USE OF NON-*SACCHAROMYCES* YEASTS FOR REDUCING THE ETHANOL CONTENTS OF RED WINE

By

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Abstract

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Yeasts isolated from Washington vineyards were investigated for their abilities to reduce alcohol contents of red wines. An initial evaluation of carbohydrate and nitrogen utilization was conducted in high sugar (>300 g/L glucose and fructose) grape musts using four non-*Saccharomyces* yeasts (*Candida californica*, *Metschnikowia pulcherrima*, *Meyerozyma caribbica*, and *Wickerhamomyces anomalus*). When inoculated into Syrah grape must six days prior to *S. cerevisiae*, non-*Saccharomyces* yeasts influenced concentrations of glycerol and volatile aromas, and lower concentrations of ethanol were observed in ferments with *C. californica* and *Mt. pulcherrima*. In a broader screening, 16 species of non-*Saccharomyces* yeasts were investigated for their ability to metabolize sugar without ethanol production when inoculated into Merlot must (310 g/L glucose and fructose) six days before *S. cerevisiae* without and with added oxygen. Five species (*Mt. chrysoperlae*, *Mt. pulcherrima, My. guilliermondii*, *Pichia kluyveri*, and *P. membranifaciens*) produced wines with significantly less ethanol without excessive acetic acid accumulation. When inoculated into Merlot must containing less sugar (266 g/L glucose and fructose) three days prior to *S. cerevisiae*, dry wines (≤2 g/L glucose and fructose) with significantly less ethanol were obtained using *Mt. pulcherrima* and *My. guilliermondii*. Synthetic media sequentially inoculated with *Mt. pulcherrima* then *S. cerevisiae*,

which contained 2.4 to 3.0% less ethanol than cultures fermented with *S. cerevisiae* alone, confirmed lower ethanol production by *Mt. pulcherrima*. Conducting larger-scale fermentations (100 L) with unsterilized Merlot grape musts, wines with *Mt. pulcherrima* contained 0.9% v/v less ethanol than wines inoculated solely with *S. cerevisiae*. Finally, sensory evaluations were conducted on Merlot wines fermented under winery conditions with *Mt. pulcherrima* or *My. guilliermondii* yeasts. *Mt. pulcherrima* generated wines with 1.1% v/v less ethanol than wines fermented only with *S. cerevisiae* which were characterized by 'estery' and fruit-associated aromas and flavors. While *My. guilliermondii* did not affect ethanol content, wines fermented with this yeast exhibited 'berry' and 'woody' aromas and/or flavors with heightened astringency. Overall, this research demonstrates the potential to produce wines with lower alcohol content without compromising wine quality using *Mt. pulcherrima* isolated from Washington vineyards.

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DEDICATION

This dissertation is dedicated to my mother. If only she were still here to see me…

Mary L. Aplin

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CHAPTER I

LITERATURE REVIEW

Introduction

The average ethanol concentration in red wines produced worldwide increased between 1984 and 2014 from being less than 12.5% to greater than 13.6% v/v, with wines containing 15% v/v becoming more common (Godden et al., 2015; Gonzalez et al., 2013). This trend is primarily due to consumer preferences for 'rich', 'full-bodied' wine styles which require extended vine hang-times to achieve optimal phenolic maturity. One consequence is that these grape musts contain increased amounts of sugar which yield wines with higher ethanol levels (Mira de Orduña, 2010). In addition, environmental climate change has influenced vine phenology such that grapes accumulate sugars faster, while also containing less acidity and altered phenolic contents (Mira de Orduña, 2010).

Increasing levels of alcohol in wine can markedly affect quality and consumer acceptance. Several studies have reported that higher ethanol content intensified sensory perceptions of 'chemical', 'woody', 'hotness', and/or 'bitterness' (Baker et al., 2016; Bindon et al., 2014; Gawel et al., 2007; Heyman et al., 2013; Villamor et al., 2013b). In addition, elevated concentrations of alcohol can mask 'fruity' and/or 'floral' attributes by decreasing volatility of specific aroma compounds (Goldner et al, 2009; Mira de Orduña, 2010; Villamor et al., 2013a; 2013b). Higher must sugar concentrations also contribute to an increased risk of sluggish or even stuck alcoholic fermentations due to ethanol concentrations that are toxic to yeast, yielding unbalanced wines with undesirable sweetness (Berthels et al., 2004; Bisson and Butzke, 2000; Coulter et al., 2008). Furthermore, high-alcohol wines often incur additional taxation and potentially rejection by health-conscious consumers over concerns related to excessive alcohol

consumption and road safety (Ciani et al., 2016; Gonzalez et al., 2013). However, Bindon et al. (2014) and Meillon et al. (2010a; 2010b) reported that consumers still prefer wine with a moderate alcohol strength (13.5% v/v). Winemakers are thus primarily interested in methods that achieve a 1 to 3% v/v reduction in alcohol concentration to compensate for changes in climate and growing practices (Ciani et al., 2016; Gonzalez et al., 2013). Though classifications vary between countries based on differing labeling and legislative requirements, Pickering (2000) and Saliba et al. (2013) noted that wines with reduced ethanol contents have been classified as dealcoholized/alcohol free (<0.5% v/v), low alcohol (0.5 to 1.2% v/v), reduced alcohol (1.2 to 6.5% v/v), and lower alcohol wine (5.5 to 10.5% v/v).

To reduce alcohol contents, various viticultural and/or enological approaches have been explored which target different aspects of production. For instance, viticultural practices aimed to delay and manage harvest date has been examined including reducing leaf area via defoliation and/or shoot trimming (Martínez de Toda et al., 2013) or applying growth regulators such as 1-napthalene acetic acid or brassinazole (Böttcher et al., 2011; Symons et al., 2006). Another approach is to remove sugars from grape musts using nanofiltration prior to fermentation, (Garcia-Martín et al., 2010) or to oxidize glucose using the enzyme glucose oxidase (Pickering et al., 1999). Ethanol in wine can also be physically removed through membrane technologies, vacuum distillation, or supercritical CO_2 extraction (Rolle et al., 2018, Schimdtke et al., 2012). However, many of these practices decreased wine quality by unintentionally altering concentrations of important organic acids, phenolics, and/or volatile aroma compounds (Garcia-Martín et al., 2010; Pickering et al., 1999; Rolle et al., 2018; Schmidtke et al., 2012).

Although these approaches require further research prior to implementation, this review will focus on different yeasts and inoculation strategies as means to reduce subsequent ethanol.

While *Saccharomyces cerevisiae* is the principal microorganism used for alcoholic fermentation, other yeasts may also be useful.

Reducing Ethanol Using Modified *S. cerevisiae*

Development of yeasts for reducing the final alcohol concentrations in wine has focused on identifying species/strains that exhibit lower ethanol yields from carbohydrate metabolism, expressed as grams ethanol produced per gram of sugar consumed. As *Saccharomyces cerevisiae* is the principal microorganism responsible for wine fermentation, it is unsurprisingly the preferred organism for many researchers seeking to reduce wine alcohol content. In fact, high yields of ethanol from sugar coupled with high tolerance to ethanol's toxic effects are key towards this species ability to dominate high sugar grape musts (Piškur et al., 2006). Although some natural variability exists between wild and commercial *S. cerevisiae* strains, the intraspecies distribution of ethanol yield is rather narrow (±0.5% v/v), resulting in comparable wine ethanol concentrations (Ciani et al., 2016; Magyar and Toth, 2011; Varela et al., 2008). In general, *S. cerevisiae* will produce 1% v/v ethanol for every 17 g/L sugar metabolized (Ciani et al., 2016).

Metabolism

Yeast have two primary pathways for metabolism of sugar; respiration or fermentation. As illustrated in Figure 1, fermentation allows for utilization of sugar to produce ethanol and CO_2 while respiration yields only CO_2. In fact, fermentation produces two molecules of ethanol and two molecules of carbon dioxide per hexose molecule while respiration converts all six carbon atoms from sugar into carbon dioxide. Respiration generates a net gain of 38 ATP (adenosine triphosphate) per hexose molecule through the process of oxidative phosphorylation compared to two produced through fermentation (Fugelsang and Edwards, 2007). Here, ATP is

generated when electrons are transferred through cytochromes to oxygen during the reoxidation of NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide) to NAD^+ and FAD. Utilizing the membrane-bound enzyme ATPase, each molecule of NADH yields 3 ATP, while each $FADH_2$ produces 2 ATP.

Because oxygen is the final electron acceptor, aerobic conditions are normally required for respiration in contrast to typically anerobic conditions during winemaking (Fugelsang and Edwards, 2007). However, inducing respiration by *S. cerevisiae* present in a grape must is difficult due to the phenomenon known as the Crabtree effect. First noted by De Deken (1966), this effect dictates that fermentation metabolic pathways are strongly favored compared to those of respiration even in the presence of oxygen (*i.e.,* aerobic fermentation) because of elevated sugar concentrations, >9 g/L (De Deken, 1966; Ribéreau-Gayon et al., 2006). If concentrations of sugars are below this value, then respiration becomes the primary metabolic pathway unlike the conditions of grape must with high amounts of sugar (Ciani et al., 2016). From an evolutionary point of view, the Crabtree effect is thought to have played a major role in the adaptation of *S. cerevisiae* to sugar rich environments, thus allowing the yeast to outcompete other microorganisms (Piškur et al., 2006). Even though fermentation metabolism is less energy efficient than respiration (*i.e.,* lower ATP yield per hexose molecule), the resultant ethanol inhibits, if not kills, other microorganisms. Furthermore, ethanol can be catabolized in the presence of oxygen once glucose is depleted, a metabolic property known as diauxic shift (Galdieri et al., 2010; Piškur et al., 2006).

The exact biochemical nature of the Crabtree effect has been long debated. Earlier studies concluded that high concentrations (>9 g/L) of glucose directly repressed production and activity of enzymes used in the Krebs cycle (Beck and von Meyenburg, 1968; De Deken, 1966; Polakis

et al., 1965). Indeed, *S. cerevisiae* utilizes a series of interlinked regulatory and signaling pathways to sense extra- and intracellular glucose levels and control gene transcription of hexose transporters and metabolism-related enzymes (reviewed by Kayikci and Nielsen, 2015). Glucose repression of respiratory enzymes has led researchers to deduce that *S. cerevisiae* has a limited capacity for respiration (Alexander and Jeffries, 1990; Postma et al., 1989). In support, Aceituno et al. (2012) determined that insufficient NADH transport from cytosol to inside mitochondria, where reoxidation to NAD^+ occurs during respiratory metabolism, limited fully aerobic respiration in *S. cerevisiae*.

The immediate induction of aerobic fermentation upon glucose pulse, deemed the short-term Crabtree effect (Dashko et al., 2014), has been attributed to overflow metabolism at the pyruvate node (Postma et al., 1989; Pronk et al., 1996; Van Urk et al., 1989). Postma et al. (1989) demonstrated that an overabundance of pyruvate and acetate produced during glycolysis have an uncoupling effect on respiration in *S. cerevisiae.* The yeast cell then increases pyruvate decarboxylase production, which leads to ethanol generation, in order to maintain growth, regenerate NAD^+ and produce ATP. This was confirmed by Van Urk et al. (1990), who observed that pyruvate decarboxylase activity increased six-fold immediately after adding glucose. In addition, Van Urk et al. (1989) observed that *S. cerevisiae* utilized mainly low-affinity facilitated-diffusion glucose transport systems, which they suggested could lead to unrestricted glucose uptake and an overabundance of pyruvate. Furthermore, Otterstedt et al. (2004) and Jansen et al. (2005) eased the Crabtree effect in *S. cerevisiae* by inducing mutations which decreased glycolytic flow, demonstrating that a high sugar consumption rate is critical for aerobic fermentation.

Growth and sugar consumption by *S. cerevisiae* is dependent upon the redox balance between NAD^+ and NADH. *S. cerevisiae* generates large pools of NADH during glycolysis when glyceraldehyde-3-phosphate is oxidized to 1, 3-bisphophoglycerate (Bakker et al., 2001). In order to maintain glycolytic flux, the yeast cell must recycle NAD^+ to prevent depletion of ATP used for cellular energy. The bulk of NAD^+ is regenerated through the subsequent production of ethanol in typical *S. cerevisiae* cells, although yeast cells also utilize glycerol and succinate as sinks for NADH oxidation.

Genetic modification

Targeted changes to the genome of *S. cerevisiae* can redirect sugar metabolism away from ethanol towards glycerol, chosen for its positive contributions to sweetness, 'smoothness', and viscosity of wine (Gawel et al., 2007; Jones et al., 2008; Kutyna et al., 2010; Noble and Bursick, 1984). Genes such as *GPD1, GPD2, PDC2, ADH1,* and *TPI1* can be modified in order to favor over-production of glycerol (Figure 1). Varela et al. (2012) concluded that the most efficient modification strategy was the overexpression of *GPD1*, which encodes for glycerol-3-phosphate dehydrogenase. In support, these authors reported that a strain of *S. cerevisiae* which contained three copies of the *GPD1* gene produced wine with up to 3.6% v/v less ethanol than those fermented with the parental strain but with a corresponding to a 5-fold increase in glycerol. However, Cambon et al. (2006) and Remize et al. (1999) also noted that overexpression of *GPD1* increased production of metabolites that negatively impact wine quality, namely acetate, acetaldehyde, and acetoin. As such, further genetic modifications were needed to reduce the accumulation of by-products responsible for sensory faults (Cambon et al., 2006; Ehsani et al., 2009; Eglington et al., 2002; Varela et al., 2012). Kutyna et al. (2010) surmised that reduced ethanol production by *GPD* mutant strains lead to surpluses in NAD^+ and that other enzymes,

primarily aldehyde dehydrogenase, were needed to restore the NAD^+/NADH balance. Other researchers have overexpressed the *GPD2* gene in *S. cerevisiae,* which encodes an isomer of glycerol-3-phosphate dehydrogenase, however these strains did not yield as much glycerol as those with *GPD1* modifications (de Barros Lopes et al., 2000; Eglington et al., 2002).

Another genetic means to increase glycerol yields is through modification to pyruvate decarboxylase genes, specifically deletion of *PDC2* (Nevoight and Stahl, 1996). Pyruvate decarboxylase catalyzes the decarboxylation of pyruvate to acetaldehyde, the latter of which is reduced to ethanol (Fig. 1). Deletion of the *PDC2* gene, which codes for the transcriptional factor Pdc2p which regulates pyruvate decarboxylase production, decreased enzyme activity by 81% (Nevoight and Stahl, 1996). *PDC2* null strains produced 366% more glycerol and decreased ethanol yield by 28%, which highlights the importance of the Pdc2p cofactor in central carbon metabolism. By also overexpressing *GPD1* in *PDC2* null strains, glycerol yield increased 707% while ethanol yield decreased 45% at the cost of an increase in acetate production (Nevoight and Stahl, 1996). Furthermore, glucose consumption rate for *PDC2* null mutants (with and without *GPD1* overexpression) was significantly decreased.

Besides overexpression of *GPD1*, researchers have redirected carbon flux towards glycerol by deleting genes involved in glycolysis. The first method involved deletion of the *ADH1* gene that codes for the primary isoform of alcohol dehydrogenase responsible for reduction of acetaldehyde to ethanol. Yeasts lacking *ADH1* exhibited impaired ethanol synthesis (63% reduction) and increased glycerol by 1433% (Drewke et al., 1990). In a different approach, Compagno et al. (1996) deleted the triose phosphate isomerase *TPI1*, which converts dihydroxyacetone phosphate to glyceraldehyde-3-phospahte. Here, *TPI1* null *S. cerevisiae* strains converted glucose to glycerol at amounts as high as 90% of the theoretical molar yield. However,

S. cerevisiae strains with deletions to *ADH1* or *TPI1* exhibited impaired growth on glucose due to reduced glycolytic flux which lead to NAD^+/NADH imbalances (Kutyna et al., 2010). Moreover, *ADH1* null strains also accumulated up to 11% w/v of acetic acid caused by the low conversion rate of acetaldehyde to ethanol (Drewke et al., 1990).

Adaptive laboratory evolution

Traditional genetic engineering of *S. cerevisiae* has limitations, namely that concerns from consumers and legislative bodies regarding use of genetically modified organisms by the food and beverage industry. To circumvent legislative issues surrounding genetic modifications, some researchers have explored adaptive laboratory evolution as a means to develop new *S. cerevisiae* strains. Here, tens to hundreds of generations of yeasts are serially cultivated under specific pressures (*e.g.*, stress conditions or growth with a poorly assimilated substrate) to select for natural genetic variants exhibiting higher fitness under cultivation conditions (Tilloy et al., 2015). Using growth on gluconate, Cadière et al. (2011; 2012) developed strain ECA5™ which exhibited a 1.5-fold increase in flux through the pentose phosphate pathway, enhanced fermentation rate and aroma production, and decreased acetate formation. More recently, Tilloy et al. (2014) serially cultivated wine strains under carbon-limited, hyperosmotic stress (1.25M to 2.4M KCl), conditions known to stimulate glycerol production via the high-osmolarity glycerol (HOG) mitogen-activated protein kinase pathway (Dihazi et al., 2004). Evolved strain K300.1(b) exhibited increased glycerol, succinate, and 2,3-butanediol production coupled with lower ethanol production compared to the ancestral EC1118 strain, which was then further enhanced after conventional breeding (Tilloy et al., 2014). In pilot-scale fermentations of Syrah must, strains evolved by Tilloy et al. (2014) reduced ethanol by up to 1.3% v/v, increased glycerol and 2,3-butanediol concentrations, and decreased acetate content.

Limitation of *S. cerevisiae*

A major drawback to using *S. cerevisiae* to reduce wine alcohol content is that the increase in concentration of alternate by-products, including glycerol, required to impact ethanol levels would affect wine sensory characteristics (Ciani et al., 2016). Assuming each 1% v/v ethanol requires 17 g/L sugar, a 2% v/v reduction in ethanol would generate concomitant glycerol concentrations well beyond the difference thresholds for sweetness (5.2 g/L) and perceived viscosity (25.8 g/L) established by Noble and Bursick (1984). While the contribution of glycerol to wine quality is often considered positive (Jones et al., 2008), the levels needed to reduce alcohol by 1 to 2% v/v may yield red wines with undesired sweetness (Ciani et al., 2016; Kutyna et al., 2010; Noble and Bursick, 1984). Concentrations of other metabolites (*i.e.*, succinate and acetate) would also be unacceptable at such elevated concentrations (Ciani et al., 2016; Jolly et al., 2014). Given its gaseous nature, carbon dioxide is perhaps the only metabolite that would not directly impact a wine's sensory characteristics. Metabolically, respiration is needed to produce more carbon dioxide without ethanol production, which is limited in *S. cerevisiae* due to the Crabtree effect (Pronk et al., 1996).

Reducing Ethanol Using Non-*Saccharomyces* Yeasts

While *S. cerevisiae* is the prototypical yeast which exhibits the Crabtree effect (Crabtree-positive), many respiratory non-*Saccharomyces* species do not, so-called Crabtree-negative yeasts. Gonzalez et al. (2013) recently proposed partial respiration of sugars using respiratory Crabtree-negative non-*Saccharomyces* yeasts to reduce wine alcohol levels. An idealized fermentation strategy utilizing non-*Saccharomyces* yeasts recreated from Gonzalez et al. (2013) is depicted in Figure 2. Early growth of these yeasts converts sugars to biomass, CO_2, and other by-products besides ethanol prior to inoculation with *S. cerevisiae*. The addition of oxygen to the

must during the non-*Saccharomyces* growth phase can increase the portion of sugar which is respired (Contreras et al., 2015; Gonzalez et al., 2013; Morales et al., 2015; Quirós et al., 2014). Following this type of strategy, researchers have produced wines with less alcohol than those fermented with *S. cerevisiae* alone (Canonico et al., 2016; Contreras et al. 2014; 2015a; 2015b; Di Maio et al., 2011; Englezos et al., 2016; Gobbi et al., 2013; Morales et al. 2015; Rossouw and Bauer, 2016; Varela et al., 2016; 2017). Due to lower ethanol tolerance and other factors (Comitini et al., 2011; Fleet, 2003; Jolly et al., 2014), non-*Saccharomyces* yeasts cannot typically metabolize all the available sugar in grape must so continued fermentation by *S. cerevisiae* is needed to reach dryness (≤2 g/L residual sugar).

In musts purposefully inoculated with non-*Saccharomyces* yeasts, *S. cerevisiae* can be co-inoculated simultaneously or sequentially following the addition of non-*Saccharomyces* starter cultures. For the purposes of ethanol reduction, sequential inoculation strategies are typically utilized to allow the metabolic action of the non-*Saccharomyces* yeast to proceed without interference by *S. cerevisiae*. The initial inoculation level of non-*Saccharomyces* yeasts affects how they compete with *S. cerevisiae* and wild yeasts (Comitini et al., 2011), while the timing interval before *S. cerevisiae* introduction affects the duration of their metabolic activity (*i.e.,* how much sugar they utilize (Ciani et al., 2016)). After *S. cerevisiae* inoculation, non-*Saccharomyces* yeast populations quickly decline due to the competitiveness of *S. cerevisiae* and the production of ethanol and other toxic metabolites (Albergaria and Arneborg, 2016; Nissen and Arneborg, 2003; Pérez-Navado et al., 2006; Salvadó et al., 2011; Wang et al., 2016).

Metabolism

While many of the pathways for sugar utilization are conserved between yeast species, the metabolic flux distribution during fermentation differ between *S. cerevisiae* and non-

Saccharomyces yeasts (Ciani et al., 2016; Flores et al., 2000). For *S. cerevisiae*, ethanol production is approximately 90 to 95% of the theoretical yield on sugar, with the remaining 5-10% explained by biomass production and alternative pathways, reflective of its primarily fermentative metabolism (Konig et. al, 2009). Non-*Saccharomyces* yeasts on the other hand will divert sugar catabolism away from ethanol and produce an abundance of secondary metabolites, like acetic acid, glycerol, ethyl acetate, acetaldehyde, and CO_2, which can explain in part the lower ethanol yield exhibited by some non-*Saccharomyces* yeasts (Ciani et al., 2000; Ciani and Maccarelli, 1998; Domizio et al., 2011; Magyar and Tóth, 2011; Tofalo et al., 2012). For example, ethanol generation was positively correlated with glycerol production for *Candida stellata*, while it was inversely related to acetic acid and ethyl acetate content for *Hanseniaspora uvarum/Kloeckera apiculata*. Glycerol production in *C. stellata* during fermentation is most likely a response to osmotic stress from high sugar concentration (Ciani et al., 2000; Wang et al., 2001). Indeed, Ciani et al. (2000) found that *C. stellata* exhibited a forty-fold increase in glycerol-3-phosphate dehydrogenase activity and a four-fold decrease in alcohol dehydrogenase activity compared to *S. cerevisiae*, which explains the low growth and ethanol production by *C. stellata* in wine fermentations (Magyar and Tóth, 2011). For *H. uvarum/K. apiculate*, the inverse relationship between ethanol and acetic acid or ethyl acetate is due to the primarily oxidative metabolism exhibited by this yeast (Quirós et al., 2014), which is Crabtree-negative (Venturin et al., 1995).

Yeasts which do not exhibit the Crabtree effect generally utilize respiration, when grown in aerobic conditions, regardless of glucose concentration. To assess yeasts for the Crabtree effect, cultures are grown under aerobic conditions in glucose-limited chemostats (De Deken, 1966; Postma et al., 1989; Van Urk et al., 1989; 1990). In chemostat cultures, fresh media is

continuously added a specified rate (dilution rate) while culture liquid is simultaneously removed at the same rate, so that microorganisms can be grown under a physiological steady state. Researchers observed that Crabtree positive organisms, like *S. cerevisiae*, respired sugars as long as glucose concentrations were maintained below a strain-specific threshold value, known as the critical dilution rate, above which aerobic fermentation occurred (De Deken, 1966; Van Dijken et al., 1993). In contrast, Crabtree negative yeasts will respire glucose under aerobic conditions, even at high dilution rates, with negligible ethanol production. Given the complexity of assessing the Crabtree phenotype, as well as the sheer number of yeasts (approximately 1500 species), most species have not been assessed for this metabolic feature even though many exhibit fermentative metabolism (Kurtzman et al., 2011). In addition, more recent analysis has determined that the balance between respiratory and fermentative metabolism varies on more of a continuum for different yeasts (Quirós et al., 2014), rather than binarily as either Crabtree positive or negative.

Crabtree-negative yeasts typically exhibit lower glucose uptake capacity than Crabtree-positive yeasts (Van Urk et al. 1989; 1990). Van urk et al. (1989) determined that yeasts not exhibiting the Crabtree effect produced hexose transport systems with 10 to 50 times greater affinity for glucose, fructose, and mannose than the facilitated-diffusion transport systems produced by *S. cerevisiae*. Yeasts with high-affinity transport systems accumulated 6-deoxyglucose against the concentration gradient, an observation consistent with energy-dependent active transport. These high-affinity transport systems contribute to the ‘weak’ or low fermentation rate observed in cultures of non-*Saccharomyces* yeasts (Van Urk et al., 1989; Bisson, 1999). Furthermore, high-affinity sugar transport systems may let Crabtree-negative

oxidative yeasts dominate *S. cerevisiae* on the surface of healthy grape berries, where sugar concentration is minimal (Barata et al., 2012b).

On its own, Crabtree status is a poor predictor of whether yeasts will exhibit reduced ethanol yield under enological conditions (Contreras et al., 2014; 2015b; Quirós et al., 2014). Quirós et al. (2014) found respiratory quotient (RQ), calculated as the ratio of CO_2 produced to amount O_2 consumed and ranged from 1 (full respiration) to ∞ (full fermentation) for hexose consumption, to be a poor predictor (Spearman coefficient of .470) of ethanol yield in synthetic grape juice media. Quirós et al. (2014) hypothesized that the differences between Chemostat conditions typically used to determine Crabtree status and their experimental setup (sugar availability, pH, cultivation time, and aeration regime) may be to blame for the low predictive ability. In addition, Rodrigues et al. (2016) determined that environmental factors besides aeration and sugar content, such as temperature and nitrogen source availability, impacted the ability of some yeasts to respire sugar more efficiently. For example, both high nitrogen availability and high temperature were positively correlated to reduced levels of ethanol in ferments with *Metschnikowia pulcherrima* or *Kluyveromyces lactis*, both of which are Crabtree negative (González Siso et al., 1996; Rodrigues et al., 2016; Schnierda et al., 2014). Further research is needed concerning metabolic diversity among non-*Saccharomyces* yeasts and how it can be applied towards producing wine with lower alcohol content.

