Ecological Survey of Spontaneously Fermented Riesling in the Finger Lakes

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ABSTRACT

Spontaneous fermentations are completed by a complex succession of non-*Saccharomyces* yeast species and *S. cerevisiae* strains that influence the final wine aroma and flavor profile. Regional yeast composition may help shape the wine characteristics typical in a region, a concept known as microbial terroir. To establish the yeast composition of Riesling grapes in the Finger Lakes American Viticultural Area (AVA), an ecological survey of vineyard and winery microbiome, with a particular focus on *S. cerevisiae* strains, was performed. Grapes, winery equipment, and spontaneous fermentations were sampled during the 2015 and 2016 vintages at three Finger Lakes AVA vineyards and the associated wineries. Yeast was isolated using culture-dependent methods, identified using the 5.8S internal transcribed spacer (ITS) rRNA region, and *S. cerevisiae* strains were characterized using a six-locus multiplex variable number of tandem repeat (VNTR) analysis. Species from the *Aureobasidium, Candida*, *Hannaella, Hanseniaspora*, *Metschnikowia*, *Meyerozyma, Pichia*, *Rhodotorula, Saccharomyces*, *Torulaspora*, *Trigonopsis, Wickerhamomyces, Zygoascus,* and *Zyosaccharomyces* genera were identified. Numerous unrelated native *S. cerevisiae* strains, and a small number of commercial strains, were commonly observed in fermentations. When commercial strains were present, they did not always become established or dominant in the fermentation, and never completely displaced the native strains. Several native *S. cerevisiae* strains are likely part of the regional microbiome and should be investigated further. The *S. cerevisiae* strains observed in fermentations appear to be influenced by regional and resident winery microbiome, and by vintage specific factors.

BIOGRAPHICAL SKETCH

Marie Guido-Miner is a Masters candidate in food science with a concentration in enology at Cornell University. She received a Bachelor of Science degree in biochemistry from the University of Rochester in 2005 and a Master of Science degree in forensic molecular biology from University at Albany in 2006. Formerly a DNA analyst at a crime lab, Marie brings her science background and passion for education to her new field of wine. She has Wine & Spirit Education Trust Level III certification and is a Wine Scholar Guild Champagne Master. Marie is currently pursuing wine writing and wine education while working in a winery tasting room.

DEDICATION

I would like to dedicate this Masters thesis to all the people who have encouraged and helped me on my journey to a second career. My wonderful husband Zac for his love and support, my family for their understanding and encouragement, my friends for their enthusiasm and laughter, my labmates for their expertise and taste in wine, my undergraduate research assistants for their time and effort, and my advisors for their direction and patience.

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Introduction

Wine fermentations have been conducted by humans for thousands of years, but yeast, particularly *Saccharomyces cerevisiae*, was only identified as the causative fermenting agent in the late 1800s (Pretorius, Curtin and Chambers 2015). At its most basic level, wine fermentation is the process of yeast metabolizing sugar into alcohol, carbon dioxide, and heat. In addition to these primary products, yeast also produce secondary metabolites during fermentation that influence the aroma and taste of the final wine. While *S. cerevisiae* is the most important wine yeast, it is not the only yeast species present during wine fermentations. In recent years, the link between regional yeast biodiversity and regional wine character, known as microbial terroir, has been explored. This review gives an overview of wine fermentation, the role of yeast in wine fermentation kinetics and aroma development; the distinctions between and debates surrounding native and commercial *S. cerevisiae* strains, spontaneous and inoculated fermentations, and wild and domesticated *S. cerevisiae* strains; theories of yeast movement; and the concept of microbial terroir.

Wine Fermentation

Wine fermentation can be divided into three kinetic phases – lag, exponential, and stationary phase. During the lag phase yeast build up biomass with little conversion of sugar into alcohol. Must sugar content drops during the exponential phase as yeast convert a majority of the sugars into alcohol. During the stationary phase the rate of alcohol creation slows and eventually stops completely (Bisson and Butzke 2000). A successful fermentation is often defined as one that achieves dryness, or less than 2 g/L of fermentable sugars remaining, in the final wine (Bisson 1999, Bisson and Butzke 2000).

Fermentation kinetics encompasses the rate at which the primary grape sugars, glucose and fructose, are metabolized and the amount of time it takes for each phase of fermentation to be completed (Zuchowska et al. 2015). Many factors influence fermentation kinetics and the chances of a successful fermentation, including the availability of nutrients (Contreras et al. 2011, Barbosa et al. 2014), fermentation temperature (Pallmann et al. 2001, Molina et al. 2007, Barbosa et al. 2014, Fernández-González, Úbeda and Briones 2015), the proportion of solids in the must (Bisson and Butzke 2000, Jolly, Varela and Pretorius 2014), and the distribution, abundance, and interactions of yeast species and strains present in the must (Fernández-Espinar et al. 2001, Bokulich et al. 2014, Fernández-González, Úbeda and Briones 2015, Zuchowska et al. 2015, Bokulich et al. 2016). Under unfavorable conditions the fermentation may become stuck or sluggish. According to Bisson (1999), a stuck fermentation retains higher residual sugar than desired at the end of alcoholic fermentation, and a sluggish fermentation takes longer than the typical 7 to 10 days to reach dryness.

Although many different yeast species can be present in must, not all are capable of successfully completing fermentation. Yeast is a diverse category including numerous unrelated species, only a portion of which are observed in food fermentations. The most important of these is *S. cerevisiae,* which is the primary yeast driving the conversion of sugars to alcohol and CO_2; for this reason 'yeast' is used as a shorthand for *S. cerevisiae* in many food-related industries (Kurtzman, Fell and Boekhout 2011). In the context of wine fermentations, yeast are often described as either *Saccharomyces* or non-*Saccharomyces* (Jolly, Varela and Pretorius 2014). Alcoholic fermentations are completed by a succession of both, typically with non-*Saccharomyces* yeast dominating the early stages, and *Saccharomyces* yeast, most often *S. cerevisiae*, becoming most active in later stages of fermentation (Romano

2003, Clemente-Jimenez et al. 2004, Swiegers et al. 2005, Tofalo et al. 2012, Chambers et al. 2015, Ubeda-Iranzo et al. 2015, Martiniuk et al. 2016). Most non-*Saccharomyces* yeast are not strong fermenters, meaning that these yeasts do not successfully complete fermentation when used alone, and wine usually cannot be fermented to dryness without the presence of *S. cerevisiae* (Bisson 1999, Jolly, Varela and Pretorius 2014, Padilla, Gil and Manzanares 2016). The term is also used to refer to non-*Saccharomyces* yeast whose fermentation kinetics are considered sluggish, or are outcompeted by or killed off in the presence of *S. cerevisiae*. However, even if *S. cerevisiae* dominates the fermentation, the presence of fructophilic non-*Saccharomyces* yeast at the end of the fermentation may assist in fermenting to dryness, as *Saccharomyces* yeast tend to be glucophilic (Bisson 1999, Masneuf-Pomarede et al. 2016).

The succession of yeast in a fermentation is affected by both abiotic (temperature, pH, ethanol tolerance, nutritional requirements, etc.) and biotic factors (starting grape material, interaction with other yeast and bacteria, killer factors, etc.), which determine what yeast species, and strains within species, will be dominant at any given point (Bisson 1999, Bisson and Butzke 2000, Pramateftaki, Lanaridis and Typas 2000, Romano 2003, Boynton and Greig 2014, García-Ríos et al. 2014, Rodríguez-Sifuentes et al. 2014, Tristezza et al. 2014). *S. cerevisiae* consistently outcompetes non-*Saccharomyces* yeast during fermentation for three primary reasons – (1) the Crabtree effect (i.e., it favors fermentation over respiration at high sugar concentrations), (2) the efficient creation of ethanol and heat, (3) high osmotic and ethanol tolerances (Bisson 1999, Swiegers and Pretorius 2005, Goddard 2008, García-Ríos et al. 2014). These factors both favor the growth of *S. cerevisiae* and create an unfavorable environment for the growth of non-*Saccharomyces* yeast species (Goddard 2008). The combination of yeast species and strains present, and their

abundance and persistence in the fermentation, will shape the final wine aroma profile with secondary metabolites produced during fermentation (Pretorius, Curtin and Chambers 2015).

Yeast Identification

Yeast Taxonomy

Yeast are defined as fungi that primarily reproduce asexually, via budding or fission, and whose sexual state is not encompassed in a fruiting body (Kurtzman, Fell and Boekhout 2011). The term yeast includes member species across two phylum – Ascomycota and Basidiomycota (Kurtzman, Fell and Boekhout 2011). The classic definition of species is based on the concept of sexual reproductive isolation, while strains are genetic variants within a species. For a number of reasons this definition is of limited value for yeast. First, asexual reproduction is a common means of propagation in yeast (Kurtzman, Fell and Boekhout 2011). Next, minor DNA sequence divergences, rather than major differences in genes or chromosomal rearrangements, are often the only barrier to sexual reproduction between yeast species (Liti, Barton and Louis 2006). In fact, interspecies hybridization is relatively common between yeast species, especially closely related species (Liti, Barton and Louis 2006, Gibson and Liti 2015). In winemaking settings, yeast with DNA from two species (double hybrids) and three species (triple hybrids) have been observed (Cappello et al. 2010). Finally, horizontal gene transfer (HGT) has been observed in many yeast species (Liti, Barton and Louis 2006, Cappello et al. 2010, Chambers and Pretorius 2010, Almeida et al. 2014, Gibson and Liti 2015). When present, HGT is often observed as a single gene transferred from a bacterium to a yeast. However, HGT between co-located *Saccharomyces* and non-*Saccharomyces* yeast has been observed both in the wild and in laboratory settings (Cappello et al. 2010). These

factors cause the taxonomical classification of yeast species to be complex and the distinction between yeast species to be somewhat fluid. Especially in closely related yeast species, designations exist on a continuum rather than as discrete categories (Liti, Barton and Louis 2006, Kurtzman, Fell and Boekhout 2011, Gibson and Liti 2015).

Yeast species characterization is further complicated by the fact that each yeast species has two taxonomically valid names, one each for its teleomorph (sexual reproduction) and anamorph (asexual reproduction) state (Kurtzman, Fell and Boekhout 2011, Jolly, Varela and Pretorius 2014). Teleomorphs produce spores which can sexually reproduce by combining, while anamorphs produce offspring by budding or through fission (Cubillos et al. 2009, Kurtzman, Fell and Boekhout 2011, Pretorius, Curtin and Chambers 2015). If both states have been characterized, the taxonomical name of the teleomorph is preferred, while anamorph name is used only if the teleomorph has not been characterized (Kurtzman, Fell and Boekhout 2011). The anamorphic state, however, is more common in yeast, and not all teleomorphs have been characterized due to the difficulty of inducing sporulation (Jolly, Varela and Pretorius 2014). Teleomorphs and anamorphs of the same yeast can be morphologically dissimilar, meaning that prior to the advent of DNA analysis many yeast species were incorrectly labeled as two different species. Similarly, a single state of a single yeast species can present a range of phenotypic differences, which prior to DNA analysis lead to the designation of multiple taxonomical names for a single species (Jolly, Varela and Pretorius 2014). This has led to the existence of multiple taxonomic synonyms for many yeast species, with the wine industry often adopting a name other than that preferred by the taxonomical community (Table 1).

Table 1 - Common synonyms for yeast names

Teleomorphic Form	**Anamorphic Form**	**Synonyms**
Candida zemplinina		*Starmerella bacillaris*
Dekkera bruxellensis	*Brettanomyces bruxellensis*	
Hanseniaspora uvarum	*Kloeckera apiculata*	
Metschnikowia pulcherrima	*Candida pulcherrima*	*Torulopsis pulcherrima*
Meyerozyma guilliermondii	*Candida guilliermondii*	*Pichia guilliermondii*
Pichia fermentans	*Candida lambica*	
Pichia kluyveri		*Hansenula kluyveri*
Pichia kudriavzevii	*Candida krusei*	*Issatchenkia occidentalis*
Pichia membranifaciens	*Candida valida*	
Pichia occidentalis	*Candida sorbose*	*Issatchenkia occidentalis*
Rhodotorula glutinis		*Cryptococcus gluntinis*
Wickerhamomyces anomalus	*Candida pelliculosa*	*Pichia anomala; Hansenula anomala*
Zygosaccharomyces bailii		*Saccharomyces bailii*

(Kurtzman, Fell and Boekhout 2011, Alessandria et al. 2015)

Yeast Species and Strain Identification

Identification of yeast species after they were first microscopically observed in the 1800s was based on morphology and physiology. Morphological distinctions were made through examinations via microscope and by growing plated colonies on differential nutritional media (Pallmann et al. 2001, Zimbro et al. 2009, Kurtzman, Fell and Boekhout 2011). Physiological distinctions were made through observing the reactions of yeast to varying environmental conditions, such as fermentation rate in glucose, pH tolerance, and ability to utilize various nitrogen sources (Schütz and Gafner 1995, Kurtzman, Fell and Boekhout 2011). While these methods are still often used as screening tools, the limitations of observational methods have been made apparent with the advent of DNA analysis. Some morphologically similar yeast colonies originally listed as a single species have been shown to be genetically

distinct, and conversely morphologically dissimilar yeast colonies originally listed as separate species have been shown to be genetically identical. In addition, distinct yeast species can have similar physiological reactions to varying environmental conditions, and different strains within a single yeast species can have different reactions to the same conditions (Pallmann et al. 2001, Fugelsang and Edwards 2007, Jolly, Varela and Pretorius 2014). DNA analysis of yeast has made identification more accurate, but highlighting the many genetic similarities between different yeast has made the species definitions less distinct (Liti, Barton and Louis 2006, Kurtzman, Fell and Boekhout 2011, Gibson and Liti 2015).

Several DNA analysis methods exist, and they have substantially evolved over the years (Esteve-Zarzoso et al. 1999, Esteve-Zarzoso et al. 2000, Pramateftaki, Lanaridis and Typas 2000, Sun et al. 2009, Mercado et al. 2010, Taylor et al. 2014, Ubeda-Iranzo et al. 2015). Commonly used methods include Sanger sequencing or restriction analysis of specific DNA amplicons, Next Generation Sequencing (NGS), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and microsatellite analysis of polymorphic DNA loci (Mercado et al. 2010, Barata, Malfeito-Ferreira and Loureiro 2012, Schoch et al. 2012, Taylor et al. 2014, Martiniuk et al. 2016). These methods have different levels of discrimination, and can attempt to distinguish between: (1) yeast and non-yeast microbes, (2) different yeast species, and (3) different strains within a yeast species. Another major factor separating different DNA analysis methods is the method of sample collection, which can be broken into two major categories: culture dependent and culture independent. Culture dependent methodologies involve adding samples to an enrichment media to encourage the growth of any yeast present in the sample prior to the isolation subsequent genetic analysis (Vaz-Moreira et al. 2011, Taylor et al. 2014). Culture independent methods perform genetic analysis directly from the sample without

prior isolation of yeast, and may or may not involve enrichment (Vaz-Moreira et al. 2011, Taylor et al. 2014).

Culture dependent methods typically enrich samples on solid gel media plates that encourage the growth of yeast colonies. The solid media may encourage all yeast growth, such as those made with Wallerstein Lab (WL) nutrient media (Zimbro et al. 2009), or may encourage the growth of specific yeast, such as non-*Saccharomyces* yeast on media with lysine as the only nitrogen source (Martin and Siebert 1992, Ubeda and Briones 2000). Samples may also be enriched in liquid media, such as grape juice, then added to solid media plates and incubated until visible yeast colonies form (Pallmann et al. 2001). A key point about culture dependent collection methods is that the yeast colonies are isolated, meaning each is processed separately regardless of the DNA analysis method chosen. This generally allows for a higher degree of specificity in identifying yeast species and strains. However, these methods can be biased towards yeast species that grow better in the enrichment media and incubation conditions, underrepresenting or missing yeast species that grow more slowly (Cocolin, Bisson and Mills 2000, Pallmann et al. 2001, Keller and Zengler 2004, Renouf, Claisse and Lonvaud-Funel 2007, Vaz-Moreira et al. 2011, Bokulich and Mills 2012, Taylor et al. 2014, Alessandria et al. 2015). For example, *Brettanomyces* species are difficult to observe via culture-dependent methods, as they grow too slowly to compete on non-selective media (Dias et al. 2003). Additionally, the ratio of *Saccharomyces* to non-*Saccharomyces* in a sample will affect the growth patterns in the enrichment media (Ubeda-Iranzo et al. 2015). However, differential growth patterns can also be exploited to favor the growth of certain classes of yeast, e.g. lysine nutrient media favors the growth of non-*Saccharomyces* yeast over *Saccharomyces* species (Martin and Siebert 1992, Ubeda-Iranzo et al. 2015). The bias introduced by differential growth patterns can be exacerbated when yeast colony

morphology is used as a screening tool for selecting colonies for DNA analysis, leading to distortions in the estimated yeast species diversity and/or abundances (Schuller and Casal 2007, Li et al. 2011, David et al. 2014).

Culture independent methods involve direct genetic analysis of a sample without prior isolation on solid media (Taylor et al. 2014, Ubeda-Iranzo et al. 2015). This is most often performed without sample enrichment, but enrichment in liquid media may be performed. In contrast to culture dependent methods, culture independent methods do not separate the microbes present in the sample prior to DNA analysis, but analyzes the mixture. Culture independent methods give a more accurate representation of the breadth of yeast present and their relative abundances, but at the cost of depth of information, as they yield taxonomic resolution to only family or order level specificity (Vaz-Moreira et al. 2011, Bokulich et al. 2012, Hanson et al. 2012, Taylor et al. 2014). The yeast present in the sample may also be grouped by genetic similarity but not necessarily by distinct taxon information; such grouping is typically referred to as operational taxonomy units (OTUs) (Martiny et al. 2006, Vaz-Moreira et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Taylor et al. 2014). Another factor of culture independent methods is that, unlike culture dependent methods, they do not distinguish between microbes that are alive or dead at the time of collection (Bokulich et al. 2013). Similarly the presence of a microbe does not necessarily mean that it is a significant contributor to the fermentation results (Bokulich and Mills 2012).

Comparisons of culture-dependent and culture-independent methods on the same set of samples show that the results do not contradict each other, but provide different levels of certainty in species identification and coverage of total biodiversity (Vaz-Moreira et al. 2011, David et al. 2014, Taylor et al. 2014). Culture dependent methods are more specific, typically identifying yeast to the species and often the

strain level (Vaz-Moreira et al. 2011, Hanson et al. 2012). In contrast, culture independent methods identify yeast to the family or genus level, occasionally to the species level, and infrequently to the strain level (Bokulich and Mills 2012). It should be noted that NGS methods are almost always used on samples collected through culture independent methods. These methods have a high throughput ability to sequence multiple DNA fragments concurrently, allowing mixtures to be separated into OTUs (Bokulich et al. 2012, Bokulich and Mills 2012, David et al. 2014, Taylor et al. 2014, Ubeda-Iranzo et al. 2015).

Regardless of the sample collection method used, the rDNA ribosomal internal transcribed spacer (ITS) region is often targeted for yeast species identification analysis. The ITS region is variable across yeast species but flanked by broadly conserved regions that encode for ribosomal subunits, which allows the use of the same amplification primers when sequencing most fungi, including those related to winemaking (White et al. 1990, Clemente-Jimenez et al. 2004, Schoch et al. 2012, Romanelli et al. 2014). Once ITS region data has been obtained from the unknown yeast sample, it can be compared against the data of known yeast species analyzed in the same way to make an identification. If the region was sequenced the data is often compared to GenBank, one of the largest publicly available database of yeast genetic sequence data, run by the National Center for Biotechnology Information (NCBI), a part of the United States' National Institute of Health (Benson et al. 2013). Yeast strains can also be differentiated using the ITS region (White et al. 1990, Clemente-Jimenez et al. 2004, Hanson et al. 2012, Schoch et al. 2012, Romanelli et al. 2014), however higher levels of discrimination can be achieved if PCR based microsatellite analysis of various loci is performed (Field and Wills 1998, Howell et al. 2004, Ayoub et al. 2006, Vaudano and Garcia-Moruno 2008, Richards, Goddard and Gardner 2009, Kurtzman 2011). Similarly, degree of relation between yeast species

and strains can be evaluated by examining the differences between sample sequence data (Kurtzman 2011). Multigene analysis of allele differences between samples can also be used to estimate relatedness, and may lead to greater intra-species power of discrimination if the genes are unlinked (Ayoub et al. 2006, Kurtzman 2011). The choice of collection and DNA analysis methods come down to the research question and the degree of discrimination between yeast that is desired (Schuller and Casal 2007).

Saccharomyces **Yeast**

Saccharomyces Genus

The feature that unites all *Saccharomyces* species is the ability to convert sugars into significant amounts of alcohol, making them strong fermenters (Kurtzman, Fell and Boekhout 2011). As mentioned previously, the distinctions between *Saccharomyces* species exist on a continuum, and hybridization between species is relatively common (Liti, Barton and Louis 2006). Species designations have changed significantly over time, and some classifications remain controversial (Kurtzman, Fell and Boekhout 2011, Borneman and Pretorius 2015). The most recent edition of *The Yeasts: A Taxonomic Study* lists eight accepted species designations: *S. arboricolus, S. bayanus, S. cariocanus, S. cerevisiae, S. kudriavzevii, S. mikatae, S. paradoxus,* and *S. pastorianus* (Kurtzman, Fell and Boekhout 2011). Since then several changes have been proposed, including: (1) Listing *S. cariocanus* as a variant within the *S. paradoxus* species instead of a separate species (Boynton and Greig 2014, Borneman and Pretorius 2015), (2) listing *S. eubayanus* as a novel *Saccharomyces* species (Libkind et al. 2011), and (3) creating two separate species from the known variants of *S. bayanus* - *S. bayanus* and *S. uvarum* (Libkind et al. 2011, Borneman and Pretorius 2015). Additionally, *S. pastorianus* and *S. bayanus* are considered hybrids of

other *Saccharomyces* species (Kurtzman, Fell and Boekhout 2011, Zuchowska et al. 2015) and as a result can be grouped separately. It is likely that species designations will continue to evolve as the study of *Saccharomyces* yeast progresses.

Not all species that completely ferment sugars to alcohol create pleasant aromas, so only a subset of *Saccharomyces* species are associated with beverage fermentations (Kurtzman, Fell and Boekhout 2011, Chambers et al. 2015). *S. cerevisiae*, *S. kudriavzevii*, *S. bayanus*, *S. uvarum*, and *S. pastorianus* are all associated with beverage fermentations to some degree (Gonçalves et al. 2011, García et al. 2012, Almeida et al. 2014, Boynton and Greig 2014, Chambers et al. 2015, Gibson and Liti 2015). Of these *S. cerevisiae* is by far the most important, with other *Saccharomyces* yeast species occurring with varying frequency.

Saccharomyces cerevisiae

S. cerevisiae is the most commonly observed yeast in alcoholic fermentations (Kurtzman, Fell and Boekhout 2011), and is infrequently isolated in natural environments (Hyma et al. 2011, Kurtzman, Fell and Boekhout 2011, Hyma and Fay 2013, Boynton and Greig 2014). It is thermotolerant, with an optimal growing temperature around 25-30°C (Beltran et al. 2008, Boynton and Greig 2014). *S. cerevisiae* associated with wine fermentations tend towards rapid fermentation, complete sugar conversion, and production of pleasantly aromatic secondary metabolites (Hyma et al. 2011, Dapporto et al. 2016). It most often present in the middle and late stages of fermentation, but can be seen earlier (Fleet 2003, Romano 2003, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Fleet 2008, Bezerra-Bussoli et al. 2013, Pinto et al. 2015).

S. cerevisiae's most simple genetic forms are the basic mating cell types, a and α, which are haploid and capable of mitotic asexual reproduction through budding,

though it is more common for them to sexually reproduce, fusing with the opposite mating type to create an a/α diploid cell. Only opposite mating type cells can fuse to create a diploid. The a/α diploid cell will reproduce asexually via mitotic budding in nutrient rich environments; when nutrients are scarce, the diploid cell will meiotically divide, creating four haploid cells, typically two a and two α cells, in a process known as sporulation. The haploid cells are contained within protective structures called ascospores, themselves surrounded by a protective sac known as an ascus. An ascus containing four ascospores is known as a tetrad, and can withstand extreme environmental conditions. When sufficient nutrients become available the ascospores are released from the ascus and germinate into haploid cells capable of mitotic budding. As mentioned previously, most of these haploids will mate and fuse into diploids shortly after germination. Diploids can be created by a fusion of haploids from the same tetrad (intra-tetrad mating) or from meiotically separate tetrads (inter-tetrad mating). If asexually reproducing haploid cells do not encounter an opposite mating type they are capable of switching mating cell type during budding, creating daughter cells of the opposite mating type. Haploids capable of switching mating types are called homothallic, while those that lack the ability are heterothallic. Diploids created by self-fertilization of homothallic haploids will be completely homozygous (Boynton and Greig 2014, Pretorius, Curtin and Chambers 2015, Knight and Goddard 2016).

The accepted life cycle of *S. cerevisiae* was determined through observation under laboratory conditions; *in vivo* it is less well understood and may be more variable (Chambers et al. 2015, Knight and Goddard 2016). In winemaking environments diploids are the most common genetic form, but haploids, polyploids, and aneuploids also exist (Legras et al. 2007, Cubillos et al. 2009, Salinas et al. 2010). The ability to sporulate and ascospore viability varies considerably among *S. cerevisiae* strains and

is tied to environmental conditions (Cubillos et al. 2009, Fernández-González, Úbeda and Briones 2015). Most strains of *S. cerevisiae* used in winemaking are homothallic and heterozygous. The frequency of homozygous strains observed indicates that in winemaking environments *S. cerevisiae* undergoes mostly asexual reproduction with some sexual reproduction, influenced significantly by homothallic mating (Legras et al. 2007, Fernández-González, Úbeda and Briones 2015). The potential for genetic diversity in populations that reproduce asexually is lower than those that reproduce sexually (Cubillos et al. 2009), though chromosomal rearrangement during mitosis can be a source of genetic variation in *S. cerevisiae* during asexual reproduction (Schuller et al. 2007). Given its multiple viable forms, multiple reproductive pathways, ability to hybridize with other yeast species, and the possible presence of horizontally transferred genes, *S. cerevisiae* is rapidly adaptable to local conditions (Bradbury et al. 2005, Cubillos et al. 2009, Salinas et al. 2010).

S. cerevisiae have shown large intra-species variation, with at least hundreds of distinct strains (Ubeda and Briones 2000, Schuller et al. 2007, Goddard et al. 2010, Charron et al. 2014). The total number of observed *S. cerevisiae* strains is uncertain, as different studies use different methods of differentiation, which can be genetic, phenotypic, or both. This uncertainty is exacerbated by the genetic complexity of *S. cerevisiae* and the speed at which genetic adaptations occur. New strains can arise from genetic variations created by mutations, recombination events during reproduction, hybridization with closely related species, and HGT from species that are not closely related (Cubillos et al. 2009, Pretorius, Curtin and Chambers 2015). Genetic variations can occur anywhere in the DNA of the strain, including in genes and gene promoter regions, which regulate gene expression (Treu et al. 2014). Strains with gene variation may create different functional products, whereas strains with variations in gene promoter regions will likely create the same functional

product but may differ in when and how much of the product is created (Treu et al. 2014).

Genetic differences between *S. cerevisiae* strains can be the cause of different phenotypic characteristics, such as nutrient requirements, fermentation efficiency, and volatile formation (Bisson and Butzke 2000, Chambers and Pretorius 2010, Contreras et al. 2011, García-Ríos et al. 2014, Fernández-González, Úbeda and Briones 2015, Zuchowska et al. 2015, García et al. 2016). Strains that are genetically similar are likely to have phenotypic similarities (Ubeda and Briones 2000), and genetic differences are likely to yield phenotypic differences (Treu et al. 2014). However, there is not a direct correlation between genetic similarity and phenotypic similarity – genetically similar strains can have different phenotypic characteristics and dissimilar strains can have similar phenotypic characteristics (Ubeda and Briones 2000, Schuller et al. 2007, Mendes et al. 2013, Gibson and Liti 2015). The relationship between genetic variations and aroma compound formation, in particular, is not well understood, as more than one gene is often involved. Only a few genes are known to have a causal relationship with aroma compound formation (Pretorius, Curtin and Chambers 2015).

Other Saccharomyces Species

S. kudriavzevii is not commonly observed, but has been isolated in commercial wine fermentations in New Zealand and Europe. It is most often isolated in natural environments, including Japan and Portugal (Kurtzman, Fell and Boekhout 2011). *S. kurdriavzevii* is more cryotolerant than *S. cerevisiae*, having an optimal growing temperature close to 10°C (Gonçalves et al. 2011, Boynton and Greig 2014).

S. bayanus is an interspecies *Saccharomyces* hybrid. It is alternatively thought of as a single hybrid with a complex genetic background on its way to speciation, or as a

grouping of genetically similar interspecific hybrids (Gibson and Liti 2015). It is commonly observed in commercial fermentation environments, specifically beer fermentations (Gonçalves et al. 2011), and has yet to be observed in nature (Libkind et al. 2011, Boynton and Greig 2014). Certain *S. bayanus* strains are known to have an additional sugar transportation mechanisms beyond that seen in *S. cerevisiae*, which may give it an advantage in stressful environments (Schütz and Gafner 1995, Zuchowska et al. 2015). *S. bayanus* tends to produce more volatile thiols then *S. cerevisiae* during fermentation (Swiegers et al. 2005, Swiegers and Pretorius 2005, Padilla, Gil and Manzanares 2016).

S. uvarum may be a variant of the hybrid *S. bayanus*, a related but separate interspecies hybrid, or a separate species (Libkind et al. 2011, Borneman and Pretorius 2015). The species distinction between *S. bayanus* and *S. uvarum* is controversial, but they are seen as distinct groups even by those who think they should be classified together (Kurtzman, Fell and Boekhout 2011). Caution should be applied when distinguishing the two, both due to the controversy and because sources used for genetic comparison, such as NCBI's GenBank, may have outdated references (Abarenkov et al. 2010, Boynton and Greig 2014, Borneman and Pretorius 2015). *S. uvarum* has been isolated from several types of commercial fermentation, including cider, and low temperature or high sugar wine fermentations (Gonçalves et al. 2011, Kurtzman, Fell and Boekhout 2011, Almeida et al. 2014, Borneman and Pretorius 2015). It has also been observed in natural environments including Patagonia (Libkind et al. 2011). *S. uvarum* is cryotolerant, and tends to produce less ethanol and acetic acid, but more glycerol, succinic acid, and volatile thiol compounds than *S. cerevisiae* (Almeida et al. 2014, Boynton and Greig 2014).

S. pastorianus is another hybrid commonly observed in commercial fermentation settings, particularly lager beers, but not observed in nature (Gonçalves et al. 2011,

Libkind et al. 2011, Almeida et al. 2014, Boynton and Greig 2014, Chambers et al. 2015, Gibson and Liti 2015, Zuchowska et al. 2015). Like *S. bayanus*, *S. pastorianus* has an additional sugar transportation mechanism (Zuchowska et al. 2015). *S. pastorianus* is more cryotolerant, and tends to produce fewer esters than *S. cerevisiae* (Gibson and Liti 2015).

Other unclassified *Saccharomyces* hybrids have been observed in both nature and commercial fermentation environments (Liti, Barton and Louis 2006, Zhang et al. 2010, Boynton and Greig 2014, Chambers et al. 2015). The number of different *Saccharomyces* hybrids observed in alcoholic fermentation, and the genetic diversity within these hybrids, indicates that hybridization events are not rare and can result in beneficial hybrid species (Gibson and Liti 2015). Studies have shown that low temperature wine and beer fermentations lead to the increased observation of *Saccharomyces* interspecies hybrids, suggesting that hybrids have an advantage over *S. cerevisiae* in low temperature conditions (García et al. 2012, Almeida et al. 2014). Natural hybridization events between *Saccharomyces* species in fermentation environments may be due to selection pressures in low temperature fermentations, combined with human selection of these hybrids as beneficial to fermentation (Gibson and Liti 2015). While improved fitness at low fermentation temperatures is one of the most important traits seen in hybrids, it is not the only one, as beneficial aroma and flavor development and desirable flocculation patterns have also been observed (Chambers et al. 2015, Gibson and Liti 2015). Hybrids tend to produce ethanol concentrations in a range between those produced by the parent strains, while nitrogen requirements and fermentation kinetics do not show a simple pattern of inheritance (García et al. 2012). Interspecies hybrids between *Saccharomyces* and non-*Saccharomyces* yeast, with similar characteristics, have also been observed (Cappello et al. 2010).

Non-*Saccharomyces* Yeast

Wine Relevant Non-Saccharomyces Yeast

Currently there are 149 recognized non-*Saccharomyces* yeast genera, with almost 1500 recognized yeast species, of which at least 40 have been observed in wine fermentations (Kurtzman, Fell and Boekhout 2011, Jolly, Varela and Pretorius 2014). Non-*Saccharomyces* yeast are less well studied than *S. cerevisiae*, so basic information like chromosome number, genome size, and life cycle mechanics are often unknown (Masneuf-Pomarede et al. 2016). As with all yeast, non-*Saccharomyces* species have somewhat fluid distinctions that have changed over time as more sensitive DNA techniques have become available.

In the context of alcoholic fermentations, non-*Saccharomyces* yeast can be divided into three broad categories – low fermenting apiculate yeast, primarily aerobic yeast, and strong fermenting yeast (Sun et al. 2009, Jolly, Varela and Pretorius 2014). As previously mentioned, most non-*Saccharomyces* yeast are not strong fermenters, and are either outcompeted by *S. cerevisiae* or are unable ferment wine to dryness (Padilla, Gil and Manzanares 2016), though the presence of fructophilic non-*Saccharomyces* yeast at the end of the fermentation may assist in metabolizing residual sugars that cannot be metabolized by *S. cerevisiae* (Masneuf-Pomarede et al. 2016). Non-*Saccharomyces* yeast are commonly present on grapes and typically dominate the early stages of wine fermentations, but can even persist after *S. cerevisiae* becomes dominant in later stages (Cocolin, Bisson and Mills 2000, Barata et al. 2008, Santamaría et al. 2008, Sun et al. 2009, Li et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Bezerra-Bussoli et al. 2013, Díaz et al. 2013). Apiculate yeast often appear first, followed by other non-*Saccharomyces* yeast of varying abundance and type (Clemente-Jimenez et al. 2004, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Padilla, Gil and Manzanares 2016). Some non-

Saccharomyces genera associated with fermentations include *Brettanomyce*s, *Candida, Hanseniaspora*, *Metschnikowia, Pichia, Rhodotorula*, *Torulaspora*, and *Zygosaccharomyces* (Cocolin, Bisson and Mills 2000, Esteve-Zarzoso et al. 2000, Fleet 2003, Clemente-Jimenez et al. 2004, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Urso et al. 2008, Pinto et al. 2015, Padilla, Gil and Manzanares 2016). Each non-*Saccharomyces* yeast species has intra-species strain diversity; only a portion of strains within a species can survive winemaking environments, and there is evidence that some adapt to winemaking environments (Masneuf-Pomarede et al. 2016).

Brettanomyces Genus

Brettanomyces species, most commonly *B. bruxelliensis*, are occasionally observed in fermentations (Esteve-Zarzoso et al. 2000, Clemente-Jimenez et al. 2004). While the aroma compounds it creates can add a desirable aromatic complexity to some styles of wine, large concentrations of these volatiles are considered undesirable, and *B. bruxelliensis* is often considered a spoilage yeast (Echeverrigaray et al. 2013).

