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The growth of *Brettanomyces bruxellensis* is a major cause of wine spoilage due to the production of the volatile phenols 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG), derivatives of *p*-coumaric acid and ferulic acid. During the winemaking process, some microorganisms can impact the concentration of the 4-EP precursors *p*-coumaric and coutaric acid (tartaric acid-esterified *p*-coumaric acid). This study investigated the effect of certain wine microorganisms on these 4-EP precursors as well as potential inhibitory relationships between *B. bruxellensis* and the wine bacteria *Oenococcus oeni. Saccharomyces cerevisiae* strains with and without phenolic acid decarboxylase (PAD) activity as well as *Oenococcus oeni* strains with and without cinnamoyl esterase (CE) activity were used to perform sequential and simultaneous Pinot noir alcoholic fermentations (AF) and malolactic fermentations (MLF). Simultaneous fermentation using PAD (+) *S. cerevisiae* and a CE (+) *O. oeni* resulted in the largest reduction of volatile phenol precursors. However, these same strains when utilized in sequential fermentations resulted in the largest number of precursors in the form most readily usable by *B. bruxellensis*. Several commercial and

non-commercial *O. oeni* were also screened for CE activity. Two of the eleven previously unscreened commercial *O. oeni* as well as one of the four non-commercial *O. oeni* exhibited cinnamoyl esterase activity, resulting in significantly higher concentrations of free hydroxycinnamic acids in these wines. The stability of the 4-EP precursor compounds during aging was investigated by aging Pinot noir wines that had undergone MLF using either a CE (+) or CE (-) *O. oeni.* Wines were adjusted to different pH and ethanol concentrations and stored at either 13 or 21°C. During 180 days of aging, the concentration of *p*-coumaric acid and coutaric acid remained relatively stable and were most impacted by the strain of *O. oeni* that had conducted MLF.

While *O. oeni* can impact *B. bruxellensis* wine spoilage through the liberation of tartaric acid-bound hydroxycinnamic acids, it has also been noted that the presence of *O. oeni* at the completion of MLF may inhibit *B. bruxellensis* growth. Experiments were conducted to determine how long this inhibition lasted as well the sensitivity of multiple *B. bruxellensis* strains. Three different *O. oeni* strains were used to conduct MLF in a Pinot noir wine. Upon completion of MLF, wines were pulled and inoculated with *B. bruxellensis* at 0, 34, and 112 days post-MLF. Growth of *B. bruxellensis* and *O. oeni* were monitored and volatile phenol (4-ethylphenol and 4-ethylguaiacol) concentrations in the wine were also measured. *O. oeni* populations in the wines were high directly after the completion of MLF and *B. bruxellensis* populations declined rapidly following inoculation. 34 days post-MLF, high populations of two *O. oeni* strains were still present in the wine but no culturable cells

were detected in the wine for the third strain. When *B. bruxellensis* was inoculated into the wine containing no culturable *O. oeni* cells, it grew well. In contrast, *B. bruxellensis* populations rapidly declined when inoculated into wine where there were still culturable *O. oeni* cells. 112 days post-MLF, no culturable *O. oeni* cells were detected in any of the wines and *B. bruxellensis* grew well in all but one of the wines. The sensitivity of different *B. bruxellensis* strains to the presence of culturable *O. oeni* post-MLF was tested by inoculating six strains of *B. bruxellensis* into wine that had undergone MLF by *O. oeni* or had not (control). Subsequent growth and volatile phenol production was tracked. The degree of inhibition on growth and volatile phenol production was strain dependent where the growth of some *B. bruxellensis* strains in wine that underwent MLF was comparable to growth in the control wines. For other strains, there was reduced or no growth in wines that had undergone MLF. All six strains of *B. bruxellensis* produced significantly less volatile phenols in wines that had undergone MLF compared to those that had not. These findings suggest that MLF may offer limited protection against *B. bruxellensis* infection due to the presence of live *O. oeni* cells post-MLF but that this may be dependent on the *B. bruxellensis* strain present. Additional research should involve screening a larger number of *B. bruxellensis* strains for their sensitivity to live *O. oeni* cells. Focusing on *B. bruxellensis* strains which have been sequenced and better classified from an -omics point of view would likely provide better insight behind variations in inhibition as well as possible mechanisms of inhibition.

Interactions between *Oenococcus oeni* and *Brettanomyces bruxellensis* during Winemaking and Consequences for Wine Quality

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Aubrey E. DuBois, Author

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Red Winemaking

Red winemaking varies depending on the specific style being produced but there are some basic, common steps. Grapes are harvested at peak ripeness, as determined by parameters such as color, flavor, sugar, and acid content. Grape clusters are then put through a destemmer to remove grape stems. In red wine production, fermentation occurs with the skins and seeds present to aid in the extraction of phenolic compounds important for wine color and mouthfeel (Bautista-Ortín et al., 2004; Canals et al., 2005; Ivanova et al., 2012). The skins and seeds are not removed until or near completion of the alcoholic fermentation. Alcoholic fermentation can be initiated via the inoculation of a commercial yeast starter culture or can occur due to yeasts naturally found in the winery and/or on the grape skins.

Alcoholic fermentation is typically conducted by the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* is commonly found in wineries and it can also be present in low numbers on grape skins close to harvest (Drumonde-Neves et al., 2016; Schuller et al., 2005; Török et al., 1996; Valero et al., 2007). *S. cerevisiae* is generally preferred because of its tolerance to the harsh wine environment, where pH can be as low as 3.0 and ethanol concentrations can exceed 13% (v/v) (Arroyo-López et al., 2010). Furthermore, *S. cerevisiae* is relatively tolerant to sulfur dioxide (SO_2), a chemical commonly used in winemaking as an antimicrobial agent, and can withstand

concentrations over 30 ppm (Henick-Kling and Park, 1994; Martini, 1993). *S. cerevisiae* primarily metabolizes the grape sugars, glucose and fructose, into ethanol and carbon dioxide. This process generates energy (ATP) and helps maintain the internal redox potential of the yeast cells via recycling NADH (Herskowitz, 1988). Additionally, the yeast produces many secondary metabolites such as esters and higher alcohols that can have significant impacts on the aroma, taste, and mouthfeel of the wine (Lilly et al., 2006; Swiegers et al., 2007; Swiegers et al., 2009).

Following alcoholic fermentation, red wines are pressed to remove grape skins and seeds. After pressing, red wines often undergo a secondary fermentation called malolactic fermentation (MLF). It is worth noting that MLF is not a true fermentation as it is not a direct metabolism of carbohydrates and organic acids into alcohols, carbon dioxide, and other by-products. Rather, it is an enzymatic conversion of malic acid to lactic acid carried out by lactic acid bacteria (LAB) as a means to generate ATP via chemiosmosis (Costantini et al., 2009; Fugelsang and Edwards, 2007). The conversion of malic acid to weaker lactic acid causes the pH to rise and thus lends a positive perception of decreased acidity (Costantini et al., 2009; Liu, 2002). MLF may be initiated with the addition of a commercial bacterial culture or via the natural LAB population of the winery. *Oenococcus oeni* is the LAB most often utilized to conduct MLF due to its acid and ethanol tolerance as well as its positive contributions to the wine's sensory attributes. Many *O. oeni* strains can enhance positive aromas such as fruit and buttery notes through its metabolic and

enzymatic activity (Bartowsky and Henschke, 2004; Bloem et al., 2008; Costello et al., 2012; Malherbe et al., 2013; Michlmayr et al., 2012; Ugliano et al., 2003; Ugliano and Moio, 2005). While other LAB present in wine such as *Pediococcus spp.* and *Lactobacillus spp* can conduct MLF, they are generally considered spoilage microorganisms. These bacteria are often more common in uninoculated MLF and are undesirable due to their production of off-aromas such as ethyl acetate (nail polish remover aroma), off-flavors such as bitterness due to acrolein production, or undesirable ropy, viscous textures due to exopolysaccharide production (Costello and Henschke, 2002; Francis and Newton, 2005; König et al., 2009; Swiegers et al., 2005; Walling et al., 2005; Wisselinka et al., 2002).

Apart from mouthfeel and taste enhancement, MLF also serves as a tool for minimizing the risk of microbial spoilage. The metabolism of malic acid and residual sugars by *O. oeni* reduces the amount of carbon sources available for spoilage microorganisms to utilize. *O. oeni* growth will also reduce the concentration of nitrogen compounds, minerals, and vitamins in the wine (Terrade and Mira de Orduña, 2009). However, the rise in pH due to MLF may allow certain spoilage yeasts and bacteria to grow more easily as many wine spoilage microbes prefer pH values > 3.50 (Davis et al., 1985; Du Toit and Pretorius, 2000). Because of this, it is important that MLF is conducted quickly and efficiently as the winemaker cannot make an addition of antimicrobial SO_2 until MLF is completed due to the SO_2 sensitivity of *O. oeni*.

Microbial Wine Spoilage

Unlike the production of many other foods and beverages, the presence of pathogenic microorganisms during wine production is not generally a concern due to wine's acidity and high ethanol content which prevent their proliferation (Jeon et al., 2015; Møretrø and Daeschel, 2006; Sugita-Konishi et al., 2001). However, some molds belonging to the *Aspergillus* and *Penicillium* genera that are found in the vineyard on the surface of damaged grapes can produce Ochratoxin A (OTA) (Mateo 2007). OTA is a mycotoxin that affects kidney and liver function and is also potentially carcinogenic. OTA can be broken down by the fermenting yeast and many common wine fining methods can reduce OTA levels in the final product (Gambuti et al., 2005, Castellari et al., 2001). Fortunately, many studies have also demonstrated that the small OTA levels found in U.S.-produced wines are not ordinarily a concern (Mateo et al., 2007).

While the growth of pathogens is not a concern in wine, several non-pathogenic, non-toxin-forming microorganisms are able to grow in the wine environment. Often, these microbes can produce metabolites that are associated with off-aromas, flavors, and textures. Microbial spoilage can occur at any point in the winemaking process, but there are specific points in which the risk of spoilage is heightened. Prior to the initiation of alcoholic fermentation, the freshly harvested grapes host a number of bacteria and yeast species (Renouf et al., 2006). If alcoholic fermentation is not

initiated soon after harvest or if it is not completed in a timely manner, some of these microbes may proliferate and produce metabolites that cause undesirable organoleptic properties (Fugelsang and Edwards, 2007; Du Toit and Pretorius, 2000; Renouf et al., 2006). The microorganisms of most concern at this stage of the process are acetic acid bacteria (*Acetobacter* and *Gluconobacter*) and the oxidative yeast *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*).

Acetic acid bacteria (AAB) are capable of metabolizing ethanol into acetaldehyde (green apple, grassy aroma) which can then be metabolized further into acetic acid (vinegar aroma). Additionally, the esterification of ethanol and acetic acid can lead to the formation of ethyl acetate (nail polish remover) (Bartowsky and Henschke, 2008; Osborne and Edwards, 2005; Swiegers et al., 2005). AABs are often found in high concentrations on damaged and/or botrytis-infected grapes (Fleet, 2003). If the initiation of alcoholic fermentation is delayed, these bacteria can proliferate and produce excessive amounts of acetic acid and ethyl acetate. They can also be problematic during wine storage as *Acetobacter* is ethanol tolerant and can therefore survive in wine (Bartowsky and Henschke, 2008). AABs are typically controlled using SO_2 that is added either prior to grape processing or later during wine aging. Removal of damaged grapes prior to fermentation can also help reduce populations of AABs present during ferment (Bartowsky and Henschke, 2008; Pretorius et al., 1999). In addition, AABs are obligate aerobes and as such, they require the presence of oxygen to perform many metabolic activities. By regularly topping-off barrels to

reduce the amount of available headspace, the risk of AAB spoilage may be further reduced (Fugelsang and Edwards, 2007).

The yeast *H. uvarum* (anamorph *Kloeckera apiculata*) is typically one of the most dominant yeasts present on grapes at harvest and during initiation of uninoculated fermentations (Mateo et al., 1991; Zott et al., 2010; Albertin et al., 2016). It is a spoilage yeast, capable of producing excessive amounts of acetic acid and ethyl acetate (Romano et al., 1992). *H. uvarum* is found in significant numbers in damaged grapes and is also associated with sour rot, grey rot, noble rot, and honeydew (Barata et al., 2012). The yeast can be isolated and identified via its ability to grow on 0.01% cyclohexamide agar. *H. uvarum* has a relatively high tolerance to both DMDC and SO_2 (Cocolin et al., 2003; Delfini et al., 2002). As such, removal of damaged grape berries prior to fermentation as well as utilizing a fermentation starter culture can help prevent issues associated with *H. uvarum* spoilage (Malfeito-Ferreria, 2011; Loureiro and Malfeito-Ferreira, 2003).

Aside from pre-fermentation, the period between the end of AF and MLF initiation also poses a serious infection risk because there is relatively little SO_2 present in the wine. Furthermore, SO_2 additions cannot be made until after MLF completion because the MLF bacteria are SO_2-sensitive (Osborne and Edwards, 2005). Additionally, a degree of residual sugars, nitrogen, and vitamins may remain in the wine after alcoholic fermentation. Quick, inoculated MLFs are suggested to add

protection during this time (Gerbaux et al., 2009). Once SO_2 can be added to the wine, it is better protected from microbial contamination. However, spoilage can still occur, particularly in barrel-aged wines. During barrel-aging, a degree of evaporation occurs through the barrel. This increases the headspace and therefore the amount of oxygen in the wine. This environment can encourage the growth of aerobic spoilage microorganisms such as film yeast and *Acetobacter*. Additionally, the concentration of SO_2 in the wine can decrease during aging due to evaporation, oxidation, and binding of SO_2 with compounds such as acetaldehyde and anthocyanins in the wine (Jackowetz and Mira de Orduña, 2013; Ferreira et al., 2014). Furthermore, the small cracks and crevices found in barrels, stainless steel, and concrete tanks may allow microbes to avoid mechanical and chemical cleaning/sanitation methods; and so during aging these microbes may proliferate (Oelesfe et al., 2008).

