonized from microbes in the soil and the ecosystems surrounding the vineyard. The soil and surrounding ecosystems have been shown to act as reservoirs for microbial communities (Zarraonaindia *et al.*, 2015; Miura *et al.*, 2019). Studies have suggested that microbes from the soil are able to transport through roots of the vines and quickly recolonize the

fruiting bodies of grapes (Compant *et al.*, 2011). Additionally, Studies have shown that many ASV's (Amplicon Sequence Variants) detected in vineyards are also found in the surrounding ecosystem, Miura *et al.* 2019 found that 45% of the fungal species detected in the vineyard were shared with the surrounding ecosystem. This implies that an environmental disturbance only applied to a small population of vines within an otherwise typical vineyard would be quickly recolonized.

1.3.1 Smoke Exposure

Due to the increasing frequency of forest fires in North America, grapes are often exposed to wildfire smoke. Wildfires burn more than 700,000 hectares of Canadian boreal forest every year and fire rates are increasing (Moritz *et al.*, 2012; Chaste *et al.*, 2018). Smoke exposure to grapes, especially post-*veraison*, can lead to wine with undesirable characteristics such as 'ashtray', 'Band-Aid', and 'medicinal' flavours creating a defect known as 'smoke-taint'. Volatile phenolic compounds (VPCs) in the smoke can be absorbed into the grape and can conjugate to glycosides rendering them tasteless and odorless until fermented; at which stage, the smoke flavours return and tarnish the wine (Kennison *et al.*, 2007; Noestheden, Dennis, *et al.*, 2018; Noestheden, Noyovitz, *et al.*, 2018; van der Hulst *et al.*, 2019). Researchers at UBCO have been investigating the use of various agricultural sprays as an in-vineyard protection strategy against the uptake of VPCs into grapes from forest fire smoke (Favell *et al.*, 2019). Neither these products, nor chronic (low-grade) smoke-exposure have been investigated with respect to their impact on the grape microbiome, including both fungi and bacteria.

1.3.2 Agricultural Sprays

The use of a currently approved agricultural sprays to mitigate smoke taint would be advantageous because they are already approved for use on crops, well-characterized, and readily

available. There are many agricultural sprays are currently used in vineyards that are potential candidates. However, these sprays have not been evaluated for their effect on the microbial communities in vineyards. Researchers at UBCO have decided to test 3 different agricultural sprays for their capacity to block the uptake of VPC's into grapes: Parka® spray, PureSpray® Oil, and Timorex Gold®.

Parka® spray, hereafter refered to as 'Biofilm 1', is an artificial phosolipid-based cuticle enhancing chemical that is frequently used in the Okanagan region to protect cherry skins from splitting open after heavy rain fall. By increasing the fatty acid composition of the cuticular layer of fruit, Parka® spray increases the elasticity and hydrophobicity of the skin while still allowing respiration and gas exchange. This protects the fruit from harsh environmental conditions and therefore increases the yield of marketable fruit (Cultiva, 2019). Alterations in the natural plant cuticle are likely to affect the natural microbial activity. Preliminary tests conducted by the Zandberg laboratory at the University of British Columbia Okanagan show that Parka spray is a promising candidate for smoke taint prevention in Pinot Noir grapes (Favell *et al.* unpublished).

PureSpray® Oil, hereafter refered to 'Oil 1', is a mineral-oil based fungicide that is used to control a variety of pests and fungal infections from mites to powdery mildew and can be used on almost any plant or vegetable. Mineral oils work against pathogenic fungi by physically preventing oxygen diffusion into the fungus and preventing growth and spore germination (Helmy, Kwaiz and El-Sahn, 2012). Although petroleum-based sprays have been used in agriculture for centuries and are frequently used in viticulture, there is a paucity of research regarding the effects of mineral oil on the microbial consortia of grapes.

Timorex Gold®, hereafter refered to as 'Oil 2', is a tea tree oil-derived bio-fungicide that is used to control powdery mildew on fruit crops. The anti-fungal activity of tea tree oil is from

the high terpene content. Terpinen-4-ol and 1,8-cineole degrade the cell and organelle membranes in fungi (Yu *et al.,* 2015). Fungicides, based on naturally derived essential oils, are a popular option for viticulturists because they are broad-spectrum, biodegradable, and eco-friendly.

1.3.3 Microbial Recovery from Environmental Disturbances

After an environmental disturbance, microbial communities are replenished from the reservoirs of microbes in the surrounding ecosystems. In the event of an environmental disturbance isolated to a few vines, microbial species from surrounding vines will recolonize the disturbed vine. Microbes in the soil can migrate through the plant via the roots and re-colonize the leaves and fruit after an environmental disturbance (Compant *et al.*, 2011). A recent study using 16S rRNA amplicon sequencing showed that 45 % of the operation taxonomic units (OTU's) on grapes were shared with the surrounding ecosystem (Miura *et al.*, 2019). This demonstrates that the surrounding ecosystem is capable of acting as a reservoir for microbial diversity allowing recolonization onto the grapes after an environmental disturbance.

1.4 Microbes During Fermentation

Primary fermentation is a complex biochemical process characterized mainly by the conversions of sugars in the grapes to ethanol and CO_2 (Fleet, 2003). After grapes have been harvested, they are destemmed, crushed, and sometimes processed further before being transferred for primary fermentation. Fermentations are most commonly inoculated, meaning a commercially sourced yeast (usually *S. cerevisiae*) is added. Alternatively, winemakers may choose a ‘spontaneous fermentation’; also called ‘uninoculated fermentations’, or ‘natural fermentations’. In spontaneous fermentations, commercial yeast strains are not added, and the must is fermented by yeast already present on the grape berries, from the air, or on surfaces from

inside the winery. These spontaneous fermentations have been shown to produce more unique and regionally representative flavor profiles; however, they increase the risk of stuck or sluggish fermentations (Heard and Fleet, 1985; Mas *et al.*, 2016; Padilla, Gil and Manzanares, 2016).

1.4.1 Microbial Interactions During Fermentation

Fungal diversity at the onset of fermentation is relatively high due to the initial presence of non-Saccharomyces species from the vineyard. Although these species do not survive through the end of fermentation, they do affect the final flavor profile of the wine (Bokulich *et al.*, 2013; Mas, Guillamón and Beltran, 2016). *Hanseniaspora,* and *Candida* are the most frequently found non-*Saccharomyces* species during this stage (Ganga and Martínez, 2004) and can produce higher amounts of ethyl acetate in wine (Fleet, 2003). *Pulcherimia* is also present but has only been found in the first few days of fermentation (Milanović, Comitini and Ciani, 2013). Other species and genera including *Metschnikowia, Rhodotorula, Pichia, Saccharomyces bayanus, Saccharomyces paradoxus,* and *Saccharomyces uvarum* are also found and have the potential to persist through all stages of fermentation (Rementeria *et al.*, 2003; Magyar and Tóth, 2011; Díaz *et al.*, 2013; Alonso-del-Real *et al.*, 2017). Recent research suggests that bacteria may survive longer into fermentations than previously thought as well. Acetic acid bacteria has been observed dominating low sulfated spontaneous fermentations, while *Lactobacillus* and other *Lactobacillaceae* have been found dominating sulfite-free fermentations (Bokulich *et al.*, 2014).

As fermentation progresses, there is a complex succession of species in the highly dynamic environment of the fermentation vessel. Some species interact competitively while others have synergistic relationships. The competitive fermentative yeast *S. cerevisiae* grows quickly consuming oxygen, releasing CO_2, and producing compounds that are toxic to other species like ethanol. Other yeast strains can be especially toxic to other species by producing

growth inhibiting peptides, and killer toxins (Schmitt and Breinig, 2006). As the fermentation environment becomes more anaerobic and toxic, many yeast and bacterial species begin to die off. As other species die off, they release nutrients and vitamins that then become available for the surviving microbes (Alexandre and Guilloux-Benatier, 2006). By the end of fermentation, there is usually a single dominant species of yeast. This is most commonly an *S. cerevisiae* species, but other species have been found dominating fermentations. Recent findings show that *S. uvarum* can compete with or even dominate over *S. cerevisiae* in controlled mixed cultures (Su *et al.*, 2019) and in winery-based spontaneous fermentations (McCarthy *et al.*, 2019; Morgan, Tantikachornkiat, *et al.*, 2019). In addition, *S. uvarum* populations exhibit a high genetic diversity in spontaneous fermentations (McCarthy *et al.* 2019), which may suggest that not all *S. uvarum* strains fit the metabolic stereotype of the species.

1.4.2 Effect of Fermentation Temperature

The fermentation temperature can affect the species dynamics during fermentation altering organoleptic profiles and what strain dominates at completion. Although colder fermentation temperatures take longer to complete, they are being explored by the wine industry to increase the production and retention of aroma compounds (Gamero *et al.*, 2013). However, this advantage does not come without risk as low temperatures can increase the chances of stuck or sluggish fermentations (Alonso-del-Real *et al.*, 2017). Some strains are more cryotolerant, especially *S. uvarum* and *S. kudriavzevii* (Alonso-del-Real *et al.*, 2017). Many studies have demonstrated that *S. uvarum* species show greater cryotolerance when fermented at lower temperatures relative to other fermentative yeasts and have been found dominating fermentations at lower temperatures (Moreira *et al.*, 2008; Tosi *et al.*, 2009; Gamero *et al.*, 2013; Su *et al.*, 2019). Different metabolic adaptations allow *S. uvarum* and *S. kudriavzevii* to ferment so successfully at lower temperatures.

Metabolic studies have shown that these cryotolerant strains differ in their lipid metabolisms as well as their metabolism of fructose (López-Malo, Querol and Guillamon, 2013).

1.5 Flavour-Active Organoleptic Compounds

1.5.1 Production of Organoleptic Compounds

Organoleptic properties of wine, which act on or involve the use of human sense organs are determined by the absolute amounts of chemical compounds as well as specific ratios of these flavour-active chemicals. These compounds are produced in many ways: they can be metabolic by-products excreted by the yeast; molecules created by enzymes produced by microbes; or compounds released by yeast autolysis (Fleet, 1993). Important compounds that determine the organoleptic properties of wine are: higher alcohols; ethyl esters; acetate esters, residual sugars, and glycerol (Capozzi *et al.*, 2015).

Some of these compounds are dependent on precursor availability in the wine must while others are limited by the metabolic enzymes produced in different amounts by different yeast strains. Higher alcohols are a main source of flavour characteristics in wine and include any alcohols with more than 2 carbons. They are formed from amino acid precursors as by-products of sugar metabolic pathways, anabolic reactions, and the Ehrlich pathway. Their production varies greatly between strains (Dickinson, Salgado and Hewlins, 2003; Pires *et al.*, 2014). Ethyl esters impart fruity characteristics and are produced by the yeast metabolism of medium chain fatty acid precursors. Esters are produced with enzymatic condensation reactions and their levels are affected by many factors including fermentation temperature, initial concentrations of medium chain fatty acid precursors, and unsaturated fatty acid concentrations in the grapes (Saerens *et al.*, 2008). Acetate esters are another important compound group for wine imparting fruity or solvent flavors

and are produced by enzymatic activity of alcohol acetyl-transferases produced by yeast which are usually limited by the expression of this gene, as opposed to available substrate in grape must (Rojas *et al.*, 2001). Glycerol is vital to give wine body and texture. It is produced by yeast through the glycolytic metabolic pathway. Glycerol production is also limited not by substrate, but by the expression of genes that produce the enzymes in this pathway (Remize, Barnavon and Dequin, 2001; Wang *et al.*, 2001).

1.5.2 Fermentative Characteristics of *S. uvarum*

Indigenous yeast strains, such as *S. uvarum*, have the potential to make more complex wines with better mouth feel due to higher concentrations of glycerol, beneficial secondary metabolites, and higher secretions of extracellular enzymes that change the wine flavor (Jolly, Varela and Pretorius, 2014; Padilla, Gil and Manzanares, 2016). By directing carbon flux into the synthesis of glycerol instead of ethanol, they produce wines with lower levels of ethanol and more body, this is advantageous because climate change is increasing the sugar content of grapes and therefore increasing the ethanol content above palatable levels (Ciani *et al.*, 2016). *Saccharomyces uvarum* strains have been shown to produce higher amounts of 2-phenylethanol and 2-phenylehtyl acetate which have floral and fruity aromas (Masneuf-Pomarède *et al.*, 2010; Gamero *et al.*, 2013). However, they can also produce wines with unpleasant levels of volatile acidity, which instills a vinegar aroma in the wine (Loureiro and Malfeito-Ferreira, 2003; Tristezza *et al.*, 2013).

The genetic history of *S. uvarum* is convulted as it has historically been classified under different names. *S. uvarum* has been previously considered a distiuct subgroup of *S. bayanus* known as *S. bayanus var. uvarum* but has since been reclassified separately from *S. bayanus* after the reinstatement of the speices *S. uvarum,* and the determination that *S. bayanus* is in fact a hybrid of *S. uvarum* and *S. eubayanus* (Magyar and Tóth, 2011). The whole genome sequence of *S.*

uvarum has been aquired (Scannell *et al.*, 2011). There is highly conserved synteny between the genomes of *S. uvarum* and *S. cerevisiae*, as 98% of the genes retain the same neighboring relationships between the two species (Masneuf-Pomarède *et al.*, 2010). This is a reflection of the co-evolution of these species in the northern hemisphere (Libkind *et al.*, 2011).

1.6 Thesis Objectives and Hypothesis

The overall objective of my thesis is to better characterize the microbial diversity in Okanagan vineyards. Within this overall objective my research will address two specific objectives: 1) To assess the response of fungal community composition and diversity on grapes in vineyards exposed to forest fire smoke and potentially prophylactic agricultural sprays. 2) To determine whether different strains of *S. uvarum*, including some that may be indigenous to the Okanagan, differ in flavour/aroma profile production as compared with commercial strains of *S. cerevisiae*.

1.6.1 The response of microbial communities on grapes in vineyards to forest fire smoke exposure and agricultural sprays.

Objective 1: To characterize the response of microbial communities on grapes in local vineyards to forest fire smoke exposure and to agricultural sprays.

Hypothesis 1a: Exposure of smoke will shift the balance of microbial populations so that those species that are more tolerant to anaerobic conditions and volatile phenolic compounds will dominate the community structure.

Rationale: Smoke exposure will limit the amount of available oxygen. The particulates in the smoke that adhere to the grape surface will also create a physical barrier preventing gas exchange. Smoke also contains compounds with anti-microbial properties that aid in the preservation of foods and thus inhibit microbial growth.

Hypothesis 1b: The use of fungicides on grapes in the vineyard will initially reduce or decimate fungal populations on grapes after application.

Rationale: Chemical fungicides are known to decrease fungal species richness and diversity (Escribano-Viana *et al.*, 2018).

Hypothesis 1c: The microbial populations on the grapes sprayed with agricultural sprays will revivify and will be indistinguishable from the control vines at the end of the experiment.

Rationale: There are strong reserves of microbes surrounding the grapes in the soil and on adjacent vines in the field. Studies have shown that after an environmental disturbance that causes microbial mortality on grapes in the vine that the microbes in the soil, on the leaves, and from ecosystems surrounding the vineyard will recolonize the grapes restoring a typical grape community structure (Miura *et al.*, 2019).

Hypothesis 1d: The three different types of sprays will have different effects on the microbial communities because they all have different modes of action and purposes.

Rationale: The time that it takes for microbial communities to return to normal will depend on how long the sprays remain adhered to the surface of the grape and the amount of rainfall during the experimental period. This will affect the length of time each spray alters the microbial community.

Hypothesis 1e: The untreated control vines at the three different vineyards will have different fungal community composition and structure at harvest.

Rationale: The three vineyards in which the experiments were conducted use different agricultural practices, two are organic and the third is traditional. It is also expected that vineyard 3, which is on the west bank of Okanagan Lake, unlike Vineyards 1 and 2, will host different fungi due to environmental differences between the two sides of the lake.

1.6.2 The fermentative performance and production of organoleptic compounds of Indigenous *Saccharomyces uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation of Chardonnay wines.

Objective 2: To compare the fermentation kinetics and production of volatile aroma/flavour active compounds of *S. uvarum* strains (indigenous to the Okanagan region) with commercially-available strains of *S. cerevisiae* when fermented in chardonnay juice at 15 °C or 25 °C.

Hypothesis 2a: *S. cerevisiae* strains, especially EC118, will complete fermentations faster at 25 °C as compared with *S. uvarum* strains.

Rationale: Commercial *S. cerevisiae* strains are robust fermenters with high alcohol tolerance and are likely to outcompete even commercial *S. uvarum* strains in speed of fermentation at 25 °C which is an optimal temperature for commercial *S. cerevisiae* strains.

Hypothesis 2b: *S. uvarum* strains will complete fermentations faster at 15 °C as compared with commercial *S. cerevisiae* strains. (Diamantidou, Zotou and Theodoridis, 2018)

Rationale: *S. uvarum* strains are more cryotolerant and are likely to ferment faster than the *S. cerevisiae* strains at lower temperatures (Gamero *et al.*, 2013; Alonso-del-Real *et al.*, 2017).

Hypothesis 2c: The locally isolated *S. uvarum* strains will produce higher amounts of glycerol and succinic acid as compared with *S. cerevisiae* strains.

Rationale: Previous research has demonstrated that *S. uvarum* strains produce more glycerol, and succinic acid (Hu, Jin, Mei, *et al.*, 2018).

Hypothesis 2d: Commercial *S. cerevisiae* strains will produce higher amounts of ethanol, acetaldehyde and acetic acid as compared with *S. uvarum*.

Rationale: Previous research has demonstrated that *S. cerevisiae* strains produce more ethanol, acetaldehyde and acetic acid than *S. uvarum* strains (Hu, Jin, Mei, *et al.*, 2018).

Hypothesis 2e: Fermentations performed at 15 °C will retain aromatic compounds better than the 25 °C fermentations for each strain.

Rationale: Fermentations of all strains conducted at cooler temperatures have been demonstrated to retain aromatic compounds and produce fruitier/more complex aromas in wine (Gamero *et al.*, 2013); at warmer fermentation temperatures there is often a loss of aromatic compounds due to evaporation (Beltran *et al.*, 2008).

Chapter 2: Fungal Community response to forest fire smoke and agricultural sprays in vineyards

2.1 Synopsis

Genetically identical vines grown in different geographical regions produce wines with different aromas and flavours, which is known as the *terroir* of a wine. Terroir is of interest to winemakers because it adds value to the final product by instilling a distinct and unique flavour/aroma to the wine. The terroir affects numerous variables of wine and the characteristics of a given vintage begin to take place while grapes are ripening on the vine. The microbial diversity in the vineyard has been shown to be associated with the *terroir* of the wine (Gilbert *et al.*, 2013). In both spontaneous and inoculated fermentations, the fungal community in the vineyard often carries on into the fermentation process. Therefore, it is valuable to understand how the microbial communities in a vineyard are structured and how they respond to environmental perturbations.

The increase in forest fires in recent years has had a large effect on wineries in North America. The duration and severity of the fire season is increasing every year and wineries are scrambling to determine how to cope with this aspect of climate change. When grapes in vineyards are exposed to large amounts of forest fire smoke, volatile phenolic compounds in the smoke absorb into the grapes and result in 'smoke tainted' wine with ash-like flavours (Noestheden *et al.*, 2017). Wineries want to know more about how smoke exposure will alter their vineyards, wine flavours, and how to mitigate these effects. Understanding how the fungal communities in vineyards change in response to agricultural sprays and forest fire smoke will help wineries to continue to produce unique, regionally specific wines representative of the Okanagan Valley.