Strain selection

Identifying non-*Saccharomyces* yeasts that result in lower ethanol contents has focused on the interspecies and intraspecies variability among yeasts with regards to fermentation products. Based on observations that many non-*Saccharomyces* yeasts exhibit a broad range of fermentation by-products, low fermentation purity, and sometimes low ethanol yield, it was

thought that the variability in these products could be exploited to produce wines with lower alcohol content (Ciani and Maccarelli, 1998; Gonzalez et al. 2013; Romano et al. 1993). In a systematic examination of yeasts conducted by Ciani and Maccarelli (1998), ethanol production was related to acetaldehyde, ethyl acetate, and acetoin in *H. uvarum/K. apiculata* and to glycerol and/or succinic acid production in *C. stellata* and *T. delbrueckii* strains. While not specifically evaluating ethanol yield, Ciani and Maccarelli (1998) observed consistently lower rates of fermentation (g of CO_2/day), decreased amounts of ethanol produced by the end of fermentation (% v/v ethanol), and lower fermentation purity (volatile acidity g/L ÷ % v/v ethanol) in the non-*Saccharomyces* yeast strains.

More recent studies have evaluated the variability in ethanol yield between non-*Saccharomyces* species and strains under enological conditions (Canonico et al., 2016; Contreras et al., 2014; 2015a; Englezos et al., 2016; Gobbi et al., 2014; Magyar and Tóth, 2011; Rossouw and Bauer, 2016; Varela et al., 2016; 2017). While comparing enological properties of wine yeasts, Magyar and Tóth (2011) noted four strains of fructophilic *C. zemplinina* (syn. *Starmerella bacillarus*) displayed ethanol yields that were approximately half those exhibited by the evaluated *S. cerevisiae* strains. In an investigation of 33 yeast strains belonging to 9 different species, Gobbi et al. (2014) showed that *H. uvarum, Zygosaccharomyces sapae, Z. bailii,* and *Z. bisporus* promoted significant reductions in ethanol yield and fermentation efficiency compared to *S. cerevisiae*. In a separate experiment, Contreras et al. (2014) identified four non-*Saccharomyces* yeast strains (two belonging to *Mt. pulcherrrima* and one each from *Schizosaccharomyces malidevorans* and *C. stellata*) that exhibited low ethanol yield in an examination of 50 different non-*Saccharomyces* strains belonging to 24 different genera. Similarly, Rossouw and Bauer (2016) found 21 different strains among 91 isolates which showed

lower ethanol yields than the control *S. cerevisiae* wine yeast. Recently, Mestre Furlani (2017) identified five strains (belonging to *H. uvarum, H. osmophila, St. bacillarus,* or *C. membranifaciens*) of enological interest for reducing ethanol content among 114 isolates based on an integrative assay of respiratory, fermentative, and physiological characteristics.

Reduced alcohol wines fermented under enological conditions have been produced using non-*Saccharomyces* yeasts that exhibited reduced ethanol yield. Contreras et al. (2014; 2015a) produced Chardonnay and Shiraz wines sequentially inoculated with *Mt. pulcherrima* then *S. cerevisiae* which contained 0.9% to 1.6% v/v less ethanol, respectively, than similar wines fermented with *S. cerevisiae* alone. Similarly, Varela et al. (2017) used this same *Mt. pulcherrima* strain to produce reduced alcohol wines (~1.0% v/v less ethanol) from red and white grape musts which contained increased levels of volatile aromas associated with positive sensory attributes. Ethanol reduction in fermentations with *Mt. pulcherrima* were enhanced by the presence of *S. uvarum* (up to 1.8% less alcohol), revealing a potential 'collaboration' between these yeast species (Contreras et al., 2015a; Varela et al., 2016). Canonico et al. (2016) produced Verdicchio wines with 1.00 to 1.64% v/v less ethanol than control wines using *H. osmophila, H. uvarum, Mt. pulcherrima,* or *St. bombicola* yeasts immobilized in alginate beads. Resulting wines did not exhibit high concentrations of negative fermentation aromas, excluding *H. uvarum* wines which contained significant amounts of ethyl acetate (Canonico et al., 2016). Similarly, Roussow and Bauer (2016) observed 1.3% and 1.1% v/v ethanol reduction in Sauvignon Blanc wines inoculated with *H. opuntiae* and *H. uvarum*, respectively. Sequential fermentations with *St. bacillarus* performed by Englezos et al. (2016) yielded Barbera wines with up to 0.7% v/v less ethanol.

In terms of mass balance, it is currently unclear what happens to sugar carbon in reduced alcohol wines. In fermentations with *S. uvarum* conducted by Varela et al. (2016), the reduction in ethanol content was explained by similar increases in glycerol and succinic acid. However, in fermentations with *Mt. pulcherrima* or *Hanseniaspora* spp. yeasts, the formation of by-products such as glycerol and succinic acid did not account for the observed reduction in alcohol (Canonico et al., 2016; Contreras et al., 2014; Varela et al., 2016; 2017). *Mt. pulcherrima* and *H. uvarum* are known Crabtree-negative yeasts (Schnierda et al., 2014; Venturin et al., 1995), while other *Hanseniaspora* spp. are oxidative species, and it is possible sugar was respired directly to CO_2 in ferments with these yeasts. Further research is needed to identify potential carbon sinks and active metabolic pathways utilized by these yeasts under enological conditions, particularly aspects of respiratory metabolism.

Other researchers have selected yeasts based on their performance under highly aerated conditions to identify yeasts which can consume grape must sugars via respiration (Contreras et al., 2015b; Morales et al., 2015; Quirós et al., 2014). In an examination of 63 yeast strains from 29 species, Quirós et al. (2014) used respiratory quotient (RQ) as an indicator of respiration capacity of yeast grown in synthetic media under fully aerobic conditions. RQ was calculated as the ratio of CO_2 produced to amount O_2 consumed and ranged from 1 (full respiration) to ∞ (full fermentation) for hexose consumption. The amount of sugar consumed via respiration (%SR) was calculated based on stoichiometry from the RQ value as $\%SR = 100/(3RQ-2)$. Here, Quirós et al. (2014) identified 15 yeast strains that exhibited RQ values between 1.0 and 1.5, from which it was expected that 40 to 100% of the consumed sugar was respired. Selected based on experimental observations (Quirós et al., 2014), *Mt. pulcherrima* strain IFI 1244 exhibited significantly lower ethanol yields in natural grape juice under conditions where air was sparged

into the culture at approximately 1.3 volumes of air per volume of culture per minute (VVM). Using this same strain and moderate aeration (0.33 VVM), Morales et al. (2015) produced white wine with up to 3.7% less ethanol than anaerobic fermentations with *S. cerevisiae* alone. Similarly, but with more mild aeration (up to 0.05 VVM), Contreras et al. (2015b) identified a strain of *T. delbrueckii* and *Z. bailii* from amongst 48 yeasts which produced wine containing 1.5% and 2.0% less ethanol, respectively, than anaerobic control wines.

Of concern when selecting yeast strains for use in aerated cultures is volatile acidity accumulation (Ciani et al., 2016; Gonzalez et al., 2013; Quirós et al., 2014). There are several reports depicting higher production of volatile acidity by *S. cerevisiae* under aerated conditions versus anaerobiosis (Contreras et al., 2015b; Morales et al., 2015; Quirós et al., 2014; Rodrigues et al., 2016). In addition, some non-*Saccharomyces* strains produce high quantities of volatile acidity when aerated, possibly indicative of a primarily fermentative metabolism (Contreras et al., 2015b; Morales et al., 2015; Quirós et al., 2014), while others produce excessive concentrations under typical fermentation conditions (Andorrà et al., 2010; Ciani and Maccarelli, 1998; Vianna et al., 2008). Indeed, Rodrigues et al. (2016) reported that acetic acid production was significantly correlated with oxygen supply for four different yeasts. When selecting non-*Saccharomyces* yeasts for reducing alcohol content, it is important to choose yeasts which produce little acetic acid even under aerated conditions (Contreras et al., 2015b; Quirós et al., 2014; Rodrigues et al., 2016). Moreover, fermentation conditions need to be controlled to minimize oxygenation after inoculation of the *S. cerevisiae* strain.

There is a risk that the strong aeration levels required for efficient yeast respiration would oxidize phenolic and aroma compounds in the grape must (du Toit et al., 2006). Even though hyperoxygenation practices are often used in grape musts for reasons of color stability (du Toit et

al., 2006; Schneider, 1998), it is unknown if the oxygen supplied through these techniques is enough to support respiratory metabolism in non-*Saccharomyces* yeasts. Given that yeast cell affinity for oxygen is approximately 1000 times higher than wine polyphenols (Salmon, 2006), aeration should be balanced with yeast oxygen consumption to keep dissolved oxygen levels at approximately 0% to prevent must oxidation (Ciani et al., 2016). Morales et al. (2015) demonstrated this was possible using controlled aeration and an appropriate strain of *Mt. pulcherrima*. The development of new aeration devices which can better detect and control oxygenation levels during fermentation would also help winemakers using non-*Saccharomyces* yeasts for ethanol reduction reduce oxidative damage and acetic accumulation.

Impacts on fermentation

The deliberate use of non-*Saccharomyces* yeasts originally gained popularity for their ability to modulate the sensory properties of wine. In fact, these yeasts often contribute positively to wine quality and aroma complexity (Fleet, 2003; Jolly et al., 2003a; 2014; Varela et al., 2017). For instance, some species enhance concentrations of esters and fusel alcohols responsible for 'fruity' and 'floral aromas (Contreras et al., 2014; Rojas et al., 2003; Viania et al., 2008). Furthermore, many non-*Saccharomyces* yeasts synthesize extracellular hydrolytic enzymes, such as glycosidases and pectinases, which enhance wine aroma and mouthfeel properties through the release of grape varietal aromas and soluble polysaccharides (Comitini et al., 2011; Cordero-Bueso et al., 2013; Rodríguez et al., 2010; Strauss et al., 2001). Maturano et al. (2012) determined that these enzymes were active throughout fermentation, even after non-*Saccharomyces* yeasts died off. However, some strains produce surface films, turbidity, and excessive concentrations of undesirable aroma compounds, like acetic acid, ethyl acetate, and

acetaldehyde (Andorrà et al., 2010; Ciani and Maccarrelli, 1998; Jolly et al., 2014; Rojas et al., 2003; Zohre and Erten, 2002).

Besides potentially altering sensory quality, growth of non-*Saccharomyces* yeasts can inhibit the subsequent fermentation conducted by *S. cerevisiae*. Proliferation of non-*Saccharomyces* yeasts during the early stages of fermentation can consume vitamins and nitrogenous compounds required by *S. cerevisiae*, leading to sluggish and even stuck fermentations (Bisson and Butzke, 2000). Bataillon et al. (1996) noted that *Kloekera apiculata* growth depleted thiamin in a grape must, resulting in sluggish fermentation conditions even though sufficient nitrogen was present. In fermentations conducted by Medina et al. (2012) and Taillandier et al. (2014), *Mt. pulcherrima, H. vineae,* and *T. delbrueckii* yeasts consumed enough yeast assimilable nitrogen (YAN) and vitamins to induce sluggish fermentations in synthetic media. Furthermore, some species/strain preferentially consume specific nitrogen sources, which can impact *S. cerevisiae* growth as well as wine aroma (Kemsawasd et al., 2015; Gobert et al., 2017, Rollero et al., 2018a; 2018b). The addition of nitrogen and vitamin mixtures at the time of *S. cerevisiae* inoculation can help compensate for nutrient consumption by non-*Saccharomyces* yeasts and improve overall fermentation kinetics (Medina et al., 2012; Lage et al., 2014).

Yeast Ecology

Novel yeast strains with enological applications can be found throughout nature based on their ability to develop communities within a multitude of atmospheric, terrestrial, and aquatic environments, or niches (Kurtzman et al., 2011). Species found within these communities can be described as autochothonous (native species acting as essential components of the environment) or allochothonous (transient species). Native yeast communities within these habitats are further defined by the physical, chemical, and nutrient requirements required for them to survive and

grow. Niches associated with vineyards and wine production, particularly the grape berry surfaces, fermenting must, and winery equipment, are an excellent source of diversity for yeasts relevant to the winemaking process (Jolly et al., 2014). Some of the non-*Saccharomyces* yeasts isolated from grapes or wine fermentations are listed in Table 1.

Classification

Yeasts can be known by two different names depending on their sexual state, which can be complicated by various factors. The teleomorphic name is used to describe the sexual state, which is capable of producing ascospores, while the anamorphic name refers to the non-sporulating vegetative state (Kurtzman et al., 2011). Taxonomic classification of yeast isolates can be difficult because long-term storage and environmental conditions can impact a yeast's ability to form ascospores (Jolly et al., 2014; Kurtzman et al., 2011). When culture-based techniques are used to identify yeast, any delays between isolation and identification may result in the misidentification of yeasts as teleomorphic or anamorphic. Furthermore, on-going changes to the taxonomic record, leading to multiple synonyms for the same yeast, can make it difficult to determine which species were investigated in older literature (Kurtzman et al., 2011). The development of genome databases and modern DNA-based identification methods have helped clarify yeast taxonomy.

Vineyard

Most yeasts associated with wine production originate from the surface of grape berries (Barata et al., 2012b; Jolly et al., 2003c; 2014). On sound grapes, population of 10^2 to 10^4 CFU/g are typical, although higher populations have been reported, particularly for damaged grapes (Barata et al., 2008b; 2012a; 2012b; Jolly et al., 2003c; Li et al., 2010; Martini et al., 1996; Martins et al., 2014; Milanović et al., 2013; Raspor et al., 2006). As noted by Barata et al.

(2008a; 2008b; 2012a), sampling technique, particularly bunch sampling without accurate separation of damaged grapes, can introduce high variability in population quantification. As berries ripen, populations climb to 10^4 to 10^6 CFU/g, most likely due to sugars leaching from the inner tissues to the surface (Barata et al., 2012a; 2012b; Martins et al., 2014). Additionally, damage to the skin from insects, birds, or disease increases nutrient availability and encourages high microbial populations (>10^6 CFU/g) and diversity, especially of spoilage yeasts (Barata et al., 2008b; 2012a; 2012b).

Grape berries are colonized by a wide diversity of yeast and yeast-like organisms. Representative genera commonly present include *Aureobasidium, Candida, Cryptococcus, Hanseniaspora, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Wickerhamomyces*, and/or others (Bourret et al., 2013; Jara et al., 2016; Jolly et al., 2003c; Li et al., 2010; Milanović et al., 2013; Settani et al., 2012). Though responsible for the primary conversion of grape sugars into ethanol and CO_2, *Saccharomyces* spp. are only present in low numbers (if at all) on the berry surface (Martini et.al., 1996; Mortimer and Polsinelli, 1999). Likewise, Renouf et al. (2005) only found *Saccharomyces* spp. in samples obtained at harvest at populations which represented less than 2% of the total population.

Species distribution on the grape berries is highly correlated with berry ripening due to changes in surface nutrient availability as fruit matures and is damaged (Barata et al., 2012b; Martins et al., 2014). On unripe berries, the dominant species are comprised of oligotrophic, oxidative basidiomycetous yeasts and the yeast-like fungi *Aureobasidium pullulans*. These organisms are incapable of fermentation and thrive in nutrient poor conditions (Davenport, 1974; Kurtzman et al., 2011). The cuticle and epicuticular waxes that protect the surface of the sound grapes create a harsh environment typically devoid of nutrients that allows basidiomycetous

yeasts to dominate (Davenport, 1974; Sabate et al., 2002). Additionally, these organisms can often be isolated from other cuticle-protected surfaces, such as leaves and shoots, as well as other vineyard substrates, like soils and bark (Sabate et al., 2002).

As grapes begin to ripen, copiotrophic oxidative and weakly fermentative ascomycetes yeasts begin to dominate berry surfaces (Barata et al., 2012b). This includes *Candida* spp., *Pichia* spp., apiculate *Hanseniaspora* spp., and respiro-fermentative yeasts like *Metschnikowia* spp. From these yeasts, *H. uvarum/K. apiculata* appears to be one of the most common and widely distributed species worldwide (Barata et al., 2012b), which may explain its predominance in spontaneously fermenting musts (Jolly et al., 2014). Barata et al. (2012a; 2012b) hypothesized that species distribution shifts towards oxidative and weakly fermentative yeasts due to the release of small amounts of juice through the skin as the cuticle softens.

Proliferation of copiotrophic strongly fermentative yeasts, including *Saccharomyces* spp., *Torulasporaa* spp., *Zygosaccharomyces* spp., *Lachancea* spp., and some *Candida* and *Pichia* species (Barata et al., 2012b), occurs primarily on damaged berries, where available nutrients are in abundance. Mortimer and Polsinelli (1999) observed *S. cerevisiae* on about 0.05% to 0.1% of sound grape berries and on 25% of damaged berries at populations of approximately 10^5 to 10^6 cells/grape. In fact, the presence of *Zygoascus hellenicus/C. steatolytica, P. terricola,* and/or *P. kudriavzevii* was suggested as a zymological indicator of grapes affect by sour rot or mealybugs (*Pseudococcus* and *Planococcus* species) which excrete honeydew (Barata et al., 2008a; 2008b; 2012a). *Torulaspora* spp. and *Zygosaccharomyces* spp. yeasts were isolated in higher frequencies from grapes affected by noble rot and sour rot than from healthy grapes (Barata et al., 2008a; 2008b; Nisiotou and Nychas, 2007). Guerzoni and Marchetti (1987) found

significantly higher populations of *Zygosaccharomyces* spp. (6.78 $\log_{10}$ CFU/g) growing on sour rotten grapes than on healthy berries (1.9 $\log_{10}$ CFU/g).

While damaged berries support high numbers of yeasts, especially strongly fermentative species (Barata et al., 2012a), other basidiomycetous and oxidative ascomycetous yeasts are still typically present. For example, Barata et al. (2008b; 2012a) observed Basidiomycetes yeasts on sour-rotten grapes at similar populations as those observed on sound grapes, albeit their overall proportion amongst total yeasts was lower. For *H. uvarum* and *C. zemplinina* yeasts, populations actually increased in damaged grapes even though highly fermentative yeasts dominated the overall microbiota (Barata et al., 2008b; 2012a). Even in grapes affected by sour rot, yeasts like *Candida* spp., *Pichia* spp., and *Hanseniaspora* spp. dominated over highly fermentative species, possibly because the aerobic conditions favor oxidative yeasts (Barata et al., 2008a; 2008b; Nisiotou and Nychas, 2007).

Even in damaged berries, isolation of spoilage yeasts is uncommon, and isolation may require special selective or enrichment media (Barata et al., 2012b). Barata et al. (2008b) only identified *Z. bailii* and *Z. bisporus* from diseased grapes through the use of *Zygosaccharomyces* differential medium (ZDM). In surveys conducted by Renouf and Lonvaud-Funel (2007) and Barbin et al. (2007), *Dekkera/Brettanomyces* spp. were detected only after enrichment steps from grapes berries. One exception is Jolly et al. (2003c), who reported that *Z. baili* was the predominant yeast species (>50% of total isolates) found on grape berry bunches sampled at one of 12 sites in South Africa. In addition, Combina et al. (2005b) found *Saccharomycodes ludwigii* in high percentage of isolates (up to 17%) from grapes collected from two vineyards in Mendoza, Argentina. No mention of grape health was made in either Jolly et al. (2003c) or Combina et al. (2005b).

Insects can serve as a vector to transport yeasts throughout the vineyard, particularly between damaged berries (Mortimer and Polsinelli, 1999). Examining damaged grapes for the presence of *S. cerevisiae*, Mortimer and Polsinelli (1999) observed a variety of insects, including fruit flies (*Drosophila*), wasps (*Vespa*), honeybees (*Apis*), and other insects, primarily around damaged clusters but also crawling over intact berries. Stamps et al. (2012) demonstrated the role that both adult and larvae stage *Drosophila melanogaster* fruit flies play in the dissemination and density of yeast populations amongst fruit. In fact, the presence of *S. cerevisiae* yeasts with the *ATF1* gene, responsible for acetate ester production, in rotten fruit was found to attract more fruit flies versus yeasts without *ATF1*, which then furthered dispersal (Buser et al., 2014; Christiaens et al., 2014). Barata et al. (2012b) hypothesized that *Drosophila* flies carrying yeasts and acetic acid bacteria were essential to causing sour rot in grapes. Stefanini et al. (2012) demonstrated that social wasp queens overwintering as adults can harbor yeast cells and pass them on to their progeny, which aided in the evolution and dispersal of *S. cerevisiae.* Some insects, such as honeybees, can even disperse yeasts locally across approximately 10 km (Goddard et al., 2010).

Migratory birds also act as a reservoir for yeasts and can transport them over vast distances (Francesca et al., 2010; 2012; 2013; 2014). Beak and cloacae swabs of birds captured in vineyards from the Campania region of Italy found *H. uvarum* and *C. albicans* yeasts (Francesca et al., 2010). In a later study surveying yeast diversity in migratory birds captured in Sicily, Francesca et al. (2012) isolated 18 different yeasts species from 349 captured birds. The detection of living *S. cerevisiae* from bird cloacae 12 hours after ingestion of inoculated feed confirmed that yeasts could be disseminated during migration. Other surveys of captured birds conducted in Ustica, Italy even found isolates of the previously undescribed species *Phaffomyces usticensis* (Francesca et al., 2014) and *Wickerhamomyces sylviae* (Francesca et al., 2013) which

can grow at 40°C and 2.5 pH, suggesting possible adaptation to the bird cloaca. Sites examined in the various studies by Francesca et al. (2010; 2012; 2013; 2014) represent important stop-over points for birds seasonally migrating between Africa and Northern Europe.

Alcoholic fermentation

Yeasts present on the grape berry surface and on cellar equipment surfaces get transferred to the grape must during crushing (Jolly et al., 2014). While non-*Saccharomyces* yeasts have been isolated from cellar surfaces, including *P. anomala, P. membranifaciens, Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Debaryomyces hansenii, K. apiculata, Mt. pulcherrima*, yeast populations resident to cellar surfaces more frequently are dominated by *S. cerevisiae* (Jolly et al., 2014; Loureiro and Malfeito-Ferreira, 2003). The proportion of yeasts in grape musts originating from cellar surfaces is often minimal however, due to hygiene practices utilized by most modern wineries which minimize contamination (Jackson, 2008).

The initial conditions of the grape must, as well as various pre-fermentation practices, can affect yeast populations. Grape musts represent a rather harsh environment for many microorganisms, due to low pH, high osmotic stress, and often the presence of added SO_2, used to limit native yeast growth (Jackson, 2008; Pretorius, 2000). Pre-fermentation practices, such as cold-settling and cold-soaking, provide non-optimal temperatures for growth of *S. cerevisiae* and may favor the growth of some non-*Saccharomyces* yeasts (Maturano et al., 2015; 2016; Pretorius, 2000; Zott et al., 2008). Furthermore, must clarification procedures utilized in the making of white wine may reduce initial yeast populations (Pretorius et al., 2000).

During fermentation there is typically a progression of predominant yeasts, particularly in spontaneous ferments which lack inoculated cultures of *S. cerevisiae* (Jolly et al., 2014). Initially, species common to grape surfaces, most prominently *H. uvarum/K. apiculata* and various

Candida spp., are found at low levels in fresh must and then quickly grow to populations between 10^6 and 10^8 cells per mL (Combina et al., 2005a; Di Maro et al., 2007; Fleet, 1993; Fleet et al., 1984; Granchi et al., 1998; Heard and Fleet, 1985; Parish and Carroll, 1985; Zott et al., 2008; 2010). Populations of non-*Saccharomyces* yeasts can sustain into the later stages of fermentation (beyond 12 days), much later than initially believed, prior to *Saccharomyces* spp. dominating at the onset of alcoholic fermentation (Combina et al., 2005a; Fleet et al., 1984; Zott et al., 2008; 2010). In fact, some researchers have detected some non-*Saccharomyces* yeasts until the end of fermentation, such as strains of *C. stellata*, *T. delbrueckii*, *C. zemplinina*, and *H. uvarum* (Combina et al., 2005a; Zott et al., 2008; 2010).

The decline of non-*Saccharomyces* yeast populations during vigorous fermentation has been attributed to several factors. The combined effects of SO_2 added at crush, low pH, and progressively increasing ethanol concentration are inhibitory for many yeast species (Fleet, 2003; Fugelsang and Edwards, 2007; Jackson, 2008). Many non-*Saccharomyces* yeasts, such as *Hanseniaspora* spp., *T. delbrueckii*, and *K. thermotolerans*, grow poorly at low levels of dissolved oxygen typically encountered during vigorous fermentation (Hansen et al., 2001; Visser et al., 1990). Furthermore, researchers have noted that high *S. cerevisiae* populations ($\geq 10^7$ CFU/mL) can induce death in multiple yeast species (Granchi et al., 1998), either through a cell-cell contact mechanism (Nissen et al., 2003) or through the production of extracellular toxic metabolites besides ethanol (Pérez-Navado et al., 2006; Wang et al., 2016). However, it must be stated that the extent to which these factors inhibit non-*Saccharomyces* yeast growth will vary between species, and even between strains of the same species.

Post-alcoholic fermentation

Non-*Saccharomyces* yeasts isolated from finished wine are typically spoilage organisms associated with barrel-aged wines (Jolly et al., 2014; Loureiro and Malfeito-Ferreira, 2003). Only a small number of yeasts can grow in finished wine due to the adverse conditions imposed by high ethanol concentration and other toxic compounds, including *Dekkera/Brettanomyces* spp., *Z. bailii, Schizosaccaromyces pombe, P. membranifaciens,* and *P. kudriazevii* (Cartwright et al., 2018; Edwards and Oswald et al., 2017; Fleet et al., 1984; Grbin and Henschke, 1999; Parish and Carroll, 1985; Zuehlke et al., 2015). Indeed, *Sc. Pombe, Brettanomyces* spp. and *Zygosaccharomyces* spp. have exhibited ethanol tolerance similar or higher than *S. cerevisiae* and may be isolated from bottled wines (Edwards and Oswald, 2017; Grbin and Henschke, 1999; Zuehlke et al., 2015). When present in bottled wines, yeasts like *Sc. pombe* and *Z. bailii* can cause secondary fermentations of any residual sugars and produce turbidity, sediment, and high levels of acetic acid and/or ethyl acetate (du Toit and Pretorius, 2000). The presence of spoilage organisms in finished wines is influenced by SO_2 concentration, barrel sanitation, cellar hygiene practices, storage temperature, and the use of filtration preceding bottling (Cartwright et al., 2018; Edwards and Oswald, 2017; Jolly et al., 2014).