Some of the most common spoilage microorganisms in wine post alcoholic fermentation are wine lactic acid bacteria (LAB) and the yeast *Brettanomyces bruxellensis* (Wedral et al., 2010; Bartowsky, 2009; Loureiro and Malfeito-Ferreira, 2003; Du Toit and Pretorius, 2000; Fleet, 2003). *Pediococcus spp.*, *Lactobacillus spp.*, and *Oenococcus spp*. are the most common lactic acid bacteria found in wine. *Pediococcus spp.* and *Lactobacillus spp.* are typically considered spoilage microbes while *Oenococcus spp.* is generally only considered so if MLF is not desired for the particular wine (Bartowsky and Borneman, 2011; Malherbe et al., 2013; Osborne and

Edwards, 2005). *O. oeni* may also be considered a spoilage bacteria if it produces excessive amounts of 2,3-butandione (diacetyl). This compound is responsible for the buttery, butterscotch aroma commonly noted in wines which have undergone MLF. In concentrations below 4 mg/L, diacetyl is often viewed positively but it is largely unwelcome at high concentrations (Bartowsky et al., 2002; Malherbe et al., 2013; Ramos et al., 1995). While most commercial strains of *O. oeni* do not produce excessive amounts of diacetyl, other wine LAB such as *Pediococcus spp.* have been reported to produce in excess of 15 mg/L (Strickland et al., 2016). *Pediococcus spp.* spoilage is often identified due to the presence of viscous, slimy, 'ropy' textures in the wine. This is caused by the production of beta-glucan, an exopolysaccharide some *Pediococcus* strains excrete following their initial growth phase (Edwards and Jensen, 1992; Osborne and Edwards, 2005; Walling et al., 2005). *O. oeni* can also impact wine mouthfeel when under certain conditions it ferments fructose into mannitol. Mannitol creates a viscous, slimy texture and is accompanied with a slight sweet taste (Richter et al., 2003; Wisselinka et al., 2002).

Brettanomyces bruxellensis

While wine LAB can be problematic due to the wide array of spoilage compounds they can produce, they are relatively sensitive to SO_2 and so maintaining sufficient free SO_2 at a pH below 3.60 should be sufficient to control their growth (Bartowsky, 2009). A more difficult wine spoilage microorganism to control is the yeast *B. bruxellensis,* due to the organism's ability to survive in wine for long periods, its

relative resistance to SO_2, and its minimal nutrient requirements. *B. bruxellensis* (teleomorph *Dekkera bruxellensis*), commonly referred to simply as 'Brett', is a spoilage yeast that is found in a number of fermentation products. *B. bruxellensis* is responsible for the production of undesirable aroma and taste compounds, often associated with the descriptors 'Band-Aid', mousy, barnyard, fecal, medicinal, smoke, and clove. (Chatonnet et al., 1995; Licker et al.,1998). It has been isolated from wine, cider, beer, sake, kimchi, dairy, and bioethanol fermentations as well as soft drinks and other foods (Steensels et al., 2015). Except for wines from particular historical French winemaking regions, *Brettanomyces* is a highly undesirable, problematic microbe in the wine industry (Crauwels et al., 2014). Depending on the strain of *B. bruxellensis*, its morphology can range from ovoid to ellipsoidal in shape and it can also form pseudohyphae (Fugelsang and Edwards, 2007). *Brettanomyces* can be isolated and identified using selective/differential Wallerstein Laboratory (WL) agar containing a relatively high concentration of cycloheximide. Most yeast are sensitive to cycloheximide, a eukaryotic protein synthesis inhibitor, however *B. bruxellensis* is relatively resistant to it (Ibeas et al., 1996).

The origin of *Brettanomyces bruxellensis* has been somewhat debated. Research utilizing enrichment techniques have demonstrated its presence in small numbers on grape skins (Renouf et al., 2007; Guerzoni and Marchetti, 1987). Others have suggested its introduction to the winery occurs primarily through the air or via fruit fly vectors (Connell et al., 2002; Fugelsang et al., 1993; Fugelsang and Edwards,

2007). The spoilage yeast has been isolated from several winery surfaces including drains, transfer houses, pumps, tank corners, and many other hard-to-sanitize areas (Joseph et al. 2007; Oelofse et al., 2008). Nonetheless, *Brettanomyces* is most commonly isolated from the wine barrels themselves, particularly older barrels. Because *Brettanomyces* can metabolize the cellobiose of the barrel wood, it can exist within tiny crevices of the barrel for extremely long periods of time (Oelofse et al., 2008). Some strains of *Brettanomyces* are also capable of biofilm formation, which through its adhesion to surfaces and production of an exopolysaccharide (EPS) matrix, allow it to be better protected from typical cleaning regimens (Joseph et al., 2007; Tristezza et al., 2010). In such scenarios, even if all organisms within a biofilm are killed, the existing EPS structure provides protection for later microorganisms to recolonize (Joseph et al., 2007). The risk of *Brettanomyces* infection is not isolated to barrel-aged red wines, as infections have also been reported in both white and sparkling wines (Fugelsang and Edwards, 2007; Ciani and Ferraro, 1997).

B. bruxellensis is quite genetically diverse, which has made the organism more difficult to study compared to other fermentation microbes such as *S. cerevisiae*. The genome of *B. bruxellensis* was fully sequenced for the first time by the Australian Wine Research Institute in 2012 (Curtin et al., 2012a). To date, five *B. bruxellensis* strains (CBS-2499, AWRI-1499, AWRI-1608, AWRI-1613, LAMAP-2480) isolated from wine and one strain (ST05.12/22) isolated from a Belgian lambic beer have been fully sequenced (Curtin and Pretorius, 2014; Crauwels et al., 2014; Godoy et al.,

2017). This data uncovered several surprising details about *B. bruxellensis*. Strains CBS-2499 and AWRI-1613 as well as the beer strain (ST05.12/22) were determined to be diploidal, while strains AWRI-1499 and AWRI-1608 were identified as triploids. Preliminary work has also suggested that LAMAP-2480 also expresses triploidy (Godoy et al., 2017). Reports have compared these ploidy findings to *S. cerevisiae*, which can be capable of meiotic chromosome segregation, and thus concluded that *B. bruxellensis* must be somewhat capable of a sexual cycle (Hellborg and Piskur, 2009; Curtin and Pretorius, 2014).

A 2007 report by Curtin et al. collected and analyzed over 240 Brett isolates from 31 winemaking regions in Australia. The authors used amplified fragment length polymorphism (AFLP) to categorize the yeast strains into 8 genotypes. Interestingly, two of the genotypes appeared to be affiliated specifically with Australia's cooler winemaking regions. The researchers also found that over 90% of the isolates collected were closely related to the triploid strains later sequenced in 2012 (Curtin et al., 2007; Curtin et al., 2012a). Building on this, microsatellite profiles of *B. bruxellensis* strains isolated from South Africa and France demonstrated comparable profiles to those of the Australian triploid strains (Albertin et al., 2014). Curtin et al. suggested that the presence of this extra set of genes may give the strain a selective advantage in wine conditions while others proposed specifically that the ploidy of *B. bruxellensis* may assist the organism when growing in high sugar, anaerobic environments (Curtin et al., 2007, Curtin et al., 2012b; Piškur et al., 2012). Genetic

differences affect nutrient uptake, metabolic byproduct production, ethanol tolerance, and sulfite tolerance, among others (Piškur et al., 2012; Curtin et al., 2014; Crauwel et al., 2014; Vigentini et al., 2014; Godoy et al., 2017). Due to the genetic diversity of this spoilage yeast, it is likely that some *B. bruxellensis* strains may pose a larger spoilage risk than others due to greater resistance to difficult environmental conditions present in wine such as low pH, high ethanol, and SO_2 as well as the ability to utilize a range of carbon sources.

Brettanomyces Growth and Metabolism

The wine environment can have a significant impact on both the growth of *B. bruxellensis* and its metabolism. Many papers have demonstrated a strong link between oxygen concentrations and *Brettanomyces* growth. Freer (2003) reported that low concentrations of oxygen resulted in the highest cell biomass compared to completely anaerobic or fully aerobic conditions. This rapid stimulation in growth caused by low levels of oxygen appeared to decrease viability shortly after (Aguilar-Uscanga et al., 2003; Ciani and Ferraro, 1997). A phenomenon known as the Custers effect (negative Pasteur effect) causes increased acetic acid production by *Brettanomyces* when in an oxygen-rich environment (Freer et al., 2003). Temperature was also shown to have a significant impact on growth. Brandam et al. (2008) reported that optimal growth rates by *Brettanomyces* were observed between 25 and 32°C. They also demonstrated that overall metabolite quantities were not changed at different temperatures, only the speed of metabolism. Ethanol tolerance of *B.*

bruxellensis is comparable to *S. cerevisiae*, up to 15-16%, although levels of 13% begin to significantly reduce growth (Barata et al., 2008; Dias et al., 2003). Higher pHs favor *B. bruxellensis* growth as it prefers slightly more basic conditions (Blomqvist et al., 2010). However, the most significant impact of higher pH is due to the relationship between SO_2 and pH. Free SO_2 in wine remains in equilibrium between sulfite, bisulfite, and the antimicrobial molecular SO_2. Le Chatelier's Principle describes this equilibrium, which can be shifted due to a pH change. A higher pH favors sulfite and bisulfite which do not have antimicrobial power, however a lower pH favors molecular SO_2 and thus provides better protection against *Brettanomyces* growth (Zuehlke and Edwards, 2013; Barata et al., 2008; Fugelsang and Edwards, 2007).

Studies regarding the nutrient requirements of *B. bruxellensis* have demonstrated that most strains can utilize a large range of carbon sources including glucose, fructose, sucrose, maltose, trehalose, and cellobiose (Crauwels et al., 2015). Some strains have also demonstrated the ability to utilize galactose, raffinose, arabinose, and lactose as additional carbon sources while others can use ethanol as its sole source (Crauwels et al., 2015; Fugelsang and Edwards, 2007; Silva et al., 2004). Although *Brettanomyces* is capable of alcoholic fermentation, redox imbalances provoke the yeast to exhibit the Custer effect, in which the typical alcohol metabolic pathway is inhibited under anaerobic conditions (Vigentini et al., 2008; Van Dijken et al., 1986). Crauwels et al. (2015) reported that some strains of *B. bruxellensis* can utilize nitrate as its sole

nitrogen source. In fact, ⅔ of the tested strains demonstrated the ability. Because of this, *B. bruxellensis* can have a competitive advantage over *S. cerevisiae* as the yeast cannot utilize nitrate alone.

Wine Spoilage by *Brettanomyces bruxellensis*

B. bruxellensis causes wine spoilage through its production of several volatile compounds. Acetic acid is often produced in small amounts through its metabolism of glucose. Concentrations of acetic acid above 1 g/L can result in undesirable vinegar aromas (Fugelsang and Edwards, 2007; Licker et al.,1998). Isovaleric acid (rancid, gym socks, goat) is formed by *B. bruxellensis* through metabolism of the amino acid L-leucine (Vigentini et al., 2013; Licker et al., 1998). While these compounds are sometimes associated with *Brettanomyces*, the major compounds responsible for *Brettanomyces* wine spoilage are the volatile phenols 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG), and 4-ethylcatechol (4-EC). 4-EC has only recently been associated with *Brettanomyces* contamination (Oelofse et al., 2008; Hesford et al., 2004) but is described as a mousey aroma. It is produced by *Brettanomyces* as a result of the metabolism of caffeic acid, a compound naturally present in grapes (Chatonnet et al., 1995; Oelofse et al., 2008).

Compared to 4-EC, there is substantially more knowledge regarding wine spoilage cause by 4-EP and 4-EG. 4-EP is often associated with barnyard, mousey, horse sweat, Band-Aid®, and medicinal aromas while 4-EG is commonly described as

having smoky, bacon, and clove characteristics (Chatonnet et al., 1992; Curtin et al., 2013). *B. bruxellensis* produces 4-EP and 4-EG through metabolism of the hydroxycinnamic acids (HCA) *p*-coumaric acid and ferulic acid respectively. Sensory thresholds for 4-EP and 4-EG were initially cited at 600 µg/L and 100µg/L respectively (Chatonnet et al., 1992; Chatonnet et al., 1995). However, recent research has indicated poor correlations regarding quantity and sensory effects of the compounds. The exact concentrations of both volatiles as well as their respective ratios to one another, in addition to the wine matrix itself, can have significant impacts on sensory thresholds (Romano et al., 2009; Conterno et al., 2006). Some have reported sensory thresholds as low as 230 µg/L for 4-EP detection and 40 µg/L for 4-EG perception (Coulter et al., 2004; Kheir et al., 2013). Wines which are fuller bodied and heavily oaked can significantly raise the sensory threshold to combined concentrations of over 1000 µg/L (Morata et al., 2013; Kheir et al., 2013; Šućur et al., 2016). Production of volatile phenols can also be impacted by wine conditions such as oxygen and ethanol (Curtin et al., 2013). In addition, a number of researchers have noted differences in volatile phenol production between *B. bruxellensis* strains (Madsen et al., 2016; Fugelsang and Zoecklein, 2003; Conterno et al., 2006; Barbin et al., 2008; Agnolucci et al., 2009; Romano et al., 2008; Harris et al., 2009; Silva et al., 2005).

Aside from wine conditions and *B. bruxellensis* strain, an additional factor that impacts the production of volatile phenols in wine is the concentration of precursor

compounds, the hydroxycinnamic acids *p*-coumaric and ferulic acid. These compounds are naturally present in wine grapes at various concentrations and are impacted by grape varietal, viticulture management practices, and climate (Nagel et al., 1979). The hydroxycinnamic acids are first converted by *B. bruxellensis* to vinylphenols via hydroxycinnamic decarboxylase before being reduced to ethylphenols via vinylphenol reductase (Couto et al., 2005; Renouf et al., 2006; Chatonnet et al., 1992). The reason *B. bruxellensis* conducts this process is still undetermined. It has been theorized that HCA degradation initiates an electron gradient, thereby reoxidizing NADH and allowing for additional ATP production (Godoy et al., 2009; Fugelsang and Edwards, 2007). Fugelsang and Edwards (2007) discussed the requirement of reducing cofactors in the enzymatic reduction activity of HCA derivatives, suggesting that the reduction of vinylphenols to ethylphenols serves as a source of NAD+ to maintain redox balance. Others have also suggested that HCAs are degraded by microorganisms as a detoxifying step because HCAs can have an antimicrobial effect (Renouf et al., 2006).

In grapes, hydroxycinnamic acids are primarily present bound to tartaric acid via an ester linkage (Oelofse et al., 2008). When bound to tartaric acid, *B. bruxellensis* cannot utilize HCAs for metabolism and production of volatile phenols (Schopp et al., 2013). These tartaric acid esters may be hydrolyzed during aging, resulting in the release of free hydroxycinnamic acids (Nagel et al., 1979). However, this process generally occurs slowly during aging and so wines can still retain high amounts of

tartaric acid-bound hydroxycinnamic acids (Kheir et al., 2013; Ginjom et al., 2011; Nagel et al., 1979). While *B. bruxellensis* cannot degrade tartaric-bound HCAs, many reports have demonstrated HCA degradation activity by wine lactic acid bacteria (Cheschier et al., 2015; Burns and Osborne, 2013; Hernández et al., 2006). Burns and Osborne (2013) observed an increase in free hydroxycinnamic acids (*p*-coumaric and caffeic acid) after MLF, noting that only one out of the four *O. oeni* strains used caused this change in concentration. However, only a small number of *O. oeni* strains were used in this study and the effect on volatile phenol production by *Brettanomyces* was not reported. Chescheir et al. (2015) screened eleven commercial *O. oeni* strains for their ability to degrade tartaric-bound HCAs and noted that only one strain, *O. oeni* VFO, was capable of this process. As a result, wines that underwent MLF with VFO had a significantly higher concentration of *p*-coumaric acid than wines that underwent MLF with other *O. oeni* strains. When *B. bruxellensis* grew in these wines, significantly higher concentrations of 4-EP and 4-EG were produced in wines where MLF was conducted by VFO. Chescheir et al. (2015) suggested winemakers avoid using strains of *O. oeni* that could degrade tartaric acid-bound HCAs in wines most susceptible to *B. bruxellensis* infection.