There have been numerous studies using amplicon-based Next Generation Sequencing (NGS) techniques to characterize the fungal communities on grapes in vineyards, as reviewed by

Stefanini and Cavalieri (2018). The use of NGS allows the direct analysis of environmentally isolated DNA including the identification of 'viable but not culturable (VBNC) speices as opposed to using culture-based techniques that only allow the analysis of the few strains that can be cultured in lab (Salma *et al.*, 2013). In this study, I extracted DNA and in a PCR reaction I used specific DNA primers that targeted the 'internal transcribed spacer 1' (ITS1) region of rRNA coding genes. This region is typically used to sequence and identify fungi in wine samples so as to ultimately determine their relative abundance and diversity (Blaalid *et al.*, 2013).

The objective of this study was to monitor the response of fungal communities on grapes growing in vineyards after exposure to heavy forest fire smoke and agricultural sprays. Experimental vines at three different vineyards were exposed to either smoke or agricultural sprays or a combination of the both treatments. NGS was used to analyze the composition of the fungal communities on grape bunches before exposure to the treatments, 14 days after exposure, and again immediately before harvest. We found no significant differences between the control and treatment vines at either of the three vineyards, however, we did see differences in the control vines between vineyards.

2.1 Materials and Methods

2.1.1 Experimental Design

The experiment was conducted across three vineyards in the Okanagan region growing Pinot Noir grapes to determine the effect of smoke exposure and agricultural sprays. At each vineyard, there were four treatments with each treatment consisting of seven vines: 1) a control treatment where grapes were sprayed with only water (These samples served as controls for the differences in the community composition of grapes between vineyards and changes throughout the ripening process); 2) grapes were sprayed with an agricultural spray (each of the three

vineyards had a one of three sprays applied); 3) grapes were exposed to heavy smoke (applied for one hour in two applications, two days apart, to mimic smoke exposure from a forest fire); 4) grapes were sprayed with one of three agricultural sprays, seven days before their initial exposure to smoke, which was two days before their second exposure. A single bunch of grapes from each vine was collected before spraying, 4 days after the second round of smoke exposure, and immediately before harvest to compare the diversity and community composition of fungi on grapes among the different treatments (Fig. 2.1).

Smoke	Spray	Number of Vines per vineyard
Yes	Yes	7
Yes	No	7
No	Yes	7
No	No	7

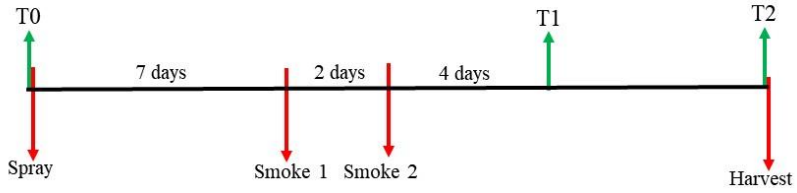

Figure 2.1 Experimental Design. Three vineyards in the Okanagan growing Pinot noir grapes were used as experimental sites. One of three sprays were tested at each vineyard. At each vineyard, there were 4 treatments including 7 untreated control vines, 7 vines that were sprayed with an agricultural spray, 7 that were exposed to simulate forest fire smoke, and 7 that were sprayed and exposed to smoke.

2.1.2 Smoke Application

To expose vines to forest fire smoke, modular enclosures were constructed out of PVC tubing wrapped in polyethylene sheets as described in detail by Noestheden *et al.* (2017). Briefly, fuel for the smoke production was locally sourced, and the composition was designed to mimic forest fire smoke. The fuel sources were collected from a *Pinus ponderosa* forests containing 20% pine needles (w/w), 30 % bark pieces cut into 3cm particles (w/w), and 50% soil organic material (w/w).

2.1.3 Sample Collection

One grape cluster was aseptically collected per vine at each timepoint and placed into sterile plastic bags using pruning shears that were sterilized with 70% ethanol between each sample. After collection of the grapes in the vineyards, samples were transported to the lab on ice, and crushed manually in the bags. Thirty mL of crushed grape must was transferred to two separate 15 mL conical centrifuge tubes, and centrifuged at 3,578 RCF for 5 minutes. The supernatant was discarded, and the pellets were rinsed with 10 mL of HPLC grade water before another centrifugation at 4,000 RMP for 5 minutes. The tissue pellet was then resuspended in 3 mL of PBS solution (Sigma-Aldrich P4417059TAB) and stored at -80 °C until DNA extraction.

2.1.4 DNA Extraction

One mL of grape must material suspended in PBS solution was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 9,740 RCF for five minutes. The supernatant was discarded, the pellet was resuspended in 500 µL of 50 mM EDTA solution at a pH of 8 (Thermo Fisher 15575-038) and transferred to a 2 mL FastPrep tube containing 300 µL volume of an equal mixture of 0.1 mm and 0.5 mm of glass beads (Scientific Industries SI-BG01, SI-BG05). Samples were vortexed on a bead beater for 2.5 minutes, placed on ice for 3 minutes, and

vortexed again for an additional minute. An aliquot of 500 µL nuclei lysis solution (Fisher PR-A7941) was added and vortexed again for another minute followed by a 15-minute incubation at 95 °C. Samples were then centrifuged at 9,740 RCF for five minutes. The supernatant (750 µL) was transferred to a new 2 mL microcentrifuge tube and 300 µL of protein precipitate solution was added (Fisher PR-17951), briefly vortexed and incubated at room temperature for 15 minutes before centrifugation at 9,740 RCF for five minutes. The supernatant (600 µL) was transferred to another 2 mL microcentrifuge tube and 90 µL of 20% (w/v) polyvinylpyrrolidione (PVP) solution (Sigma-Aldrich PV40-50G) was added and pulse-vortexed for 20 seconds, then centrifuged again at 9,740 RCF for 10 minutes at 5 °C. The supernatant (600 µL) was transferred to another 2 mL microcentrifuge tube to which 360 µL of chilled 2-propanol was added. The tubes were inverted 10 times and left to incubate for 15 minutes at room temperature before centrifugation at 9,740 RCF for two minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL chilled 70 % ethanol and centrifuged again at 9,740 RCF for two minutes. The supernatant was discarded again, the pellet was air dried, resuspended in 100 µL of TE buffer, and stored at -80 °C until further analysis.

2.1.5 PCR

The total genomic DNA content and the purity of each sample was analyzed by the absorbance ratio of A260/280 nm (Nanodrop Thermo Fisher Scientific, United States) and diluted to 35 ng/µL. The fungal ITS1 region of the rRNA gene was amplified using the forward primer BITS: 5'-ACCTGCGGARGGATCA-3', and the reverse primer B58S3: 5'-GAGATCCRTTGYTRAAAGTT-3' (Bokulich and Mills, 2013). The primers where modified with CS1 and CS2 linker overhang sequences to allow for index library preparations according to the Illumina protocol. The amplicon PCR was set up as follows: 0.5 µL of 10mM mixtures of

the forwards and reverse primers, 12.5 µL of KAPA HiFi Hotstart Readymix (Roche Diagnostics, Basel Switzerland), 2.5 µL of DNA template, and 9.0 µL of molecular grade water for a total reaction volume of 25 µL. The thermocycler conditions were as follows: 95 °C for 2 min (1 cycle); 95 °C for 40 seconds, 55 °C for 40 seconds, 72 °C for one minute (25 cycles); 72 °C for 5 min (1 cycle).

PCR amplification was confirmed by gel visualization in 1.5% agarose gel with SYBR™ Safe DNA gel stain (Life Technologies, Carlsbad, CA, USA) on a Gel Logic 400 imaging system (Mandel, Rochester, NY, USA). Amplicon products were diluted based on the intensity of the gel band. Indexing PCR primers where complimentary to the CS1/CS2 linker sequences attached during the amplification PCR, and also contained P5/P7 Illumina adapter sequences. Each sample was barcoded with a unique combination of forward and reverse primer barcodes for downstream demultiplexing. The index PCR was set up as follows: 0.5 µL each of forward and reverse primers, 12.5 µL of KAPA HiFi Hotstart Readymix, 2.5 µL of DNA template, and 9.0 µL of molecular grade water. The thermocycler conditions were as follows: 95 °C for 1 min (1 cycle); 95 °C for 30 seconds, 62 °C for 30 seconds, 68 °C for 1.5 min (12 cycles); 68 °C for 5 min (1 cycle).

2.1.5 NGS Data Analysis

Amplified and indexed DNA sequences were submitted to the IBEST Genomics Resources Core facility at the University of Idaho (Moscow, ID, USA) sequencing. Sequencing data was collected on an Illumina MiSeq Desktop Sequencer (Illumina® Inc., San Diego, CA, USA) on a 250 x 2 bp run. The demultiplexed sequence files were processed using DADA2 (Callahan *et al.*, 2016). DADA2 was ran as described in the https://benjjneb.github.io/dada2/ITSworkflow.html tutorial. Briefly, sequences with ambiguous

base calls were removed, primers were trimmed, sequences were denoised and errors corrected before merging paired end reads. This analysis outputs an Amplicon Sequence Variation (ASV) table that was imported into Qiime2. A Native Baes classifier was trained using the most recent release of the UNITE fungal database (Nilsson *et al.*, 2019) to assign taxonomy to the sequence variants. α-diversity Simpson and Shannon indices were calculated in Qiime2. To analyze β-diversity, a Bray-Curtis dissimilarity metric was used.

2.1.6 Statistical Analysis

The packages qiiime2R (version 0.99.34) and phyloseq (version 1.32.0) were used to facilitate the importation of Qiime2 artifacts into R. For statistical analysis, each vineyard at Timepoint 1 and Timepoint 2 were analyzed individually. A Kruskal-Wallace Pairwise test with a Benjamin and Hochberg correction was performed on the Shannon and Simpson α-diversity metrics to determine any significant differences in α-diversity composition between treatments within each vineyard at each timepoint. Results were considered significant if the q-value was below 0.05. A Bray-Curtis dissimilarity metric (non-phylogenic) was used to compare β-diversity (fungal composition) between treatments within each vineyard. A pairwise Permutational Analysis of Variance (PERMANOVA) was used to analyze differences between treatments in the β-diversity metrics within each vineyard, differences were considered significant if the p-value < 0.05. Principle coordinate analysis (PCoA) plots were constructed to visualize β-diversity. Heatmaps were constructed to visualize the top 20 most abundant features in each vineyard at each timepoint. To analyze the differences in species abundances, a differential abundance analysis was performed using the ALDEX2 plugin within Qiime2 for significant β-diversity pairwise comparisons (Fernandes *et al.*, 2013). Differences in species abundance was considered significant if the expected p-value of the Wilcoxon rank test < 0.001. (Fernandes *et al.*, 2013).

2.2 Results

The fungal community composition was analyzed at 3 vineyards at 2 timepoints after exposure to simulated forest fire smoke and agricultural sprays (Fig 2.1). To determine the effects of the agricultural sprays and smoke exposure, each vineyard was analyzed individually and treatments within each vineyard were compared to the control vines for that respective vineyard at that timepoint. This was to control for the differences in the community composition of grapes between vineyards and changes throughout the ripening process.

A total of 8,848,794 ITS sequences were recovered from 258 samples with an average read length of 187 base pairs before merging paired end reads. The merged sequences were clustered into 2,892 Amplicon Sequence Variants (ASV's). The ASV's were classified using a dynamic taxonomic classifier and identified 3 phyla, 11 classes, 20 orders, 38 families, 46 genera, and 56 species of fungi. The most abundant phylum was Ascomycota which comprised 60% of the total reads, followed by Basidiomycota which represented 38% of the sequences obtained (Fig 2.2a). A total of 306 ASV's were shared between all of the vineyards (Fig 2.2b).

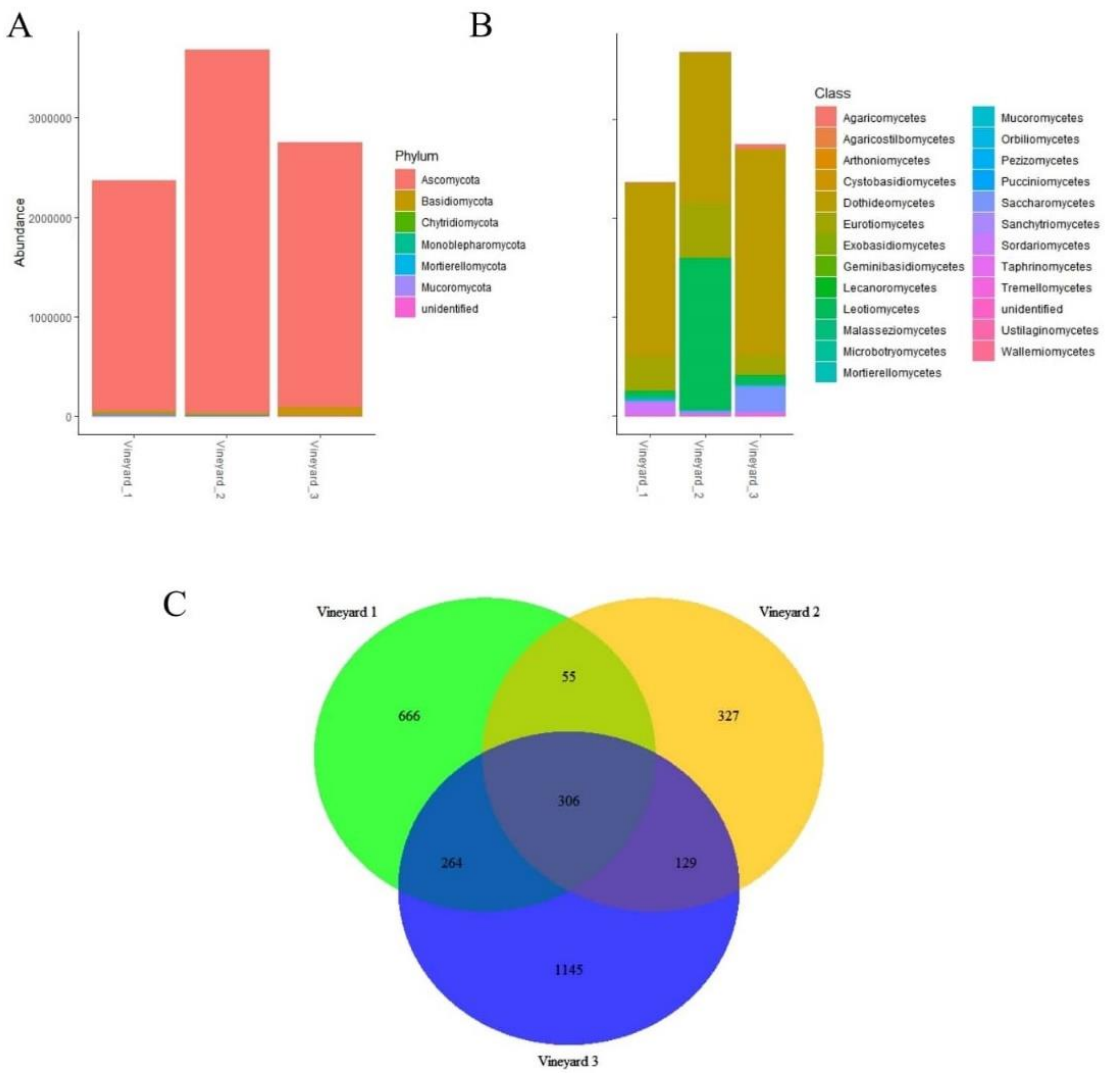

Figure 2.2 The relative abundance and distribution of identified ASVs between three vineyards in the Okanagan Region. a) Phylum abundances by vineyard. b) Class abundances by vineyard. c) Venn diagram of the number of shared and unique ASV's found between all three vineyards.

2.2.1 Differences between treatments

There were no significant differences in α-diversity or β-diversity between any of the treatments and their respective control vines for any timepoint at any of the vineyards (Table A.2, A.3). The Shannon and Simpson α-diversity metrics did not show any discernible patterns within each vineyard between treatments or timepoints (Fig 2.3). Based on the PCoA plots for

timepoints one and two (Fig 2.4), there are no overt trends visible at either post-treatment timepoint. To visualize the fungal community composition of treatments at each winery at each timepoint, heatmaps were constructed displaying the log10 percent abundance of the top 20 ASV's in each vineyard broken down by treatment (Fig A.1, A.2, A.3). Although not significant, there appear to be some trends in the heatmaps. The ASV identified as *Botryotinia pelargonii* appears to have decreased abundance in the treatment that was sprayed with Oil 2 and exposed to simulated forest fire smoke.

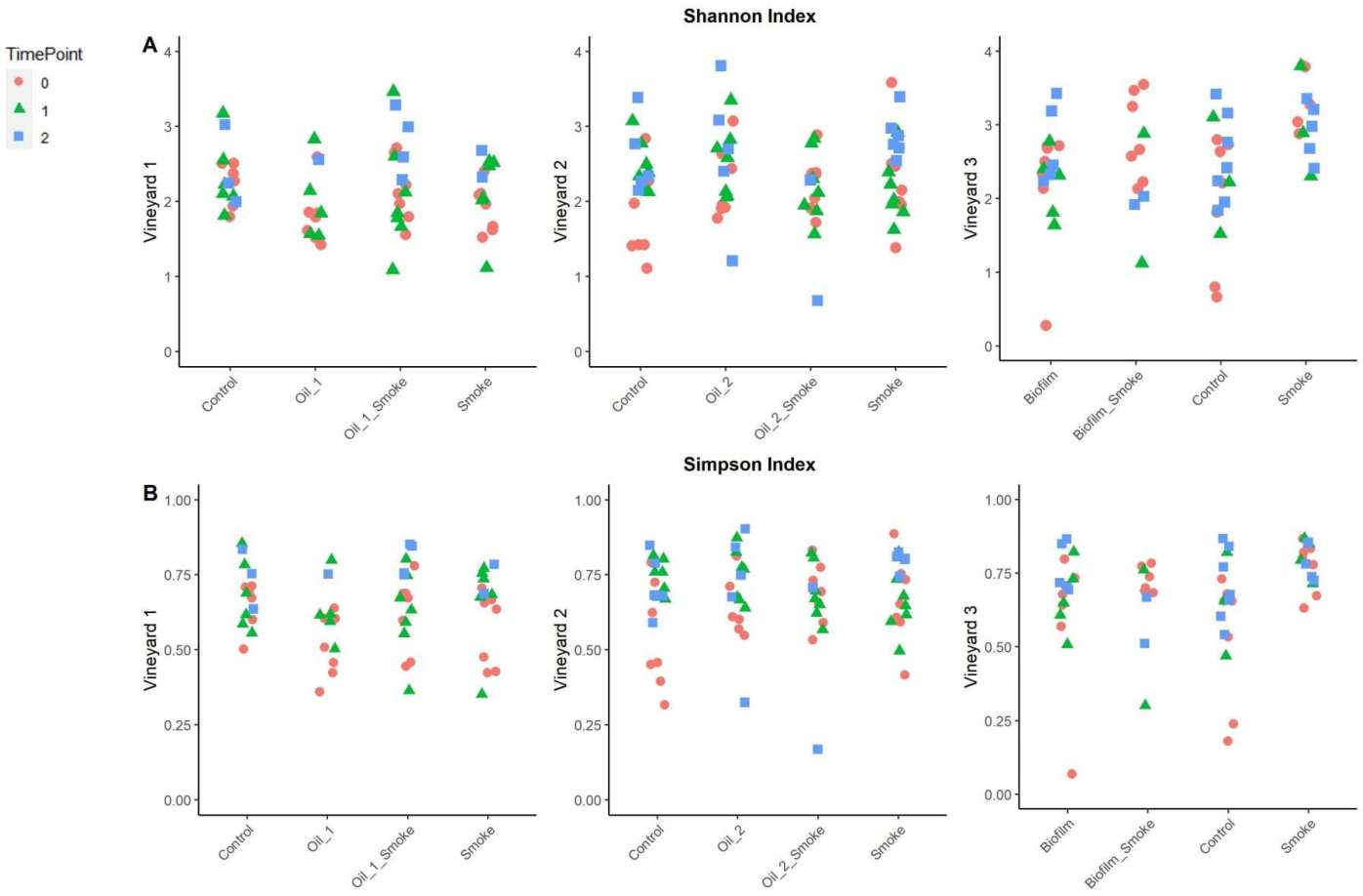

Figure 2.3 Alpha diversity indices of fungi at three wineries after treatments of simulate forest fire smoke, agricultural sprays, and both smoke and sprays (n=7 per treatment). After the application of treatments, grapes were sampled three times throughout the ripening process. For each timepoint both the Shannon diversity indices (a) were calculated, as well as the Simpson diversity indices (b). No significant differences were found between treatments.