Some yeasts which spoil wine aging in bulk tanks and barrels can form a film on the surface, sometimes referred to as mycoderma (du Toit and Pretorius, 2000; Fugelsang and Edwards, 2007). Film forming yeasts are primarily oxidative organisms, such as *C. krusei, C. vini, P. membranifaciens, P. farinose, P. vini,* and *P. kudriazevii* (du Toit and Pretorius, 2000). Development of film yeasts may appear initially as “floating flowers” and can develop into a thick pellicle which looks “mold-like” or “chalky” in appearance (Fugelsang and Edwards, 2007). Besides causing cosmetic problems, film yeasts can synthesize high levels of spoilage

compounds such as acetic acid, ethyl acetate, and acetaldehyde (du Toit and Pretorius, 2000; Fugelsang and Edwards, 2007). Due to their oxidative metabolism, the development of film yeasts is highly dependent on oxygen exposure and occurs more often in partially filled tanks or barrels (du Toit and Pretorius, 2000). Growth of film yeasts can be prevented by maintaining properly topped tanks and barrels, thereby depriving the yeasts of oxygen, and cellaring at temperatures below 15°C (Fugelsang and Edwards, 2007).

‘Microbial terroir’

Proposed by Bokulich et al. (2013), ‘microbial terroir’ refers to specific wine growing regions which harbor defined microbiota that contribute to regionally distinct wine characteristics. By sequencing 16S rRNA along with internal transcribed spacer ribosomal sequence, Bokulich et al. (2013) showed that microbiomes in eight vineyards from four different wine growing regions in California exhibited defined biogeography. The factors contributing to regionally distinct microbial communities were primarily microclimate, but also predominant grape cultivars and vintage. In fact, Bokulich et al. (2016) later demonstrated that wine chemical composition correlated with regional biogeography patterns. Similarly, Jara et al. (2016) showed that microbial populations on grapes from Chilean vineyards varied with latitude due to local differences in relative local humidity and rainfall. Furthermore, Jara et al. (2016) noted good aromatic properties amongst some of the *Metschnikowia* and *Hasneniaspora* isolates. This highlights that regional yeast surveys can yield novel yeast strains with interesting characteristics for enological use.

Characteristics of Yeasts for Industrial Application

A recent survey of yeast diversity in Washington state vineyards by Bourret et al. (2013) isolated 53 different species, including 18 that have not been reported in North America

previously. In an evaluation of yeast growth, glucose and fructose consumption, and nitrogen utilization, White (2016) demonstrated that *Mt. pulcherrima*, *W. anomalus*, *C. californica*, and *W. anomalus* strains isolated by Bourret et al. (2013) may have enological applications.

Metschnikowia pulcherrima

Mt. pulcherrima is a Crabtree-negative yeast which is encountered in high populations on grapes and in fermenting musts (Bourret et al., 2013; Jara et al., 2016; Jolly et al., 2003c; Li et al., 2010; Milanović et al., 2013; Schnierda et al., 2014; Settani et al., 2012). Besides the ethanol reduction capabilities mentioned previously, these yeasts are known to produce high concentrations of esters (Clemente-Jimenez et al., 2004; Contreras et al., 2014; Parapouli et al., 2010; Whitener et al., 2017), particularly the 'pear-scented' compound ethyl caprylate. In fact, Jolly et al. (2003a; 2003b) noted that inoculation of *Mt. pulcherrima* enhanced overall quality scores in Chenin Blanc and Sauvignon Blanc wines. Concerning enological application, this yeast produces both β-glucosidase and β-xylosidase, enzymes which catalyze the release of varietal aromas (Comitini et al., 2011; Parapouli et al., 2010; Rodríguez et al., 2007; 2010). In mixed cultures with *S. cerevisiae*, Sadoudi et al. (2012; 2017) showed that *Mt. pulcherrima* reduced acetic acid production. Additionally, some strains of *Mt. pulcherrima* are known to inhibit other yeasts growing in media through the production of pulcherrimin, a pigment that irreversibly binds iron (Sipiczki, 2006).

Wickerhamomyces anomalus

W. anomalus (syn. *Hansenula anomala* or *P. anomala*) is another Crabtree-negative yeast which has exhibited useful aromatic properties for winemaking in mixed cultures with *S. cerevisiae* (Fredlund et al., 2002; Domizio et al., 2011; Izquierdo Cañas et al., 2014). Inoculation of *W. anomalus* into 'Airén' white grape must yielded wines with more intense fruity aromas

which were preferred over the *S. cerevisiae* control wine (Izquierdo Cañas et al., 2011). Mazuela red wines sequentially inoculated with *W. anomalus* then *S. cerevisiae* contained higher levels of acetate and ethyl esters than those fermented with *S. cerevisiae* alone (Izquierda Cañas et al., 2014). In preference testing, Mazuela wines with *W. anomalus* were noted for their fruity/floral characteristics and were preferred by 71.5% of tasters. Similar to *Mt. pulcherrima*, strains of this yeasts are strong producers of β-glycosidase enzymes (Cordero-Bueso et al., 2013; Madrigal et al., 2013; Sabel et al., 2013).

Meyerozyma caribbica

Little is known regarding the impact of *My. caribbica* on wine quality. In fact, White (2016) was the first to describe *My. caribbica* growth, sugar consumption, and nitrogen utilization in Chardonnay grape must and synthetic media. Here, in mixed culture with *S. cerevisiae*, White (2016) noted that *My. caribbica* increased the perception of 'banana' in Chardonnay wines. Besides grape wine, however, *My. caribbica* has been used to enhance aroma production in other fermented products, such as pineapple wine and cachaça, a sugarcane spirit from Brazil (Duarte et al., 2013; Ribeiro et al., 2015). Ribeiro et al. (2015) noted that pure cultures of *P. caribbica* (syn. *My. caribbica*) increased concentrations of 2-phenylethanol, 2-methyl-1propanol, 3-methyl-butanol, ethyl acetate, and phenylethyl acetate in a sugarcane and pineapple fruit wine. *P. caribbica* pineapple wines were dry (<2 g/L residual sugar) and contained 77.42 g/L ethanol. These fruit wines were liked 'moderately', 'very much', or 'extremely' by >60% of panelists (Riberio et al., 2015). Duarte et al. (2013) produced the base for cachaça, a Brazilian sugarcane spirit, using a mixed culture of *P. caribbica* and *S. cerevisiae*. Compared to cachaça produced with *S. cerevisiae* alone, co-culture of *P. caribbica* and *S.*

cerevisiae increased concentrations of ethyl hexanoate, 2-phenylethanol, linalool, ethyl butyrate, phenylethyl acetate, diethylsuccinate, and geraniol (Duarte et al., 2013).

Candida californica

Very little has been reported about the influence of *C. californica* on wine fermentation or wine quality. Eder et al. (2017) reported that a strain of *C. californica* isolated from spontaneously fermenting Isabella (*Vitis labrusca* L.) grape must was tolerant to 7.5% v/v ethanol in an agar assay. White (2016) was the first to demonstrate this yeast could grow and consume glucose and fructose in Chardonnay must. Chardonnay wines produced with *C. californica* were characterized by 'citrus' and 'green apple' aromas.

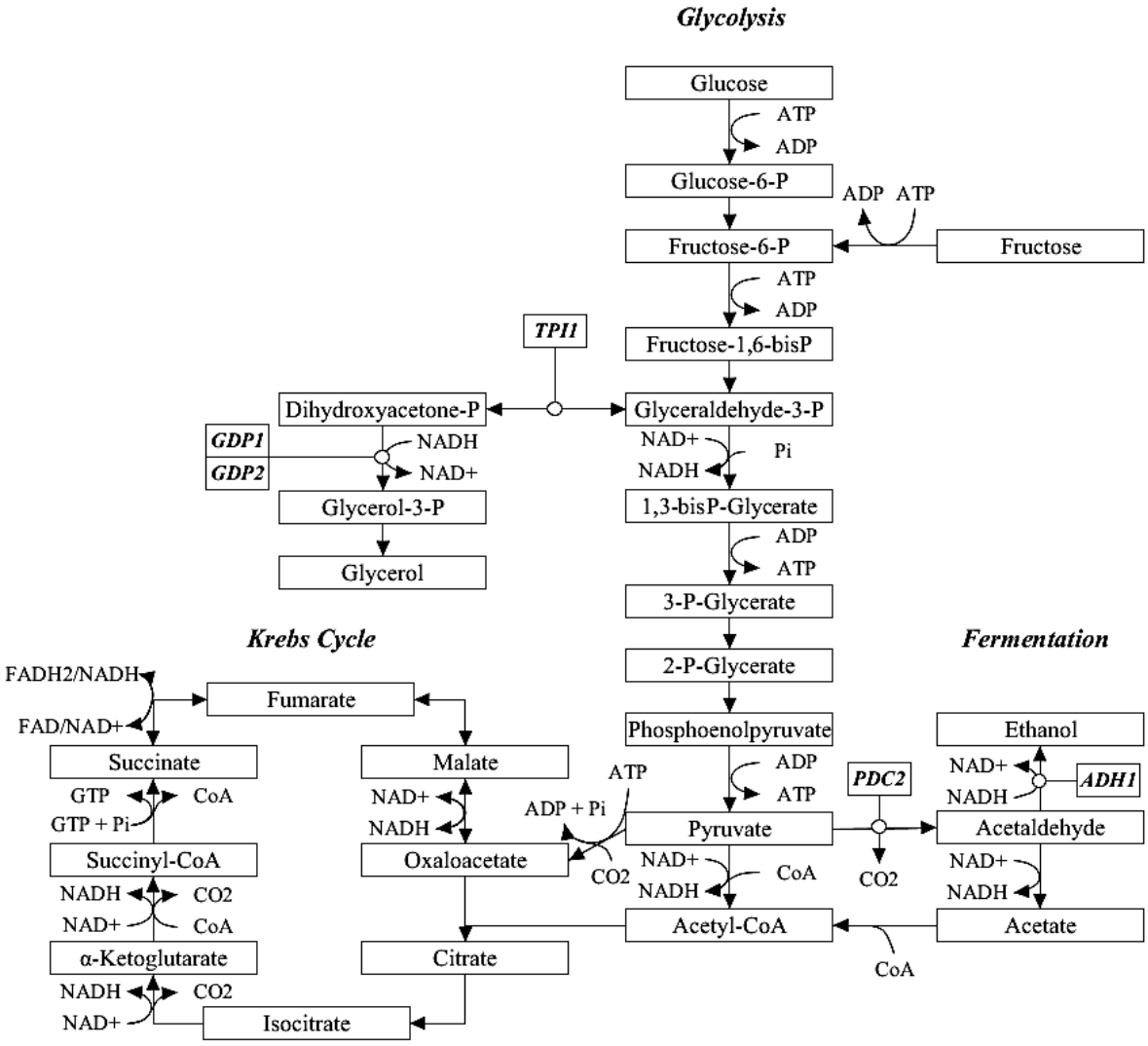

Figure 1. Metabolic pathways utilized by yeasts for the degradation of hexoses into ethanol and other by-products. Genetically modified strains of *S. cerevisiae* with changes to genes *GPD1*, *GPD2*, *TPI1*, *PDC2*, and *ADH1* have been explored for ethanol reduction. Redrawn from Ciani et al. (2016), Flores et al. (2000), and Fugelsang and Edwards (2007).

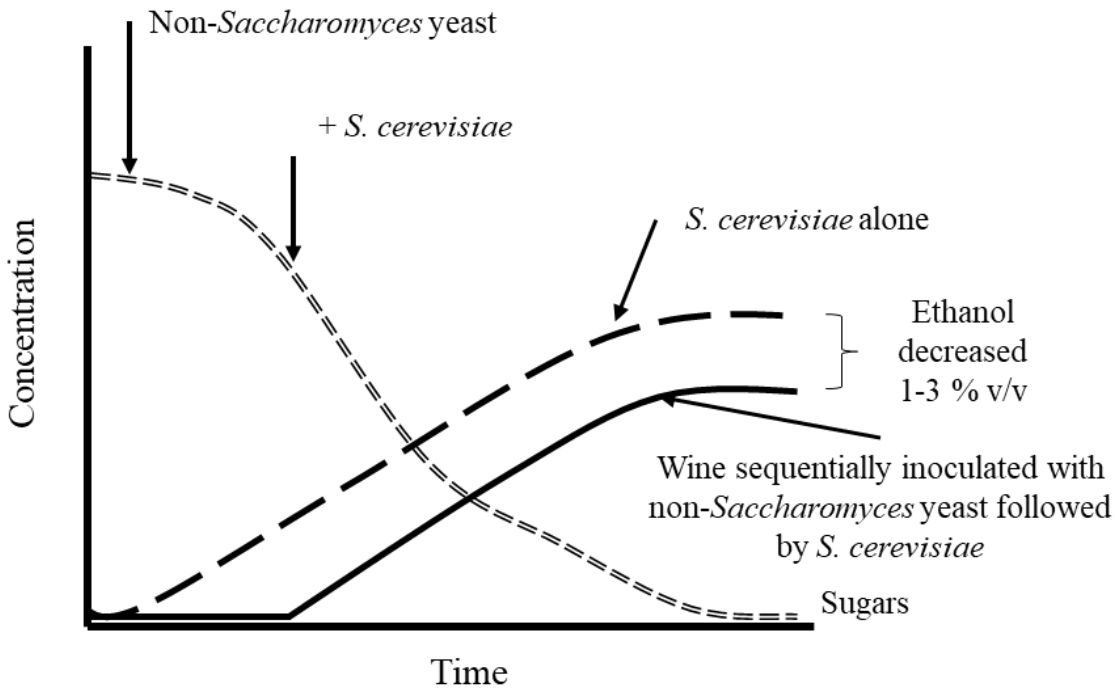

Figure 2. Idealized representation of the expected accumulation of ethanol in a fermentation sequentially inoculated with Crabtree-negative non-*Saccharomyces* yeasts followed by *S. cerevisiae* (continuous line). Oxygen applied during aerated phase prior to *S. cerevisiae* addition allows non-*Saccharomyces* yeasts to respire sugars, rather than ferment them to ethanol. Aeration should be discontinued once *S. cerevisiae* is inoculated to reduce acetic acid production. In contrast, the higher expected evolution of ethanol in a fermentation inoculated solely with *S. cerevisiae* is represented by a dashed line. For simplicity, sugar consumption (double-dashed line) has been assumed to follow the same pattern in both inoculation schemes. Redrawn from Gonzalez et al. (2013).

Table 1. Teleomorphs, anamorphs, and synonyms of some of the non-*Saccharomyces* yeasts reported on grapes and in wine (Barata et al., 2012b; Bourret et al., 2013; Jolly et al., 2014; Li et al., 2010; Zott et al., 2008).

Teleomorphic Form	Anamorphic Form	Synonyms
Citeromyces matritensis	*Candida globosa*	*Pichia hansenii*
Debaryomyces hansenii	*Candida famata*	*Pichia hansenii*
Dekkera bruxellensis	*Brettanomyces bruxellensis*	
Hanseniaspora guilliermondii	*Kloeckera apis*	
Hanseniaspora osmophila	*Kloeckera corticis*	
Hanseniaspora uvarum	*Kloeckera apiculata*	
Hanseniaspora vineae	*Kloeckera africana*	
Lachancea kluyveri	–*	*Saccharomyces kluyveri*
Lachancea thermotolerans	*Candida dattila*	*Kluyveromyces thermotolerans*
Metschnikowia chrysoperlae	–*	
Metschnikowia fructicola	–*	
Metschnikowia pulcherrima	*Candida pulcherrima*	
Meyerozyma caribbica	*Candida fermentati*	*Pichia caribbica*
Meyerozyma guilliermondii	*Candida guilliermondii*	*Pichia guilliermondii*
Milleronzyma farinose	–*	
Pichia fermentans	*Candida lambica*	
Pichia kluyveri	–*	*Hansenula kluyveri*
Pichia kudriazevii	*Candida krusei*	*Issatchenkia orientalis*
Pichia membranifaciens	*Candida valida*	*Zygosaccharomyces bisporus*
Pichia occidentalis	*Candida sorbose*	*Issatchenkia occidentalis*
Pichia terricola	–*	*Issatchenkia terricola*
Saccharomycodes ludwigii	–*	
Schizosaccharomyces pombe	–*	
Starmerella bacillaris	*Candida zemplinina*	
Starmerella bombicola	*Candida bombicola*	*Torulopsis bombicola*
Torulaspora delbrueckii	*Candida colliculosa*	
Wickerhamomyces anomalus	*Candida pelliculosa*	*Pichia anomala*
Yamadazyma mexicana	*Candida entomaea*	*Pichia mexicana*
Yarrowia lipolytica	*Candida deformans*	*Candida oleophila*
Zygoascus hellenicus	*Candida steatolytica*	
Zygosaccharomyces bailii	–*	*Saccharomyces bailii*
Zygosaccharomyces rouxii	–*	*Saccharomyces rouxii*
Zygotorulaspora florentina	–*	*Zygosaccharomyce florentinus*
–†	*Candida californica*	
–†	*Candida railenensis*	

* No known anamorphic form.

† No known teleomorphic form.

CHAPTER II

GROWTH AND METABOLISM OF NON-*SACCHAROMYCES* YEASTS ISOLATED FROM WASHINGTON STATE VINEYARDS IN MEDIA AND HIGH SUGAR GRAPE MUSTS

Abstract

Utilization of carbohydrates and amino acids/ammonium by selected non-*Saccharomyces* yeasts and impacts on alcoholic fermentation was evaluated using high sugar grape musts (>301 g/L). Consumption patterns of single cultures were ascertained in Merlot grape must during six days of growth. Here, isolates consumed between 41% (*C. californica*) and 73% (*Meyerozyma carribica*) of available amino acids and 18% (*Metschnikowia pulcherrima*) and 73% (*C. californica*) of ammonium compared to >90% by *S. cerevisiae*. Furthermore, non-*Saccharomyces* yeasts yielded higher amounts of residual sugar (≥258 g/L glucose + fructose) and less ethanol (≤15.6 g/L) than *S. cerevisiae* (50.2 g/L glucose +fructose, 70.0 g/L ethanol) in Merlot must after six days of incubation. To minimize problems associated with nutrient depletion before alcoholic fermentation, *S. cerevisiae* was inoculated six days after non-*Saccharomyces* yeasts with additional YAN supplementation. Syrah ferments inoculated with *C. californica* or *Metschnikowia pulcherrima* contained lower concentrations of residual sugar and ethanol compared to those with only *S. cerevisiae*. Furthermore, the presence of non-*Saccharomyces* yeasts influenced concentrations of glycerol and volatile aroma compounds. These results suggested potential use of some non-*Saccharomyces* yeasts towards reducing alcohol concentrations without risking slower alcoholic fermentations.

Introduction

Yeasts found on wine grapes (*Vitis vinifera*) at the time of harvest are referred to as "native", "wild", or, more commonly, "non-*Saccharomyces*" (Fugelsang and Edwards, 2007). As could be expected, non-*Saccharomyces* yeasts comprise a heterogeneous group representing several genera such as *Aureobasidium, Candida, Cryptococcus, Hanseniaspora, Issatchenkia, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Wickerhamomyces*, and others (Bourret et al., 2013; Fleet, 1993; Jolly et al., 2003b; Li et al., 2010). Healthy, undamaged grape berries have reported viable populations which range from 10^2 to 10^6 CFU/mL (Jolly et al., 2003b; Li et al., 2010).

The impacts of these yeasts on overall wine quality have long been the subject of debate. In fact, non-*Saccharomyces* yeasts can contribute either positively or negatively to the sensory profile of wines depending on a number of factors (Fleet, 2003; Jolly et al., 2003a; Viana et al., 2008). For instance, some species synthesize various esters responsible for desirable 'fruity' and 'floral' aromas (Clemente-Jimenez et al., 2004; Izquierdo Cañas et al., 2014; Viana et al., 2008; Zohre and Erten, 2002). Furthermore, some of these yeasts produce extracellular hydrolytic enzymes such as glucosidases, proteases, and/or pectinases which can affect wine quality through enhancement of grape varietal aromas, mouthfeel, and/or other properties (Comitini et al., 2011; Cordero-Bueso et al., 2013). In contrast, other species produce undesirable surface films, turbidity, or excessive concentrations of ethyl acetate, acetic acid (volatile acidity or VA), and/or acetaldehyde that lessen or devastate quality (Andorrà et al., 2010; Ciani and Maccarelli, 1998; Rojas et al., 2003; Zohre and Erten, 2002).

Besides potentially altering sensory quality, growth of non-*Saccharomyces* yeasts can affect alcoholic fermentation conducted by *Saccharomyces cerevisiae*. On the one hand, non-

Saccharomyces yeasts may consume key nutrients present in grape musts important for *S. cerevisiae* (Bataillon et al., 1996; Medina et al., 2012). As an example, Bataillon et al. (1996) noted that these yeasts can quickly deplete a grape must of thiamin, thereby causing stuck or sluggish fermentations. More recently, Medina et al. (2012) reported that *Hanseniaspora viniae* and *Metschnikowia pulcherrima* yeasts consumed enough nutrients to slow fermentations. Moreover, non-*Saccharomyces* yeasts may preferentially consume specific nitrogen sources, potentially impacting subsequent growth and fermentation by *S. cerevisiae* (Gobert et al., 2017; Kemaswasd et. al., 2015; Rollero et al., 2018a, 2018b). On the other hand, co-inoculation of specific non-*Saccharomyces* yeasts may reduce the risks of problem fermentations. As some species of non-*Saccharomyces* yeasts are fructophilic (Ciani and Fatichenti, 1999; Magyar and Tóth, 2011), co-inoculation with glucophilic *S. cerevisiae* (Berthels et al., 2004) may result in lower residual concentrations of fructose, reducing the risk of stuck alcoholic fermentations (Schutz and Gafner, 1995). Furthermore, Gonzalez et al. (2013) suggested that successive inoculation of yeasts can reduce the final alcohol contents of wines. Here, early inoculation of non-*Saccharomyces* yeasts transforms sugar to produce biomass and by-products besides ethanol before addition of *S. cerevisiae* (Quiros et al., 2014). This could prove particularly useful to winemakers sourcing grapes from hot, sunny regions where high sugar musts lead to wines exceeding 15% v/v alcohol (Mira de Orduña, 2010).

In a survey of grapes obtained from two vineyards located in Washington, 53 species of yeasts were isolated including *Candida californica, Mt. pulcherrima, Meyerozyma caribbica,* and *Wickerhamomyces anomalus* (Bourret et al., 2013). Frequently isolated from grapes, strains of *Mt. pulcherrima* have been identified that produce wines with reduced ethanol contents (Canonico et al., 2016; Contreras et al., 2014, 2015a, 2015b; Varela et al., 2016) and increased

concentrations of fruit-associated esters (Clemente-Jimenez et al., 2004; Jolly et al., 2003a; Rodríguez et al., 2010; Zohre and Erten, 2002). Similarly, *W. anomalus* (synonym *Pichia anomala*) has also been isolated during fermentation (Fredlund et al., 2002) and enhances 'fruity' and 'floral' sensory properties (Izquierdo Cañas et al., 2014; Varela, 2016). Although few reports describe *C. californica* on grapes, this species can metabolize glucose to varying degrees (Lachance et al., 2011). Another species, *My. caribbica* (synonym *Pichia caribbica*) was found to produce desirable volatile aromas in fermented pineapple beverages (Ribeiro et. al., 2015).

While present on regionally-grown grapes, little is known regarding the impacts (if any) of the non-*Saccharomyces* isolates described by Bourret et al. (2013) on alcoholic fermentation or resultant wine quality. In earlier work by White (2015), pure cultures of *C. californica*, *Mt.pulcherrima*, *My. caribbica*, and *W. anomalus* yeasts yielded high concentrations of residual sugar, consumed similar concentrations of ammonium and amino acids as *S. cerevisiae*, and, in some cases produced significant amounts of acetic acid. However, experiments involving inoculation of single pure cultures were conducted over a long period of time (56 days). As such, it was possible that the non-*Saccharomyces* could metabolize some glucose and fructose without inhibiting alcoholic fermentation or producing excessive VA in grape musts if inoculated only a few days before *S. cerevisiae*. Thus, the objectives of this study were to determine compositional changes to grape musts by several yeast isolates including consumption of sugars and amino acids as well as the production of various metabolites (VA, alcohol, and aroma/flavor compounds) when sequentially inoculated with *S. cerevisiae*.

Material and Methods

Yeast strains

C. californica P01C003, *Mt. pulcherrima* P01A016, *My. caribbica* P46A001, and *W. anomalus* P01A017 were previously isolated from vineyards located at the Irrigated Agriculture Research and Extension Center (Prosser, WA) as described by Bourret et al. (2013). *S. cerevisiae* strains ECA5 and D254 were obtained as active dry cultures from Lallemand Inc. (Montréal, Quebec, Canada). Strain ECA5 was selected given its low nitrogen requirements, while D254 is commonly used in commercial red wine fermentations (Cadière et al., 2011; G. Specht, personal communication). All yeasts were maintained on yeast peptone dextrose (YPD) agar slants.

To prepare starter cultures, single colonies grown on YPD agar were transferred to yeast/mold (YM) broth (Becton, Dickinson, and Company, Sparks, MD). Upon reaching late exponential growth, cells were harvested by centrifugation at 2000 x *g* for 20 min and washed twice in 0.2 M Na_2HPO_4 (pH 7.0) buffer prior to inoculation.