While Chescheir et al. (2015) screened eleven *O. oeni* strains, there is a large number of additional commercial *O. oeni* strains that have not been characterized for the ability to degrade tartaric acid-bound HCAs. In addition, winemakers do not always inoculate their wines with commercial bacteria cultures but rather rely on the

naturally-present lactic acid bacteria to perform MLF. In addition to being more unreliable, this practice also means that the winemaker has little control over which bacteria species or strain will conduct MLF. As such, winemakers cannot regulate what the specific characteristics, such as flavor and aroma production, the bacteria conducting MLF possesses. To date, only commercial *O. oeni* strains have been tested for the ability to degrade tartaric acid-bound HCAs and so it is unknown whether this trait exists in naturally-occurring populations of *O. oeni.* Therefore, an objective of this study is to screen a number of commercially available *O. oeni* strains as well as non-commercial *O. oeni* strains isolated from wines during MLF. In addition to previous reports from Chescheir et al. (2015) and Burns and Osborne (2013), results from this study will provide more information on how widespread the ability to degrade tartaric acid-bound HCAs is amongst *O. oeni* strains.

Control of *Brettanomyces*

Because *Brettanomyces* wine spoilage can cause significant economic losses (Puig et al., 2011; Loureiro and Malfeito-Ferreira, 2003), preventing its growth in wine is critical. The control of *Brettanomyces* can be divided into two main categories: prevention methods to inhibit the proliferation of the yeast, and remedial measures which serve to stop a current infection as well as to correct any undesirable wine qualities which have occurred. Prevention begins at harvest by processing only disease-free grapes, which can reduce the risks of microbial spoilage (Du Toit et al., 2005). Arguably the most basic preventative measure is proper sanitation and

cleaning regimens in the winery. Regular cleaning of the winery removes large organic material and helps reduce mold and biofilm buildup (Du Toit et al., 2005). Barrels, especially those received secondhand, commonly harbor *Brettanomyces* (Malfeito-Ferreira, 2011). New barrels may also be particularly susceptible to contamination by *Brettanomyces* due to both their cellobiose contents and the quicker evaporation of SO_2 (Wedral et al., 2010). Additionally, the porosity of the barrels makes complete sanitation quite difficult because microbes can effectively hide out deep in these pores. Malfeito-Ferreira et al. (2004) discovered culturable *B. bruxellensis* 8 mm deep into oak barrels. Similarly, other researchers demonstrated the presence of *B. bruxellensis* cells as far as 8 mm into French oak staves (Cartwright et al., 2016). Barrel sanitation may include the use of hot water, steam, and/or ozone treated water (Alejandra Aguilar Solis, 2014; Wilker et al., 1997; Malfeito-Ferreira, 2004). Recently, microwave radiation, high power ultrasonics, and dry ice have also demonstrated promising antimicrobial effects in the winery setting (Costantini et al., 2016; Schmid et al., 2011 González-Arenzana et al., 2012; Jiranek et al., 2008).

Aside from specific cleaning and sanitation regimes, SO_2 management is another basic step in spoilage prevention (Agnolucci et al., 2010; Zuehlke and Edwards, 2013; von Cosmos et al., 2016). Maintaining the appropriate concentration of free SO_2 post-fermentation and during aging can prevent the majority of spoilage issues, so it is important that SO_2 is added to the wine as soon as possible. Uninoculated

fermentations are often associated with sluggish or stuck fermentations (Agnolucci et al., 2009). When a fermentation is slow or stuck, there are still many nutrients available for *Brettanomyces* to utilize. Since the wine cannot have SO_2 added until the completion of fermentation, it is further vulnerable to *Brettanomyces* infections. For these reasons, it is strongly suggested fermentations be carried out by robust, quick starter inoculums.

While SO_2 is the most widely used chemical for microbial inhibition in wine, two additional compounds, dimethyl dicarbonate (DMDC) and chitosan, have demonstrated promising inhibition of *B.* bruxellensis growth during the winemaking process. DMDC is a compound sold by the tradename Velcorin™ and is highly effective in treating microbial contamination (Zuehlke et al., 2013). DMDC can be used during aging but is also commonly used at bottling to prevent secondary fermentations from occurring in bottle (Zuehlke et al., 2015). Following application of DMDC, antimicrobial effects are immediate. The compound quickly decomposes within a few hours into insignificant amounts of carbon dioxide and methanol which have no sensory impacts on the wine (Zuehlke et al., 2013).

Chitosan is a chitin-derived polysaccharide isolated from *Aspergillus niger* that has effective antimicrobial properties. A 2013 study by Ferreira et al. looked at the antimicrobial capacity of chitosan at different molecular weights against *Brettanomyces*. They found minimum inhibitory concentrations as low as 0.2 mg/ml

when using low molecular weight chitosan. Contrastingly, Gómez-Rivas et al. (2004) found concentrations of at least 1 mg/ml could significantly slow the lag phase of *Brettanomyces* but the overall final cell populations were not changed. Others have shown concentrations of 0.2 g/L were able to kill most wine-related spoilage microorganisms while *S. cerevisiae* was able to withstand more than 2 g/L before showing signs of inhibition (Bağder et al., 2015).

Microbial Interactions in Wine

Although the use of chemical antimicrobials such as SO_2 remain the most widely used method for the control of spoilage microorganisms in the wine industry, there is increasing interest in developing control strategies that minimize the use of chemical preservatives due to consumer concerns (du Toit and Pretorius, 2000). One alternative is to utilize antagonistic relationships between wine microorganisms. For example, some commercially available *S. cerevisiae* strains possess the killer phenotype that inhibits the growth of sensitive yeast which may be present during fermentation (Pérez-Nevado et al., 2006). Another example is the inhibition of *O. oeni* via peptides produced by certain *S. cerevisiae* strains during alcoholic fermentation (Osborne and Edwards, 2007). However, there have been few reports of studies investigating interactions between *B. bruxellensis* and other wine microorganisms. Renouf et al. (2006) noted that when *B. bruxellensis* and *S. cerevisiae* were inoculated together in grape juice, *S. cerevisiae* dominated the fermentation with *B. bruxellensis* growth

occurring mainly after the completion of alcoholic fermentation when *S. cerevisiae* populations were in decline.

There have been some reports regarding MLF influencing the growth and volatile phenol production of *B. bruxellensis.* In 2005, Renouf et al. noted that wines which had relatively long MLF finishing times ended up with a much higher biomass of *B. bruxellensi*s if infected. This was partly attributed to the fact that SO_2 was not added to the wine until much later, increasing the risks of *B. bruxellensis* infection. In contrast, Gerbaux et al. (2009) reported that wines which had undergone MLF had considerable less volatile phenol concentrations than wines which had not undergone MLF. However, no population data for *B. bruxellensis* and *O. oeni* was provided by Gerbaux et al. (2009) making it difficult to conclude whether *B. bruxellensis* populations were impacted by *O. oeni*. Chescheir (2014) investigated whether the reduced volatile phenol content reported by Gerbaux et al. (2009) was due to interactions between *O. oeni* and *B. bruxellensis*. Chescheir (2014) noted that when *B. bruxellensis* was inoculated into wine that had recently undergone MLF, its population declined rapidly. Additional experiments determined that the inhibition of *B. bruxellensis* was relieved if *O. oeni* was removed from the wine post-MLF or if only non-viable *O. oeni* cells were present (Chescheir, 2014). The author concluded that the inhibition of *B. bruxellensis* was likely due to cell-cell contact with live *O. oeni* cells.

While the study by Chescheir (2014) reported inhibition of *B. bruxellensis* by *O. oeni,* how long this inhibition lasted post-MLF was not determined. In addition, only one *B. bruxellensis* strain was used. *B. bruxellensis* is known to display a large degree of genotypic and phenotypic diversity (Godoy et al., 2017; Curtin et al., 2014; Crauwel et al., 2014; Vigentini et al., 2013; Piškur et al., 2012). Testing additional strains of *B. bruxellensis* for their sensitivity to the presence of live *O. oeni* post-MLF is therefore necessary to determine the practical significance of what was reported by Chescheir (2014).

Because *B. bruxellensis* is the main spoilage yeast present during winemaking, significant effort is expended by the winemaker to prevent infection and growth of this yeast in wine. Control tools are limited to the use of SO_2 while rigorous sanitation is one of the best approaches to prevent spoilage issues. This study investigates two additional strategies that could be utilized to aid in the control of *B. bruxellensis* during winemaking. Firstly, methods to minimize the concentration of the volatile phenol precursor, *p*-coumaric acid, in wine will be investigated. This will include the use of *S. cerevisiae* strains that can deplete *p*-coumaric acid during alcoholic fermentation as well screening *O. oeni* strains for their ability to degrade tartaric-bound *p*-coumaric acid, resulting in increased free *p*-coumaric acid content in wines post-MLF. Secondly, the inhibition of *B. bruxellensis* by *O. oeni* will be further explored, including how long this inhibition lasts post-MLF as well as the sensitivity

of additional *B. bruxellensis* to *O. oeni*. Therefore, the specific objectives of the research are:

1) Investigate factors impacting the concentration of volatile phenol precursors in wine focusing on impact of microbes present during winemaking
2) Investigate influence of malolactic fermentation on *B. bruxellensis* growth and volatile phenol production, specifically interactions between *O. oeni* and *B. bruxellensis*

Chapter 2

Changes in free and tartaric acid-bound hydroxycinnamic acids due to *Saccharomyces cerevisiae* and *Oenococcus oeni* during winemaking

Abstract

This study investigated changes in the concentration of 4-ethyphenol precursor compounds during alcoholic (AF) and malolactic fermentation (MLF). *Saccharomyces cerevisiae* strains with and without phenolic acid decarboxylase (PAD) activity as well as *Oenococcus oeni* strains with and without cinnamoyl esterase (CE) activity were used to perform sequential and simultaneous Pinot noir AF and MLF. Simultaneous fermentation using PAD (+) *S. cerevisiae* and a CE (+) *O. oeni* resulted in the largest reduction of volatile phenol precursors, likely due to *S. cerevisiae* metabolism of free hydroxycinnamic acids liberated by the CE (+) *O. oeni*. However, these same strains when utilized in sequential fermentations resulted in the largest number of precursors in the form most readily usable by *B. bruxellensis*. Several commercial and non-commercial *O. oeni* were also screened for CE activity. Two of the eleven previously unscreened commercial *O. oeni* as well as one of the four non-commercial *O. oeni* exhibited cinnamoyl esterase activity, resulting in significantly higher concentrations of free hydroxycinnamic acids in these wines. The stability of the 4-EP precursor compounds during aging was investigated by using Pinot noir wines that had undergone MLF using either a CE (+) or (-) *O. oeni* and then adjusting the wines to different pH and ethanol concentrations before aging at

either 13 or 21°C. During 180 days of aging. the concentration of *p*-coumaric acid and coutaric acid remained relatively stable and were most impacted by the strain of *O. oeni* that had conducted the MLF. These findings highlight the importance of careful consideration when choosing which microorganisms to utilize in wine fermentations as they have the most significant impact on volatile phenol precursors present in the wine.

Introduction

Brettanomyces bruxellensis spoils wine via the production of undesirable aroma compounds. While *B. bruxellensis* can produce a number of compounds which have negative sensory impacts in wine, it is most known for its production of volatile phenols 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) (Romano et al., 2008; Loureiro and Malfeito-Ferreira, 2005; Chatonnet et al., 1992). These compounds are commonly described as being reminiscent of barnyard, horsey, medicinal, Band-Aid, plastic, smokey, bacon, and clove (Chatonnet et al., 1992; Licker et al.,1998). 4-EP and 4-EG are produced from the hydroxycinnamic acids *p*-coumaric acid and ferulic acid respectively through a two-step enzymatic process (Chatonnet et al., 1992). The hydroxycinnamic acid is first converted to a vinylphenol via hydroxycinnamic decarboxylase before being reduced to an ethylphenol via vinylphenol reductase. *ρ*-Coumaric acid and ferulic acid are naturally present in grapes and are typically found as esters of tartaric acid (coutaric and fetaric acid, respectively). During winemaking, these tartaric acid esters may be hydrolyzed resulting in release of the free

hydroxycinnamic acids (Nagel et al., 1979). However, this process generally occurs slowly during aging so wines can still retain high amounts of tartaric acid ester-bound hydroxycinnamic acids (Ginjom et al., 2011; Nagel et al., 1979).

B. bruxellensis infections most often occur during aging, particularly when performed in barrels (Oelofse et al., 2008; Wedral et al., 2010). The yeast can withstand the harsh wine conditions, surviving on little nutrients for a relatively long period of time. It is often hard to detect and winemakers may not realize they have a *Brettanomyces* infection until the wine has already been spoiled. As such, significant research efforts have focused on the control of *B. bruxellensis*. Common methods to prevent and control *Brettanomyces* include sanitation and cleaning regimens which use hot water, steam, ozone, velcorin (DMDC), and chitosan (Alejandra Aguilar Solis, 2014; Wilker et al., 1997; Malfeito-Ferreira, 2004). The most common and effective tool winemakers have is SO_2 (Du Toit and Pretorius, 2000).

Aside from control methods using chemical additives, an additional strategy to prevent *Brettanomyces* spoilage is to minimize the amount of the volatile precursor compounds, hydroxycinnamic acids, in the wine. Grape variety, ripeness, sun exposure, geographical origin, and vintage can all impact concentrations of hydroxycinnamic acids present in the wine (Nagel et al., 1979). During fermentation, some microorganisms may also impact the concentration of hydroxycinnamic acids. For example, Morata et al. (2013) suggested the utilization of *Saccharomyces*

cerevisiae strains which possessed phenolic decarboxylase (PAD) activity so that the yeast could metabolize *p*-coumaric acid. This metabolism would produce vinylphenols that have an affinity for binding with anthocyanins, thereby making the precursors unavailable to *B. bruxellensis.* Additional research by Burns and Osborne (2013) and Chescheir et al. (2015) demonstrated that *Oenococcus oeni* may have cinnamoyl esterase (CE) activity which results in the degradation of tartaric acid-bound *p*-coumaric acid and an increase of free *p*-coumaric acid in the wine. Because *B. bruxellensis* cannot degrade tartaric acid ester-bound hydroxycinnamic acids (Schopp et al., 2013), this meant that if *B. bruxellensis* grew in wines that had undergone MLF with a CE (+) *O. oeni* strain then significantly higher volatile phenols would be produced (Chesceir et al., 2015). To date, few *O. oeni* have been characterized for CE activity and so the frequency of this trait is not well known.