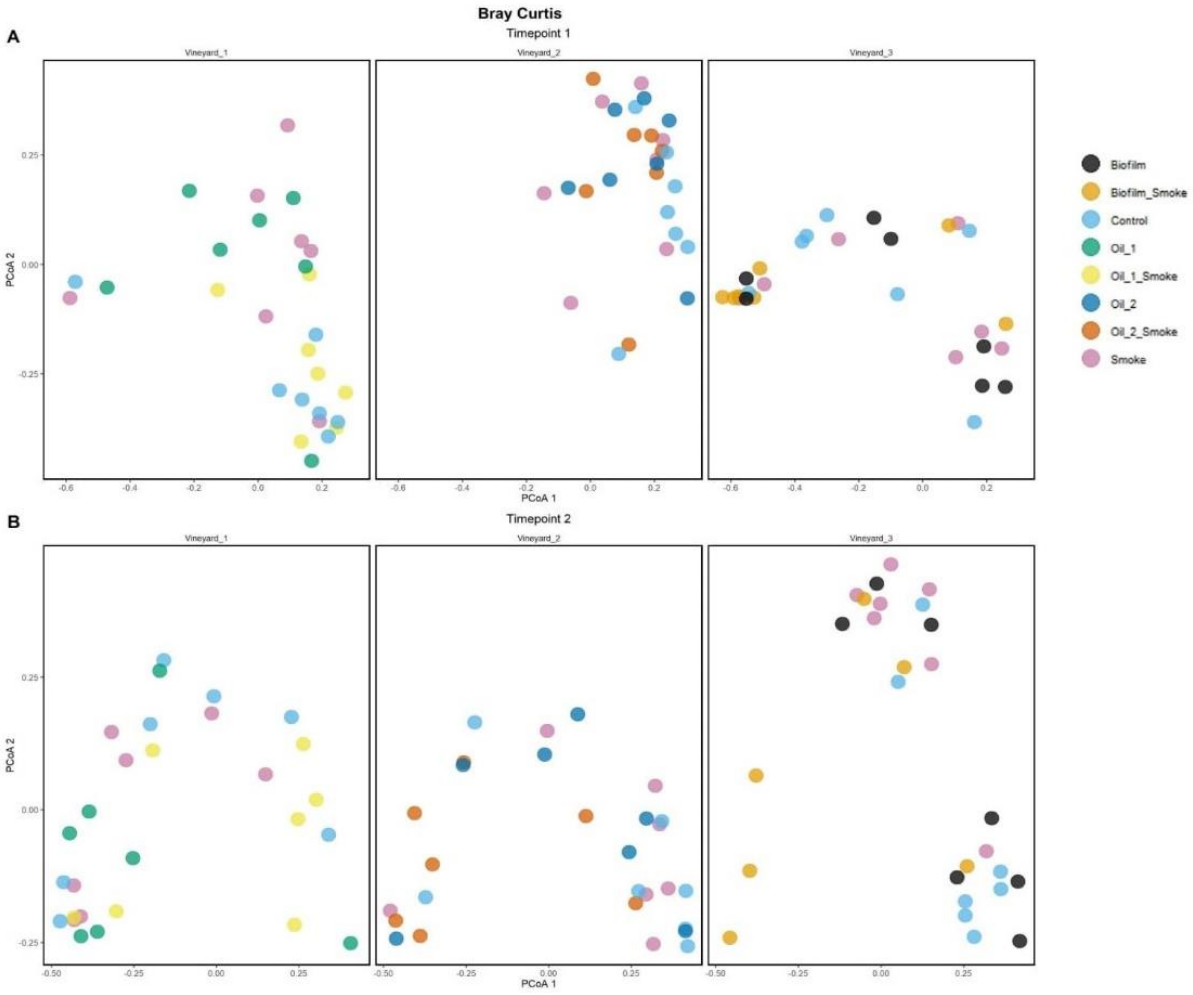

Figure 2.4 Principle coordinate analysis (PCoA) ordination plots were constructed on a Bray-Curtis distance matrix for the fungal communities on grapes treated with different agricultural sprays and exposed to simulated forest fire smoke (n=7 per treatment). Each datapoint represents the fungal community composition of a single grape bunch. Grapes were collected four days after the second smoke exposure (a) and at harvest (b).

2.2.2 Differences between vineyards

Overall, Vineyards 1 and 2 only shared 55 ASV's despite being geographically closer to each other than Vineyard 3, which shared 264 ASV's with Vineyard 1 and 129 ASV's with Vineyard 2. There were significant differences in β-diversity between the control vines of the vineyards at all 3 timepoints (Table A.4); however, there were not any significant differences in

α-diversity at any of the timepoints (Table A.5). When visualized in a PCoA plot, the control vines from Vineyard 2 at timepoint 0 show a strong separation from the other 2 vineyards along the PCoA 1 axis which explains 45% of the total variation (Fig 2.5a). A pairwise comparison of β-diversity showed a significant difference between Vineyard 1 and Vineyard 2 (PERMANOVA, *pseudo-F* = 8.67, $p < 0.01$) as well between Vineyard 3 and Vineyard 2 (PERMANOVA, *pseudo-F* = 8.93, $p < 0.01$) at the first timepoint (T0). At the second timepoint, 14 days later, there were still significant differences in the β-diversity of the control vines between Vineyard 1 and Vineyard 2 (PERMANOVA *pseudo-F*=4.56, $p < 0.01$) as well as between Vineyard 3 and Vineyard 2 (PERMANOVA *pseudo-F*=4.42, $p < 0.01$). This can be seen in the PCoA plot along the PCoA 2 axis explaining 40% of the variation (Fig 2.5b). At the time of harvest, the only significant difference between the control vines was between Vineyard 1 and Vineyard 3.

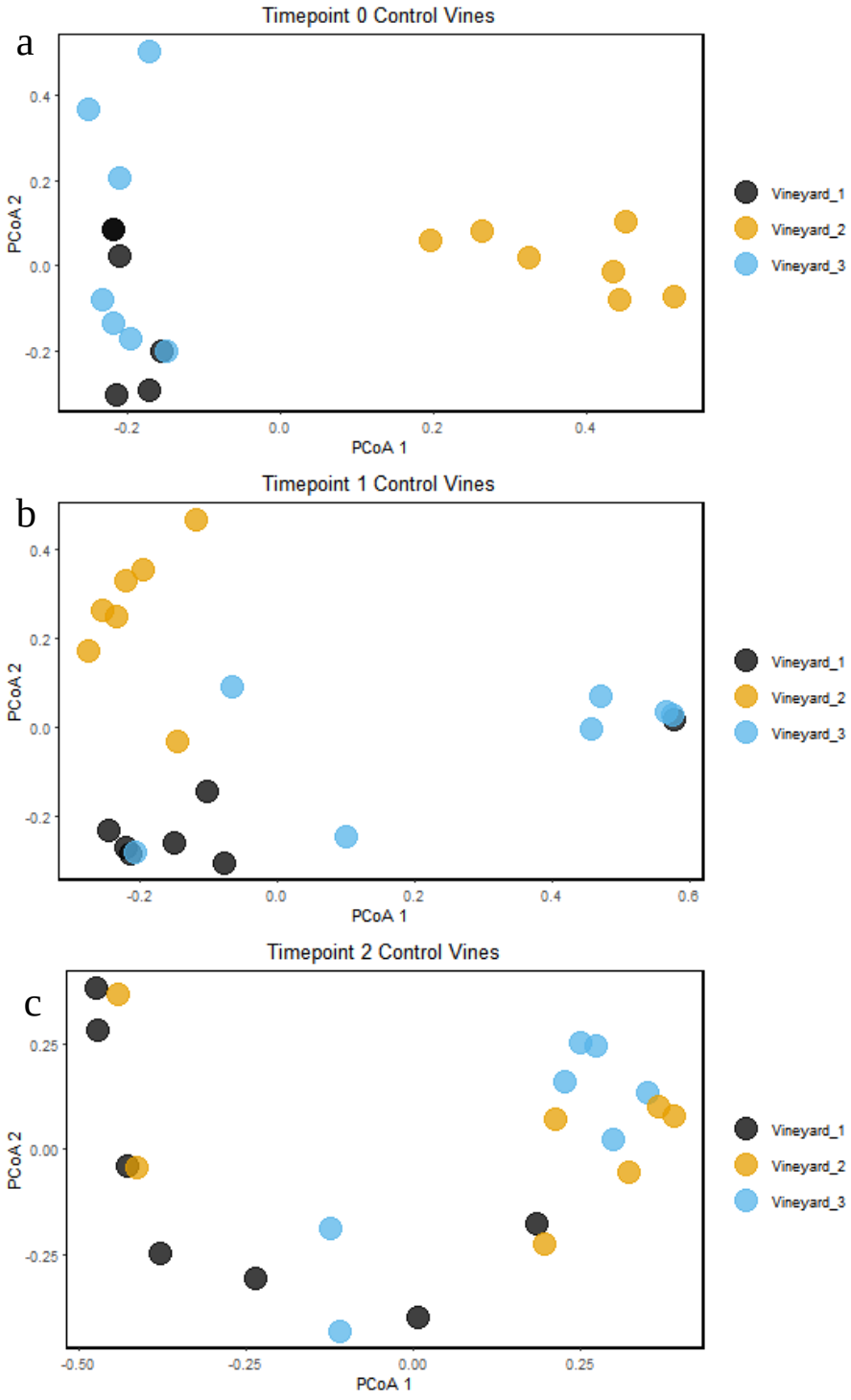

Figure 2.5 Principle coordinate analysis (PCoA) ordination plots were constructed on a Bray-Curtis distance matrix for the fungal communities on grapes at three vineyards in the Okanagan region throughout the ripening process (n=7 per treatment). Each datapoint represents the fungal community composition of a single grape bunch. Initial samples were taken early in the season (a), 14 days later (b), and again at harvest (c).

To visualize what species could be causing the pronounced separation of Vineyard 2 from the other vineyards at the first two timepoints and between Vineyard 1and Vineyard 3 at harvest, heatmaps were constructed showing the relative abundance of the top 30 most abundant features in the control vines at each timepoint (Fig 2.6). A pairwise differential abundance analysis was used to determine what ASV's were significantly different between the vineyards at these timepoints (Table A.6a-A.6e). At timepoint 0, Vineyard 2 contained higher relative abundance of *Erysiphe necator*, *Chalastospora gossypii*, as well as an ASV identified to the genus *Alternaria*, than compared to Vineyards 1 and 3 (Fig A.4-A.5, Tables A.6a-A.6b). At timepoint one, 14 days later, there were still significant differences between Vineyard 2 and the other two vineyards. The ASV's driving these differences were *Erysiphe necator* and *Alternaria rosae* which were both found in higher abundance in Vineyard 2 (Fig A.6-A.7, Tables A.6c-A.6d). At the time of harvest, the composition of the fungal community had shifted and the only significant pairwise comparison for β-diversity was now between Vineyard 1 and Vineyard 3. The species driving the differences between these vineyards were *Hanseniaspora uvarum* and *Metschnikowia sinensis* (Fig A.8, Table A.6e).

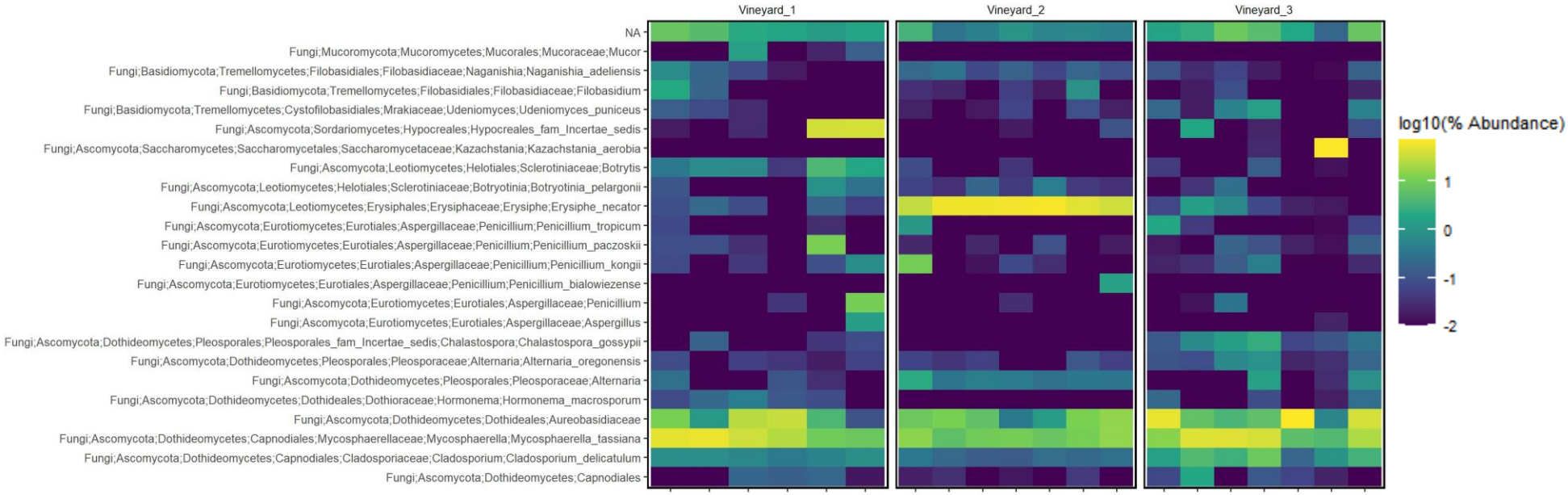

Figure 2.6 The log 10 relative abundance of the top 30 most numerous ASV's found on the control vines at three vineyards in the Okanagan early in ripening at timepoint 0 (a), 14 days later (b), and at harvest (c). Each column reperesnts one of 7 vines per treatment.

b

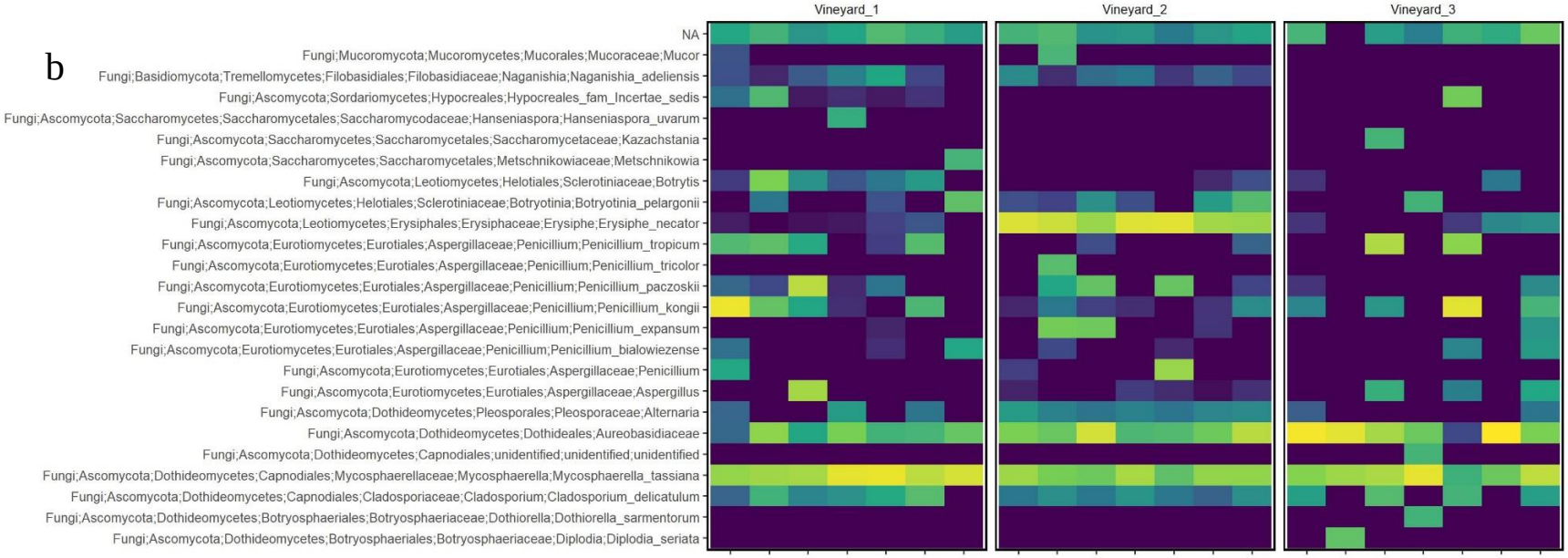

Figure 2.6 Continued.

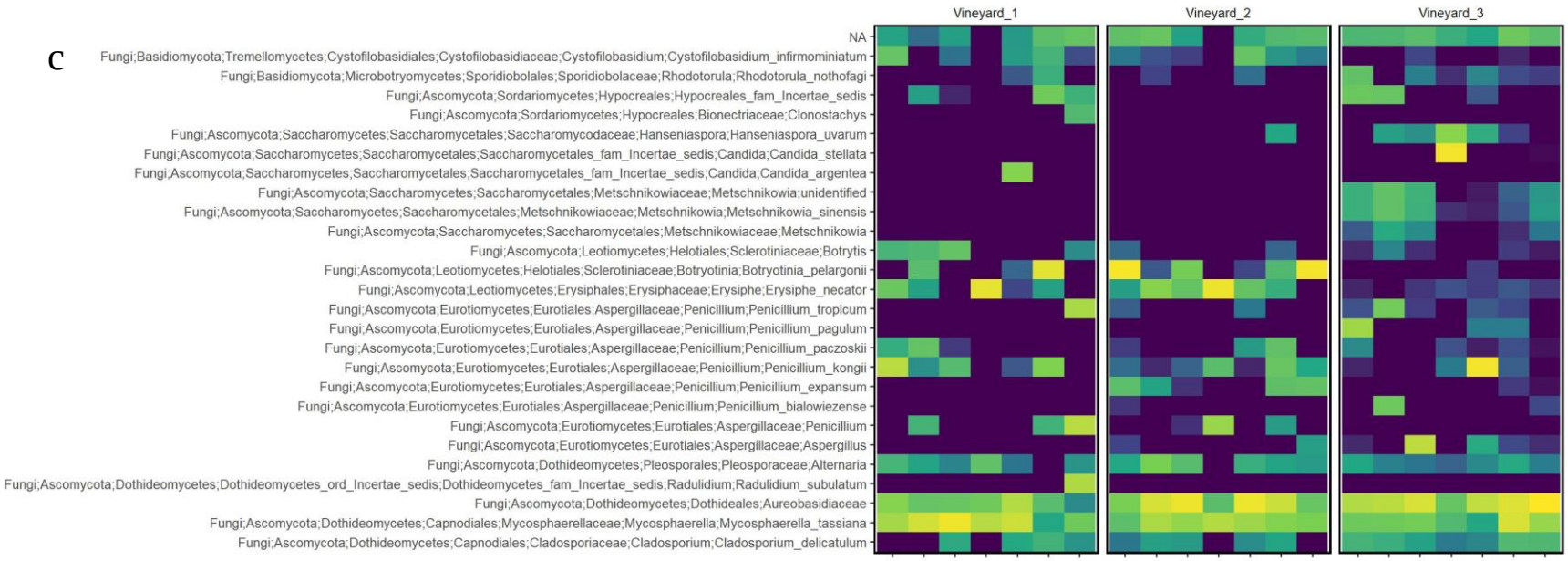

Figure 2.6 Continued

2.3 Discussion

2.3.1 Differences between treatments

The phylum Ascomycota was found in relatively high abundance in the sequences that I recovered. This was expected based on previous studies characterizing fungal communities in Vineyards. These studies found yeast from the Ascomycota phylum comprised between 23% - 96% of the recovered fungal sequences where the class *Dithideomycetes* comprised the vast majority of Ascomycota sequences; this class was also dominant in my study (Fig 2.2a, 2.2b) (Bokulich *et al.*, 2013; Pinto *et al.*, 2014; Miura *et al.*, 2017).