Merlot grape must

Grape juice concentrate (Merlot) was obtained from the California Concentrate Company (Acampo, CA) and reconstituted with distilled water according to manufacturer's instructions. The must was adjusted to 155 g/L of glucose and fructose (each), pH 3.58 with tartaric acid, and 300 mg N/L yeast assimilable nitrogen (150 mg/L amino N and 150 mg/L ammonium) using a mixture of Fermaid® K and diammonium phosphate (Scott Laboratories, Petaluma, CA) before sterile-filtration through 0.45 µm polyvinylidene fluoride cartridges (MilliporeSigma, Bellerica, MA) into previously sterilized 4 L Celstir fermenters. A suspension of powdered cellulose (Sigmacell® Type 20, Sigma-Aldrich, St. Louis, MO), sterilized at 121°C for 15 min, was added at a rate of 1 g/L prior to inoculation with *C. californica, Mt. pulcherrima, My. caribbica, S.*

cerevisiae D254, or *W. anomalus* starter cultures (10^5 CFU/mL). Fermentations were conducted at 21°C, in triplicate, with constant mixing using magnetic stirring bars for six days.

Chardonnay grape must (pH 3.34, 133 g/L glucose, 141 g/L fructose, 137 mg N/L yeast assimilable nitrogen) was obtained from a regional winery. After removal of SO_2 by H_2O_2, the must was sterile-filtered through 0.2 μm Nylon® membranes housed in an autoclaved filter housing (Pall, Port Washington, NY). Diammonium phosphate (50 mg/L) and powdered cellulose (1 g/L, Sigmacell® Type 20, Sigma-Aldrich, St. Louis, MO) were aseptically added before transfer into sterile 100 mL milk dilution bottles. The cellulose was prepared as a suspension and sterilized at 121°C for 15 min. Chardonnay musts were inoculated, in triplicate, with starter cultures of *C. californica, C. oleophila*, *My. caribbica, Mt. pulcherrima, S. cerevisiae* ECA5, or *W. anomalus* to achieve initial populations between 10^4 and 10^5 CFU/mL and incubated statically at 18°C.

Syrah grape must

Syrah grapes were obtained from the Irrigated Agriculture Research and Extension center (Prosser, WA). Potassium metabisulphite (10 mg/L SO_2) was added immediately after crushing/destemming and before storage of the must at -20°C in 5-gallon containers. Upon thawing, the must (pH 3.51, 5.56 g/L titratable acidity, 152 g/L glucose, 149 g/L fructose, 253 mg N/L yeast assimilable nitrogen) was distributed into 1L sterile bottles after addition of Fermaid® K (0.33 g/kg) and potassium metabisulphite (30 mg/L SO_2). Bottles were either not inoculated or inoculated with *C. californica, Mt. pulcherrima, My. caribbica, S. cerevisiae* D254, *W. anomalus* at 10^5 CFU/mL in triplicate. Fermentation "water bubble" locks were added to musts inoculated with *S. cerevisiae* while uninoculated or those with non-*Saccharomyces* yeasts were sealed with gas-porous stoppers (Whatman® Bugstopper, Maidstone, U.K.). After six days,

all musts were inoculated with *S. cerevisiae* D254 and sealed with fermentation locks with additional Fermaid® K (0.66 g/L) added two days later. Fermentations were conducted at 23°C and stirred for 30 seconds each day. Once soluble solids reached 0° to 3°Brix, free-run wine was collected into sterilized milk dilution bottles to complete fermentation. Wines were considered "dry" when reducing sugar concentrations reached <2 g/L as determined by the Clinitest® method (Ough and Amerine, 1988).

Analytical methods

Culturability was evaluated by spiral plating (Autoplate 4000, Spiral Biotech, Bethesda, MD) using either Wallenstein Laboratory agar (WL, Becton, Dickinson, and Company, Franklin Lakes, NJ) for total yeasts or lysine agar (Oxoid, Hamphshire, England) for non-*Saccharomyces* yeasts after incubation for 3 to 5 days at 28°C. Populations of *S. cerevisiae* in Syrah musts were estimated based on the difference between plate counts on WL and lysine agar media.

Amino acids, glucose/fructose, and ethanol were quantified with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with UV-VIS diode array and refractive index detectors. Samples were filtered through 0.22 µm polyethersulfone membranes (MilliporeSigma) into crimp-top vials. Amino acids were quantified using an Agilent ZORBAX Eclipse Plus-C18 column (4.6 x 150 mm, 3.5 µm particle size) after pre-column *o*-phthaldialdehyde derivatization according to Henderson and Brooks (2010) with a detection limit of 0.5 mgN/L (White, 2016). Yeast assimilable nitrogen was calculated as the sum of primary amino nitrogen, determined according Dukes and Butzke (1998), and ammonium measured with an ammonia ion-selective electrode (Denver Instruments, Orville, NY, USA). Glucose and fructose concentrations for Chardonnay and Syrah fermentations were measured enzymatically

(Yellowline Kit, r-Biopharm, Darmstadt, Germany) while volatile acidities were determined by Cash still (Ough and Amerine, 1988).

Volatile aromas were extracted by headspace solid-phase microextraction (HS-SPME) using a 65 µm polydimethysiloxane-divinylbenzene fiber (Supelco, Bellefonte, PA) following the procedures of Clary et al. (2006). Compounds were analyzed with an Agilent HP 6890 gas chromatograph fitted with a 0.32 mm x 60.0 m, 1.0 µm thickness DB-1MS column (Phenomenex, Torrence, CA) coupled to a HP 5973 Mass Selective Detector. Fibers were desorbed for 3 min at 250°C in the injection inlet operating in splitless mode. The carrier gas was helium flowing at 0.7 mL/min. The oven temperature was programmed to hold at 33°C for 5 min, increase at 2.0°C/min to 50°C and then 5.0°C/min to 225°C and held for 5 min. The mass spectrometer was operating in electron impact mode at 70 eV (150°C ion source). Compounds were identified using the Wiley/NIST library while quantification was accomplished using 4-methyl-2-pentanol and 2-octanol as internal standards.

Statistical methods

Statistical analyses were performed by ANOVA using XLSTAT while mean separations were accomplished by Tukey's HSD (Addinsoft, New York, NY).

Results and discussion

Singular inoculation of non-*Saccharomyces* yeasts compared to *S. cerevisiae*

Sugar and ethanol concentrations were measured in Merlot musts inoculated with *C. californica*, *Mt. pulcherrima*, *My. caribbica*, *S. cerevisiae,* and *W. anomalus* (Table 2). After six days, concentrations of residual sugars in musts inoculated with non-*Saccharomyces* yeasts were ≥258 g/L, higher amounts compared to those with *S. cerevisiae* (52 g/L). Furthermore,

concentrations of ethanol reflected sugar utilization where lower amounts were produced by non-*Saccharomyces* yeasts (<15.6 g/L) compared to *Saccharomyces* (70.6 g/L).

Besides sugar and ethanol concentrations, consumption of amino acids and ammonium varied with yeast inocula (Table 3). *S. cerevisiae* consumed the highest concentrations of amino acids and ammonium (96%) while *C. californica* (60%), *My. caribbica* (51%), *W. anomalus* (54%), or *Mt. pulcherrima* (33%) utilized less (Table 3). Unlike previous observations by White (2015), the non-*Saccharomyces* yeasts left appreciable amounts (>25%) of ammonium, alanine, arginine, and tryptophan yet consumed most of the available histidine, isoleucine, and lysine. In contrast, Medina et al. (2012) noted that strains of *Mt. pulcherrima* and *H. uvarum* metabolized most of the available nitrogen in a white grape must (178 mg N/L YAN, 210 g/L glucose/fructose) within three days.

While non-*Saccharomyces* yeasts utilized less nitrogen in six days compared to the 56 day incubation period utilized by White (2015), further nitrogen supplementation may be required in sequential inoculations with *S. cerevisiae*. Assuming that 21°Brix grape musts require 200 mg N/L YAN to complete fermentation, Bisson and Butzke (2000) recommended an additional 25 mg N/L for every 1°Brix increase. Given that the Merlot grape must inoculated with non-*Saccharomyces* yeasts contained ≥258 g/L fermentable sugar (approximately ≥23.5°Brix), 263 mg N/L may be required for fermentations to proceed to dryness (≤2 g/L residual sugar). Residual amino and ammonium nitrogen content in Merlot musts inoculated with non-*Saccharomyces* yeasts ranged from 102 mg N/L (*C. californica*) to 171 mg N/L (*Mt. pulcherrima*) (Table 3). Additional experimentation was conducted using a Syrah must containing a similar concentration of fermentable sugars (301 g/L) but with extra YAN supplementation at the time of inoculation of *S. cerevisiae*.

Sequential inoculation of non-*Saccharomyces* followed by *S. cerevisiae*

As observed with fermentations conducted by White (2015) which were filter-sterilized prior to inoculation, non-*Saccharomyces* yeasts achieved high culturable populations in the unsterilized Syrah must (Fig. 3). Here, populations of *C. californica*, *Mt. pulcherrima*, *My. caribbica,* and *W. anomalus* exceeded $>10^7$ CFU/mL. However, a low population of an unidentified strain of *S. cerevisiae* emerged on day 4 prior to subsequent inoculation of *S. cerevisiae* D254 on day 6. Non-*Saccharomyces* yeasts were not recoverable by day 12, an observation in agreement with others (Comitini et al., 2011; Contreras et al., 2014; Rodríguez et al., 2010).

Sugar consumption by *C. californica, Mt. pulcherrima, My. caribbica, S. cerevisiae,* and *W. anomalus* and concentrations of residual sugar, ethanol, and acetic acid in the resultant wines depended on the yeasts present (Table 4). Inoculation of non-*Saccharomyces* yeasts yielded dry wines by day 18 (<2 g/L glucose and fructose) where those inoculated with *S. cerevisiae* alone contained 0.78 to 1.0 g/L more residual sugar than those with non-*Saccharomyces* yeasts. Regarding concentrations of ethanol after fermentation, wines produced with *C. californica* or *Mt. pulcherrima* contained less (0.8 to 0.9% v/v) than those fermented with only *S. cerevisiae*. While Contreras et al. (2014; 2015a) and Varela et al. (2016) also reported decreases in ethanol production through inoculation of different strains of *Mt. pulcherrima*, this is the first report regarding the influence of *C. californica*. The presence of *My. caribbica* nor *W. anomalus* did not affect final alcohol contents, in contrast to Contreras et al. (2015b) studying other strains of the latter species.

Besides ethanol, growth of non-*Saccharomyces* yeast affected concentrations of glycerol as well as titratable and volatile acidities in the Syrah wines (Table 4). Wines produced with *Mt.*

pulcherrima and *My. caribbica* contained 1.5 and 1.8 g/L more glycerol respectively than wines initially inoculated with *S. cerevisiae*. Inoculation of *C. californica, Mt. pulcherrima*, and *My. caribbica* increased titratable acidity compared to wines obtained from *S. cerevisiae* alone, partly due to differences in volatile acidities, similarly noted by Rodríguez et al. (2010) and Contreras et al. (2014). However, inoculation of these yeasts into the Syrah grape musts did not increase resultant concentrations of volatile acidity beyond the rejection sensory threshold of 0.7 g/L established by Bandion and Valenta (1977). In fact, concentrations were 0.44 g/L for *W. anomalus*, in agreement with Rojas et al. (2003) and Cordero-Bueso et al. (2013) but below the 0.755 g/L reported by White (2015). In mixed cultures, *S. cerevisiae* commonly dominates non-*Saccharomyces* yeasts within a short time period (Toro and Vazquez, 2002), thereby potentially lessening acetic acid production by these yeasts. However, some non-*Saccharomyces* yeasts were not influenced by *S. cerevisiae* as evidenced by Andorrà et al. (2010) who reported elevated concentrations inoculating *Hanseniaspora uvarum* or *Candida zemplinina* with *S. cerevisiae*.

Gonzalez et al. (2013) first suggested that sequential inoculation of Crabtree-negative non-*Saccharomyces* yeasts followed by *S. cerevisiae* could be used to reduce the final ethanol concentration of wines. Some of the non-*Saccharomyces* yeasts studied (*e.g.*, *Mt. pulcherrima* or *W. anomalus*) are classified as Crabtree-negative in which respiration is the preferred metabolic pathway regardless of glucose concentration (De Deken, 1966; Fredlund et al., 2002; Gonzalez et al., 2013; Schnierda et al., 2014). Such yeasts are often characterized by high affinity, energy-dependent glucose transport systems that result in slower accumulation of carbohydrates and less efficient conversion of sugar to ethanol (Van Urk et al., 1989). *S. cerevisiae* is Crabtree-positive and will exhibit fermentative metabolism regardless of the presence/absence of oxygen when the glucose concentration is >9 g/L (Fugelsang and Edwards, 2007; Van Urk et al., 1989). In

support, others have reported that the ethanol contents were reduced by 0.9 to 1.6% v/v when musts were inoculated with *Mt. pulcherrima* before adding *S. cerevisiae* (Canonico et al., 2016; Contreras et al., 2014; Varela et al., 2016). Varela et al. (2016) suggested that decreased alcohol content of fermentations by *Saccharomyces uvarum* were due to increased production of glycerol and succinic acid, although changes in glycerol and succinic acid content in Syrah fermentations (data not shown) were not sufficient to explain the observed ethanol reduction. To date, the metabolic preference of *C. californica* or *My. caribbica*, Crabtree-positive or negative, has not been studied.

Compared to wines inoculated with *S. cerevisiae* alone, the addition of non-*Saccharomyces* yeasts increased the amount of higher alcohols (*i.e.*, fusel oils) in Syrah wines (Table 5). Concentrations of higher alcohols were greatest in wines with *Mt. pulcherrima* and *C. californica* present which produced more 2-methyl-1-propanol and 2- and 3-methyl-1-butanol than wines fermented solely with *S. cerevisiae*. Conversely, smaller increases in concentrations of 1-propanol (all species) and 1-hexanol (*C. californica, My. caribbica,* and *W.* anomalus) were noted in wines inoculated with non-*Saccharomyces* yeasts. Other authors have similarly noted increased concentrations of higher alcohols in wines made with *Mt. pulcherrima* (Contreras et. al., 2014; Gobert et al., 2017) and *W. anomalus* (Izquierdo Cañas et al., 2014). Competition for specific nitrogen sources may explain the increased concentration of higher alcohols observed (Gobert et al., 2017; Rollero et al., 2018) given their formation through transamination reactions involving amino acids (Fugelsang and Edwards, 2007). When present in moderate concentrations (<400 mg/L), higher alcohols can contribute beneficially to wine complexity (Rapp and Mandery, 1986).

Besides differences in higher alcohols, esters were more concentrated in Syrah wines inoculated initially with *S. cerevisiae* than those inoculated with non-*Saccharomyces* yeasts (Table 5). Primarily, *S. cerevisiae* wines contained increased concentrations of 2- and 3-methylbutyl acetate. While Comitini et al. (2011) and Varela et al. (2016) reported wines inoculated with *Mt. pulcherrima* to contain lower concentrations of esters, Contreras et al. (2014) and Izquierdo Cañas et al. (2014) noted that wines fermented with *Mt. pulcherrima* or *W. anomalus* had higher concentrations. Despite the elevated ester content however, *S. cerevisiae* wines may not be sensorily perceived as 'fruitier' compared to wines inoculated with *C. californica* and *Mt. pulcherrima* due to higher amounts of ethanol. In fact, increased concentrations of ethanol can decrease the perception of 'fruity' notes in wine (Goldner et al., 2009).

While non-*Saccharomyces* yeast can impart desirable sensory qualities, growth of these yeasts can also produce volatile compounds that negatively affect wine quality. Here, concentrations of such aroma compounds as ethyl acetate ('nail polish remover') and hexanoic acid ('cheesy') in wines with non-*Saccharomyces* yeasts were higher than in wines with *S. cerevisiae* alone (Table 5). Because ethyl acetate contributes unfavorably to the sensory profile of wines at concentrations above 150 mg/L, wines produced with non-*Saccharomyces* yeasts in this study will likely possess some negative sensory attributes (Fugelsang and Edwards, 2007; Rapp and Mandery, 1986). *W. anomalus* and *Mt. pulcherrima* have been reported to produce 200 mg/L ethyl acetate in Chardonnay and Bobal wines yet lower amounts in Shiraz (Contreras et al., 2014; Varela et al., 2016), suggesting an impact of specific winemaking practices on synthesis. In agreement, Rojas et al. (2001) observed greater ethyl acetate concentrations in aerated cultures

of *P. anomala* (*W. anomalus*) while Comitini et al. (2011) noted increased ethyl acetate when the proportion of *S. cerevisiae* to non-*Saccharomyces* yeast inoculum was decreased.

Conclusions

This study examined the enological properties of four non-*Saccharomyces* yeast strains native to Washington vineyards during fermentations. For the first time, growth of *C. californica* and *My. caribbica* under winemaking conditions was described. When grown in pure culture for six days, non-*Saccharomyces* yeasts yielded high concentrations of residual sugar, low concentrations of ethanol, and consumed amounts of ammonium and amino acids which would warrant additional must nutrient supplementation prior to sequential *S. cerevisiae* inoculation. When successively inoculated into Syrah must with *S. cerevisiae* with additional nutrient supplementation, non-*Saccharomyces* yeasts increased concentrations of compounds beneficial to wine quality (titratable acidity, glycerol, and higher alcohols) without significantly increasing acetic acid content or fermentation time. Notably, *C. californica* and *Mt. pulcherrima* inoculation yielded wines with less alcohol, an average of 0.8 and 0.9% v/v, respectively. In any case, the reduced alcohol wines made with *C. californica* or *Mt. pulcherrima* require sensory evaluation in order to fully evaluate the impact of these yeasts on wine quality in addition to optimizing processing conditions (*i.e.*, temperature and oxygen availability).

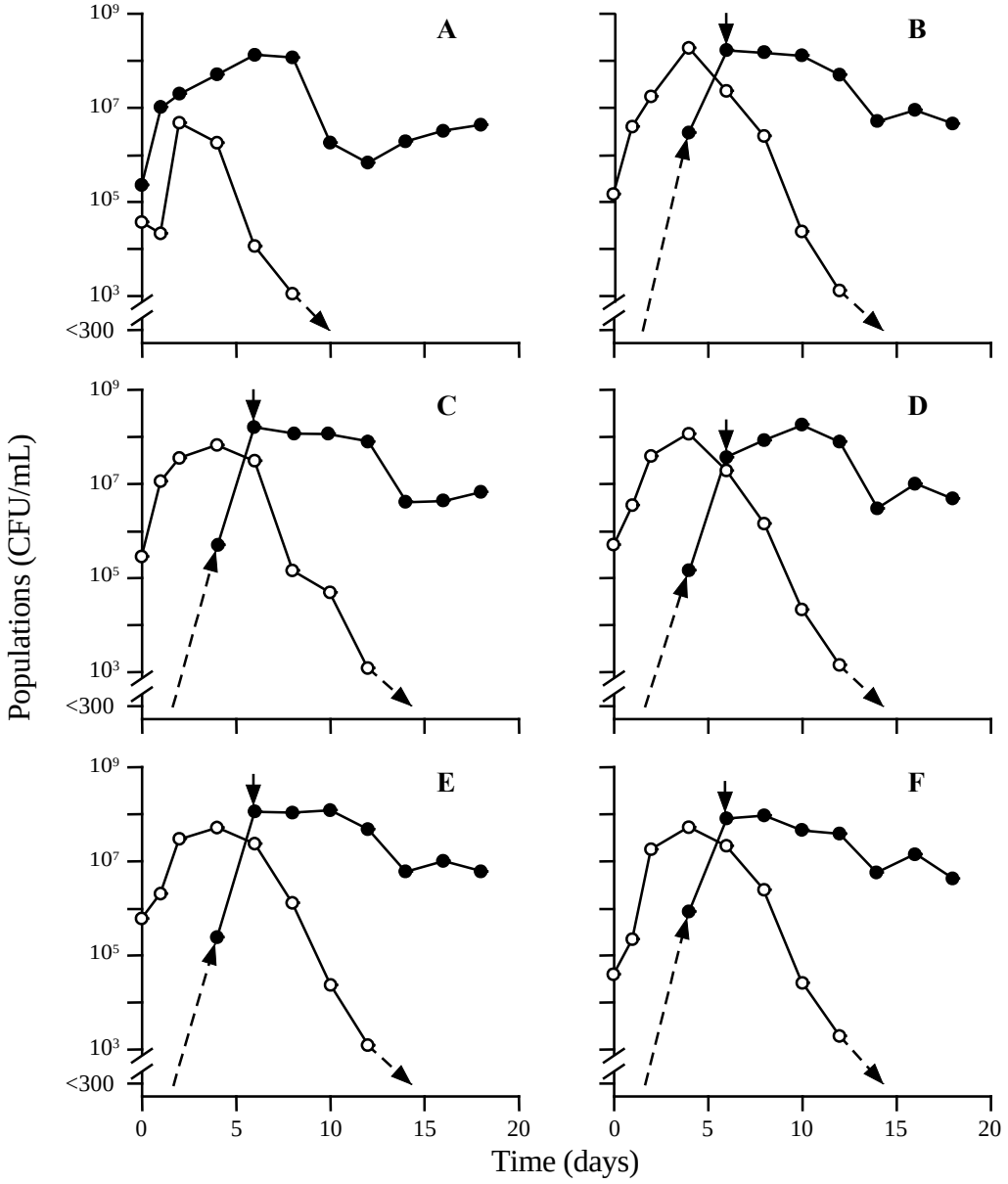

Figure 3. Culturable populations of *Saccharomyces cerevisiae* (●) and non-*Saccharomyces* (○) yeast in a Syrah must inoculated either solely with *S. cerevisiae* D254 (A), sequentially with *C. californica* (B), *Mt. pulcherrima* (C), *My. caribbica* (D), or *W. anomalus* (E) followed by *S. cerevisiae* D254, or left initially uninoculated (F) before *S. cerevisiae* addition. Sequential inoculation of D254 (B-F) indicated by ↓.

Table 2. Chemical composition of Syrah wines produced by *S. cerevisiae* alone or by sequential inoculation with non-*Saccharomyces* followed by *S. cerevisiae* D254 six days later.

Yeast	Glucose + Fructose (g/L)	Ethanol (g/L)
S. cerevisiae	52.0 ± 7.45^{a}	70.6 ± 1.93^{c}
C. californica	266 ± 2.24^{b}	3.00 ± 1.18^{a}
Mt. pulcherrima	258 ± 6.81^{b}	15.6 ± 3.30^{b}
My. caribbica	284 ± 0.97^{c}	12.9 ± 0.90^{b}
W. anomalus	268 ± 5.88^{bc}	10.5 ± 2.89^{b}

[a-d] Mean values within columns with different superscripts are significantly different ($p \leq 0.05$).

Table 3. Concentrations of amino acids and ammonium before and after 6 days of yeast growth in Merlot grape must. Values are means of triplicate fermentations.

Nitrogen Compound	Pre-inoculation (mg N/L)	Post-inoculation (mg N/L)				
		S. cerevisiae	*C. californica*	*Mt. pulcherrima*	*My caribbica*	*W. anomalus*
Ala	4.81	1.95[a]	4.24[b]	4.25[b]	3.36[b]	4.26[b]
Ammonium	150	nd	40.0[a]	123[d]	96.2[c]	76.1[b]
Arg	71.0	3.85[a]	41.6[d]	33.7[c]	19.4[b]	29.0[c]
Asp	0.80	nd	0.72	nd	nd	nd
Glu	0.75	1.01[a]	0.62[a]	nd	nd	nd
Gly	0.65	1.49[a]	1.20[a]	0.72[a]	nd	0.76[a]
His	2.94	nd	nd	nd	nd	nd
Ile	1.15	nd	nd	nd	nd	nd
Leu	3.71	nd	nd	nd	nd	nd
Lys	nd	nd	0.51	nd	nd	nd
Met	0.50	1.06[a]	0.95[a]	0.74[a]	nd	0.78[a]
Phe	nd	nd	nd	nd	nd	nd
Ser	2.01	nd	1.80	nd	nd	nd
Thr	1.14	0.96[a]	0.99[a]	nd	nd	nd
Trp	2.83	nd	9.82[c]	8.19[bc]	5.57[a]	7.54[b]
Tyr	nd	nd	nd	nd	nd	nd
Val	nd	nd	nd	nd	nd	nd
Total	256	10.3[a]	102[b]	171[d]	125[c]	119[c]

[a-d] Post-inoculation mean values within a row with different superscripts are significantly different ($p \leq 0.05$).
nd: not detected.

Table 4. Chemical composition of Syrah wines produced by *S. cerevisiae* alone or by sequential inoculation with non-*Saccharomyces* followed by *S. cerevisiae* D254 six days later.

Inoculated Yeast	Glucose + Fructose (g/L)	Ethanol (% v/v)	Glycerol (g/L)	Titratable Acidity (g/L)	Volatile Acidity (g/L)
S. cerevisiae	1.46 ± 0.506^{b}	16.4 ± 0.1^{b}	11.2 ± 0.1^{a}	6.84 ± 0.04^{a}	0.238 ± 0.005^{a}
C. californica	0.686 ± 0.260^{ab}	15.6 ± 0.3^{a}	12.0 ± 0.2^{ab}	7.31 ± 0.04^{bc}	0.286 ± 0.005^{bc}
Mt. pulcherrima	0.461 ± 0.058^{a}	15.5 ± 0.1^{a}	12.7 ± 0.1^{b}	7.61 ± 0.04^{c}	0.243 ± 0.008^{ab}
My. caribbica	0.554 ± 0.090^{a}	15.9 ± 0.2^{ab}	13.0 ± 0.8^{b}	7.19 ± 0.12^{b}	0.322 ± 0.009^{c}
W. anomalus	0.612 ± 0.077^{a}	16.4 ± 0.1^{b}	12.4 ± 0.1^{ab}	6.59 ± 0.12^{a}	0.442 ± 0.028^{d}
*S. cerevisiae**	0.826 ± 0.159^{ab}	16.2 ± 0.1^{b}	12.6 ± 0.1^{b}	7.21 ± 0.18^{b}	0.315 ± 0.010^{c}

* Inoculated six days after crush.
[a-d] Mean values within columns with different superscripts are significantly different ($p \leq 0.05$).

Table 5. Concentration of volatile compounds in Syrah wines inoculated initially with non-*Saccharomyces* or *S. cerevisiae* yeasts.