Candida Genus

The *Candida* genus is phylogenetically diverse, and teleomorph yeast with unclear lineage are often grouped within it (Kurtzman, Fell and Boekhout 2011). *Candida* species are most abundant in the early stages of fermentation (Romano 2003, Clemente-Jimenez et al. 2004, Jolly, Varela and Pretorius 2014), though they can persist longer in cooler fermentations, as temperatures $\leq$ 15-20°C enhances their ethanol tolerance (Di Maro, Ercolini and Coppola 2007); they can be both beneficial and detrimental to wine quality. The *Candida* species pairs *C. oleophila*/*C. railenensis* and *C. zemplinina*/*C. stellata* are closely related and can be difficult to distinguish;

(Kurtzman, Fell and Boekhout 2011, Tofalo et al. 2012, Jolly, Varela and Pretorius 2014) as such any yeast identified as any of these species will be identified with both names to indicate that it may belong to either species. These pairs have all been observed natural environments, such as decaying fruit, *Drosophila* fruit flies, North American Oak trees, and South American Beech trees, as well as in fermentations (Kurtzman, Fell and Boekhout 2011).

C. zemplina/*C. stellata* is a strong fermenter that has been associated with positive aroma development and improved mouthfeel in wine, although detrimental aromas has also been reported (Constantí et al. 1997, Torija et al. 2001, Clemente-Jimenez et al. 2004, Combina et al. 2005, Cordero-Bueso et al. 2011, Tofalo et al. 2012, Bokulich et al. 2014, Jolly, Varela and Pretorius 2014, Canonico, Comitini and Ciani 2015, Teixeira, Caldeira and Duarte 2015, Garofalo et al. 2016, Padilla, Gil and Manzanares 2016). Some strains of *C. zemplinina/C. stellata* have been found to be tolerant of ethanol concentrations up to 14% alcohol by volume, much higher than is typical for non-*Saccharomyces* yeast (Tofalo et al. 2009), and it is generally more fructophilic and osmotolerant than *S. cerevisiae* (Romano 2003, Bokulich et al. 2012, Tofalo et al. 2012, Jolly, Varela and Pretorius 2014, Alessandria et al. 2015, Teixeira, Caldeira and Duarte 2015). *C. zemplinina*/*C. stellata* are considered to be important yeast in Sherry fermentations, and were frequently observed in spontaneous fermentations of grapes from the Priorat and Andarax valley regions of Spain, the Tuscany region of Italy (Clemente-Jimenez et al. 2004), and the northern Apulia region of southern Italy (Garofalo et al. 2016).

Hanseniaspora Genus

Hanseniaspora species are low fermenting apiculate yeast; like *Candida* species, they are frequently found on grapes and in abundance during the early stages of

fermentation (Fleet 2003, Romano 2003, Clemente-Jimenez et al. 2004, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Jolly, Varela and Pretorius 2014, Pinto et al. 2015), and are observed more frequently and later in lower-temperature fermentations (Di Maro, Ercolini and Coppola 2007). The *Hanseniaspora* genus can be broken into two species groups – one containing *valbyensis*, *guilliermondii*, and *uvarum*, and a second consisting of *vineae*, *osmophila*, and *occidentalis* (Giorello et al. 2014). Species of interest include *H. uvarum* and *H. valbyensis*.

H. uvarum is the most important species within the *Hanseniaspora* genera. It is commonly observed on grapes and in the early stages of fermentations worldwide, and is frequently the most abundant non-*Saccharomyces* yeast identified in fermentations (Constantí et al. 1997, Esteve-Zarzoso et al. 2000, Bujdoso, Egli and Henick-Kling 2001, Torija et al. 2001, Beltran et al. 2002, Combina et al. 2005, Baffi et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Bezerra-Bussoli et al. 2013, Sun et al. 2014, Alessandria et al. 2015, Drumonde-Neves et al. 2017). *H. uvarum*'s high genetic strain diversity is likely due to its range of ecological niches, as it has been documented on insects, soils, and various fruits (Kurtzman, Fell and Boekhout 2011), Masneuf-Pomarede et al. 2016), and multiple strains have been observed on grapes in the Finger Lakes (Bujdoso, Egli and Henick-Kling 2001). It was once classified as a spoilage yeast, but undesirable compounds are correlated to total ethanol production, so negative impact is minimal in typical wine fermentations where *H. uvarum* only thrives early in fermentation (Romano 2003), Ciani and Maccarelli 1997). Some *H. uvarum* strains are also known to produce beneficial aroma compounds (Baffi et al. 2011, Jolly, Varela and Pretorius 2014, Belda et al. 2016). *H. valbyensis* is observed in similar natural environments, is known to contribute

positively to cider, and may contribute positively to wine fermentations (Kurtzman, Fell and Boekhout 2011).

Metschnikowia Genus

Metschnikowia species have been observed on grapes and in fermentations, typically the early or middle stages, but are observed in different frequencies in different locations (Clemente-Jimenez et al. 2004, Combina et al. 2005, Li et al. 2011, Díaz et al. 2013, Garofalo et al. 2016). The main species of interest in fermentations is *M. pulcherrima*, which can contribute beneficial aroma compounds and may result in lower ethanol concentrations (Jolly, Varela and Pretorius 2014, Canonico, Comitini and Ciani 2015, Belda et al. 2016, Padilla, Gil and Manzanares 2016). Some *M. pulcherrima* strains have been shown to have an antimicrobial effect against an array of non-*Saccharomyces* yeasts, and other strains may inhibit *S. cerevisiae* growth (Fleet 2003, Oro, Ciani and Comitini 2014). *M. chrysoperlae* and *M. fructicola* are two closely related species (Kurtzman, Fell and Boekhout 2011). For the purposes of this review it will be assumed that if *M. pulcherrima* is identified it may include these two species.

Pichia Genus

Pichia is a large genus that encompasses 20 species, including some previously classified as members of the *Candida* and *Issatchenkia* genera (Kurtzman, Fell and Boekhout 2011). *Pichia* species are primarily aerobic fermenters and are most often observed in the middle stage of fermentation (Clemente-Jimenez et al. 2004, Di Maro, Ercolini and Coppola 2007, Sun et al. 2009, Jolly, Varela and Pretorius 2014). *Pichia* species also tend to contribute to positive aroma characteristics (Jolly, Varela and Pretorius 2014, Belda et al. 2016), although some are considered spoilage yeast

(Echeverrigaray et al. 2013). The *Pichia* species groups *P. cecembensis*, *P. occidentalis*, and *P. kudriavzevii*, are closely related and can be difficult to distinguish, so are often identified as *P. cecembensis*/*P.occidentalis*/*P. kudriavzevii* (Kurtzman, Fell and Boekhout 2011, Tofalo et al. 2012, Jolly, Varela and Pretorius 2014).
Several *Pichia* species have been observed in fermentation and natural environments. The contribution of *P. cecembensis*/*P.occidentalis*/*P. kudriavzevii* to fermentations is unclear (Kurtzman, Fell and Boekhout 2011). *P. fermentans* has been shown to reduce the total acetic acid produced by *S. cerevisiae* (Padilla, Gil and Manzanares 2016). *P. kluyveri* has been observed in the late stage of fermentations (Bezerra-Bussoli et al. 2013, Díaz et al. 2013), and has been shown to increase positive aroma characteristics in fermentations with *S. cerevisiae* (Ciani et al. 2010, Padilla, Gil and Manzanares 2016). *P. membranifaciens* has occasionally been observed in wine fermentations (Díaz et al. 2013) is typically considered a spoilage yeast, but produces a killer toxin that may help control *B. bruxellenis* populations (Ciani and Comitini 2011). In 2006 a novel species closely related to *P. membranifaciens*, *Candida californica*, was described (Wu, Robert and Bai 2006); due to possibilities for confusion, either species will be referred to as *P. membranifaciens*/*C. californica*.

Rhodotorula Genus

The *Rhodotorula* genus is composed of basidiomycetous yeast species, which have occasionally been observed in wine fermentations and intact grapes (Díaz et al. 2013, Setati et al. 2013). The closely related *R. glutinis*/*Rh. babjevae* species pair is most often seen in natural environments, especially apples, but also grapes (Kurtzman, Fell and Boekhout 2011), and can produce off-aromas in wine (Alessandria et al. 2015). A Chinese isolate of *R. mucilaginosa*, one of the most widely distributed

basidiomycetous yeast in natural environments (Kurtzman, Fell and Boekhout 2011), was found to contribute to beneficial aroma compounds (Wang et al. 2017).

Torulaspora Genus

Torulaspora species have occasionally been observed in wine fermentations and intact grapes (Esteve-Zarzoso et al. 2000, Clemente-Jimenez et al. 2004, Díaz et al. 2013, Setati et al. 2013). *T. delbrueckii* is the only species of interest to wine fermentations, and some strains appear to have genetically adapted to winemaking conditions (Masneuf-Pomarede et al. 2016). *T. delbrueckii* is generally considered to have a positive impact on wine quality, though some undesirable aromas have been reported (Ciani and Maccarelli 1997, Cordero-Bueso et al. 2011, Jara, Rojas and Romero 2015, Belda et al. 2016, Padilla, Gil and Manzanares 2016).

Zygosaccharomyces Genus

The fructophilic *Zygosaccharomyces* species have occasionally been observed in wine fermentations (Esteve-Zarzoso et al. 2000, Clemente-Jimenez et al. 2004, Jolly, Varela and Pretorius 2014). *Z. bailii*, a highly osmotolerant, glucophilic fermenter rarely appears on sound grapes, but is associated with mummified or sour rot infected berries (Barata et al. 2008, Tofalo et al. 2009, Kurtzman, Fell and Boekhout 2011). For these reasons, *Z. bailii* is often considered a spoilage yeast (Barata et al. 2008), but it has also been shown to increase the formation of desirable aroma compounds in cofermentations with *S. cerevisiae* (Padilla, Gil and Manzanares 2016). *Z. parabailii* is a closely related novel species, whose impact on fermentations is unclear but likely similar to *Z. bailii* (Suh et al. 2013).

Aroma Compounds

Different yeast species, and different strains within species, create different types and concentrations of aroma compounds during fermentation. The combination of yeast, grape material, and fermentation conditions will affect the aroma compounds created and shape the final wine aroma profile (Swiegers and Pretorius 2005, Leeuwen and Seguin 2006, Carrau et al. 2008, Gayevskiy and Goddard 2012, Jolly, Varela and Pretorius 2014, Pretorius, Curtin and Chambers 2015); even small differences will affect a wine's sensory profile (Swiegers et al. 2005, Carrau et al. 2008). The presence of non-*Saccharomyces* yeast in fermentations has been positively linked to increased perception of wine complexity (Belda et al. 2016), defined as a layering of diverse aroma compounds within a single wine – the more aromas, the more complex the wine (Swiegers et al. 2005). Yeast create aroma compounds during fermentation through de novo synthesis of secondary metabolites and the enzymatic release of bound aroma precursors from grape material (Romano 2003, Swiegers and Pretorius 2005, Molina et al. 2007, Padilla, Gil and Manzanares 2016). All yeast can create aroma compounds through both mechanisms, but secondary metabolites are primarily produced by *Saccharomyces* yeast, while non-*Saccharomyces* yeast contribute more via enzymatic activity (Swiegers and Pretorius 2005, Baffi et al. 2011, Jolly, Varela and Pretorius 2014, Bisotto et al. 2015, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Padilla, Gil and Manzanares 2016). The major aroma compounds produced by yeast as secondary metabolites are esters, higher alcohols, carbonyl compounds, volatile acids, volatile phenols, and sulfur compounds (Combina et al. 2005, Swiegers and Pretorius 2005, Molina et al. 2007).

Esters

Esters are one of the largest and most important groups due to their contribution of fruity aromas (Swiegers and Pretorius 2005, Cordente et al. 2007). Energy is required to produce esters, so production is low during the lag phase and increases during the exponential and stationary phases (Swiegers and Pretorius 2005, Cordente et al. 2007, Molina et al. 2007). Ester production stops with fermentation, but esterase-induced cleavage continues (Swiegers and Pretorius 2005), leading to a slow dissipation of fruity aromas. There is an inverse correlation between the concentrations of esters and higher alcohols, as higher alcohols are precursor molecules for ester formation (Swiegers and Pretorius 2005). *S. cerevisiae* tends to produce more esters than non-*Saccharomyces* yeast (Cordente et al. 2007), though *W. anomalus*, *H. uvarum*, and *M. pulcherrima* are all known to produce esters during fermentation (Jolly, Varela and Pretorius 2014). While esters primarily contribute positive aromas, some are unpleasant; of these, ethyl acetate, the ester of ethanol and acetic acid, is the most common. All yeast can produce ethyl acetate, but high concentrations are often associated with non-*Saccharomyces* yeast, including *H. uvarum* and *C. zemplinina*/*C. stellata* (Ciani and Maccarelli 1997, Romano 2003, Belda et al. 2016).

Higher Alcohols

Higher alcohols are the largest group of secondary metabolites formed during fermentation, although only a portion are aromatic (Swiegers and Pretorius 2005). Higher alcohol concentrations of 300 mg/L or less are considered positive, as they are perceived to increase wine complexity, while at concentrations of 400 mg/L or higher they are considered negative, and are perceived as being too pungent (Swiegers and Pretorius 2005).

Carbonyl Compounds

There are two primary carbonyl compounds important to winemaking – acetaldehyde and diacetyl. Acetaldehyde is as the last major precursor formed by fermenting yeasts during ethanol creation. Ethanol can be oxidized to reform acetaldehyde, so acetaldehyde concentrations tend to increase after the end of fermentation as the wine is slowly oxidized over time (Swiegers et al. 2005, Swiegers and Pretorius 2005). The sensory threshold of acetaldehyde is approximately 100 mg/L, and though the apple or nutty aroma (Swiegers et al. 2005, Swiegers and Pretorius 2005) it contributes at sensory threshold can be beneficial to wine complexity, higher concentrations are detrimental to all but specific wines styles, such as Sherry and Madeira (Swiegers and Pretorius 2005). Diacetyl, which is perceived as buttery or toasty, can be produced or metabolized by yeast (Swiegers and Pretorius 2005, Jolly, Varela and Pretorius 2014), certain non-*Saccharomyces* yeast are known to increase diacetyl concentrations (Jolly, Varela and Pretorius 2014).

Volatile Acids

Acetic acid is the most common volatile acid produced during fermentation; concentrations of up to 0.7 g/L are acceptable, and may give a desirable freshness, while higher concentrations lead to objectionable, vinegar-like aromas (Swiegers and Pretorius 2005). *S. bayanus* and *S. uvarum* tend to produce less acetic acid than *S. cerevisiae*, but there is great variation between strains (Swiegers et al. 2005). The production of acetic acid by non-*Saccharomyces* yeast is variable, with *H. uvarum* producing a wide range (Romano 2003) and *T. delbrueckii* reportedly lowering acetic acid concentrations (Jara, Rojas and Romero 2015).

Volatile Phenols

Volatile phenols include several types of compounds, including vinylphenols and ethylphenols, which add complexity in small amounts but undesirable medicinal or barnyard aromas at higher concentrations. Volatile phenols can be produced as secondary metabolites or released from grape material via enzymatic reactions. Some *S. cerevisiae* strains can create small concentrations of volatile phenols, but *Brettanomyces* yeast are the primary source in wine (Swiegers and Pretorius 2005).

Sulfur Compounds

The broad category of sulfur compounds includes aroma compounds formed through a variety of pathways, not all of which have been completely elucidated. Generally, sulfur compounds created as secondary metabolites have a low detection threshold and are often associated with unpleasant aromas like rotten eggs, cabbage, and rubber (Swiegers et al. 2005, Swiegers and Pretorius 2005). Hydrogen sulfide, which smells like rotten eggs, is one of the most important of these. *S. cerevisiae* strains can be placed into three groups by hydrogen sulfide production - non-producing, producing, and condition-dependent, and also releases the compound through autolysis following cell death (Swiegers and Pretorius 2005). Non-*Saccharomyces* yeast can also contribute to hydrogen sulfide production – both directly, as a byproduct of fermentation, and indirectly, through autolysis release (Swiegers et al. 2005).

Enzymatically Released Aroma Compounds

The major aroma compounds released enzymatically from bound aroma precursors during fermentation are thiols, terpenes, and volatile phenols, though type and concentration vary by cultivar (Romano 2003, Swiegers et al. 2005, Swiegers and

Pretorius 2005, Molina et al. 2007, Zott et al. 2011). Aroma precursors can be bound to several different compounds, but glycoside- and cysteine-bound are of particular interest in winemaking (Padilla, Gil and Manzanares 2016). The release of aroma precursors is associated with an increase in the perceived varietal character of some wines, particularly Sauvignon Blanc and Riesling (Jackson and Lombard 1993, Swiegers and Pretorius 2005, Swiegers et al. 2007, Fleet 2008, Santamaría et al. 2008, Ciani et al. 2010, Belda et al. 2016, Padilla, Gil and Manzanares 2016, Wang et al. 2017). Thiols, sulfur compounds generally associated with positive aromas like tropical fruit and grapefruit (Swiegers et al. 2005, Padilla, Gil and Manzanares 2016), are of particular importance in Sauvignon Blanc, and can have a significant impact on the aroma profile of Riesling as well (Padilla, Gil and Manzanares 2016). Thiols are often cysteine-bound in grape material and can be hydrolyzed by lyases, particularly β-lyase (Swiegers et al. 2005, Swiegers and Pretorius 2005, Zott et al. 2011, Belda et al. 2016, Padilla, Gil and Manzanares 2016). While enzyme production is very strain specific, non-*Saccharomyces* yeast usually release more thiols than *S. cerevisiae* (Romano 2003, Swiegers et al. 2005, Swiegers and Pretorius 2005, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Padilla, Gil and Manzanares 2016), with *Pichia*, *Wickerhamomyces*, and *Hanseniaspora* species evincing the greatest potential for glycosidase expression (Belda et al. 2016). Notable β-lyase producers include strains of *C. zemplinina*, *P. kluyveri*, and *M. pulcherrima* (Padilla, Gil and Manzanares 2016). Among *Saccharomyces* yeast, *S. bayanus* appears to release more volatile thiols during fermentation than hybrids of *S. cerevisiae* and *S. bayanus*, which release more volatile thiols than *S. cerevisiae* (Swiegers et al. 2005, Swiegers and Pretorius 2005, Padilla, Gil and Manzanares 2016).

Terpenes, particularly terpenols and C_{13}-norisoprenoids, are the most important glycoside-bound compounds (Padilla, Gil and Manzanares 2016). Terpenes, which

generally correspond to floral aromas, and the C_{13}-norisoprenoid 1,1,6,-trimethyl-1,2,-dihydronapthalene (or TDN), which is perceived as a petrol aroma, are particularly important in Riesling sensory profiles (Jackson and Lombard 1993, Swiegers and Pretorius 2005, Fleet 2008, Padilla, Gil and Manzanares 2016). Glycoside-bound terpenes can be hydrolyzed by glycosidases, such as β-glucosidase (Swiegers and Pretorius 2005, Sabel et al. 2014, Belda et al. 2016, Padilla, Gil and Manzanares 2016). Terpenes can also be released through acid hydrolysis after fermentation (Hernández et al. 2003, Belda et al. 2016). Many non-*Saccharomyces* yeast can produce β-glucosidase, but production is strain specific (Swiegers and Pretorius 2005, Sabel et al. 2014). *Metschnikowia* species and *A. pullulans* may also produce a glycosidase capable of hydrolyzing bound terpenes (Belda et al. 2016). Microvinification experiments showed that the perception of typical Riesling character and fruitiness increased in fermentations with both non-*Saccharomyces* and *S. cerevisiae* (Benito et al. 2015), and both *T. delbrueckii* and certain strains of *S. cerevisiae* have also been shown to create terpenes as a secondary metabolite (Swiegers et al. 2005, Swiegers and Pretorius 2005). While the production of glucosidases is lower in *S. cerevisiae* than non-*Saccharomyces* species, *Saccharomyces* hybrids can have higher glycosidase activity than *S. cerevisiae* (Swiegers et al. 2005, Bisotto et al. 2015).

Volatile phenols can be released via enzymatic reaction as well as secondary metabolite formation. 4-methyl-4-sulfanylpentan-2-one (4MSP) and 3-sulfanylhexan-1-ol (3SH) are important volatile phenols found as cysteine-bound aroma precursors in grape material (Zott et al. 2011). Specific strains of *M. pulcherrima* and *T. delbrueckii* in mixed fermentations with *S. cerevisiae* produced higher concentrations of 3SH than control fermentations with only *S. cerevisiae* (Zott et al. 2011).

Effects of Yeast on Mouthfeel

In addition to aroma compounds, yeast can produce other compounds that affect wine quality via taste or mouthfeel during fermentation. Key among these is glycerol, which is produced by both *S. cerevisiae* (García et al. 2016), and non-*Saccharomyces* yeast (Swiegers and Pretorius 2005, Ciani et al. 2010, Cordero-Bueso et al. 2011, Jolly, Varela and Pretorius 2014). *C. zemplinina*/*C. stellata* is known for high glycerol production, which can contribute to a smooth or viscous mouthfeel, an increased perception of sweetness, and increased complexity (Jolly, Varela and Pretorius 2014). The sensory threshold for glycerol to contribute to perceived wine sweetness is 5.2 g/L, and 25.8 g/L to contribute to perceived viscosity (Swiegers et al. 2005). Other compounds, such as succinic acid, affect the perceived acidity of the final wine. In high concentrations succinic acid can cause a wine to taste bitter, decreasing quality (Jolly, Varela and Pretorius 2014). Succinic acid can be produced, in increasing concentrations, by *S. cerevisiae,* other *Saccharomyces* species (Swiegers et al. 2005), and non-*Saccharomyces* yeast (Jolly, Varela and Pretorius 2014).

Effect of Strain Variations on Aroma Compound Production

While the differences in aroma compound production between yeast species and strains can be generalized, there is significant variation among strains within a species (Romano 2003, Ganga and Martínez 2004). Research has shown that different *S. cerevisiae* strains have different fermentation kinetics; secondary metabolite compositions, including the concentrations of acetaldehyde, higher alcohols, and hydrogen sulfide; and enzyme production, including β-gluosideases and of β-lyase (Hernández et al. 2003, Romano 2003, Swiegers et al. 2005, Swiegers and Pretorius 2005, Swiegers et al. 2007, Carrau et al. 2008, Jolly, Varela and Pretorius 2014). *S. cerevisiae* strains that are genetically similar tend to produce a similar range

of secondary metabolites, but the opposite is not true; genetically different strains do not always produce different secondary metabolites (Ubeda and Briones 2000). Similarly, fermentation kinetics do not always correlate to aroma compound formation; for example, two strains may have similar fermentation kinetics under the same conditions but produce different aroma compounds (Carrau et al. 2008). One factor complicating the determination of genetic and phenotypic relationships is epigenetics, the stable, inheritable phenotypic characteristics that are not linked to genetic variations (Berger et al. 2009). *S. cerevisiae* strains that appear genetically identical but phenotypically different (Gibson and Liti 2015), may be explained by changes in gene expression caused by epigenetic factors (Garcia and Jarosz 2014). This is, however, a new area of study, and the extent to which epigenetic changes contribute to the phenotypic diversity of *S. cerevisiae* strains remains unknown (Halfmann et al. 2012). One such epigenetic factor that has been observed in *S. cerevisiae* are prion interactions (Halfmann et al. 2012, Garcia and Jarosz 2014, Jarosz et al. 2014). Prions are defined as "proteins that convert between structurally and functionally distinct states, at least one of which is self-perpetuating" (Garcia and Jarosz 2014). *S. cerevisiae* strains with prion-mediated epigenetic changes have vastly different phenotypes for fermentation efficiency (Halfmann et al. 2012, Garcia and Jarosz 2014, Jarosz et al. 2014).

Phenotypic variations can also be caused by differential gene expression due to environmental factors. In other words, if the same *S. cerevisiae* strain is exposed to different environmental conditions, the gene expression can differ. For example, variations in temperature and nutrient availability can lead to phenotypic changes in the type and concentration of aroma compounds created by a single strain (Chambers and Pretorius 2010, Barbosa et al. 2014, García-Ríos et al. 2014, Treu et al. 2014). Variable gene expression, prompted by epigenetic and environmental factors,

may help explain the greater than expected amount of phenotypic diversity seen in *S. cerevisiae* strains (Mendes et al. 2013, Treu et al. 2014). It is difficult to tease out the cause of any given phenotypic variation observed in wine, however, as fermenting wine is a complex matrix where fermentation conditions and microbe interactions are constantly changing (Ubeda and Briones 2000, Chambers and Pretorius 2010, Hyma et al. 2011, Charron et al. 2014).

Strains within non-*Saccharomyces* species have also shown great variation in fermentation kinetics and aroma compound production. Strains within non-*Saccharomyces* yeast species have been less extensively researched, so caution should be applied in extrapolating the results of aroma compound production in one strain to all strains within that species. For example, while the production of β-glucosidase and β-lyase is widespread across non-*Saccharomyces* yeast species, it appears to be extraordinarily strain specific. The same species can have some strains with high activity and others with little to no activity (Romano 2003, Swiegers et al. 2005, Swiegers and Pretorius 2005, Sabel et al. 2014, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Padilla, Gil and Manzanares 2016). This applies to direct production of metabolites as well. For example, hydrogen sulfide production is less widespread across non-*Saccharomyces* yeast, but production still is still quite variable among strains within a species (Belda et al. 2016).

Effect of Yeast Interactions on Aroma Compound Production

Interspecies yeast interactions during fermentation are complex, appear to be strain specific (Ciani et al. 2010), and can have both physiological and metabolic effects (Ciani et al. 2010). Physiologically, the interaction between non-*Saccharomyces* yeast species, or between *S. cerevisiae* and a non-*Saccharomyces* yeast species, may lead to growth inhibition in one or both of the interacting species (Fleet 2003, Romano

2003, Oro, Ciani and Comitini 2014, Satora et al. 2014). While it is typical for *S. cerevisiae* to outcompete, or inhibit the growth of, non-*Saccharomyces* yeast through cell-to-cell contact or the increasing level of ethanol produced (Jara, Rojas and Romero 2015), there have been instances of non-*Saccharomyces* yeast inhibiting the growth of *S. cerevisiae* (Fleet 2003). These effects even extend to dead yeast cells, which can release nutrients to living yeast via autolysis (Jolly, Varela and Pretorius 2014).

Metabolically, species and strain interactions can increase, decrease, or change the types and concentrations of aroma compounds created by each yeast. However, these changes are complex - the differences in metabolite production of mixed yeast fermentations cannot be effectively recreated by blending single yeast fermentations together in the same proportions as the relative abundance of the yeast species in the mixed fermentation (Ciani et al. 2010). For example, a specific non-*Saccharomyces* species may produce an undesirable aroma compound when fermenting in isolation, but when other yeast species or strains are present it may not produce this compound to the same degree or at all (Ciani and Maccarelli 1997, Ciani et al. 2010, Padilla, Gil and Manzanares 2016). The interaction between *S. cerevisiae* and non-*Saccharomyces* yeast can also influence the production of ethanol, glycerol, acids, and aroma compounds by both yeast types (Ciani and Maccarelli 1997, Swiegers et al. 2005, Canonico, Comitini and Ciani 2015, Wang et al. 2017). The interactions of multiple yeast species and strains during fermentation are not well understood, however, so the wine aroma profile of commercial fermentations may not reflect the results observed in laboratory experiments (Ciani, Beco and Comitini 2006, Bokulich et al. 2016, Padilla, Gil and Manzanares 2016).

Effect of Fermentation Conditions on Aroma Compound Production

Fermentation conditions can also influence the physiological and metabolic outcomes of, and the interactions between, yeast species and strains. Grape competition, nutrient availability, must pH, ethanol concentration, and fermentation temperature can all affect yeast (Ciani and Maccarelli 1997, Romano 2003, Swiegers et al. 2005, Molina et al. 2007, Carrau et al. 2008, Chambers and Pretorius 2010, Canonico, Comitini and Ciani 2015, Belda et al. 2016, García et al. 2016).

Precursors in grapes affect the types and concentrations of aroma compounds in the final wine (Ubeda and Briones 2000, Swiegers and Pretorius 2005, Canonico, Comitini and Ciani 2015), and typical sugar concentrations increase the production of glycerol and acetic acid by *S. cerevisiae*, favor the growth of non-*Saccharomyces* yeast (like *H. uvarum*, *C. zemplinina*/*C. stellata*, and *T. delbrueckii*) over *S. cerevisiae*, and decreases the efficiency of glycosidase activity (Swiegers and Pretorius 2005, García et al. 2016). Low nitrogen availability is highly correlated to an increase in hydrogen sulfide production, regardless of the yeast species or strain, though sensitivity varies (Ciani and Maccarelli 1997, Swiegers et al. 2005, Swiegers and Pretorius 2005, Belda et al. 2016). Nutrient availability will also impact yeast fermentation kinetics and the likelihood of successful completion (García et al. 2016). Ethanol tolerance is often the trigger for yeast death as ethanol concentration increases during fermentation. This favors *S. cerevisiae* over non-*Saccharomyces* yeast, though some non-*Saccharomyces* species and strains have atypical ethanol tolerances, allowing them to survive later into the fermentation (Goddard 2008, García et al. 2016).

Fermentation temperature can be argued to have the greatest effect on yeast kinetics, metabolism, and interactions. Fermentation temperature ranges are based on the optimal temperature for *S. cerevisiae* strain growth in must, but also take aroma formation into consideration. *S. cerevisiae* starts to outcompete non-

Saccharomyces yeast at 20°C (Goddard 2008) , and other *Saccharomyces* species around 25-30°C (Beltran et al. 2008, Boynton and Greig 2014). The higher fermentation temperature range also corresponds to the quickest fermentation rates and highest biomass accumulations. As fermentation temperature decreases, yeast require more cell membrane lipids to maintain ethanol tolerance and growth (Beltran et al. 2008). This leads to slower fermentation rates, but also a higher long-term yeast viability especially at temperatures below 15°C (Ciani, Beco and Comitini 2006, Molina et al. 2007, Beltran et al. 2008). Low fermentation temperatures also favor non-*Saccharomyces* growth (Pallmann et al. 2001, Goddard 2008), increased ester formation (Leeuwen and Seguin 2006, Molina et al. 2007, Beltran et al. 2008), reduced higher alcohol and acetic acid formation (Beltran et al. 2008), and increased retention of terpenes (Beltran et al. 2008). Higher fermentation temperatures have been linked to greater concentrations of acetaldehyde, volatile thiols (Swiegers and Pretorius 2005), and increased glucosidase activity (Hernández et al. 2003). Sequential dominance of *S. cerevisiae* strains during fermentation may also be related to preferential growth at different temperatures (Gonçalves et al. 2011). The differences in aroma compounds produced appears to be due to differential gene expression at different fermentation temperatures (Beltran et al. 2008, Rodríguez-Sifuentes et al. 2014, Treu et al. 2014). Overall, lower fermentation temperatures result in more prominent fruity and fresh aromas, while higher temperatures produce more floral aromas and selected fruit aromas, such as banana and pineapple (Molina et al. 2007).

Spontaneous Fermentation

Native Versus Commercial S. cerevisiae

Wine fermentations can be 'spontaneous' (Martiniuk et al. 2016), effected by the yeast naturally present in the environment, or inoculated with commercial yeast purposefully added to the grape must. The yeast naturally present in the environment have been called 'native,' 'wild,' 'natural', and 'feral,' among other things (Pretorius, Curtin and Chambers 2015). Inoculated fermentations typically involve the addition of commercial yeast products containing one or several strains, usually of *S. cerevisiae*, that have been isolated, cultured, and produced in bulk. The use of commercial yeasts has spread widely since their introduction in 1963 (Reed and Chen 1978, Pretorius, Curtin and Chambers 2015), ostensibly to ensure consistent fermentation with predictable kinetics and secondary metabolite production (Chambers and Pretorius 2010, Barbosa et al. 2014). Currently there are over 200 different commercial yeast products on the market (Mendes et al. 2013, Barbosa et al. 2014, García-Ríos et al. 2014), the majority consisting of a single *S. cerevisiae* strain (Bradbury et al. 2005, Chambers and Pretorius 2010, Barbosa et al. 2014), though single non-*S. cerevisiae* strains (Bradbury et al. 2005, Padilla, Gil and Manzanares 2016), combinations of *S. cerevisiae* and a non-*S. cerevisiae* strains (Padilla, Gil and Manzanares 2016), and interspecific hybrids of *Saccharomyces* species are also available (Bradbury et al. 2005, Chambers et al. 2015).

Most commercial yeast strains were isolated as native yeasts from successful wine fermentations, although some were bred in laboratories for specific phenotypic traits. Genetically screening yeast strains for positive aroma compound production is challenging and time consuming, as the genetic basis of aroma compound production is known in only a few instances, and multiple genes are usually involved (Chambers et al. 2015, Pretorius, Curtin and Chambers 2015). Native yeast strains are selected

for commercial development on the basis of specific phenotypic criteria, including temperature, ethanol, or sulfur dioxide tolerance, ability to complete alcoholic fermentation, and desirable aroma formation (Chambers and Pretorius 2010, Barbosa et al. 2014, Treu et al. 2014). The development of yeast strains based on any one phenotypic characteristic can have the unintended consequence of selecting for other, less desirable characteristics. For example, potassium bisulfite is commonly used as an antioxidant and antimicrobial in must, and commercial yeast products are selected for tolerance to sulfur dioxide (Mendes et al. 2013, Barbosa et al. 2014, Treu et al. 2014). The unintended consequence of this selection is that commercial yeast products tend to have increased growth in the presence of potassium bisulfite (Mendes et al. 2013).

Independent analysis of multiple commercial yeast products produced by different companies indicate that several genetically indistinguishable products are marketed under different names (Fernández-Espinar et al. 2001, Bradbury et al. 2005, Boynton and Greig 2014). For example, Lalvin EC1118 and Red Star Premier Cuvée, both marketed as Prise de Mousse yeast strains, are genetically indistinguishable (Bradbury et al. 2005). Surprisingly, strains that appear to be genetically indistinguishable often are marketed as different yeasts by the same producer, (Fernández-Espinar et al. 2001), though is possible that some are actually distinct strains that cannot be distinguished by the analysis method used. It is also possible that prion-mediated inheritable epigenetic factors could cause genetically identical *S. cerevisiae* strains to exhibit different phenotypic expressions, though the extent of differential gene expression due to epigenetic changes is currently unknown (Halfmann et al. 2012, Garcia and Jarosz 2014, Jarosz et al. 2014, Gibson and Liti 2015). Other commercial products appear to be very closely related strains; for example, Bradbury et al. suggest that Lalvin DV10, a single *S. cerevisiae* strain, is a

mutant derived from Lalvin EC1118, another single *S. cerevisiae* strain, given their genetic similarity (Bradbury et al. 2005).

Different commercial yeast products can produce different secondary metabolites, release different volatile compounds from grapes, or produce different concentrations of these compounds (Chambers and Pretorius 2010). However, the development of both desirable and undesirable aromas depend on a combination of the yeast strain and the fermentation conditions, as previous discussed (Barbosa et al. 2014). When a fermentation is inoculated it is assumed that the commercial yeast product will outcompete the native yeast species and strains, but this is not always the case (Valero et al. 2007, Barrajón et al. 2009). The successful inoculation of the commercial yeast is dependent on a number of factors, most importantly the relative temperatures of the inoculum and the must (Barrajón et al. 2009). Even if an inoculation is successful the commercial yeast may not dominate the fermentation (Valero et al. 2007). Multiple *S. cerevisiae* strains are frequently observed in fermentations, even those that have been inoculated (Mercado et al. 2010). It is possible to see fermentations with a single dominant *S. cerevisiae* strain, several co-dominant strains, or a plurality of strains (Valero et al. 2007). Therefore, while inoculation with a commercial yeast product can increase the likelihood of beneficial aroma development, it cannot guarantee it.