It was expected that the application of agricultural sprays (hypotheses 1b) and the exposure of smoke in this experiment would have altered the microbial community composition, especially considering that two of the three sprays used in the experiment are known fungicides frequently utilized by vineyards to manage pathogenic fungi. Exposure to dense forest fire smoke was also predicted (hypothesis 1a) to alter the fungal community through oxygen depletion and the deposition of particulate matter from the smoke. It is possible that the treatments applied to the vines did not have any effect on the fungal communities present, however if we assume there was an effect that was not detected, there are a few possible reasons for my results where no differences between treatments were found. These reasons will be discussed in the remaining paragraphs of this section.

It is not possible to determine if the DNA recovered from the grapes was from living or dead fungi. One of the trade-offs of using a culture-independent technique such as NGS to characterize microbial populations is that the analyzed DNA is extracted directly from environmental samples. This is beneficial because most fungal species found in-situ are not able

to be cultured in a lab and are therefore absent from culture-based methods. However, when using NGS to characterize environmental samples, there is no guarantee that the recovered DNA sequences are derived living microorganisms, which can lead to the overestimation of species diversity (Lundberg *et al.*, 2012; Lebeis and Bonardi, 2014; Tantikachornkiat *et al.*, 2016). Furthermore, the persistence of extracellular DNA in a given environment is heavily dependent on conditions and decay times are poorly characterized (Nagler *et al.*, 2018). The time between treatment application and sample collection was designed to allow time for the changes in the fungal community composition to become apparent in the environmental DNA, but it is also possible that the experimental design allowed too much time to pass and the grapes were re-colonized by healthy fungi before our samples were taken.

Another important factor to consider, and perhaps the most impactful, is that the agricultural sprays and smoke exposure treatments were only applied to 7 vines per treatment, these experimental vines were surrounded by untreated vines in the vineyard. Therefore, it is possible that the microbial community re-population was greatly accelerated due to the surrounding healthy vines compared to a vineyard-wide disturbance. The soil and surrounding ecosystems have been shown to act as reservoirs for microbial communities. Studies have shown that many ASV's detected in vineyards are also found in the surrounding ecosystem have been shown to act as reservoirs for microbial communities. Studies have shown that many ASV's detected in vineyards are also found in the surrounding ecosystem (Zarraonaindia *et al.* 2015). Miura *et al.* 2019 found that 45% of the fungal species detected in the vineyard were shared with the surrounding ecosystem. Additionally, studies have suggested that microbes from the soil are able to transport through roots of the vines and quickly recolonize fruiting bodies (Compant *et*

al., 2011). This implies that an environmental disturbance only applied to a small population of vines within an otherwise typical vineyard would be quickly recolonized.

An additional consideration for the smoke exposure treatments is that naturally occurring forest fires were emitting large amounts of smoke the year the experiment was conducted, therefore there was already a significant amount of smoke exposure to grapes vines before the experiment began as well as during the experiment. As forest fires are increasing in severity every year, it is not likely that an in-vineyard experiment on smoke exposure will be able to be conducted in the absence of this confounding factor.

2.3.2 Differences between vineyards

The significant differences between Vineyard 2 and the other two vineyards at the first two timepoints is most likely driven by vineyard management practices as opposed to environmental conditions. Vineyard 1 and Vineyard 2 were only 1.2 km apart making it unlikely that there were sufficient geographical variations to induce the observed differences (Fig. 2.1). Additionally, Vineyards 1 and 3 did not have any significant differences in β-diversity at the first two timepoints despite being 5.67 km apart on the other side of Lake Okanagan on a slope facing the opposite direction than the first two wineries. The most abundant species driving the significant difference was *Erysiphe necator* which is a pathogenic fungus that causes what is known as 'powdery mildew'. This pathogen thrives in warmer humid climates and causes crop loss, delayed ripening, as well as off-flavors in wine (Stummer *et al.*, 2003; Huang *et al.*, 2017). A study conducted by Martins *et al.* in 2014 found that vineyard management practices had a significant effect on the fungal community growing on grapes in the vineyard. Therefore, because the vineyards that participated in this study declined to share with us any of their

management practices, it is impossible to determine any other correlations between fungal community composition and management practices.

The significant differences in β-diversity found at harvest between Vineyards 2 and 3 is more likely to be caused by geographical variation, or a combination of geographical variation and vineyard management. Vineyards 1 and 2 are organic, while Vineyard 3 is traditional. The main species contributing to the separation of Vineyards 1 and 3 at harvest are *Hanseniaspora uvarum*, *Metschnikowia sinensis*, as well as an ASV only identified to the family Aureobasidiaceae (likely dominated by *Aureobasidium pullulans*). *Hanseniaspora uvarum* and *Metschnikowia sinensis* are not unexpected winery resident yeasts and typically increase in abundance throughout ripening when found (Nisiotou and Nychas, 2007; Barata *et al.*, 2008; Barata, Malfeito-Ferreira and Loureiro, 2012). Variations in species succession likely also contributed to the diverging community compositions (Liu and Howell, 2020).

Many studies have looked at how the fungal community composition on grapes differ across spatial distributions in ecosystems (Bokulich *et al.*, 2013; Drumonde-Neves *et al.*, 2017; Mezzasalma *et al.*, 2017; Miura *et al.*, 2017; Liu and Howell, 2020). These differences were found in scales larger than the distances between the vineyards in this study; however, Vineyard 3 is situated on a slope facing south-east while the other two vineyards face the north-west. These results indicate a robustness to microbial communities found in vineyards through environmental disturbances. However, further research should be conducted on a larger scale to determine if a more widespread an environmental disturbance has a greater affect as opposed to the localized treatments applied in this study.

Chapter 3: Unique organoleptic compounds produced by indigenous *Saccharomyces uvarum* and *Saccharomyces cerevisiae* strains during alcoholic fermentation of Chardonnay wines

3.1 Synopsis

Each wine growing region around the world hosts unique communities of indigenous yeasts, which may enter fermentations and contribute to the final flavor profile of wines. Winemakers are increasingly interested in harnessing these indigenous yeasts to produce more unique and complex wines that can be considered truly local. One of these yeasts is *Saccharomyces uvarum*, which is typically described as a cryotolerant yeast that produces relatively high levels of glycerol and the rose-scented volatile compounds 2-phenylethanol and 2-phenylethyl acetate. Recent findings show that *S. uvarum* populations exhibit high genetic diversity in uninoculated fermentations; this may suggest a high diversity in metabolic function within the species as well. *Saccharomyces cerevisiae* is one of the first domesticated yeasts and is still the primary species used in inoculated fermentations. The objective of this study was to compare the fermentation kinetics and production of organoleptic compounds by indigenous and commercial *Saccharomyces* strains (both *S. cerevisiae* and *S. uvarum*) at different temperatures. I conducted laboratory-scale fermentations of Chardonnay juice at 15 °C and 25 °C, monitoring fermentation kinetics and the production of organoleptic compounds by 11 *Saccharomyces* yeast strains (six indigenous *S. uvarum*, one commercial *S. uvarum*, one indigenous *S. cerevisiae*, and three commercial *S. cerevisiae*). The indigenous *S. uvarum* strains showed comparable kinetics to commercially sourced strains at both temperatures. Organoleptic compound production among the strains was more variable at 15 °C than 25 °C. Furthermore, the organoleptic profiles produced by the indigenous *S. uvarum* strains tended to depart from *S. cerevisiae* strains more at 15 °C than at 25 °C. Indigenous *S. uvarum* strains produced relatively high levels of 2-phenylethyl acetate and 2-phenylethanol whereas these compounds in commercially sourced

strains of both *S. cerevisiae* and *S. uvarum* were found at much lower levels. Production of glycerol in the indigenous *S. uvarum* strains used in this study did not differ from the commercial strains. Our data demonstrate that the high genetic diversity among the different indigenous *S. uvarum* strains was reflected in their unique fermentation property profiles, especially at a lower fermentation temperature.

3.2 Materials and Methods

3.2.1 Yeast Strains Used

Eleven *Saccharomyces* yeast strains were chosen for this study: seven *S. uvarum* strains (six indigenous and one commercial), and four *S. cerevisiae* strains (one indigenous and three commercial) (Table 3.1). The *S. uvarum* and *S. cerevisiae* strains of indigenous origin were isolated from uninoculated fermentations conducted at commercial wineries in the Okanagan Valley wine region of Canada between the 2013 and 2017 vintages. Each strain was typed using 11 hypervariable microsatellite loci, as described previously (Morgan *et al.*, 2019). The microsatellite profiles of all strains are provided in the supplementary material (Table B1, B2).

Table 3.1. *Saccharomyces* strains used in controlled Chardonnay fermentations at two different temperatures.

Strain name	Species	Yeast type	Source
Velluto BMV58™	*S. uvarum*	Commercial	(Lallemand, Edwardstown, SA, Australia)
P01H01	*S. uvarum*	Indigenous	(unpublished data)
2015 Strain 1	*S. uvarum*	Indigenous	(McCarthy *et al.*, 2019; Morgan, Mccarthy, *et al.*, 2019)
2015 Strain 3	*S. uvarum*	Indigenous	(McCarthy *et al.*, 2019; Morgan, Mccarthy, *et al.*, 2019)
2015 Strain 163	*S. uvarum*	Indigenous	(McCarthy *et al.*, 2019; Morgan, Mccarthy, *et al.*, 2019)
2017 Strain 097	*S. uvarum*	Indigenous	(McCarthy *et al.*, 2019)
2017 Strain 151	*S. uvarum*	Indigenous	(McCarthy *et al.*, 2019)
Fermol Mediterranée	*S. cerevisiae*	Commercial	(AEB Group, Lodi, CA, USA)
Premier Classique ǂ	*S. cerevisiae*	Commercial	(Red Star Yeasts, Milwaukee, WI, USA)
Lalvin EC-1118	*S. cerevisiae*	Commercial	(Lallemand, Montréal, QC, Canada)
UBC47	*S. cerevisiae*	Indigenous	(unpublished data)

ǂ Formerly known as Red Star Montrachet

All indigenous *S. uvarum* strains used in this study except for P01H01 were isolated from uninoculated Chardonnay fermentations conducted at a single commercial winery during the 2015 and/or 2017 vintages. The indigenous *S. uvarum* strain '2015 Strain 1' was a dominant strain in both 2015 and 2017 fermentations (McCarthy *et al.*, 2019, Morgan *et al.*, 2019). During previous laboratory-controlled experiments, this strain was able to compete with a commercial *S. cerevisiae* strain at low temperatures, and produced a unique aroma profile (Morgan *et al.*, 2020). The indigenous *S. uvarum* yeast strain '2015 Strain 3' was also a dominant strain in both 2015 and 2017 at the same commercial winery (McCarthy *et al.*, 2019, Morgan *et al.*, 2019). The indigenous strain '2015 Strain 163' was found in both vintages as well but was not considered a dominant strain in either vintage, whereas the indigenous strains '2017 Strain 151' and '2017 Strain 097' were found to be dominant in 2017, but were not isolated at all in 2015 (McCarthy *et al.*, 2019). The indigenous *S. uvarum* strain P01H01 was originally isolated in 2013 and

dominated Pinot Gris fermentations at a different Okanagan winery (unpublished data). The commercial *S. uvarum* strain Velluto BMV58 (Lallemand Inc, Edwardstown, SA, Australia) was used as a commercially sourced comparison. This strain was originally isolated in Spain (https://www.lallemandwine.com/en/australia/products/catalogue/wine-yeasts/66/velluto-bmv58/ accessed 2020-07-28) and was the only pure *S. uvarum* strain available commercially for wine fermentations when this study was conducted.

The *S. cerevisiae* strain UBC47 is a putative indigenous strain originally isolated from uninoculated fermenting musts in the Okanagan Valley. The *S. cerevisiae* strains EC-1118 (Lallemand Inc., Petaluma, CA, USA), Fermol Mediterranée (AEB Group, Lodi, CA, USA), and Montrachet Premier Classique (Red Star Yeasts, Milwaukee, WI, USA) are commercially available. The commercial *S. cerevisiae* strain EC-1118 functioned as a standard against which the other yeast strains, particularly the indigenous and non-traditional yeast strains, were compared. This is because EC-1118 is a very commonly used yeast strain in wine research, and its fermentation kinetics have been well-studied (Egli *et al.*, 1998; Callejón *et al.*, 2012; Lee *et al.*, 2012).

3.2.2 Experimental Design and Sampling

The 11 *Saccharomyces* strains were each inoculated into sterile-filtered Chardonnay juice for single-strain fermentations under controlled conditions. The fermentations were conducted at both 15 °C and 25 °C, for a total of 22 treatments (n = 3 per treatment). Chardonnay juice (Cellarmaster Wines, Surry Hills, NSW, Australia) was diluted to 23 °Brix with water (as measured by a refractometer), and sterile filtered by successive filtration beginning with 11 µm

pore nitrocellulose filters and ending with 0.22 µm pore nitrocellulose filters (GSWP04700 Millipore Sigma, Burlington MA, USA).

A single colony of each strain was aseptically transferred to a flask containing liquid YPD medium (yeast extract 10 g/L, bacteriological peptone 20 g/L, dextrose 20 g/L) and incubated overnight at 25 °C. The starter cultures were used to inoculate the sterile Chardonnay juice at 10^6 cells/mL, as approximated by absorbance at 600 nm by spectrophotometer. The 500 mL fermentation flasks (GL45, Schott) were sealed with custom airlocks 3D-printed made from cyanate ester (CE) 220 carbon resin with aluminum tops, filled with 5 mL of sterile water and containing a central sampling port closed with a silicone septum (https://www.carbon3d.com/case-studies/tthandadelaide/, accessed 2020-06-29).

Fermentations were conducted with constant agitation inside incubators at 80 RPM. A negative control of a flask of juice left uninoculated was included in each round of fermentations at both temperatures for chemical composition comparisons and to confirm the absence of contaminants. Fermentation vessels were fitted with 3D printed one-way airlock inserts to allow for gas release without contamination. Fermentation progress was measured in weight loss of CO_2 as described previously (Ergun and Ferda Mutlu, 2000). Due to the limited space in each incubator and the large number of strains, it was not feasible to conduct every fermentation at once. To address this issue, this experiment was conducted in three batches, with one replicate from each strain represented in each batch. To keep the duration of fermentations consistent, all fermentations of each batch were ended when the EC-1118 replicate fermentation at 15 °C reached completion as determined by weight loss of CO_2 and fermentation was no longer progressing, this was determined to be 25 days during the first round of fermentations and kept constant for subsequent replicates.

3.2.3 Chemical Analysis

An aliquot of 15 mL of fermented Chardonnay wine was sampled from each flask at the termination of the fermentation and stored at -80 °C for chemical analysis using both GC-MS and enzyme kits. A comprehensive table of properties for all of the chemical compounds analyzed in this study can be found in the Appendix (Table B3).

Standards for compounds were run for retention time and mass spectral matching (Table 3.2). Standard solutions were prepared from high purity solutions obtained from: Alfa Aesar (Ward Hill, MA, USA; (99%) 3-methylbutanoic acid, 2-methylbutanoic acid, octanoic acid, 2-methylpropanol, hexanol, benzalcohol, 2-methyl butanol, 3-methyl butanol, ethyl-2-methyl propanoate, ethyl butanoate, ethyl-2-methyl butanoate, ethyl-3-methyl butanoate; Aldrich (Milwaukee, WI, USA; ≥99%) hexyl acetate, 2-methylbutyl acetate , 2-phenylethyl acetate, hexanoic acid, methionol, 2-phenylethanol, ethyl hexanoate, ethyl octanoate, ethyl decanoate; Fluka (Buchs, SG, Switzerland; ≥99%) ethyl acetate; Acros Organics (Fair Lawn, New Jersey, USA) ethyl propanoate.

Table 3.2. Volatile compounds analyzed in controlled Chardonnay fermentations using Headspace-GCMS. If more than one value for Kovat's Retention Index was available in the NIST database, the range is displayed.

Compound Category	Compound	Standard Ran	Retention Time (min)	Kovat's Retention Index (polar/TempRamp)	Quantitation Ion	Confirmation Ions
Acetates	ethyl acetate	Yes	3.57	880-900	43	61, 29
	hexyl acetate	Yes	10.14	1255-1301	88	228, 101
	2-methylbutyl acetate	Yes	5.71	1132	72	57, 69, 74
	2-phenylethyl acetate	Yes	27.92	1785-1835	104	65, 91
Acids	hexanoic acid	Yes	29.78	1857	60	73, 87, 157
	3-methylbutanoic acid	Yes	22.69	1660	60	61, 74, 87
	2-methylbutanoic acid	Yes	22.72	1660	74	60, 73, 87
	acetic acid	No	16.28	1400-1465	60	43,
	octanoic acid	Yes	34.59	2070	60	73, 115
Alcohols	2-methylpropanol	Yes	4.75	1075-1122	74	41, 43
	hexanol	Yes	12.76	1320-1362	56	45, 55
	methionol	Yes	24.20	1708-1738	106	61, 73
	benzalcohol	Yes	30.73	1834-1895	79	51, 77, 108
	2-phenylethanol	Yes	9.19	1874-1931	55	70,
	2-methyl butanol	Yes	7.11	1194-1202	57	55, 56, 70
	3-methyl butanol	Yes	7.74	1190-1240	55	42, 56, 57, 70
Ethyl Esters	ethyl-2-methyl propanoate	Yes	3.77	961	116	43,
	ethyl butanoate	Yes	4.55	1008-1035	88	101, 116
	ethyl-2-methyl butanoate	Yes	4.37	1053	102	87, 115
	ethyl-3-methyl butanoate	Yes	4.67	1067	88	115, 130
	ethyl hexanoate	Yes	8.49	1220-1267	88	99, 115
	ethyl octanoate	Yes	15.04	1442-1477	88	101, 172
	ethyl decanoate	Yes	20.75	1639-1678	101	157, 200
	ethyl propanoate	Yes	3.92	80-170	57	43, 102
Formates	ethyl formate	No	3.37	822-848	28	45.1, 32
Aldehydes	acetaldehyde	No	3.14	689-744	44	29
Amines	ethananolamine	No	2.97	1402-1427	28	43, 44
Flavenoids	5,7 Dihydroxy 3,3',4',5',6,8 hexamethoxyflavone	No	29.96	1880	28	32, 44

3.2.3.1 GCMS Analysis

A 6 mL aliquot of fermented Chardonnay juice was mixed with 2 g of NaCl into borosilicate glass headspace vials. A pooled QC mixture was created using equal volumes from all samples and injected between every 10 sample injections to monitor instrument stability. Prior to injection, samples were incubated at 70 °C for 10 minutes, pressurized to 19 kPa, and

equilibrated for 0.2 minutes. A 1 mL portion of headspace gas was collected using a headspace autosampler attached to a Thermo Scientific Trace 1300 gas chromatograph. Compound separation was performed on a Zebron™ ZB-WAX GC capillary column (60 m x 0.25 mm x 0.25 μm, cat. 7KG-G007-11) The chromatography conditions were as described by Haggerty *et al.*, (2016). In short, A constant flow rate of 1.7 mL/min was used with an inlet temperature of 250 °C. The temperature program started at 80 °C for 10 minutes, increased to 130 °C at a rate of 10 °C/minute, was held constant for 5 minutes, increased to 151 °C at a rate of 2 °C/minute, increased to a final temperature of 240 °C at a rate of 20 °C/minute and was held for 5 minutes. The chromatograph was coupled to a Thermo ISQ mass spectrometer. The transfer line temperature was set to 250 °C. The MS source temperature was set to 230 °C and an ionization energy of 70 eV. The eluting compounds were analyzed using in full scan mode with a range of 25-500 m/z with a 0.2 second dwell time. Peaks were identified using a combination of comparison to standards injected separately, as well as putative identifications based on spectral matches in the NIST database, and Kovat's Retention indices. Each peak area was normalized to the total ion current (TIC) of the injection to produce relative quantitative values used in downstream statistical analysis.