Compound (mg/L)	*S. cerevisiae*	*Mt. pulcherrima*	*My caribbica*	*C. californica*	*W. anomalus*
Higher Alcohols					
1-Propanol	4.00^{a}	8.33^{b}	13.1^{cd}	13.8^{d}	11.1^{c}
2-Methyl-1-propanol	32.5^{a}	140^{c}	106^{b}	152^{c}	95.3^{b}
2&3-Methyl-1-butanol	143^{a}	188^{c}	141^{a}	165^{b}	129^{a}
1-Hexanol	4.01^{a}	4.13^{a}	5.73^{b}	6.27^{b}	5.74 ^{b}
1-Octanol	2.11^{a}	3.81^{a}	2.56^{a}	4.08^{a}	0.76^{a}
2-Phenylethanol	30.4^{ab}	34.7^{b}	27.8^{ab}	26.0^{ab}	24.0^{a}
Esters					
Ethyl Acetate	69.3^{a}	168^{b}	297^{c}	344^{cd}	399^{d}
2&3-Methylbutyl Acetate	4.61^{b}	3.78^{a}	3.23^{a}	3.17^{a}	3.33^{a}
Hexyl Acetate	0.024^{c}	0.007^{a}	0.012^{b}	0.007^{ab}	0.011^{b}
Diethyl Succinate	0.208^{a}	0.309^{b}	0.193^{a}	0.317^{b}	0.205^{a}
2-Phenylethyl Acetate	0.150^{a}	0.165^{a}	0.157^{ab}	0.197^{b}	0.143^{a}
Ethyl Butanoate	0.428^{b}	0.409^{b}	0.316^{a}	0.273^{a}	0.306^{a}
Ethyl Hexanoate	0.202^{bc}	0.231^{c}	0.181^{b}	0.140^{a}	0.139^{a}
Ethyl Octanoate	0.518^{c}	0.422^{abc}	0.440^{bc}	0.343^{a}	0.381^{bc}
Acids					
Hexanoic Acid	3.56^{a}	6.72^{c}	5.04^{abc}	6.26^{bc}	4.11^{ab}
Octanoic Acid	2.98^{ab}	3.37^{b}	3.16^{ab}	3.24^{b}	2.71^{a}

$^{a-d}$ Mean values within rows with different superscripts are significantly different ($p \leq 0.05$).

CHAPTER III

EVALUATION OF NON-*SACCHAROMYCES* YEASTS ISOLATED FROM WASHINGTON STATE VINEYARDS TO REDUCE FINAL ALCOHOL CONTENTS OF MERLOT WINES

Abstract

Yeasts previously isolated from Washington vineyards were investigated for their abilities to reduce resultant alcohol contents of wines. Sixteen species of non-*Saccharomyces* yeasts were inoculated into a high sugar grape must (310 g/L) prior to addition of *S. cerevisiae* on day 6. Although many fermentations did not reach dryness (>2 g/L residual sugar), inoculation of *Metschnikowia chrysoperlae, Mt. pulcherrima, Meyerozyma guillermondii, Pichia kluyveri,* or *P. membranifaciens* resulted in wines with lower concentrations of ethanol without production of excessive levels of acetic acid. Dry wines containing less alcohol were also obtained through inoculation of *Mt. pulcherrima* or *My. guilliermondii* into a grape must that contained a lower amount of sugar (266 g/L). Single culture inoculation into a synthetic grape juice medium (SGJM) confirmed that *Mt. pulcherrima* and *My. guilliermondii* exhibited slower sugar uptake and yielded lower amounts of ethanol and glycerol than *S. cerevisiae*. Ferments of SGJM inoculated on day 0 with *Mt. pulcherrima* and on day 3 with *S. cerevisiae* achieved alcohol concentrations 2.4% to 3.0% v/v lower than those with *S. cerevisiae* alone. Conducting larger-scale fermentations (100 L) with unsterilized Merlot grape musts, wines with *Mt. pulcherrima* contained 0.9% v/v less ethanol than wines inoculated solely with *S. cerevisiae*. This research demonstrates the potential use of *Mt. pulcherrima* to produce wines with reduced alcohol contents.

Introduction

Viticultural systems that yield 'rich', 'full-bodied' red wines often involve extended vine ripening periods prior to harvest to achieve phenolic maturity, practices which increase must sugar concentrations and subsequent alcohol contents (Godden et al., 2015; Gonzalez et al. 2013). As evidence, average alcohol concentrations of red wines from Australia increased from <12.5% in 1984 to up to 13.6% v/v in 2014, and wines containing >15% v/v are a more common occurrence (Godden et al., 2015). With an increase in alcohol, wines possess intensifying perceptions of 'hotness' or 'bitterness' (Baker et al., 2016; Heymann et al., 2013) and fruity attributes tend to be masked (Goldner et al., 2009; Villamor et al., 2013b). Higher sugar concentrations also contribute to increased risks of sluggish or even stuck fermentations due to higher ethanol, yielding wines with undesirable sweetness (Berthels et al., 2004; Bisson and Butzke, 2000). Furthermore, wines with higher alcohol content may incur additional taxation or even rejection by health-conscious consumers (Gonzalez et al., 2013).

To reduce alcohol contents without adversely affecting quality, various winemaking approaches have been explored. Such approaches have included removal of sugar from musts by nanofiltration (García-Martín et al., 2010), dealcoholization after fermentation using membrane processes (Rolle et al., 2018a), or application of genetically-engineered yeast strains capable of reduced ethanol yield (Tilloy et al., 2014; Varela et al., 2012). However, these approaches can lower wine quality or present consumer and/or regulatory concerns. As a consequence, Gonzalez et al. (2013) proposed the use of selected non-*Saccharomyces* yeasts which convert fermentable sugar to by-products other than ethanol prior to inoculation of *S. cerevisiae*. In fact, several species and strains of non-*Saccharomyces* can produce wines containing less alcohol (Canonico et al., 2016; Contreras et al., 2014; 2015a; 2015b; Englezos et al., 2016; Rossouw and Bauer,

2016; Varela et al., 2016; 2017), likely because these exhibit respiration under aerobic conditions regardless of glucose concentration (De Deken et al., 1966). Due to lower ethanol tolerances or other reasons (Comitini et al., 2011; Fleet, 2003), non-*Saccharomyces* yeasts cannot typically metabolize all available sugar so continued fermentation by *S. cerevisiae* is critical to achieve dryness (≤2 g/L glucose and fructose).

One question associated with commercial use of non-*Saccharomyces* yeasts is the potential contributions to flavor and aroma profile of wines which can be positive or negative (Jolly et al., 2003a; Varela et al., 2017; Viana et al., 2008). On the one hand, some species synthesize esters responsible for desirable 'fruity' or 'floral' aromas (Contreras et al., 2014; Rojas et al., 2003; Viana et al., 2008) and/or extracellular enzymes which enhance grape varietal aromas or breakdown polysaccharides important for filtration and/or mouthfeel (Comitini et al., 2011; Cordero-Bueso et al., 2013). On the other hand, however, certain strains synthesize high amounts of ethyl acetate, acetic acid, and/or other compounds with undesirable aromas and potential regulatory concerns (Andorrà et al., 2010; Ciani et al., 2006; Rojas et al., 2003).

A preliminary evaluation of non-*Saccharomyces* yeasts isolated from Washington state vineyards identified strains which may be applicable industrially. Here, the presence of *Candida californica* or *Metschnikowia pulcherrima* in a high sugar Syrah grape must lowered subsequent ethanol concentrations by 0.8 to 0.9% v/v (Aplin et al. 2019b). While other strains of *Mt. pulcherrima* have produced wines with less ethanol in other studies (Canonico et al., 2016; Contreras et al., 2014; Morales et al., 2015), additional non-*Saccharomyces* yeasts not typically used in winemaking may also exhibit similar abilities. As such, the objective of this study was to screen additional yeasts isolated by Bourret et al. (2013) for their abilities to reduce wine alcohol content using synthetic grape juice media and grape musts.

Materials and Methods

Yeasts

Candida californica P01C003, *C. oleophila* P40C006, *C. railenensis* RcaA001, *C. saitoi* P45A002, *Hanseniaspora uvarum* P34A006, *Issatchenkia orientalis* J5-6-5, *Metschnikowia chrysoperlae* P40A002, *Mt. pulcherrima* P01A016, *Meyerozyma caribbica* P46A001, *My. guilliermondii* P40D002, *Pichia fermentans* M1-3-1, *P. kluyveri* P01C002, *P. membranifaciens* P43C010, *Wickerhamomyces anomalus* P01A017, and *Yamadazyma mexicana* P45C009 were previously isolated from vineyards located at the Irrigated Agriculture Research and Extension Center (Prosser, WA) as described by Bourret et al. (2013). *Kluyveromyces marxianus* HA 63 (NRRL Y-8281) was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). *Torulaspora delbrueckii* NS-TD and *Saccharomyces cerevisiae* D254 were obtained from Lallemand Inc. (Montréal, Quebec, Canada). All yeasts were maintained on yeast peptone dextrose (YPD) agar slants containing 10 g/L yeast extract (Becton, Dickinson, and Company, Sparks, MD), 20 g/L peptone (Becton, Dickinson, and Company), 20 g/L dextrose (Thermo Fisher Scientific, Waltham, MA), and 15 g/L agar (Acros Organics, Morris, NJ).

Starter cultures were prepared by transferring single colonies grown on YPD agar to yeast/mold (YM) broth (Becton, Dickinson, and Company, Sparks, MD). Once late exponential growth was reached, cells were harvested by centrifugation at 2000 x *g* for 20 min, washed twice using 0.2 M Na_2HPO_4 (pH 7.0) buffer, and resuspended in phosphate buffer prior to inoculation. Cultures of *S. cerevisiae* in active dry form were rehydrated with distilled water according to manufacturer's instructions.

Vinification I

Merlot grape juice concentrate was obtained from the California Concentrate Company (Acampo, CA) and reconstituted with distilled water according to manufacturer's instructions. The must was adjusted to 155 g/L of glucose and fructose (each), pH 3.58 with tartaric acid, and 300 mg N/L yeast assimilable nitrogen (YAN) using a mixture of Fermaid® K and diammonium phosphate (Scott Laboratories, Petaluma, CA). Sterile filtration was accomplished using 0.45 µm polyvinylidene fluoride cartridges (MilliporeSigma, Bellerica, MA) into previously sterilized 6 L Celstir fermenters. A suspension of powdered cellulose (Sigmacell® Type 20, Sigma-Aldrich, St. Louis, MO), sterilized at 121°C for 15 min, was added at a rate of 1 g/L prior to inoculation with non-*Saccharomyces* yeast starter cultures (10^5 CFU/mL). Fermentations were conducted, in triplicate, at 21°C with constant mixing using magnetic stirring bars with and without aeration. For aerated samples, oxygen was sparged into the must at a rate of 75 mL/min, equivalent to 0.025 VVM (volume of O_2 in liters per volume of juice in liters per min), through a stainless-steel diffuser with rate measured using a flow meter (ADM 2000, Agilent Technologies, Santa Clara, CA). After six days, 100 mL aliquots were transferred to sterile milk dilution bottles for inoculation with *S. cerevisiae* (10^6 CFU/mL). Fermentations proceeded for an additional 30 days prior to analysis.

Vinification II

Additional Merlot grape juice concentrate was reconstituted and adjusted to 266 g/L glucose and fructose, pH 3.62 with tartaric acid, and 325 mg/L YAN using a mixture of Fermaid® K and diammonium phosphate. The juice (300 mL) was sterile-filtered through 0.45 µm polyvinylidene fluoride cartridges into 500 mL bottles and powdered cellulose, prepared as a sterilized suspension as described previously, was added at a rate of 1 g/L. Yeast starter cultures

were prepared and inoculated, in triplicate, to initial populations of 10^6 CFU/mL. Vessels were closed with gas-porous Bugstoppers® (Whatman, Maidstone, U.K.) and incubated at 21°C on a rotary shaker (100 rpm). After 72 hr, a starter culture of *S. cerevisiae* was inoculated to yield 10^6 CFU/mL while the stoppers were replaced by fermentation bubble locks and shaking was discontinued. Fermentations proceeded to dryness (<2 g/L reducing sugar) as measured by the Clinitest® method (Ough and Amerine, 1988).

Vinification III

A synthetic grape juice medium (SGJM) was prepared without added sulfites as described by Wang et al. (2003) with some modifications. Similar to Wang et al. (2003), the SGJM contained 250 g/L fermentable sugars (125 g/L each of glucose and fructose), 3.0 g/L each tartaric and malic acid, 0.22 g/L citric acid, and the same concentrations of vitamins/minerals. However, YAN was adjusted to 300 mg N/L by altering the concentrations of alanine (193 mg/L), arginine (587 mg/L), aspartic acid (61 mg/L), glutamic acid (245 mg/L), glycine (18 mg/L), histidine (132 mg/L), isoleucine (79 mg/L), leucine (96 mg/L), lysine (114 mg/L), methionine (35 mg/L), phenylalanine (79 mg/L), proline (6097 mg/L), serine (140 mg/L), threonine (125 mg/L), tryptophan (53 mg/L), tyrosine (61 mg/L), valine (534 mg/L), and $(NH_4)_2HPO_4$ (178 mg/L). The pH was adjusted to 3.5 using potassium hydroxide prior to sterile filtration through 0.22 μm PES Express™ Plus bottle-top filters (MilliporeSigma, Bellerica, MA). SGJM media (400 g) were aseptically added to sterilized 500 mL bottles. In addition, powdered cellulose (Sigmacell® Type 20, Sigma-Aldrich, St. Louis, MO) was prepared as a suspension in 0.2M phosphate buffer and sterilized at 121°C for 15 min before being added to all bottles at 1 g/L.

For single inoculum experiments, bottles containing SGJM were inoculated, in triplicate, with starter cultures of *Mt. pulcherrima*, *My. guilliermondii*, or *S. cerevisiae* at 10^6 CFU/mL. Bottles were sealed with gas-porous stoppers and incubated at 25°C on a rotary shaker (100 rpm) for seven days. For sequential inoculation experiments, bottles of SGJM were inoculated with *Mt. pulcherrima*, *My. guilliermondii*, or *S. cerevisiae* at 10^6 CFU/mL without and with added low population microflora potentially found on grapes consisting of *H. uvarum*, *P. kluyveri*, and *T. delbrueckii* (10^3 CFU/mL each). Bubble locks were added to bottles inoculated with *S. cerevisiae* while those with non-*Saccharomyces* yeasts were sealed with gas-porous stoppers. *S. cerevisiae* was inoculated (10^6 CFU/mL) into cultures containing non-*Saccharomyces* yeasts on day 3 and then sealed with bubble locks. Fermentations were conducted in triplicate at 25°C on a rotary shaker (100 rpm) and monitored until fermentative activity ceased (constant weight for three days). Prior to sampling, fermentations were mixed for one min using a magnetic stir plate.

Vinification IV

Merlot grapes were hand-harvested in 2017 from vineyards located at the WSU Irrigated Agriculture Research and Extension Center (Prosser, WA) and crushed/destemmed using standard methods. Immediately after crushing, 20 mg/L total SO_2 and 0.2 g/kg diammonium phosphate were added to 37.5 kg of must (271 g/L glucose and fructose, pH 3.39, 5.94 g/L titratable acidity, 206 mg/L YAN) in 100L stainless steel fermenters. Musts were inoculated, in triplicate, with either *S. cerevisiae* or *Mt. pulcherrima* (10^6 CFU/mL) and fermented without lids. After 72 hr, *S. cerevisiae* was sequentially added (10^6 CFU/mL) into musts initially inoculated with *Mt. pulcherrima* and lids were replaced on all fermenters. Fermentations were conducted at cellar temperature with daily punch downs, while soluble solids were monitored with an Anton Paar DMA35 density meter (Anton Paar USA Inc., Ashland, VA). At approximately 18° Brix,

0.2 g/kg Fermaid® K was added. Wine was pressed-off using a hydraulic bladder press (Speidel, Ofterdingen, Germany) when soluble solids reached approximately 2°Brix. Once dry (<2 g/L reducing sugar), wine was racked and moved to 3° to 4°C after addition of 50 mg/L total SO_2 until analysis.

Analytical methods

Culturability was monitored by spiral plating (Autoplate® 4000, Spiral Biotech, Bethesda, MD) on either Wallenstein Laboratory agar (WL, Becton, Dickinson, and Company, Franklin, MD) for total yeasts or lysine agar (Oxoid, Hampshire, England) for non-*Saccharomyces* yeasts after two days at 28°C. In sequentially inoculated fermentations, populations of *S. cerevisiae* were estimated based on the difference between plate counts on WL and lysine agar media. In Merlot grape must (vinification IV conditions), low populations of non-*Saccharomyces* yeasts were detected by spread plating 0.5 mL of fermenting grape juice on lysine agar.

Glucose, fructose, ethanol, glycerol, and organic acids were quantified with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) according to Eyéghé-Bickong et al. (2012) with some modifications. Compounds were separated using an Aminex HPX-87H column (300 x 7.8 mm, BIO-RAD, Hercules, CA) equilibrated at 60°C using 0.005M H_2SO_4 as the mobile phase flowing at 0.6 mL/min. Samples were filtered through 0.22 µm PES syringe filters (MilliporeSigma) into crimp top vials prior to analysis. Volatile acidity (Cash Still) and reducing sugars (Clinitest) was measured following standard methods (Ough and Amerine, 1988). YAN was calculated as the sum of primary amino nitrogen according to Dukes and Butzke (1998) and ammonium by an ion-selective probe (Denver Instruments, Orville, NY).

Yeast biomass was determined as cellular dry weight in pre-dried (105°C overnight), pre-weighed aluminum weigh pans. Following homogenization on a stir plate for one min, 10 mL

samples were aseptically transferred to centrifuge tubes using sterilized volumetric pipettes. Cell biomass was centrifuged at 4000 x *g* and washed twice with Milli-Q water then transferred to pre-dried aluminum weigh pans to dry overnight at 105°C. The weight of the biomass was obtained by subtracting the pan weight from the dried weight. Yeast biomass was expressed as g per liter with the concentration of cellulose added to fermentations (1 g/L) subtracted.

Statistical methods

Statistical analyses were performed by ANOVA while means were separated by Fisher's least significant difference test using XLSTAT software (Addinsoft, New York, NY).

Results and Discussion

Vinification I

Non-*Saccharomyces* yeasts were sequentially inoculated with *S. cerevisiae* into a high sugar grape must (310 g/L glucose + fructose) to evaluate sugar utilization and ethanol production without or with aeration (Table 6). While ferments inoculated solely with *S. cerevisiae* contained high amounts of residual sugar (8.67 g/L), many wines with non-*Saccharomyces* yeasts contained ≤2 g/L including *C. saitoana* (1.56 g/L), *H. uvarum* (0.918 g/L), *My. guilliermondii* (1.02 g/L), *P. fermentans* (0.647 g/L), or *P. kluyveri* (0.643 g/L). Ethanol contents in wines produced with non-*Saccharomyces* yeasts were similar to those fermented by *S. cerevisiae* alone (16.1% v/v), excepting ferments conducted with *C. oleophila* or *W. anomalus*, which contained less (15.4 and 15.1% v/v, respectively), and *P. fermentans* which produced more (16.4% v/v). When aerated, however, most wines with non-*Saccharomyces* yeasts typically contained more residual sugar (2.15 to 17.7 g/L) than those with *S. cerevisiae* alone (2.01 g/L) or *P. fermentans* (1.64 g/L) but generated the same or less alcohol with the lowest being those with *K. marxianus* (13.2%), *P. membranifaciens* (13.6%), or *W. anomalus*

(13.7%) than those with *S. cerevisiae* (15.9 g/L). In fact, inoculation of *H. uvarum*, *Mt. pulcherrima*, *My. guilliermondii*, or *P. kluyveri* resulted in wines with less alcohol and similar amounts of residual sugar as with inoculation of *S. cerevisiae* alone.

Besides sugar and ethanol, concentrations of acetic acid formed during fermentation varied widely (Table 6). Without aeration, wines inoculated with *S. cerevisiae* alone produced the lowest concentrations of acetic acid (0.452 g/L) while those with *Mt. pulcherrima*, *My. caribbica*, or *My. guilliermondii* wines contained levels below the sensory threshold of 0.7 g/L (Fugelsang and Edwards, 2007). With aeration, all treatments produced higher amounts of acetic acid in agreement with others (Contreras et al, 2015b; Morales et al., 2015; Quirós et al, 2014). Under these conditions, only wines with *Mt. pulcherrima* contained significantly less acetic acid than those from *S. cerevisiae* alone (0.773 g/L vs. 1.38 g/L). In fact, wines with *C. californica*, *C. oleophila*, *H. uvarum*, *I. orientalis*, or *W. anomalus* all produced high amounts of acetic acid (>1.38 g/L) regardless of fermentation condition. High production of volatile acidity was also noted in red and white grape musts inoculated with *C. oleophila* (Aplin et al., 2019b), *H. uvarum* (Andorrà et al., 2010; Ciani et al., 2006), or *W. anomalus* (Aplin et al., 2019b; Rojas et al., 2003).

Given the high amount of sugar in the Merlot grape must, it was not surprising that some wines did not complete fermentation or that many had elevated levels of acetic acid. Assuming that 21°Brix grape musts require 200 mg N/L YAN to complete fermentation, Bisson and Butzke (2000) recommended an additional 25 mg N/L for every 1°Brix increase. If valid, the Merlot grape must (approximately 27.7°Brix) should have required 360 mg N/L but only contained 300 mg N/L. Moreover, early growth of non-*Saccharomyces* yeasts consumed additional nutrients that may have hampered *S. cerevisiae* (Aplin et al., 2019b; Gobert et al., 2017; Medina et al.,

2012; Rollero et al., 2018a). Even so, some non-*Saccharomyces* species, *C. californica, C. oleophila, H. uvarum,* and *W. anomalus,* consistently produced acetic acid so further research involving these yeasts was discontinued.

Vinification II

Additional experimentation was conducted without aeration using a Merlot must that contained a lower amount of fermentable sugars (266 g/L) than vinification I but with additional YAN supplementation (325 mg N/L). Based on previous sugar utilization and decreased ethanol production, *Mt. chrysoperlae, Mt. pulcherrima, My. guilliermondii*, *P. kluyveri*, and *P. membranifaciens* were selected for further evaluation. A strain of *T. delbrueckii*, a yeast associated with fermenting grape musts (Contreras et al., 2015a), was also included.

Under vinification II conditions, inoculation of non-*Saccharomyces* yeasts generally yielded less residual sugar, ethanol, and acetic acid in wines compared to those produced with *S. cerevisiae* alone (Table 7). Though residual sugar concentrations in ferments with non-*Saccharomyces* yeasts (0.416 to 1.19 g/L) were less than those produced with *S. cerevisiae* (1.28 g/L), all wines were considered dry (<2 g/L). While the presence of most non-*Saccharomyces* yeasts exhibited only slight reductions to final alcohol concentrations (13.0% to 13.4% v/v), inoculation of *My. guilliermondii* or *Mt. pulcherrima* resulted in far less alcohol (12.3% or 11.7% v/v, respectively). The ability of *Mt. pulcherrima* to reduce final alcohol concentrations have been previously reported (Aplin et al., 2019b; Contreras et al., 2014; Varela et al., 2016; 2017) but this was the first report describing the impact of *My. guilliermondii*. Moreover, no wines contained acetic acid at levels above the sensory threshold of 0.7 g/L (Fugelsang and

Edwards, 2007). In fact, inoculation of non-*Saccharomyces* yeast generated less acetic acid compared to wines with *S. cerevisiae* alone with the exception of those with *T. delbrueckii*.

Gonzalez et al. (2013) first proposed the use of respiratory non-*Saccharomyces* yeasts as a means to lowering alcohol contents through metabolism of fermentable sugar to other by-products. Being Crabtree-negative, these yeasts would rely upon respiration pathways as opposed to fermentation towards metabolism of sugar regardless of the availability of glucose. In support, ethanol reductions of 0.7% to 1.8% v/v have been reported after inoculation of *C. californica, H. opuntiae, H. uvarum, Mt. pulcherrima, S. uvarum, Starmerella bacillaris,* and *St. bombicola* (Aplin et al., 2019b; Canonico et al., 2016; Contreras et al., 2014; 2015a; Englezos et al., 2015; Rossouw and Bauer, 2016; Varela et al., 2016; 2017). While ethanol reductions may correspond to increases in glycerol and succinic acid (Varela et al., 2016), other by-products such as biomass, CO_2, and water have been suggested but with limited evidence (Gonzalez et al., 2013; Quirós et al., 2014).

Vinification III

Yeasts which produced wines under vinification II conditions with >1% v/v reduction in ethanol were further characterized using a well-defined grape juice medium (SGJM). Here, *Mt. pulcherrima*, *My. guilliermondii,* and *S. cerevisiae* were individually inoculated into SGJM to evaluate growth, sugar utilization, and metabolite production. All yeasts grew well in SGJM, developing populations $\geq 10^8$ CFU/mL within two days (Fig. 4A). Even though all yeasts achieved high populations, *S. cerevisiae* produced more biomass (7.10 g/L) than either *Mt. pulcherrima* (4.52 g/L) or *My. guilliermondii* (2.10 g/L) after seven days of growth (Fig. 4B). Furthermore, Fig. 4C illustrates that *Mt. pulcherrima* and *My. guilliermondii* consumed less sugar (117 or 221 g/L residual sugar, respectively) than *S. cerevisiae* (4.78 g/L), in agreement

with previous findings (Aplin et al., 2019b; Comitini et al., 2011; Cordero-Bueso et al., 2013). With regards to other by-products (Fig. 4D, E, and F), *S. cerevisiae* generated the most ethanol (13.7% v/v) and glycerol (5.44 g/L), while *Mt. pulcherrima* produced the most succinic acid (1.09 g/L). Fermentations conducted by *My. guilliermondii* produced the least amount of ethanol (1.10% v/v), glycerol (1.70 g/L), or succinic acid (0.365 g/L). Ethanol yields per g sugar consumed for *Mt. pulcherrima* (0.372 g/g) and *My. guilliermondii* (0.301 g/g) were significantly lower than those exhibited by *S. cerevisiae* (0.422 g/g) while higher yields of glycerol and succinic acid were noted for the non-*Saccharomyces* yeasts (data not shown).

Metabolically, *Mt. pulcherrima* and *My. guilliermondii* yeasts are both Crabtree-negative, whereas glucose concentration in media does not inhibit respiration (De Deken et al., 1966; Qi et al., 2014; Schnierda et al., 2014). Sugar transport in such yeasts is therefore often governed by high affinity, energy-dependent systems that restrict sugar uptake (Van Urk et al., 1989). As such, reduced ethanol production coupled with higher production of other metabolites may be indicative of increased flux through respiratory pathways. In contrast, *S. cerevisiae* is Crabtree-positive and exhibits faster sugar transport as well as faster rates of glycolysis and fermentation regardless of the presence/absence of oxygen in high glucose environments (Van Urk et al., 1989; 1990).