Spontaneous Versus Inoculated Fermentations

Non-*Saccharomyces* yeast are commonly present on grapes, and typically dominate the early stages of both spontaneous and inoculated fermentations (Barata et al. 2008, Santamaría et al. 2008, Barata, Malfeito-Ferreira and Loureiro 2012, Bezerra-Bussoli et al. 2013). In inoculated fermentations, the commercial yeast strain typically dominates the middle and late stages of fermentation. Spontaneous fermentations

are also eventually dominated by *S. cerevisiae,* but usually involve a more complex succession of yeast species and strains, with varying lifespans (Schuller et al. 2005, Díaz et al. 2013, Jolly, Varela and Pretorius 2014). Low fermenting apiculate non-*Saccharomyces* yeast often appear first, followed by varying abundances and types of other non-*Saccharomyces* yeast that may or may not coexist with assorted *S. cerevisiae* strains (Clemente-Jimenez et al. 2004, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Padilla, Gil and Manzanares 2016). Factors such as ethanol, sugar, and SO_2 tolerance, nutrient availability, fermentation temperature, yeast interactions, and the initial amount of yeasts present on grapes affect the ability of non-*Saccharomyces* yeast to persist in fermentations (Bisson and Butzke 2000, Torija et al. 2001, Tofalo et al. 2009, Cavazza, Poznanski and Guzzon 2010, Cordero-Bueso et al. 2011, Jolly, Varela and Pretorius 2014, Padilla, Gil and Manzanares 2016).

The persistence of non-*Saccharomyces* species in fermentation appears to be partially dependent on how quickly *S. cerevisiae* biomass forms; the slower *S. cerevisiae* biomass grows, the longer non-*Saccharomyces* species persist (Ciani, Beco and Comitini 2006). Non-*Saccharomyces* yeast may or may not be completely displaced by *S. cerevisiae*, even following inoculation (Ciani and Maccarelli 1997, Cocolin, Bisson and Mills 2000, Fleet 2008, Li et al. 2011, Bezerra-Bussoli et al. 2013, Díaz et al. 2013, Liu et al. 2016, Patrignani et al. 2017). In some cases non-*Saccharomyces* yeast or native *S. cerevisiae* may even overtake the *S. cerevisiae* inoculate (Urso et al. 2008).

Factors that tend to favor non-*Saccharomyces* over *S. cerevisiae* include low fermentation temperatures, high initial sugar concentration, and minimal additions of SO_2 (Bisson and Butzke 2000, Ciani et al. 2010, Bokulich et al. 2012). Fermentation temperature appears to be a key fermentation condition for the behavior of non-

Saccharomyces yeast. The ethanol tolerances of many non-*Saccharomyces* yeast appear linked to fermentation temperature, with lower temperatures increasing the tolerance (Di Maro, Ercolini and Coppola 2007, Ciani et al. 2010). There are significant strain variations that can affect how fermentation conditions impact non-*Saccharomyces* growth. For example, it is assumed that a significant reduction in non-*Saccharomyces* yeast population can be achieved with sulfur dioxide addition rates of 50ppm or higher, as most non-*Saccharomyces* yeast are sulfur dioxide sensitive (Bisson and Butzke 2000). However, the growth of some strains of *C. zemplinina*/*C. stellata* seem to be favored at these higher SO_2 concentrations (Torija et al. 2001).

Successfully inoculated fermentations have some distinct advantages over spontaneous fermentations. The nutritional requirements of the dominant yeast strain are known, decreasing the risk of a stuck or sluggish fermentation and the development of unpleasant aroma compounds (Bisson 1999). Displacing native non-*Saccharomyces* yeast can limit the risk of forming unpleasant aromas (Swiegers and Pretorius 2005, Benito et al. 2015, Belda et al. 2016). Commercial yeast products also complete fermentation in a relatively short period of time, typically within two weeks (Bisson 1999).

Spontaneous fermentations also have perceived benefits, as they are thought to improve mouthfeel and increase aromatic complexity (Santamaría et al. 2008, Jolly, Varela and Pretorius 2014, Benito et al. 2015, Chambers et al. 2015, Jara, Rojas and Romero 2015, Belda et al. 2016, Belda et al. 2016, Martiniuk et al. 2016). Spontaneous fermentations often include multiple *S. cerevisiae* strains, with sequential strain replacement over the course of the fermentation, and may or may not involve a dominant strain at any given point (Schuller et al. 2005, Schuller et al. 2012, Tristezza et al. 2014). The presence of higher *S. cerevisiae* strain diversity has

been linked to an increased chance of successful fermentation (Schuller et al. 2012). In contrast, it has been hypothesized that native strains have adapted to local conditions and will produce more successful fermentations when used with local grapes (Hernández et al. 2003, García et al. 2016, Drumonde-Neves et al. 2017). There is some support for the latter idea; a study of *S. cerevisiae* strain inoculation implantation rates showed that native strains had a higher rate of successful implantation than commercial strains isolated from other regions (Esteve-Zarzoso et al. 2000). In consumer sensory tests, the wines inoculated with native strains were rated similarly or better than those inoculated with commercial strains (Esteve-Zarzoso et al. 2000). Other studies have also shown a connection between the presence of native *S. cerevisiae* strains and positive organoleptic characteristics (Martínez et al. 2007, Valero et al. 2007, Orlic et al. 2010).

Spontaneous fermentations also contain a higher diversity of non-*Saccharomyces* yeast species than inoculated fermentations, which has been correlated with an increase in perceived wine complexity (Santamaría et al. 2008, Jolly, Varela and Pretorius 2014, Benito et al. 2015, Chambers et al. 2015, Jara, Rojas and Romero 2015, Belda et al. 2016, Belda et al. 2016, Martiniuk et al. 2016). Non-*Saccharomyces* yeast have also been linked to higher enzyme production, which leads to higher concentrations of terpenes and thiols released from grape material (Ciani et al. 2010, Belda et al. 2016, Padilla, Gil and Manzanares 2016), and a greater perception of varietal aromas (Swiegers et al. 2007, Belda et al. 2016); spontaneous fermentations may lead to greater varietal typicity, or trueness-to-type. Greater diversity of non-Saccharomyces species in a fermentations increase the chance of enzymatic activity, which may increase aroma precursor release (Belda et al. 2016). Having multiple yeast species in a fermentation may also result in more efficient consumption of both glucose and fructose, due to the variable affinity and ability of the different yeast to

use these sugar sources (Ciani and Comitini 2011). There are also winemakers who value 'natural' or 'non-interventionist' wine as more representative of the expression of the wine's place of origin (Jolly, Varela and Pretorius 2014, Pretorius, Curtin and Chambers 2015).

There are also risks associated with spontaneous fermentation, which are less likely to ferment to dryness (Bisson 1999, Bisson and Butzke 2000, Chambers et al. 2015). Spontaneous fermentations are typically longer than inoculated fermentations (Drumonde-Neves et al. 2017), and annual variation in native yeast populations can lead to unpredictability (Martínez et al. 2007). Spontaneous fermentations are also associated with a higher risk of deleterious aroma production, which may be due to the higher diversity of non-*Saccharomyces* yeast species (Benito et al. 2015, Belda et al. 2016). If large numbers of non-*Saccharomyces* yeast die, the autolysis of their cells may contribute excessive hydrogen sulfide (Swiegers and Pretorius 2005). Choosing spontaneous over inoculated fermentation is the balance between the potential rewards of increased complexity, varietal aroma characteristics, and regional character and the risks of increased detrimental aroma compounds, overall unpredictability, and problematic fermentation kinetics.

There are four typical progressions of problematic fermentations: 1. long lag time followed by a normal fermentation, 2. normal lag time followed by a slow fermentation, 3. long lag time followed by a slow fermentation, and 4. normal lag time followed by a normal fermentation with an abrupt arrest near the end of sugar consumption (Bisson and Butzke 2000).

Not all problematic fermentations are sluggish or become stuck; some simply have a slow progression of successive yeast species or strains over time (Bisson and Butzke 2000). Long lag times are typical of spontaneous fermentations, as they typically have low levels of *S. cerevisiae* in the initial must due to the rarity of this species on grapes

(Bisson and Butzke 2000, Drumonde-Neves et al. 2017). Long lag times are not necessarily the sign of a poor ferment, but if they result in inadequate biomass accumulation, the fermentation can remain problematic (Bisson and Butzke 2000).

Similarly, slow fermentation rates are not necessarily detrimental, as longer fermentations tend to produce higher levels of esters (Bisson and Butzke 2000), but also increased risk of undesirable aroma compound formation (Benito et al. 2015, Belda et al. 2016). Long fermentations can indicate nutrient deficiency, non-optimal fermentation conditions, mild temperature shock, or competition with bacteria or non-*Saccharomyces* yeasts (Bisson and Butzke 2000, Bokulich et al. 2016). Nitrogen deficiency is the most common source of nutrient driven stuck or sluggish fermentations, but phosphate, thiamine, or other micronutrient deficiencies can also be a cause, as can a lack of oxygen in the early stages of yeast biomass production (Bisson 1999). In addition to nutrient deficiencies, abrupt arrest of fermentation can also be the result of a toxic metabolite (i.e. ethanol), or non-*Saccharomyces* killer toxin accumulation (Bisson 1999, Bisson and Butzke 2000, Rodríguez-Sifuentes et al. 2014).

Inhibition of *S. cerevisiae* growth by non-*Saccharomyces* yeast is a major concern in spontaneous fermentations, as high concentrations of non-*Saccharomyces* yeast can increase the risk of a stuck fermentation. The main risks to *S. cerevisiae* from non-*Saccharomyces* yeast are depletion of essential nutrients, competition, and killer toxins (Bisson 1999). These risks are specific to species and strain, with some strains increasing the chance of a stuck fermentation and others helping to mitigate the risk. An example of these issues can be seen in the complicated interaction of *H. uvarum* and *S. cerevisiae*. *H. uvarum* is generally more sensitive to ethanol, but also more efficient at metabolizing thiamine than *S. cerevisiae,* which requires thiamine to grow (Bisson 1999). In most fermentations, *S. cerevisiae* will inhibit the growth of *H.*

uvarum with ethanol production before *H. uvarum* can inhibit the growth of *S. cerevisiae* by metabolizing thiamine. However, low fermentation temperatures can increase the ethanol tolerance of *H. uvarum* (Ciani et al. 2010) which produce high enough levels of acetic acid to decrease *S. cerevisiae* thiamine uptake, though acetic acid production is strain specific (Bisson 1999, Jolly, Varela and Pretorius 2014). Additionally, the initial concentration of thiamine in must is variable, and sulfur dioxide can react with thiamine to make it unavailable for yeast to metabolize. Lastly, certain *H. uvarum* strains produce a killer toxin that certain *S. cerevisiae* strains are sensitive to, which can inhibit the growth of *S. cerevisiae* (Bisson 1999). In short, *H. uvarum* can inhibit the growth *S. cerevisiae,* but only under the right circumstances. Conversely, non-*Saccharomyces* yeast can also help alleviate stuck fermentations. Strains of *Z. bailii* and *T. delbrueckii* are tolerant to high ethanol, low nutrient conditions and can efficiently metabolize fructose, the fermentable sugar most likely to remain if a fermentation has halted prior to dryness (Rodríguez-Sifuentes et al. 2014); this may help prevent spontaneous fermentations from becoming stuck. Even commercial yeast strains can result in sluggish or stuck fermentations, especially with high initial sugar concentrations and low osmotolerance yeast products (Fernández-González, Úbeda and Briones 2015), inoculation does not completely mitigate the risk of a problematic fermentation.

Spontaneous Fermentation Debate

Spontaneous fermentations are defined by the lack of intentional inoculation, but there are vigorous debates about whether yeast origin, particularly for *S. cerevisiae* strains, is integral to the definition of spontaneous fermentation. There are two points of contention: (1) the physical origin of yeast, and (2) the similarity of yeast to

commercial yeast products (Ciani et al. 2004, Santamaría et al. 2005, Valero et al. 2007, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Bokulich et al. 2013). The debate over source centers on whether yeast is primarily contributed by the grapes, the winery environment, or a combination of the two. Few would argue that all yeast species are contributed by the vineyard environment alone, as grapes encounter many potential sources of yeast during processing (Jolly, Varela and Pretorius 2014, Drumonde-Neves et al. 2017). The source of *S. cerevisiae*, rather than other yeast species, is key, as some contend that a fermentation is only spontaneous if *S. cerevisiae* present in the fermentation comes from the vineyard environment (Ciani et al. 2004). Others argue that only intentional use of commercial yeast qualifies as inoculation, and spontaneous fermentations can include yeast contributed by winery surfaces (Martiniuk et al. 2016). These points of view can be modified by the second part of the spontaneous fermentation debate - whether the *S. cerevisiae* strains present in the fermentation are derived from commercial yeast products. Some argue that a true spontaneous fermentation will only include native *S. cerevisiae* strains, and that the inclusion of any strains genetically matching or similar to commercial yeast product makes it an inoculated fermentation regardless of the physical source of the yeast. Other argue that as long as *S. cerevisiae* strains matching or similar to commercial yeast products do not dominate the fermentation, then a case can be made for the 'spontaneous' label.

The question of an uninoculated fermentation being considered spontaneous is often framed in terms of the extent to which people believe that the source of *S. cerevisiae* is grape derived or winery derived (Ciani et al. 2004, Santamaría et al. 2005, Valero et al. 2007, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Bokulich et al. 2013).

A more nuanced and less debated point is the source of non-*Saccharomyces* yeast. They are generally considered to be grape-derived, although winery surfaces may also harbor and contribute non-*Saccharomyces* yeast (Bokulich et al. 2013, Drumonde-Neves et al. 2017). Since non-*Saccharomyces* yeast are rarely the driving force in fermentations, they are often considered less important than *S. cerevisiae* in the fermentation spontaneity debate. In any case, the increased presence and influence of non-*Saccharomyces* yeast is considered to be a characteristic of uninoculated fermentations (Santamaría et al. 2008).

Vineyard Contributions to Spontaneous Fermentations

The first step in assessing vineyard contributions to uninoculated fermentations is determining the presence and relative abundance of yeasts on grapes. Factors affecting yeast presence can be categorized as grape, vineyard, and vintage variation. Ripe, undamaged grapes have total yeast counts around 10^6 to 10^7 CFU/mL (Schuller et al. 2005). *Vitis vinifera* and *Vitis rotundifolia* grapes show different yeast compositions (Diezmann and Dietrich 2009), and different grape varieties within *Vitis vinifera* also show different yeast compositions to a lesser degree (Barata, Malfeito-Ferreira and Loureiro 2012, Bokulich et al. 2014). Regardless of grape type, a majority of the yeast present are non-*Saccharomyces* yeast species (Cordero-Bueso et al. 2011, Setati et al. 2013). Intact grapes favor the growth of low fermenting apiculate and primarily aerobic non-*Saccharomyces* yeast (Setati et al. 2013).The abundance and diversity of yeast species and strains increases on damaged grapes, in comparison with intact grapes (Combina et al. 2005, Valero et al. 2007, Francesca et al. 2010, Setati et al. 2013). *S. cerevisiae* is rarely observed on intact grapes (on ~1% of grapes) (Clemente-Jimenez et al. 2004, Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013, Taylor et al. 2014, Drumonde-Neves et al. 2017), but are

observed on up to ~25% of damaged grapes (Schuller et al. 2005, Valero et al. 2007, Cubillos et al. 2009, Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013).

Aseptically collected intact grapes, processed using sterile laboratory equipment, have resulted in successful fermentations, suggesting that undamaged grapes can contain enough *S. cerevisiae* for initiation (Clemente-Jimenez et al. 2004, Schuller et al. 2005, Valero et al. 2007, Martiniuk et al. 2016). In a typical grape harvest, *S. cerevisiae* is observed in maximum concentrations of 10-100 CFU/g on berries (Setati et al. 2013).

Viticultural practices, such as choice of harvest time, can also impact yeast presence (Chambers and Pretorius 2010). Grapes collected prior to the commercial harvest date of the vineyard were less likely to result in successful fermentations than those collected post-harvest. This appears to be due to an increase in yeast population, specifically in observed concentrations of *S. cerevisiae*, thought to be caused as skin damage, juice release, and insect exposure increased with maturation time (Schuller et al. 2005, Valero et al. 2007). Successful fermentations of grapes collected after the commercial harvest date had a higher number and different types of *S. cerevisiae* strains than those collected prior to harvest (Schuller et al. 2005, Valero et al. 2007). This suggests that a succession of *S. cerevisiae* strains are present on grapes over the course of ripening. Ripeness also affects the initial concentration of sugar, which has repercussions on yeast kinetics and succession patterns in the resulting fermentations (Chambers and Pretorius 2010). Fungicides and pesticides also appear to affect the types and abundances of yeasts that grow on grapes. Heavier rainfall is correlated with observations of higher abundances of non-*Saccharomyces* yeast (Valero et al. 2007), and less *S. cerevisiae* strain diversity (Schuller et al. 2005), both thought to be caused by the increased use of fungicide sprays. However, there are conflicting reports on the effects of fungicide and pesticide use, as interactions are

complex and not completely understood (Cubillos et al. 2009). Weather conditions generally affect yeast species and strain, but different environmental conditions favor different yeast, making it hard to establish an overall correlation (Bokulich et al. 2014).

Great variation in observed *S. cerevisiae* strains across vintages, both within a single vineyard and across larger areas (Pramateftaki, Lanaridis and Typas 2000, Torija et al. 2001, Valero et al. 2007, Barata, Malfeito-Ferreira and Loureiro 2012, Díaz et al. 2013, Setati et al. 2013, Bokulich et al. 2014, Alessandria et al. 2015, Pinto et al. 2015, Sun et al. 2015, Belda et al. 2016, Drumonde-Neves et al. 2017). While the same *S. cerevisiae* strain has been observed across vintages in the same vineyard, or in fermentations at the same winery, such repetition is uncommon (Vezinhet et al. 1992, Pramateftaki, Lanaridis and Typas 2000, Schuller et al. 2005, Martiniuk et al. 2016). Differences in weather and viticultural practices between vintages may contribute to the observed variations in *S. cerevisiae* strains in a single vineyard across multiple vintages, including the fact that few strains were observed in multiple years (Schuller et al. 2005, Bokulich et al. 2014, Martiniuk et al. 2016).

The vineyards themselves also appear to be a source of variation in *S. cerevisiae* strain observance. Experiments using aseptically collected grapes showed that different lots of grapes from the same vineyard, in the same vintage, yielded different combinations of *S. cerevisiae* strains, patterns of strain dominance, and strain succession during the resulting fermentations (Schuller et al. 2005, Setati et al. 2013). These differing patterns were attributed to different starting abundance and strain type present on the grapes, meaning that *S. cerevisiae* strains are inconsistently spread over grapes in a single vineyard. Within a single vineyard, the size and age of the vineyard appears to be a factor that influences the degree of *S. cerevisiae* strain diversity observed (Schuller and Casal 2007, Valero et al. 2007,

Cubillos et al. 2009). There is also evidence of differing strain diversity and abundance between fermentations of grapes from different vineyards, even those close enough geographically to control for weather variation (Valero et al. 2007, Martiniuk et al. 2016).

An analysis of *S. cerevisiae* strains obtained in aseptically collected grape fermentations showed that the extent of the phylogenetic differences between strains observed in a single vineyard over multiple vintages is similar to those observed between multiple vineyards in a single vintage (Schuller and Casal 2007). Thus, both vineyard and vintage contribute to variations in *S. cerevisiae* strain diversity. There does not appear to be a direct correlation between successful fermentation (as a proxy for abundance of *S. cerevisiae*) and either vineyard or vintage (Valero et al. 2007). This points to a natural amount of variation in types and abundances of yeast present in any uninoculated fermentation, even when controlling for variations in grape type, vineyard, and vintage (Clemente-Jimenez et al. 2004, Schuller et al. 2005, Valero et al. 2007). Although there have been cases where the same *S. cerevisiae* strains were observed in multiple vintages and/or vineyards, these instances are uncommon (Vezinhet et al. 1992, Pramateftaki, Lanaridis and Typas 2000, Schuller et al. 2005, Martiniuk et al. 2016). Overall, the presence of *Saccharomyces* species on grapes and in aseptically created musts supports the idea that the grape can be a source, but are not necessarily the only source, of *Saccharomyces* in uninoculated fermentations (Bokulich et al. 2014, Taylor et al. 2014, Pinto et al. 2015).

Winery Contributions to Spontaneous Fermentations

Because as few as 100-1000 cells/mL of *S. cerevisiae* can produce a successful fermentation (Bisson and Butzke 2000), even a small concentration of residual *S.*

cerevisiae in a winery can initiate wine production. If a winery is performing both inoculated and uninoculated fermentations, the likelihood of transferring commercial yeast into uninoculated fermentations must also be considered. Research has consistently shown yeast colonizing winery equipment and surfaces (Ciani et al. 2004, Santamaría et al. 2005, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Bokulich et al. 2013), but specifics vary. In some studies, *S. cerevisiae* was more likely than non-*Saccharomyces* yeast to be present on winery surfaces (Santamaría et al. 2008, Jolly, Varela and Pretorius 2014), while others found the opposite (Beltran et al. 2002, Bokulich et al. 2013). Similarly, yeast persistence on winery surfaces over time varies, with some work showing the same yeast species or strains consistently (Ciani et al. 2004), while others found differing populations (Beltran et al. 2002, Bokulich et al. 2013), and evidence that strains can fluctuate over time (Mercado et al. 2007, Bokulich et al. 2013). The extent to which *S. cerevisiae* strains observed on winery surfaces contribute to an uninoculated fermentation also appears to fluctuate; Mercado et al (2007) found that 30-60% of the total number of *S. cerevisiae* strains observed in uninoculated fermentations matched those seen on winery surfaces, and the influence of winery-observed strains did not correlate with individual fermentation lots or vintage.

Modern winery protocols typically call for regular cleaning and/or sanitizing of winery equipment and surfaces that directly contact grapes, usually both before and after contact, to minimize the transfer of undesirable microbes including yeast (Jolly, Varela and Pretorius 2014). It is possible that different cleaning and sanitation practices in the wineries studied accounts for some of the conflicting data (Santamaría et al. 2008, Bokulich et al. 2013, Jolly, Varela and Pretorius 2014). In wineries that perform both inoculated and spontaneous fermentations, commercial *S. cerevisiae* strains used for inoculated lots are often identified in spontaneous

fermentations produced in the same facility and vintage (Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Martiniuk et al. 2016). Commercial strains have also been found to linger, showing up in spontaneous fermentations years after they were used, providing evidence for the colonization of winery surfaces (Martiniuk et al. 2016). Not all commercial *S. cerevisiae* will be observed on winery surfaces (Santamaría et al. 2008), and even when identified, they may not be present in every spontaneous fermentation, nor become dominant over other *S. cerevisiae* strains present in the fermentation (Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Martiniuk et al. 2016). *S. cerevisiae* strains genetically similar to, but not matching, commercial strains have also been observed on winery surfaces and in spontaneous fermentations (Martiniuk et al. 2016). These similar strains have been observed in cases where the 'parent' commercial strain has not been observed in the winery or other spontaneous fermentations. It is postulated that an identifiable commercial strain colonized winery surfaces and then mutated over time, adapting to the winery environment (Martiniuk et al. 2016). The proportion of commercial and similar strains was generally higher in fermentations performed in wineries, rather than laboratory settings, adding to the evidence for the colonization of winery surfaces by commercial strains (Martiniuk et al. 2016).

Interactions Between Vineyard and Winery Contributions

There is compelling evidence that commercial *S. cerevisiae* strains move from wineries to the surrounding vineyards, which has led to the 'escaped commercial strain' hypothesis (Schuller and Casal 2007, Valero et al. 2007, Goddard et al. 2010, Salinas et al. 2010, Martiniuk et al. 2016). The range of the observed movement varies, but appears to be limited to the immediately adjacent vineyards (Salinas et al. 2010, Hyma and Fay 2013, Martiniuk et al. 2016), with winery waste water runoff the

suspected vector (Schuller and Casal 2007). The persistent use of commercial yeast products by a winery did not appear to necessarily increase the frequency of observing commercial *S. cerevisiae* strains in the surrounding vineyards (Schuller et al. 2007, Valero et al. 2007). This supports the idea that factors outside of the use of commercial yeast inoculation affect vineyard yeast populations, including the grape, vineyard, and vintage variations discussed previously.

Despite strong support for the existence of commercial strains in vineyards, there is mixed evidence regarding their effect on the population structure of native yeasts, although there does appear to be some influence (Martínez et al. 2007, Schuller and Casal 2007, Cubillos et al. 2009). In vineyards where *S. cerevisiae* matching commercial strains were observed, genetically distinct native strains were also found, showing that commercial strains do not completely supplant native strains (Schuller and Casal 2007, Schuller et al. 2007, Valero et al. 2007, Goddard et al. 2010, Salinas et al. 2010). The discovery in some vineyards of *S. cerevisiae* strains similar to, but not matching, commercial strains used in the associated winery indicates the possibility commercial strains dispersed to vineyards will persist and adapt to local conditions over time (Martínez et al. 2007, Salinas et al. 2010). However, the commercial strains observed in vineyards were subject to the same fluctuations in observation and abundance as native strains, and may not outcompete native strains (Schuller et al. 2007). The presence of commercial strains appears to decrease the overall number of distinct *S. cerevisiae* strains and their relative abundances, but not in all cases (Cubillos et al. 2009, Salinas et al. 2010). There is evidence of less variation between the *S. cerevisiae* strains observed in the winery and in affiliated vineyards than between different vineyards (Martiniuk et al. 2016). Similarly, the presence of commercial strains may be related to lower overall allelic diversity in *S. cerevisiae* strains (Cubillos et al. 2009, Salinas et al. 2010) and decreased diversity in non-

Saccharomyces species in the vineyard (Ganga and Martínez 2004, Cubillos et al. 2009, Salinas et al. 2010), indicating closer phylogenetic relationships, but this was not always the case (Schuller and Casal 2007, Salinas et al. 2010).

Based on research to date, it appears that *S. cerevisiae* strains in spontaneous fermentations arise from a combination of vineyard and winery sources, with variable ratios in different fermentations. The use of commercial strains in a winery can influence the population structure of the native yeast population on grapes in adjacent vineyards, but the interactions are unclear. The lack of clear cause and effect in these observed variations suggests that not all of the factors influencing the source of yeast present in spontaneous fermentations have been elucidated.

Yeast Biogeography

Biogeography Overview

Yeast populations can be described and analyzed using biogeography, the study of organism distribution via time and space in a given area (Hanson et al. 2012). The goal is to map the location and abundance of organism populations in an area, and to understand the mechanism behind the distribution and composition of the observed populations (Martiny et al. 2006). Biogeographical patterns, which are shaped by a combination of species-specific evolutionary (time) and regional specific ecological (space) forces (Martiny et al. 2006, Hanson et al. 2012), can be applied to yeast species and strains associated with winemaking to understand how regional differences in yeast populations occur.

Species-specific evolution is shaped by mutation, selection, gene flow and genetic drift. Naturally occurring mutations, which are random changes in genetic code, fuel genetic diversity within a species, creating strains. This genetic diversity can lead to differential fitness, or improved ability to reproduce and survive, in local conditions –

also known as selection. Strains with increased fitness are 'selected for', while strains with decreased fitness are 'selected against', impacting strain diversity and relative abundances within a population. Selection can cause strains to become more or less genetically similar, depending on the conditions creating differential fitness. Since mutations are random, different populations will have unique genetic variations in the form of different combinations of strain diversities and abundances. 'Gene flow' refers to the changes to overall species genetic diversity due to interactions between different populations. Finally, 'genetic drift' denotes different strains proliferating due to chance events, as opposed to differential fitness (Martiny et al. 2006, Hanson et al. 2012, Morrison-Whittle and Goddard 2015).

Regional specific ecology, the species diversity and relative abundances in a given area, are shaped by similar forces – speciation, selection, dispersal and drift. Speciation can be defined as the accumulation of mutations resulting in the reproductive isolation of two populations that were previously able to interbreed (Hanson et al. 2012). In this context, selection refers to the overall fitness of a species, compared to other species, in response to local conditions. Species selection will account for both diversity and the relative abundances of species. Dispersal is the movement and establishment of a species to a new region, where newly introduced species will impact local species diversity and abundances. Finally, drift occurs when different species proliferate due to chance events, as opposed to differential fitness (Hanson et al. 2012).

Distinct biogeographic regions are created through drift and selection favoring genetic diversity. In contrast, dispersal and selection events favor genetic homogeneity of both species and strains, reducing the differences between regions and lessening the creation of distinctive biogeographic patterns (Martiny et al. 2006). In addition, interspecies hybridization and HGT between yeast species are sources of

genetic diversity, and can influence speciation and selection pressures (Liti, Barton and Louis 2006, Cappello et al. 2010, Chambers and Pretorius 2010, Hanson et al. 2012, Almeida et al. 2014, Gibson and Liti 2015). Yeast also have short generational intervals and high population densities which speed the rate of genetic diversification (Martiny et al. 2006). These factors would tend to favor the creation of distinct biogeographic patterns.

Yeast Domestication

Another factor affecting the yeast genetic diversity is the possibility of domestication (Boynton and Greig 2014, Dapporto et al. 2016). The fact that *S. cerevisiae* is only commonly observed in winemaking environments, and rarely in natural environments, has led to an extensive debate as to whether the species is fully domesticated, partially domesticated, or influenced by humans but not domesticated (Diamond 2002, Goddard et al. 2010, Schuller et al. 2012, Chambers et al. 2015, Zeder 2015). The multiple possible definitions for 'domestication' contributes to the debate. Zeder's proposed definition is "a sustained multigenerational, mutualistic relationship in which one organism assumes a significant degree of influence over the reproduction and care of another organism in order to secure a more predictable supply of a resource of interest, and through which the partner organism gains advantage over individuals that remain outside this relationship, thereby benefitting and often increasing the fitness of both the domesticator and the target domesticate" (Zeder 2015). Any species that is not domesticated is typically defined as a wild species, but as with domestication there are several possible definitions (Schuller et al. 2012, Zeder 2015). The term wild yeast has been used to describe any yeast species or strain that is naturally present on grapes, in addition to any yeast that is not domesticated (Pretorius, Curtin and Chambers 2015). To avoid confusion,

this review will refer to yeast species and strains that are not domesticated as 'wild yeast', while 'native yeast' will refer to yeast species and strains that are naturally present on grapes. By Zeder's definition, *S. cerevisiae* is partially domesticated, as strains that fit and do not fit this definition of domestication have been observed (Goddard et al. 2010, Gonçalves et al. 2011). The genetic basis for these distinctions is not completely clear, as gene flow between defined domesticated and wild strain occurs regularly (Goddard et al. 2010, Schuller et al. 2012, Almeida et al. 2014, Dapporto et al. 2016).

The debate is further complicated because winemakers do not have access to phylogenetic analysis data, so the production of pleasant aromas and fermentation success are often used as a proxy for determining domestication; yeast that exhibit these characteristics are colloquially known as wine yeast (Hyma et al. 2011, Dapporto et al. 2016). Conversely, any *S. cerevisiae* strain that produces unpleasant aromas or inconsistent fermentation is considered wild regardless of its genetic makeup (Hyma et al. 2011, Dapporto et al. 2016). This can be technically inaccurate, as some domesticated strains can produce unpleasant wine aromas, and some wild strains, pleasant ones.

There is good evidence, including genetic analysis, that the *S. cerevisiae* strains associated with winemaking were domesticated from wild strains through selective pressures imposed by humans during winemaking and other human related fermentations (Cubillos et al. 2009, Goddard et al. 2010, Gonçalves et al. 2011, Hyma et al. 2011, Almeida et al. 2014, Boynton and Greig 2014, Chambers et al. 2015, Dapporto et al. 2016). The specific conditions of winemaking – high sugar and ethanol concentrations at different times and the presence of sulfur dioxide – as well as humans selection of strains that produce pleasant aromas, have helped shape domesticated strains (Treu et al. 2014). As domestication tends to decrease genetic

variation but increase phenotypic variation (Boynton and Greig 2014, Dapporto et al. 2016) the domestication hypothesis is supported by the fact that *S. cerevisiae* associated with winemaking are genetically distinct from strains found in non-winemaking environments and less genetically diverse as a group, while retaining a high phenotype-to-genotype diversity ratio (Hyma et al. 2011, Mendes et al. 2013).

There is also evidence that non-human related selective pressures still affect *S. cerevisiae* strains, indicating partial, rather than complete, domestication. *S. cerevisiae* found in winemaking environments can display characteristics detrimental to winemaking despite human selection for strains that contribute positive characteristics. For example, the production of acetic acid and ethyl acetate are undesirable traits for winemaking, but are known to attract *Drosophila* fruit flies (Knight and Goddard 2015). The ability to attract insects may increase the fitness of *S. cerevisiae* strains, even those found in winemaking environments, and help explain why traits detrimental to winemaking persist in *S. cerevisiae* strains (Chambers et al. 2015, Dapporto et al. 2016). If the domestication of *S. cerevisiae* was complete, traits undesirable to winemaking would be rare, if not completely absent.

Partial domestication is also supported by the observation of *S. cerevisiae* from non-winemaking environments. While comparatively rare, *S. cerevisiae* has been isolated worldwide from such places as trees, soil, and wild fruit (Goddard et al. 2010, Zhang et al. 2010, Hyma and Fay 2013, Charron et al. 2014). These strains display markedly different kinetic and metabolic characteristics, and a distinct set of genetic variations, from those isolated in winemaking environments. The survival of *S. cerevisiae* outside fermentations is poorly understood, as their metabolism and growth patterns in natural environments are unclear (Boynton and Greig 2014, Chambers et al. 2015, Knight and Goddard 2016). The genetic diversity between *S. cerevisiae* strains isolated in wine and non-wine related environments is due to the different selective

pressures on the two groups (Zeder 2015). *S. cerevisiae* strains isolated from winemaking environments are much more likely to show evidence of domestication than those isolated from natural environments. However, there is some evidence that natural environment *S. cerevisiae* strains have been affected by human activities, but not to the degree that indicates domestication (Charron et al. 2014). The selective pressures imposed during fermentations favor such a specific set of genetic variations that the differences between domesticated wine, and wild non-wine, *S. cerevisiae* strains may actually be greater than the differences in wild non-wine *S. cerevisiae* strains and other *Saccharomyces* species (Borneman and Pretorius 2015). However, there is a degree of genetic and phenotypic overlap between domesticated and wild strains, possibly due to similar local adaptations, convergent evolution, and gene flow between groups (Dapporto et al. 2016). Any domestication of *S. cerevisiae* populations would affect the creation of biogeographic patterns, favoring a reduction in distinctions between regions (Martiny et al. 2006, Boynton and Greig 2014, Dapporto et al. 2016).

In contrast with *S. cerevisiae*, the possible domestication of non-*Saccharomyces* yeast has not been extensively researched, as non-*Saccharomyces* yeast are considered wild with few exceptions (Masneuf-Pomarede et al. 2016). In examining phenotypic characteristics of non-*Saccharomyces* species for evidence of adaptation to winemaking conditions, some species, such as *T. delbrueckii*, show signs of domestication similar to that seen with *S. cerevisiae* (Masneuf-Pomarede et al. 2016). An examination of the genetic diversity of *T. delbrueckii* strains also shows a range similar to that seen in *S. cerevisiae*, which indicates adaptation to winemaking conditions (Masneuf-Pomarede et al. 2016). Other non-*Saccharomyces* yeast species show evidence supporting their wild status. Genetic diversity in *H. uvarum* strains is consistent with variations due to geographic distance or time, and no evidence of

adaptation to winemaking environments on a large scale (Masneuf-Pomarede et al. 2016). Each non-*Saccharomyces* yeast species will have a different set of selective pressures, a unique biogeographic pattern, and will contribute differently to the larger pattern of the combined biogeographic region.

Yeast Biogeography Research Framework

With these complications in mind, framing the investigation of yeast biogeography patterns with one or more of the following null hypotheses can be useful: (1) there are no regional differences in yeast species diversity or relative abundances, (2) regional differences are due solely to regional isolation, (3) regional differences are due solely to ecological niches, (4) regional differences are due to both regional isolation and ecological niches (Martiny et al. 2006, Hanson et al. 2012, Taylor et al. 2014, Morrison-Whittle and Goddard 2015). Since regional differences have been found to exist, the other null hypotheses are useful for exploring the causes of differentiation. The regional isolation null hypothesis assumes that drift is the primary cause of differentiation – the accumulation of mutations in the absence of selective pressures from the local conditions. Since different regions will have a different set of random mutations, each population will be different, and gene flow and dispersal is assumed to be localized. (Martiny et al. 2006, Morrison-Whittle and Goddard 2015). The distance-decay model, that the differences between yeast populations are correlated to the distance between them, is often used to test this hypothesis (Martiny et al. 2006, Hanson et al. 2012).