3.2.3.2 Enzyme Kit Analysis

Residual sugars (glucose and fructose) were quantified using the Megazyme® D-Fructose/D-Glucose Assay Kit (K-FRUGL, Wicklow, Ireland) following the manufacturer's specifications with the following modification: to increase the accuracy and precision of the measurements, instead of sequential addition of enzymes with measurements taken in between the additions, I recorded separate absorbance measurements in three different wells as follows. The first well contained 200 µL of HPLC grade water, 10 µL of sample diluted 1:100 in HPLC

grade water, 10 µl of Solution 1 (buffer), and 10 µL of Solution 2 ($NADP^+$/ATP). The second well contained the same as the first well plus 2 µL of Solution 3 (HK/G6P-DH). The third well contained the same as the second well plus 2 µL of suspension 4 (PGI). As such, for each individual sample there were 3 wells: a blank well with buffer reagents; a second well measuring the absorbance for glucose and fructose; and a third well measuring the absorbance for fructose alone. A six-point standard curve ranging from 200 µg/mL to 6.25 µg/mL was made using the standards provided with the kit. Two technical replicate measurements were performed on each sample. Absorbance data was collected using plate reader (Varioskan Lux, Thermo Scientific Walthan, MA USA).

Glycerol was quantified using the Megazyme® Glycerol Kit (K-GCROL). Samples were diluted 1:100 in HPLC grade water. A six-point standard curve ranging from 6.25 µg/mL to 200 µg/mL was made using the standards provided with the kit. Two technical replicate measurements were performed on each sample.

Ethanol was quantified using the Megazyme® Ethanol Kit (K-ETOH). Samples were diluted 1:1000 in HPLC grade water. A six-point standard curve was made using the standard provided in the kit ranging from 20 µg/mL to 150 µg/mL. Two technical replicate measurements were performed on each sample and averaged for analysis.

3.2.4 Statistical Analysis

All statistical analysis was performed in R 3.6.1 (R Core Team 2019). All tests were considered significant at $p \leq 0.05$. The reproducible code is provided in full in the supplementary materials.

3.2.5 Kinetics Analysis

The *growthcurver* R package (Sprouffske, 2018) was used to fit a logistical curve and calculate the area under this logistical curve for each sample by taking the integral of the logistic equation (Liccioli, Chambers and Jiranek, 2011). This value provides a metric, which includes all parameters of the growth curve including initial population size, maximum growth rate, and carrying capacity into a single value. A one-way analysis of variance (ANOVA) was used to analyze the differences in the logistical curve area between strains within each temperature group (Table B4, B5). A student's *t*-test was used to analyze differences for each strain between temperature treatments.

3.2.6 Compound Analysis

A principle component analysis (PCA) ordination was used to visualize the distribution of strains within each temperature treatment, in terms of their production of organoleptic compounds. To analyze the difference in organoleptic compounds among strains within a temperature treatment, a one-way ANOVA was performed on the normalized peak areas for each compound, followed by a Tukey HSD *post hoc* analysis using the *agricolae* R package (Felipe de Mendiburu, 2020), adjusted for multiple comparisons (Table 3.3, B5, B5). To analyze changes in the aroma profile of a single strain when fermented at 15 °C as compared to 25 °C, a student's *t*-test was performed for each compound using R base package (R Core Team 2019).

3.3 Results

3.3.1 Fermentation Properties

All strains completed fermentations more slowly at 15 °C than at 25 °C, and most *S. uvarum* strains had comparable kinetics to most *S. cerevisiae* strains at 25 °C (Fig. 3.1, ANOVA

results in Table B4, B5). *S. cerevisiae* strain EC-1118 had a statistically significantly larger area under the logistical curve (AUC) value than most other strains at both temperatures, except for *S. uvarum* '2017 strain 151' at 15 °C and BMV58 at 25 °C, where the AUC metric did not differ significantly from EC-1118. At 15 °C, however, *S. uvarum* '2017 strain 151' exhibited a significantly larger AUC value than the indigenous *S. cerevisiae* strain UBC47 and the commercial *S. cerevisiae* strains Fermol Mediterranée and Premier Classique. There were no statistically significant differences found in the residual sugar concentrations between strains except for the commercial *S. cerevisiae* strain Premier Classique, which consumed less glucose than EC-1118 at 15 °C (Table 3.3). Additionally, there were no significant differences found in glycerol concentrations within each temperature treatment. The indigenous *S. cerevisiae* strain UBC47, however, produced less glycerol at 15 °C than at 25 °C. Lastly, strains did not differ significantly in the final concentrations of ethanol.

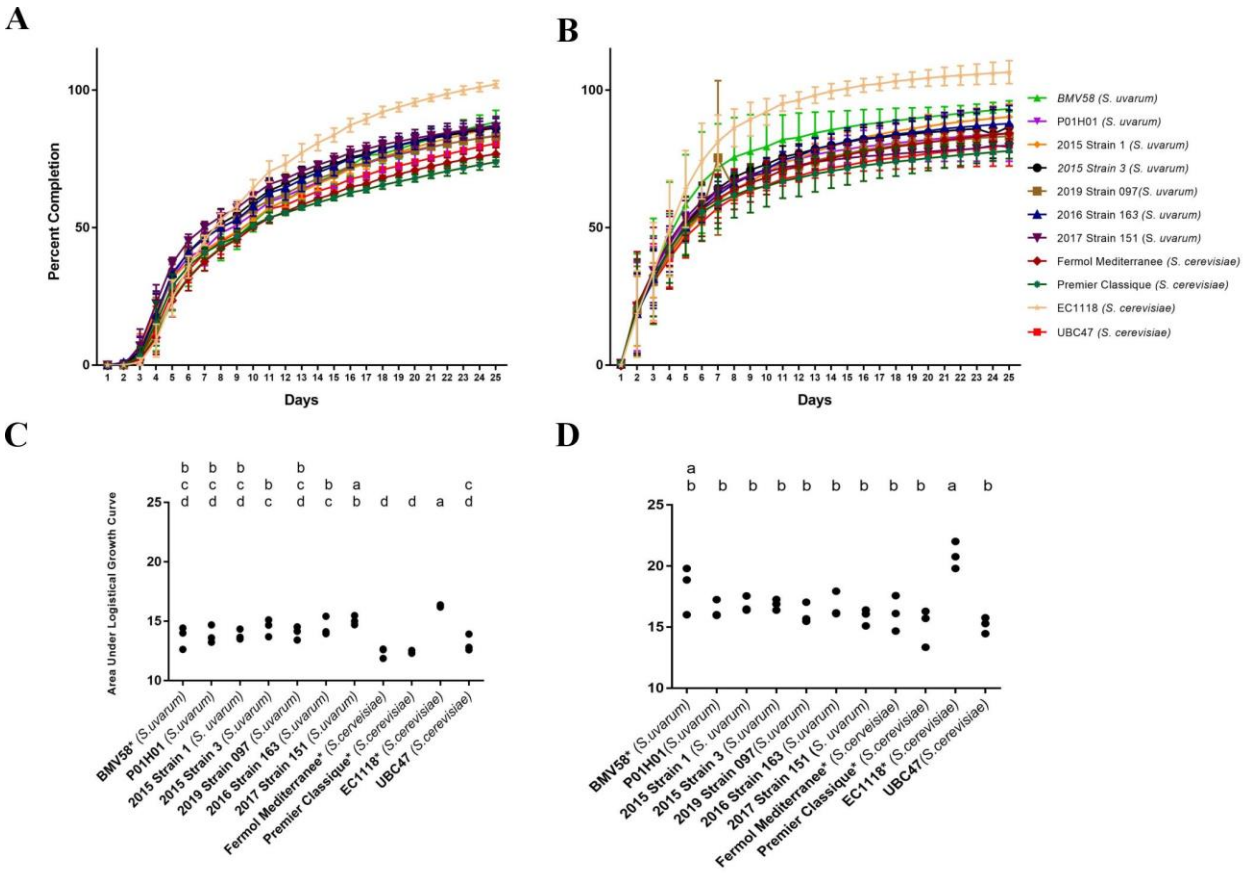

Figure 3.1 Fermentation kinetics of different *S. cerevisiae* and *S. uvarum* strains incubated at **A)** 15 °C and **B)** 25 °C, showing percent completion of fermentation ± SD (n = 3 per treatment). Scatterplots of the area under the calculated logistical curves for each replicate fermentation were calculated for the fermentations at **C)** 15 °C and **D)** 25 °C. Fermentation progression was measured by weight loss as a function of CO_2 production during alcoholic fermentation. Fermentation vessels were weighed daily. Differences in the areas were analyzed using 2-way ANOVA followed by a Tukey HSD *post hoc* test. If letter codes for any given strains do not share a letter, then the strains are significantly different at $p \leq 0.05$. An asterisk (*) indicates that the strain is of commercial origin.

Table 3.3 Concentration of fermentation-related compounds identified in Chardonnay wines fermented by 11 *Saccharomyces* yeast strains at two different temperatures (15 °C and 25 °C) (*n* = 3 per treatment). Samples were taken at the termination of fermentation and presented as the mean ± SD. Data were analyzed using an ANOVA followed by a Tukey HSD *post hoc* test. Strains in the same column that do not share a letter code were significantly different at $p < 0.05$ as calculated by a Tukey HSD *post hoc* test.

		Residual Glucose (g/L)		Residual Fructose (g/L)		Glycerol (g/L)		Ethanol (g/L)	
	Strain Name	15 °C	(25 °C	15 °C	25 °C	15 °C	25 °C	15 °C	25 °C
S. uvarum	BMV58‡	4.1 ± 1.1 ^{ab}	4.5 ± 2.2 ^{a}	25.9 ± 11.5 ^{a}	31.9 ± 14.4 ^{a}	10.5 ± 1.9 ^{a}	11 ± 0.6 ^{a}	71.3 ± 7.7 ^{a}	84.2 ± 33.2 ^{a}
	P01HO1	5.8 ± 2.5 ^{ab}	9 ± 6.0 ^{a}	34.3 ± 16.5 ^{a}	39.5 ± 25.2 ^{a}	9.2 ± 1.3 ^{a}	10.6 ± 1.6 ^{a}	73.4 ± 23.6 ^{a}	70.5 ± 19.5 ^{a}
	2015 Strain 1	8.9 ± 2.8 ^{ab}	9 ± 3.2 ^{a}	38.6 ± 14.5 ^{a}	39.3 ± 17.1 ^{a}	7.8 ± 0.6 ^{a}	8.1 ± 0.7 ^{a}	57.6 ± 15.5 ^{a}	66 ± 13.8 ^{a}
	2015 Strain 3	4.8 ± 1.6 ^{ab}	7.7 ± 2.7 ^{a}	29.1 ± 11.6 ^{a}	42.2 ± 14.9 ^{a}	11.9 ± 0.9 ^{a}	11.1 ± 1.0 ^{a}	67.6 ± 3.9 ^{a}	73.2 ± 15.6 ^{a}
	2019 Strain 097	3.7 ± 2.3 ^{ab}	3.6 ± 2.6 ^{a}	25.2 ± 15.9 ^{a}	29.3 ± 18.6 ^{a}	6.5 ± 5.1 ^{a}	9.4 ± 2.6 ^{a}	66.2 ± 10.1 ^{a}	83.3 ± 29.3 ^{a}
	2016 Strain 163	1.5 ± 0.9 ^{ab}	1.4 ± 1.1 ^{a}	9.1 ± 5.9 ^{a}	13.6 ± 9.8 ^{a}	7.2 ± 0.9 ^{a}	8.5 ± 1.2 ^{a}	75.3 ± 13.7 ^{a}	74.5 ± 2.2 ^{a}
	2017 Strain 151	3.8 ± 0.4 ^{ab}	8 ± 3.4 ^{a}	29.9 ± 13.4 ^{a}	35.8 ± 10.9 ^{a}	9 ± 1.2 ^{a}	8.8 ± 0.9 ^{a}	66.5 ± 10.3 ^{a}	68.5 ± 10.1 ^{a}
S. cerevisiae	Fermol Med.‡	3.4 ± 1.4 ^{ab}	8 ± 6.1 ^{a}	29.8 ± 16.5 ^{a}	45.1 ± 26.6 ^{a}	9 ± 2.0 ^{a}	9.5 ± 1.3 ^{a}	64.3 ± 11.2 ^{a}	80.4 ± 28.3 ^{a}
	Premier Classique‡	7.9 ± 3.2 ^{a}	7.9 ± 1.9 ^{a}	35.1 ± 16.5 ^{a}	47.8 ± 20.3 ^{a}	7.5 ± 1.5 ^{a}	8.1 ± 0.9 ^{a}	54 ± 25.8 ^{a}	75.2 ± 20.6 ^{a}
	EC-1118‡	5 ± 1.2 ^{b}	6.5 ± 2.5 ^{a}	22.1 ± 15.8 ^{a}	41.5 ± 19.6 ^{a}	10 ± 1.1 ^{a}	10.1 ± 0.8 ^{a}	70.6 ± 10.1 ^{a}	69.2 ± 9.7 ^{a}
	UBC47	8.1 ± 5.1 ^{ab}	9.3 ± 4.7 ^{a}	38.3 ± 18.2 ^{a}	40.9 ± 21.9 ^{a}	6.9 ± 0.9 ^{a}	9.1 ± 0.9 ^{a}	60.3 ± 19.6 ^{a}	52.3 ± 5.9 ^{a}

‡ Indicates commercially sourced strains

3.3.2 Yeast Production of Organoleptic Compounds

All four *S. cerevisiae* strains, as well as the commercial *S. uvarum* strain BMV58, had similar production of volatile aroma compounds. For example, at both temperatures, the *S. cerevisiae* strains and BMV58 all produced lower levels of 2-phenyl ethyl acetate than the six indigenous *S. uvarum* strains (Fig. 3.2, Fig. 3.3). Similarly, at 15 °C BMV58 produced the most 2-methyl-butyl acetate, followed by two commercial *S. cerevisiae* strains, Premier Classique and EC-1118 (Fig. 3.2). Additionally, at 15 °C Fermol Mediterranée and EC-1118 produced higher levels of ethyl octanoate than the other strains followed by Premier Classique, BMV58, and UBC47.

There was also high variation among the six indigenous *S. uvarum* strains with respect to the production of volatile aroma compounds (Fig. 3.2, Fig. 3.3). For example, 2-phenylethanol, a compound often reported as characteristic of *S. uvarum*, was produced in higher concentrations

by the indigenous *S. uvarum* strains ‘2019 strain 97’ and ‘2017 strain 151’ than other *S. uvarum* indigenous strains. In addition, the indigenous *S. uvarum* strains ‘2015 strain 3’, ‘2019 strain 97’, ‘2016 strain 163’, and ‘2017 strain 151’ also produced more methionol than all other strains at 15 °C but not at 25 °C. Additionally, ‘2019 strain 97’, and ‘2016 strain 163’, and ‘2015 strain 1’ produced more acetaldehyde than all other strains at 15 °C. At colder temperatures, the unique organoleptic profiles of the indigenous *S. uvarum* strains were more apparent with higher variation in the production of ethyl esters and higher alcohols. While at warmer temperatures, the organoleptic profiles were more homogenous.

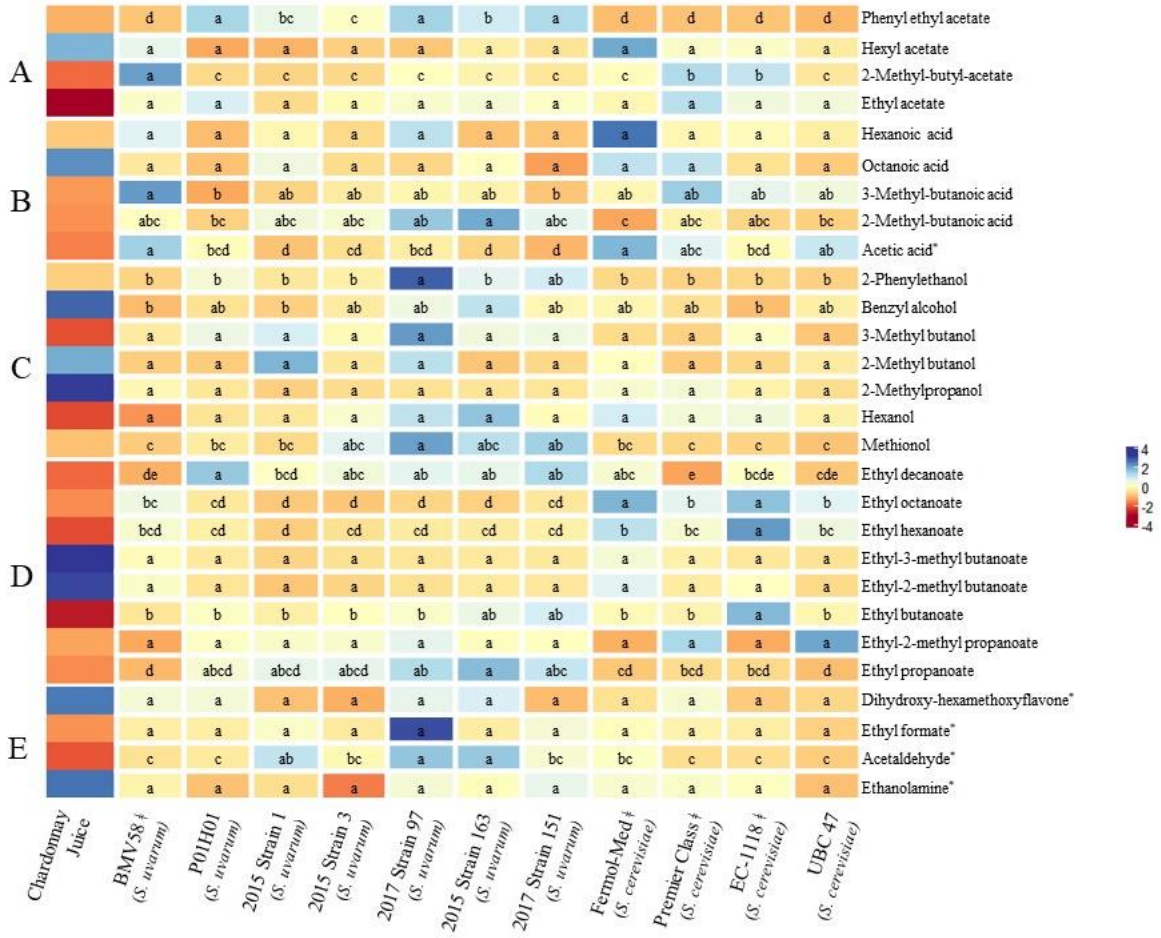

Figure 3.2. Heatmap showing the abundances of analyzed compounds relative to unfermented Chardonnay juice organized by class of compound for fermentations conducted at 15 °C by different strains of *S. cerevisiae* and *S. uvarum*. Darker blue values indicate an increase in concentration compared to unfermented chardonnay juice while red colors indicate a decrease. Group A, acetates; group B, acids; group C, alcohols; group D, ethyl esters, and group E, all other compounds. If letter codes for any given strains do not share a letter, then the strains are significantly different at $p \leq 0.05$ as indicated by a Tukey HSD *post hoc* test. * Indicates putative identifications. ǂ Indicates commercially sourced yeast strains.