Because neither *Mt. pulcherrima* nor *My. guilliermondii* can consume all sugar present in a natural grape must, sequential inoculation with *S. cerevisiae* is required needed to complete fermentation (Gonzalez et al., 2013). However, species compatibility with potential native microflora must be also assessed prior to industrial application. To this end, *Mt. pulcherrima* and *My. guilliermondii* were evaluated in sequential inoculations with *S. cerevisiae* with and without the presence of yeasts typically found in grape musts. Similar to the approach of Contreras et al.

(2015a), *H. uvarum*, *P. kluyveri*, and *T. delbrueckii* were inoculated at low populations (10^3 CFU/mL) on day 0.

Growth and sugar utilization by *S. cerevisiae* alone and co-inoculated with non-*Saccharomyces* yeasts were similar to previous trends (Fig. 5). Ferments with *S. cerevisiae* alone finished the quickest, achieving populations $\geq 10^8$ CFU/mL by day 2 and consuming all available sugar by day 11 (Fig. 5A). In contrast, prior inoculation of non-*Saccharomyces* yeasts (Fig. 5B-F) delayed completion of fermentation where SGJM inoculated with background microflora followed by *S. cerevisiae* finished fermenting by day 18 (Fig. 5B), while those with *Mt. pulcherrima* or *My. guilliermondii* finished by day 24 (Fig. 5C-F). In all sequentially inoculated wines, *S. cerevisiae* quickly exerted dominance and established higher culturable populations than non-*Saccharomyces* yeasts within two days. In fact, non-*Saccharomyces* yeasts were unrecoverable after day 11 (Fig. 5B, E) or 18 (Fig. 5F) but persisted in those inoculated with *Mt. pulcherrima* (Fig. 5C, D). Though wines with non-*Saccharomyces* yeasts contained some residual sugar (0.450 to 3.12 g/L), all were considered dry except those with *My. guilliermondii* containing background microflora (3.12 g/L).

The presence of *Mt. pulcherrima* or *My. guilliermondii* affected the final concentration of ethanol and other metabolites in sequentially inoculated SGJM (Table 8). Alcohol amounts in ferments inoculated with *Mt. pulcherrima* without (11.3% v/v) or with (11.9% v/v) added background microflora were lower than wines produced with *S. cerevisiae* alone (14.3% v/v), in agreement with results under vinification II conditions. Significant reductions in alcohol contents were also observed in wines with *My. guilliermondii* with background microflora (12.3% v/v), but not without background microflora (13.7% v/v). Besides ethanol, more biomass was produced in ferments inoculated with *Mt. pulcherrima* and background microflora (5.13 g/L)

above other treatments (3.64 to 4.12 g/L). *Mt. pulcherrima* inoculation decreased glycerol content (5.23 to 5.43 g/L) in comparison to treatments inoculated day 0 with *My. guilliermondii* or *S. cerevisiae* (6.04 to 6.28 g/L). With regards to organic acids, non-*Saccharomyces* yeasts generated more succinic acid (0.497 to 0.794 g/L) than *S. cerevisiae* alone (0.347 g/L). Acetic acid however was decreased in wines with *Mt. pulcherrima* (0.176 to 0.213 g/L), in agreement with Sadoudi et al. (2012), while other treatments contained concentrations above the sensory threshold of 0.7 g/L. Ferments conducted by Varela et al. (2016; 2017) using *S. uvarum* suggested that increases to glycerol and succinic acid can justify reductions in alcohol content. However, changes to by-products measured in the current study (biomass, glycerol, succinic acid, and acetic acid) do not explain the decreased ethanol concentration in wines with *Mt. pulcherrima* and *My. guilliermondii*, suggesting these yeasts may utilize other carbon sinks.

The persistence of *Mt. pulcherrima* or *My. guilliermondii* when co-inoculated with *S. cerevisiae* was correlated with the final alcohol concentration in resultant wines. As evidence, limited ethanol reduction was observed in ferments with *My. guilliermondii*, where non-*Saccharomyces* yeasts died-off before the completion of alcoholic fermentation, in contrast to ferments with *Mt. pulcherrima*. Furthermore, *My. guilliermondii* wines with added background yeasts contained less alcohol than wines inoculated with *My. guilliermondii* alone, where non-*Saccharomyces* yeasts persisted seven fewer days. Early growth of non-*Saccharomyces* yeasts is known to impact *S. cerevisiae* during wine fermentation through preferential consumption of vitamins (Bataillon et al., 1996; Medina et al., 2012) and/or nitrogenous compounds (Gobert et al., 2017; Rollero et al., 2018a) required for growth. Additionally, *Mt. pulcherrima* produces pulcherrimin, which depletes iron in growth media (Sipiczki, 2006), while many *Hanseiaspora* and *Pichia* species produce killer toxins with antifungal properties (Fleet, 2003).

Vinification IV

Based on ethanol reduction and yeast persistence when co-inoculated with *S. cerevisiae* in SGJM, *Mt. pulcherrima* was selected for vinification trials in Merlot grape must under winery conditions. As previously observed using SGJM, non-*Saccharomyces* yeasts achieved high culturable populations ($\geq 10^7$ CFU/mL) in the unsterilized Merlot must prior to sequential inoculation of *S. cerevisiae* on day 3 (Fig. 6). In contrast to previous results in SGJM, *S. cerevisiae* populations did not exceed populations of non-*Saccharomyces* yeasts until day 11 in fermentations with *Mt. pulcherrima*. Once *S. cerevisiae* dominated ferments inoculated with *Mt. pulcherrima*, non-*Saccharomyces* yeast populations quickly decreased to undetectable levels, in agreement with others (Aplin et al., 2019b; Comitini et al., 2011; Contreras et al., 2014; Sadoudi et al., 2012).

Inoculation of *Mt. pulcherrima* resulted in wines that contained less ethanol yet were similar in general chemical composition (Table 9). Specifically, *Mt. pulcherrima* reduced final alcohol content by 0.9% v/v compared to wines fermented with *S. cerevisiae* alone where residual sugar was below the detection limit (≈0.07 g/L). Slight increases to titratable acidity were observed in wines with *Mt. pulcherrima*, although amounts of volatile acidity were similar and concentrations of succinic acid were lower compared to *S. cerevisiae* wines. In agreement, Varela et al. (2017) produced Merlot wine from a must sterilized by dimethyl dicarbonate containing 0.9% v/v less ethanol but using a different strain of *Mt. pulcherrima*.

Conclusions

This study evaluated non-*Saccharomyces* yeasts isolated from Washington vineyards for their abilities to reduce final ethanol content in wine when inoculated in grape musts. Of the species examined, inoculation of either *Mt. pulcherrima* or *My. guilliermondii* into musts

followed by *S. cerevisiae* led to significant reductions in alcohol contents. In fact, inoculation of *Mt. pulcherrima* in an unsterilized Merlot must produced wines with 0.9% v/v less ethanol than wines inoculated with *S. cerevisiae* alone without elevated concentrations of acetic acid. Additional research is needed to ascertain the sensory profile of reduced alcohol wines produced with *Mt. pulcherrima*.

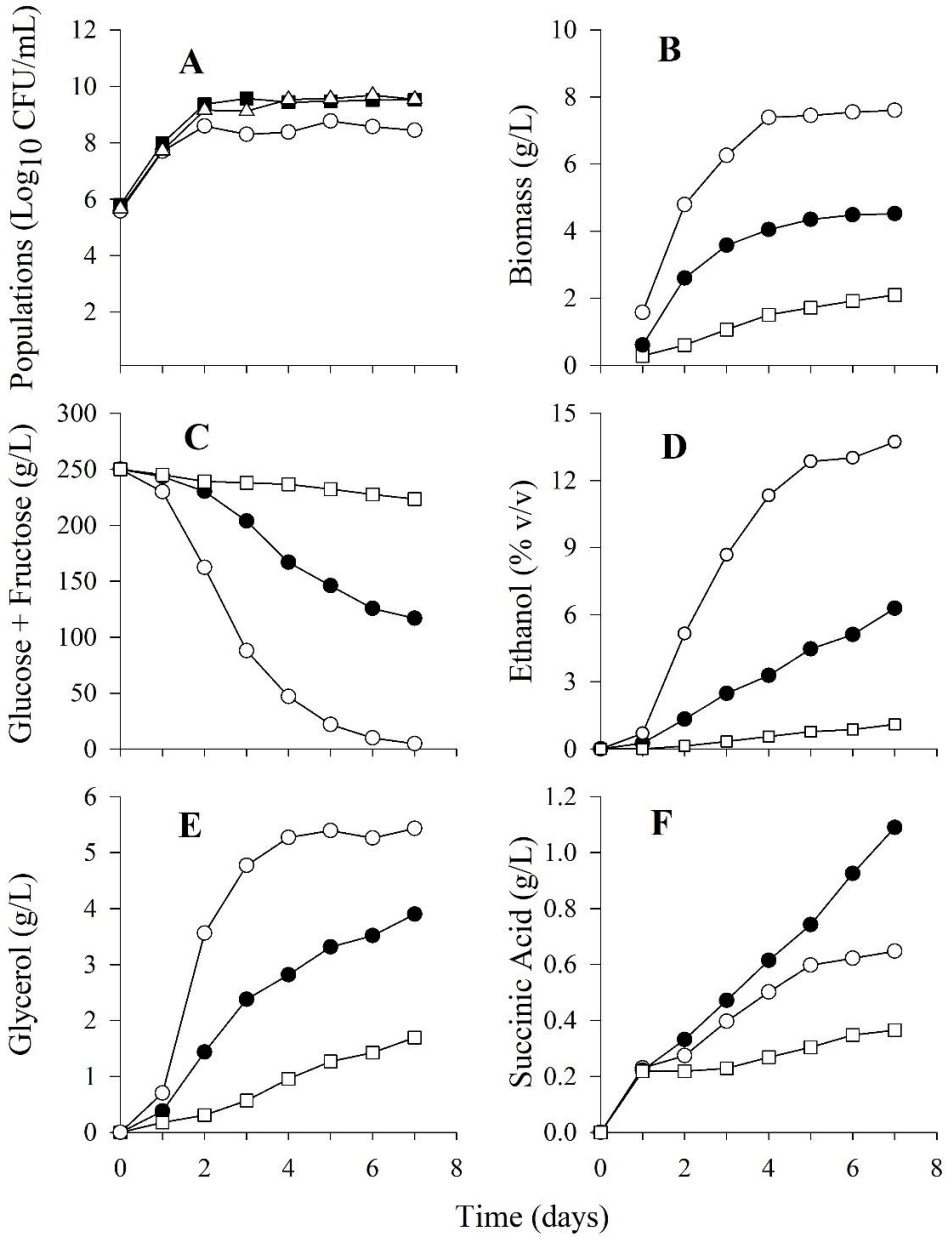

Figure 4. Culturable populations (A), biomass (B), glucose + fructose (C), ethanol (D), glycerol (E), and succinic acid (F) after single inoculations of *S. cerevisiae* (○), *Mt. pulcherrima* (●), or *My. guilliermondii* (□) on day 0 in sterilized SGJM fermented under vinification III conditions.

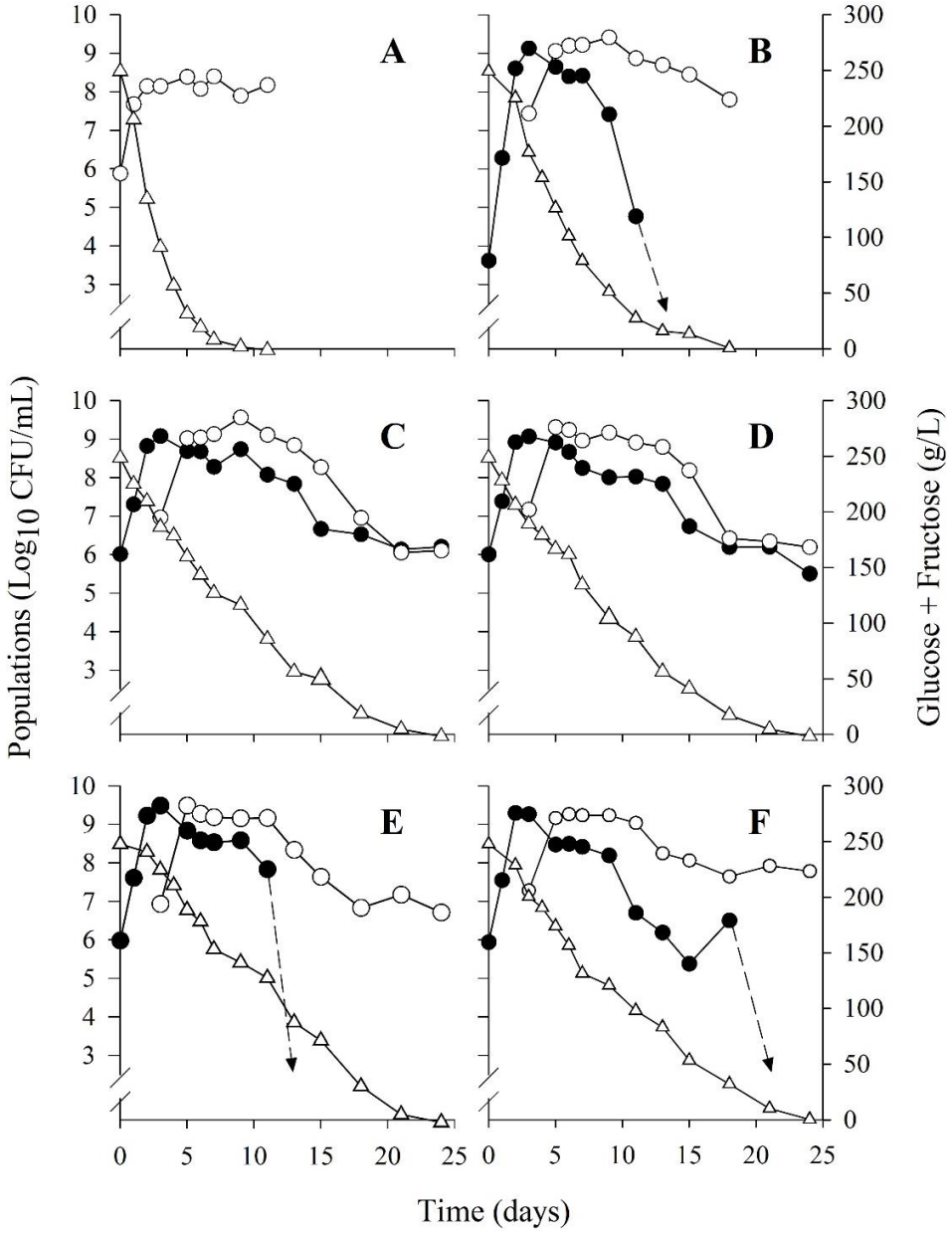

Figure 5. Culturable populations of *S. cerevisiae* (○) and non-*Saccharomyces* yeasts (●) as well as concentrations of glucose + fructose (△) in sterilized SGJM fermented under vinification III conditions. Media were inoculated on day 0 with *S. cerevisiae* alone (A), on day 3 alone (B), or on day 3 into musts previously inoculated with *Mt. pulcherrima* (C, D) or *My. guilliermondii* (E, F) on day 0. *H. uvarum*, *P. kluyveri*, and *T. delbrueckii* (10^3 CFU/mL each) were inoculated into media B, D, and F on day 0 to simulate potential microflora present in an unsterilized grape must.

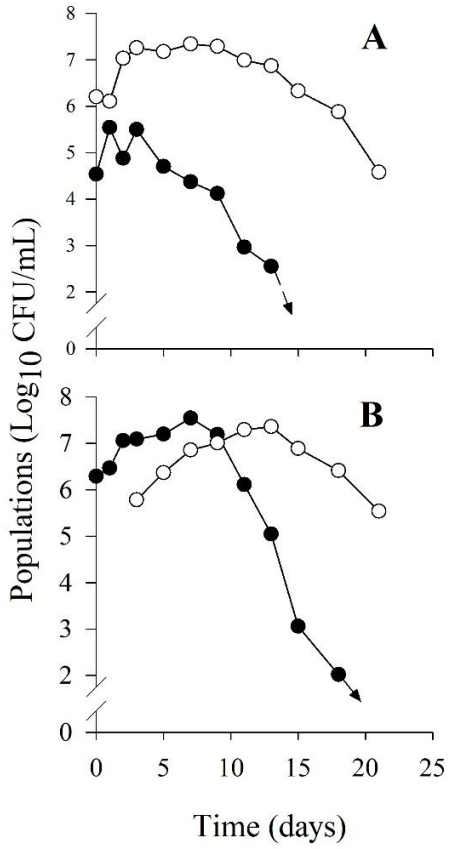

Figure 6. Culturable populations of *S. cerevisiae* (○) and total non-*Saccharomyces* yeasts (●) in non-sterile Merlot grape musts fermented under vinification IV conditions. Grape musts were inoculated on day 0 with *S. cerevisiae* alone (A) or on day 3 into musts inoculated with *Mt. pulcherrima* (B) added on day 0.

Table 6. Composition of Merlot wines produced from sterilized musts under vinification I conditions and either non-aerated or aerated.

	Non-Aerated			Aerated		
Yeast	Glucose + Fructose* (g/L)	Ethanol (% v/v)	Acetic acid (g/L)	Glucose + Fructose* (g/L)	Ethanol (% v/v)	Acetic acid (g/L)
S. cerevisiae	8.67^{g}	16.1^{cd}	0.452^{a}	2.01^{ab}	15.9^{hi}	1.38^{bc}
Non-*Saccharomyces*†						
C. californica	2.18^{abc}	15.7^{bc}	1.44^{g}	8.56^{cd}	14.9^{def}	2.34^{f}
C. oleophila	5.78^{ef}	15.4^{ab}	1.38^{g}	17.7^{e}	14.5^{cd}	1.88^{e}
C. railenensis	4.38^{cde}	15.8^{cd}	1.07^{de}	15.2^{e}	14.6^{cd}	1.35^{bc}
C. saitoana	1.56^{ab}	16.1^{def}	1.05^{g}	8.09^{d}	15.2^{efg}	1.68^{cde}
H. uvarum	0.918^{ab}	16.1^{def}	1.39^{g}	2.38^{ab}	15.2^{efg}	1.80^{de}
I. orientalis	2.38^{abcd}	16.1^{de}	1.43^{g}	4.44^{abc}	16.1^{i}	1.47^{bcd}
K. marxianus	15.7^{h}	15.6^{bc}	1.19^{f}	17.4^{e}	13.2^{a}	2.52^{f}
Mt. chrysoperlae	7.84^{fg}	15.8^{cd}	0.734^{bc}	9.51^{d}	14.5^{cd}	1.16^{ab}
Mt. pulcherrima	3.23^{bcd}	15.6^{bc}	0.645^{b}	5.68^{abcd}	14.9^{def}	0.773^{a}
My. caribbica	19.1^{i}	16.1^{def}	0.623^{b}	5.77^{abcd}	15.7^{ghi}	1.12ab
My. guilliermondii	1.02^{ab}	15.5^{bc}	0.670^{b}	5.93^{bcd}	14.8^{cde}	1.48^{bcd}
P. fermentans	0.647^{a}	16.4^{f}	1.03^{de}	1.64^{ab}	15.8^{hi}	1.70^{cde}
P. kluyveri	0.643^{a}	15.8^{cd}	1.14^{ef}	2.15^{ab}	14.3^{bc}	1.92^{e}
P. membranifaciens	4.77^{de}	16.1^{de}	1.00^{d}	7.79^{cd}	13.6^{a}	2.47^{f}
W. anomalus	14.8^{h}	15.1^{a}	1.38^{g}	8.89^{d}	13.7^{ab}	2.32^{f}
Y. mexicana	3.28^{bcd}	16.3^{ef}	0.843^{c}	3.52^{ab}	15.4^{gh}	1.38^{bc}

$^{a-i}$ Mean values within columns with different letters are significantly different ($p \leq 0.05$).
* Sugar concentration in the grape must prior to inoculations was 310 g/L.
† Non-*Saccharomyces* yeasts added on day 0 with *S. cerevisiae* was inoculated on day 6.

Table 7. Composition of Merlot wines produced from sterilized musts under vinification II conditions without aeration. Values are means of triplicate fermentations.

Yeast	Glucose + Fructose* (g/L)	Ethanol (% v/v)	Acetic Acid (g/L)
S. cerevisiae	1.28[c]	13.7[e]	0.60[d]
Non-*Saccharomyces*[†]			
Mt. chrysoperlae	1.09[c]	13.3[d]	0.51[c]
Mt. pulcherrima	0.416[a]	11.7[a]	0.23[a]
My. guilliermondii	0.508[a]	12.3[b]	0.24[a]
P. kluyveri	1.05[bc]	13.3[d]	0.33[b]
P. membranifaciens	0.611[ab]	13.0[c]	0.25[a]
T. delbrueckii	1.19[c]	13.4[d]	0.62[d]

[a-f] Mean values within columns with different letters are significantly different ($p \leq 0.05$).
* Sugar concentration in the grape must prior to inoculations was 266 g/L.
[†] Non-*Saccharomyces* yeasts added on day 0 with *S. cerevisiae* was inoculated on day 3.

Table 8. Composition of synthetic wines produced from sterilized SGJM under vinification III conditions without aeration.

Presence of Additional Yeasts	Yeast Strain	Biomass (g/L)	Ethanol (% v/v)	Glycerol (g/L)	Succinic Acid (g/L)	Acetic Acid (g/L)
No	*S. cerevisiae*	4.11[a]	14.3[c]	6.04[b]	0.347[a]	1.00[c]
	Mt. pulcherrima[†]	4.12[a]	11.3[a]	5.23[a]	0.703[d]	0.213[a]
	My. guilliermondii[†]	3.78[a]	13.7[c]	6.16[b]	0.497[b]	0.966[c]
Yes*	*S. cerevisiae*[†]	3.71[a]	13.6[c]	5.37[a]	0.794[e]	0.816[b]
	Mt. pulcherrima[†]	5.13[b]	11.9[ab]	5.43[a]	0.702[d]	0.176[a]
	My. guilliermondii[†]	3.64[a]	12.3[b]	6.28[b]	0.546[c]	0.812[b]

* *H. uvarum*, *P. kluyveri*, and *T. delbrueckii* (10^3 CFU/mL each) were inoculated on day 0 to simulate microflora present in an unsterilized grape must. Sugar concentration in the grape must prior to inoculations was 250 g/L.

[†] Non-*Saccharomyces* yeasts added on day 0 with *S. cerevisiae* was inoculated on day 3.

[a-e] Mean values within columns with different letters are significantly different ($p \leq 0.05$).

Table 9. Composition of synthetic wines produced from sterilized SGJM under vinification III conditions without aeration.

Compound	*S. cerevisiae*	*Mt. pulcherrima*[†]
Glucose + Fructose* (g/L)	nd	nd
Ethanol (% v/v)	15.3[b]	14.4[a]
Glycerol (g/L)	9.96[a]	10.2[a]
pH	3.45[a]	3.41[a]
Titratable acidity (g/L)	6.80[a]	7.22[b]
Volatile acidity (g/L)	0.603[a]	0.599[a]
Succinic acid (g/L)	3.09[b]	2.77[a]

[nd] Not detected (below limit of detection <0.07 g/L).
[a-b] Mean values within rows with different letters are significantly different ($p \leq 0.05$).
* Sugar concentration in the grape must prior to inoculations was 271 g/L.
[†] *Mt. pulcherrima* was added on day 0 while *S. cerevisiae* was inoculated on day 3.

CHAPTER IV

CHEMICAL AND SENSORY PROFILES OF MERLOT WINES PRODUCED WITH NON-*SACCHAROMYCES* YEASTS ISOLATED FROM WASHINGTON STATE VINEYARDS

Abstract

Inoculation of selected non-*Saccharomyces* yeasts with *Saccharomyces cerevisiae* as means to produce Merlot wines with reduced ethanol contents from high °Brix musts was investigated. Fermentations of grape musts (25.4° Brix, pH 3.50, and 4.23 g/L titratable acidity) were conducted in 300 L stainless steel tanks using *Metschnikowia pulcherrima* (strains P01A016 and NS-MP) and *Meyerozyma guilliermondii* (strain P40D002) with subsequent inoculation of *S. cerevisiae* (strain Syrah) prior to chemical and descriptive sensory analyses. Wine previously inoculated with *Mt. pulcherrima* contained 13.8% (P01A016) or 13.9% (NS-MP) v/v ethanol, respectively, lower amounts compared to wines inoculated only with *S. cerevisiae* that contained 14.9% v/v. The presence of *My. guilliermondii* had no impact on production of ethanol but did produce greater amounts of esters such as 2- and 3-methylbutyl acetate, 2-phenylethyl acetate, and ethyl acetate than other wines while all wines with non-*Saccharomyces* yeasts yielded higher concentrations of alcohols such as 2-methyl-1-propanol and 2- and 3-methyl-1-butanol. Descriptive sensory analysis revealed that relative to *S. cerevisiae* wines, ferments with *Mt. pulcherrima* P01A016 enhanced ‘fruity’/‘berry’ aromas and ‘dried fruit’/‘estery’ aromas and flavors, while *My. guilliermondii* P40D002 increased ‘woody’ aroma, ‘berry’ aroma and flavor, and ‘astringent’ mouthfeel. This research indicates the potential for commercial application of these yeasts towards the production of reduced alcohol wines expressing positive sensory attributes.

Introduction

The average ethanol contents of red wine worldwide have increased from less than 12.5% to upwards of 13.6% v/v between 1984 and 2014 (Godden et al., 2015). This trend has been driven primarily by consumer demand for 'rich', 'full-bodied' wine styles which require prolonged vine hang-time to achieve phenolic ripeness (Godden et al., 2015; Goldner et al., 2009). However, an increase in ethanol can impact wine quality by intensifying sensory perceptions of 'chemical', 'woody', 'hotness', and/or 'bitterness' (Baker et al., 2016; Gawel et al., 2007; Heymann et al., 2013; Villamor et al., 2013b). In addition, elevated alcohol contents can mask some attributes such as 'fruity' and/or 'floral' by decreasing volatility of certain aroma compounds (Goldner et al., 2009; Mira de Orduña, 2010; Villamor et al., 2013a; 2013b**)**. Furthermore, high-alcohol wines can also face rejection by health-conscious consumers regarding concerns linked to excessive ethanol consumption (Gonzalez et al., 2013).