Tartaric acid ester-bound hydroxycinnamic acids may undergo hydrolysis during wine aging but the significance of this compared to degradation by *O. oeni* during MLF is not known. Madsen et al. (2016) noted that during aging the *p*-coumaric acid content of a Cabernet Sauvignon increased such that when *B. bruxellensis* grew in the wines relatively similar concentrations of volatile phenols were produced whether the wine had undergone MLF with a CE (+) *O. oeni* strain or not. However, this study was only conducted under one condition and with one wine (pH 3.50, 12.5% v/v ethanol). Conditions that may have impacted acid hydrolysis during aging such as storage temperature, pH, and ethanol, were not investigated.

This study investigated the impact of using a PAD (+/-) *S. cerevisiae* and CE (+/-) *O. oeni* on the hydroxycinnamic acid content of a wine. Additional commercial and non-commercial strains of *O. oeni* were screened for cinnamoyl esterase activity to determine the frequency of this trait. Finally, the stability of free and tartaric acid ester-bound *p*-coumaric acid during wine aging was studied under a number of conditions to help determine the significance of MLF being conducted by CE (+) *O. oeni* with respect to volatile phenol production by *B. bruxellensis.*

Materials and Methods

Microorganisms

The non-commercial *S. cerevisiae* strain OSU-2 was originally isolated from Pinot noir grapes and shown to have high *p*-coumaric acid decarboxylase activity (Hall, 2012). The commercial *S. cerevisiae* strains Enoferm CSM and RC212 were sourced from Lallemand (Montreal, Canada). Strain OSU-2 was prepared from a frozen stock (-80°C) culture by inoculation into acidic grape juice (AGJ) broth (2.5 mg/L manganese sulfate, 125 mg/L magnesium sulfate, 5g/L yeast extract, 1 ml/l 5% (w/w) Tween 80, 250 ml/l white grape juice, pH 3.5) and incubation at 25°C for 48 hours before use. CSM and RC212 were prepared from freeze-dried cultures according to manufacturer's instructions.

O. oeni strain Viniflora Oenos (VFO) was sourced from Chr. Hansen (Hørsholm, Denmark). *O. oeni* strains Lalvin VP41, and Enoferm® Alpha were sourced from Lallemand. Additional strains currently not commercially available (strains 201, 202, 203, 301, 302, 304, 401, 402, 405, 501, and 502) were also provided by Lallemand. Four 'native' *O. oeni* strains (0294, 0652, 0651, 0238) were provided by ETS Laboratories (St. Helena, CA). These strains were determined by ETS Labs to be non-commercial strains (native) after comparison to available commercial strains of *O. oeni* by microsatellite multi-locus variable number tandem repeat (VNTR) analysis. *B. bruxellensis* strain UCD VEN # 2049 was obtained from the UC Davis Department of Viticulture and Enology Culture Collection housed in the UC Davis Department of Viticulture and Enology, University of California, Davis, CA 95616 U.S.A. All microorganisms were prepared as described by Chescheir et al. (2015) for storage in glycerol (15%) at -80°C until needed. Microorganisms were prepared from frozen cultures by inoculation into AGJ broth and grown at 25°C for five (*B. bruxellensis*) or seven (*O. oeni*) days. Cells were harvested by centrifugation (4,000 x g for 20 minutes) and re-suspended in 0.2 M phosphate buffer (pH 7.0) prior to inoculation.

Winemaking

Pinot noir wines were produced at the Oregon State University Research Winery from grapes harvested at Woodhall Vineyard (Alpine, Oregon, USA) in 2015 and 2016. After harvest, grapes were stored overnight at 4°C before being destemmed. Approximately 25 kg of grapes were then divided into 40 L plastic fermenters. In

2015, *p*-coumaric acid (Sigma-Aldrich St. Louis, MO) dissolved in a 95% v/v ethanol solution was added to each fermenter at a rate of 2 mg/L. After mixing, samples were taken for pH, sugar content (°Brix), titratable acid (TA), and yeast assimilable nitrogen (YAN) analysis. Basic juice parameters of the 2015 Pinot noir must were 24.8°Brix, pH 3.42, 6.4 g/L titratable acid and 110 mg/L YAN. In 2016, the grapes at harvest had very high sugar content (27 °Brix) and so an addition of distilled water was made to each fermenter to reduce the initial Brix to approximately 24 °Brix. The other basic grape parameters were pH 3.40, 4.35 g/L titratable acid, and 143 mg/L YAN. In 2015 and 2016, the yeast nutrient Fermaid K (Lallemand) was added to each tank at a rate of 0.25 g/L.

Impact of S. cerevisiae *and* O. oeni *on volatile phenol precursor concentrations*

The 2015 experimental design is shown in Figure 2.1. In brief, one set of three tanks was inoculated with *S. cerevisiae* strain Enoferm CSM (Lallamend) while an additional set of three tanks was inoculated with *S. cerevisiae* strain OSU-2. In a third set of tanks, a simultaneous fermentation was induced by the inoculation of OSU-2 and *O. oeni* VFO at the same time. Yeast and bacteria were inoculated at approximately 1 x 10^6 cfu/mL. Fermenters were placed in a temperature-controlled room set at 27°C and punched-down twice daily. Fermentations were monitored by assessing °Brix (Anton-Paar DMA 35N Density Meter (Graz, Austria)) and malic acid was assessed in the simultaneous ferments (Vintessential Enzymatic L-Malic Acid Test Kit; Victoria, Australia). When all alcoholic fermentations (and MLF in the

case of the simultaneous fermentations) were complete (< 0.25 g/L reducing sugar, < 50 mg/L malic acid for simultaneous fermentations), wines were pressed and placed in a cold room at 4°C for 48 hours to cold settle. Following cold settling, wines were racked and then filtered through a plate and frame filter fitted with 20 cm x 20 cm Beco K-1 3.0μm nominal filter sheets (Langenlonsheim, Germany). Wine was then filtered through a 1.0μm nylon cartridge (G.W. Kent, Ypsilanti, Michigan, USA) and a 0.45μm sterile polyethersulfone cartridge (Merck-Millipore, MA, USA) in succession and dispensed into sterilized one gallon carboys. Wines that had undergone a simultaneous fermentation were stored at 25°C while the other wines were inoculated for MLF by the addition of either *O. oeni* strain VFO or strain Alpha and held at 25°C until MLF was completed (< 50 mg/L malic acid). At the completion of MLF (malic acid < 50 mg/L), samples were taken and frozen at -80°C for later HPLC analysis of hydroxycinnamic acid content.

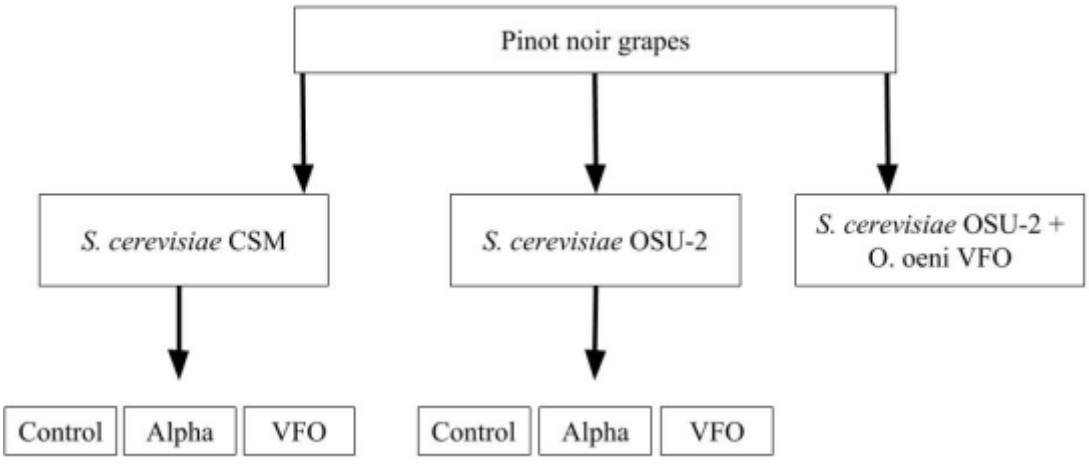

Figure 2.1 Experimental design for determining the impact of *S. cerevisiae* and *O. oeni* on volatile phenol precursor concentrations.

Cinnamoyl esterase activity screening of O. oeni

Sterile-filtered 2015 Pinot noir wine fermented by CSM that had not undergone MLF was utilized to determine the ability of various *O. oeni* strains to degrade tartaric-bound hydroxycinnamic acids. In addition, Pinot noir wine produced in 2016 using *S. cerevisiae* RC212 and fermented as previously described was also used. 10 mL of either 2015 or 2016 Pinot noir wine was dispensed into 20 mL sterilized test tubes and inoculated with *O. oeni* at approximately 1 x 10^6 cfu/mL. *O. oeni* cultures were prepared for inoculation as previously described. Treatments were performed in triplicate and a non-inoculated control was also prepared. *O. oeni* strain VFO, known to be able to degrade tartaric-esterified hydroxycinnamic acids, was utilized as a positive control (Cheschier et al., 2015). Wines were incubated at 25°C and monitored weekly for malic acid (enzymatic test kit, Vintessentials). At the end of MLF (malic acid < 50 mg/L), samples were taken and frozen at -80°C for later HPLC analysis of hydroxycinnamic acids.

Impact of pH, temperature, and ethanol on Pinot noir wine hydroxycinnamic acid content during aging

2016 Pinot noir wine prepared as previously described was inoculated for MLF with either *O. oeni* Alpha or *O. oeni* VFO. At the completion of MLF (malic acid < 50 mg/L), wines were assessed for pH and ethanol using standard methods. The Pinot noir wine ethanol content was 13.1 % (v/v) and pH 3.80. Portions of the wines were

then adjusted to different pH and ethanol contents as illustrated in Figure 2.2. The pH was adjusted with 25% (v/v) phosphoric acid while ethanol content was adjusted using 95% (v/v) Everclear (Luxco Inc.; St. Louis, Missouri, USA). Free SO_2 concentrations were assessed by aeration-oxidation method and appropriate SO_2 additions (in the form of a 10% w/v potassium metabisulfite solution) were made to achieve 30 mg/L free SO_2. All wines were then sterile-filtered using a 0.45μm sterile polyethersulfone cartridge (G.W. Kent) and dispensed into 375 mL screw-capped (Stelvin™, Amcor, Zurich) wine bottles. A set of each of the wine treatments (VFO or Alpha, pH 3.60 or 3.80, 13 or 15% ethanol) was then stored at either 13°C or 21°C. At 0, 30, 100, and 180 days post-bottling, three bottles of each treatment were pulled and samples were taken and frozen at -80°C for later HPLC analysis of hydroxycinnamic acids.

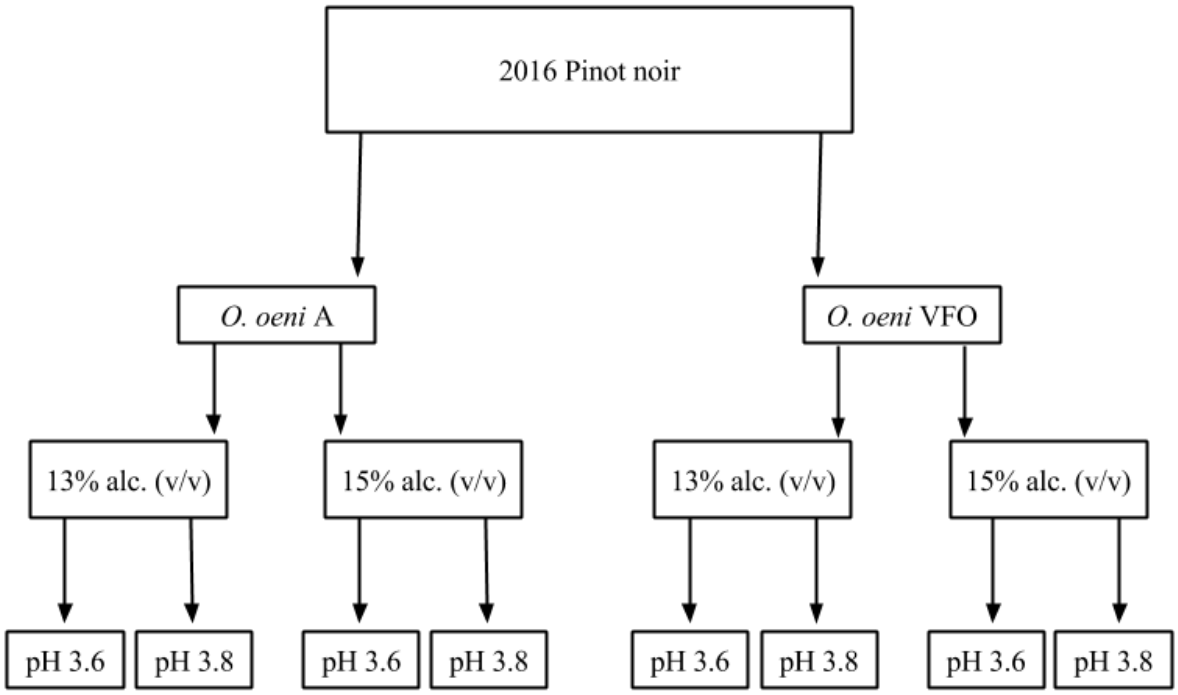

Figure 2.2 Experimental design scheme for determining impact of pH, temperature, and ethanol on Pinot noir wine hydroxycinnamic acid content during aging

HPLC analysis of hydroxycinnamic acid content

Hydroxycinnamic acids (free and tartaric acid-bound) were determined by HPLC-DAD as described by Burns and Osborne (2013). Prior to HPLC analysis, samples were centrifuged using an Allegra X-22 instrument (Beckman Coulter, Brea, CA) at 10,000 *g* for 10 min. 20 µL aliquots were injected and hydroxycinnamic acids were detected by scanning from 200 to 400 nm. Identification and quantification of hydroxycinnamic acids was performed at 320 nm based on UV-visible spectra and retention times of known standards obtained from Sigma-Aldrich. Calibration curves were prepared for *p*-coumaric and caffeic acid quantification. Due to the lack of

available standards, coutaric and caftaric acid were reported as *p*-coumaric and caffeic acid equivalents.

Statistical Analysis

Statistical analysis of free and bound hydroxycinnamic acid content in wines using PAD (+/-) *S. cerevisiae* and CE (+/-) *O. oeni* was performed using a two-tail Welch's t-test comparison of means to determine significance ($p<0.05$). Statistical analysis of the cinnamoyl esterase activity of commercial and non-commercial *O. oeni* strains was performed using a two-tail student t-test comparison of the means to determine significance ($p<0.05$).