The ecological niche null hypothesis assumes that selection for increased fitness to local environmental conditions is the primary cause of differentiation, and can be modified to include assumptions about gene flow and drift. One modification, known as the Baas-Becking hypothesis, assumes that there are no limits to gene flow and

dispersal, that 'everything is everywhere', and differences are only due to selection (Martiny et al. 2006, Hanson et al. 2012, Morrison-Whittle and Goddard 2015). Under Baas-Becking it would be expected that two regions with similar environmental conditions would produce similar yeast populations regardless of the physical distance between the areas. Another modification assumes that there are significant limits on gene flow and dispersal, and that historical dispersion events have a significant impact on diversity between regions. In this hypothesis, regions with similar environmental conditions will only produce similar yeast populations if they were exposed to the same dispersion events. A distance-decay model can also be used with these hypotheses to estimate the importance of gene flow and dispersal (Martiny et al. 2006, Gayevskiy and Goddard 2012, Hanson et al. 2012, Taylor et al. 2014, Morrison-Whittle and Goddard 2015).

The first step in yeast biogeography research is establishing the yeast present in a region through an ecological survey (Taylor et al. 2014). The scope and type of ecological survey performed can have profound ramifications on the conclusions. The methodology used for yeast identification affects the taxonomic resolution and coverage of total biodiversity that can be achieved. Culture-dependent methodologies tend to yield higher taxonomic resolution but less biodiversity coverage than culture-independent methodologies (Vaz-Moreira et al. 2011, Hanson et al. 2012). Similarly, higher taxonomic resolution is typically gained through multigene analysis methods rather than by single gene analysis (Ayoub et al. 2006, Kurtzman 2011). Single gene analysis of the ribosomal internal transcribed spacer (ITS) region is both specific enough for distinguishing between yeast species, and sensitive enough to a broad range of fungi to identify most yeast species important in wine fermentations (Schoch et al. 2012). Multigene analysis of the alleles present at several unlinked loci can result in better discrimination between strains within a

species than an analysis of the nucleotide differences in a single gene, but must be tailored to each yeast species of interest (Ayoub et al. 2006, Kurtzman 2011).

When examining the resulting data, the higher the taxonomic resolution, the higher the likelihood of detecting a relationship between distance and yeast diversity and the effect of drift. The size of the region tested also influences the likelihood of detecting distance effects. Additionally, if the same region is tested on both micro and macro scales, different distance effects may be observed. Survey timing also matters, as there is evidence that selection, mutation, dispersal and drift change both seasonally and over longer periods (Hanson et al. 2012). Once the parameters of an ecological survey have been established, the results can be evaluated in several ways, including: (1) richness – the number of distinct taxonomic groups present in an area, (2) composition – the identity of the groups, to the taxonomic resolution conveyed by the methodology, and their relative abundances, and (3) genetic diversity – types and extent of strain diversity within a yeast species and their relative abundances (Martiny et al. 2006, Hanson et al. 2012). Ecological survey results from different regions can be compared to determine whether regional differences in yeast diversity or relative abundances exist.

Following the ecological survey is the task of elucidating the effects of numerous environmental factors and their complex, poorly understood interactions. This is easier in small ecological survey areas (Martiny et al. 2006), but it is difficult to control for environmental factors over greater distances and between regions, so distance effects are likely to be overestimated and the effects of drift may be underestimated. This is one of the most serious challenges in interpreting the comparison of ecological surveys of different regions (Hanson et al. 2012).

Only after environmental factors have been accounted for can the effects of yeast dispersal can be assessed (Martiny et al. 2006), generally through the distance-decay

model. Greater distances make historical dispersal events more evident, while recent local dispersal ranges are best assessed using shorter distances. At intermediate distances, ~10-3000 km or ~6-1900 miles, both aspects of dispersal may be discernable (Martiny et al. 2006). It should be noted that dispersal patterns may differ by strain (Hanson et al. 2012).

Regional Ecological Surveys

Ecological surveys of yeast populations, including some of only *S. cerevisiae* strains, have been performed in numerous winemaking regions (Torija et al. 2001, Clemente-Jimenez et al. 2004, Combina et al. 2005, Barata et al. 2008, Chavan et al. 2009, Sun et al. 2009, Zhang et al. 2010, Baffi et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Bezerra-Bussoli et al. 2013, Díaz et al. 2013, Bokulich et al. 2014, Alessandria et al. 2015, Pinto et al. 2015, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Garofalo et al. 2016, Garofalo et al. 2016, Drumonde-Neves et al. 2017). Variations in yeast identification methodology prevent universal comparison between surveys, which vary by region size, depth of testing (number of samples analyzed), length of study time, and types of strains identified. In comparable surveys, there is strong support for regional differences in yeast population richness, composition, and genetics, both between winemaking regions and within different parts of the same region (Bokulich et al. 2014, Knight and Goddard 2015).

As discussed above, a variety of environmental factors influence the types and abundances of yeast species and strains in wineries, vineyards, and the immediately adjacent areas; the effects are complex and not fully elucidated. A study specifically looking at the effect of environmental conditions on yeast biogeography in a small geographic region seem to confirm these general conclusions (Bokulich et al. 2014). Differences in environmental conditions, such as the amount of precipitation and

maximum daily temperature, correlated to the observation of differences in yeast ecological surveys of different regions physically close to each other. However, it was difficult to establish a correlation between any one environmental condition and a discrete, consistent change in yeast population composition (Bokulich et al. 2014), underscoring the multiplicity of factors, with complex interactions, that cause overestimation of distance effects and underestimation of drift effects (Martiny et al. 2006, Hanson et al. 2012).

Yeast Dispersal Mechanisms

Yeast are immobile and cannot actively disperse (Martiny et al. 2006, Goddard et al. 2010, Boynton and Greig 2014, Chambers et al. 2015). Trees are also passively dispersed, and can be used as a model for the expected dispersal pattern of yeast – most progeny will be observed in close proximity to the parent, with a few travelling longer distances. The initial population size affects this pattern of dispersal, with higher population densities translating to further dispersal distances (Martiny et al. 2006). The vectors for movement will also affect dispersal distances and resulting patterns. There appear to be multiple vectors for yeast dispersal (Goddard et al. 2010, Zhang et al. 2010, Charron et al. 2014, Dapporto et al. 2016).

S. cerevisiae have been observed on or in several insects, such as *Drosophila* fruit flies (Charron et al. 2014), social wasps (Dapporto et al. 2016), and bees (Goddard et al. 2010). These insects are commonly observed on or near damaged fruit, including fruit being processed at wineries. Bees in New Zealand have been linked as yeast dispersal vectors in small areas, up to 10 kilometers (Goddard et al. 2010). It is likely that the range of other insects as vectors for *S. cerevisiae* dispersal are similarly localized.

Humans are another likely vector for yeast dispersal (Valero et al. 2007, Goddard et al. 2010). Over short distances the human movement of pomace containing yeast, waste water, and other equipment harboring yeast populations may be a dispersal mechanism (Valero et al. 2007, Blanco, Orriols and Losada 2011). Over long distances, the historic movement of humans and the grapevines and winemaking equipment they moved with them from region to region, along with any yeast present on these items is a potential dispersal mechanism. Multiple *S. cerevisiae* strains have been identified on new French oak barrels, lending support for a human dispersal model, even if local grapes and new equipment is used (Goddard et al. 2010). Genetic analysis of *S. cerevisiae* strains isolated from grapes and wineries around the world also supports human influence on yeast migration (Legras et al. 2007, Knight and Goddard 2015). The dispersal of non-*Saccharomyces* yeast has been little studied, and is likely to have similar mechanisms but different dispersal patterns and vectors. Additionally, there are likely other vectors that have not been explored thoroughly, such as non-insect animals and air currents (Valero et al. 2007, Francesca et al. 2010, Adams et al. 2013, Chambers et al. 2015, Dapporto et al. 2016).

Current Yeast Biogeographic Research

Current research on yeast biogeography can be separated into two groups – research that focuses on *S. cerevisiae* and research that also includes non-*Saccharomyces* yeasts. While the exact number of *S. cerevisiae* strains is unknown, it numbers in at least the hundreds; the reference database of strains used by Ubeda and Briones in 2000 included almost 400 distinct strains (Ubeda and Briones 2000). Regional ecological surveys have shown that many regions independently contain large numbers of *S. cerevisiae* strains, including New Zealand (Goddard et al. 2010, Gayevskiy and Goddard 2012, Knight and Goddard 2015), Spain (García et al. 2016),

Portugal (Schuller et al. 2005), Italy (Azzolini et al. 2013), Canada (Martiniuk et al. 2016), China (Li et al. 2011), Bosnia and Herzegovina (Orlic et al. 2010), and France (Vezinhet et al. 1992). Knight and Goddard found that any vineyard or winery samples that yielded *S. cerevisiae* had on average 4.7 unique strains (Knight and Goddard 2015). While varying methodologies make it difficult to compare results across studies, there is strong support for the existence of regional differences in *S. cerevisiae* populations.

Wild S. cerevisiae Biogeographical Patterns

Wild *S. cerevisiae* strains are primarily found in natural, non-winemaking environments (Goddard et al. 2010, Hyma and Fay 2013) and have been isolated worldwide from trees, flowers, soil, and decaying fruit, among other sources (Goddard et al. 2010, Zhang et al. 2010, Hyma and Fay 2013, Charron et al. 2014). Contrary to expectations of *S. cerevisiae* thriving in high sugar environments, wild *S. cerevisiae* are more frequently observed on trees than on decaying fruit (Charron et al. 2014). Trees are the most common source of wild *S. cerevisiae* among natural environments, particularly oaks and beech trees, which occupy the same ecological niche in the northern and southern hemisphere, respectively (Boynton and Greig 2014, Charron et al. 2014).

Two possible explanations for the prevalence *S. cerevisiae* on trees rather than sugar-rich decaying fruit are the safe environment provided by tree bark when fruit is not available, and the potential for *Saccharomyces* to thrive in a large number of environments using different metabolic pathways (Boynton and Greig 2014). The overall abundances of wild *S. cerevisiae* populations varies seasonally, increasing during the period between April and August/September, with a sharp drop off in abundance at the end of summer (Charron et al. 2014). Populations also appear to be

temperature sensitive, with the frequency of isolation increasing as the temperature rises (Boynton and Greig 2014, Charron et al. 2014). It is possible that, whatever the reason for the decrease, wild strains have an ecological incentive to colonize other surfaces as summer ends.

Both wild and domesticated *S. cerevisiae* strains can be observed in vineyards and surrounding oak trees, but only wild strains were observed on trees not immediately adjacent to vineyards (Hyma and Fay 2013). There is evidence of gene flow between wild and domesticated *S. cerevisiae* strains from sources of wild strains near vineyards, indicating some blurring between domesticated and wild strains based on geography (Goddard et al. 2010, Hyma and Fay 2013, Almeida et al. 2014, Knight and Goddard 2015). Gene flow between wild and domesticated *S. cerevisiae* strains show that it is possible for either group to serve as a reservoir of genetic diversity for the other group (Knight and Goddard 2015). In the small distances between the vineyard and surrounding trees insects serve as a possible vector for *S. cerevisiae* strain dispersal (Goddard et al. 2010, Libkind et al. 2011).

Other *Saccharomyces* species may also add to the genetic diversity of wild *S. cerevisiae*. Gene flow between *Saccharomyces* species via spontaneous hybridization has been observed with relative frequency in the wild (Liti, Barton and Louis 2006, Zhang et al. 2010, Almeida et al. 2014). For example, co-located *S. cerevisiae* and *S. paradoxus* can form interspecific hybrids in the wild, although only a small percentage of these hybrids are viable (Liti, Barton and Louis 2006).

An overall analysis of ecological surveys of wild *S. cerevisiae* strains shows that the differences between populations appears to be related more to ecological niche than to regional isolation, and observed differences do not appear to correlate to distance (Liti et al. 2009, Zhang et al. 2010, Hyma and Fay 2013).

Domesticated S. cerevisiae Biogeographical Patterns

Domestication of a species, by definition, involves the manipulation of genetic variation in favor of desired phenotypic characteristics, predisposing domesticated species to be ecologically, not geographically, driven. In the context of domesticated *S. cerevisiae,* this would suggest that regions with similar fermentation traditions would have genetically similar strains, and regions with different fermentation traditions genetically distinct strains, regardless of physical proximity (Boynton and Greig 2014). Researchers tested this hypothesis by comparing phylogenetic differences between *S. cerevisiae* strains isolated from varying types of fermentations, and other environments, worldwide (Legras et al. 2007), including wine related *S. cerevisiae* strains isolated from spontaneous fermentations in multiple winemaking regions, and over 20 commercial strains. Of the wine-related strains, 95% were phylogenetically grouped together; this grouping also included all the strains associated with cider fermentation. Most of the remaining 5% of the wine related strains were phylogenetically distinct from this main grouping, and included strains isolated from fermentations in Austria and California; these were similar to bread related strains. The remaining wine related strains were all isolated in the United States, and were grouped with strains isolated from oak trees or Chinese distilling and rice wine fermentations. Phylogenetically distinct groupings of distillation, palm wine, laboratory, beer, fermented milk and sake strains were also observed (Legras et al. 2007).

These findings agree with multiple studies suggesting that specific fermentation process, a type of ecological niche, is more likely to correlate to phylogenetic similarity than the geographic location of isolated strains (Legras et al. 2007, Diezmann and Dietrich 2009, Hyma et al. 2011, Hyma et al. 2011, Hyma and Fay 2013, Barbosa et al. 2014, Borneman and Pretorius 2015, Morrison-Whittle and

Goddard 2015, Pretorius, Curtin and Chambers 2015). Genetically identical non-commercial strains have even been isolated from different geographic areas (Legras et al. 2007). While this appears to favor a Baas-Becking 'everything is everywhere' hypothesis, the studies also show that ecological niche is not the only factor in phylogenetic similarity as not all wine-related strains were grouped together.
The influence of geography is more evident when analyzing the genetic differences between the wine related *S. cerevisiae* strains, where some genetically distinct subgroups correlated with the original isolation location (Guillamón, Barrio and Querol 1996, Legras et al. 2007, Martínez et al. 2007, Goddard et al. 2010, Jeyaram et al. 2011, Hyma and Fay 2013, Borneman and Pretorius 2015, Knight and Goddard 2015). For example, multiple strains from within the Champagne region of France were genetically more similar to each other than strains from other places (Legras et al. 2007). A similar genetic similarity was observed in strains observed in California, and the United States generally (Legras et al. 2007). Several studies of *S. cerevisiae* strain-level gene structure indicate that there is gene flow between populations from different regions (Gayevskiy and Goddard 2012, Knight and Goddard 2015). The gene flow appears to follow human movement in relationship to fermentation activities; regions with more exchanges of grapes or equipment have higher levels of genetic similarities in respective *S. cerevisiae* strains (Knight and Goddard 2015). This dovetails with studies showing that *S. cerevisiae* strains from neighboring geographical areas are not necessarily more genetically similar than strains from a greater distance away (Liti, Barton and Louis 2006, Liti et al. 2009).

As the distance between regions does not correspond to the degree of genetic similarity between the *S. cerevisiae* strains of the regions, there is not a traditional distance-decay relationship at the regional level (Liti, Barton and Louis 2006, Cubillos et al. 2009, Liti et al. 2009). However, the evidence for a distance-decay relationship

changes depending on the scale and sizes of the ecological surveys being compared. At small distances, as between different vineyards within the same region, there was evidence of genetic similarity directly correlated with geographic distance (Schuller et al. 2012). At even smaller distances there is good evidence to support the movement of commercial *S. cerevisiae* strains from wineries to immediately adjacent vineyards, with commercial strains found most frequently closer to the winery. Research on this subject effectively examined the dispersal limits of *S. cerevisiae* strains in very small overlapping regions and found evidence of a distance-decay relationship.

Gene flow within a region appears to have human and insect dispersal vectors (Knight and Goddard 2015). At these distances, there is also evidence that other ecological niche factors, such as the grape cultivar from which a strain was isolated, may contribute to genetic similarities – i.e., in neighboring vineyards different grape cultivars may harbor genetically dissimilar strains (Cubillos et al. 2009, Schuller et al. 2012, Dapporto et al. 2016). The dissipation of distance-decay correlations at larger geographic scales indicates that multiple factors affect gene flow and dispersal (Schuller et al. 2012). The fact that the genetic differences in *S. cerevisiae* strains between regions are not correlated to distance may also indicate multiple independent domestication events, distortions due to founder group population differences, the introduction of strains from several different regions, region-specific historical mutation events or reproductive strategies, and/or factors not previously considered to drive regional evolution (Legras et al. 2007, Cubillos et al. 2009, Diezmann and Dietrich 2009, Liti et al. 2009, Goddard et al. 2010, Borneman and Pretorius 2015). Genetic analysis of *S. cerevisiae* strains associated with global winemaking shows several novel strain lineages, indicating multiple independent domestication events (Legras et al. 2007, Goddard et al. 2010).

Phylogenetic analysis of commercially developed *S. cerevisiae* strains shows conflicting results, with some finding no distinct commercial grouping (Legras et al. 2007), but others indicating genetic similarities between commercial strains, or between commercial strains and native *S. cerevisiae* strains, isolated in Europe (Vezinhet et al. 1992, Martínez et al. 2007, Mercado et al. 2010, Pretorius, Curtin and Chambers 2015). Suggested explanations for these genetic similarities include the possibility that commercial strains were developed from native strains isolated in the same regions of Europe, that bias towards certain phenotypic traits in commercial strain development resulting in genetic similarities, or that convergent evolution of commercial and native strains in response to the same selective pressures is imposed by winemaking conditions (Martínez et al. 2007, Mercado et al. 2010).

Surprisingly, commercial *S. cerevisiae* strains have been observed on surfaces and spontaneous fermentations in wineries where they were never used for inoculation (Martiniuk et al. 2016), and in wineries where the identified strain had not been used for five years (Scholl et al. 2016). The isolation of commercial strains in places with no obvious causal dispersal vectors indicates that dispersal forces are not fully understood.

Non-Saccharomyces Yeast Biogeographical Patterns

Biogeographic research into the total yeast populations is a more complex question than *S. cerevisiae* strain distribution patterns, as there are more species involved. Only a small portion of all non-*Saccharomyces* yeast species are usually observed in winemaking environments, but it is common to observe multiple yeast species in a single sample. The parameters of ecological surveys of total yeast populations and analysis of their results must take this complexity into account.

The method of species identification determines how ecological surveys are evaluated. Culture-dependent methodologies focus on yeast composition and genetic diversity over richness and relative abundances, but offer a higher degree of taxonomical resolution, often identifying yeast to the species level. Culture-independent methodologies focus on yeast richness, composition, and relative abundances over genetic diversity. As they offer a lower taxonomical resolution, yeast composition may only be defined by operational taxonomical units that rarely identify particular species.

Ecological surveys of total yeast populations have been completed in many winemaking regions worldwide, including Italy (Alessandria et al. 2015, Garofalo et al. 2016), Spain (Torija et al. 2001, Clemente-Jimenez et al. 2004, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016), China (Sun et al. 2009, Li et al. 2011, Sun et al. 2014), Brazil (Baffi et al. 2011, Bezerra-Bussoli et al. 2013), Portugal (Barata et al. 2008, Barata, Malfeito-Ferreira and Loureiro 2012, Pinto et al. 2015), the Azores Archipelago (Drumonde-Neves et al. 2017), India (Chavan et al. 2009), Argentina (Combina et al. 2005), New Zealand (Zhang et al. 2010), Switzerland (Díaz et al. 2013), and the United States (Bokulich et al. 2014, Bokulich et al. 2016). Similar to ecological surveys of *S. cerevisiae* strains, total yeast ecological survey results from each region cannot always be directly compared due to differences in identification methodologies. Overall, there is strong support for the existence of regional differences in yeast species composition.

Taylor et al. examined the reproducibility of culture-dependent ecological survey results by retesting the samples using culture-independent methods (Taylor et al. 2014), and found that both methods yielded the same differences in regional yeast composition, showing that either method can be used to assess yeast biogeography (Gayevskiy and Goddard 2012, Taylor et al. 2014). Culture-independent methods

were also found to reveal more operational taxonomy units (OTUs) than the culture-dependent method – hundreds of OTUs versus 10s of yeast species (Taylor et al. 2014). However, it is unclear what portion of the additional OTUs were relevant to wine fermentations.

Support for regional differences in yeast species composition exists at several different distance levels – between subregions of a single winemaking region (Bokulich et al. 2014, Pinto et al. 2015, Sun et al. 2015, Belda et al. 2016, Drumonde-Neves et al. 2017), between vineyards in a single subregion (Barata, Malfeito-Ferreira and Loureiro 2012, Alessandria et al. 2015), and between and within vineyard rows in a single vineyard (Setati et al. 2013). More study is needed to assess whether a distance-decay relationship defines yeast species composition differences at each scale.

Yeast species composition differences have also been observed in a single region across vintages (Pramateftaki, Lanaridis and Typas 2000, Torija et al. 2001, Barata, Malfeito-Ferreira and Loureiro 2012, Díaz et al. 2013, Bokulich et al. 2014, Belda et al. 2016), but the extent of difference across vintages was not constant between regions. Some studies found that vintage variation had a greater effect when comparing vineyards within a subregion then when comparing subregions (Bokulich et al. 2014, Drumonde-Neves et al. 2017). The scale of areas compared, therefore, impacts interpretation of results.

While regional differences in yeast composition are well supported, the factors driving those differences are unclear. The reasons for this confusion are two-fold: the number of yeast species in the populations being studied, and the number of ecological factors known to influence these species. As discussed previously, different environmental conditions including climate, weather (Combina et al. 2005, Drumonde-Neves et al. 2017), grape cultivar (Torija et al. 2001, Renouf, Claisse and

Lonvaud-Funel 2007, Bezerra-Bussoli et al. 2013, Díaz et al. 2013, Bokulich et al. 2014), and viticultural practices (Combina et al. 2005, Cordero-Bueso et al. 2011, Setati et al. 2013, Patrignani et al. 2017) will affect which yeast species are present on grapes. Given the lack of research on non-*Saccharomyces* yeast, the impact of environmental conditions on each species is largely unknown. Some known factors include human interactions with vineyards, which have been found to increase yeast species richness and change relatively abundances (Drumonde-Neves et al. 2017); microbiome diversity, as relative abundances of non-*Saccharomyces* yeast appear to be connected to *H. uvarum* abundance, where higher *H. uvarum* levels correlated with lower levels of other non-*Saccharomyces* yeasts (Combina et al. 2005); and grape damage, which significantly increases both yeast species diversity and abundances (Schuller et al. 2005, Valero et al. 2007, Cubillos et al. 2009, Barata, Malfeito-Ferreira and Loureiro 2012). The effect of grape damage on yeast composition may be a key factor in the differences observed within a single vineyard. Despite these clear links to differences in yeast composition in samples with different environmental conditions (Bokulich et al. 2014), the effect of any of these ecological niche conditions is inconsistent both across and within studies, showing a complex set of interactions affecting a complex set of yeast. The sheer number of yeast composition variations linked to environmental conditions makes it difficult to separate the impact of ecological niche from geographic isolation, though it is likely that both forces have shaped yeast biogeographic patterns observed throughout the world (Martiny et al. 2006, Hanson et al. 2012)

Microbial Terroir

Overview

Yeast biogeography analysis describes the differences in yeast populations between areas and assess their causes. As discussed previously, differences in the yeast composition of a fermentation can result in differences in the aromatic profile, expression of grape varietal character, mouthfeel, and perceived complexity of the final wine. This has led to speculation that regional differences in yeast species richness, composition and genetic diversity could cause differences in aromatic profile, expression of grape varietal character, mouthfeel, and perceived complexity of wines from specific regions, a concept referred to as 'microbial terroir' (Valero et al. 2007, Bokulich et al. 2014, Chambers et al. 2015, Knight and Goddard 2015, Belda et al. 2016, Bokulich et al. 2016). As with other aspects related to wine yeast, there is a substantial debate surrounding microbial terroir, and research into this concept is relatively recent and quite active (Valero et al. 2007, Barata, Malfeito-Ferreira and Loureiro 2012, Chambers et al. 2015).

Microbial terroir is related to the more general concept of wine terroir – that the unique sensory properties of a wine are directly related to its region of origin (Douglas, Cliff and Reynolds 2001, Willwerth, Reynolds and Lesschaeve 2015, Bokulich et al. 2016). Terroir itself is defined various ways, but it is consistently related to the regional variables that affect grape development (soil, climate, grape varietal, etc.), and may also include the enological traditions of a region (Willwerth, Reynolds and Lesschaeve 2015, Bokulich et al. 2016). Even if attributed causes vary, the fact that different regions, subregions, and even individual wineries can produce wines with different chemical compositions and sensory properties has been well documented (Fischer, Roth and Christmann 1999, Douglas, Cliff and Reynolds 2001, Willwerth, Reynolds and Lesschaeve 2015, Bokulich et al. 2016). Proponents of

microbial terroir suggest that the yeast composition of an area is another factor influencing regional wine differences (Knight et al. 2015, Bokulich et al. 2016).

As research on regional yeast composition is recent and evolving, there is no consensus on a definition of microbial terroir, which is a substantial part of debates over the concept. One possible definition is a set of stable, distinct *S. cerevisiae* strains associated with a region across vineyards and vintages (Barata, Malfeito-Ferreira and Loureiro 2012). Another possible definition is a regionally-distinct yeast composition, including both *S. cerevisiae* and non-*Saccharomyces* yeast, with broad similarities, but not necessarily the same composition, observed across vineyards and vintages (Bokulich et al. 2014). Yet another proposed definition incorporates the effect of regional yeast composition on both grape development and fermentation outcomes (Knight et al. 2015, Pinto et al. 2015). The evidence supporting and contesting each aspect of these definitions is explored below.

Regional Yeast Composition Uniqueness and Stability

There is strong evidence for regionally variable yeast composition, however, evidence of gene flow mediated by human-vectored dispersal, between regionally located native *S. cerevisiae* strains (Gayevskiy and Goddard 2012, Knight and Goddard 2015) has raised questions about the uniqueness of different regional *S. cerevisiae* compositions. The primary evidence for unique regional yeast compositions are studies of New Zealand (Knight and Goddard 2015) and California (Bokulich et al. 2014) wine regions, where ecological surveys of multiple subregions within the larger winemaking region found unique yeast composition in each subregion. The New Zealand study focused exclusively on *S. cerevisiae*, and suggested that genetic relationships between regional *S. cerevisiae* composition were linked to dispersal patterns by human and insect vectors (Knight and Goddard 2015). The California

study examined both *Saccharomyces* and non-*Saccharomyces* yeast composition, and found that each subregion had a unique yeast composition related to a combination of the local conditions, vintage, and climate (Bokulich et al. 2014). The California study also concluded that when the yeast compositions of each subregion were combined, the resulting regional yeast composition was distinct from other regions (Bokulich et al. 2014).

There is conflicting research on the extent of yeast composition variation within subregions. In New Zealand, *S. cerevisiae* strain composition was relatively homogenous within subregions (Knight and Goddard 2015). However, several studies of individual vineyards in close proximity to each other have shown distinct total yeast compositions, but no clear distance-decay relationship (Pramateftaki, Lanaridis and Typas 2000, Valero et al. 2007, Francesca et al. 2010, Schuller et al. 2012, Bokulich et al. 2014, Martiniuk et al. 2016). Several studies have suggested that it is uncommon to observe the same *S. cerevisiae* strains between fermentations at the same winery using grapes from different vineyards, suggesting variation within subregions (Vezinhet et al. 1992, Pramateftaki, Lanaridis and Typas 2000, Schuller et al. 2005, Valero et al. 2007, Martiniuk et al. 2016). Intra-vineyard variation in yeast composition was also found, both within and between vineyard rows (Valero et al. 2007, Setati et al. 2013, Bokulich et al. 2014). Yeast composition variations within a single vineyard are supported by the fact that spontaneous fermentations from different grape lots have evinced different yeast compositions at the beginning of fermentation (Clemente-Jimenez et al. 2004, Schuller et al. 2005, Bokulich et al. 2014).

At these short distances, microvariations in environmental conditions, the age and size of the vineyard, and grape variety differences may be contributing to variations in yeast composition – especially at the strain level (Schuller and Casal 2007, Valero

et al. 2007, Cubillos et al. 2009, Schuller et al. 2012, Dapporto et al. 2016). Additionally, as discussed above, viticultural practices and grape damage has been shown to significantly change the yeast composition on grapes. The soundness of the sampled grapes likely impacts the amount of intra-vineyard yeast composition variation observed, such that researchers should be cautious in comparing vineyards if grape sampling was not a controlled factor. (Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013).

The effect of 'escaped commercial yeast' on intra-vineyard *S. cerevisiae* composition also needs to be addressed. All microbial terroir definitions assume that *S. cerevisiae* strains inhabiting an area are native and impacted by local conditions (Knight and Goddard 2016). As previously discussed, there is compelling evidence for the existence of commercial *S. cerevisiae* strain in vineyards immediately adjacent to wineries. However, the effect of these 'escaped commercial yeasts' on the richness, composition, and genetic diversity of the native *S. cerevisiae* population is unclear. The consensus is that there is some impact, but that commercial strains do not replace native strains (Schuller et al. 2005, Schuller and Casal 2007, Schuller et al. 2007, Valero et al. 2007, Santamaría et al. 2008, Cubillos et al. 2009, Goddard et al. 2010, Salinas et al. 2010, Zhang et al. 2010, Blanco, Orriols and Losada 2011, Gayevskiy and Goddard 2012, Hyma and Fay 2013, Knight and Goddard 2015, Knight and Goddard 2016, Martiniuk et al. 2016).

There is also conflicting research on the stability of yeast composition in the same region across vintages. In some cases vineyards are thought to have relatively stable total yeast compositions over time (Bokulich et al. 2014), where other vineyards show significant variations, particularly in *S. cerevisiae* strain composition, across vintages (Vezinhet et al. 1992, Pramateftaki, Lanaridis and Typas 2000, Schuller et al. 2005, Valero et al. 2007, Martiniuk et al. 2016). Observing the same *S. cerevisiae*

strain in the same vineyard or fermentations at the same winery across vintages occurs, but is uncommon (Vezinhet et al. 1992, Schuller et al. 2005, Martiniuk et al. 2016). In one study, the *S. cerevisiae* strain genetic diversity within a single vineyard over multiple vintages was equivalent to the genetic diversity observed across multiple vineyards within a single vintage (Schuller and Casal 2007). Differences in weather and viticultural practices between vintages may contribute to this variability (Schuller et al. 2005, Valero et al. 2007, Martiniuk et al. 2016). Bokulich et al. found that environmental conditions, such as amount of precipitation and maximum daily temperature, correlated to differences in the observed microbial ecology (Bokulich et al. 2014). These environmental conditions would also effect the viticultural management practices employed and the extent of grape damage, further complicating the relationship. Environmental variations at the vineyard level appear to contribute more to overall variations in yeast compositions then the conditions at the regional level (Bokulich et al. 2014). The unexplained fluctuations over time lead to questions about the stability of any observed regional yeast compositions (Valero et al. 2007, Barata, Malfeito-Ferreira and Loureiro 2012). The survival of *S. cerevisiae* outside of fermentations is poorly understood, so it is unclear how or if yeast overwinters in the vineyard (Knight and Goddard 2015). Further, the dispersal patterns and mechanisms of non-*Saccharomyces* yeast are also largely unknown (Hanson et al. 2012).

Regional Yeast Composition Effect on Wine Sensory Properties

The effect of regional yeast composition on wine sensory properties can be considered in two ways – the effect of the yeast on grape development, and the effect on fermentation (Pinto et al. 2014, Knight et al. 2015). The focus of most research involving yeast and grapes addresses the former, with very little work

reported on the effect yeast can have on grapes. Ecological surveys have established that the yeast populations are present in grape-adjacent areas, such as grapevine bark and soil (Morrison-Whittle and Goddard 2015, Knight and Goddard 2016). A longitudinal study found that the yeast composition in grape-adjacent areas changes over time, and those changes were correlated to specific periods of grape development (Morrison-Whittle and Goddard 2015). This could indicate a causational effect, or changes could be seasonally linked and not effect grape development at all; further research is required to determine if a causational link can be established.

The effects of regional yeast composition on fermentation sensory outcomes are of great interest to winemakers, but are poorly understood. Wine typicity can be defined at the regional, subregional, or vineyard level, as chemical and sensory differences have been observed in wines from each of these different size levels (Fischer, Roth and Christmann 1999, Douglas, Cliff and Reynolds 2001, Willwerth, Reynolds and Lesschaeve 2015, Bokulich et al. 2016). Sensory attributes of subregions within a region have been shown to be interrelated, such that wines from different subregions could be differentiated by sensory attributes, and overlapping sensory attributes of subregions could be attributed to regional sensory typicity (Douglas, Cliff and Reynolds 2001, Willwerth, Reynolds and Lesschaeve 2015). These sensory attributes were found to be consistent over several vintages (Douglas, Cliff and Reynolds 2001, Willwerth, Reynolds and Lesschaeve 2015). To link typicity with yeast identified in regional ecological surveys, research must show that relevant yeasts affect the development of key sensory characteristics during fermentation. Several studies have established that *S. cerevisiae* and non-*Saccharomyces* yeast have an impact the sensory properties of the final wine. Ecological surveys have also established that regional differences in yeast composition include changes to these fermentatively important yeast, both in the types present and their relevant

abundances (Bokulich et al. 2014, Knight et al. 2015). Therefore, regional yeast compositions could be impacting fermentation kinetics, the formation of aroma compounds, and other important wine sensory properties.

Yeast interactions within fermentations are complex, and it can be difficult to predict their effect on sensory properties of the final wine. One study minimized the complexities inherent in such testing by focused on the effect of regionally observed native *S. cerevisiae* strains on sensory properties in isolated fermentations (Knight et al. 2015). The resulting wines were analyzed quantitatively for various aroma compounds and for several measures of wine quality, then grouped by similarity. Wines fermented with *S. cerevisiae* strains from a single region were shown to be more similar then wines fermented with *S. cerevisiae* strains from different regions (Knight et al. 2015), suggesting a causational link between regional *S. cerevisiae* strain differences and differential wine expression (Knight et al. 2015).

It should be noted that this causational link is just the first step in establishing a relationship between total yeast composition differences and regional wine characteristics. No single aroma compound tested was related to the differences produced by the different strains, leading to the conclusion that the link between strains and volatiles produced is complex even in a mono-strain fermentation (Knight et al. 2015). These interactions will become even more complex as the effect of total yeast compositions are evaluated. Additionally, it is unclear the degree to which the observed sensory differences would be perceived by consumers, or the persistence of these differences over time (Knight et al. 2015).

Regional yeast composition is not the only factor that could influence variations in regional wine characteristics, as grape material and fermentation conditions also affect aroma compound formation (Swiegers and Pretorius 2005, Leeuwen and Seguin 2006, Carrau et al. 2008, Gayevskiy and Goddard 2012, Jolly, Varela and

Pretorius 2014, Pretorius, Curtin and Chambers 2015). In the original concept of terroir, the starting grape material was thought to be the primary source of variations between regions, with enological practices also impacting the final aroma profile (Fischer, Roth and Christmann 1999). As grapes, fermentation conditions, and winery environment affect wine aroma profile, they should be accounted for when assessing the impact of the regional yeast composition. As discussed previously, the yeast active in fermentation appear to derive from a combination of vineyard and winery related sources. Winery related yeast are not currently considered part of the regional yeast composition under any proposed definition of microbial terroir, but to assess the concept of microbial terroir the influence of winery related yeast must be considered. At the very least, the conflicting results of the importance of vineyard, vintage, and winery variation reveals that yeast composition is not the only factor in differentiating regional wine sensory characteristics, and that further investigation is required to elucidate the changes caused by the regional yeast composition.