Since most commercial strains of *S. cerevisiae* yield similar amounts of alcohol after fermentation (Varela et al., 2008), Gonzalez et al. (2013) proposed the use of selected respiratory non-*Saccharomyces* yeasts as a means to reduce ethanol contents. Here, early growth of these non-*Saccharomyces* yeasts diverts sugar metabolism from ethanol into biomass and other by-products (Aplin et al., 2019a; Quirós et al., 2014). In fact, prior studies have identified species of non-*Saccharomyces* yeasts that produce wines with less alcohol (Aplin et al., 2019a; 2019b; Canonico et al., 2016; Contreras et al. 2014; Englezos et al., 2016; Rossouw and Bauer, 2016; Varela et al., 2016; 2017) by expressing Crabtree-negative metabolism whereas sugars are respired under aerobic conditions regardless of glucose concentration (De Deken et al., 1966). However, non-*Saccharomyces* yeasts are typically incapable of consuming all the available sugar

in a grape must and therefore require subsequent inoculation of *S. cerevisiae* to complete fermentation (Aplin et al., 2019b; Comitini et al., 2011; Jolly et al., 2014).

Within the industry, the use of non-*Saccharomyces* yeasts has gained popularity through alteration of the sensory profiles of wines (Jolly et al., 2014). On the one hand, these yeasts can positively contribute to wine quality and complexity (Jolly et al., 2003a; 2003b; Varela et al., 2017). For instance, some species of yeasts produce various esters responsible for 'fruity' and/or 'floral' aromas (Contreras et al., 2014; Rojas et al., 2003; Viana et al., 2008). Furthermore, some non-*Saccharomyces* yeasts synthesize glycosidases and pectinases that influence wine quality by catalyzing the release of varietal grape aromas as well as polysaccharides (Comitini et al., 2011; Domizio et al., 2011). On the other hand, some species decrease wine quality through production of undesirable amounts of ethyl acetate, acetic acid, and/or acetaldehyde (Ciani and Maccarelli, 1998; Rojas et al., 2003). Furthermore, early growth of non-*Saccharomyces* yeasts in grape musts may consume key nutrients important for *S. cerevisiae* growth, resulting in sluggish or stuck fermentations (Bataillon et al., 1996; Medina et al., 2012; Rollero et al., 2018).

Evaluating a number of different species isolated from Washington state vineyards (Bourret et al., 2013), Aplin et al. (2019a; 2019b) noted that strains of *Metschnikowia pulcherrima* and *Meyerozyma guilliermondii* produced wines with reduced alcohol contents. While the sensory effects of different strains of *Mt. pulcherrima* have been previously characterized (Jolly et al., 2003a; 2003b; Benito et al, 2015; Rodríguez et al., 2010; Varela et al., 2017), the impacts of *My. guilliermondii* on wine quality have not been described. As such, the objective of this research was to evaluate the influence of certain non-*Saccharomyces* yeasts on ethanol production and wine quality produced under pilot-scale winery conditions.

Material and Methods

Yeast strains

Mt. pulcherrima P01A016 and *My. guilliermondii* P40D002 were previously isolated from vineyards located at the Irrigated Research and Extension Center (Prosser, WA) as described by Bourret et al. (2013). Strains of *Mt. pulcherrima* (NS-MP) and *S. cerevisiae* (Syrah) were acquired from Lallemand Inc. (Montréal, Quebec, Canada). All yeasts were maintained on yeast peptone dextrose (YPD) agar plates containing 10 g/L yeast extract (Becton, Dickinson, and Company, Sparks, MD), 20 g/L peptone (Becton, Dickinson, and Company), 20 g/L dextrose (Thermo Fisher Scientific, Waltham, MA), and 15 g/L agar (Acros Organics, Morris, NJ).

Non-*Saccharomyces* yeast starter cultures were prepared by inoculating yeast/mold (YM) broth (Becton, Dickinson, and Company, Sparks, MD) from single colonies grown on YPD agar. Upon reaching late exponential phase, cells were harvested by centrifugation at 2000 x *g* for 20 min, washed twice with 0.2 M Na_2HPO_4 (pH 7.0) buffer, then resuspended in buffer prior to inoculation. Active dry yeast cultures of *S. cerevisiae* were rehydrated with distilled water according to manufacturer's instructions.

Merlot grape must

Merlot grapes were hand-harvested 9/22/2016 from vineyards located at the WSU Irrigated Agriculture Research and Extension Center (Prosser, WA) and crushed/destemmed using standard methods. Immediately after crushing, 20 mg/L total SO_2 were added to 37.5 kg of must (264 g/L glucose and fructose, pH 3.50, 4.23 g/L titratable acidity, 198 mg yeast assimilable nitrogen/L) placed in 300 L stainless steel, jacketed tanks without lids present. Diammonium phosphate was added at a rate of 0.2 g/kg to raise YAN content to approximately

250 mg N/L. Musts were then either uninoculated or inoculated at 10^6 colony forming units/mL (CFU/mL) with *Mt. pulcherrima* P01A016, *Mt. pulcherrima* NS-MP, *My. guilliermondii* P40D002, or *S. cerevisiae* Syrah, in triplicate. After 72 hr, lids were placed on all tanks just after inoculation of *S. cerevisiae* (10^6 CFU/mL) into all treatments not previously inoculated with this yeast. Fermentations were conducted at cellar temperature (approximately 20°C), without heating or chilling, with 1-minute daily punch-downs using a stainless-steel punch down tool. At approximately 18° Brix, 0.2 g/kg Fermaid-K was added to all tanks while fermentations were pressed at 0° Brix using a hydraulic bladder press (Speidel, Ofterdingen, Germany). Once dry (<2 g/L reducing sugars), wines were racked and moved to 3° to 4°C after addition of 50 mg/L SO_2. After 9 months, enough $K_2S_2O_5$ was added to achieve 0.8 mg/L molecular SO_2 prior to bottling without filtering. Bottled wines were stored for at least 4 months at 4°C before all analyses.

Chemical and microbiological analyses

Yeast culturability was monitored by spiral plating using an Autoplate 4000 (Spiral Biotech, Bethesda, MD) and both Wallenstein Laboratory nutrient agar for total yeast populations (Becton, Dickinson, and Company, Franklin Lakes, NJ) and lysine agar for non-*Saccharomyces* yeasts (Oxoid, Hampshire, England). Plates were incubated at 28°C for two days prior to enumeration. *S. cerevisiae* populations were estimated as differences between culturabilities on the two media.

Volatile acidity (Cash Still), titratable acidity, reducing sugars (Clinitest® method), and free and total SO_2 (aeration/oxidation) were measured following standard methods (Ough and Amerine, 1988). Yeast assimilable nitrogen (YAN) was calculated as the sum of primary amino

nitrogen according to Dukes and Butzke (1998) and ammonium by an ion-selective probe (Denver Instruments, Orville, NY).

Concentrations of glucose, fructose, glycerol, and organic acids were quantified by an Agilent 1100 HPLC system equipped with an Aminex HPX-87H column (300 x 7.8 mm, BIO-RAD, Hercules, CA) equilibrated at 60°C with 0.005M H_2SO_4 as mobile phase flowing at 0.6 mL/min. Volatile aroma compounds were analyzed by headspace solid-phase microextraction coupled with gas chromatography-mass spectroscopy (Aplin et al. 2019b).

Sensory analyses

Merlot wines were evaluated by a trained panel (n=10, four males and six females aged 24 to 40) consisting of regular wine drinkers recruited from the Washington State University community. Panelists received 12 hours of training across six weeks using feedback calibration through Compusense Cloud 8.8 sensory acquisition software (Guelph, Ontario, Canada). Wines were evaluated on basic taste, mouthfeel, and aroma/flavor attributes representative of the Merlot wines selected by a small focus group of experienced wine drinkers where attributes and references were detailed in Table 10. Samples (40 mL) were presented to panelist in covered three-digit coded ISO standard wine glasses at room temperature in individual tasting booths under white light at the Washington State University Sensory Evaluation Facility. Ten wines were assessed in duplicate in a randomized order over four evaluation sessions. Five wines were evaluated each session and fresh bottles were opened each day to prevent oxidative changes to wines. To avoid fatigue, panelists were required to take a five-minute break between samples and instructed to rinse their palate with water and an unsalted cracker. Panelist response were collected on a 15 cm unstructured line scale with anchor points ‘low’ (10% of the scale) and ‘high’ (90% of the scale) using Compusense software.

Statistical methods

Statistical analyses of chemical analyses were performed by ANOVA while means separations were accomplished by Fisher's LSD using XLSTAT software (Addinsoft, New York, NY). For sensory data, three-way ANOVA was performed to analyze panelist, treatment, and replicate interactions while means were separated using Fisher's Least Significant Difference (LSD) *post-hoc* test. Differences were considered significant when $p \leq 0.05$. Principal component analysis (PCA) of covariance for panel data was performed using XLSTAT. Mean attribute ratings of Merlot wines are listed in Table 11.

Results

Merlot grape musts were inoculated with *S. cerevisiae* alone or with *Mt. pulcherrima* P01A016, *Mt. pulcherrima* NS-MP, *My. guilliermondii* P40D002, or left uninoculated on day 0 followed by *S. cerevisiae* Syrah on day 3 (Fig. 7). Maximal *S. cerevisiae* populations (7.2×10^7 CFU/mL) were achieved by day 3 in ferments inoculated with this yeast alone on day 0 (Fig. 7A), in comparison to ferments inoculated with *S. cerevisiae* on day 3 (Fig. 7B-D) where maximal population (4.9×10^7 to 7.3×10^7 CFU/mL) were observed on day 7. With regards to non-*Saccharomyces* yeasts, musts inoculated with P01A016, NS-MP, or P01A016 yeasts achieved higher maximal populations on lysine agar ($\geq 10^7$ CFU/mL) than uninoculated ferments (7.6×10^6 CFU/mL) or those with *S. cerevisiae* (7.5×10^5 CFU/mL). Non-*Saccharomyces* yeast culturability quickly decreased from high populations ($\geq 10^6$ CFU/mL) to undetectable levels (<300 CFU/mL) in ferments where *S. cerevisiae* was introduced on day 3 (Fig. 7B-E). Even so, in musts inoculated with non-*Saccharomyces* yeasts (Fig. 7C-E), cells were recoverable on lysine agar for an additional four days in contrast to ferments with *S. cerevisiae* alone (Fig. 7A, B).

Chemical compositions of final wines depended on the yeast species present during fermentation (Table 12). While ferments inoculated with *S. cerevisiae* on day 0 achieved dryness by day 13 (≤2 g/L reducing sugar), wines inoculated with non-*Saccharomyces* yeasts or *S. cerevisiae* added on day 3 required approximately three more days. As such, residual concentrations of glucose and fructose did not statistically vary between wines (0.109 to 0.130 g/L), similar to the amounts of glycerol (10.0 to 10.1 g/L) and succinic acid (1.73 to 1.91 g/L). While wines inoculated with non-*Saccharomyces* yeasts contained slightly higher titratable acidities and decreased pH, volatile acidities were similar (P01A016 and P40D002) or less (NS-MP) than wines inoculated with *S. cerevisiae* alone (0.37 g/L). In fact, wines without inoculated non-*Saccharomyces* yeasts but inoculated with *S. cerevisiae* on day 3 contained the highest concentrations volatile acidity (0.45 g/L). However, wines inoculated with P01A016 or NS-MP contained 1.0 to 1.1% v/v less ethanol than ferments inoculated with *S. cerevisiae* alone (14.9% v/v). The addition of P40D006 to grape musts did not affect final alcohol concentration (15.0% v/v). Both strains of *Mt. pulcherrima* were also able to metabolize some of the malic acid present.

Compared to wines inoculated with *S. cerevisiae* alone, Merlot wines inoculated with non-*Saccharomyces* yeasts exhibited different concentrations of volatile aromas (Table 13). Higher alcohols (*i.e.*, fusel oils) were more concentrated in wines produced with non-*Saccharomyces* yeasts, driven by increased amounts of 2-methyl-1-propanol (61.4 to 70.4 mg/L) and 2- and 3-methyl-1-butanol (137 to 165 mg/L). The highest concentration of fusel alcohols was produced by NS-MP, with slightly lower amounts produced by P01A016 and P40D002. Elevated concentrations of 2-methyl-1-propanol (64.5 mg/L) were also noted in uninoculated wine. With regards to esters, concentrations of specific compounds differed between yeast

treatments while total amounts (excluding ethyl acetate) were similar. Ester concentrations did not vary significantly between P01A016 and *S. cerevisiae* wines. Ferments with P40D002 exhibited higher concentrations of 2- and 3-methyl-butyl acetate (1.02 mg/L) and 2-phenylethyl acetate (0.073 mg/L) compared to wines inoculated with *S. cerevisiae* alone. Similar increases to 2-phenylethyl acetate content were observed in NS-MP (0.054 mg/L) and uninoculated (0.058 mg/L) treatments. Ethyl octanoate was most concentrated in *S. cerevisiae* ferments (0.541 mg/L). High levels of ethyl acetate were observed in P40D002 (148 mg/L) and uninoculated (145 mg/L) wines in contrast other treatments (52.3 to 73.1 mg/L). Volatile acids, namely hexanoic acid, were lower in uninoculated and P01A016 ferments compared to *S. cerevisiae* wines. Octanoic acid concentrations were similar in all wines.

While analysis of variance results for sensory attributes revealed significant differences between the wines (Table 14), no differences were attributed to yeast inoculation. A significant panelist effect ($p<0.05$) was observed for all attributes except 'green', 'earthy', 'sweaty', 'vegetal' aromas and 'earthy' and 'vegetal' flavors. In addition, a replicate effect was noted for 'ethanol' flavor and 'sulfur' aroma and flavor. It is possible that, even with training and use of reference samples, panelists used different portions of the scale. The variance introduced by panelists may have obscured possible differences between yeast treatments.

Principal component analysis of covariance was used to visualize possible trends within mean evaluations of sensory attributes for Merlot wines inoculated with non-*Saccharomyces* and *S. cerevisiae* wine yeasts (Fig. 8). The first principal component (F1) accounted for 47.92% of the co-variance, while the second (F2) explained a further 23.55%. Wines inoculated with *S. cerevisiae* alone on day 0 were more characterized as 'bitter', 'viscous', and 'sour' with a higher ethanol burn ('hot'), as well possess more 'earthy', 'yeasty', and 'pungent' aromas. Inoculation

of P40D002 produced wines with more ‘berry’ aroma and flavor and ‘woody’ aroma as well as increased ‘astringency’. While NS-MP wines were characterized by ‘estery’, ‘fruity’, ‘yeasty’, ‘woody’, and ‘ethanol’ flavors and/or aromas, those fermented with P01A016 exhibited more ‘fruit’, ‘dried fruit’ and ‘ester’ aromas and flavors. Uninoculated wines produced wines with similar sensory properties as those with P01A016, but with increased ‘chemical’ and ‘sulfur’ flavors and a ‘rougher’ mouthfeel.

Discussion

Non-*Saccharomyces* yeasts grew well in the non-sterile Merlot grape must without hampering subsequent fermentation by *S. cerevisiae*. While non-*Saccharomyces* yeast culturability $\geq 10^7$ CFU/mL in ferments with P01A016 and NS-MP were similar to fermentations of other unsterilized grape musts inoculated with *Mt. pulcherrima* (Aplin et al., 2019a; 2019b; Contreras et al., 2014, 2015), this is the first report describing growth of *My. guilliermondii* under such conditions. Researchers have suggested that early growth of non-*Saccharomyces* yeasts may reduce vitamin and/or nutrient content in grape must (Bataillon et al., 1996; Medina et al., 2012), which can inhibit subsequent growth of *S. cerevisiae* and yield sluggish, or even stuck, fermentations (Andorrà et al., 2010; Contreras et al., 2014; Englezos et al., 2016; Varela et al., 2016). Despite high populations of non-*Saccharomyces* yeasts in ferments with P01A016, NS-MP, or P40D002, growth of the sequentially inoculated *S. cerevisiae* strain was not inhibited and wines reached dryness within three days of musts inoculated with *S. cerevisiae* alone. In fact, an antagonistic effect between *S. cerevisiae* and non-*Saccharomyces* yeasts present in the grape musts was observed, where culturability on lysine agar quickly decreased after *S. cerevisae* inoculation. Similar antagonistic effects have been reported by others (Comitini et al., 2011;

Contreras et al., 2014; 2015a; Jolly et al., 2003b, Rodríguez et al., 2010), thought to be due to the competition between yeasts for nutrients or the production of ethanol and other toxic metabolites.

Sequentially inoculating *S. cerevisiae* 72 hours after *Mt. pulcherrima* strains P01A016 or NS-MP generated Merlot wines containing 1.1 and 1.0% v/v less ethanol, respectively, than wines inoculated solely with *S. cerevisiae*. Other studies have reported that ethanol amounts were reduced 0.9 to 1.6% v/v in musts with *Mt. pulcherrima* utilizing inoculation protocols which may have negative consequences concerning fermentation management (Aplin et al., 2019b; Canonico et al., 2016; Contreras et al., 2014; Varela et al., 2016, 2017). For example, Contreras et al. (2014) timed the inoculation of *S. cerevisiae* to after *Mt. pulcherrima* had consumed 50% of the sugar in Chardonnay and Shiraz grape must prior, which delayed *S. cerevisiae* addition until day 17 and day 9, respectively. A long delay before *S. cerevisiae* inoculation may be difficult for wineries to manage because extensive growth of *Mt. pulcherrima* can deplete grape must of nutrients required by *S. cerevisiae* (Aplin et al., 2019b; Gobert et al., 2017; Medina et al., 2012). Canonico et al. (2016) immobilized *Mt. pulcherrima* in alginate beads, a process which the authors admit may substantially increase fermentation management costs. In Merlot wines produced by Varela et al. (2017), *Mt. pulcherrima* reduced ethanol content by 0.9% v/v after sterilization of grape must by dimethyl dicarbonate, which can reduce contributions of native yeasts towards wine complexity (Fleet, 2003; Medina et al., 2013).

It is unclear what metabolite *Mt. pulcherrima* produced in lieu of ethanol during Merlot fermentation. While ethanol reduction was correlated to increased amounts of glycerol and succinic acid in fermentations with *S. uvarum* conducted by Varela et al. (2016; 2017), Merlot wines produced with P01A016 and NS-MP contained similar concentrations of glycerol and succinic acid as those produced with *S. cerevisiae* alone. Metabolically, *Mt. pulcherrima* is

classified as Crabtree-negative, whereas respiration is not inhibited by glucose concentration. Conversion of sugar carbon directly to CO_2 via respiration could explain the reduction in alcohol content. Further research is needed to determine which carbon sinks are utilized by *Mt. pulcherrima*.

The presence of *My. guilliermondii* P40D002 did not affect ethanol content, in contrast to previous observations in sterile-filtered Merlot grape must (Aplin et al., 2019a). When singly inoculated into synthetic media in earlier work (Aplin et al., 2019a), *My. guilliermondii* exhibited significantly lower ethanol production with slower sugar utilization than both *Mt. pulcherrima* and *S. cerevisiae*. While glucose and fructose concentrations were not measured during fermentation in the present work, it is possible that P40D002 was unable to consume sufficient sugar to impact ethanol content. Furthermore, Contreras et al. (2015a) demonstrated in ferments with *Mt. pulcherrima* that native yeast microflora can inhibit inoculated non-*Saccharomyces* species.

Descriptive sensory analysis revealed that wines produced with non-*Saccharomyces* and *S. cerevisiae* yeasts exhibited significant differences in aroma, mouthfeel, taste and flavor characteristics. However, panelist responses for sensory attributes were highly variable, as evidenced by the significant ($p<0.05$) panelist effect for most measured traits. As such differences in wine sensory attributes could not be attributed directly to yeast inoculation by the statistical methods used. Panelists are known to be a significant source of variation in descriptive analysis and they tend to have differences in use of scale and definition of attributes (Bende and Nordin, 1997; Landon et al., 2008; McMahon et al., 2017). It is possible that high variance among panelist responses is obscuring yeast-related differences, which could be decreased with

further training (Bende and Nordin, 1997). To visualize possible trends which may be hidden by panelist response variance, PCA was performed on the sensory data.

Wines inoculated with non-*Saccharomyces* yeasts were generally characterized by higher scores for positive sensory attributes compared to wines fermented with *S. cerevisiae* alone. Wines fermented with either P01A016 or NS-MP had relatively higher scores for 'fruity' and 'estery' aroma, while wines produced with P01A016 also exhibited high scores for 'berry' aroma, 'dried fruit' aroma and flavor, and 'estery' flavor, compared to *S. cerevisiae* wines with similar concentrations of esters responsible for 'fruity/estery' aromas (Jackson, 2008). Increases in ethanol concentration are known to decrease perception of 'fruity' sensory attributes in wine (Goldner et al., 2009; Villamor et al., 2013a; 2013b). As ferments inoculated with *S. cerevisiae* alone contained 1.0 to 1.1% v/v more ethanol than those with *Mt. pulcherrima*, it is possible that the increased alcohol content masked some of the expected fruity attributes in *S. cerevisiae* wines. In addition, the increased levels of fusel alcohols in *Mt. pulcherrima* wines, which are characterized by 'fruity', 'floral', and 'pungent' aromas (Konig et al., 2009) may have contributed to higher 'fruity' ratings. In fact, fusel alcohols, which were most concentrated in NS-MP samples, may explain why these wines were characterized by high scores for 'ethanol' aroma and flavor despite having reduced levels of alcohol. In other studies, strains of *Mt. pulcherrima* were shown to increase fruity and floral aromas (Benito et al., 2015; Rodríguez et al., 2010; Varela et al., 2017) as well as overall quality scores in Chenin blanc and Sauvignon blanc wines (Jolly et al. 2003a, 2003b).

Researchers have shown that higher levels of ethanol can increase the perception of mouthfeel and taste properties, such as 'hotness', 'viscosity', 'bitterness', and 'astringency' (Baker et al., 2016; Gawel et al., 2007; Heymann et al., 2013). Wines inoculated with *S.*

cerevisiae alone had higher ratings for ‘hotness’, ‘viscosity’, and ‘bitterness’ compared to reduced alcohol wines produced with *Mt. pulcherrima*. Elevated ratings for ‘hotness’, ‘viscosity’, ‘bitterness’ and ‘astringency’ (P40D002 only) were also observed in uninoculated wines and those produced with P40D002, both of which contained similar ethanol concentrations as *S. cerevisiae* wines (14.9 to 15.0% v/v ethanol). Scores for ‘roughness’ were lowest in wines inoculated with *S. cerevisiae* alone, possibly due to the lower concentrations of fusel alcohols present in these wines (Varela et al., 2017).

Previous studies have not described the impact of *My. guilliermondii* on wine sensory properties. Despite high concentrations of ethyl acetate (148 mg/L), wines produced with P40D002 elicited lower scores for ‘chemical’ and ‘pungency’ aroma than those produced with *S. cerevisiae* alone (52.3 mg/L ethyl acetate). Ethyl acetate is associated with the negative aroma of ‘nail-polish remover’ when present at concentrations ≥150 mg/L (Jackson, 2008). In addition, P40D002 wines were characterized by ‘woody’ aroma and ‘berry’ aroma and flavor, possibly due to increased concentrations of 2- and 3-methyl-butyl acetate, 2-phenylethyl acetate, and fusel alcohols associated with fruity and floral descriptors (Jackson, 2008). Though non sensory evaluations were performed, Benito et al. (2011) noted elevated amounts of vinylphenolic pyranoanthocyanins, highly stable wine pigments which increase color stability, as well as increased ethyl acetate and higher alcohols in wines inoculated with *Pichia guilliermondii* (synonym *My. guilliermondii*).

Conclusions

This work described Merlot wines produced with *Mt. pulcherrima* and *My. guilliermondii* yeasts at pilot-scale (300L) under winery conditions. Inoculation of *Mt. pulcherrima* P01A016 and NS-MP generated wines characterized by ‘estery’ and ‘fruity’ descriptors containing up to

1.1% v/v less ethanol than wines inoculated with *S. cerevisiae* alone. While *My. guilliermondii* P40D002 did not impact ethanol content, wines made with this yeast exhibited sensory descriptors that suggest possible applications for wine aroma modulation. Future research concerning processing conditions (*i.e.*, temperature and oxygen availability) is needed to optimize ethanol reduction in fermentations with non-*Saccharomyces* yeasts.