Results

Impact of S. cerevisiae *and* O. oeni *on volatile phenol precursor concentrations*

Experiments were conducted to investigate changes in 4-EP precursor compounds due to fermentations with a yeast strain with the ability to degrade free *p*-coumaric acid in combination with an *O. oeni* strain that could degrade tartaric acid-bound hydroxycinnamic acids. In wines which had not undergone MLF, no significant difference was noted in coutaric acid or *p*-coumaric acid concentrations in wines which had undergone AF with a PAD (+) *S. cerevisiae* strain (OSU-2) versus a PAD (-) *S. cerevisiae* strain (CSM) (Table 2.1). However, significantly less coutaric acid was present in the wine produced via a simultaneous fermentation with *S. cerevisiae* OSU-2 and *O. oeni* VFO (Table 2.1).

No significant differences in *p*-coumaric or coutaric acid concentrations was also noted in wines that underwent MLF with *O. oeni* Alpha, a CE (-) strain (Table 2.1). However, when *O. oeni* VFO conducted MLF, significant differences in coutaric and *p*-coumaric acid concentrations were found compared to wines that underwent MLF with *O. oeni* Alpha. For example, when MLF was conducted by VFO after alcoholic fermentation, concentrations of *p*-coumaric acid in wine fermented by CSM was 1.61 mg/L while in wine fermented by OSU-2 2.07 mg/L of *p*-coumaric acid was present. In contrast, less than 0.4 mg/L *p*-coumaric acid was present in all wines where *O. oeni* Alpha conducted MLF (Table 2.1). Overall, the lowest levels of total potential 4-EP precursors (coutaric and *p*-coumaric acid) were noted in the simultaneous fermentations using OSU-2 and VFO, with a concentration of 1.14 mg/L at the end of the fermentations.

Table 2.1 Concentration (mg/L) of 4-ethylphenol precursor compounds (coutaric and *p*-coumaric acid) in 2015 Pinot noir wine produced by *S. cerevisiae* OSU-2 or CSM at the beginning and end of malolactic fermentation conducted by *O. oeni* Alpha or VFO

	Start of MLF		End of MLF	
	Coutaric acid	*p*-coumaric acid	Coutaric acid	*p*-coumaric acid
OSU-2 No MLF	1.90 ± 0.14^{a}	0.38 ± 0.03^{a}	1.75 ± 0.02^{a}	0.35 ± 0.02^{a}
CSM No MLF	1.73 ± 0.04^{a}	0.48 ± 0.004^{b}	1.54 ± 0.06^{b}	0.37 ± 0.02^{a}
OSU-2/VFO Simultaneous	0.56 ± 0.26^{b}	0.59 ± 0.12^{c}	0.54 ± 0.22^{c}	0.60 ± 0.12^{b}
OSU + Alpha	1.90 ± 0.14^{a}	0.38 ± 0.03^{a}	1.66 ± 0.14^{ab}	0.32 ± 0.07^{a}
OSU + VFO	1.90 ± 0.14^{a}	0.38 ± 0.03^{a}	0.12 ± 0.05^{d}	2.07 ± 0.05^{c}
CSM + Alpha	1.73 ± 0.04^{a}	0.48 ± 0.004^{b}	1.64 ± 0.05^{b}	0.27 ± 0.05^{a}
CSM + VFO	1.73 ± 0.04^{a}	0.48 ± 0.004^{b}	0.08 ± 0.02^{d}	1.61 ± 0.08^{d}

[a-d]Mean values with different superscript letters within a column are significantly different at $p \leq 0.05$, n=3.

Cinnamoyl esterase activity screening of O. oeni

A number of commercial and non-commercial *O. oeni* strains were screened for their ability to degrade tartaric acid-bound hydroxycinnamic acids. While for the majority of strains there was no change in hydroxycinnamic acid content (free or tartartic acid-bound), some *O. oeni* strains caused significant changes (Table 2.2 and 2.3). For example, when compared to the concentration of caftaric acid in the 2015 control wine (37.3 mg/L), there was a decrease to 1.1 mg/L in wines which had undergone

MLF with commercial strain 405. This corresponded to a significant increase in caffeic acid concentrations to 35.8 mg/l post-MLF. Similar trends were seen in concentrations of coutaric and *p*-coumaric acid when comparing strain 405 wines to the control. Strain 405 wines contained 1.6 mg/L coutaric acid post-MLF (compared to 7.8 mg/ml in the control) and 5.2 mg/l *p*-coumaric acid (contrasting with 0.5 mg/l in the control wines). Wines where MLF was conducted by strain 502 also had significantly higher concentrations of caffeic and *p*-coumaric acids and corresponding lower concentrations of coutaric and caftaric acid (Table 2.2). As expected, this result also occurred in wines where MLF was conducted by *O. oeni* VFO, a strain known to have cinnamoyl esterase activity (Chescheir et al., 2015). Of the non-commercial strains tested, significant differences in free and bound hydroxycinnamic acids were present when strain 0238 conducted the MLF (Table 2.3).

Table 2.2 Concentration (mg/L) of hydroxycinnamic acids in 2015 Pinot noir at the completion of MLF after inoculation with commercial strains of *O. oeni.*

	Caftaric acid	Coutaric acid	Caffeic acid	*p*-coumaric acid
Control No MLF	37.3 ± 0.5[a]	7.8 ± 0.1[a]	3.6 ± 0.1[a]	0.5 ± 0.1[a]
VFO	1.1 ± 0.1[b]	1.5 ± 0.1[b]	33.8 ± 0.7[b]	5.1 ± 0.2[b]
201	36.3 ± 0.4[a]	7.6 ± 0.1[a]	3.6 ± 0.1[a]	0.5 ± 0.1[a]
202	37.1 ± 0.2[a]	7.8 ± 0.2[a]	3.5 ± 0.1[a]	0.4 ± 0.1[a]
203	36.4 ± 0.4[a]	7.9 ± 0.1[a]	3.7 ± 0.1[a]	0.4 ± 0.1[a]
301	36.3 ± 1.1[a]	7.8 ± 0.1[a]	3.6 ± 0.2[a]	0.4 ± 0.1[a]
302	36.8 ± 0.3[a]	7.8 ± 0.2[a]	3.6 ± 0.1[a]	0.4 ± 0.1[a]
304	36.3 ± 0.3[a]	7.6 ± 0.2[a]	3.9 ± 0.1[a]	0.6 ± 0.1[a]
401	36.6 ± 0.5[a]	7.9 ± 0.1[a]	3.6 ± 0.1[a]	0.5 ± 0.1[a]
402	34.3 ± 0.7[a]	7.7 ± 0.1[a]	4.6 ± 0.3[c]	0.5 ± 0.1[a]
405	1.1 ± 0.1[b]	1.6 ± 0.1[b]	35.8 ± 0.3[b]	5.2 ± 0.2[b]
501	37.3 ± 0.3[a]	8.0 ± 0.2[a]	3.4 ± 0.3[a]	0.5 ± 0.1[a]
502	0.9 ± 0.2[b]	1.1 ± 0.3[b]	34.5 ± 1.2[b]	5.5 ± 0.3[b]

[a-c] Mean values with different superscript letters within a column are significantly different at $p \leq 0.05$, n=3.

Table 2.3 Concentration (mg/L) of hydroxycinnamic acids in 2016 Pinot noir at the completion of MLF after inoculation with non-commercial strains of *O. oeni.*

	Caftaric acid	Coutaric acid	Caffeic acid	*p*-coumaric acid
Control No MLF	21.6 ± 0.9[a]	4.3 ± 0.3[a]	2.9 ± 0.4[a]	0.5 ± 0.1[a]
0294	22.1 ± 0.4[a]	4.1 ± 0.1[a]	3.8 ± 0.8[b]	0.5 ± 0.2[a]
0652	20.7 ± 0.3[a]	3.6 ± 0.1[b]	4.1 ± 0.1[b]	0.6 ± 0.1[a]
0651	21.4 ± 0.4[a]	3.4 ± 0.3[b]	3.1 ± 0.1[a]	0.4 ± 0.1[a]
0238	8.9 ± 1.2[b]	1.9 ± 0.8[c]	13.3 ±2.6[c]	1.45 ± 0.1[b]

[a-c] Mean values with different superscript letters within a column are significantly different at $p \leq 0.05$, n=3.

Impact of time, pH, temperature, and ethanol on Pinot noir wine hydroxycinnamic acid content

Concentrations of coutaric and *p*-coumaric acid were monitored during aging at two different temperatures in Pinot noir wines adjusted to two different ethanol and pH values (Tables 2.4-2.7). At the beginning of aging (Day 0), there were large differences in the hydroxycinnamic acid content of the wines depending on whether MLF was conducted by *O. oeni* VFO (Table 2.4, 2.5) or Alpha (Table 2.6, 2.7). In wines where VFO conducted MLF, the coutaric and *p*-coumaric acid concentrations were 0.05 mg/L and 7.47 mg/L respectively. In wines where MLF was conducted by Alpha, the coutaric and *p*-coumaric acid concentrations were 9.09 mg/L and 1.81 mg/L respectively. After 180 days aging, the coutaric and *p*-coumaric acid concentrations in all wines had changed slightly regardless of storage temperature (13 or 20°C), ethanol content (13 or 15%), or pH (3.60 or 3.80) (Table 2.4-2.7).

Table 2.4 Concentration (mg/L) of coutaric and *p*-coumaric acid in Pinot wine at pH 3.6 or 3.8 and ethanol concentrations of 13 or 15% during aging for 0-180 days at 13°C. Malolactic fermentation was conducted by O. oeni VFO.

Ethanol	Time	pH 3.60		pH 3.80	
		Coutaric acid	*p*-coumaric acid	Coutaric acid	*p*-coumaric acid
13 %	Day 0	0.05 ± 0.01*	7.47 ± 0.09	0.05 ± 0.01	7.47 ± 0.09
	Day 30	0.06 ± 0.02	7.50 ± 0.93	0.09 ± 0.02	8.13 ± 1.20
	Day 100	0.15 ± 0.02	9.49 ± 1.69	0.11 ± 0.02	8.38 ± 1.31
	Day 180	0.09 ± 0.03	7.77 ± 0.77	0.07 ± 0.01	7.20 ± 0.22
15 %	Day 0	0.05 ± 0.01	7.47 ± 0.09	0.05 ± 0.01	7.47 ± 0.09
	Day 30	0.08 ± 0.04	8.73 ± 1.48	0.08 ± 0.03	9.71 ± 1.23
	Day 100	0.10 ± 0.06	9.19 ± 1.59	0.07 ± 0.04	8.92 ± 1.40
	Day 180	0.07 ± 0.01	7.81 ± 0.03	0.06 ± 0.02	7.58 ± 0.16

*Standard deviation, n=3.

Table 2.5 Concentration (mg/L) of coutaric and *p*-coumaric acid in Pinot wine at pH 3.6 or 3.8 and ethanol concentrations of 13 or 15% during aging for 0-180 days at 20°C. Malolactic fermentation was conducted by *O. oeni* VFO.

Ethanol	Time	pH 3.60		pH 3.80	
		Coutaric acid	*p*-coumaric acid	Coutaric acid	*p*-coumaric acid
13 %	Day 0	0.05 ± 0.01*	7.47 ± 0.09	0.05 ± 0.01	7.47 ± 0.09
	Day 30	0.12 ± 0.05	7.99 ± 0.21	0.13 ± 0.07	8.52 ± 0.48
	Day 100	0.05 ± 0.01	7.43 ± 0.32	0.23 ± 0.08	7.17 ± 0.90
	Day 180	0.07 ± 0.02	7.61 ± 0.08	0.27 ± 0.29	6.32 ± 0.69
15 %	Day 0	0.05 ± 0.01	7.47 ± 0.09	0.05 ± 0.01	7.47 ± 0.09
	Day 30	0.06 ± 0.03	7.92 ± 0.38	0.14 ± 0.12	7.84 ± 0.55
	Day 100	0.11 ± 0.03	7.76 ± 0.61	0.12 ± 0.03	8.16 ± 0.10
	Day 180	0.07 ± 0.01	7.86 ± 0.03	0.06 ± 0.01	7.82 ± 0.08

*Standard deviation, n=3.

Table 2.6 Concentration (mg/L) of coutaric and *p*-coumaric acid in Pinot wine at pH 3.6 or 3.8 and ethanol concentrations of 13 or 15% during aging for 0-180 days at 13°C. Malolactic fermentation was conducted by *O. oeni* Alpha.

		pH 3.60		pH 3.80	
Ethanol	Time	Coutaric acid	*p*-coumaric acid	Coutaric acid	*p*-coumaric acid
13 %	Day 0	9.09 ± 1.26*	1.81 ± 0.58	9.09 ± 1.26	1.81 ± 0.58
	Day 30	9.09 ± 0.42	1.42 ± 0.14	8.91 ± 0.37	1.15 ± 0.06
	Day 100	9.25 ± 1.55	1.61 ± 0.43	9.19 ± 1.46	1.66 ± 0.37
	Day 180	8.43 ± 1.54	1.17 ± 0.07	7.55 ± 0.03	1.11 ± 0.04
15 %	Day 0	9.09 ± 1.26	1.81 ± 0.58	9.09 ± 1.26	1.81 ± 0.58
	Day 30	9.21 ± 1.42	1.24 ± 0.27	9.88 ± 1.03	1.26 ± 0.50
	Day 100	8.92 ± 1.93	1.30 ± 0.36	9.64 ± 0.87	1.51 ± 0.23
	Day 180	7.50 ± 0.06	1.20 ± 0.06	9.20 ± 1.53	1.27 ± 0.08

*Standard deviation, n=3.

Table 2.7 Concentration (mg/L) of coutaric and *p*-coumaric acid in Pinot wine at pH 3.6 or 3.8 and ethanol concentrations of 13 or 15% during aging for 0-180 days at 20°C. Malolactic fermentation was conducted by *O. oeni* Alpha.

		pH 3.60		pH 3.80	
Ethanol	Time	Coutaric acid	*p*-coumaric acid	Coutaric acid	*p*-coumaric acid
13 %	Day 0	9.09 ± 1.26*	1.81 ± 0.58	9.09 ± 1.26	1.81 ± 0.58
	Day 30	8.96 ± 1.08	1.34 ± 0.12	9.64 ± 0.62	1.22 ± 0.19
	Day 100	7.48 ± 0.07	1.34 ± 0.02	8.58 ± 1.12	1.15 ± 0.43
	Day 180	7.40 ± 0.03	1.35 ± 0.04	7.42 ± 0.02	1.43 ± 0.06
15 %	Day 0	9.09 ± 1.26	1.81 ± 0.58	9.09 ± 1.26	1.81 ± 0.58
	Day 30	10.28 ± 0.62	1.41 ± 0.09	8.46 ± 0.85	1.14 ± 0.30
	Day 100	8.52 ± 0.51	1.40 ± 0.04	9.05 ± 1.26	1.28 ± 0.12
	Day 180	7.38 ± 0.03	1.33 ± 0.04	7.34 ± 0.07	1.34 ± 0.06

*Standard deviation, n=3.