When examining the evidence in relationship to the proposed microbial terroir definitions very different conclusions can be drawn. Microbial terroir defined as a set of stable, distinct *S. cerevisiae* strains associated with a region across vineyards and vintages (Barata, Malfeito-Ferreira and Loureiro 2012) is not supported by current research. Multiple studies observed fluctuations in regional yeast compositions, as well as the rarity of observing the same *S. cerevisiae* strain across vineyard in a region, and across vintages within a vineyard. The uncertainty about *S. cerevisiae* survival between fermentations in natural environments, dispersal patterns, and the effect of commercial yeast on native yeast population structure all casts doubt on this proposed microbial terroir hypothesis.

Microbial terroir defined as a regionally distinct yeast composition, including both *S. cerevisiae* and non-*Saccharomyces* yeast, with broad similarities observed across

vineyards and vintages but not necessarily the exact same yeast composition (Bokulich et al. 2014), has some support from current literature. There is good evidence for a correlation between regional yeast composition variations and regional wine sensory characteristics. Theoretical links between regional yeast composition and these characteristics have been shown for non-*Saccharomyces* yeast. Further, region-specific *S. cerevisiae* strains developed differential wine sensory characteristics, and wines fermented with strains from a single region could be grouped by similar wine sensory characteristics. While causation has not been shown, there is enough circumstantial evidence that further research into microbial terroir is justified. For similar reasons, microbial terroir defined as incorporating the effects of regional yeast composition on both grape development and fermentation outcomes (Knight et al. 2015, Pinto et al. 2015) seems plausible.

The idea of microbial terroir is an attractive one, as the mystique of regional wine expression is deeply entrenched in the mind of wine enthusiasts and winemakers alike. Spontaneous fermentations have already been linked to increases in perceived complexity and varietal aroma characteristics; further research may link the expression of a unique regional character to unique blend of yeast species and strains. While further research is needed to determine whether a causal link exists, but there is good circumstantial evidence for the inclusion of regional yeast composition in the factors that influence terroir.

Ecological Survey of Spontaneously Fermented Riesling in the Finger Lakes

Abstract

Spontaneous wine fermentations are completed by a complex succession of non-*Saccharomyces* yeast species and *S. cerevisiae* strains that influence final wine aroma and flavor profile. Regional yeast composition may shape the wine characteristics typical to a region, a concept known as microbial terroir. This work represents an ecological survey investigating yeast composition of Riesling grapes in the Finger Lakes AVA, with a particular focus on *S. cerevisiae* strains. Grapes, winery equipment, and spontaneous fermentations were sampled during the 2015 and 2016 vintages at three Finger Lakes AVA wineries. Yeast was isolated using culture-dependent methods, identified using the 5.8S internal transcribed spacer (ITS) rRNA region, and *S. cerevisiae* strains were characterized using a six-locus multiplex variable number of tandem repeat (VNTR) analysis. Species from the *Aureobasidium, Candida*, *Hannaella, Hanseniaspora*, *Metschnikowia*, *Meyerozyma, Pichia*, *Rhodotorula, Saccharomyces*, *Torulaspora*, *Trigonopsis, Wickerhamomyces, Zygoascus,* and *Zyosaccharomyces* genera were identified, as well as over 100 *S. cerevisiae* strains. Most *S. cerevisiae* were native strains, with only a few profiles matching commercial strains. Commercial related strains observed in the fermentations did not always become established or dominant, and never completely displaced native strains. *S. cerevisiae* strains observed appear to be influenced by both the regional and resident winery microbiome, as well as vintage-specific factors; several native strains show potential to be part of the regional microbiome.

Introduction

Spontaneous wine fermentations are completed by yeast species and strains naturally present in grape juice or musts, in contrast to inoculated fermentations, where commercial yeast is intentionally added (Pretorius, Curtin and Chambers 2015, Martiniuk et al. 2016). The combination of yeast species and strains present, and their abundance, persistence, and interactions in a fermentation, shape the final wine sensory profile through compounds formed during wine production (Fleet 2003, Romano 2003, Swiegers and Pretorius 2005, Leeuwen and Seguin 2006, Carrau et al. 2008, Chambers and Pretorius 2010, Ciani et al. 2010, Gayevskiy and Goddard 2012, Jolly, Varela and Pretorius 2014, Oro, Ciani and Comitini 2014, Satora et al. 2014, Pretorius, Curtin and Chambers 2015, Pretorius, Curtin and Chambers 2015). Spontaneous fermentations produce wines that are generally perceived as more complex, with more positive aroma attributes and better mouthfeel than inoculated wines (Santamaría et al. 2008, Jolly, Varela and Pretorius 2014, Benito et al. 2015, Chambers et al. 2015, Jara, Rojas and Romero 2015, Belda et al. 2016, Belda et al. 2016, Martiniuk et al. 2016). Wine complexity can be defined as a layering of diverse aroma compounds within a single wine – the more aromas present, the more complex the wine (Swiegers et al. 2005). In spontaneous fermentations, this complexity is thought to be caused by a higher diversity of non-*Saccharomyces* yeast species and native *S. cerevisiae* strains, as strains may release different secondary metabolites when interacting in combination (Esteve-Zarzoso et al. 2000, Swiegers et al. 2005, Martínez et al. 2007, Valero et al. 2007, Santamaría et al. 2008, Ciani et al. 2010, Orlic et al. 2010, Bokulich and Mills 2013, Jolly, Varela and Pretorius 2014, Benito et al. 2015, Benito et al. 2015, Chambers et al. 2015, Jara, Rojas and Romero 2015, Belda et al. 2016, Belda et al. 2016, Martiniuk et al. 2016). Persistence of non-*Saccharomyces* yeast in fermentations has been also been correlated to the

perception of increased wine quality (Domizio et al. 2007). Riesling, in particular, has shown greater typicity and fruitiness in fermentations where both non-*Saccharomyces* and *S. cerevisiae* were present (Benito et al. 2015). This may be due to the ability of some non-*Saccharomyces* yeast species to release bound aroma precursors from grapes via enzyme production (Jackson and Lombard 1993, Romano 2003, Swiegers et al. 2005, Swiegers and Pretorius 2005, Molina et al. 2007, Fleet 2008, Santamaría et al. 2008, Ciani et al. 2010, Baffi et al. 2011, Zott et al. 2011, Jolly, Varela and Pretorius 2014, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Padilla, Gil and Manzanares 2016, Padilla, Gil and Manzanares 2016, Wang et al. 2017). The release of aroma precursors, such as thiols and norisoprenoids, has been linked to increased perception of typical varietal aromas (Jackson and Lombard 1993, Swiegers and Pretorius 2005, Swiegers et al. 2007, Fleet 2008, Santamaría et al. 2008, Ciani et al. 2010, Belda et al. 2016, Padilla, Gil and Manzanares 2016, Wang et al. 2017). Specific fermentation conditions also contribute to yeast interactions and metabolic production of compounds (Barbosa et al. 2014). The choice of a spontaneous versus inoculated fermentation represents a balance between the rewards of increased complexity, varietal aroma characteristics, and regional character, and the risks of problematic fermentation kinetics, potentially detrimental aroma compounds, and overall unpredictability.

Spontaneous fermentations involve a more complex succession of yeast species and strains (Schuller et al. 2005, Díaz et al. 2013, Jolly, Varela and Pretorius 2014). Non-*Saccharomyces* yeast dominate the early fermentation stages, usually starting with low-fermenting apiculate non-*Saccharomyces* yeast, followed by varying abundances and types of primarily aerobic and strong-fermenting non-*Saccharomyces* yeast (Romano 2003, Clemente-Jimenez et al. 2004, Swiegers et al. 2005, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Sun et al. 2009, Tofalo et al. 2012,

Jolly, Varela and Pretorius 2014, Chambers et al. 2015, Ubeda-Iranzo et al. 2015, Martiniuk et al. 2016, Padilla, Gil and Manzanares 2016). The decrease in non-*Saccharomyces* yeast species diversity and abundance during fermentation occurs for numerous biotic and abiotic reasons, although some species and strain survive better than others (Bisson 1999, Bisson and Butzke 2000, Pramateftaki, Lanaridis and Typas 2000, Romano 2003, Díaz et al. 2013, Boynton and Greig 2014, García-Ríos et al. 2014, Jolly, Varela and Pretorius 2014, Rodríguez-Sifuentes et al. 2014, Tristezza et al. 2014, Masneuf-Pomarede et al. 2016). Some non-*Saccharomyces* genera associated with fermentations include *Brettanomyce*s, *Candida, Hanseniaspora*, *Metschnikowia, Pichia, Rhodotorula*, *Torulaspora*, and *Zygosaccharomyces* (Cocolin, Bisson and Mills 2000, Esteve-Zarzoso et al. 2000, Fleet 2003, Clemente-Jimenez et al. 2004, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Urso et al. 2008, Pinto et al. 2015, Padilla, Gil and Manzanares 2016). *S. cerevisiae* typically dominates the late stages of fermentation, although non-*Saccharomyces* yeast have also been observed in their non-dominant fermentation stage (Cocolin, Bisson and Mills 2000, Romano 2003, Clemente-Jimenez et al. 2004, Swiegers et al. 2005, Barata et al. 2008, Santamaría et al. 2008, Sun et al. 2009, Li et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Tofalo et al. 2012, Bezerra-Bussoli et al. 2013, Díaz et al. 2013, Chambers et al. 2015, Ubeda-Iranzo et al. 2015, Martiniuk et al. 2016). Spontaneous fermentations frequently include multiple *S. cerevisiae* strains, with sequential replacement of strains over the course of the fermentation, and may or may not involve a dominant strain at any point during the fermentation (Schuller et al. 2005, Mercado et al. 2010, Schuller et al. 2012, Tristezza et al. 2014). Similar to yeast species succession, *S. cerevisiae* strain succession during fermentation is complex and not well understood, but may be partially related to the preferential growth of different strains at different temperatures (Gonçalves et al. 2011). Fermentations

with a single dominant *S. cerevisiae* strain, several co-dominant strains, or a plurality of strains have been observed (Valero et al. 2007). The presence of higher *S. cerevisiae* strain diversity has been linked an increase in the chance of a successful spontaneous fermentation (Schuller et al. 2012), possibly due to an increase in sugar consumption efficiency (Ciani and Comitini 2011).

During fermentation numerous biotic and abiotic factors affect the succession of yeast species and strains (Bisson 1999, Bisson and Butzke 2000, Pramateftaki, Lanaridis and Typas 2000, Torija et al. 2001, Romano 2003, Tofalo et al. 2009, Cavazza, Poznanski and Guzzon 2010, Cordero-Bueso et al. 2011, Boynton and Greig 2014, García-Ríos et al. 2014, Jolly, Varela and Pretorius 2014, Rodríguez-Sifuentes et al. 2014, Tristezza et al. 2014, Padilla, Gil and Manzanares 2016). Factors that tend to favor non-*Saccharomyces* include low fermentation temperatures, low ethanol concentrations, high initial sugar concentration, and minimal additions of SO_2 (Bisson and Butzke 2000, Di Maro, Ercolini and Coppola 2007, Goddard 2008, Ciani et al. 2010, Ciani et al. 2010, Bokulich et al. 2012, García et al. 2016). The persistence of non-*Saccharomyces* species in fermentation appears to be partially dependent on when *S. cerevisiae* reaches peak biomass; the lower the final *S. cerevisiae* concentration and the longer it takes to reach peak biomass, the longer the non-*Saccharomyces* species persist (Ciani, Beco and Comitini 2006). Long lag times are typical of spontaneous fermentations, as they generally have low initial levels of *S. cerevisiae* due to their rarity on grapes (Bisson and Butzke 2000, Drumonde-Neves et al. 2017). Non-*Saccharomyces* yeast species are more commonly observed on grapes than *Saccharomyces* species, and *S. cerevisiae* is rarely observed on grapes (Cordero-Bueso et al. 2011, Setati et al. 2013). However, as little as 100 to 1000 cells/mL of *S. cerevisiae* present initially can produce a fermentation in which *S. cerevisiae* eventually dominates (Bisson and Butzke 2000). Basidiomycetous yeast, such as

Cryptococcus and *Rhodotorula*, are more often observed on unripe grapes, while ascomycetous yeast, such as *Candida, Hanseniaspora, Metschnikowia*, and *Pichia*, are more often observed on ripe grapes (Barata, Malfeito-Ferreira and Loureiro 2012). Ascomycetous yeast are more commonly observed in fermentations, but basidiomycetous yeast have occasionally been identified (Díaz et al. 2013, Setati et al. 2013). Undamaged ripe grapes favor apiculate and primarily aerobic yeast, while damaged ripe grapes favor strongly fermentative yeast, such as *Saccharomyces* and *Zygosaccharomyces* (Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013).

Ecological studies have reported regional differences in yeast species and strain diversity and composition (Torija et al. 2001, Clemente-Jimenez et al. 2004, Combina et al. 2005, Barata et al. 2008, Chavan et al. 2009, Sun et al. 2009, Zhang et al. 2010, Baffi et al. 2011, Li et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Díaz et al. 2013, Bokulich et al. 2014, Sun et al. 2014, Alessandria et al. 2015, Pinto et al. 2015, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Bokulich et al. 2016, Garofalo et al. 2016, Drumonde-Neves et al. 2017). Support for regional differences in yeast species composition exists at several levels – between subregions of a single winemaking region (Bokulich et al. 2014, Pinto et al. 2015, Sun et al. 2015, Belda et al. 2016, Drumonde-Neves et al. 2017), between vineyards in a single subregion (Barata, Malfeito-Ferreira and Loureiro 2012, Alessandria et al. 2015), and between and within vineyard rows in a single vineyard (Valero et al. 2007, Setati et al. 2013, Bokulich et al. 2014) (Torija et al. 2001, Schuller et al. 2005). Intra-vineyard yeast species and *S. cerevisiae* strain composition variations may be caused by microvariations in environmental conditions, the age and size of the vineyard, and grape variety differences (Schuller and Casal 2007, Valero et al. 2007, Cubillos et al. 2009, Schuller et al. 2012, Dapporto et al. 2016). Additionally, the intra-vineyard variations in grape ripeness and damage can significantly change the yeast

composition observed (Combina et al. 2005, Schuller et al. 2005, Valero et al. 2007, Francesca et al. 2010, Setati et al. 2013).

The extent of variability in *S. cerevisiae* strain diversity and composition between and within regions is unclear (Vezinhet et al. 1992, Ubeda and Briones 2000, Schuller et al. 2005, Schuller et al. 2007, Goddard et al. 2010, Orlic et al. 2010, Li et al. 2011, Gayevskiy and Goddard 2012, Schuller et al. 2012, Azzolini et al. 2013, Charron et al. 2014, Knight and Goddard 2015, García et al. 2016, Martiniuk et al. 2016). On a global scale, *S. cerevisiae* strains observed in fermentations sourcing grapes from different vineyards within the same region are typically more phylogenetically similar to each other than to *S. cerevisiae* strains observed in fermentations sourcing grapes from different regions. However, this observation does not denote a strict distance-decay relationship, as the distance between vineyards within a region, or between regions, does not predict the phylogenetic similarity of the *S. cerevisiae* strains observed in the associated fermentations (Pramateftaki, Lanaridis and Typas 2000, Schuller and Casal 2007, Valero et al. 2007, Francesca et al. 2010, Gayevskiy and Goddard 2012, Schuller et al. 2012, Bokulich et al. 2014, Taylor et al. 2014, Knight and Goddard 2015, Martiniuk et al. 2016). Variations in *S. cerevisiae* diversity, composition, and succession patterns have even been observed in spontaneous fermentations from different lots of grapes from the same vineyard, in the same vintage (Torija et al. 2001, Clemente-Jimenez et al. 2004, Schuller et al. 2005, Setati et al. 2013, Bokulich et al. 2014). The soundness of the sampled grapes likely impacts the amount of intra-vineyard yeast composition variation observed (Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013).

Differences in yeast diversity and composition have also been observed across vintages to varying degrees (Pramateftaki, Lanaridis and Typas 2000, Torija et al. 2001, Barata, Malfeito-Ferreira and Loureiro 2012, Díaz et al. 2013, Bokulich et al.

2014, Belda et al. 2016). Vintage variations in weather and viticultural practices, as well as vineyard variations in size, age, and grape varieties planted, appear to affect yeast diversity and composition in complex ways (Combina et al. 2005, Schuller et al. 2005, Schuller and Casal 2007, Valero et al. 2007, Cubillos et al. 2009, Cordero-Bueso et al. 2011, Setati et al. 2013, Bokulich et al. 2014, Martiniuk et al. 2016, Drumonde-Neves et al. 2017, Patrignani et al. 2017). Phylogenetic differences in *S. cerevisiae* strains observed in fermentations sourcing grapes from a single vineyard over multiple vintages can be as high as in fermentations sourcing grapes from nearby vineyards in a single vintage, or they can be relatively stable (Vezinhet et al. 1992, Pramateftaki, Lanaridis and Typas 2000, Schuller et al. 2005, Schuller and Casal 2007, Valero et al. 2007, Bokulich et al. 2014, Martiniuk et al. 2016, Drumonde-Neves et al. 2017). This is exacerbated by the observation of *S. cerevisiae* strain succession patterns on grapes during ripening (Schuller et al. 2005), and the increase in both yeast species and *S. cerevisiae* strain diversity on damaged grapes (Combina et al. 2005, Valero et al. 2007, Francesca et al. 2010, Setati et al. 2013). This suggests a natural amount of variation in the types and abundances of *S. cerevisiae* strains present in any spontaneous fermentation, even when controlling for variations in vineyard and vintage (Clemente-Jimenez et al. 2004, Schuller et al. 2005, Valero et al. 2007).

Considerable *S. cerevisiae* strain diversity across vineyards and vintages makes it uncommon for the same *S. cerevisiae* strain to be observed in multiple fermentations (Vezinhet et al. 1992, Pramateftaki, Lanaridis and Typas 2000, Torija et al. 2001, Schuller et al. 2005, Schuller and Casal 2007, Valero et al. 2007, Martiniuk et al. 2016). Shared *S. cerevisiae* strains are more common when a winery completes both spontaneous and inoculated fermentations (Constantí et al. 1997, Ciani et al. 2004, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Martiniuk et al. 2016), when

a region as a whole practices more inoculated fermentations (Torija et al. 2001, Salinas et al. 2010), or when the *S. cerevisiae* strains are part of the resident winery yeast population (Mercado et al. 2007).

Winery equipment and surfaces harbor yeast, which can be transferred to juice or must via direct contact (Jolly, Varela and Pretorius 2014, Drumonde-Neves et al. 2017). The type, persistence, and extent of the yeast on winery equipment and surfaces varies considerably from winery to winery (Beltran et al. 2002, Ciani et al. 2004, Santamaría et al. 2005, Mercado et al. 2007, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Bokulich et al. 2013, Jolly, Varela and Pretorius 2014) (Mercado et al. 2007, Santamaría et al. 2008, Bokulich et al. 2013). Differences in the cleaning and sanitation practices of the individual wineries may account for some of the variability (Santamaría et al. 2008, Oćon et al. 2010, Bokulich et al. 2013, Jolly, Varela and Pretorius 2014). The observation of the same yeast species on winery equipment over time indicates that it has become part of the resident winery microbiome (Sabate et al. 2002, Santamaría et al. 2005, Mercado et al. 2007, Jolly, Varela and Pretorius 2014). Additionally, the higher the frequency of observation the more likely the yeast species will be unintentionally transferred to fermentations. Given the ease that yeast can be transferred from grapes to equipment, and vice versa, fewer observations could be the result of yeast being deposited yearly rather than persisting across vintages (Bokulich et al. 2013, Jolly, Varela and Pretorius 2014, Drumonde-Neves et al. 2017). Both non-*Saccharomyces* yeast species and *S. cerevisiae* strains (native and commercial) have been observed on winery equipment (Martini, Ciani and Scorzetti 1996, Renouf, Claisse and Lonvaud-Funel 2007, Oćon et al. 2010, Bokulich et al. 2013, Drumonde-Neves et al. 2017). 'Escaped commercial yeast' can become part of the resident winery or regional vineyard microbiome, and can influence spontaneous fermentations unintentionally if transferred into the must

(Schuller and Casal 2007, Valero et al. 2007, Goddard et al. 2010, Salinas et al. 2010, Martiniuk et al. 2016). The presence of a commercial *S. cerevisiae* strain in a fermentation does not always indicate its outcompeting native *S. cerevisiae* strains or dominating fermentations, but it can influence wine microbiome (Valero et al. 2007, Urso et al. 2008, Barrajón et al. 2009, Mercado et al. 2010). There are vigorous debates about how many yeast species and strains observed in spontaneous fermentations are contributed by the winery environment (Ciani et al. 2004, Santamaría et al. 2005, Valero et al. 2007, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Bokulich et al. 2013). However, the consensus is that the starting yeast species and strain diversity, composition, and abundances in a spontaneous fermentation are a blend of those found on the grapes and those unintentionally added to the must during winery processing (Ciani et al. 2004, Santamaría et al. 2005, Valero et al. 2007, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Bokulich et al. 2013, Jolly, Varela and Pretorius 2014, Drumonde-Neves et al. 2017).

As yeast species diversity and composition in a fermentation can influence the final wine characteristics, and different regions have distinct yeast, efforts to link the two are ongoing. This concept of 'microbial terroir,' the idea that regional differences in yeast composition lead to distinct regional wine characteristics (Valero et al. 2007, Bokulich et al. 2014, Chambers et al. 2015, Knight and Goddard 2015, Belda et al. 2016, Bokulich et al. 2016), is currently under substantial debate. In particular, questions surrounding the influence of regional yeast composition on regional wine characteristics, and the importance of commercial *S. cerevisiae* strains in the resident winery microbiome (Valero et al. 2007, Barata, Malfeito-Ferreira and Loureiro 2012, Chambers et al. 2015), are of continued interest. The very definition of microbial terroir lacks consensus, with one definition favoring a set of stable, distinct *S. cerevisiae* strains associated with a region across vineyards and vintages (Barata,

Malfeito-Ferreira and Loureiro 2012), and another, that of a regionally distinct yeast composition, including both *S. cerevisiae* and non-*Saccharomyces* yeast, with broad similarities observed across vineyards and vintages (Bokulich et al. 2014). A third proposed definition incorporates the effect of regional yeast composition on both grape development and fermentation outcomes (Knight et al. 2015, Pinto et al. 2015).

To initiate study of Finger Lakes AVA microbial terroir, an ecological survey of yeast species and *S. cerevisiae* strains associated with spontaneously fermenting Riesling sourced from a single vineyard was performed. Grapes, fermentations, and winery equipment from three wineries were sampled during two vintages, and their yeast composition identified. To best assess the *S. cerevisiae* strain diversity in spontaneously fermented Finger Lakes Riesling, a culture-dependent methodology was used for species identification. Culture-dependent methodologies focus on examining yeast composition and genetic diversity, and offer a high degree of taxonomical resolution, but have higher limits of detection (Oćon et al. 2010). This approach was chosen to give a clearer picture of the richness and composition of the major non-*Saccharomyces* yeast species and *S. cerevisiae* strains.

Materials and Methods

Sample Selection and Processing

Samples were collected from three Finger Lakes AVA wineries with spontaneously fermenting, single-vineyard Riesling over the 2015 and 2016 vintages. All wineries regularly performed spontaneous fermentations using single-vineyard Riesling grapes, and also performed inoculated fermentations in the same production space. Three types of samples were collected – grapes from each vineyard used for

fermentation, must from the corresponding fermentations, and swabs from winery equipment.

Grape Samples

Grapes were collected from 7 vineyards (V1-V7) approximately one week prior to harvest. Each grape sample contained an average of 95 sound, undamaged berries, collected using a modified random sampling plan that avoided outer panels. Samples were placed in a plastic bag and manually crushed to allow contact between the skins and the juice immediately prior to further processing.

Winery Fermentation Samples

During fermentation, 50mL juice samples were collected from tank sample valves after discarding the initial 1-2mL. Samples were drawn within the first week post-harvest, following each reduction of 5°Brix, and at 5% residual sugar; additional samples were collected in extended fermentations (Appendix 1). Juice samples were vortexed immediately prior to further processing.

Winery Equipment Samples

Pumps, hoses, cleaning brushes, presses, crushers, destemmers, fermentation tanks, settling tanks, sorting tables, and conveyor belts were sampled using a swab moistened with peptone liquid. The swabs were placed in a sterile tube with additional peptone liquid, then vortexed and the liquid wrung out of the swab as it was removed from the tube immediately prior to further processing. A selection of equipment from each winery was swabbed each year after the start of the harvest season but prior to the time the tracked fruit lots were processed (Appendix 2). All equipment was cleaned according to individual winery procedures prior to sampling.

Sample Processing

All samples were serially diluted and spot plated in duplicate onto three different media plate types, then incubated at room temperature for 14 days. Grape and winery swab samples were plated onto Wallenstein Lab (WL) nutrient media with either propionate (WL+P) or cycloheximide (WL+C) added. Fermentation samples were plated onto WL, WL+C, and lysine media. Resulting microbe colonies were counted and described at two time points during incubation – after 2 to 4 days and after 10 to 14 days. Observed colonies were separated by morphology, and all morphologically unique colonies were isolated. Isolation was performed using the procedure outlined by Fugelsang and Edwards (Fugelsang and Edwards 2007). Isolated colonies were maintained on WL media at approximately 2°C until further processing for identification.

Yeast Identification

DNA was extracted, quantitated, amplified, and sequenced from pure cultures. Colonies were resuspended using YM broth; cellular material was placed into 250 µl of YM broth using a sterile transfer loop. The Qiagen DNAeasy Blood Tissue Kit Supplementary protocol (Qiagen, Valencia, CA) for the purification of total DNA from yeast was used with the following modifications: the first centrifugation step was not performed; after pelleting the spheroblasts the supernatant was discarded; centrifugation after the Buffer AW2 addition was performed for 4 minutes at 19,090 x g; and an extra centrifugation of 1 minute at 19,090 x g was performed after the Buffer AW2 addition. Included in each set of extracted samples was a known *Saccharomyces cerevisiae* sample as a positive control, and YM broth with no added DNA as a negative control.

DNA quantitation was performed using a Qubit Fluorometer utilizing the protocol laid out in the Qubit dsDNA HS assay kit (Thermo Fischer Scientific, Waltham, MA). The 5.8S internal transcribed spacer (ITS) rRNA region was amplified with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al. 1990), in a 50 µl final volume reaction for all samples that had a measurable amount of DNA, with a target of 25 ng of DNA when possible (White et al. 1990, Bokulich et al. 2012, Schoch et al. 2012, Ramírez-Castrillón et al. 2014, Romi et al. 2014, Sofia et al. 2015) using Promega GoTaq Colorless Master Mix (Promega, Madison, WI). Amplification was performed on a Bio-Rad 1000 thermocycler (Bio-Rad, Hercules, CA) with the following amplification protocol: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 s, extension at 72°C for 1 min, and a final extension step of 72°C for 7 min (White et al. 1990, Bokulich et al. 2012, Irinyi et al. 2015). The amplicons were purified when necessary with a QiaQuick PCR Purification Kit and eluted into Buffer EB using the manufacturer's protocol (Qiagen, Valencia, CA). Aliquots of 3 µl of purified amplicon were electrophoretically separated in a 1% (w/v) agarose gel in a 1X TAE buffer and stained with SYBR Safe at 100V for 30 minutes. The bands were visualized under a UV light, and analyzed using a BioRad Molecular Imager Gel Doc XR+ with Image Lab Software (Bio-Rad, Hercules, CA). A 10,000 bp ladder (Bionexus, Oakland, CA) was used as a size standard.

Samples with a single detectable gel band were submitted to the Cornell University Genomics Facility for Sanger sequencing of the rRNA ITS region. Samples were submitted in 96-well plates and each reaction contained between 70 and 200 ng of DNA, depending on fragment size, and 8 pmoles of either ITS1 or ITS4 primer in a final reaction volume of 18 µl. Submitted samples were cyclosequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer Scientific, Waltham,

MA). Resulting ITS sequence data was compared to known sequence data from the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm for species identification (Altschul et al. 1997, Benson et al. 2013). Results with a sequence similarity identity value of 90% of higher were identified to the species level. Results with identity values lower than 90% were reported to the genus level when a taxonomic consensus could be achieved.

Because certain non-*Saccharomyces* yeast species were difficult to distinguish the following closely related species were treated as a single operational taxonomy unit, indicating that the identified isolate may be any of the grouped species: *Candida oleophila/Candida railenensis, Candida zemplinina/Candida stellata*, *Pichia cecembensis/Pichia occidentalis/Pichia kudriazveii, Pichia fermentans/Pichia kluyveri*, *Pichia membranifaciens/Candida californica*, *Rhodotorula gluntinis/Rhodosporidium babjavae , Trigonopsis californica/Trigonopsis cantarellii*, and *Zygoascus hellenicus/Zygoascus meyerae* (Kurtzman, Fell and Boekhout 2011, Tofalo et al. 2012, Jolly, Varela and Pretorius 2014). Additionally, two rarely observed but closely related species of *Metschnikowia pulcherrima* - *M. chrysoperlae* and *M. fructicola* – may have been present but misidentified as *M. pulcherrima* (Kurtzman, Fell and Boekhout 2011).

S. cerevisiae Strain Identification

Possible *S. cerevisiae* or *Saccharomyces* species samples were submitted to ETS Laboratories for DNA microsatellite strain analysis using a six-locus multiplex of a variable number of tandem repeat (VNTR) loci. Five loci were *S. cerevisiae* specific - Sc8132X (Howell et al. 2004, Vaudano and Garcia-Moruno 2008), YOR267C (Vaudano and Garcia-Moruno 2008), C5, C11 and C12b – and used to distinguish between strains. One locus was a ribosomal region conserved across yeast species acting as an

internal yeast standard (IYS), and an amplification positive control. Any sample that developed a VNTR profile was identified as *S. cerevisiae* regardless of the identity result from the NCBI GenBank database. If the NCBI GenBank database and VNTR results were conflicting, the sample was not included in the results.

Samples sharing at least 80% VNTR profile alleles with commercial *S. cerevisiae* strains in an internal ETS database were reported as genetically similar. The reported commercial strains and all sample VNTR profiles were subsequently grouped as matching, similar, potentially similar, or genetically distinct based on the number of shared alleles between the strains (Table 2). Alleles were considered identical if there was a difference of $\leq$3 base pairs, with the following exceptions: locus SC-2 alleles 211 and 214, locus SC-4 alleles 366 and 369, and locus SC-5 316 and 319. These alleles are distinct from each other, but within the 3 base pair allowable range, so a 2 base pair acceptable variance was used for these allele pairs.

Table 2 – Genetic Similarity Criteria for Matching, Similar, Potentially Similar, and Genetically Distinct VNTR profiles of *S. cerevisiae* Strains

Genetic Similarity	Number of shared alleles	Allowed allele additions/deletions
Matching	All	0
Similar	At least 1, at 4 or more loci	2
Potentially Similar	At least 1, at 4 or more loci	4
Genetically Distinct	No shared alleles at 2 or more loci	>4

For matching, similar, and potentially similar profiles both columns criteria must be met. For genetically distinct profiles satisfying either column criteria is sufficient.

Analysis of Fermentations Over Time

All fermentations with more than two sampling points were assessed for the persistence and dominance of the identified yeast species. For non-*Saccharomyces* yeast both general persistence and the individual persistence of each identified species was determined. The general persistence of non-*Saccharomyces* yeast was defined as the last sampling time point where any non-*Saccharomyces* yeast was identified, while the persistence of individual non-*Saccharomyces* yeast species was described by its observational range during fermentation stage, defined as early (up to 21 days post-harvest), middle (21-49 days), and late (>49 days). General dominance of non-*Saccharomyces* yeast was defined as more than 50% of isolates in a single fermentation sample identified as non-*Saccharomyces* yeast, and individual dominance was defined as more than 50% identified as a particular non-*Saccharomyces* species. *S. cerevisiae* was analyzed for the time required to dominate over non-*Saccharomyces* yeast, determined by the first fermentation sampling point where it comprised over 50% of the identified isolates. *S. cerevisiae* strains were assessed for both persistence, the observed range of isolates in a fermentation, and dominance, samples where a *S. cerevisiae* strain was more than 50% of isolates and three or more isolates were present. Additionally, it was determined if each fermentation sample was dominated by native or commercial related strains, based on which strain type had the greater number of isolates in any sample containing three or more isolates.

Results

Yeast Species Identification

A total of 891 unique colonies (337 in 2015 and 554 in 2016) were isolated, of which 670 (284 in 2015 and 386 in 2016) were yeast. Each sample yielded an average of 10 morphologically distinct colonies.

Twenty-three different yeast species were identified (Table 3) and an additional three were observed as unique and distinct, but not identified. While most of the yeast isolates were identified to the species level, 62 isolates (31 each year) could only be identified to the genus level. Isolates identified to the genus level were either *Candida*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, or *Saccharomyces*. Of the 670 isolates determined to be yeast, 234 (123 in 2015 and 111 in 2016) were identified as a non-*Saccharomyces* yeast species or genera.

Table 3 – Identified Yeast Species Isolated from Grape, Fermentation, and Equipment Samples

Aureobasidium pullulans	*Pichia fermentans/Pichia kluyveri*
Candida boidnii	*Pichia membranifaciens/Candida californica*
Candida flavescens	*Rhodotorula gluntinis/Rhodosporidium babjavae*
Candida oleophila/Candida railenensis	*Rhodotorula mucilaginosa*
Candida zemplinina/Candida stellata	*Saccharomyces cerevisiae*
Candida flavescens	*Torulaspora delbrueckii*
Hannaella oryzea	*Trigonopsis californica/Trigonopsis cantarellii*
Hanseniaspora uvarum	*Wickerhamomyces anomalus*
Hanseniaspora valbyensis	*Zygoascus hellenicus/Zygoascus meyerae*
Metschnikowia pulcherrima	*Zyosaccharomyces bailii*
Meyerozyma guilliermondii	*Zygosaccharomyces parabailii*
Pichia cecembensis/Pichia occidentalis/Pichia kudriazveii	

S. cerevisiae Strain Identification

A total of 437 isolates, 162 from 2015 and 275 from 2016, were sent to ETS Laboratories for strain analysis. Of these, 415 (159 from 2015 and 256 from 2016) were confirmed to be *S. cerevisiae* and strain VNTR profiles were developed; all were from fermentation isolates except four from 2016 equipment swab isolates. VNTR profiles were not developed for the remaining 22 isolates; two were a closely related *Saccharomyces* species distinct from *S. cerevisiae*, six were removed due to discrepancies in the NCBI GenBank and VNTR analyses, one was determined to not be yeast, and the remaining 13 could not be analyzed. The later 14 were removed from the *S. cerevisiae* strain analysis but included in the overall data as *S. cerevisiae* or a *Saccharomyces* species based on the NCBI GenBank analysis.

The 415 VNTR strain profiles yielded 171 matching profile groups (MPG), each with a variable number of isolates; these groups were given unique designations Cornell 1 to Cornell 171, abbreviated as C1 to C171 (Table 4). Seventy MPG were unique to 2015, and 90 to 2016, with 11 observed in both vintages (Table 5).

ETS Laboratories reported 25 commercial *S. cerevisiae* strains with at least 80% allelic similarity to one or more sample profile (Table 6). Four commercial strain profile pairs matched, two groups of commercial profiles were similar, and two groups were potentially similar (Table 7). The remaining commercial strains were genetically distinct. It should be noted that two distinct VNTR profiles for Fermol Premier Cru were listed on two different reports, differing by the presence or absence of the SC-1 locus 174 allele; they will be described as Fermol Premier Cru (w 174) and Fermol Premier Cru (wo 174), respectively. While it is likely that the commercial product contains a mixture of Fermol Premier Cru (w 174) and Fermol Premier Cru (wo 174), there are other possible explanations, such as the commercial yeast product

changing over time, so they were considered related but separate *S. cerevisiae* strains.