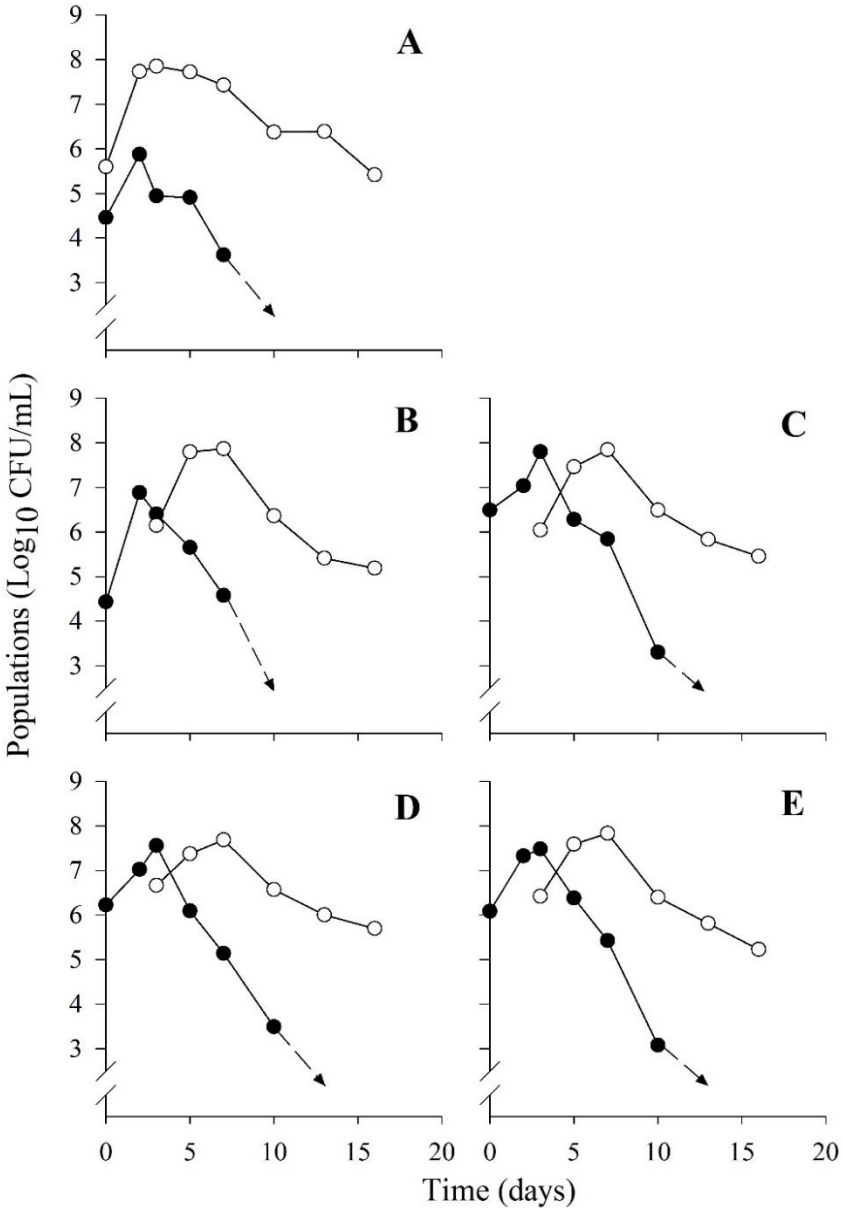

Figure 7. Culturable populations of *Saccharomyces cerevisiae* (○) and non-*Saccharomyces* yeasts (●) in Merlot grape must inoculated with *S. cerevisiae* alone on day 0 (A) or on day 3 into musts left initially uninoculated (B), inoculated with *Mt. pulcherrima* P01A016 (C), *Mt. pulcherrima* NS-MP (D), or *My. guilliermondii* P40D002 (E).

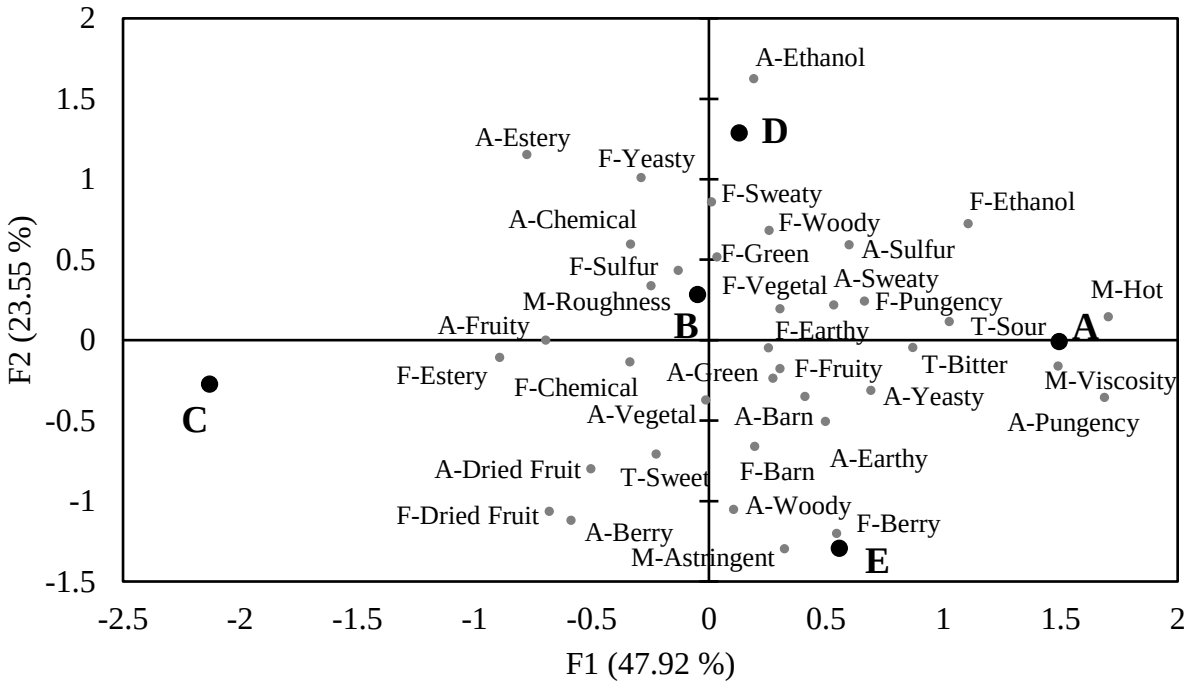

Figure 8. Principal component analysis of trained panel (n=10) data for Merlot wines inoculated with *S. cerevisiae* alone on day 0 (A), on day 3 alone (B), or on day 3 in musts inoculated with *Mt. pulcherrima* P01A016 (C), *Mt. pulcherrima* NS-MP (D), or *My. guilliermondii* P40D002 (E) on day 0. 'A-' denotes aroma attributes, 'F-' denotes in-mouth flavor attributes, 'M-' denotes mouthfeel, and 'T-' denotes taste attributes.

Table 10. Attributes, description, and reference standard evaluated by panelists for Merlot wine trained panel.

Attribute	Description	Reference*
Aroma and Flavors		
Estery	Aromatics associated with esters, often appear as fruity. Some of the potential perceptions may include banana, like banana nerds, and exotic fruits	2 ml of ester solution into 50 mL of base wine. Ester solution =0.5 g of isoamyl acetate, 0.2 g of ethyl hexanoate, and 0.2 g of ethyl octanoate in 100 mL of redistilled ethanol.
Pungency	Perception of irritation, prickling, or burn in the nasal cavity	0.25 g horseradish in 25 mL base wine
Fruity	Aromatics associated with an overall rating of fruit including tree and stone fruit	1 Cm^2 each of fresh apple and canned peach soaked in wine with 5 g of unsweetened applesauce per 40 mL of base wine
Dried Fruit	Aroma associated with dried fruits such as raisins or prunes.	10 crushed California seedless raisins per 40 mL of base wine
Green	Aromatic associated with grass or unripe fruit	30 mm piece of wheat grass ground in 25 mL of base wine
Yeasty	Aromatic associated with fresh yeast	280 g/L yeast proofed in 25 mL base wine with 7 g sucrose diluted 1:12.5 in base wine
Ethanol	The aroma associated with ethanol, can cause some irritation in the nose	60 mL of 100 proof ethanol in 240 mL of base wine
Berry	The aroma associated with fresh berries, such as strawberry, raspberry and blackberry	5 mL fresh strawberry/raspberry/blackberry juice per 40 ml base wine
Barn	The aroma associated with a barn, the smell of animal or leather	4EP: 4EG in a proportion of 10:1, 1 mg/L in base wine
Woody	Aromatic associated with oak or wood, can include some sweet notes such as vanilla	Three oak chips (approximately 2 g) in 10 ml of deionized water + 5 ml of base wine. Submerged 24 hr at room temp, 15 mL of base wine added prior to training
Sulfur	The Aromatic associated with sulfur. Can create notes of rotten egg, rubber tire or burnt matches	1 ml of 95% ethanol and 0.5 mL of SO_2 solution in 15 mL base wine

*Carlo Rossi Burgundy was used as base wine for all standards.

Table 10 (continued).

Attribute	Description	Reference*
Sweaty	A pungent, sour aromatic associated with sweaty, perspiration generated foot odor and certain aged cheeses such as Romano	0.2 mL Hexanoic acid stock (10g/L) in 100 ml of base wine
Chemical	Aromatics associated with solvents. Often similar to how nail polish or vinegar smells	4 ml of vinegar and 30 µL of nail polish remover into 100 ml of base wine
Vegetal	The aromatics associated with vegetables and undergrowth.	2.5 g chopped asparagus + 2.5 g chopped green bean per 40 mL of base wine
Earthy	The musky aromatic associated with mushroom or fresh potting soil	2.5 g of fresh mushrooms into 40 ml of base wine
Taste		
Sweet	Taste stimulated by sucrose and other sugars, such as fructose or glucose	26.67 g sucrose/ L base wine*
Bitter	Taste simulated by bitter substances, such as quinine, caffeine or hops	1.5 mg quinine sulfate/L base wine
Sour	Taste simulated by acids such as citric or malic	2.5 g tartaric acid/ L base wine
Mouthfeel		
Viscosity	The degree to which wine resists flow when moved from side to side in the mouth	25.8g/L glycerol base wine
Astringency	The drying sensation in the mouth after consuming a wine with tannins	0.78 g tannic acid + 0.35g alum in 300 ml base wine
Burning	The warmth elicited by alcohol, also can be considered a burning sensation	60 mL of 100 proof ethanol in 240 mL of base wine
Roughness	A roughening sensation felt on mouth surfaces when the different surfaces come in contact with each other	Addition of 5 g of coffee grounds into 500 mL of base wine

*Carlo Rossi Burgundy was used as base wine for all standards.

Table 11. Mean scores for sensory attributes of Merlot wines inoculated with *S. cerevisiae* and non-*Saccharomyces* yeasts.

	Inoculated Yeasts				
Attribute	*S. cerevisiae*	*S. cerevisiae**	P01A016*	NS-MP*	PD04002*
Aroma					
Estery	3.05[a]	2.91[a]	3.54[a]	3.35[a]	2.46[a]
Pungency	3.01[a]	1.81[b]	1.49[b]	2.17[ab]	2.46[a]
Fruity	2.64[a]	3.31[a]	3.36[a]	3.19[a]	3.19[a]
Dried Fruit	2.01[ab]	2.935[a]	2.48[ab]	1.785[b]	2.410[ab]
Green	1.37[a]	1.43[a]	1.18[a]	1.40[a]	1.57[a]
Yeasty	2.34[a]	1.71[a]	1.63[a]	1.65[a]	1.87[a]
Ethanol	4.03[ab]	4.84[a]	3.87[b]	4.76[ab]	3.91[ab]
Berry	2.51[b]	2.94[ab]	3.23[ab]	2.77[ab]	3.44[a]
Earthy	1.34[a]	1.21[a]	0.985[a]	1.17[a]	1.54[a]
Woody	1.80[a]	1.69[a]	1.78[a]	1.45[a]	2.12[a]
Sulfur	2.29[a]	2.01[a]	1.71[a]	2.24[a]	1.91[a]
Sweaty	1.57[a]	1.20[a]	1.17[a]	1.75[a]	1.61[a]
Chemical	2.78[a]	2.95[a]	2.88[a]	2.58[a]	2.21[a]
Vegetal	0.710[b]	1.00[ab]	0.945[ab]	1.22[ab]	1.48[a]
Barn	1.43[a]	1.15[a]	1.09[a]	1.17[a]	1.41[a]
Taste					
Sweet	4.28[a]	4.04[a]	4.49[a]	4.06[a]	4.42[a]
Bitter	4.19[a]	3.97[a]	3.49[a]	4.06[a]	4.19[a]
Sour	5.18[ab]	5.45[a]	4.37[b]	5.12[ab]	5.27[ab]
Mouthfeel					
Viscosity	6.57[a]	6.00[ab]	5.20[b]	5.78[ab]	6.05[ab]
Hot	6.71[a]	6.34[ab]	5.25[b]	6.29[ab]	6.42[ab]
Astringent	5.06[a]	4.74[a]	4.89[a]	4.65[a]	5.46[a]
Roughness	3.30[b]	3.89[ab]	3.71[ab]	4.16[a]	3.98[ab]

**S. cerevisiae* inoculated on day 3.
[a-b] Mean values within rows with different superscripts are significantly different ($p \leq 0.05$).

Table 11 (continued)**.**

	Inoculated Yeasts				
Attribute	*S. cerevisiae*	*S. cerevisiae**	P01A016*	NS-MP*	PD04002*
Flavor					
Estery	2.37[a]	2.53[a]	3.08[a]	2.41[a]	2.37[a]
Pungency	2.26[a]	2.37[a]	1.72[a]	2.26[a]	2.24[a]
Fruity	3.56[a]	3.36[a]	3.21[a]	2.99[a]	3.17[a]
Dried Fruit	1.92[ab]	2.35[ab]	2.57[a]	1.75[b]	2.34[ab]
Green	1.41[a]	1.21[a]	1.31[a]	1.48[a]	1.12[a]
Yeasty	1.55[ab]	1.74[ab]	1.74[ab]	1.96[a]	1.31[b]
Ethanol	4.97[a]	4.71[ab]	3.99[b]	4.91[a]	4.59[ab]
Berry	3.00[ab]	3.15[ab]	2.58[ab]	2.33[b]	3.23[a]
Earthy	1.16[a]	1.58[a]	1.04[a]	1.33[a]	1.48[a]
Woody	1.58[a]	1.44[a]	1.33[a]	1.76[a]	1.34[a]
Sulfur	1.96[a]	2.15[a]	2.05[a]	2.14[a]	1.88[a]
Sweaty	1.43[a]	1.77[a]	1.38[a]	1.73[a]	1.25[a]
Chemical	2.26[a]	2.59[a]	2.64[a]	2.56[a]	2.64[a]
Vegetal	1.20[a]	1.23[a]	0.870[a]	0.975[a]	0.925[a]
Barn	1.45[a]	1.03[a]	1.25[a]	0.990[a]	1.37[a]

**S. cerevisiae* inoculated on day 3.
[a-b] Mean values within rows with different superscripts are significantly different ($p \leq 0.05$).

Table 12. Chemical composition of Merlot wines inoculated with *S. cerevisiae* and non-*Saccharomyces* yeasts.

	Inoculated Yeasts				
Constituent	*S. cerevisiae*	*S. cerevisiae**	P01A016*	NS-MP*	P40D002*
Ethanol (% v/v)	14.9[b]	14.9[b]	13.8[a]	13.9[a]	15.0[b]
Glucose + fructose (g/L)	0.109[a]	0.114[a]	0.119[a]	0.112[a]	0.130[a]
pH	3.31[cd]	3.29[c]	3.23[b]	3.21[a]	3.29[cd]
Titratable acidity (g/L)	5.56[a]	5.61[a]	6.09[b]	6.09[b]	6.81[c]
Volatile acidity (g/L)	0.37[bc]	0.45[d]	0.35[ab]	0.33[a]	0.40[c]
Malic acid (g/L)	2.22[b]	2.18[b]	1.38[a]	1.36[a]	2.29[b]
Succinic acid (g/L)	1.75[a]	1.73[a]	1.91[a]	1.74[a]	1.82[a]
Glycerol (g/L)	10.0[a]	10.1[a]	10.1[a]	10.1[a]	10.1[a]

**S. cerevisiae* inoculated on day 3.

[a-d] Mean values within rows with different superscripts are significantly different ($p \leq 0.05$).

Table 13. Concentration of volatile compounds in Merlot wines inoculated with *S. cerevisiae* and non-*Saccharomyces* yeasts.

	Inoculated Yeasts				
Constituent	*S. cerevisiae*	*S. cerevisiae**	P01A016*	NS-MP*	P40D002*
Higher Alcohols (mg/L)					
1-Propanol	2.52^{a}	3.98^{a}	5.33^{a}	3.36^{a}	4.60^{a}
2-Methyl-1-propanol	29.0^{a}	64.5^{b}	61.4^{b}	64.5^{b}	70.4^{b}
2&3-Methyl-1-butanol	108^{a}	112^{a}	137^{b}	165^{b}	144^{b}
1-Hexanol	1.18^{a}	1.20^{a}	0.975^{a}	0.949^{a}	1.18^{a}
1-Octanol	1.84^{a}	0.662^{a}	1.52^{a}	0.735^{a}	1.23^{a}
2-Phenylethanol	36.5^{a}	37.3^{a}	37.9^{a}	37.8^{a}	45.3^{a}
Esters (mg/L)					
Ethyl acetate	52.3^{a}	145^{b}	73.1^{a}	64.1^{a}	148^{b}
2&3-Methylbutyl acetate	0.712^{a}	0.912^{ab}	0.808^{ab}	0.858^{ab}	1.02^{b}
Hexyl acetate	0.007^{a}	0.007^{a}	0.012^{a}	0.009^{a}	0.009^{a}
Diethyl succinate	1.24^{b}	0.787^{a}	0.992^{ab}	1.00^{ab}	1.05^{ab}
2-Phenylethyl acetate	0.016^{a}	0.058^{bc}	0.043^{ab}	0.054^{bc}	0.073^{c}
Ethyl butanoate	0.223^{a}	0.166^{a}	0.193^{a}	0.190^{a}	0.187^{a}
Ethyl hexanoate	0.089^{a}	0.024^{a}	0.073^{a}	0.038^{a}	0.037^{a}
Ethyl octanoate	0.541^{b}	0.353^{a}	0.388^{ab}	0.348^{a}	0.355^{a}
Acids (mg/L)					
Hexanoic acid	4.05^{c}	2.39^{a}	2.79^{ab}	3.90^{bc}	3.09^{abc}
Octanoic acid	4.15^{a}	3.13^{a}	3.79^{a}	3.69^{a}	3.63^{a}

**S. cerevisiae* inoculated on day 3.

$^{a\text{-}c}$ Mean values within rows with different superscripts are significantly different ($p \leq 0.05$).

Table 14. Significance and F ratios from analysis of variance of trained panel evaluations of aroma, taste, mouthfeel, and flavor sensory attributes in Merlot wines inoculated with *S. cerevisiae* and non-*Saccharomyces* yeasts.

Source of Variation	Pr > F (*p*)	Panelist (*F*)	Yeast (*F*)	Replicate (*F*)	Panelist*Yeast (*F*)
df		9	4	1	36
Aroma					
Estery	0.108	5.21***	0.895	0.709	0.567
Pungency	0.350	3.13**	1.96	0.716	0.532
Fruity	0.0004†	7.81***	0.695	0.038	1.70
Dried Fruit	0.056	5.69***	1.478	0.393	0.597
Green	0.709	1.58	0.306	0.005	0.759
Yeasty	0.095	4.56**	0.861	0.685	0.768
Ethanol	<0.0001†	17.5***	2.10	2.92	0.707
Berry	0.018†	4.99***	1.39	0.043	1.15
Earthy	0.778	0.852	0.847	1.33	0.772
Woody	0.096	4.59**	0.952	3.88	0.659
Sulfur	0.496	3.97**	0.374	4.94*	0.221
Sweaty	0.054	1.91	0.957	0.590	1.61
Chemical	<0.0001†	12.2***	0.911	0.756	1.12
Vegetal	0.190	1.66	2.01	0.017	1.15
Barn	0.044†	4.72**	0.949	1.06	0.952
Taste					
Sweet	0.014†	7.03***	0.356	0.041	0.815
Bitter	0.0002†	12.5***	0.934	0.478	0.678
Sour	0.001†	9.02***	1.37	0.527	1.057

† Differences are significant ($p<0.05$).
* $p<0.05$
** $p<0.01$.
*** $p<0.0001$.

Table 14 (continued).

Source of Variation	Pr > F (*p*)	Panelist (*F*)	Yeast (*F*)	Replicate (*F*)	Panelist*Yeast (*F*)
df		9	4	1	36
Mouthfeel					
Viscosity	0.041†	4.23**	1.46	0.177	1.06
Hot	0.008†	7.06***	1.62	0.824	0.803
Astringent	<0.0001†	16.6***	1.01	0.857	1.05
Roughness	<0.0001†	18.5***	1.55	0.482	1.25
Flavor					
Estery	0.094	5.25***	0.740	2.99	0.550
Pungency	0.001†	9.88***	0.857	0.157	0.958
Fruity	0.222	3.72**	0.235	0.872	0.749
Dried Fruit	0.001†	8.09***	1.72	2.06	1.12
Green	0.007†	7.26***	0.635	0.022	0.949
Yeasty	0.0003†	8.26***	1.29	2.35	1.57
Ethanol	<0.0001†	16.5***	1.71	6.72*	1.67*
Berry	0.013†	4.29**	1.64	3.40	1.29
Earthy	0.017†	1.96	1.33	0.21	1.90*
Woody	0.287	3.01**	0.740	0.961	0.771
Sulfur	0.088	4.12**	0.114	8.66**	0.764
Sweaty	0.020†	2.41*	1.43	2.41	1.69*
Chemical	<0.0001†	22.3***	0.676	0.097	1.31
Vegetal	0.802	0.753	0.645	0.122	0.827
Barn	0.118	4.32**	1.20	1.27	0.703

† Differences are significant ($p<0.05$).
* $p<0.05$
** $p<0.01$.
*** $p<0.0001$.

CHAPTER V

CONCLUSIONS

The studies in this dissertation examined enological properties of non-*Saccharomyces* yeasts isolated from Washington state vineyards during fermentations in high sugar grape musts and media. Growth of some of the non-*Saccharomyces* yeasts investigated in this dissertation had not previously been described under winemaking conditions. An initial evaluation of four species (*C. californica, Mt. pulcherrima, My. caribbica,* and *W. anomalus*) determined that sequential inoculation of non-*Saccharomyces* yeasts into high sugar Syrah must (301 g/L glucose and fructose) followed by *S. cerevisiae* after six days increased concentrations of compounds beneficial to wine quality without drastically increasing acetic acid content or fermentation time. In fact, inoculation of *C. californica* or *Mt. pulcherrima* resulted in wines containing 0.8 and 0.9% v/v less ethanol, respectively, than wines inoculated with *S. cerevisiae* alone.

In a broader screening, 16 different non-*Saccharomyces* yeasts were investigated for their abilities to metabolize sugar without ethanol formation. Of these, five species (*Mt. chrysoperlae, Mt. pulcherrima, My. guilliermondii*, *P. kluyveri*, and *P. membranifaciens*) reduced ethanol contents when inoculated into high sugar Merlot must (310 g/L glucose and fructose) six days prior to *S. cerevisiae* without production of excessive levels of acetic acid. When inoculated into sterile-filtered Merlot musts with lower amounts of sugar (266 g/L) three days before *S. cerevisiae*, dry wines (≤2 g/L glucose and fructose) with significantly less ethanol were obtained using *Mt. pulcherrima* and *My. guilliermondii* yeasts. Single inoculum cultures in SGJM confirmed that *Mt. pulcherrima* and *My. guilliermondii*, both Crabtree-negative yeasts, exhibited slower sugar consumption and yielded lower amounts of ethanol and glycerol than *S. cerevisiae*. When inoculated into SGJM three days before *S. cerevisiae* with and without a consortium of

native yeasts, *Mt. pulcherrima* produced wines with 2.4 and 3.0% v/v less ethanol, respectively, than wines fermented solely with *S. cerevisiae*. As a proof-of-concept, pilot scale fermentations of unsterilized Merlot must inoculated with *Mt. pulcherrima* generated wines containing 0.9% v/v less ethanol than wines produced with *S. cerevisiae* alone without increasing acetic acid content.

Finally, the sensory aspects of Merlot wines produced under winery conditions using *Mt. pulcherrima* and *My. guilliermondii* yeasts were examined. Inoculation of *Mt. pulcherrima* P01A016 generated wines with 1.1% v/v less ethanol than those inoculated with *S. cerevisiae* alone. These wines were characterized by generally positive sensory attributes, like 'estery' flavor and fruity characteristics. While *My. guilliermondii* did not affect ethanol content, wines fermented with this yeast exhibited 'berry', 'earthy', and 'woody' aromas and/or flavors which suggest possible applications for wine aroma modulation. Overall, this research demonstrates that reduced alcohol wines with good sensory properties can be produced using non-*Saccharomyces* yeasts isolated from Washington vineyards.

CHAPTER VI

FUTURE RESEARCH

While this dissertation identified several non-*Saccharomyces* yeasts that may be utilized for reducing wine alcohol content, particularly *Mt. pulcherrima* P01A016, several questions remain unanswered concerning their application to winemaking practices. For instance, most of the non-*Saccharomyces* yeasts which reduced wine alcohol content do not exhibit the Crabtree effect (*i.e., Mt. pulcherrima* and *My. guilliermondii*), meaning respiration is the preferred metabolic pathway for sugar carbon. However, the methods used in this dissertation could not determine what metabolites were produced in lieu of ethanol. While it is suspected that the carbon in sugar was diverted towards CO_2, the methods used in this dissertation did not quantify CO_2 production. Further experimentation is needed to evaluate both CO_2 and ethanol production in fermentations with Crabtree-negative yeasts. Furthermore, genetic studies on non-*Saccharomyces* yeasts should be performed to identify any as of yet unknown metabolic processes utilized by these yeasts, as well as examining underlying interactions with other yeasts, like *S. cerevisiae*.

Besides metabolite characterization, fermentation processes utilizing non-*Saccharomyces* yeasts evaluated in this dissertation need to be optimized, particularly with respects to fermentation temperature. Researchers have shown that various species of non-*Saccharomyces* yeasts survive longer at lower temperatures (Ciani et al., 2006; Erten, 2002; Heard and Fleet, 1988). Manipulating fermentation temperature may be a useful technique for encouraging the growth of yeasts which can reduce ethanol content. However, timing of sequential *S. cerevisiae* inoculation would have to be further managed around sugar consumption by other yeasts,

nutrient utilization, and production of compounds associated with negative sensory properties, such as ethyl acetate and acetic acid.

In addition to fermentation temperature, the use of aeration during fermentation needs further examination. As Crabtree-negative yeasts most likely rely on respiration to divert sugar carbon away from ethanol, additional oxygen should increase the proportion of sugar which is respired instead of fermented because oxygen is the final electron acceptor for oxidative metabolism (Fugelsang and Edwards, 2007). In fact, some non-*Saccharomyces* yeast species require discrete amounts of oxygen to grow (Hansen et al., 2001). Specific oxygen requirements for maintaining respiration with selected yeast strains in grape must need to be determined, while also managing production of spoilage compounds. Moreover, potential interactive effects between native microflora and *S. cerevisiae* would need to be ascertained to manage nutrient content and prevent sluggish/stuck fermentations in aerated must. Furthermore, methods to supply grape must with sufficient oxygenation at production scale would need to be further explored. Specifically, devices which let winemakers better measure and control oxygenation levels in fermenting musts are needed to manage yeast metabolite production and prevent oxidation of grape must components.

CHAPTER VII

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