Discussion

The concentration of the 4-EP precursors *p*-coumaric acid and coutaric acid can be impacted throughout the winemaking process. In this study, *S. cerevisiae* strains with and without phenolic acid decarboxylase (PAD) activity were utilized to determine their impact on *p*-coumaric acid concentration. Several studies have demonstrated *S. cerevisiae* decarboxylating activity on hydroxycinnamic acids (Smit et al., 2003; Swiegers et al., 2005; Chatonnet et al., 1993). Morata et al. (2013) suggested that use of yeast with this property could lead to reduced volatile phenol production as vinylphenols produced from the metabolism of *p*-coumaric acid can react with anthocyanins and form stable color compounds, thus reducing the concentration of volatile phenol precursors. In this study, there was unexpectedly no significant difference in *p*-coumaric acid concentrations between wines fermented using PAD (+) (OSU-2) or PAD (-) (CSM) strains of *S. cerevisiae*. Kosel et al. (2014) reported decreased HCA conversion to vinylphenol derivatives by *S. cerevisiae* in the presence of high ethanol concentrations. In the present study, wines produced with PAD (+) *S. cerevisiae* OSU-2 also had the highest alcohol concentration of the wines studied (data not shown). It is possible that high ethanol concentrations were responsible for the lack of HCA conversion in these wines. However, it is more likely that the lack of HCA conversion was simply due to the relatively low *p*-coumaric acid levels in the wine. *p*-Coumaric acid levels differ significantly depending on grape varietal, vintage, geographical origin, grape ripeness, and sun exposure (Nagel et al., 1979). Goldberg et al., (1999) reported an average concentration of 2.4 mg/L *p*-coumaric

acid in Pinot noir produced in the Pacific Northwest. Previous Pinot noir vintages as well as Pinot noir used in this study made using grapes from Woodhall Vineyard at the OSU Pilot Winery have had consistently low levels of *p*-coumaric acid, near 1 mg/L (Schopp et al., 2013; Burns and Osborne, 2013; Chescheir et al., 2015).

Numerous studies have demonstrated the antimicrobial effects of hydroxycinnamic acids (Herald and Davidson, 1983; Tunçel and Nergiz, 1993; Wansi et al., 2010; Teodoro et al., 2015; Bisogno et al., 2007; Aziz et al., 1998). Therefore, it has been suggested that PAD activities by microorganisms may serve to convert the antimicrobial compounds to less toxic substances (Sánchez-Maldonado et al., 2011; Campos et al., 2009; Stead, 1993). Because levels of *p*-coumaric acid were relatively low, it is possible concentrations were not high enough to exhibit the antimicrobial activity on *S. cerevisiae* which would necessitate the conversion to vinylphenols.

Aside from using PAD (+) *S. cerevisiae* strains, an additional way that the concentrations of *p*-coumaric and coutaric acid may change during winemaking is through the action of *O. oeni*. Cheschier et al. (2015) reported that certain strains of *O. oeni* can degrade tartaric acid-bound hydroxycinnamic acids via cinnamoyl esterase (CE) activity, resulting in a higher concentration of *p*-coumaric acid in wine after MLF. This finding is supported by the results from the current study as wine where MLF was conducted by *O. oeni* VFO, a strain known to be CE (+), contained a significantly higher concentration of *p*-coumaric acid compared to wine where MLF

was conducted by the CE (-) bacteria *O. oeni* Alpha. A corresponding decrease in coutaric acid was noted in wines where VFO conducted MLF as would be expected. When the CE (+) *O. oeni* VFO was used in combination with the PAD (+) yeast *S. cerevisiae* OSU-2 to perform a simultaneous fermentation, a reduction in coutaric acid was noted but the expected increase in *p*-coumaric acid concentration did not occur. This may be due to the metabolism of *p*-coumaric acid by OSU-2 into 4-vinylphenol but this hypothesis was not confirmed as 4-vinylphenol was not measured in this study. It is possible that the higher concentration of *p*-coumaric acid present in the wine due to the action of *O. oeni* VFO stimulated the PAD (+) *S. cerevisiae* to initiate its HCA decarboxylating activity, thereby reducing the final concentrations of both coutaric and coumaric acid. Again, this would have to be confirmed through analysis of 4-vinylphenol and its anthocyanin derivatives. Overall, the use of a simultaneous inoculation of a PAD (+) *S. cerevisiae* and a CE (+) *O. oeni* reduced the total amount of 4-EP precursors and may be an additional strategy to minimize the risk of *Brettanomyces* wine spoilage.

To date, *O. oeni* VFO is one of the few strains that have been reported to have cinnamoyl esterase activity in wine (Cheschier et al., 2015). Aside from the ten strains that Cheschier et al. (2015) tested, an additional eleven commercial strains of *O. oeni* were screened in the present study and two of these strains exhibited cinnamoyl esterase activity. Furthermore, of the 4 non-commercial strains screened, one demonstrated cinnamoyl esterase activity. This finding shows that the CE trait

exists among native strains. Fortunately, commercial producers are screening for this trait so it can be avoided. However, if a winemaker is conducting a native fermentation, there is no real control over the strain performing malolactic fermentation and as such, there is an increased risk of a CE (+) strain performing the MLF which can potentially lead to an increase in volatile phenol precursors usable by *Brettanomyces*. This is one additional reason why the use of well-characterized commercial *O. oeni* strains are recommended for conducting MLF.

While there is clear evidence that the bacteria used to conduct MLF can change the concentration of *p*-coumaric acid in the wine post-MLF, there is still some debate over the significance of this regarding *Brettanomyces* wine spoilage. Chescheir et al. (2015) reported that when *B. bruxellensis* grew in wine where MLF was conducted by *O. oeni* VFO, significantly higher concentrations of 4-EP and 4-EG were produced compared to wines where MLF was conducted by CE (-) *O. oeni* strains. Chescheir et al. (2015) concluded that use of *O. oeni* strains that can degrade tartaric acid ester-bound hydroxycinnamic acids should be avoided in barrel-aged red wines that are most at risk for spoilage by *Brettanomyces*. In contrast, Madsen et al. (2016) suggested that the level of volatile phenols in wine depends more on strain differences of *B. bruxellensis* than on cinnamoyl esterase activity of *O. oeni*. Madsen et al. (2016) noted that during the aging of wine over a 180-day period, there was hydrolysis of coutaric acid to *p*-coumaric acid but that the conversion due to MLF

bacteria was unlikely to be as significant to the production of volatile phenols by *B. bruxellensis* as reported by Cheschier et al. (2015).

To determine if changes in *p*-coumaric acid content during aging negated the impact of MLF by CE (+) *O. oeni,* a wine aging study was conducted with Pinot noir wine that underwent MLF with either a CE (+) or CE (-) *O. oeni*. While Madsen et al. (2016) investigated changes in *p*-coumaric acid during aging under one condition (Cabernet sauvignon wine, pH 3.50, 12.6 (v/v) % alcohol), the current study determined the effect of different ethanol contents, pH, and storage temperatures on a wine's hydroxycinnamic acid content. Unlike Madsen et al. (2016), concentrations of *p*-coumaric acid in Pinot noir did not differ during aging with high *p*-coumaric acid being present in wines that underwent MLF with a CE (+) *O. oeni* while low concentrations were present in wines that underwent MLF with a CE (-) *O. oeni*. Ethanol content, pH, and storage temperature had no effect on *p*-coumaric and coutaric acid concentrations, demonstrating the relative stability of these compounds under these conditions.

While others have shown that coutaric may be converted to *p*-coumaric through acid hydrolysis (Kallithraka et al., 2009; Zafrilla et al., 2003), the degree and speed of coutaric acid degradation by the CE (+) *O. oeni* VFO is significantly higher compared to its degradation over time by hydrolysis alone. For example, while Kallithraka et al. (2009) did note a decrease in the tartaric esters of HCAs, over 65% of the caftaric

acid and 60% of the initial coutaric acid remained nine months after bottling. In contrast, nearly 100% of the caftaric and coutaric acid was converted by *O. oeni* VFO during MLF (Cheschier et al., 2015).

A potential explanation for some of the differences between results from the current study and previous reports regarding changes in *p*-coumaric acid concentrations during aging may be due to differences in how the wine was prepared prior to aging. While in the present study wines were sterile-filtered prior to aging, Madsen et al. (2016) did not sterile filter their wines and reported populations of yeast and lactic acid bacteria in their control wines. As certain *S. cerevisiae* strains and lactic acid bacteria can degrade *p*-coumaric acid (Smit et al., 2013; Sánchez-Maldonado et al., 2011; Curiel et al., 2010; Swiegers et al., 2005; Chatonnet et al., 1993), it cannot be concluded that the background microorganisms present in the Madsen et al. (2016) study did not have a significant impact on total hydroxycinnamic acid and volatile phenol concentrations. In fact, changes in the *p*-coumaric acid content of the control wines was noted during aging but no explanation for these changes was given. In the same manner, Kallithraka et al. (2009) did not report any information regarding microorganisms used for fermentations or the use of sterile filtration pre-bottling and so the impact of microbes on the results is unknown.

Conclusions

The present study demonstrated that overall, wine parameters do not appear to have a large impact on the degradation of tartaric acid-bound hydroxycinnamic acids whether due to acid hydrolysis or other mechanisms. While the amount of bound HCAs changed slightly during aging, the degree and speed of tartaric acid-bound HCA degradation does not compare to degradation noted when *O. oeni* strains possessing cinnamoyl esterase activity are used for malolactic fermentation. The use of a CE (+) strain in sequential AF/MLF fermentations lead to an increase in available 4-EP precursors while during a simultaneous fermentation the use of a PAD (+) *S. cerevisiae* and a CE (+) *O. oeni* reduced the total amount of 4-EP precursors. Additionally, utilizing native rather than inoculated malolactic fermentations may increase the risk of MLF being performed by a CE (+) strain. While the strain of *B. bruxellensis* that infects the wine may significantly impact the concentration of volatile phenols present, the winemaker has no control over what strain this might be. In contrast, the winemaker has control of the *O. oeni* strain used for MLF and so this choice is an important step in strategies to prevent wine spoilage by *B. bruxellensis*.

Chapter 3

Impact of *Oenococcus oeni* on *Brettanomyces bruxellensis* growth and volatile phenol production

Abstract

The effect of the timing of a *Brettanomyces bruxellensis* infection relative to the end of malolactic fermentation (MLF) on *B. bruxellensis* growth and volatile phenol production was investigated. MLF was carried out by one of three strains. Upon completion, treatments were pulled and inoculated with *B. bruxellensis* at 0, 34, and 112 days post-MLF. Growth of *B. bruxellensis* and *O. oeni* were monitored and volatile phenol (4-ethylphenol and 4-ethylguaiacol) concentrations in the wine were also measured. *O. oeni* populations in the wines were still high directly after the completion of MLF and *B. bruxellensis* populations declined rapidly after inoculation. 34 days post-MLF, high populations of two *O. oeni* strains were still present in the wine but no culturable cells were detected in the wine for the third strain. When *B. bruxellensis* was inoculated into the wine containing no culturable *O. oeni* cells, it grew well. In contrast, *B. bruxellensis* populations rapidly declined when inoculated into wine where there were still culturable *O. oeni* cells. 112 days post-MLF, no culturable *O. oeni* cells were detected in any of the wines and *B. bruxellensis* grew well in all but one of the wines. The sensitivity of different *B. bruxellensis* strains to the presence of culturable *O. oeni* post-MLF was tested by inoculating six different strains of *B. bruxellensis* into the control wines (No MLF) or wine that had undergone

MLF by *O. oeni*. Subsequent growth and volatile phenol production was tracked. The degree of inhibition on growth and volatile phenol production was strain dependent where the growth of some *B. bruxellensis* strains in wine that underwent MLF was comparable to growth in the control wine. For other strains, there was reduced or no growth in wines that had undergone MLF. All six strains of *B. bruxellensis* produced significantly less volatile phenols in wines that had undergone MLF compared to those that had not. These findings suggest MLF may offer limited protection against *B. bruxellensis* infection due to the presence of live *O. oeni* cells post-MLF but that this may be dependent on the *B. bruxellensis* strain present.

Introduction

Brettanomyces bruxellensis is considered one of the most problematic wine spoilage yeasts because it is difficult to control and can lead to significant financial losses due to prevention and remediation measures (Puig et al., 2011; Loureiro and Malfeito-Ferreira, 2003). Through its production of volatile phenols 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG), it lends undesirable aromas of barnyard, horsey, Band-Aid, medicinal, smokey, and clove to the wine (Šućur et al., 2016; Chatonnet et al., 1992; Chescheir, 2014). Winemakers are limited in the tools available to prevent infection and growth of *B. bruxellensis* in wine. Robust tank/barrel cleaning and sanitation programs coupled with the use of the antimicrobial sulfur dioxide (SO_2) is the typical strategy a winery will employ to minimize the risk of *B. bruxellensis* spoilage, yet *Brettanomyces* spoilage still frequently occurs (Puig et al., 2011). Furthermore, there

are large differences reported in strain sensitivity to SO_2 with some strains of *B. bruxellensis* demonstrating resistance to this antimicrobial agent (Zuehlke et al., 2013).

Wine is particularly susceptible to *B. bruxellensis* infection during the window between the end of MLF and SO_2 additions. The raised pH post-MLF as well as lack of antimicrobial SO_2 provide an inviting environment for *Brettanomyces* proliferation. When MLF is performed uninoculated to allow the winery's residential bacteria to execute MLF, sluggish or stuck fermentations often ensue (Fugelsang and Edwards, 2007). Because SO_2 is effective against MLF bacteria, it cannot be added until the completion of MLF. As such, Gerbaux et al. (2009) suggested that a rapid MLF initiated by a commercial starter would allow SO_2 to be added to the wine sooner and would therefore be helpful in the prevention of a *Brettanomyces* infection. Gerbaux et al. (2009) also noted decreased volatile phenol production by *Brettanomyces* in wines which had undergone MLF compared to those that hadn't. The cause of MLF resulting in a reduction in volatile phenols in the wine was not reported by Gerbaux et al. (2009). One possibility is that the presence of *O. oeni* in the wine resulted in reduced growth of *B. bruxellensis* as *B. bruxellensis* has been reported to be a poor competitor with another common wine microorganism, *Saccharomyces cerevisiae* (Renouf et al., 2006). However, no population data for *B. bruxellensis* and *O. oeni* was provided by Gerbaux et al. (2009), making it difficult to conclude whether *B. bruxellensis* populations were impacted by *O. oeni*. Subsequent studies by

Chescheir (2014) investigated this inhibition and it was noted that not only did MLF result in a decrease in volatile phenol production but that *B. bruxellensis* growth was also inhibited as well. The greatest inhibition was noted in wines containing culturable *O. oeni* but the mechanism behind the inhibition remains unknown. In the 2014 study, *B. bruxellensis* was inoculated directly at the end of MLF but researchers did not investigate the length of the inhibitory effect post-MLF. Finally, Chescheir (2014) also only used one *B. bruxellensis* strain and so it is unknown if other *B. bruxellensis* strains are also sensitive to the presence of *O. oeni* cells at the end of MLF. Therefore, the objectives of this study were to investigate the effect of timing of *B. bruxellensis* infection post-MLF on the growth and volatile phenol production. Additionally, several more *B. bruxellensis* strains from different geographical regions were tested for their sensitivity to the presence of *O. oeni* cells at the end of MLF.