Table 4 – Matching *S. cerevisiae* Profile Groups

Matching Profile Group	Number of Isolates Per Group	Total Number of Isolates
C1	36	36
C2	26	26
C3	23	23
C4	20	20
C5, C6, C7	15	45
C8*	12	12
C9	11	11
C10, C11	6	12
C12, C13	5	10
C14 to C21	4	32
C22 to C30	3	27
C31 to C50	2	40
C51 to C171*	1	121

*Matching profile groups C8, C93, and C103 contained isolates from winery equipment swab samples, all others from fermentation samples

Table 5 – *S. cerevisiae* Matching Profile Group Observation by Vintage

Vintage Observed	Matching Profile Group
2015 only	C7, C9, C14, C15, C21-C23, C28-C32, C34, C36-C38, C42, C44, C47, C51, C53-C60, C62-C72, C85, C91, C92, C94, C99-C101, C111, C114, C119, C120, C130, C131, C133, C134, C136-C141, C161-C167, C170, C171
2016 only	C1-C4, C8*, C13, C16, C17, C19, C20, C24-C26, C39, C40, C41, C43, C49, C50, C52, C61, C73-C84, C86-C90, C93*, C95-98, C102-C110*, C112, C113, C115-C118, C121-C129, C132, C135, C142-C160, C168, C169
2015 and 2016	C5, C6, C10-C12, C18, C27, C33, C35, C45, C46, C48

*Matching strain groups C8, C93, and C103 contained isolates from winery equipment swab samples, all others from fermentation samples

Table 6 – Commercial *S. cerevisiae* Strains Identified by ETS Laboratory Screening

Anchor Vin7	Fermol Complet Killer	Lallemand Enoferm M2	Lalvin Rhone 4600
Erboferm Rouge	Fermol Premier Cru	Lallemand Enoferm RP15	Lalvin W15
Erboferm Structure	Fermol Sauvignon	Lalvin CY3079	Premium Blanc 12V
Erboferm TM Freddo	Laffort Zymaflore Xpure	Lalvin ICV D21	Red Star Pasteur Red
Fermol Arome Plus	Lallemand Enoferm CSM	Lalvin ICV D254	Vitilevure 3001
Fermol Blanc	Lallemand Enoferm L2226	Lalvin ICV GRE	Vitilevure Elixir

Table 7 – Matching, Similar, and Potentially Similar Commercial *S. cerevisiae* Strains

Commercial Strain	Matches	Similar	Potentially Similar
Erboferm Structure	Red Star Pasteur Red	NA	NA
Erboferm TM Freddo	Lalvin Rhone 4600	Fermol Blanc, Fermol Complet Killer	NA
Fermol Blanc	NA	Erboferm TM Freddo, Lalvin Rhone 4600, Fermol Complet Killer	NA
Fermol Complet Killer	NA	Erboferm TM Freddo, Lalvin Rhone 4600, Fermol Blanc	NA
Fermol Premier Cru (w 174)	Lalvin ICV D254	Fermol Premier Cru (wo 174)	Lalvin CY3079, Lalvin ICV D21
Fermol Premier Cru (wo 174)	NA	Fermol Premier Cru (w 174)	Lalvin CY3079, Lalvin ICV D21
Lallemand Enoferm M2	Lalvin W15	NA	NA
Lalvin CY3079	NA	NA	Fermol Premier Cru (w 174), Fermol Premier Cru (wo 174), Lalvin ICV D254

Lalvin ICV D21	NA	NA	Fermol Premier Cru (w 174), Fermol Premier Cru (wo 174), Lalvin ICV D254
Lalvin ICV D254	Fermol Premier Cru (w 174)	Fermol Premier Cru (wo 174)	Lalvin CY3079, Lalvin ICV D21
Lalvin Rhone 4600	Erboferm TM Freddo	NA	NA
Lalvin W15	Lallemand Enoferm M2	NA	NA
Premium Blanc 12V	NA	NA	Vitilevure 3001
Red Star Pasteur Red	Erboferm Structure	NA	NA
Vitilevure 3001	NA	NA	Premium Blanc 12V

The comparison of C1-C171 to the commercial strains yielded 43 MPG that were at least potentially related to a commercial strain - 9 matching a commercial strain, 22 similar to a commercial strain, and 12 potentially similar to a commercial strain groups (Table 8). The remaining 128 MPG were genetically distinct from the reported commercial strains, and were considered native strains (Table 9).

Table 8 – Matching Profile Groups Matching, Similar, or Potentially Similar to a Commercial Strain

MPG	Matches	Similar	Potentially Similar
C2	Erboferm TM Freddo/Lalvin Rhone 4600	Fermol Blanc, Fermol Complet Killer	NA
C3	Fermol Premier Cru (w 174)/Lalvin ICV D254	NA	Lalvin ICV D21, Lalvin CY3079
C4	Fermol Blanc	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Complet Killer	NA

C5	NA	NA	Premium Blanc 12V
C6	Vitilevure 3001	NA	Premium Blanc 12V
C7	Lalvin ICV GRE	NA	
C8	Fermol Premier Cru (wo 174)	Fermol Premier Cru (w 174)/Lalvin ICV D254	Lalvin ICV D21
C12	NA	Fermol Sauvignon	NA
C14	NA	Lalvin W15, Lallemand Enoferm M2	NA
C18	NA	NA	Laffort Zymaflore Xpure
C23	Erboferm Structure/Red Star Pasteur Red	NA	NA
C27	NA	Anchor Vin7	NA
C28	NA	Lallemand Enoferm L2226	NA
C33	NA	Lallemand Enoferm CSM	NA
C45	NA	NA	Lalvin D254
C46	NA	NA	Premium Blanc 12V
C48	NA	NA	Lalvin CY3079, Premium Blanc 12V
C50	NA	NA	Fermol Sauvignon
C81	NA	NA	Fermol Sauvignon
C105	NA	NA	Fermol Sauvignon
C113	NA	Fermol Premier Cru (w 174)/Lalvin ICV D254	Lalvin ICV D21, Lalvin CY079
C114	NA	NA	Laffort Zymaflore Xpure, Lalvin CY3079, Lalvin D254
C115	NA	NA	Laffort Zymaflore Zpure
C117	NA	Premium Blanc 12V	Vitilevure 3001
C118	NA	NA	Premium Blanc 12V
C119	NA	Vitilevure 3001	NA
C120	NA	Vitilevure 3001	Premium Blanc 12V
C121	NA	NA	Vitilevure 3001

C122	NA	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc	Fermol Complet Killer
C123	NA	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet Killer	NA
C124	NA	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc	Fermol Complet Killer
C125	NA	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet Killer	NA
C126	NA	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet Killer	NA
C127	NA	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet Killer	NA
C128	NA	Fermol Blanc, Fermol Complet Killer	Erboferm TM Freddo/Lalvin Rhone 4600
C129	NA	Anchor Vin7	NA
C130	Lallemand Enoferm L2226	NA	NA
C131	NA	Lallemand Enoferm L2226	NA

C133	NA	Erboferm Structure/Red Star Pasteur Red	NA
C134	NA	Lalvin ICV GRE	NA
C135	NA	Lalvin ICV GRE	NA
C143	Lallemand Enoferm RP15	NA	NA
C144	NA	Lallemand Enoferm RP15	NA

NA – not applicable, none identified

Table 9 – Matching Profile Groups Considered Native and Commercial Related

Type	Matching Profile Group
Native *S. cerevisiae* strains	C1, C9-C11, C13, C15-C17, C19-C22, C24-C26, C29-C32, C34-C44, C47, C49, C51-C80, C82-C104, C106-C112, C116, C132, C136-C142, C145-C171
Commercial related *S. cerevisiae* strains	C2-C8, C12, C14, C18, C23, C27, C28, C33, C45, C46, C48, C50, C81, C105, C113-C115, C117-C131, C133-C135, C143, C144

Of the 171 MPG, 103 could be categorized into 27 similar profile groups (SPG). The remaining 68 MGP were not similar to any other groups (Appendix 3). The isolates in the SPG were given unique identifiers – S1 to S27 (Table 10). SPG in both vintages were split evenly between two types – five that contained a MPG observed in both vintages, and five deemed similar due to the combination of MPG they encompassed (Table 11).

Of the 171 MPG, 138 could be further categorized into 6 potentially similar profile groups (PSPG). The remaining 33 MPG were genetically distinct (Appendix 3). The isolates in the PSPG were given unique identifiers - PS1 to PS6 (Table 12). Two PSPG encompassed MPG and SPG from 2015 only, while the remaining PSPG contained combinations of MPG and SPG found in both vintages (Table 13).

Table 10 – Similar Profile Groups That Contain More Than One Matching Profile Group

Similar Profile Group	Included MPG	Number of Isolates
S1	C1, C13, C20, C24, C25, C26, C39, C40, C73, C74, C75, C76, C77, C78, C79, C80, C81, C82, C83, C84, C85, C86	72
S2	C5, C18, C45, C46, C47, C48, C114, C115, C116, C117, C118	32
S3	C2, C4, C122, C123, C124, C125, C126, C127, C128	53
S4	C15, C51, C52, C53	7
S5	C43, C95, C96, C97	5
S6	C3, C8, C113	36
S7	C6, C119, C120	17
S8	C7, C134, C135	17
S9	C12, C104, C105	7
S10	C17, C71, C72	6
S11	C22, C170, C171	5
S12	C28, C130, C131	5
S13	C44, C102, C103	4
S14	C62, C63, C64	3
S15	C10, C61	7
S16	C21, C55	5
S17	C23, C133	4
S18	C27, C129	4
S19	C35, C59	3
S20	C37, C70	3
S21	C41, C87	3
S22	C42, C94	3
S23	C49, C121	3
S24	C50, C155	3
S25	C88, C89	2
S26	C139, C140	2
S27	C143, C144	2

Table 11 – Similar Profile Group Observation by Vintage

Vintage Observed	Similar Profile Group
2015 only	S11, S12, S14, S16, S17, S20, S22, S26
2016 only	S3, S5, S6, S21, S23, S24, S25, S27
2015 and 2016	S1, S2, S4, S7, S8, S9, S10, S13, S15, S18, S19

Table 12 – Potentially Similar Profile Groups That Contain More Than One Matching Profile Group

Potentially Similar Profile Group	Including Matching and Similar Profile Groups	Total Number of Isolates
PS1	C16, C19, C34, C36, C38, C57, C58, C60, C65, C66, C67, C68, C69, C90, C91, C92, C93, C98, C99, C100, C101, C106, C108, C109; S1, S5, S9, S10, S13, S14, S15, S19, S20, S21, S22, S25	151
PS2	S2, S6, S7, S23	88
PS3	C9, C111, C112	13
PS4	C31, C32, C54, C56; S16	11
PS5	C132, C168; S12	7
PS6	C30, C167	4

Table 13 – Potentially Similar Profile Groups by Vintage

Vintage Observed	Potentially Similar Profile Group
2015 only	PS4, PS6
2016 only	NA
2015 and 2016	PS1, PS2, PS3, PS5

The inclusion of any commercial related *S. cerevisiae* strain (CRS) in a SPG or PSPG may indicate a possible genetic relationship of the commercial strain to all strains in the larger group, even if a direct comparison did not show a possible relationship. This tiered comparison increased the potential for unrelated strains to be associated

with commercial strains, but limited the possibility of missing a sample strain that is truly related to a commercial strain. The analysis yielded 10 SPG and 3 PSPG possibly related to commercial strains (Tables 14 and 15).

Table 14 – Similar Profile Groups Encompassing a Commercial Related Matching Profile Group

Similar Profile Group	Commercial Related Matching Profile Group	Similar Commercial Strain	Potentially Similar Commercial Strain
S2	C117	NA	Premium Blanc 12V
S3	C2, C4, C122, C123, C124, C125, C126, C127, C128	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc	Fermol Complet Killer
S6	C3, C8, C113	Fermol Premier Cru (w 174)/Lalvin ICV D254, Fermol Premier Cru (wo 174)	NA
S7	C6, C119, C120	Vitilevure 3001	NA
S8	C7, C134, C135	Lalvin ICV GRE	NA
S9	C12, C105	NA	Fermol Sauvignon
S12	C28, C130, C131	Lallemand Enoferm L2226	NA
S17	C23, C133	Erboferm Structure/Red Star Pasteur Red	NA
S18	C27, C129	NA	Anchor Vin7
S27	C143, C144	Lallemand Enoferm RP15	NA

NA – not applicable

Table 15 – Potentially Similar Profile Groups Encompassing a Commercial Related Matching Profile Group

Potentially Similar Profile Group	Commercial Related Matching Profile Group	Potentially Similar Commercial Strain
PS1	C12, C105	Fermol Sauvignon
PS2	C117, C3, C8, C113, C6, C119, C120	Fermol Premier Cru (w 174)/Lalvin ICV D254, Fermol Premier Cru (wo 174), Premium Blanc 12V, Vitilevure 3001
PS5	C28, C130, C131	Lallemand Enoferm L2226

Grapes

A total of 11 grape samples (5 in 2015 and 6 in 2016) were collected from seven vineyards (Table 16), yielding 74 unique colonies isolates. Each sample yielded an average of seven morphologically distinct colonies, of which 51 were identified as one of seven different yeast species. Yeast species diversity was higher in 2015 than 2016, and no unique yeast species were observed in 2016 (Table 17). It should be noted that 19 isolates could only be identified to the genus level, all belonging to *Hanseniaspora*. Most isolates, 35 out of 51, were identified as *H. uvarum* or as in the *Hanseniaspora* genus.

Yeast types from grape samples varied by vineyard, but all included *H. uvarum* or an isolate from the *Hanseniaspora* genus (Table 17). Other species were observed less frequently and inconsistently across vineyards, and across years within the same vineyard. In vineyards tested in two consecutive vintages, only *H. uvarum* was consistently observed both years (Table 17).

Table 16 – Grape Samples Collection by Vineyard, Winery, and Vintage

Vintage	Vineyard	Processing Winery
2015	1	A
	2	
	3	
	4	B
	5	
2016	1	A
	2	
	3	
	4	B
	6	C
	7	

Table 17 – Non-*Saccharomyces* Yeast Species Observed on Grape Samples from Vineyards 1-7 in 2015 and 2016

Vineyard	V1		V2		V3		V4		V5	V6	V7
Vintage	15	16	15	16	15	16	15	16	15	16	16
C. zemplinina/C. stellata									X		X
C. flavescens					X						
H. uvarum	X	X	X		X	X	X	X	X		X
*Hanseniaspora**		X				X	X		X	X	X
M. pulcherrima	X										
P. cecembensis/ P. occidentalis /P. kudriazveii									X		
P. fermentans/ P. kluyveri									X		
R. glutinis/R. babjavae					X						

**Hanseniaspora* to the genus level was identified
Vintage 15 = 2015, 16 = 2016
No yeast was observed on grapes from V2 in 2016

Winery Equipment

Of the 23 equipment swabs (11 in 2015 and 12 in 2016) collected (Appendix 2), 19 yielded an average of seven morphologically distinct colonies (Table 18); of these, 97

were unique, and 65 were identified as yeast. Fifteen different yeast species and one unique unidentified species (Unknown Yeast 1) were observed (Table 19). *S. cerevisiae* strains C93 and C103 were found on the conveyor belt swab from Winery A, and both isolates from pump and hose swab from Winery C were identified as strain C8. It should be noted that 11 isolates could only be identified to the genus level – 10 *Hanseniaspora* and one *Pichia*.

Only *H. uvarum* and *T. delbrueckii*, the two species that accounted for the overall highest percentage of isolates identified, were observed on equipment swab samples both years. While the specific yeast species varied between years, the overall diversity was the same, with nine unique species identified each year. However, the 2015 samples contained on average more yeast species per piece of equipment than the 2016 swabs. Five yeast species were observed on equipment at more than one winery (Table 20). It should be noted that there were no more than four yeast species were identified on any one piece of equipment, regardless of winery and vintage.

Table 18 – Winery Equipment Swab Samples Collected by Winery and Vintage

Winery	Vintage	Presses	Tanks	Pumps	Conveyor Belts/Sorting Tables	Siphon	Cleaning Brush
A	2015	2*	1	1	NA	1	NA
	2016	1	2	1	2	NA	NA
B	2015	2	3*	1*	NA	NA	NA
C	2016	1	2	1	1	NA	1

*Includes sample where no colonies were isolated
NA – not applicable, no equipment of this type swabbed

Table 19 – Yeast Species Observed on Equipment Swab Samples by Equipment Type and Vintage

Yeast Species Observed	Presses	Tanks	Pumps	Conveyor Belts/Sorting Tables	Siphon	Cleaning Brush
C. boidnii	NA	NA	NA	2016	NA	NA
C. zemplinina/C. stellata	NA	NA	NA	2016	NA	NA
H. oryzea	NA	2015	NA	NA	NA	NA
H. uvarum	2015	2015	2015	2016	2015	NA
M. pulcherrima	2016	NA	NA	NA	NA	NA
M. guilliermondii	NA	NA	NA	2016	NA	NA
P. cecembensis/P. occidentalis/P. kudriavzevii	NA	2015	NA	NA	2015	NA
P. fermentans/P. kluyveri	NA	NA	NA	NA	2015	NA
P. membranifaciens/C. californica	NA	NA	2015	NA	NA	NA
R. mucilaginosa	NA	2016	NA	NA	NA	NA
S. cerevisiae	NA	NA	2016	2016	NA	NA
T. delbrueckii	2015 and 2016	2016	2015	NA	NA	NA
T. californica/T. cantarellii	2015	NA	NA	NA	NA	NA
W. anomalus	NA	NA	NA	NA	NA	2016
Z. hellenicus/Z. meyerae	NA	2015	NA	NA	NA	NA
Unknown Yeast 1	NA	2015	NA	NA	NA	NA

Presses, tanks, and pumps were tested in both vintages, the siphon was only tested in 2015, and conveyor belt/sorting tables and the cleaning brush were only tested in 2016.

NA – not applicable, not identified

Table 20 – Yeast Species Observed on Winery Equipment Swab Samples by Winery and Vintage

Yeast Species Observed	Observed at Winery A	Observed at Winery B	Observed at Winery C
C. boidnii	NA	NA	2016
C. zemplinina/C. stellata	2016	NA	NA
H. oryzea	NA	2015	NA
H. uvarum	2015 and 2016	2015	NA
M. pulcherrima	2016	NA	NA
M. guilliermondii	2016	NA	2016
P. cecembensis/P. occidentalis/P. kudriavzevii	2015	NA	NA
P. fermentans/P. kluyveri	2015	NA	2016
P. membranifaciens/C. californica	2015	NA	NA
R. mucilaginosa	NA	NA	2016
S. cerevisiae	2016	NA	2016
T. delbrueckii	2015 and 2016	2015	2016
T. californica/T. cantarellii	NA	2015	NA
W. anomalus	NA	NA	2016
Z. hellenicus/Z. meyerae	2015	NA	NA
Unknown Yeast 1	2015	NA	NA

Equipment at Winery A was tested in both vintages, equipment at Winery B was only tested in 2015, and equipment at Winery C was only tested in 2016.
NA – not applicable, not identified

Fermentations

Ten wines (five in each vintage) using grapes from one of the sampled vineyards were tracked throughout fermentation (F1-F10), for a total of 64 fermentation samples (25 in 2015, and 39 in 2016) (Table 21, Appendix 2). F9 was unique, as Winery A used grapes from a subsection of V2 identified by the proprietor as having unique organoleptic properties. The grapes from this subsection were harvested after the

grapes used for F7. One sample from F1 was not included in the research results due to a labelling error. Samples were not taken after the first week of F4, or from the fermentations using grapes from V4 and V7 in 2016 as the winemakers chose to inoculate these fermentations, disqualifying them from further inclusion in the research. Samples from F4 taken prior to inoculation were used for non-*Saccharomyces* species identification, but not *S. cerevisiae* strain comparisons.

Table 21 – Tracked Fermentations

Fermentation	Grapes from	Processed by	Vintage	Number of Samples Collected
F1	V1	Winery A	2015	5[#]
F2	V2			7
F3	V3			6
F4	V4	Winery B	2015	2[+]
F5	V5			5
F6	V1	Winery A	2016	8
F7	V2			9
F8	V3			7
F9	V2*			8
F10	V6	Winery C	2016	7

*F9 used grapes from a subsection of V2
[+]F2 was only tracked until the end of the first week of fermentation
[#]F1 had one sample removed from analysis due to a labelling error

Fermentation samples yielded an average of 12 morphologically distinct colonies, for a total of 720, 554 of which were identified as yeast. Sixteen different yeast species were identified, along with two unique, distinct but unidentified species (Unknown Yeasts 2 and 3). Additionally, 33 isolates could only be identified to the genus level – 23 *Hanseniaspora*, four *Saccharomyces*, three *Metschnikowia*, and two *Candida*. Each fermentation showed a different pattern of identified yeast species, but all included *S. cerevisiae* and most also contained *H. uvarum* (Table 22).

Table 22 – Yeast Species Observed in Fermentation Samples by Fermentation

Fermentation	1	2	3	4	5	6	7	8	9	10
A. pullulans			X							
C. boidnii		X								
C. oleophila/C. railenensis		X								X
C. zemplinina/C. stellata					X				X	
Candida species		X		X						
H. uvarum		X	X	X		X	X	X	X	X
H. valbyensis							X		X	
Hanseniaspora genus	X	X	X	X		X		X	X	X
M. pulcherrima		X	X	X				X		
Metschnikowia species			X	X				X		
P. cecembensis/P. occidentalis/P. kudriavzevii				X	X		X			X
P. fermentans/P. kluyveri				X						
P. membranifaciens/C. californica	X	X	X	X			X		X	
S. cerevisiae	X	X	X	X	X	X	X	X	X	X
Saccharomyces species					X		X		X	
T. californica/T. cantarellii	X		X				X	X		
Z. bailii					X					
Z. parabailii								X		
Unknown Yeast 2			X							
Unknown Yeast 3			X							

Yeast species diversity was higher in 2015 than in 2016; 2015 also had a higher percentage of isolates identified as non-*Saccharomyces*. Eight yeast species were observed in both years; the remaining yeast species were only seen in a single vintage (Table 23).

Table 23 – Yeast Species Observed in Fermentation Samples by Vintage

Vintage	Yeast Species Observed
2015	*A. pullulans, C. boidnii, P. fermentans/P. kluyveri, Z. bailii,* Unknown Yeast 1, Unknown Yeast 2
2016	*H. valbyensis, Z. parabailii*
Both vintages	*C. oleophila/C. railenensis, C. zemplinina/C. stellata, H. uvarum, M. pulcherrima*, *P. cecembensis/P. occidentalis/P. kudriavzevii, P. membranifaciens/C. californica, S. cerevisiae, T. californica/T. cantarellii*

Fruit from five different vineyards was used for tracked fermentations (F1-F10) (Table 21) and included in a comparison of non-*Saccharomyces* yeast observed (Table 24). V1, V2, and V3 includes non-*Saccharomyces* yeast observed in fermentations from both vintages, while V5 results are only from 2015, and V6 from 2016.

Tracked fermentations were processed at three different wineries – for both vintages in Winery A, during 2015 in Winery B, and 2016 in Winery C. The non-*Saccharomyces* yeast results for fermentations processed at each winery was aggregated and compared (Table 25). Isolates identified as belonging to the *Saccharomyces* genus were also observed in fermentations at Winery B, and were determined to likely be a non-*S. cerevisiae Saccharomyces* species or a *S. cerevisiae* hybrid (Table 25).

Most of the fermentation sample isolates, 428 out of 554, were identified as *S. cerevisiae*. Of these, VNTR strain profiles were developed for 411, and 169 MPG were identified. Each fermentation had a unique pattern of multiple *S. cerevisiae* strains. On average, each fermentation contained 21 strains, ranging from three to 47 distinct strains (Table 26). All but one fermentation contained CRS, with at least one and as many as three matching commercial strains (Table 27). It should be noted that C8 included isolates from both fermentation and equipment swab samples.

Table 24 – Yeast Species Observed in Fermentations with Fruit Sourced from Different Vineyards

Fermentation Fruit Sourced from	V1	V2	V3	V5	V6
A. pullulans			X[a]		
C. boidnii		X[a]			
C. oleophila/C. railenensis		X[a]			X
C. zemplinina/C. stellata				X	
Candida species		X[a]			
H. uvarum	X[b]	X[c]	X[c]		X
H. valbyensis		X[b]			
Hanseniaspora species	X[c]	X[c]	X[c]		X
M. pulcherrima		X[a]	X[c]		
Metschnikowia species			X[c]		
P. cecembensis/P. occidentalis/P. kudriavzevii		X[b]		X	X
P. fermentans/P. kluyveri		X[a]			
P. membranifaciens/C. californica	X[a]	X[c]	X[a]		
S. cerevisiae	X[c]	X[c]	X[c]	X	X
Saccharomyces species		X*[b]		X[+]	
T. californica/T. cantarellii	X[a]	X[b]	X[c]		
Z. bailii				X	
Z. parabailii			X[b]		
Unknown Yeast 2			X[a]		
Unknown Yeast 3			X[a]		

**Saccharomyces* species designation given due to NCBI GenBank analysis having less than a 90% identity value

[+]*Saccharomyces* species designation given due to likelihood of *S. cerevisiae* hybrid or distinct *Saccharomyces* species

[a]Yeast species observed in 2015 fermentation only

[b]Yeast species observed in 2016 fermentation only

[c]Yeast species observed in both 2015 and 2016 fermentations

Table 25 – Yeast Species Observed in Fermentations by Processing Winery and Vintage

Fermentation Processing Winery and Vintage	Winery A 2015	Winery A 2016	Winery B 2015	Winery C 2016
A. pullulans	X			
C. boidnii	X			
C. oleophila/C. railenensis	X			X
C. zemplinina/C. stellata			X	
Candida species	X		X	
H. uvarum	X	X	X	X
H. valbyensis		X		
Hanseniaspora species	X	X	X	X
M. pulcherrima	X	X	X	
Metschnikowia species	X	X	X	
P. cecembensis/P. occidentalis/P. kudriavzevii		X	X	X
P. fermentans/P. kluyveri	X		X	
P. membranifaciens/C. californica	X	X	X	
S. cerevisiae	X	X	X	X
Saccharomyces species		X*	X[+]	
T. californica/T. cantarellii	X	X		
Z. bailii			X	
Z. parabailii		X		
Unknown Yeast 2	X			
Unknown Yeast 3	X			

*Identity value of less than 90% in NCBI GenBank analysis
[+]Likely *S. cerevisiae* hybrid or distinct *Saccharomyces* species

All of the tracked fermentations used for *S. cerevisiae* strain analysis contained at least one MPG in common with at least one other fermentation (Table 28). No fermentation shared all its MPG with another fermentation, and all had at least five MPG not seen in any other fermentation (Table 29). Additional fermentations shared SPG and PSPG (Tables 30 and 31).

Table 26 – Matching *S. cerevisiae* Profile Groups by Fermentation

Fermentation	Number of MPG	Number of Isolates	Matching Profile Groups
1	25	35	C5#, C12+, C14+, C23*, C31-C34, C36, C38, C42, C44, C47, C51, C54, C57, C58, C60, C71, C94, C101, C133+, C136-C138
2	40	60	C9, C11, C14+, C15, C18#, C21, C27+, C28+, C29-C32, C35, C37, C38, C45#, C48#, C53, C56, C62-C70, C91, C100, C114#, C130*, C131+, C140, C161, C163-C167
3	19	28	C5, C9-C11, C15, C23*, C27+, C30, C46#, C47, C55, C59, C72, C85, C92, C99, C139, C141, C162
4	3	5	C22, C170, C171
5	6	31	C6*#, C7*, C111, C119+, C120+#, C134+
6	46	72	C1, C2*+, C4*+, C5#, C11, C12+, C13, C16, C18#, C19, C20, C24-C26, C39-C41, C48#, C52, C61, C75, C77, C80, C81#, C84, C87, C88, C106, C107, C109, C110, C112, C116, C127+, C128+#, C132, C142, C144+, C149, C150, C153-C155, C160, C168, C169
7	31	65	C1, C2*+, C4*+, C10, C13, C17, C20, C24, C25, C33+, C40, C45, C76, C83, C86, C96, C97, C105#, C115#, C117+#, C118#, C124+#, C135+, C143*, C145-C148, C151, C152
8	20	27	C1, C2*+, C4*+, C10, C12+, C17, C27+, C35, C43, C78, C79, C82, C90, C98, C102, C104, C126+, C129+, C158, C159
9	19	47	C1, C2*+, C4*+, C5#, C16, C20, C43, C46#, C50#, C73, C74, C89, C95, C108, C122+#, C123+, C125+, C156, C157
10	6	40	C3*#, C6*#, C8*+#, C49, C113+#, C121#

*Indicates MPG matches a commercial strain

+Indicates MPG is similar to a commercial strain

#Indicates MPG is potentially similar to a commercial strain

Table 27 – Commercial Related *S. cerevisiae* Strains Observed in Fermentations

Fermentation	Matching Commercial Strain	Similar Commercial Strain	Potentially Similar Commercial Strain
F1	Erboferm Structure/Red Star Pasteur Red	Erboferm Structure/Red Star Pasteur Red, Fermol Sauvignon, Lallemand Enoferm M2, Lalvin W15	Premium Blanc 12V
F2	Lallemand Enoferm L2226	Anchor Vin7, Lallemand Enoferm M2, Lallemand Enoferm L2226, Lallemand Enoferm L2226, Lalvin W15	Laffort Zymaflore Xpure, Lalvin D254, Lalvin CY3079, Premium Blanc 12V, Laffort Zymaflore Xpure, Lalvin CY3079, Lalvin D254
F3	Erboferm Structure/Red Star Pasteur Red	Anchor Vin7	Premium Blanc 12V
F4	NA	NA	NA
F5	Lalvin ICV GRE, Vitilevure 3001	Lalvin ICV GRE, Vitilevure 3001	Premium Blanc 12V
F6	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet Killer, Fermol Sauvignon, Lallemand Enoferm RP15	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Sauvignon, Laffort Zymaflore Xpure, Lalvin CY3079, Premium Blanc 12V
F7	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Lallemand Enoferm RP15	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet Killer, Lallemand Enoferm CSM, Lalvin ICV GRE, Premium Blanc 12V	Fermol Complet Killer, Fermol Sauvignon, Laffort Zymaflore Zpure, Premium Blanc 12V, Vitilevure 3001
F8	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc	Anchor Vin7, Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet	

		Killer, Fermol Sauvignon	
F9	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc	Erboferm TM Freddo/Lalvin Rhone 4600, Fermol Blanc, Fermol Complet Killer	Fermol Complet Killer, Fermol Sauvignon, Premium Blanc 12V
F10	Fermol Premier Cru (w 174)/Lalvin ICV D254, Fermol Premier Cru (wo 174), Vitilevure 3001	Fermol Premier Cru (w 174)/Lalvin ICV D254	Lalvin ICV D21, Lalvin CY3079, Premium Blanc 12V, Vitilevure 3001

NA – not applicable, not identified

Table 28 – Matching Profile Groups Observed in More than One Fermentation

Fermentations Observed in	Matching Profile Groups
F1, F2	C14^{+}, C31, C32, C38
F1, F3	C23*, C47
F1, F7	C33^{+}
F2, F3	C9, C15, C30
F2, F6	C18$^{\#}$, C48$^{\#}$
F2, F7	C45$^{\#}$
F2, F8	C35
F3, F9	C46$^{\#}$
F5, F10	C6*$^{\#}$
F6, F7	C13, C24, C25, C40
F6, F9	C16
F7, F8	C17
F8, F9	C43
F1, F6, F8	C12^{+}
F2, F3, F6	C11
F2, F3, F8	C27^{+}
F3, F7, F8	C10
F6, F7, F9	C20
F1, F3, F6, F9	C5$^{\#}$
F6, F7, F8, F9	C1, C2*$^{+}$, C4*$^{+}$

*Indicates MPG matches a commercial strain

$^{+}$Indicates MPG is similar to a commercial strain

$^{\#}$Indicates MPG is potentially similar to a commercial strain

Table 29 – Matching Profile Groups Unique to Each Fermentation

Fermentation	Unique Matching Strain Groups
F1	C34, C36, C42, C44, C51, C54, C57, C58, C60, C71, C94, C101, C133^{+}, C136-C138
F2	C18$^{\#}$, C21, C28^{+}, C29, C37, C53, C56, C62-C70, C91, C100, C114$^{\#}$, C130*, C131^{+}, C140, C161, C163-C167
F3	C55, C59, C72, C85, C92, C99, C139, C141, C162
F5	C7*, C111, C119^{+}, C120$^{+\#}$, C134^{+}
F6	C19, C26, C39, C41, C52, C61, C75, C77, C80, C81$^{\#}$, C84, C87, C88, C106, C107, C109, C110, C112, C116, C127^{+}, C128$^{+\#}$, C132, C142, C144^{+}, C149, C150, C153-C155, C160, C168, C169
F7	C76, C83, C86, C96, C97, C105$^{\#}$, C115$^{\#}$, C117$^{+\#}$, C118$^{\#}$, C124$^{+\#}$, C135^{+}, C143*, C145-C148, C151, C152
F8	C78, C79, C82, C90, C98, C102, C104, C126^{+}, C129^{+}, C158, C159
F9	C50$^{\#}$, C73, C74, C89, C95, C108, C122$^{+\#}$, C123^{+}, C125^{+}, C156, C157
F10	C3*$^{\#}$, C8*$^{+\#}$, C49, C113$^{+\#}$, C121$^{\#}$

Table 30 – Fermentations Where Similar Profile Groups Were Observed

Fermentations	Similar Profile Groups
F1	S22
F2	S12[+], S14, S20
F4	S11
F6	S21
F10	S6[+], S23
F1, F3	S17[+]
F1, F8	S13
F2, F3	S16, S26
F5, F7	S8[+]
F5, F10	S7[+]
F6, F7	S27[+]
F6, F9	S24, S25
F2, F3, F8	S18, S19
F7, F8, F9	S5
F1, F2, F3, F6	S4
F1, F3, F7, F8	S10
F1, F6, F7, F8	S9[#]
F3, F6, F7, F8	S15
F6, F7, F8, F9	S3[+#]
F3, F6, F7, F8, F9	S1
F1, F2, F3, F6, F7, F9	S2[#]

[+]Indicates SPG is similar to a commercial strain

[#]Indicates SPG is potentially similar to a commercial strain

Table 31 – Fermentations Where Potentially Similar Profile Groups Were Observed

Fermentations	Potentially Similar Profile Groups
F2, F3	PS6
F2, F6	PS5[#]
F1, F2, F3	PS4
F2, F3, F5	PS3
F1, F2, F3, F6, F7, F8, F9	PS1[#]
F1, F2, F3, F5, F6, F7, F9, F10	PS2[#]

[#]Indicates PSPG is potentially similar to a commercial strain

Changes in Yeast Species and S. cerevisiae Strain Composition Over Time During Fermentation

The early stages of the fermentations were dominated by non-*Saccharomyces* yeast, ranging from 95% on the first day to 70% during week one, and just under 50% in week three. *S. cerevisiae* tended to dominate in the middle and late fermentation stages, and occasionally as soon as by the end of week two (Figs. 1 and 2). It should be noted that F1 sampling completed at 39 days into fermentation, so F1 did not yield any late stage fermentation sampling points. The largest proportion of the non-*Saccharomyces* yeast were identified as *H. uvarum* or belonging to the *Hanseniaspora* genus.

Non-*Saccharomyces* yeast persistence ranged widely (Table 32), though each non-*Saccharomyces* yeast species had one of two persistence patterns: either continuous or non-continuous observation, the latter with at least one sampling point containing only *S. cerevisiae*. Fermentations with a non-continuous pattern could also be broken into two types – those with the same or different non-*Saccharomyces* yeast species observed on either side of the *Saccharomyces* only sample (Table 32).