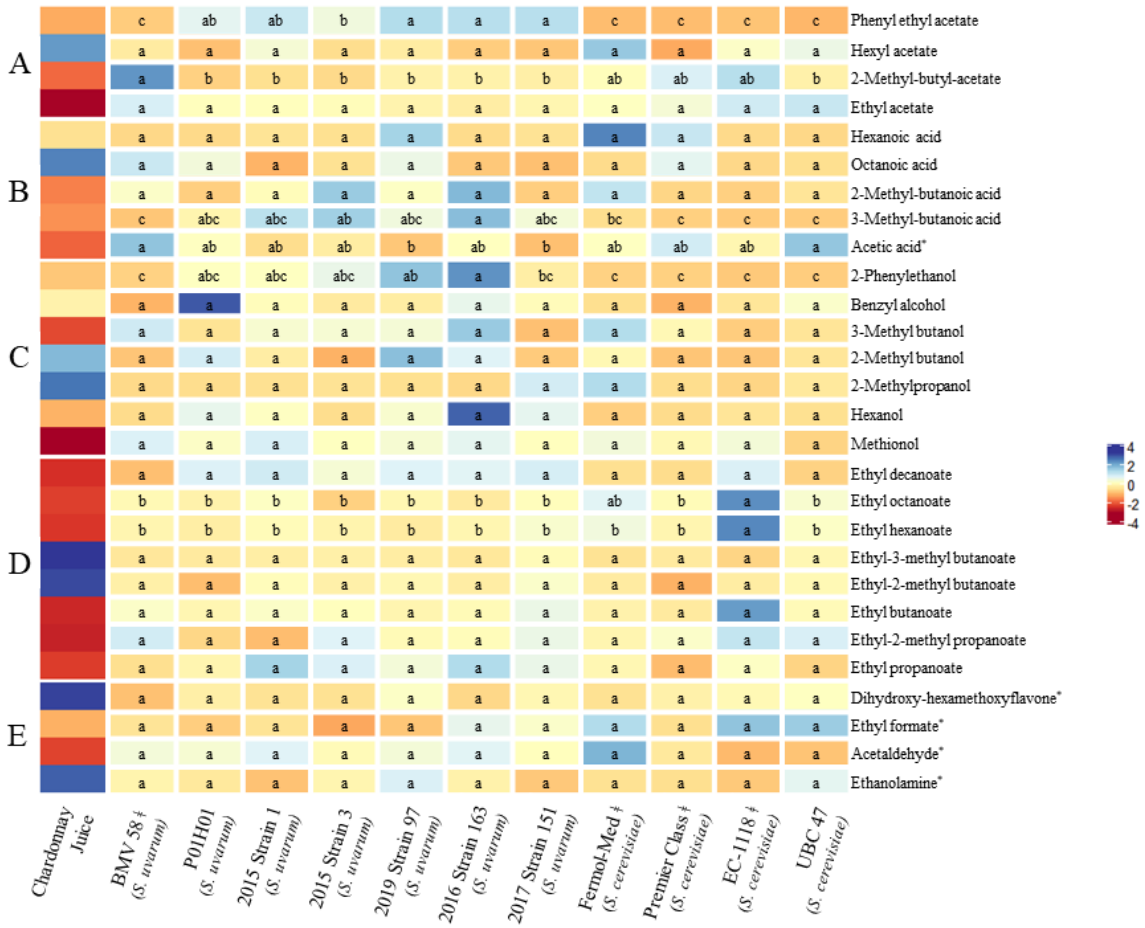

Figure 3.3. Heatmap showing the abundances of analyzed compounds relative to unfermented Chardonnay juice organized by class of compound for fermentations conducted at 25 °C by different strains of *S. cerevisiae* and *S. uvarum*. Darker blue values indicate an increase in concentration compared to unfermented chardonnay juice while red colors indicate a decrease. Group **A**, acetates; group **B**, acids; group **C**, alcohols; group **D**, ethyl esters; and group **E**, all other compounds. If letter codes for any given strains do not share a letter, then the strains are significantly different at $p \leq 0.05$ as indicated by a Tukey HSD *post hoc* test. * Indicates putative identifications. ǂ Indicates commercially sourced yeast strains.

Table 3.4 Percent change in relative abundance of analytes in *Saccharomyces* fermentations conducted at 15 °C as compared to 25 °C. Positive values indicate a higher abundance in the 15 °C fermentations compared to the 25 °C fermentations, and negative values indicate a lower relative abundance. Values are the average of 3 biological replicates per treatment. Results of paired *t*-tests are shown in brackets (each compound/strain analyzed separately). Significant results are in bold ($p \leq 0.05$).

	S. uvarum	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*
	BMV58‡	P01H01	2015 Strain 1	2015 Strain 3	2017 Strain 097	2015 Strain 163	2017 Strain 151	Fermol Mediterranee‡	Premier Classique‡	EC-1118‡	UBC47
Phenylethyl acetate	-32% (0.06)	**32% (0.01)**	-23% (0.36)	-17% (0.38)	10% (0.29)	-10% (0.3)	8% (0.29)	-16% (0.54)	15% (0.08)	-7% (0.63)	-23% (0.14)
Hexyl acetate	24% (0.68)	-35% (0.44)	-125% (0.22)	-20% (0.76)	-47% (0.36)	18% (0.64)	14% (0.74)	4% (0.96)	49% (0.21)	-8% (0.92)	-48% (0.25)
2-methyl-butyl-acetate	29% (0.15)	21% (0.07)	7% (0.71)	14% (0.43)	**30% (0.03)**	22% (0.07)	11% (0.18)	22% (0.21)	**37% (0.02)**	25% (0.29)	12% (0.49)
Ethyl acetate	-34% (0.07)	0% (0.96)	-40% (0.1)	-18% (0.4)	-7% (0.49)	-2% (0.84)	-11% (0.27)	-23% (0.35)	2% (0.95)	**-30% (0.03)**	-34% (0.18)
Hexanoic acid	97% (0.19)	38% (0.4)	88% (0.16)	80% (0.39)	65% (0.31)	60% (0.17)	48% (0.19)	69% (0.33)	38% (0.76)	94% (0.44)	93% (0.41)
Octanoic acid	-40% (0.57)	-60% (0.44)	**74% (0.04)**	24% (0.46)	-40% (0.65)	61% (0.14)	3% (0.89)	66% (0.22)	33% (0.53)	36% (0.56)	15% (0.68)
2-methyl-butanoic acid	61% (0.16)	1% (0.99)	-2% (0.9)	-17% (0.59)	46% (0.27)	24% (0.26)	30% (0.15)	-11% (0.6)	52% (0.07)	41% (0.41)	**36% (0.02)**
3-methyl-butanoic acid	71% (0.13)	-4% (0.83)	12% (0.61)	-8% (0.71)	37% (0.34)	-4% (0.88)	29% (0.06)	17% (0.59)	**76% (0.03)**	69% (0.28)	**61% (0.03)**
Acetic acid*	-28% (0.31)	-31% (0.15)	**-83% (0.05)**	-57% (0.1)	17% (0.54)	-118% (0.13)	-61% (0.25)	34% (0.04)	-34% (0.45)	-20% (0.48)	-49% (0.34)
2-phenylethanol	-55% (0.41)	24% (0.61)	-119% (0.47)	-109% (0.15)	41% (0.08)	-91% (0.37)	68% (0.36)	8% (0.92)	-63% (0.57)	72% (0.11)	-55% (0.61)
Benzyl alcohol	-19% (0.78)	-119% (0.24)	-40% (0.41)	18% (0.8)	48% (0.07)	47% (0.15)	21% (0.28)	36% (0.17)	34% (0.4)	-61% (0.4)	7% (0.85)
3-methyl butanol	-7% (0.7)	59% (0.11)	48% (0.51)	27% (0.7)	63% (0.07)	9% (0.88)	70% (0.31)	-39% (0.57)	6% (0.93)	61% (0.25)	14% (0.85)
2-methyl butanol	5% (0.93)	-99% (0.4)	83% (0.43)	60% (0.27)	59% (0.53)	-137% (0.49)	27% (0.52)	56% (0.16)	-2% (0.97)	29% (0.69)	45% (0.23)
2-methylpropanol	32% (0.45)	7% (0.93)	-41% (0.66)	-17% (0.84)	-5% (0.95)	6% (0.94)	-73% (0.28)	-16% (0.7)	45% (0.26)	27% (0.53)	-21% (0.81)
Hexanol	-50% (0.07)	-46% (0.25)	-21% (0.45)	-32% (0.18)	8% (0.79)	-14% (0.47)	-20% (0.42)	-1% (0.98)	1% (0.98)	-49% (0.13)	-38% (0.06)
Methionol	-29% (0.73)	-24% (0.82)	-60% (0.63)	76% (0.27)	**73% (0.05)**	2% (0.98)	58% (0.06)	37% (0.5)	5% (0.92)	-18% (0.75)	-104% (0.32)
Ethyl decanoate	-78% (0.22)	14% (0.24)	-52% (0.08)	-12% (0.68)	-17% (0.48)	-19% (0.11)	3% (0.87)	18% (0.52)	**-166% (0.02)**	**-48% (0.03)**	-55% (0.23)
Ethyl octanoate	16% (0.1)	-33% (0.22)	**-152% (<0.01)**	**-72% (0.03)**	-87% (0.02)	-80% (0.03)	-49% (0.13)	35% (0.15)	21% (0.11)	-6% (0.8)	15% (0.33)
Ethyl hexanoate	28% (0.19)	16% (0.24)	-29% (0.13)	-3% (0.87)	15% (0.07)	7% (0.35)	-2% (0.77)	35% (0.07)	**29% (0.01)**	16% (0.37)	26% (0.16)
Ethyl-3-methyl butanoate	31% (0.46)	7% (0.93)	-42% (0.65)	-17% (0.84)	-4% (0.96)	8% (0.91)	-13% (0.88)	46% (0.21)	17% (0.81)	18% (0.71)	-23% (0.79)
Ethyl-2-methyl butanoate	27% (0.49)	36% (0.6)	-45% (0.56)	-15% (0.83)	-9% (0.89)	10% (0.86)	-14% (0.84)	42% (0.24)	48% (0.5)	27% (0.51)	-18% (0.82)
Ethyl butanoate	19% (0.35)	39% (0.07)	24% (0.38)	34% (0.15)	40% (0.06)	44% (0.04)	39% (0.09)	38% (0.06)	**40% (0.01)**	27% (0.23)	32% (0.16)
Ethyl-2-methyl propanoate	-283% (0.41)	-22% (0.85)	2% (0.99)	-92% (0.67)	-22% (0.88)	-72% (0.7)	-101% (0.46)	-190% (0.42)	5% (0.97)	-361% (0.19)	10% (0.93)
Ethyl propanoate	-156% (0.12)	-5% (0.82)	-51% (0.29)	-26% (0.46)	15% (0.6)	4% (0.84)	2% (0.86)	-148% (0.21)	-20% (0.66)	-111% (0.09)	-118% (0.08)

	S. uvarum	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. uvarum*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*
	BMV58‡	P01H01	2015 Strain 1	2015 Strain 3	2017 Strain 097	2015 Strain 163	2017 Strain 151	Fermol Mediterranee‡	Premier Classique‡	EC-1118‡	UBC47
Dihydroxy-hexamethoxyflavone*	66% (0.16)	20% (0.73)	2% (0.97)	-5% (0.91)	0% (0.99)	52% (0.06)	-44% (0.3)	19% (0.58)	16% (0.79)	-36% (0.52)	-41% (0.63)
Ethyl formate*	-6% (0.92)	7% (0.9)	17% (0.68)	24% (0.49)	70% (0.34)	-54% (0.07)	-1% (0.97)	-53% (0.4)	4% (0.9)	-99% (0.14)	**-173% (0.03)**
Acetaldehyde*	-57% (0.43)	-39% (0.14)	10% (0.35)	-5% (0.84)	32% (0.11)	23% (0.19)	6% (0.77)	-76% (0.35)	-19% (0.65)	22% (0.25)	-1% (0.96)
Ethanolamine*	25% (0.31)	15% (0.8)	36% (0.1)	-17% (0.79)	14% (0.45)	30% (0.19)	**49% (0)**	39% (0.07)	39% (0.06)	**44% (0.01)**	-13% (0.82)
Glucose	2% (0.98)	-55% (0.46)	-12% (0.76)	-31% (0.41)	-61% (0.19)	-137% (0.32)	-113% (0.16)	1% (0.97)	0% (1)	6% (0.9)	-15% (0.78)
Fructose	-16% (0.79)	-15% (0.78)	-23% (0.6)	-88% (0.26)	-45% (0.3)	-51% (0.45)	-20% (0.59)	-36% (0.45)	-2% (0.96)	-49% (0.54)	-7% (0.88)
Ethanol	-26% (0.42)	4% (0.88)	-18% (0.57)	2% (0.87)	-8% (0.6)	-25% (0.44)	-3% (0.82)	-39% (0.33)	-15% (0.52)	1% (0.93)	13% (0.56)
Glycerol	-46% (0.44)	-15% (0.32)	-5% (0.68)	-1% (0.93)	6% (0.41)	-5% (0.75)	3% (0.79)	-8% (0.6)	-4% (0.61)	-19% (0.2)	**-32% (0.05)**

‡ indicates commercially sourced strains; *indicates putatively identified compounds.

3.3.3 Overall Characteristics

PCA plots were used to visualize the overall variation in the dataset (Fig. 3.4). When looking at the full dataset, there are strong trends showing separation of temperature treatments along the PC2 axis (Fig. 3.4a) explaining 14.62% of the variation in the data. The PC1 axis of the full dataset, which explains 20.68% of variation in the data, showed a pronounced separation when colored by species (Fig. 3.4b). At 15 °C, there is separation of *S. cerevisiae* and *S. uvarum* strains along the PC1 axis (Fig. 3.4d) with the exception of three datapoints. When colored by strain in Fig. 3.4c, it can be seen that these three outliers are the commercial *S. uvarum* strain BMV58 that is grouping with *S. cerevisiae* strains. The axis of PC1 covers a wider range of variation in the 15 °C treatment of 26.82% variation compared to 19.69% of the variation in the data for the 25 °C treatment. At 25 °C, there is vertical separation between most *S. cerevisiae* and *S. uvarum* strains along the PC2 axis (16.9% of variation explained) (Fig. 3.4e, Fig. 3.4f). As observed in the 15 °C fermentations, BMV58 is again grouping closer to the *S. cerevisiae* strains than the other *S. uvarum* strains.

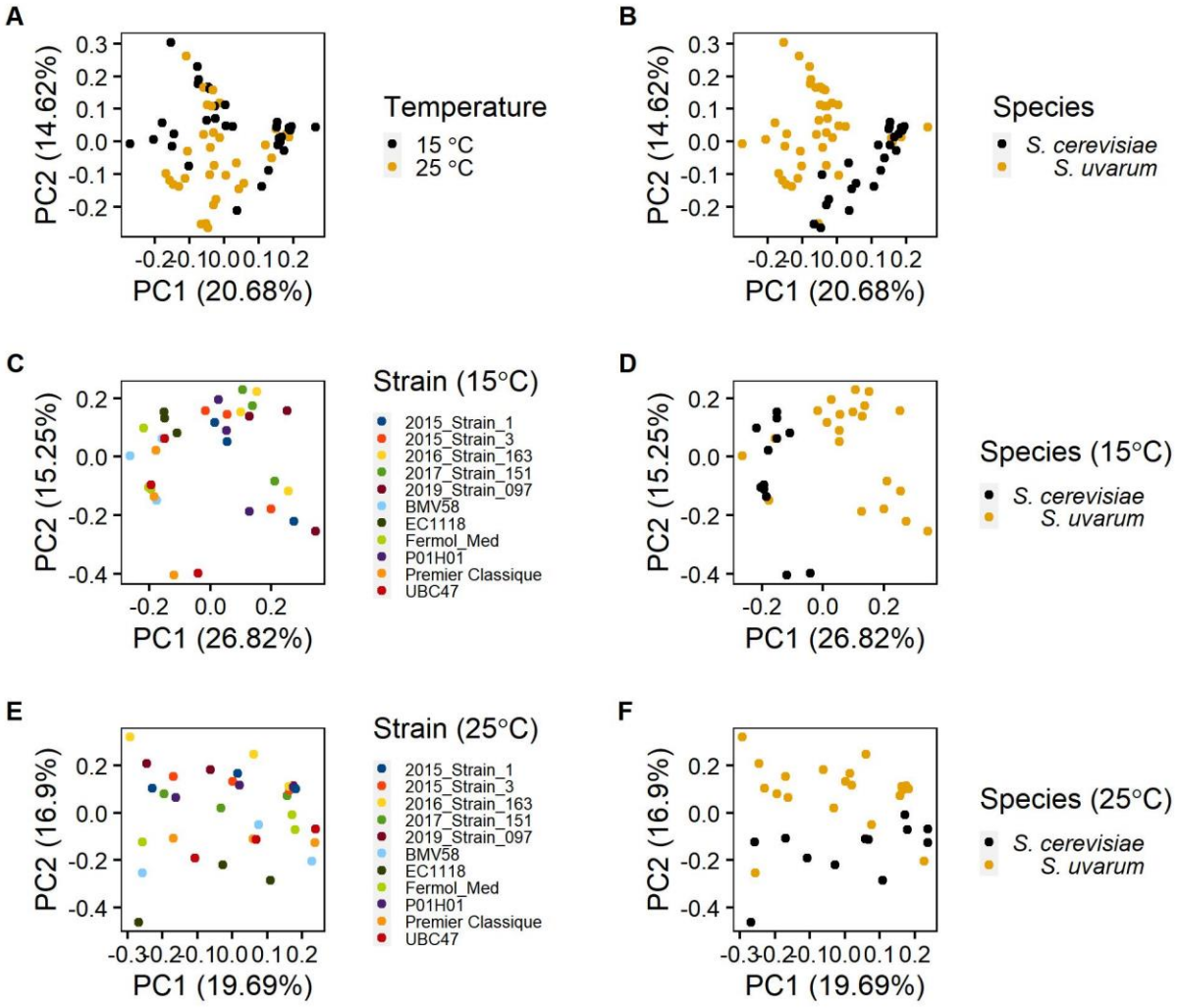

Figure 3.4 Principle component analysis (PCA) ordinations where each individual point represents all chemicals analyzed and area under logistical growth curves from Chardonnay juice fermented with different strains of *S. cerevisiae* and *S. uvarum*. a) All fermentations plotted, in which fermentation temperature is distinguished by color. b) All fermentations plotted, in which yeast species are distinguished by color. c) Only the 15 °C fermentations, distinguishing points by strains. d) Only the 15 °C fermentations, distinguishing points by species. e) Only the 25 °C fermentations, distinguishing points by strains. f) Only the 25 °C fermentations, distinguishing points by species.