Materials and Methods

Microorganisms

S. cerevisiae Enoferm CSM and RC212 were sourced from Lallemand (Montreal, Canada). *O. oeni* strains Enoferm Alpha, Lalvin VP41, and Enoferm Beta were sourced from Lallemand. *B. bruxellensis* strain UCD VEN # 2049 was obtained from the UC Davis Department of Viticulture and Enology Culture Collection housed in the UC Davis Department of Viticulture and Enology, University of California, Davis, CA 95616 U.S.A. *B. bruxellensis* strain Copper Mountain was provided by Dr. Alan Bakalinsky (Oregon State University); strains B1b and E1 were provided by Dr

Charles Edwards (Washington State University; Pullman, Washington, USA) and were originally isolated from Washington wines as described by Jensen et al., (2009); strain AWRI-1499 was sourced from the Australian Wine Research Institute (Adelaide, Australia), and strain 643 was provided by E. & J. Gallo Winery (Modesto, CA).

O. oeni and *B. bruxellensis* cultures were prepared as described by Chescheir et al. (2015) for storage in glycerol (15%) at -80°C until needed. Microorganisms were prepared from frozen cultures by inoculation into acidic grape juice broth (AGJ) (2.5 mg/L manganese sulfate, 125 mg/L magnesium sulfate, 5g/L yeast extract, 1 mL/L 5% (w/w) Tween 80, 250 mL/L white grape juice, pH 3.5) and grown at 25°C for five (*B. bruxellensis*) or seven (*O. oeni*) days. Cells were harvested by centrifugation (4,000 x g for 20 minutes) and re-suspended in 0.2 M phosphate buffer (pH 7.0) prior to inoculation. *S. cerevisiae* CSM and RC212 were used as freeze-dried cultures direct from the manufacturer.

Timing of B. bruxellensis *infection post-MLF*

Pinot noir wine was prepared in 2015 with grapes sourced from Woodhall Vineyard (Alpine, Oregon, USA). After harvest, grapes were stored overnight at 4°C before being destemmed. Approximately 25 kg of grapes were then divided into 40 L plastic fermenters and after mixing, samples were taken for pH, sugar content (°Brix), titratable acid (TA), and yeast assimilable nitrogen (YAN). Basic juice parameters of

the 2015 Pinot noir must were 24.8°Brix, pH 3.42, 6.4 g/L titratable acid, and 110 mg/L YAN. The yeast nutrient Fermaid K (Lallemand) was added to each tank at 0.25 g/L. A set of three tanks was inoculated with *S. cerevisiae* CSM at approximately 1 x10^6 cfu/mL after rehydration according to manufacturer's recommendations. Fermenters were placed in a temperature-controlled room set at 27°C and punched-down twice daily. Fermentations were monitored by assessing °Brix (Anton-Paar DMA 35N Density Meter (Graz, Austria)). When all alcoholic fermentations were complete (< 0.25 g/L reducing sugar as measured by Clinitest®), wines were pressed and placed in a cold room at 4°C for 48 hours to cold settle. Following cold settling, wines were racked and then filtered through a plate and frame filter fitted with 20 cm x 20 cm Beco K-1 3.0μm nominal filter sheets (Langenlonsheim, Germany). Wines were then filtered through a 1.0μm nylon cartridge (G.W. Kent, Ypsilanti, Michigan, USA) and a 0.45μm sterile polyethersulfone cartridge (Merck-Millipore, Massachusetts, USA) in succession and dispensed into 1 and 3-gallon sterilized glass carboys. Sterile filtered wine was stored at 4°C until needed. Basic wine parameters were pH 3.71, 7.5 g/L titratable acid, 14% (v/v) ethanol.

Sterile Pinot noir wine was transferred aseptically into sterile 1-liter glass media storage bottles (Kimble Chase, Rockwood, Tennessee, USA), with 1000 mL aliquoted into each bottle. The wines were warmed to room temperature before inoculation with either *O. oeni* Alpha, *O. oeni* Beta, or *O. oeni* VP41 at

approximately 1 x 10^6 cfu/mL in duplicate for MLF, after preparation as previously detailed. Wines were incubated at 25°C and samples were taken weekly and monitored for malic acid (Vintessential Enzymatic L-Malic Acid Test Kit; Victoria, Australia). A set of bottles were not inoculated for MLF and served as the control. At the completion of MLF (malic acid < 50 mg/L), duplicate wines were aseptically combined and mixed before transfer to sterile milk dilution bottles, with 100 mL aliquoted into each. A set of three wines from each treatment were inoculated with *B. bruxellensis* UCD-2049 at approximately 1x10^4 CFU/mL. These represent the 'Day 0' treatment. The remaining wine was stored at 25°C until needed. After 34 and 112 further days, another set of wines from each treatment was pulled and *B. bruxellensis* UCD-2049 was inoculated at approximately 1x10^4 CFU/mL.

B. bruxellensis and *O. oeni* populations were monitored weekly for up to 90 days post-inoculation by plating on either YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar, pH 6.50) for *B. bruxellensis* or de Man, Rogosa, and Sharpe (MRS) plates containing 100 mg/L cycloheximide (20 g/L tryptone, 5 g/L peptone, 5 g/L yeast extract, 5 g glucose, 1 mL/L 5% (w/w) Tween 80, 250 mL/L apple juice, 100 mg/L cycloheximide, 20 g/L agar) for *O. oeni*. Plates were incubated for 5 (YPD) or 7 (MRS) days at 25°C before being counted. Aside from plating, samples (1 mL) were also taken for analysis of hydroxycinnamic acids by HPLC-DAD. On the final day of sampling for each treatment, 50 mL samples were pulled

from each replicate for analysis of volatile phenols by GC-MS. All samples were stored at -12°C until needed for analysis.

Impact of O. oeni *on various* Brettanomyces bruxellensis *strains*

Pinot noir wine was produced in 2016 with grapes sourced from Woodhall Vineyard (Alpine, OR) following the same protocols as outlined previously for 2015 wine production. In 2016, the grapes at harvest had very high sugar content (27 °Brix) and so an addition of distilled water was made to each fermenter to reduce the initial Brix to approximately 24 °Brix. The other basic grape parameters were pH 3.40, 4.35 g/L titratable acid, and 143 mg/L YAN. The yeast nutrient Fermaid K (Lallemand) was added to each tank at 0.25 g/L prior to inoculation with *S. cerevisiae* RC212 (Lallemand) at approximately 1 x 10^6 cfu/mL after rehydration according to manufacturer's recommendations. At the completion of alcoholic fermentation, wines were pressed and placed in a cold room at 4°C for 48 hours to cold settle. Following cold settling, wines were racked and then filtered through a plate and frame filter fitted with 20 cm x 20 cm Beco K-1 3.0μm nominal filter sheets (Beco). Wine was then filtered through a 1.0μm nylon cartridge (G.W. Kent) and a 0.45μm sterile polyethersulfone cartridge (Merck-Millipore) in succession and dispensed into 3-gallon sterilized glass carboys. Sterile filtered wine was stored at 4°C until needed. Basic wine parameters were pH 3.65, 6.9 g/L titratable acid, 13.1% (v/v) ethanol.

When needed, wine was removed from cold storage and aseptically separated into sterile 0.5 L glass media storage bottles (Kimble Chase, Rockwood, Tennessee, USA). After being warmed to room temperature, wine was inoculated with *O. oeni* VP41 at approximately 1 x 10^6cfu/mL to induce MLF after preparation in AGJ broth as previously described. A set of three 500 mL bottles of wine were not inoculated with *O. oeni* to act as a control. At the completion of MLF (malic acid < 50 mg/L) wine was dispensed into 100 mL milk dilution bottles and a set of three bottles was inoculated with either *B. bruxellensis* strain UCD 2049, Copper Mountain, B1b, AWRI-1499, 643, or E1 at approximately 10^4 cfu/mL after preparation in AGJ broth (pH 3.50) as previously described. All wines were incubated at 25°C, sampled weekly, and plated on YPD agar to monitor *B. bruxellensis* culturable cells. Samples (1 mL) were also taken for analysis of hydroxycinnamic acids by HPLC-DAD. On the final day of sampling for each treatment, 50 mL samples were pulled from each replicate for analysis of volatile phenols by GC-MS. All samples were stored at -12°C until needed for analysis.

Hydroxycinnamic acid analysis

Hydroxycinnamic acids (free and tartaric acid-bound) were determined by HPLC-DAD as described by Burns and Osborne (2013). Prior to HPLC analysis, samples were centrifuged using an Allegra X-22 instrument (Beckman Coulter, Brea, CA) at 10,000 *g* for 10 min. 20 µL aliquots were injected and hydroxycinnamic acids detected by scanning from 200 to 400 nm. Identification and quantification of

hydroxycinnamic acids was performed at 320 nm based on UV-visible spectra and retention times of known standards obtained from Sigma-Aldrich. Calibration curves were prepared for *p*-coumaric quantification. Due to the lack of available standards, coutaric acid is reported as *p*-coumaric acid equivalents.

Volatile phenol analysis

Wine volatile phenols were quantified by ethylene glycol-polydimethylsiloxane based stir bar sorptive extraction and gas chromatography–mass spectrometry as described by Zhou et al. (2015).

Statistical Analysis

Statistical analysis of the volatile phenols was performed using a two-tail Welch's t-test comparison of the means to determine significance ($p<0.05$).

Results

While no *O. oeni* culturable cells were detected in the control wine that did not undergo MLF, relatively high *O. oeni* populations were still present in the wines that had undergone MLF (Figure 3.1A). *O. oeni* Alpha and VP41 were detected at approximately 10^5 cfu/mL and 10^4 cfu/mL, of *O. oeni* Beta was detected. High culturable populations of *O. oeni* strain Alpha and VP41 remained in the wines until 55 days post-MLF when populations declined until after 75 days no culturable *O. oeni* cells were detected (Figure 3.1A). *O. oeni* strain Beta culturable cell populations

declined below detectable levels much faster as no detectable cells were noted four days after the end of MLF (Figure 3.1A). When *B. bruxellensis* was inoculated into these wines directly after the end of MLF (Day 0) culturable cell counts of *Brettanomyces* decreased below detectable levels in all treatments including the control wine that had not undergone MLF (Figure 3.1B).

Wines that were inoculated with *B. bruxellensis* 34 days post-MLF still contained high populations of *O. oeni* strain Alpha and VP41 but no culturable cells of *O. oeni* strain Beta were detected (Figure 3.2A). Both *O. oeni* Alpha and VP41 remained at populations above 10^5 cfu/mL until about 65 days post-MLF where populations began to decline until no culturable cells were detected (Figure 3.2A). Growth data for *B. bruxellensis* in wines inoculated 34 days post-MLF can be found in Figure 3.2B. After an initial dip in culturable cells, growth of *B. bruxellensis* in the control reached high concentrations above 10^7 cfu/mL, reaching stationary phase by about 65 days post-MLF. In wines containing *O. oeni* Alpha, *B. bruxellensis* populations rapidly declined after inoculation and were not detected over the course of the experiment (> 90 days). This also occurred in wines containing VP41 (Figure 3.2B). However, in wines were *O. oeni* Beta had conducted MLF, *B. bruxellensis* grew in a similar manner to the control wine with populations of over 10^7 cfu/mL being reached (Figure 3.2B).

By 112 days post-MLF there were no detectable *O. oeni* culturable cells present in the wines no matter which *O. oeni* strain had been used (Figure 3.3A). *B. bruxellensis* grew well in the control wine and in wine where MLF had been carried out by *O. oeni* Alpha (Figure 3.3B). In wines where *O. oeni* Beta had performed MLF, there was an initial decline in *B. bruxellensis* growth and an increased lag phase with populations of the yeast never reaching 10^6 cfu/mL (Figure 3.3B). In wines where *O. oeni* VP41 was used for MLF, *B. bruxellensis* populations declined rapidly when inoculated 112 days post-MLF as had occurred after 0 and 34 days post-MLF and no culturable cells of *B. bruxellensis* were detected during the experiment (Figure 3.3B).

Ninety days post-*B. bruxellensis* inoculation the wines were assessed for volatile phenol concentration. The concentrations in the wine reflected the differences noted in *B. bruxellensis* growth (Table 3.1). For example, in wines inoculated with *B. bruxellensis* 34 days post-MLF, strong growth was noted in the control and *O. oeni* Beta wines. These wines with strong *B. bruxellensis* growth also had significantly higher volatile phenol concentrations, 102.14 and 142.25 µg/L respectively, than wines where *B. bruxellensis* had grown poorly (*O. oeni* Alpha and VP41-inoculated wines). This trend was also seen in the wines inoculated 112 days post-MLF. Strong growth was seen in the control and *O. oeni* Alpha wines and correspondingly, 102.99 and 146.77 µg/L of volatile phenols were present (Table 3.1). Additionally, in *O. oeni* Beta wines inoculated with *B. bruxellensis* 112 days post-MLF there was only a slight

increase in volatile phenols, which appeared to relate to the comparatively low growth of *B. bruxellensis* which occurred in those wines (Figure 3.3B).