Figure 1 – Yeast Species Observed as a Percentage of Isolates Identified by Fermentation Stage

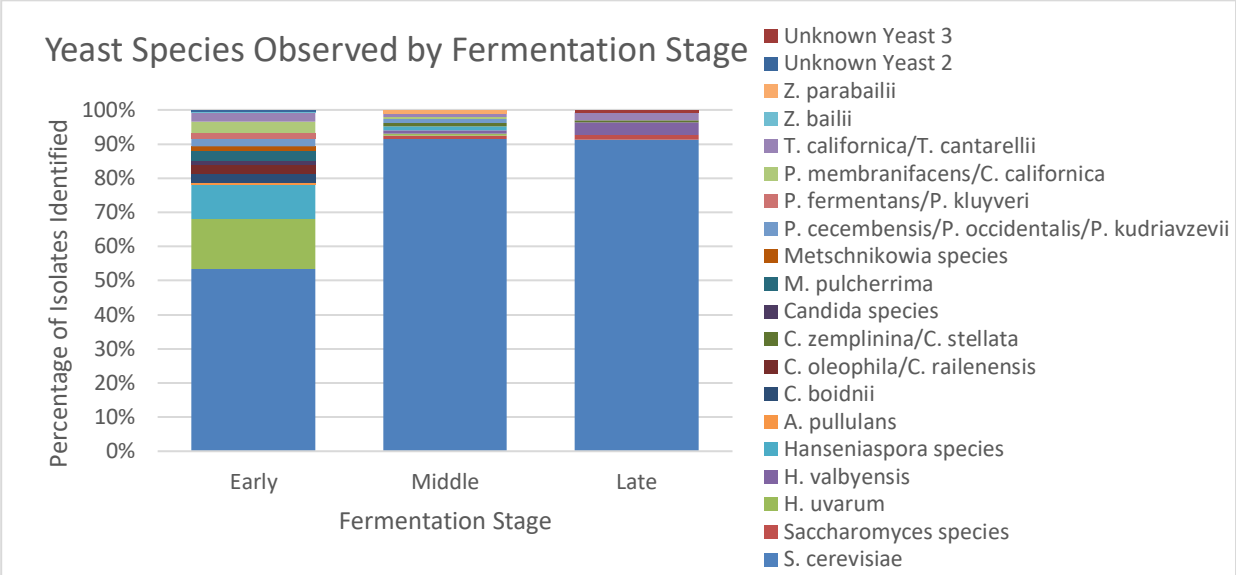

Figure 2 - Yeast Species Observed as a Percentage of Isolates in the First Two Weeks of Fermentation

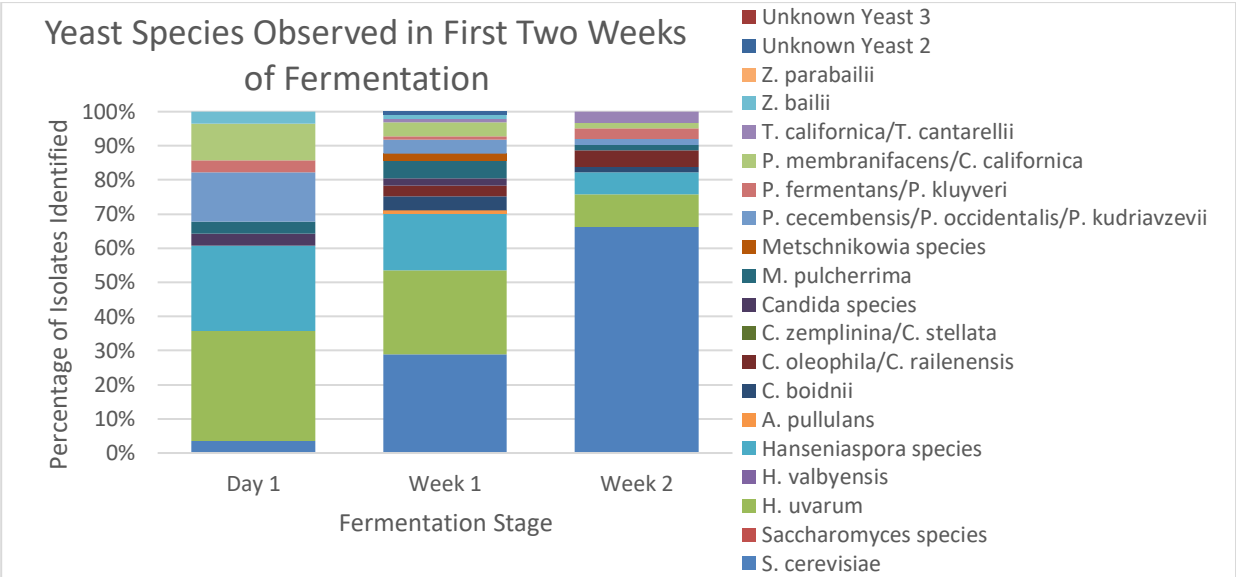

Table 32 – Non-*Saccharomyces* Yeast Species Fermentation Persistence Patterns

Fermentation	Stage Persisted To	Observed Continuously	Observed Non-continuously	
			Same Species	Different Species
1	Middle		X	
2	Early	X		
3	Late			X
5	Middle	X		
6	Early	X		
7	Late			X*
8	Late		X	
9	Late		X	X
10	Early	X		

*Non-continuous persistence of different non-*Saccharomyces* yeast at two time points

Non-*Saccharomyces* yeast species represented by five or more identified isolates, and in two or more fermentations, were used for persistence trend analysis. Seven of the fifteen non-*Saccharomyces* yeast species observed in the fermentation samples met these criteria (Fig. 3). The remaining non-*Saccharomyces* yeast species were examined individually, and the results were interpreted with caution due to the limited number of observations. Each yeast species had its own frequency of observation during the fermentation stages.

S. cerevisiae strain diversity was higher in the middle than in the early or late stages of fermentation regardless of vintage (Table 33). In 2015, there was more strain diversity in the early stage than in the late stage, while the converse was true in 2016.

Figure 3 – Non-*Saccharomyces* Yeast Species Observational Trends by Fermentation Stage

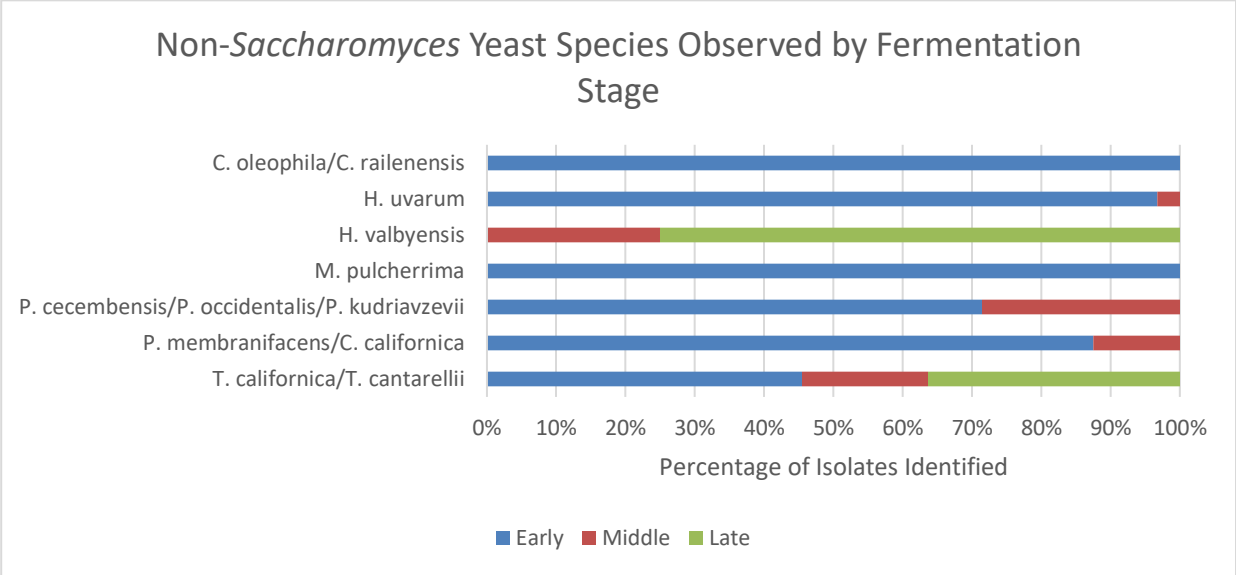

Table 33 – *S. cerevisiae* Strain Diversity by Fermentation Stage

Fermentation Stage	Total Isolates	Total Number of MPG	Matching Profile Groups Observed
Early	100	57	C1, C3, C5, C6, C8, C9, C11, C12, C14, C15, C18, C20-C24, C27, C28, C30-C32, C34, C36-C38, C42-C44, C48, C60, C66, C68, C70-C72, C86, C89, C91, C92, C94, C99, C101, C107, C116, C133, C135, C137, C138, C160-C162, C170, C171
Middle	185	124	C1-C10, C13-C18, C20, C21, C24-C26, C28, C29, C31-C35, C38-C41, C43, C45-C47, C50, C51, C53-C55, C57, C58, C61, C67, C69, C73-C77, C79-C81, C83, C85, C87, C90, C96-C98, C100, C102, C104, C105, C108-C113, C115, C117-C119, C124, C125, C131, C132, C134, C136, C139-C143, C145-C154, C156, C158, C163-C165, C167-C169
Late	127	62	C1-C8, C10-C12, C15, C16, C18, C19, C21, C25-C27, C29, C35, C48-C50, C56, C59, C62-C65, C78, C82, C84, C88, C95, C106, C114, C120-C123, C126-C130, C144, C155, C157, C159, C166

MPG containing more than one isolate were analyzed to determine patterns linked to fermentation stage or number of fermentations (Table 34). There was a fairly even

distribution of MPG observed in most fermentation stages. However, there were fewer MPG observed in the late, or the middle and late, fermentation stages. A majority of MPG were observed in only one or two fermentations; the nine MPG observed in multiple fermentations were also observed throughout all fermentation stages.

Table 34 – Observation of Matching Profile Groups with Multiple Isolates by Fermentation Stage and Number of Fermentations

Fermentation Stage	Number of Fermentations MPG was Observed in			
	1	2	3	4
Early	C22, C36, C37, C42, C44	C23*, C30	NA	NA
Middle	C39, C41	C13, C17, C33^{+}, C40, C45$^{\#}$, C46$^{\#}$, C47	NA	NA
Late	C19, C49	NA	NA	C4*$^{+}$
Early and Middle	C28, C34	C9, C14^{+}, C24, C31, C32, C38, C43	C20	NA
Early and Late	NA	C48$^{\#}$	C11, C27^{+}	NA
Middle and Late	C7*, C26, C29, C50$^{\#}$	C16, C25, C35	NA	C2*$^{+}$
All Stages	C3*$^{\#}$, C8*$^{+\#}$, C21	C6*$^{\#}$, C12^{+}, C15, C18$^{\#}$	C10, C12^{+}	C1, C5$^{\#}$

*Indicates MPG matches a commercial strain
$^{+}$Indicates MPG is similar to a commercial strain
$^{\#}$Indicates MPG is potentially similar to a commercial strain
NA – not applicable

CRS were observed in every fermentation stage, though numbers varied over time. CRS diversity was lower in the early than the middle and late stages, but the proportion of CRS among all strains was lower in the early and middle stages of fermentation and highest in the late stage. This pattern is consistent for all CRS, although most pronounced for CRS matching commercial strains (Table 35). Each tracked fermentation generally followed this pattern of increasing *S. cerevisiae* strain diversity over time, with a higher proportion of CRS present in the late stage.

Table 35 – Native and Commercial Related Strain Comparison by Fermentation Stage

	MPG		SPG		PSPG	
Fermentation Stage	CRS	N to CRS Ratio	CRS	N to CRS Ratio	CRS	N to CRS Ratio
Early	4	14:1	9	6:1	13	4:1
Middle	7	18:1	17	7:1	27	5:1
Late	8	8:1	18	4:1	24	3:1

CRS – Commercial related *S. cerevisiae* strains
N – Native *S. cerevisiae* strains

In most fermentations persistent *S. cerevisiae* strains were a mix of native and CRS; the exceptions being F5, which contained only CRS, and F10, which contained only native strains (Table 36). All fermentations had at least two persistent *S. cerevisiae* strains, and six had at least one persistent strain that matched a commercial strain. Half of these persistent strains were observed continuously, while three fermentations had non-continuous observation of commercial strain(s).

S. cerevisiae strain dominance was analyzed in two ways - native versus CRS, and by individual strains (Table 37). F5 and F10 were the only fermentations to not have at least one sampling point dominated by native strains. Five of the fermentations had no dominant strain at any sampling point. The remaining four fermentations contained two dominant strains each; two contained both a native and CRS dominant strain, while the other two contained only CRS dominant strains.

Tracked fermentations were also compared for unique and shared *S. cerevisiae* strains (Tables 38 and 39), and results for fermentations processed at each winery was aggregated and compared (Table 40).

Table 36 – *S. cerevisiae* Strain Persistence Over Time by Fermentation

Fermentation	Persistent Strain	Native or Commercial?	Number of Time Points Observed	Stages Observed	Observation Pattern
F1+	C5	P Similar C	3	E, M	Continuous
	C14	Similar C	3	E, M	Continuous
	C34	N	2	E, M	Non-C
F2	C18	P Similar C	3	E, M, L	Non-C
	C21	N	3	E, M, L	Non-C
	C29	N	2	M, L	Non-C
	C30	N	2	E	Non-C
F3	C5	P Similar C	4	E, M, L	Continuous
	C9	N	2	E, M	Non-C
F5*	C6	Match C	4	M, L	Continuous
	C7	Match C	4	M, L	Continuous
F6	C1	N	4	E, M, L	Non-C
	C4	Match C	2	M, L	Continuous
	C12	Similar C	2	L	Continuous
	C13	N	2	M	Non-C
	C16	N	2	M, L	Non-C
	C26	N	2	M, L	Continuous
F7	C1	N	4	E, M	Continuous
	C2	Match C	4	M, L	Continuous
	C4	Match C	2	M, L	Non-C
	C10	N	2	M, L	Non-C
	C24	N	2	E, M	Non-C
F8*	C1	N	2	L	Continuous
	C2	Match C	2	M, L	Non-C
	C4	Match C	3	M, L	Continuous
	C10	N	3	M, L	Continuous
	C17	N	2	M	Continuous
F9	C1	N	3	E, M	Non-C
	C2	Match C	3	M, L	Continuous
	C4	Match C	4	M, L	Continuous
	C5	P Similar C	3	M, L	Non-C
	C50	N	2	M, L	Non-C
F10	C3	N	4	E, M, L	Continuous
	C6	N	3	E, M, L	Non-C
	C8	N	3	E, M, L	Non-C

N – Native, Match C – Matches a commercial strain, Similar C – Similar to a commercial strain, P Similar C – Potentially similar to a commercial strain

E – Early stage, M – Middle stage, L – Late stage
Non-C – Non-continuous
[+]Fermentation did not have any late stage samples used for analysis
*Fermentation did not have any early stage samples used for analysis

Table 37 – *S. cerevisiae* Strain Dominance Over Time by Fermentation

Fermentation	Sampling Point Stage	Number of Observed MPG	Dominant Strain Type	Dominant Strain	Strain Type
F1[+]	Early	6	Native	NA	NA
	Early	5	Native		
	Early	8	CRS		
	Middle	10	Native		
F2	Early	1	INC	NA	NA
	Early	3	Native		
	Early	4	CRS		
	Early	7	Native		
	Middle	11			
	Middle	8			
	Late	12			
F3	Early	4	Native	NA	NA
	Early	6			
	Middle	8			
	Middle	3	CRS		
	Late	3			
F5*	Middle	4	CRS	C7	Match C
	Middle	3	CRS		
	Late	3	CRS	NA	NA
	Late	2	CRS	C6	Match C
F6	Early	6	Native	NA	NA
	Early	11			
	Middle	7			
	Middle	10			
	Late	11			
	Late	9	CRS		
F7	Early	2	Native	C1	N
	Early	4		NA	NA
	Middle	5			
	Middle	16			
	Middle	4			
	Middle	4	CRS	NA	NA
	Late	2		C2	Match C

	Late	2		NA	NA
F8*	Middle	5	Native	NA	NA
	Middle	9			
	Late	6			
	Late	7	CRS		
F9	Early	3	Native	C1	N
	Early	2	INC	C1	N
	Middle	4	Native	NA	NA
	Middle	6	CRS	C4	Match C
	Middle	5		NA	NA
	Late	7			
	Late	3			
F10	Early	2	INC	NA	NA
	Early	4	CRS	C3	Match C
	Middle	3		NA	NA
	Middle	2		C3	Match C
	Late	4			
	Late	1		C8	Match C

INC – inconclusive, <3 MPG isolates observed
Match C – Matches a commercial strain
NA – Not applicable
[+]Fermentation did not have any late stage samples used for analysis
*Fermentation did not have any early stage samples used for analysis

Table 38 – Unique Matching *S. cerevisiae* Profile Groups Observed More Than Once in Fermentations with Fruit Sourced from the Same Vineyard

Vineyard	Matching Profile Groups
V1	C19, C26, C34, C36, C39, C41, C42, C44
V2	C21, C28[+], C29, C37, C45[#], C50[#]
V3	NA
V5	C7*
V6	C3*[#], C8*[+#], C49

*Indicates MPG matches a commercial strain
[+]Indicates MPG is similar to a commercial strain
[#]Indicates MPG is potentially similar to a commercial strain

Table 39 – Shared Matching *S. cerevisiae* Profile Groups Between Fermentations with Fruit Sourced from Different Vineyards

Vineyard	Matching Profile Groups
V1, V2	C13, C14^{+}, C16, C18$^{\#}$, C20, C24, C25, C31, C32, C33^{+}, C38, C40, C48$^{\#}$
V1, V3	C12^{+}, C23*, C47
V2, V3	C9, C10, C15, C17, C27^{+}, C30, C35, C43, C46$^{\#}$
V5, V6	C6*$^{\#}$
V1, V2, V3	C1, C2*$^{+}$, C4*$^{+}$, C5$^{\#}$, C11

*Indicates MPG matches a commercial strain
$^{+}$Indicates MPG is similar to a commercial strain
$^{\#}$Indicates MPG is potentially similar to a commercial strain

Table 40 – Matching, Similar, and Potentially Similar Profile Groups by Fermentation Processing Winery

Fermentation Processed at	Matching Profile Groups	Similar Profile Groups	Potentially Similar Profile Groups
Winery A	C1, C2*$^{+}$, C4*$^{+}$, C5$^{\#}$, C9-C11, C12^{+}, C13, C14^{+}, C15-C17, C18$^{\#}$, C19-C21, C23*, C24-C26, C27^{+}, C28^{+}, C29-C32, C33^{+}, C34-C44, C45$^{\#}$, C46$^{\#}$, C47, C48$^{\#}$, C50$^{\#}$-C57, C59-C80, C81$^{\#}$, C82-C92, C94-C102, C104, C105$^{\#}$, C106-C110, C112, C114$^{\#}$, C115$^{\#}$, C116, C117$^{+\#}$, C118$^{\#}$, C122$^{+\#}$, C123^{+}, C124$^{+\#}$, C125^{+}, C126^{+}, C127^{+}, C128$^{+\#}$, C129^{+}, C130*, C131^{+}, C132, C133^{+}, C135^{+}, C136-C142, C143*, C144^{+}-C169	S1, S2$^{\#}$, S3$^{+\#}$, S4, S5, **S8**$^{+}$, S9$^{\#}$, S10, S12^{+}-S16, S17^{+}, S18$^{\#}$-S22, S24-S27^{+}	PS1$^{\#}$, **PS2**$^{\#}$, **PS3**, PS4, PS5, PS6
Winery B	**C6**$^{*\#}$, C7*, C22, C111, C119^{+}, C120$^{+\#}$, C134^{+}, C170, C171	**S7**$^{+}$, **S8**$^{+}$, S11	**PS2**$^{\#}$, **PS3**
Winery C	C3*$^{\#}$, **C6**$^{*\#}$, C8*$^{+\#}$, C49, C113$^{+\#}$, C121$^{\#}$	S6^{+}, **S7**$^{+}$, S23	**PS2**$^{\#}$

*Indicates profile group matches a commercial strain
$^{+}$Indicates profile group is similar to a commercial strain
$^{\#}$Indicates profile group is potentially similar to a commercial strain
Bolded profile groups are shared by at least 2 wineries

Discussion

Grapes

Only non-*Saccharomyces* yeast, and more ascomycetous than basidomycetous species, were observed on the grapes (Table 17). The lack of *S. cerevisiae* is consistent with previous studies showing that it is infrequently observed on sound grapes (Clemente-Jimenez et al. 2004, Cordero-Bueso et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013, Setati et al. 2013, Taylor et al. 2014, Drumonde-Neves et al. 2017). Observing fewer basidomycetous yeast species was also expected as they are more commonly found on unripe grapes, while apiculate and aerobic non-*Saccharomyces* yeast are more common on ripe grapes (Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013).

Non-*Saccharomyces* yeast species composition on grapes showed more variation between vintages than vineyards in the same vintage (Table 17). Non-*Saccharomyces* composition variation was expected as variations have previously been observed between vineyards (Barata, Malfeito-Ferreira and Loureiro 2012, Alessandria et al. 2015) and across vintages, although the extent of the variations was different for each region (Pramateftaki, Lanaridis and Typas 2000, Torija et al. 2001, Barata, Malfeito-Ferreira and Loureiro 2012, Díaz et al. 2013, Bokulich et al. 2014, Belda et al. 2016). The limited variation observed in grape samples between vineyards may be due to the fact that V1, V2, and V3 were all similarly maintained by the same vineyard manager. Different viticultural practices, including spraying schedules and the ripeness of grapes at harvest, have been found to influence the yeast species composition on grapes (Combina et al. 2005, Cordero-Bueso et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013, Patrignani et al. 2017).

Winery Equipment

Yeast was present in most equipment samples, with a high percentage of isolates identified as non-*Saccharomyces* yeast species, and few *S. cerevisiae* (Table 19). This is consistent with previous research showing that winery equipment and surfaces commonly harbor both non-*Saccharomyces* and *Saccharomyces* yeast (Bokulich et al. 2013, Drumonde-Neves et al. 2017), but that the type, persistence, and extent of yeast colonization varies considerably from winery to winery (Beltran et al. 2002, Ciani et al. 2004, Santamaría et al. 2005, Mercado et al. 2007, Santamaría et al. 2008, Oćon et al. 2010, Blanco, Orriols and Losada 2011, Bokulich et al. 2013, Jolly, Varela and Pretorius 2014). The observation of *S. cerevisiae* was notable, since as few as 100 to 1000 cells/mL of *S. cerevisiae* present in the initial must can produce a successful fermentation (Bisson and Butzke 2000).

There was no apparent correlation between the equipment type swabbed and yeast species observed, apart from the widespread observation of *H. uvarum* on many equipment types (Table 19). Winery A had more yeast species diversity (Table 20) but this may be since more samples were taken at this winery. Differences in cleaning and sanitation practices of individual wineries may account for some of the variability in winery yeast composition (Santamaría et al. 2008, Oćon et al. 2010, Bokulich et al. 2013, Jolly, Varela and Pretorius 2014).

Fermentations

As expected, each fermentation contained a unique non-*Saccharomyces* yeast species and *S. cerevisiae* strain composition, with an average of five different non-*Saccharomyces* yeast species (Table 22) and 20 different *S. cerevisiae* strains (Table 26). No single yeast species or strain was observed in all the fermentations (Table 34), as expected from fermentations using different lots of grapes (Setati et al. 2013).

The amount of yeast diversity observed was in line with that found in ecological surveys of other regions (Vezinhet et al. 1992, Ubeda and Briones 2000, Torija et al. 2001, Clemente-Jimenez et al. 2004, Combina et al. 2005, Schuller et al. 2005, Barata et al. 2008, Chavan et al. 2009, Sun et al. 2009, Goddard et al. 2010, Orlic et al. 2010, Zhang et al. 2010, Baffi et al. 2011, Li et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Gayevskiy and Goddard 2012, Azzolini et al. 2013, Bezerra-Bussoli et al. 2013, Díaz et al. 2013, Bokulich et al. 2014, Alessandria et al. 2015, Pinto et al. 2015, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Garofalo et al. 2016, Garofalo et al. 2016, Knight and Goddard 2016, Martiniuk et al. 2016, Drumonde-Neves et al. 2017).

Non-*Saccharomyces* yeast species were more diverse and dominated the early stages of fermentation, particularly during the first week. As a group, non-*Saccharomyces* yeast persisted until the middle or late stages of most fermentations, despite the increasing dominance of *S. cerevisiae* (Figs. 1, 2, 3; Table 32). This is consistent with previous research showing that even in inoculated fermentations non-*Saccharomyces* yeast commonly persist to the middle stage of fermentation (Barata et al. 2008, Santamaría et al. 2008, Barata, Malfeito-Ferreira and Loureiro 2012, Barata, Malfeito-Ferreira and Loureiro 2012, Bezerra-Bussoli et al. 2013), and have also been observed in late stages (Ciani and Maccarelli 1997, Cocolin, Bisson and Mills 2000, Ganga and Martínez 2004, Fleet 2008, Fleet 2008, Li et al. 2011, Bezerra-Bussoli et al. 2013, Díaz et al. 2013, Bokulich et al. 2015, Sun et al. 2015, Liu et al. 2016, Martins Tahim 2016, Patrignani et al. 2017). The observed succession of apiculate non-*Saccharomyces* yeast followed by varying abundances and types of other non-*Saccharomyces* yeast, with an overall decrease in yeast species diversity as *S. cerevisiae* dominates, supports the accepted yeast succession pattern in both spontaneous and inoculated fermentations (Fleet 2003, Romano 2003, Clemente-

Jimenez et al. 2004, Swiegers et al. 2005, Ciani, Beco and Comitini 2006, Di Maro, Ercolini and Coppola 2007, Fleet 2008, Gayevskiy and Goddard 2012, Tofalo et al. 2012, Bezerra-Bussoli et al. 2013, Díaz et al. 2013, Benito et al. 2015, Chambers et al. 2015, Pinto et al. 2015, Ubeda-Iranzo et al. 2015, Martiniuk et al. 2016, Martins Tahim 2016, Padilla, Gil and Manzanares 2016).

Domination by *S. cerevisiae* by the middle stage of fermentation coincided with peak strain diversity (Figs. 1, 2; Table 33). It is common for both spontaneous and inoculated wine fermentations to contain multiple *S. cerevisiae* strains (Constantí et al. 1997, Esteve-Zarzoso et al. 2000, Howell et al. 2006, Valero et al. 2007, Goddard et al. 2010, Blanco, Orriols and Losada 2011, Li et al. 2011, Gayevskiy and Goddard 2012, Francesca et al. 2014, Sun et al. 2015). High strain diversity likely confers positive benefits, as it has been correlated with higher rates of spontaneous fermentation success (Schuller et al. 2012), possibly due to an increase in sugar consumption efficiency (Ciani and Comitini 2011). Additionally, the presence of multiple *S. cerevisiae* strains during fermentation has been linked to increased perception of wine complexity (Blanco, Orriols and Losada 2011). The creation of different secondary metabolites and metabolic changes brought by the strains interacting is thought to be the cause of the perceived increase in complexity (Hernández et al. 2003, Romano 2003, Swiegers et al. 2005, Swiegers and Pretorius 2005, Swiegers et al. 2007, Carrau et al. 2008, Francesca et al. 2014, Jolly, Varela and Pretorius 2014).

Non-Saccharomyces Yeast

Three fermentation persistence trends were observed in the non-*Saccharomyces* yeast species: (1) observed in the early stage of fermentation, (2) observed in the late stage of fermentation, and (3) observed in all three stages with no clear stage

association (Fig. 3, Table 32). Yeast species observed in the early stage are generally those with a high initial concentration, a short lag time, easily outcompeted by other yeast species, or with low tolerance to changing conditions (e.g. increasing ethanol concentrations) (Fernández-Espinar et al. 2001, Bokulich et al. 2014, Fernández-González, Úbeda and Briones 2015, Zuchowska et al. 2015, Bokulich et al. 2016). Late stage observations suggest yeast species that can survive unfavorable growth conditions in early fermentation to benefit from the more favorable conditions that follow (Ganga and Martínez 2004, Goddard 2008, Santamaría et al. 2008, Ciani et al. 2010, Tofalo et al. 2012, Jolly, Varela and Pretorius 2014, Padilla, Gil and Manzanares 2016), or species introduced later to the fermentation, likely from a winery source. Species without a clear fermentation stage association can tolerate a wide range of fermentation conditions (Tofalo et al. 2009, Cordero-Bueso et al. 2011, García-Ríos et al. 2014, Rodríguez-Sifuentes et al. 2014, Fernández-González, Úbeda and Briones 2015, Masneuf-Pomarede et al. 2016, Drumonde-Neves et al. 2017).

When the same yeast species was identified in samples taken before and after a sample in which it was not observed, it is reasonable to assume that the species was present in the fermentation during the sampling point but not detected, as yeast species are unlikely to die off and be reintroduced to the fermentation. The observational break could be caused if the yeast species population was hovering close to the limit of detection of the isolation technique throughout fermentation, if it population size was fluctuating around the limit of detection, or if multiple strains of the same yeast species were present, each with a different population peak time, and the sample captured a low point in all strain populations (Cocolin, Bisson and Mills 2000, Dias et al. 2003, Schuller and Casal 2007, Vaz-Moreira et al. 2011, Bokulich et al. 2015, Ubeda-Iranzo et al. 2015, Drumonde-Neves et al. 2017). In fermentations where different yeast species were identified on each side of the

observational break, it is reasonable to assume that the changing fermentation conditions were more favorable to the new yeast species than the one originally observed.

H. uvarum was the most commonly identified non-*Saccharomyces* yeast species across all samples (Tables 17, 19, 22) and was the only yeast species observed in grapes from the same vineyard in two vintages (Table 17), indicating that it was part of the stable regional microbiome. This was expected, as *H. uvarum* is the most abundant yeast observed on grapes, is commonly found on winery equipment (Sabate et al. 2002, Combina et al. 2005, Li et al. 2010, Kurtzman, Fell and Boekhout 2011, Rantsiou et al. 2013, Sun et al. 2014, Alessandria et al. 2015, Garofalo et al. 2016, Drumonde-Neves et al. 2017, Patrignani et al. 2017), and is nearly ubiquitous in spontaneous and inoculated fermentations worldwide (Constantí et al. 1997, Bujdoso, Egli and Henick-Kling 2001, Torija et al. 2001, Beltran et al. 2002, Combina et al. 2005, Sun et al. 2009, Brežná et al. 2010, Zhang et al. 2010, Li et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Bezerra-Bussoli et al. 2013, Bokulich et al. 2014, Alessandria et al. 2015, Pinto et al. 2015, Drumonde-Neves et al. 2017). *H. uvarum* was continuously observed in the early stages of fermentations, in agreement with literature; it was also expected for some *H. uvarum* to survive longer in the favorable, low temperatures typical of these Riesling fermentations (Cocolin, Bisson and Mills 2000, Fleet 2003, Di Maro, Ercolini and Coppola 2007, Barata et al. 2008, Fleet 2008, Barata, Malfeito-Ferreira and Loureiro 2012).

The relative abundances of *H. uvarum* and other non-*Saccharomyces* yeast on grapes appeared to be correlated, with more *H. uvarum* predicting fewer isolates of other non-*Saccharomyces* yeast species (Table 17). The same correlation has been observed in grapes analyzed from Argentina (Combina et al. 2005). However, given the small number of yeast isolates obtained and the lack of controls for other factors,

such as differences in viticultural practices, there is not enough evidence to show a causational effect.

It is unclear if *H. uvarum* was part of any winery's resident microbiome or independently deposited by grapes each year. While it was the only yeast species to be observed on grapes, equipment, and fermentations processed in the same winery in the same vintage (Tables 17, 20, 25), it was not consistently observed on the all the same pieces of equipment when they were sampled in both vintages (Table 20). It is possible that the *H. uvarum* was being transferred from the grapes onto the equipment they were processed by, and vice versa. Since *H. uvarum* is almost ubiquitous on grapes and winery surfaces it is unclear if *H. uvarum* isolates were from transfers or from unique sources.

C. boidnii, C. zemplinina/C. stellata, *P. cecembensis/P. occidentalis/P. kudriavzevii*, and *T. californica/T. cantarellii* were likely part of the stable regional microbiome, given the frequency, variety of sources and vintages in which they were observed (Tables 17, 20, 25). All were observed in both vintages, in samples associated with at least 2 different wineries, and in at least 2 different types of samples. The influence of these non-*Saccharomyces* yeast may increase the perception of Riesling typicity through the enzymatic release of bound aroma precursors (Jackson and Lombard 1993, Swiegers and Pretorius 2005, Swiegers et al. 2007, Fleet 2008, Santamaría et al. 2008, Ciani et al. 2010, Belda et al. 2016, Padilla, Gil and Manzanares 2016, Wang et al. 2017). The observation of *C. boidnii* was notable, as it is more commonly associated with tree sap and drosophilid flies, but not unique to winery environments in the Finger Lakes region (Kurtzman, Fell and Boekhout 2011, Garofalo et al. 2016). Observation in only the early stage of fermentation was also consistent with what is typical for the *Candida* genus (Kurtzman, Fell and Boekhout 2011) (Fig. 1, Table 22).

C. zemplinina/C. stellata and *P. cecembensis/P. occidentalis/P. kudriavzevii* were observed in both grape samples and the corresponding fermentations (Tables 17, 21, 22, 24), indicating that these non-*Saccharomyces* yeast were likely part of the regional microbiome and influenced the aroma profile of the resulting wines. *C. zemplinina/C. stellata* was observed on all three samples types (Tables 17, 19, 22), and persisted to the middle and late stages of fermentation (Fig. 1, Table 32). It is unclear if there was direct transfer from one sample type to another, as it was not consistently observed in the chain of samples leading to fermentation. This finding agrees with the frequent observation of *C. zemplinina/C. stellata* on intact and damaged grapes and winemaking environments (Barata et al. 2008, Kurtzman, Fell and Boekhout 2011, Barata, Malfeito-Ferreira and Loureiro 2012, David et al. 2014, Alessandria et al. 2015, Teixeira, Caldeira and Duarte 2015). Being fructophilic, osmotolerant, and ethanol tolerant, this species has been found in the late stages of fermentation, potentially providing both kinetic and aromatic advantages (Constantí et al. 1997, Torija et al. 2001, Fleet 2003, Clemente-Jimenez et al. 2004, Combina et al. 2005, Cordero-Bueso et al. 2011, Kurtzman, Fell and Boekhout 2011, Tofalo et al. 2012, Bokulich et al. 2014, Jolly, Varela and Pretorius 2014, Oro, Ciani and Comitini 2014, Canonico, Comitini and Ciani 2015, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Garofalo et al. 2016, Padilla, Gil and Manzanares 2016). Interestingly, a distinct strain of *C. zemplinina* was isolated in the Niagara winegrowing region in Canada; its observation in fermentations in the nearby Finger Lakes region may indicate that it is part of a larger regional distribution (Kurtzman, Fell and Boekhout 2011). *P. cecembensis/P. occidentalis/P. kudriazveii* was observed less widely and only persisted to the middle stage of fermentation (Fig. 3, Table 22). It is unclear if there was transfer of *P. cecembensis/P. occidentalis/P. kudriazveii* from the equipment to the fermentation, or if the overlap in sample types was due to

independent depositions, as it was only observed on equipment in 2015 and in one fermentation in 2016 (Tables 19, 23). This is consistent with previous research showing the yeast to be regularly observed in winemaking environments and fermentations, although with varying frequencies in different winemaking regions (Clemente-Jimenez et al. 2004, Combina et al. 2005, Di Maro, Ercolini and Coppola 2007, Sun et al. 2009, Kurtzman, Fell and Boekhout 2011, Díaz et al. 2013, Jolly, Varela and Pretorius 2014) . Its contribution to fermentation flavor development is unclear (Kurtzman, Fell and Boekhout 2011).

The presence of *T. californica/T. cantarellii* in multiple fermentations and on equipment (Tables 19, 22) was notable both because it is infrequently observed in fermentations and is typically thought of as a spoilage yeast (Kurtzman 2007, Kurtzman, Fell and Boekhout 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Echeverrigaray et al. 2013). It was observed throughout all fermentation stages (Fig. 3), consistent with previous observations of its ability to persist in fermentations, having even been observed in bottled wines (Barata, Malfeito-Ferreira and Loureiro 2012, Echeverrigaray et al. 2013). It should be noted that *T. californica* is a recently described species closely related to *T. cantarellii* that has been observed in California wine fermentations (Kurtzman, Fell and Boekhout 2011), and not unique to the Finger Lakes region. However, the presence of *T. californica/T. cantarellii* in F3 and F8 may indicate that contributed to the unique aroma profile of the wines produced using V3 grapes (Table 21).