3.4 Discussion

3.4.1 Fermentation Properties

The importance of fermentation temperature has been emphasized in the literature (Alonso-del-Real *et al.*, 2017; Deed *et al.*, 2017; Demuyter *et al.*, 2004; Diamantidou *et al.*, 2018; Morgan *et al.*, 2020; Naumov *et al.*, 2000). In general, *S. uvarum* has been described as a cryotolerant or cryophilic strain, with its kinetic performance usually favored over *S. cerevisiae* at lower temperatures (≤ 15 °C), but not at higher temperatures (> 20 °C) (Alonso-del-Real *et al.*, 2017; Masneuf-Pomarède *et al.*, 2010; Naumov *et al.*, 2000; Stribny *et al.*, 2015). Thus, our expectation from previous studies was that *S. uvarum* would finish fermentations more quickly at 15 °C than *S. cerevisiae* and that it would have slower overall fermentation kinetics at 25 °C as compared with *S. cerevisiae*. In contrast to the findings of these previous studies, all our *S. uvarum* strains exhibited slower fermentation kinetics at 15 °C and *S. uvarum* strains had comparable kinetics to most *S. cerevisiae* strains at 25 °C. In support of our results, one study reported the optimal growth temperature of *S. uvarum* to be in the range of 23.6 - 26.2 °C, which indicates that it may not be a reliably cryotolerant organism for all of its strains (Salvadó *et al.*, 2012). Similar to our findings, a recent study published by Morgan *et al.* (2020) investigated the fermentative performance of an indigenous *S. uvarum* strain (‘2015 strain 1’ from this current study) and a commercial *S. cerevisiae* strain (Lalvin QA23), and found no difference in fermentation rate between the two strains at different fermentation temperatures; specifically, both strains fermented well at a higher temperature (24 °C), and both exhibited sluggish fermentations at a lower temperature (15 °C).

In this study, *S. cerevisiae* strain EC-1118 finished fermentations more quickly than most other strains at both temperatures, indicating it may have a broader temperature range than

indicated in the literature (Trinh *et al.*, 2011; Lu *et al.*, 2017; Gao *et al.*, 2019; Binati *et al.*, 2020). Surprisingly, I found two *S. uvarum* strains, one at 15 °C and the other at 25 °C, which had similar kinetics to that of EC-1118. One of these *S. uvarum* strains, BMV58, which had similar fermentation kinetics to EC-1118 at 25 °C, was the only commercially available *S. uvarum* strain at the time this study was conducted. It is marketed for red wine fermentations and is claimed to produce high levels of glycerol and floral/fruity aromas. The other *S. uvarum* strain, '2017 strain 151', had a similar kinetic performance to that of EC-1118 at 15 °C (https://www.lallemandwine.com/en/australia/products/catalogue/wine-yeasts/66/velluto-bmv58/). These findings may indicate that the generalization of fermentation performance at certain temperatures, based on species alone, may not be appropriate and further studies should be conducted to determine the different strain performances at different temperatures. These results also indicate that indigenous *S. uvarum* strains may be better able to conduct and complete alcoholic fermentation than previously thought, particularly at temperatures >20 °C.

Although the temperature of fermentation did affect the rate at which strains fermented, significant differences in the metabolism of sugars (specifically glucose and fructose) and the subsequent rate of ethanol production among strains, or between fermentation temperatures within a single strain, were not found. This result is contradictory to previous literature pertaining to ethanol production by both *S. uvarum* and *S. cerevisiae,* which have found final levels of ethanol production to be higher at colder fermenting temperatures (Gao *et al.*, 2019; Veloso *et al.*, 2019). It is unclear as to why these fermentations do not appear to show significant shifts in sugar and ethanol profiles between 15 °C and 25 °C. It may be that higher temperatures affect fermentation enzymes responsible for alcoholic output differently than those associated with primary growth of yeasts (Samoticha *et al.*, 2019). In this study I did not observe *S. uvarum*

strains to be high glycerol producers, in contrast with previous studies that have shown *S. uvarum* to produce more glycerol than *S. cerevisiae* (Magyar and Tóth, 2011; Hu *et al.*, 2018; Moreira *et al.*, 2008).

3.4.2 Yeast Production of Organoleptic Compounds

Acetate esters impart fruity, sweet, and tropical flavors to wine. Similar to our results, others found that *S. uvarum* strains produced higher levels of acetate esters, especially 2-phenylethyl acetate, compared with *S. cerevisiae* (Stribny *et al.*, 2015). At both fermentation temperatures I observed a significant increase in the relative amounts of 2-phenylethyl acetate by the indigenous *S. uvarum* compared to the *S. cerevisiae* strains and the commercial *S. uvarum*, BMV58. Both 2-phenylethyl acetate and 2-phenylethanol (a higher alcohol) are known to impart a rose-like scent to wine, which is an established characteristic of *S. uvarum* (Gangl *et al.*, 2009; Tosi *et al.*, 2009; Gamero *et al.*, 2013). Interestingly, the commercial *S. uvarum* did not exhibit this same increased production of these compounds, but instead produced an organoleptic profile that was similar to the *S. cerevisiae* strains used in this study. Previous studies have observed a decrease in the concentrations of acetate esters when fermentations were conducted at lower temperatures (Gamero *et al.*, 2013); in this study, however, I observed wide variation in the changes in relative concentration of acetate esters between temperature treatments. All strains tended toward increasing 2-methyl-butyl acetate production at 15 °C compared to 25 °C, although this was only significant for ‘2017 strain 097’ and Premier Classique. There was a decrease in ethyl acetate at 15 °C compared to 25 °C for most strains although only significant for EC-1118 (Table 3.4).

Higher alcohols are another major contributor to aromatic profile, especially 2-phenylethanol, which imparts a desirable rose-like aroma, as described above. Indigenous *S.*

uvarum strains produced more 2-phenylethanol than the commercial strains of both *S. cerevisiae* and *S. uvarum*, supporting results from previous studies (Masneuf-Pomarède *et al.*, 2010; Stribny *et al.*, 2015). This significant difference was observed at both temperatures. The commercial *S. uvarum* strain BMV58 did not share this beneficial trait with the other indigenous *S. uvarum* strains.

High levels of volatile acidity is undesirable in wines, adding a sharp nail-polish like smell and is a concerning trait exhibited by *S. uvarum* strains in previous studies (Loureiro and Malfeito-Ferreira, 2003; Tristezza *et al.*, 2013). Molina *et al.* (2007) found increased production of octanoic acid, 2-methyl butanoic, and acetic acid at colder fermentation temperatures, similar to our results. Further research is necessary to determine if the level of these compounds is above the aroma threshold.

3.4.3 Overall Characteristics

The PCA clustering of temperature treatments (Fig. 3.4A) was expected, as lower fermentation temperatures have been shown to alter yeast metabolism, which in turn changes the final characteristics of the wine (Llauradó *et al.*, 2005; Beltran *et al.*, 2008). Additionally, colder fermentations retain organoleptic compounds better than warmer fermentations. The clustering of fermentations by species is equally prevalent in the full dataset, indicating that species-related characteristics exist regardless of fermentation temperature (Fig 3.4B).

A separation by species was more prevalent in the 15 °C fermentations compared to the 25 °C fermentations. The former also exhibited greater variation along the PC1 axis than the 25 °C fermentations, as well as greater variation among replicates. The greater biological variation at 15 °C was not unexpected, since low-temperature fermentations tend to preserve aroma compounds, producing more complex organoleptic profiles (Heard and Fleet, 1988; Torija *et al.*,

2003; Su *et al.*, 2019). The indigenous strains '2015 Strain 163', '2017 Strain 151', and UBC47 showed substantial biological variation at both temperatures. This is in contrast to the commercial strain EC-1118, whose replicates grouped tightly together (Fig 3.4C). These results indicate that indigenous strains may be less consistent than commercial strains.

The commercial *S. uvarum* strain BMV58 grouped more closely with the *S. cerevisiae* strains than the indigenous *S. uvarum* strains at both temperatures, meaning it produced an organoleptic profile more similar to the *S. cerevisiae* strains than to the other strains of its own species (Fig 3.4C, 3.4E). This is not surprising because BMV58 performed more similarly to the *S. cerevisiae* strains in terms of fermentation properties and volatile composition. These similarities imply that the expected advantages of using an *S. uvarum* strain for fermentation are not necessarily actualized in this commercial version, as compared to the indigenous *S. uvarum* strains I tested. Further research on *S. uvarum* strains such as '2017 strain 151' is warranted, due to its ability to perform alcoholic fermentation at a similar rate to EC-1118, but at the same time produce typical *S. uvarum* compounds such as 2-phenylethanol.

Chapter 4: Conclusion

The aim of thesis was to better understand the diversity and function of the fungal community composition and function of indigenous yeasts in the Okanagan region. To accomplish this, I focused on 2 main research objectives. First, I explored the resilience of fungal communities in vineyards after agricultural spray application and exposure to simulated forest fire smoke. I then explored how indigenous and commercial strains of *S. uvarum* and *S. cerevisiae* performed in small-scale controlled Chardonnay fermentations.

In Chapter 2, I analyzed the fungal communities at 3 different vineyards after exposure to agricultural sprays and forest fire smoke. At each vineyard, an agricultural spray was tested as well as simulated forest fire smoke. There were 4 treatments in which one group of vines were exposed only to smoke, another group only to spray, a third group exposed to both, and a fourth control group. I collected grapes at different points in the ripening process and analyzed the fungal community composition using amplicon-based NGS DNA sequencing techniques. No significant changes were observed in fungal community composition after the application of 3 different agricultural sprays, after heavy smoke exposure, or after exposure to both sprays and smoke. This was attributed to the fast re-colonization of fungi from the surrounding untreated vines in the vineyard. I did however observe significant differences between vineyards when only looking at the control vines. Vineyard 2 contained a high abundance of a pathogenic fungus *Erysiphe necator* at the first two timepoints compared to Vineyards 1 and 3. At harvest, the *Erysiphe necator* population was no longer significantly present. There were significant differences between vineyards 2 and 3 at harvest driven by *Hanseniaspora uvarum* and *Metschnikowia sinensis* populations. These differences at harvest were attributed to geographical variation and differences in vineyard management practices. In order to better determine how

these environmental perturbations are changing the fungal diversity, a larger scale experiment would need to be conducted in which the experimental vines were more isolated from the surrounding untreated vines. One limitation of this experimental design was that the experimental vines were in very close proximity to the rest of the untreated vines in the vineyard. This made it difficult to determine if the fungal population was perturbed by the treatments and quickly recolonized by fungi from surrounding vines.

In Chapter 3, I explored how indigenous yeast strains that were isolated in the Okanagan compared to commercially sourced yeast strains. In this experiment, I fermented Chardonnay juice with indigenous strains and commercial strains at both 15 °C and 25°C. I analyzed the rate of fermentation, consumption of fructose and glucose, as well as the production of ethanol and organoleptic compounds. Our results reveal that indigenous *S. uvarum* were able to complete fermentation at similar rates to commercial strains (both of *S. uvarum* and *S. cerevisiae* heritage), and produced unique organoleptic profiles, particularly at 15 °C. Indigenous *S. uvarum* strains favored the production of 2-phenylethyl acetate and 2-phenylethanol and displayed increased variation among replicate fermentations, suggesting that indigenous strains may not be as stable or consistent as commercial strains across vintages. Nevertheless, production of glycerol in indigenous *S. uvarum* strains did not differ from the commercially sourced strains. The commercial *S. uvarum* strain BMV58 did not display traits consistent with the indigenous *S. uvarum* strains; its fermentation kinetics at lower temperatures did not differ from commercial *S. cerevisiae* strains and its profile of organoleptic compounds was more similar to *S. cerevisiae* strains. These results highlight the need to further investigate the fermentative potential of other indigenous strains as well as better characterize commercial strains under different fermentation conditions. This experiment was limited by the availability of a more sensitive analysis method

for the volatile compounds that would allow the detection of more compounds and determine absolute quantitative values.

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Appendices

Appendix A: Supplementary material for Chapter 2

Table A 1 Metadata table for all grapes samples collected from three vineyards in the Okanagan region that were exposed to artificial forest fire smoke, agricultural sprays, or both smoke and sprays. Grape bunches were collected at 3 different timepoints (n = 7 per treatment per timepoint).

Due to the large size of this table it has been made available on OSF at:
https://osf.io/47ctj/?view_only=c69d6c6294dd4159aec9d7b8ae7a9112

Table A 2 Statistical results for pairwise comparison of Shannon and Simpson α-diversity indices for fungal communities on grapes in three vineyards between treatments of smoke exposure and agricultural sprays throughout ripening (n=7 per treatment per timepoint).

Due to the large size of this table it has been made available on OSF at:
https://osf.io/47ctj/?view_only=c69d6c6294dd4159aec9d7b8ae7a9112

Table A 3 The results of Pairwise PERMANOVA of the Bray-Curtis β-diversity index between treatments of smoke exposure, spray exposure within each vineyard (n=7 per treatment).

Due to the large size of this table it has been made available on OSF at:
https://osf.io/47ctj/?view_only=c69d6c6294dd4159aec9d7b8ae7a9112

Table A 4 The results of a pairwise PERMANOVA of the Bray-Curtis β-diversity index between control vines of the three vineyards at each timepoint (n=7 per treatment).

Timepoint	Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
T0	Vineyard 1	Vineyard 3	13	999	1.13	0.335	0.34
T0	Vineyard 1	Vineyard 2	13	999	8.67	0.003	0.0045
T0	Vineyard 3	Vineyard 2	14	999	8.93	0.001	0.003
T1	Vineyard 1	Vineyard 3	14	999	2.04	0.052	0.052
T1	Vineyard 1	Vineyard 2	14	999	4.56	0.003	0.0045
T1	Vineyard 3	Vineyard 2	14	999	4.42	0.002	0.0045
T2	Vineyard 1	Vineyard 3	14	999	2.53	0.009	0.027
T2	Vineyard 1	Vineyard 2	14	999	1.81	0.085	0.13
T2	Vineyard 3	Vineyard 2	14	999	1.30	0.154	0.15

Table A 5 The results of a pairwise PERMANOVA test of the Shannon and Simpson α-diversity indices between the control vines of each vineyard at each timepoint (n=7 per treatment).

Timepoint	Metric	Group 1	Group 2	H	p-value	q-value
T0	Shannon	Vineyard 1 (n=6)	Vineyard 3 (n=7)	0.02	0.89	0.89
T0	Shannon	Vineyard 1 (n=6)	Vineyard 2 (n=7)	2.47	0.12	0.35
T0	Shannon	Vineyard 3 (n=7)	Vineyard 2 (n=7)	0.10	0.75	0.89
T0	Simpson	Vineyard 1 (n=6)	Vineyard 3 (n=7)	1.31	0.25	0.48
T0	Simpson	Vineyard 1 (n=6)	Vineyard 2 (n=7)	1.00	0.32	0.48
T0	Simpson	Vineyard 3 (n=7)	Vineyard 2 (n=7)	0.00	0.95	0.95
T1	Shannon	Vineyard 1 (n=7)	Vineyard 3 (n=7)	0.92	0.34	0.34
T1	Shannon	Vineyard 1 (n=7)	Vineyard 2 (n=7)	1.80	0.18	0.27
T1	Shannon	Vineyard 3 (n=7)	Vineyard 2 (n=7)	1.80	0.18	0.27
T1	Simpson	Vineyard 1 (n=7)	Vineyard 3 (n=7)	0.49	0.48	0.48
T1	Simpson	Vineyard 1 (n=7)	Vineyard 2 (n=7)	0.92	0.34	0.48
T1	Simpson	Vineyard 3 (n=7)	Vineyard 2 (n=7)	2.55	0.11	0.33
T2	Shannon	Vineyard 1 (n=7)	Vineyard 3 (n=7)	0.00	0.95	0.95
T2	Shannon	Vineyard 1 (n=7)	Vineyard 2 (n=7)	0.10	0.75	0.95
T2	Shannon	Vineyard 3 (n=7)	Vineyard 2 (n=7)	0.20	0.65	0.95
T2	Simpson	Vineyard 1 (n=7)	Vineyard 3 (n=7)	0.33	0.57	0.85
T2	Simpson	Vineyard 1 (n=7)	Vineyard 2 (n=7)	1.18	0.28	0.83
T2	Simpson	Vineyard 3 (n=7)	Vineyard 2 (n=7)	0.04	0.85	0.85

Table A 6 The results of the differential abundance analysis between vineyards that showed a significant difference in β-diversity metrics between control vines (n=7 per treatment).

Due to the large size of this table it has been made available on OSF at:
https://osf.io/47ctj/?view_only=c69d6c6294dd4159aec9d7b8ae7a9112

a

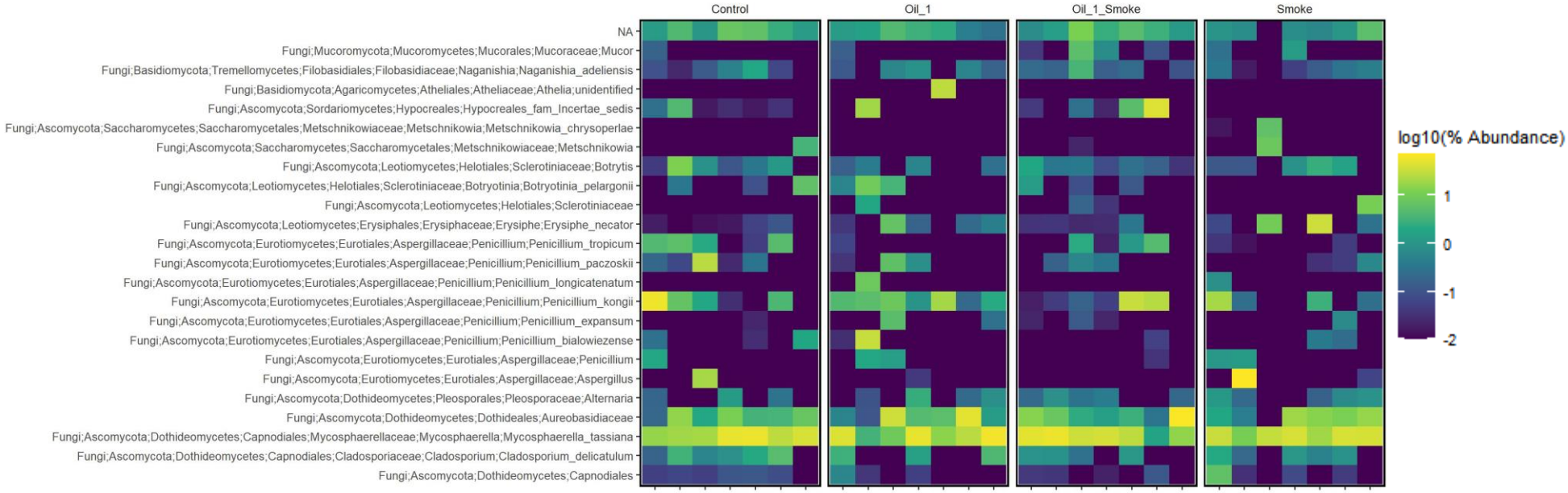

Figure A 1 The log 10 relative abundance of the top 30 most numerous ASV's found at Vineyard 1 at timepoint 1 (a), and at harvest (b) (n=7 per treatment, each column represents one vine from each treatment). Lighter values indicate a higher abundance than darker values. Figure continued on next page

b

Figure A1 Continued.

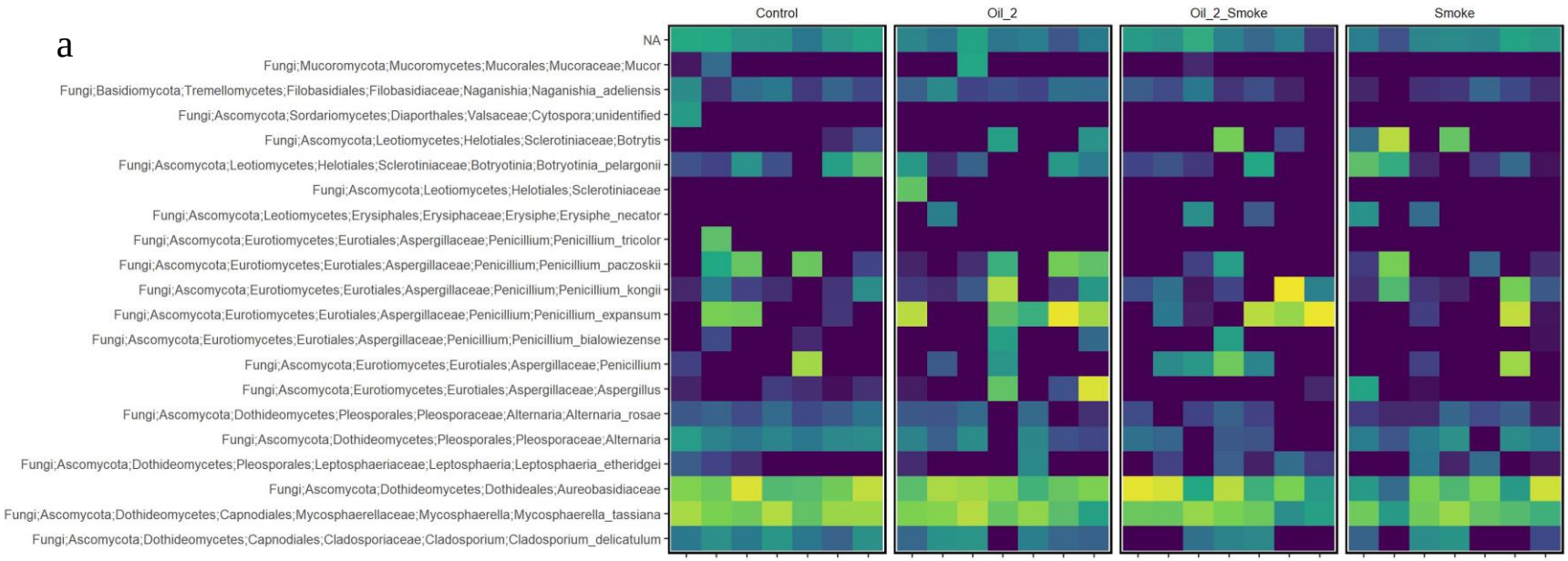

Figure A 2 The log 10 relative abundance of the top 30 most numerous ASV's found at Vineyard 2 at timepoint 1 (a), and at harvest (b) (n=7 per treatment, each column represents one vine from each treatment). Lighter values indicate a higher abundance than darker values. Figure continued on next page.

b

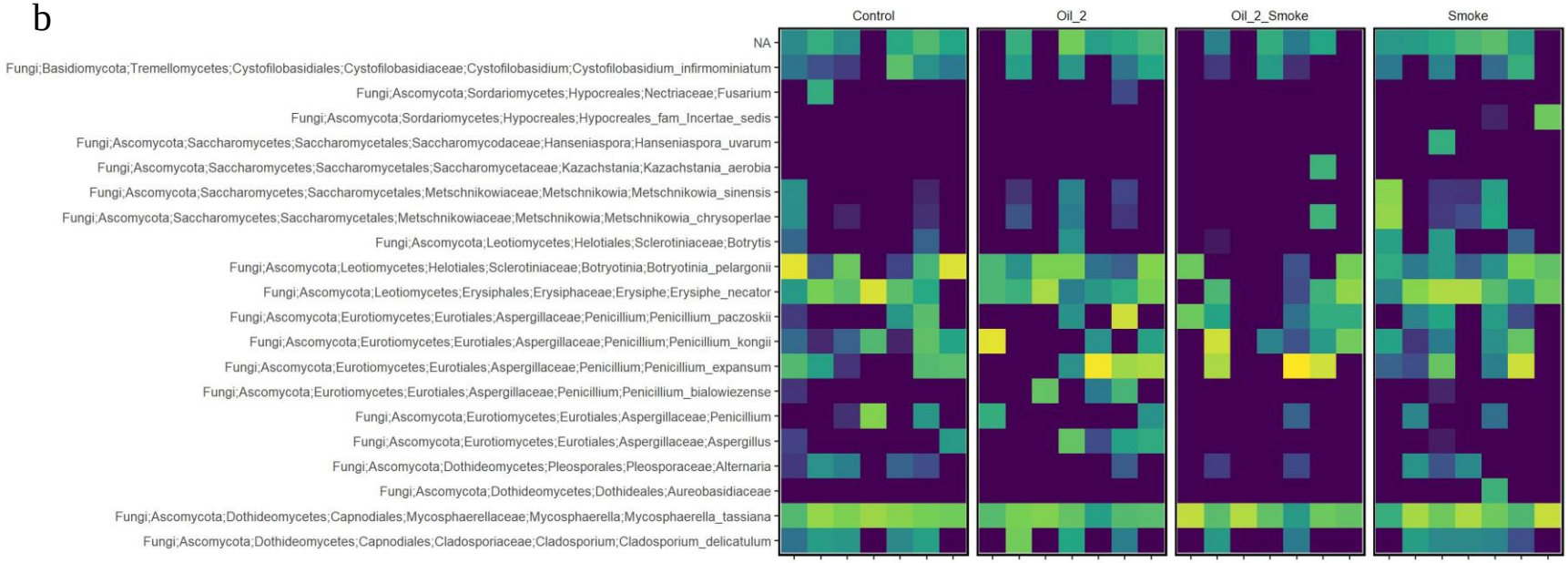

Figure A2 Continued.

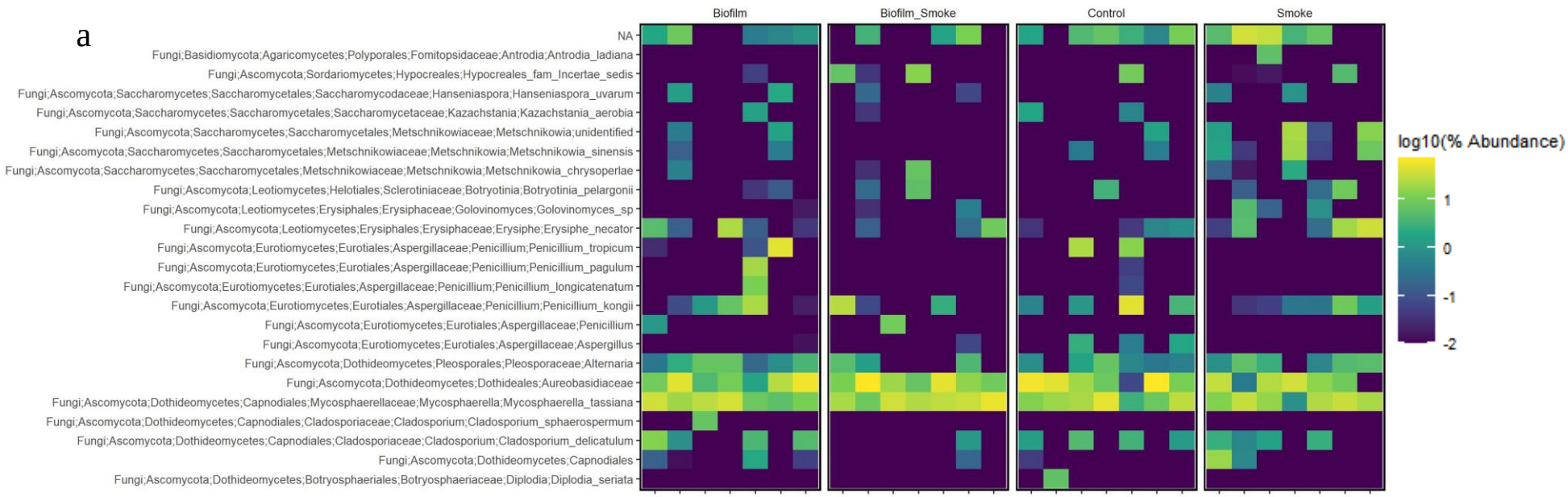

Figure A 3 The log 10 relative abundance of the top 30 most numerous ASV's found at Vineyard 3 at timepoint 1 (a), and at harvest (b) (n=7 per treatment, each column represents one vine from each treatment). Lighter values indicate a higher abundance than darker values. Figure continued on next page.

b

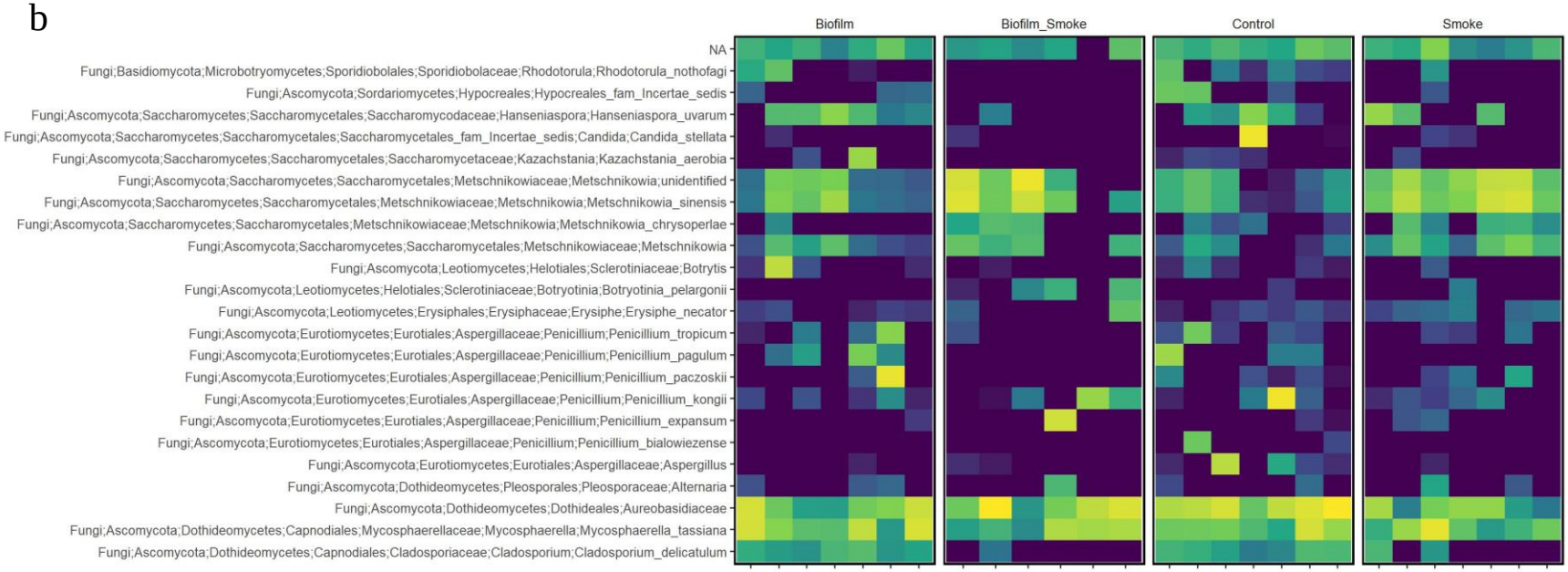

Figure A3 Continued.

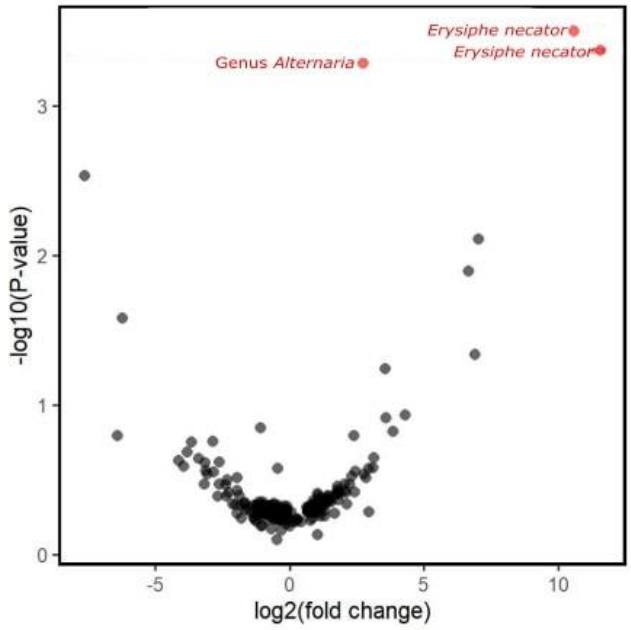

Figure A 4 'Volcano plot' of statistical significance against the log-fold change in relative abundance of ASV's between the control vines of Vineyards 1 and 2 at timepoint 0 demonstrating the most significant differences found in the differential abundance analysis. Annotated datapoints in red represent ASV's that were significantly different.

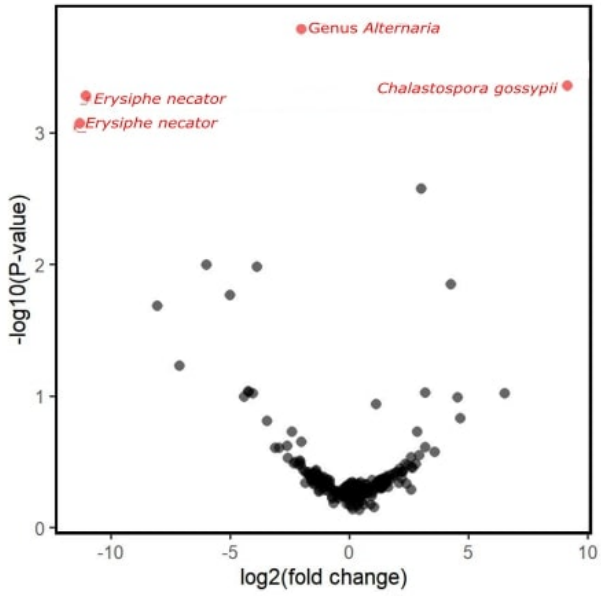

Figure A 5 'Volcano plot' of statistical significance against the log-fold change in relative abundance of ASV's between the control vines of Vineyards 2 and 3 at timepoint 0 demonstrating the most significant differences found in the differential abundance analysis. Annotated datapoints in red represent ASV's that were significantly different.

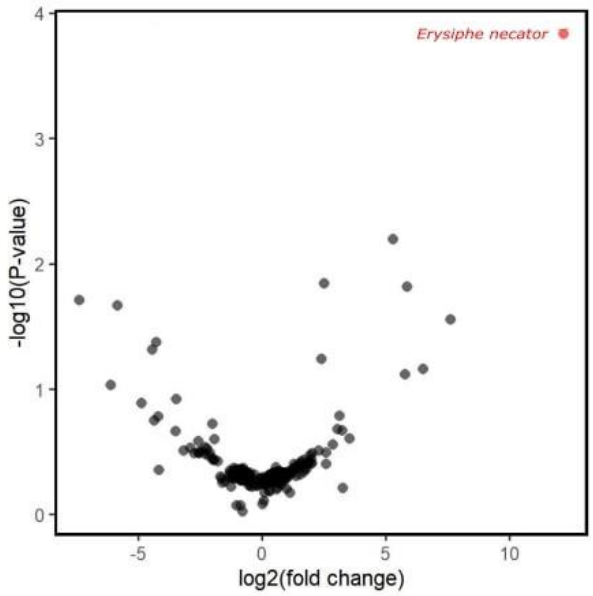

Figure A 6 'Volcano plot' of statistical significance against the log-fold change in relative abundance of ASV's between the control vines of Vineyards 1 and 2 at timepoint 1 demonstrating the most significant differences found in the differential abundance analysis. Annotated datapoints in red represent ASV's that were significantly different.

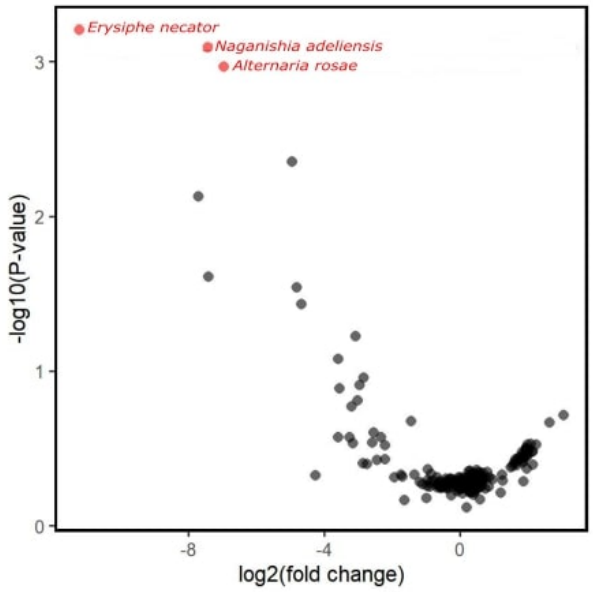

Figure A 7 'Volcano plot' of statistical significance against the log-fold change in relative abundance of ASV's between the control vines of Vineyards 2 and 3 at timepoint y demonstrating the most significant differences found in the differential abundance analysis. Annotated datapoints in red represent ASV's that were significantly different. V3 V2

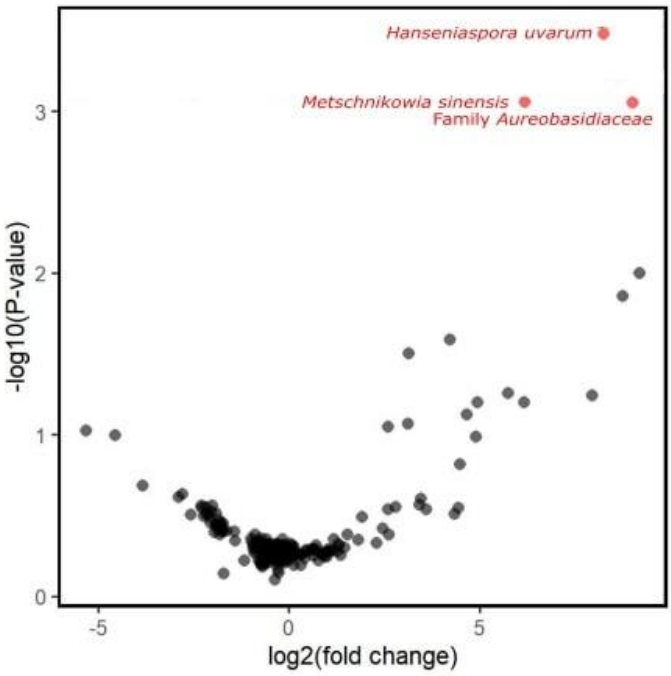

Figure A 8 'Volcano plot' of statistical significance against the log-fold change in relative abundance of ASV's between the control vines of Vineyards 1 and 3 at timepoint 2 demonstrating the most significant differences found in the differential abundance analysis. Annotated datapoints in red represent ASV's that were significantly different.