M. pulcherrima was likely part of the regional microbiome, as it was observed on all three sample types and in both vintages, but may be specifically associated with Winery A (Tables 17, 19, 20, 22, 25). Its observation in multiple sample types is consistent with previous findings that *M. pulcherrima* is widely found on grapes and in the early stage of fermentations, although observational frequency varies by

winemaking regions and vintage specific environmental conditions (Clemente-Jimenez et al. 2004, Combina et al. 2005, Di Maro, Ercolini and Coppola 2007, Kurtzman, Fell and Boekhout 2011, Li et al. 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Díaz et al. 2013, Jolly, Varela and Pretorius 2014, Pinto et al. 2015, Garofalo et al. 2016, University of California at Davis n.d.). However, it is unclear if there was direct transfer from one sample type to another as it was not consistently observed in the chain of samples leading to a single fermentation. The presence of this yeast may potentially benefit fermentations kinetically and aromatically (Constantí et al. 1997, Torija et al. 2001, Fleet 2003, Clemente-Jimenez et al. 2004, Combina et al. 2005, Cordero-Bueso et al. 2011, Kurtzman, Fell and Boekhout 2011, Tofalo et al. 2012, Bokulich et al. 2014, Jolly, Varela and Pretorius 2014, Oro, Ciani and Comitini 2014, Canonico, Comitini and Ciani 2015, Teixeira, Caldeira and Duarte 2015, Belda et al. 2016, Garofalo et al. 2016, Padilla, Gil and Manzanares 2016). *M. pulcherrima* was observed in both V3 and V8, indicating a potential association with V3 (Table 21), but was also observed in other fermentations processed at Wineries A and B. This correlation may mean that *M. pulcherrima* was part of a stable yeast population in V3, but only sometimes part of the regional yeast composition. Its presence in a Winery A settling tank in 2016 (Table 20) also brings up the possibility that *M. pulcherrima* was part of the resident Winery A microbiome, making the conclusions about stability and vineyard association more uncertain.

The results indicate that *C. oleophila/C. railenensis*, *P. fermentans/P. kluyveri*, and *P. membranifaciens/C. californica* were likely part of the regional microbiome, but with a high rate of variability. While all the yeast species were observed in both vintages (Table 17, 20, 23), they all had less evidentiary support for inclusion in the stable regional microbiome than the previously discussed non-*Saccharomyces* yeast species. *C. oleophila/C. railenensis* was only observed in two fermentations, but they were

processed at different wineries in different vintages. While this yeast is not commonly observed in fermentations, it is often observed on surfaces where *Drosophila* flies are present making them a possible vector for their introduction to these fermentations. While *Candida* genus members are often observed during early fermentation, the effect of *C. oleophila/C. railenensis* on fermentations is unknown but it has also been isolated in Ontario, suggesting that it might be part of a broader regional microbiome (Kurtzman, Fell and Boekhout 2011). The low number of *P. fermentans/P. kluyveri* isolates identified make it difficult to draw conclusions, but its presence on samples of all types and in fermentations at both wineries sampled in 2015 indicates a regional presence. The observation of *P. fermentans/P. kluyveri* on a settling tank siphon could indicate a transfer from a previous fermentation to the equipment rather than from the equipment to a tracked fermentation. This is consistent with previous research showing that *P. fermentans/kluyveri* is widely observed on fruit and regularly in spontaneous fermentations (Clemente-Jimenez et al. 2004, Combina et al. 2005, Kurtzman, Fell and Boekhout 2011, Li et al. 2011, Díaz et al. 2013, Garofalo et al. 2016). While sometimes considered a spoilage yeast, this species is not of great concern, as its creation of detrimental aroma compounds is limited in fermentations (Ciani and Maccarelli 1997, Padilla, Gil and Manzanares 2016), and some strains can produce beneficial aroma compounds (Ciani et al. 2010, Baffi et al. 2011, Jolly, Varela and Pretorius 2014, Belda et al. 2016, Padilla, Gil and Manzanares 2016). It is unclear if *P. membranifaciens/C. californica* was part of the regional microbiome, part of Winery A's resident microbiome, or both. It was frequently observed in the early stages of fermentations processed at Winery A (Fig. 3, Table 25, 32), particularly F2, F7, and F9, indicating a potential stable association with V2. Its observation in 2015 on equipment and all processed fermentations at Winery A supports inclusion in the resident winery microbiome, while its presence in

a Winery B fermentation and in all V2 fermentations supports the idea of it being part of the regional microbiome. Its presence was notable as a spoilage yeast detrimental to wine quality (Clemente-Jimenez et al. 2004, Wu, Robert and Bai 2006, Di Maro, Ercolini and Coppola 2007, Sun et al. 2009, Kurtzman, Fell and Boekhout 2011, Díaz et al. 2013, Díaz et al. 2013, Jolly, Varela and Pretorius 2014). However, not all Winery A fermentations contained *P. membranifaciens/C. californica*, and it was not observed on Winery A equipment in 2016 which would be expected if its presence was due solely to the winery environment. This correlation may mean that *P. membranifaciens/C. californica* was part of a stable yeast population in V2, but only sometimes part of the regional yeast composition or the resident winery microbiome at Winery A.

C. flavescens and *R. glutinis*/*Rh. Babjevae* were observed on 2015 V3 grapes (Table 17), suggesting they were underripe at the time of sampling, as basidiomycetous yeast species are observed in high concentrations on unripe grapes (Kurtzman, Fell and Boekhout 2011, Barata, Malfeito-Ferreira and Loureiro 2012, Díaz et al. 2013, Setati et al. 2013). The yeast does not appear to have had a direct influence on the fermentations.

Results suggest that *T. delbrueckii* was a common resident winery yeast species in the Finger Lakes, but it was unclear if it influenced regional wine sensory profiles. *T. delbrueckii* was widely observed in equipment samples at all three wineries and in both vintages, but was not found in any other sample type (Table 20). This is consistent with observations of *T. delbrueckii* in other regions (Oćon et al. 2010, Bokulich et al. 2013), and its apparent adaptation to winemaking environments (Masneuf-Pomarede et al. 2016). *T. delbrueckii* is generally considered to have a positive impact on wine quality, so its presence on winery equipment of little concern

(Ciani and Maccarelli 1997, Cordero-Bueso et al. 2011, Jara, Rojas and Romero 2015, Belda et al. 2016, Padilla, Gil and Manzanares 2016).

H. oryzea, *M. guilliermondiii*, *R. mucilaginosa*, *W. anomalus* and *Z. hellenicus/Z. meyerae* did not appear to significantly influence Finger Lakes fermentations directly, as they were only infrequently observed on equipment samples (Tables 19, 20). All are all found in a wide variety of natural environments so their observation was not novel (Kurtzman, Fell and Boekhout 2011). *W. anomalus* is commonly observed in high stress environments, such as wine fermentations, and is considered a spoilage yeast, detrimental to wine quality (Kurtzman, Fell and Boekhout 2011).

The observation of *H. valbyensis* in the middle and late stages of fermentation (Fig. 3, Tables 22, 32) was unusual for members of the *Hanseniaspora* genus (Kurtzman, Fell and Boekhout 2011). *H. valbyensis* is more commonly found in cider fermentations, typically in the early stage of fermentation (Xu, Zhao and Wang 2006), although it has been found to survive until the late stage of spontaneous cider fermentations in Spain (Valles et al. 2007). This persistence could be because the fermentation parameters under which it occurred– no sulfur dioxide addition, low fermentation temperatures, and lower final ethanol concentrations- favor *H. valbyensis* growth (Valles et al. 2007) It is also notable that it was only observed fermentations using grapes from V2, though further research is needed to determine if there was a connection between vineyard and yeast species (Table 24).

The presence of *Z. bailii* and *Z. parabailii*, in F5 and F9 respectively (Table 22), may indicate a higher proportion of damaged grapes were included in the grapes used for fermentation. *Z. bailii* is a fructophilc yeast tolerant of a large range of fermentation conditions, often persisting to the late stage of fermentation, and more likely to be observed when damaged grapes are present. Typically considered a spoilage yeast due to its association with damaged grapes, there is also evidence for creating

beneficial secondary metabolites (Esteve-Zarzoso et al. 2000, Clemente-Jimenez et al. 2004, Barata et al. 2008, Tofalo et al. 2009, Kurtzman, Fell and Boekhout 2011, Jolly, Varela and Pretorius 2014, Padilla, Gil and Manzanares 2016). *Z. parabailii* is a closely related species, whose impact on fermentations is unclear but likely similar to *Z. bailii* (Suh et al. 2013).

The observation of *A. pullulans* in a fermentation sample (Table 22) was unusual as it does not typically survive long in fermentations (Fleet 2003). It has been observed in a variety of winery and vineyard environments, despite being most commonly associated with grapes (Sabate et al. 2002, Fleet 2003, Dimakopoulou et al. 2008, Barata, Malfeito-Ferreira and Loureiro 2012, Bokulich et al. 2013, Setati et al. 2013, Bokulich et al. 2014, Sun et al. 2014, Alessandria et al. 2015, Pinto et al. 2015, Pinto et al. 2015), so its observation was not novel. The cause of this unusual result was unclear given that it was only observed once.

The remaining non-*Saccharomyces* yeast species were unidentified as they were not represented in the NCBI GenBank database. This could indicate rare species, species with unique rRNA ITS region mutations, or interspecies hybrids (Liti, Barton and Louis 2006, Wu, Robert and Bai 2006, Schoch and Seifert 2012, Schoch et al. 2012, Irinyi and Meyer 2015, Masneuf-Pomarede et al. 2016). Interspecies hybridization in winemaking settings is relatively common, especially between closely related species and in low temperatures like those used in spontaneous Riesling fermentations (Liti, Barton and Louis 2006, Cappello et al. 2010, García et al. 2012, Almeida et al. 2014, Gibson and Liti 2015). Unknown Yeast 1 was only observed on equipment, making it unclear if it had any impact on fermentation. Unknown Yeasts 2 and 3 were both observed once in F3 (Table 22), in the early and late stages, respectively indicating different tolerances for the winemaking environment.

Saccharomyces Yeast

Over 100 unique *S. cerevisiae* strains were identified (Table 4). This was consistent with previous research reporting a large amount of diversity in the *S. cerevisiae* strains in regional ecological surveys from a variety of regions (Vezinhet et al. 1992, Ubeda and Briones 2000, Schuller et al. 2005, Schuller et al. 2007, Goddard et al. 2010, Orlic et al. 2010, Li et al. 2011, Gayevskiy and Goddard 2012, Schuller et al. 2012, Azzolini et al. 2013, Charron et al. 2014, Knight and Goddard 2015, García et al. 2016, Martiniuk et al. 2016). The genetic complexity of *S. cerevisiae* and the possibility of hybridization events makes estimates of relatedness somewhat subjective (Ayoub et al. 2006, Kurtzman 2011). Strains encompassed by SPG were likely related; those encompassed by PSPG may or may not be related, but the number of shared alleles made it a possibility worth considering.

The multi-gene analysis may have also contributed to the number of strains observed as it can increase *S. cerevisiae* strain discrimination power over sequence comparisons of the ITS region (Ayoub et al. 2006, Kurtzman 2011). It should be noted that a different type of phylogenetic analysis may show relatedness between strains considered distinct, or conversely show no relationship between profiles deemed similar or potentially similar (Ayoub et al. 2006, Kurtzman 2011). For example, a profile with more than 2 alleles at every locus could be a *S. cerevisiae* strain in a polypoid state with similar strains present in the data set; it would be missed by this analysis method since the number of additional alleles would classify it as a distinct strain.

Both native and CRS *S. cerevisiae* were observed, but most isolates were native strains (Tables 8, 9, 26, 27). This conforms with previous research showing both native and commercial strains present within the same fermentation (Valero et al. 2007, Mercado et al. 2010). While most strains were only observed once, those

observed multiple times were a mix of native and commercial related strains found in anywhere from one to four fermentations (Tables 28, 34). Additionally, several commercial yeast products were found to have matching or similar profiles (Table 7), agreeing with previous analyses that found several products marketed under different commercial names are genetically indistinguishable or very similar (Fernández-Espinar et al. 2001, Bradbury et al. 2005, Bradbury et al. 2005, Boynton and Greig 2014), It should be noted that ETS Laboratories used an internal standard to determine the sample profiles' similarity to commercial strains, raising the possibility that an *S. cerevisiae* strain described as native was actually similar or potentially similar to a commercial strain that did not meet the internal similarity standard, or that it matched or was related to a commercial strain absent from the database. Further, different types of phylogenetic analysis may lead to different conclusions about the relatedness of commercial strains (Ayoub et al. 2006, Kurtzman 2011).

Native strains generally gave way to commercial related strains as fermentations progressed, though native strains persisted into late stages (Tables 34, 35). Both native and commercial related *S. cerevisiae* strains were observed in every fermentation, all including at least one MPG matching a commercial strain (Tables 26, 27). As expected, native strains were more likely to be observed in the early or middle stage, while CRS were more common in the middle or late stage of fermentation (Table 35) (Reed and Chen 1978, Fleet 2008, Mendes et al. 2013). Additionally, the lowest ratio of native to CRS was in the late stage of fermentations (Tables 33, 35), suggesting that while commercial strains generally outcompeted native strains they did so more slowly than expected and there were several instances where native strains were in the majority. This could indicate the slow buildup of CRS over time, the unintentional addition of strains in the middle or late

stage of fermentation via contact with the winery environment, CRS less well adapted to the fermentation, or a combination of these factors. Previous work shows that the presence of commercial strains does not preclude native strains from persisting to the late stage of fermentation, which was also the case for this data (Tables 35, 36) (Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Martiniuk et al. 2016).

It was common for fermentations to have persistent *S. cerevisiae* strains, but only a small portion of observed strains were persistent in any given fermentation (Tables 34, 36). Each fermentation had at least 2 persistent *S. cerevisiae* strains, which could be observed continuously or with observational gaps; gaps occurred for the same reasons as non-*Saccharomyces* yeast species. Though most persistent strains were native, the persistent CRS that did occur had longer, more sustained persistence. This supports the idea that once a commercial strain becomes established it will persist in the fermentation, but that not all commercial strains become established in a given fermentation (Barrajón et al. 2009, Martiniuk et al. 2016).

Less than half of the fermentations contained dominant strains, but when dominant strains were present, there were always at least two (Table 37). Dominant strains were always persistent, although only a fraction of the observed persistent strains were dominant in any fermentation (Tables 36, 37). Most fermentations with a dominant strain had co-domination by two strains that were dominant during different stages of fermentation, with numerous other strains present throughout. It should be noted that in F10 the co-dominant strains were closely related (Fermol Premier Cru (wo 174) and Fermol Premier Cru (w 174)/Lalvin ICV D254) and may be slightly different forms of the same strain. Both co-domination and a plurality of *S. cerevisiae* strains have been previously observed in spontaneous fermentations (Constantí et al. 1997, Torija et al. 2001, Schuller et al. 2005, Valero et al. 2007,

Blanco, Orriols and Losada 2011). The succession of several *S. cerevisiae* strains as the fermentation progresses is common, even though the succession process is complex and not well understood, especially in spontaneous fermentations (Fleet 2003, Fleet 2008, Ciani et al. 2010).

A majority of the dominant strains matched commercial strains (Table 37). The only dominant native strain, C1, was also the only strain to be dominant in two fermentations. In both F7 and F9, C1 was dominant earlier in the fermentation than the observed commercial strain, following the general pattern of native strains found earlier and commercial strains found later in fermentation. It should be noted that at two sampling time points in F7 strains matching Erboferm TM Freddo/Lalvin Rhone 4600 (C2) and Fermol Blanc (C4) together appeared to be dominant over other strains, but individually neither strain comprised more than 50% of the isolates observed, and thus were not classified as dominant. If they had been, Fermol Blanc would be the only CRS matching a commercial strain to be observed in more than one fermentation. Similarly in F2, F5, and F10 the number of isolates for one strain were equal to all other observed strains, meaning it was not deemed dominant. Two of these instances were of MPG that matched commercial strains, Vitilevure 3001 (C6) in F5 and Fermol Premier Cru (w 174)/Lalvin ICV D254 (C3) in F10, while the remaining was a native strain, C37 in F2. It was interesting to note that even in fermentations with only dominant commercial strains there was a succession of these strains, rather than a single dominant strain throughout.

The amount of *S. cerevisiae* strain overlap between fermentations was higher than expected, but can be explained by fermentation conditions of the samples. About 19% of all identified strains were observed in at least two fermentations (Table 28). It is uncommon to see *S. cerevisiae* strain overlap in unrelated spontaneous fermentations within a single region. However, the frequency of overlapping strains

in a region increases when the fermentations sampled are processed by the same winery, source grapes from the same vineyards, or are processed in the same vintage (Vezinhet et al. 1992, Pramateftaki, Lanaridis and Typas 2000, Torija et al. 2001, Schuller et al. 2005, Schuller and Casal 2007, Martiniuk et al. 2016). Strain overlap, particularly of commercial strains (Mercado et al. 2007), also increases when inoculated fermentations are performed near spontaneous fermentations, both at the winery (Constantí et al. 1997, Ciani et al. 2004, Santamaría et al. 2008, Blanco, Orriols and Losada 2011, Martiniuk et al. 2016) and regional (Torija et al. 2001, Salinas et al. 2010) level. All wineries in this study performed both spontaneous and inoculated fermentations, and within the Finger Lakes region inoculation of fermentations was and is common, so the higher than expected strain overlap fits the context of this research.

Winery and Vineyard Influences

Both the vineyard and winery environments appeared to be contributing to *S. cerevisiae* strain composition and diversity in spontaneous fermentations, as their presence in multiple fermentations suggests a role in regional or resident winery microbiome (Ciani et al. 2004, Santamaría et al. 2005, Mercado et al. 2007, Gayevskiy and Goddard 2012, Bokulich et al. 2013, Bokulich et al. 2014, Knight and Goddard 2015, Knight et al. 2015, Bokulich et al. 2016). The limited overlap of strains in fermentations processed at different wineries (Table 40) supported the influence of resident winery microbiomes. All but one shared *S. cerevisiae* strain were in wines processed at the same winery (Tables 28, 40). The remaining shared strain was found in Wineries B and C, which shared equipment and fermentation space, and perhaps had a shared resident winery microbe population. This was especially likely as it was a commercial strain, Vitilevure 3001 (C6), used by Winery C in inoculated

fermentations, that was present in both vintages. Conversely, the limited overlap (7%, Tables 5, 28) of strains in fermentations processed at the same winery in different vintages, lended support to the influence of vineyard specific factors. More *S. cerevisiae* strain diversity was observed in 2016 than in 2015 (Table 5), in direct contrast to the trend in non-*Saccharomyces* diversity over the two vintages (Tables 17, 19, 23); ripeness and damage in grapes is correlated to increases in both *S. cerevisiae* strains and non-*Saccharomyces* species diversity (Combina et al. 2005, Valero et al. 2007, Francesca et al. 2010, Barata, Malfeito-Ferreira and Loureiro 2012, Setati et al. 2013). Additionally, V1, V2, and V3 had limited variations in viticultural practices as they were maintained by the same vineyard manager, leading to the conclusion that vintage specific vineyard factors influenced the observed strain diversity (Combina et al. 2005, Drumonde-Neves et al. 2017). The fact that over half of the shared strains were in fermentations processed at the same winery in the same vintage, but each fermentation also contained at least 5 unique strains (Tables 28, 29), may suggest either winery or vintage specific regional vineyard factors. Fluctuating resident winery microbe populations (Mercado et al. 2007, Bokulich et al. 2013) may partially account for the lower than expected vintage overlap in fermentations processed at the same winery. An uneven microbe distribution in vineyards may account for the high number of unique strains observed, as even lots of grapes from the same vineyard in a single vintage can result in different *S. cerevisiae* strain composition and succession patterns (Torija et al. 2001, Schuller et al. 2005).

The influence of vineyard specific factors was further supported by the yeast species and *S. cerevisiae* strain diversity observed in single-vineyard fermentations. Each mono-vineyard fermentation had different levels of diversity, even when controlling for vintage and processing winery. Fermentations associated with V2 and V3 had

higher overall yeast species diversity than the V1, V5, and V6 fermentations (Tables 17, 24). *S. cerevisiae* strain diversity was higher in V1 and V2 > V3 > V5 and V6 fermentations (Table 39). However, when comparing the fermentations in each vintage, those associated with V5 and V6 had fewer yeast species and *S. cerevisiae* strain diversity than those from V1, V2, and V3. The amount of diversity was relatively consistent in fermentations across vintages but sourcing from the same vineyard, though composition varied by vintage (Table 38). V2 and V3 fermentations had more yeast species diversity and vintage specific composition changes than those from V1. However, V1 fermentations had an apparent inverse relationship between the number of yeast species and *S. cerevisiae* strains observed. The source of the variation was unclear, as non-*Saccharomyces* yeast interactions and winery and vineyard specific factors may have contributed to the differences in diversity.

S. cerevisiae strains that are not part of the resident winery microbiome may still be transferred by vectors in the winery environment. At Winery A there were *S. cerevisiae* strains that overlapped between all possible pairs of fermentations in each vintage (Tables 28, 40). The vintage specific overlapping strains were primarily native, with a few CRS including 3 that matched commercial strains. Erboferm Structure/Red Star Pasteur Red (C23) was found in two of the three fermentations processed in 2015, while Erboferm TM Freddo/Lalvin Rhone 4600 (C2) and Fermol Premier Cru (w174)/Lalvin ICV D254 (C3) were found in all of the 2016 fermentations. Across vintages, all but one possible pairs of fermentations – F2 and F9 – contained overlapping strains. These strains were primarily CRS, but none matched commercial strains. Strains observed in multiple Winery A fermentations could indicate that they were part of the regional or resident winery microbiome, however the limited amount of vintage overlap suggested that they were either transitory or non-dominant members of the microbiome. The overlapping strains in the same vintage

may have been transferred through interactions with the winery environment, even if they were not part of the winery microbiome. Previous researchers observing similar lack of consistency in fermentations at the same winery, using fruit from the same vineyard, or processed in the same vintage, have suggested that some combination of the three variables contribute to non-*Saccharomyces* yeast species composition (Sabate et al. 2002, Mercado et al. 2007, Blanco, Orriols and Losada 2011, Gayevskiy and Goddard 2012, Bokulich et al. 2013, Díaz et al. 2013, Setati et al. 2013, Bokulich et al. 2014, Francesca et al. 2014, Alessandria et al. 2015, Belda et al. 2016, Drumonde-Neves et al. 2017).

The observational patterns of several *S. cerevisiae* strains helped discern their source. Native strains C1, C10, C11, and CRS C20, CRS C5, C12, C27, Erboferm TM Freddo/Lalvin Rhone 4600 (C2), and Fermol Blanc (C4) were all observed frequently in Winery A fermentations (Tables 28, 40). Native strains C1 and C20 were likely influenced by regional vineyard factors, as they were only observed in 2016 and not limited to single-vineyard fermentations (Tables 5, 39). Erboferm TM Freddo/Lalvin Rhone 4600 (C2) and Fermol Blanc (C4) were also only observed in 2016, but are commercial strains with was a greater chance of late introduction to fermentations making it likely influenced by winery factors. C27 was observed in both vintages and exclusively associated with V3 fermentations (Tables 5, 28, 38), indicating that vineyard factors were a likely influence. Native C35 and CRS C45 were observed less frequently, but were found exclusively in V2 fermentations (Tables 28, 38). There were also strains observed exclusively in fermentations processed at Wineries B and C (Tables 26, 38, 39, 40), but their associations with specific vineyards or wineries were unclear.

The comparison of V2 fermentations suggested an uneven distribution of non-*Saccharomyces* yeast species and *S. cerevisiae* strains within a single vineyard (Tables

22, 24, 28, 38). F2 and F7 were of grapes from across V2, while F9 used grapes from a subsection. Though F9 had similar levels of non-*Saccharomyces* yeast species diversity as F2 and F7, it had a different species composition and less *S. cerevisiae* strain diversity with different strain compositions. F9 did not share any strains with F2, and only four (C1, C2, C4, and C20) with F7. As previously indicated, none of these strains were likely to be solely influenced by V2. It should also be noted that F9 did not contain C45, a possible V2 specific *S. cerevisiae* strain found exclusively in F2 and F7. The lack of this strain, as opposed to the gain of other distinct strains, may be partly responsible for organoleptic differences the winemaker had historically noted in wines from this subsection of V2. The non-*Saccharomyces* species and *S. cerevisiae* strain composition differences may be attributed to an uneven distribution within the vineyard (Schuller et al. 2005, Setati et al. 2013) or different proportions of damaged grapes included in the fermentations (Combina et al. 2005, Valero et al. 2007, Francesca et al. 2010, Setati et al. 2013). This lends support to the hypothesis that vineyard, winery, and vintage specific factors are not the only factors that can affect *S. cerevisiae* strain composition in a fermentation (Clemente-Jimenez et al. 2004, Schuller et al. 2005, Valero et al. 2007).

S. cerevisiae SPG and PSPG may resemble one another due to an accumulation of mutations, as both resident winery and regional vineyard strains will naturally mutate over time (Martiny et al. 2006, Hanson et al. 2012, Martiniuk et al. 2016). SPG exclusive to one winery were more likely to be related to resident winery strains, while those observed in multiple wineries were likely related to regional vineyard strains.

PSPG have a tentative genetic relationship, and can support conclusions drawn from SPG, but are not strong enough to independently draw conclusions about the encompassed strains. Winery A had one exclusive SPG, S2, which linked all

but one fermentation processed across both vintages (Table 40). Exclusive PSPG, PS4, linked all the fermentations processed at Winery A in 2015, and PS1 linked all fermentations processed at Winery A in both vintages (Table 40). A majority of the strains in the SPG and PSPG were native, suggesting that a group of native strains might have become established in a winery, vineyard, or both. Fermentations from Wineries A and B shared strains from S8 and PS3, Wineries B and C shared strains from S7, and all three wineries shared potentially similar profile group PS2 (Table 40). Given that the strains in these SPG either matched or were similar to commercial strains (Lalvin ICV GRE and Vitilevure 3001), it is possible that there were deposited independently in each winery, where separate mutations occurred over time. This is more likely than an 'escaped commercial strain' being present in multiple regional vineyards, not all of which are adjacent to a winery (Schuller and Casal 2007, Valero et al. 2007, Goddard et al. 2010, Salinas et al. 2010, Hyma and Fay 2013, Martiniuk et al. 2016). PS3 contained native strains found in fermentations from both vintages of Winery A and one 2015 fermentation from Winery B, suggesting a possible regional strain group (Table 40). PS2 contained numerous CRS including some that match commercial strains, and linked all but one tracked fermentation over both vintages. This detracts from the idea that 'escaped commercial strains' were not influencing the regional vineyard microbiome. Due to the tentative nature of the genetic connections, and limited observations of strains in PS2 and PS3, further study would be needed to draw definitive conclusions.

Comparison of Sample Types

The limited overlap in yeast species and lack of overlap in *S. cerevisiae* strains on grapes and their subsequent fermentations (Tables 17, 22, 26) does not preclude the possibility of vineyard contribution. Grape sampling methods like the exclusion of

damaged grapes from samples (Combina et al. 2005, Valero et al. 2007, Francesca et al. 2010, Setati et al. 2013), additional grape ripening between vineyard and fermentation sampling (Schuller et al. 2005, Valero et al. 2007, Barata, Malfeito-Ferreira and Loureiro 2012), uneven intravineyard distribution of yeast (Schuller et al. 2005, Schuller and Casal 2007, Valero et al. 2007, Setati et al. 2013), and the difficulty of extracting yeast from the surface of grapes and equipment (Martini, Ciani and Scorzetti 1996, Combina et al. 2005, Renouf, Claisse and Lonvaud-Funel 2005), all add a degree of uncertainty.

Additionally, the culture dependent isolation methodology may have contributed false negatives for yeast species present in amounts under the limit of detection in both grape and fermentation samples. It could have led to underrepresentation of yeast species less favored for growth on the gel media, yeast species being present on grapes but may not have survived in the must (Bisson 1999, Bisson and Butzke 2000, Cocolin, Bisson and Mills 2000, Pramateftaki, Lanaridis and Typas 2000, Pallmann et al. 2001, Romano 2003, Keller and Zengler 2004, Renouf, Claisse and Lonvaud-Funel 2007, Oćon et al. 2010, Vaz-Moreira et al. 2011, Bokulich and Mills 2012, Boynton and Greig 2014, García-Ríos et al. 2014, Rodríguez-Sifuentes et al. 2014, Taylor et al. 2014, Tristezza et al. 2014, Alessandria et al. 2015), and could have led to growth pattern distortions due to the ratio of non-*Saccharomyces* to *Saccharomyces* yeast (Ubeda-Iranzo et al. 2015). Fermentations are complex environments and species interactions are difficult to predict, so other unidentified factors may have been at work.

Disparities in yeast species observed on grapes and their subsequent fermentations were not fully accounted for by the equipment swabs (Tables 19, 20). There was inconsistent overlap in non-*Saccharomyces* yeast species observed on the winery equipment and the fermentations processed at those wineries (Table 25). At all

wineries *H. uvarum* was widespread, but as previously discussed it is unclear if the yeast was being transferred between sample types or was being independently deposited. At Winery A in 2015, *P. membranifaciens/C. californica* was the only yeast species to account for some of the difference between the grape and fermentation samples. At Winery B in 2015, and Wineries A and C in 2016, there were no clear examples of non-*Saccharomyces* yeast species transfer between the equipment and fermentations.

S. cerevisiae strains found on equipment were not always found in fermentations processed using that equipment (Tables 20, 25). At Winery A two native strains, C93 and C103, were observed on a conveyor belt in 2016 (Table 19). Neither were observed in any fermentation processed at Winery A that vintage, indicating that they were not transferred to the fermentations despite potentially being part of the resident winery microbiome. At Winery C in 2016 Fermol Premier Cru (w 174)/Lalvin D254 (C8), was observed on both the connector ports of the pump and hose and F10, suggesting the strain was transferred from the equipment to the fermentation, and was likely part of the resident winery microbiome. Since Fermol Premier Cru (w 174)/Lalvin D254 was part of SPG S6, which contained strains exclusive to Winery C fermentations, where Lalvin D254 has been used for inoculations, the connection seems clear. Wineries are dynamic systems for yeast movement, and both equipment and fermentation liquid can exchange yeast (Bokulich et al. 2013). In short, there was some transfer of yeast from equipment to fermentations, but not all yeast present on equipment was transferred nor became part of the stable resident winery microbiome.

Conclusion

Spontaneously fermented Rieslings from the Finger Lakes contained a distinct composition of yeast species and strains, influenced by both winery and vineyard factors, with significant variation between vintages. Over 100 native *S. cerevisiae* strains were observed, and were more robust than expected when compared to commercial related strains.

The influence of vineyard specific factors was suggested by several facts: (1) the observed winery equipment yeast composition did not completely account for the variations observed between grape and subsequent fermentation samples, (2) the limited overlap in of *S. cerevisiae* strains in fermentation samples across vintages included native strains observed in fermentations sourcing from the same vineyard in both years, and (3) each fermentation contained at least five unique *S. cerevisiae* strains. That winery specific factors were also influential was suggested by (1) the limited overlap in yeast species observed on grapes and in subsequent fermentation samples, (2) the lack of *S. cerevisiae* observed on grape samples, and (3) the higher than expected overlap of *S. cerevisiae* strains observed across fermentations in the same vintage at a single winery. Neither vineyard or winery specific factors alone could fully explain the observed populations, suggesting that both contributed to the yeast species and strain composition observed.

C. boidnii, C. zemplinina/C. stellata, *H. uvarum*, *M. pulcherrima*, *P. cecembensis/P. occidentalis/P. kudriavzevii*, and *T. californica/T. cantarellii* were all observed several times in multiple sample types across both vintages, supporting their inclusion in the Finger Lakes microbiome. *C. boidnii* and *T. californica/T. cantarellii* in particular are uncommonly observed in wine fermentations, as is *H. valbyensis*, which was observed less frequently but was notably present during late fermentation. *C. zemplinina/C. stellata* and *P. cecembensis/P. occidentalis/P. kudriavzevii* from the

Finger Lakes may be part of a multi-regional microbiome distribution with the Ontario Canada wine region, as supported by their identification on grape and fermentation samples in both regions. However, strain level analysis of the observed non-*Saccharomyces* yeast would be needed to establish if a close genetic relationship exists. Further research is required to determine the influence of these yeast species on Finger Lakes Riesling flavor development.

The observed abundance of native *S. cerevisiae* strains was consistent with previous research showing similarly large *S. cerevisiae* strain diversity in other wine regions. Native *S. cerevisiae* strains were observed more often than CRS in fermentations, but were less often persistent or dominant. Overall, CRS never completely displaced native strains and outcompeted them more slowly than expected, indicating the robustness, in particular, of C1, C3, C6, C8, C10, C16, C21, C29, and C50, all of which were all persistent in at least one fermentation through late fermentation. Further research into the kinetics of and contribution to Riesling flavor development of these native strains is warranted. Additionally, a comprehensive phylogenetic analysis of all observed native strains from the Finger Lakes and those observed in other wine regions would give context to the worldwide uniqueness of the *S. cerevisiae* strains.

APPENDICES

Appendix 1 - Fermentation Samples Collected
Fermentation 1 – 10/20/15*, 10/26/15, 11/5/15, 11/12/15, 11/30/15
Fermentation 2 – 10/9/15, 10/15/15, 10/20/15, 10/26/15, 11/5/15, 11/12/15, 11/30/15
Fermentation 3 – 10/9/15, 10/15/15, 10/26/15, 11/5/15, 11/12/15, 11/30/15
Fermentation 4 – 9/30/15, 10/6/15
Fermentation 5 – 10/15/15, 11/5/15, 11/12/15, 12/10/15, 12/18/15
Fermentation 6 – 10/19/16, 10/24/16, 11/3/16, 11/9/16, 11/16/16, 11/28/16, 12/7/16, 12/16/16
Fermentation 7 - 10/12/16, 10/19/16, 10/24/16, 11/3/16, 11/9/16, 11/16/16, 11/28/16, 12/7/16, 12/16/16
Fermentation 8 – 10/19/16, 10/24/16, 11/3/16*, 11/9/16, 11/16/16, 11/28/16, 12/7/16, 12/16/16
Fermentation 9 – 10/19/16, 10/24/16, 11/3/16, 11/9/16, 11/16/16, 11/28/16, 12/7/16, 12/16/16
Fermentation 10 – 10/12/16, 10/19/16, 10/24/16, 11/3/16, 11/16/16, 11/28/16, 12/7/16
*Excluded from analysis due to recording error

Appendix 2 - Equipment Swab Samples Collected by Winery
Winery A – inside of press 10/9/15, bottom of press 10/9/15*, pump 10/9/15, settling tank 10/9/15, siphon of settling tank 10/9/15, sorting tables 10/12/16, conveyor belts 10/12/16, centrifugal pump 10/12/16, fermentation tank 10/12/16, settling tank 10/12/16, press 10/12/16
Winery B – press 09/30/15, settling tank 9/30/15*, press 10/12/15* (no colonies only mold), settling tank 1 10/12/15, settling tank 2 10/12/15* (no colonies), pump* 10/12/15
Winery C – pump/hose 9/30/16, cleaning brush 9/30/16, press 9/30/16, crusher/destemmer conveyor 9/30/16, tank 9/30/16, settling tank 9/30/16
*no colonies were isolated from these equipment swab samples

Appendix 3 – Listing of Matching Strain Groups Not Similar or Potentially Similar to Any Other Matching Strain Group
The following matching S. cerevisiae strain groups were not similar to any other observed S. cerevisiae strains: C9, C11, C14, C16, C19, C29 to 34, C36, C38, C54, C56 to C58, C60, C65 to C69, C90 to C93, C98 to C101, C106 to C112, C132, C136 to C138, C141, C142, C145 to C154, and C156 to C169.
The following matching S. cerevisiae strain groups were not potentially similar to any other observed S. cerevisiae strains: C11, C14, C29, C33, C107, C110, C136 to C138, C141, C142, C145 to C154, C156 to C166, and C169.

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