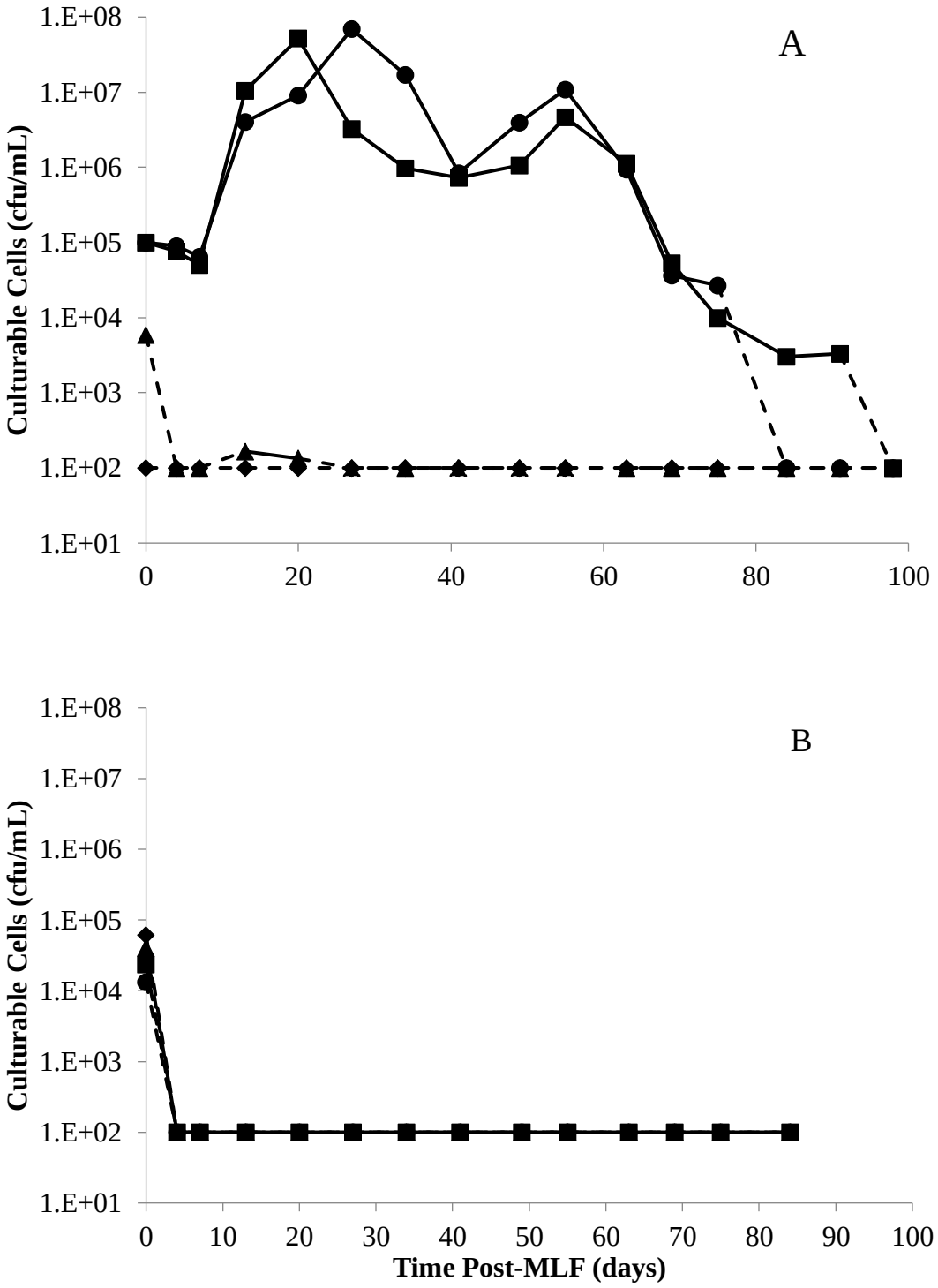

Figure 3.1 Culturable *O. oeni* (A) and *B. bruxellensis* (B) cells in Pinot noir wine that did not undergo MLF (♦) and wine that underwent MLF with *O. oeni* Alpha (▪), *O. oeni* Beta (▲), and *O. oeni* VP41 (●). *B. bruxellensis* was inoculated 0 days after the completion of MLF. Data points represent mean of replicates, n=3.

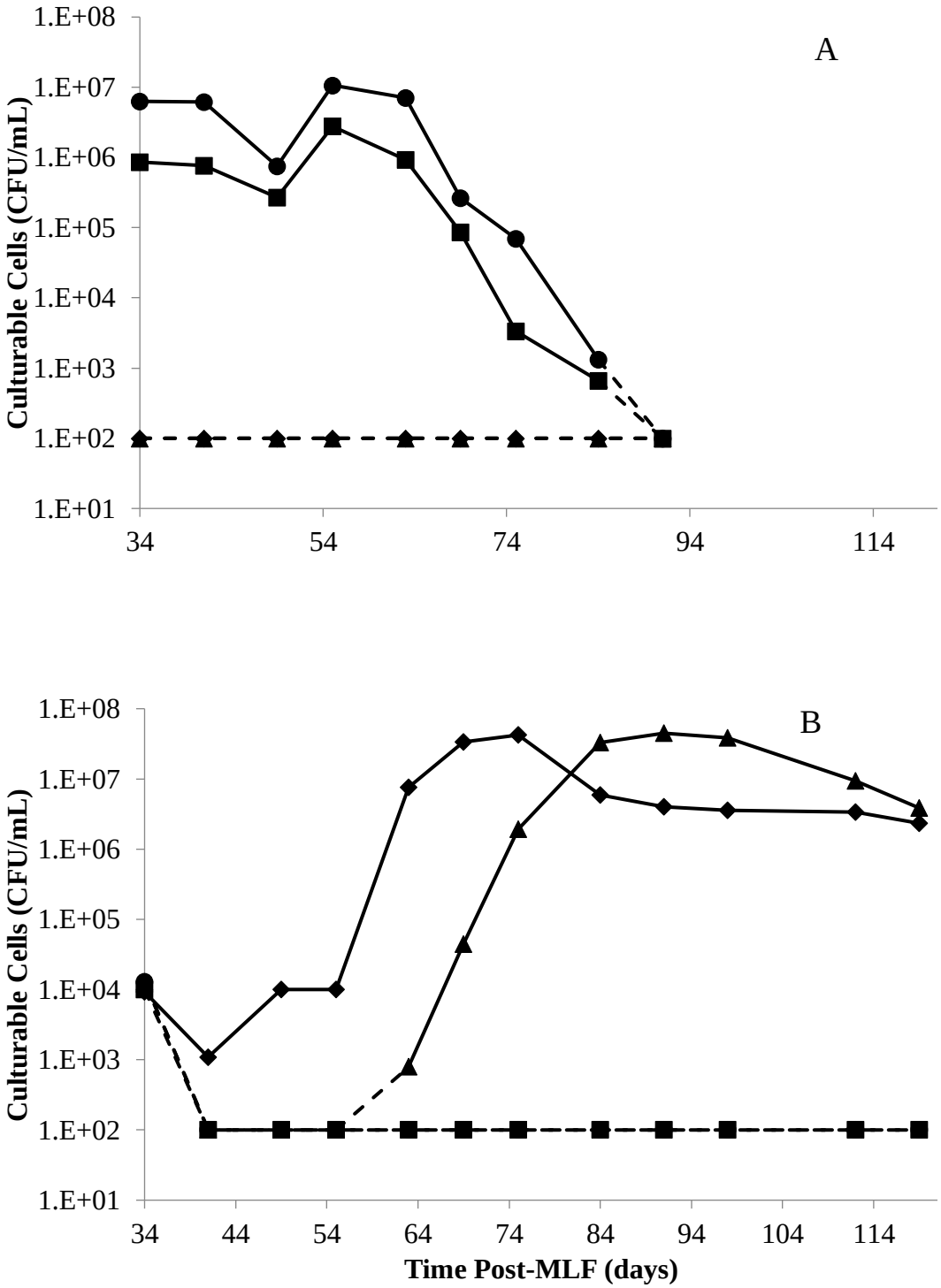

Figure 3.2 Culturable *O. oeni* (A) and *B. bruxellensis* (B) cells in Pinot noir wine that did not undergo MLF (♦) and wine that underwent MLF with *O. oeni* Alpha (▪), *O. oeni* Beta (▲), and *O. oeni* VP41 (●). *B. bruxellensis* was inoculated 34 days after the completion of MLF. Data points represent mean of replicates, n=3.

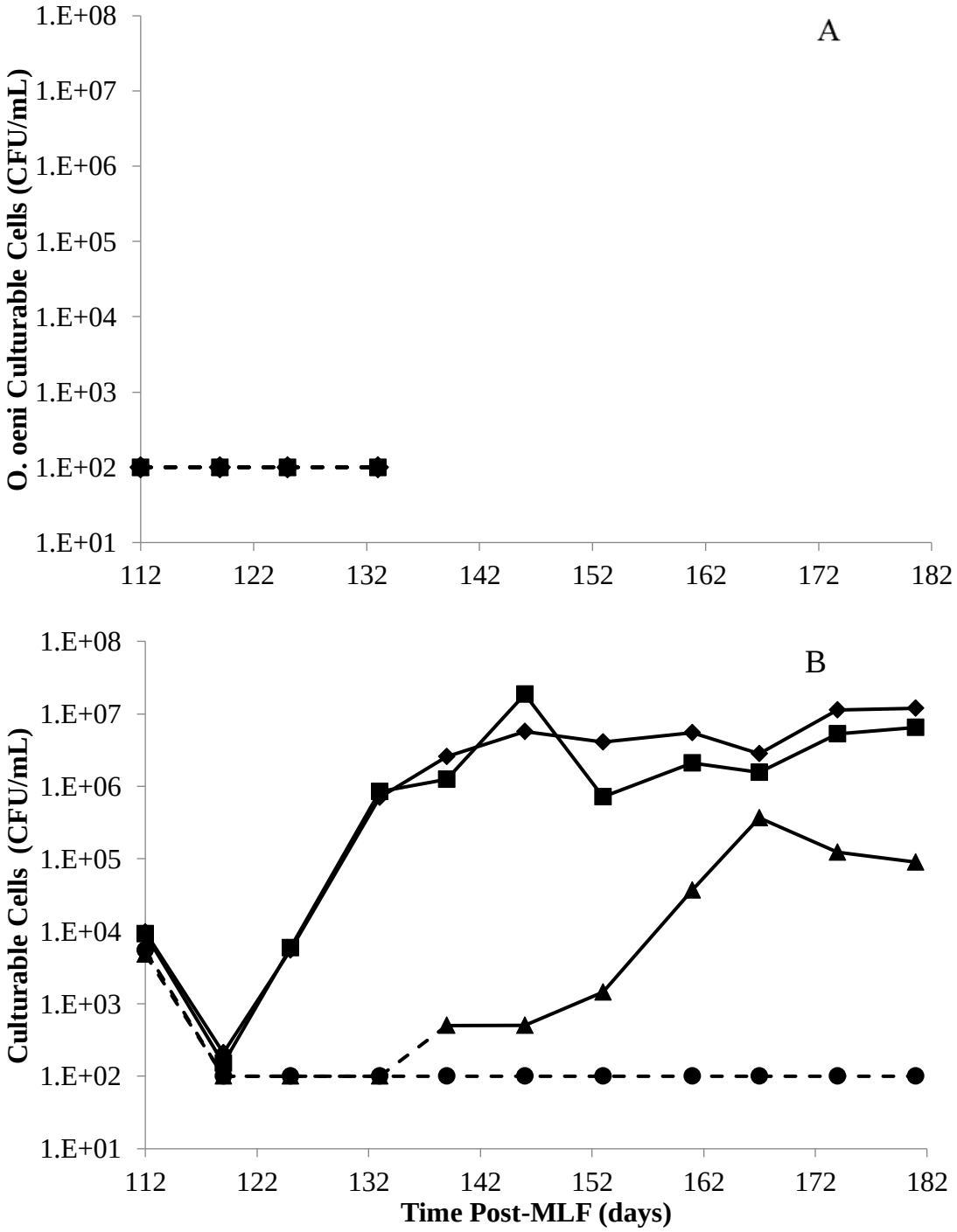

Figure 3.3 Culturable *O. oeni* (A) and *B. bruxellensis* (B) cells in Pinot noir wine that did not undergo MLF (♦) and wine that underwent MLF with *O. oeni* Alpha (▪), *O. oeni* Beta (▲), and *O. oeni* VP41 (●). *B. bruxellensis* was inoculated 112 days after the completion of MLF. Data points represent mean of replicates, n=3.

Table 3.1 Concentration (μg/l) of volatile phenols (4-ethylguaiacol + 4-ethylphenol) on the final day of sampling in 2015 Pinot noir wines inoculated with *B. bruxellensis* 0, 34, and 112 days post-MLF conducted by three different *O. oeni* strains.

	0 Days Post-MLF	34 Days Post-MLF	112 Days Post-MLF
Control	0.65 ± 0.02^{a}	102.14 ± 7.41^{a}	102.99 ± 2.99^{a}
O. oeni Alpha	0.55 ± 0.03^{a}	1.45 ± 0.30^{b}	146.77 ± 8.45^{b}
O. oeni Beta	0.51 ± 0.03^{b}	142.25 ± 5.16^{c}	3.56 ± 0.83^{c}
O. oeni VP41	0.44 ± 0.01^{c}	1.52 ± 0.46^{b}	1.57 ± 0.41^{c}

[a-b]Mean values with different superscript letters within a column are significantly different where $p \leq 0.05$, n=3.

A number of *B. bruxellensis* strains were screened for their reaction to the presence of high populations of *O. oeni* VP41 at the end of MLF. Strains 643, B1b, E1, and AWRI-1499 grew well in wines that had not undergone MLF (Figure 3.4, 3.5). Similar growth was also seen for these *B. bruxellensis* strains when inoculated into wines that had undergone MLF with *O. oeni* VP41, although there was a slight increase in lag phase compared to their respective controls (Figure 3.5). In contrast, *B. bruxellensis* strain UCD-2049 populations declined rapidly after inoculation into wine that had undergone MLF with *O. oeni* VP41 but grew well in the control wine that had not undergone MLF (Figure 3.5). For strain Cooper Mt., there was a prolonged lag phase compared to the other *B. bruxellensis* strains in both the control and MLF wines (Figure 3.5). However, growth recovered quicker in the control wine that had not undergone MLF than in the wine where *O. oeni* VP41 was present.

As was seen in the MLF timing experiment, *B. bruxellensis* growth and volatile phenol production followed a similar trend. In the wines which had strong growth of *B. bruxellensis*, higher concentrations of volatile phenols were noted compared to wines were weak to no growth of *B. bruxellensis* had occurred (Table 3.2). However, for all *B. bruxellensis* strains there were lower concentrations of volatile phenols in wines that had undergone MLF with *O. oeni* VP41when compared to the control wines (Table 3.2). For example, *B. bruxellensis* strain B1b grew to populations > 10^6 cfu/mL in wines that did or did not undergo MLF and yet 135.95 μg/L of volatile phenols were present in wines that had not undergone MLF while only 108.43 μg/L was present in wines where *O. oeni* VP41 conducted MLF (Table 3.2).

Table 3.2 Concentration (μg/l) of volatile phenols (4-ethylguaiacol and 4-ethylphenol) on the final day of sampling in 2016 Pinot noir wine inoculated with various *B. bruxellensis* strains following the end of MLF conducted with *O. oeni* VP41.

	UCD-2049	Copper Mt.	AWRI 1499
Control (No MLF)	98.69 ± 13.98 [a]	113.81 ± 9.12[a]	123.71 ± 6.63[a]
+ *O. oeni* VP41	0.49 ± 0.03[b]	72.19 ± 60.56[b]	99.30 ± 4.34[b]
	643	B1b	E1
Control (No MLF)	117.69 ± 1.18[a]	135.95 ± 8.09[a]	129.23 ± 13.20[a]
+ *O. oeni* VP41	104.18 ± 11.69[b]	108.43 ± 23.43[b]	106.72 ± 6.44[b]

[a-b]Mean values with different superscript letters within a column are significantly different where $p \leq 0.05$, n=3.

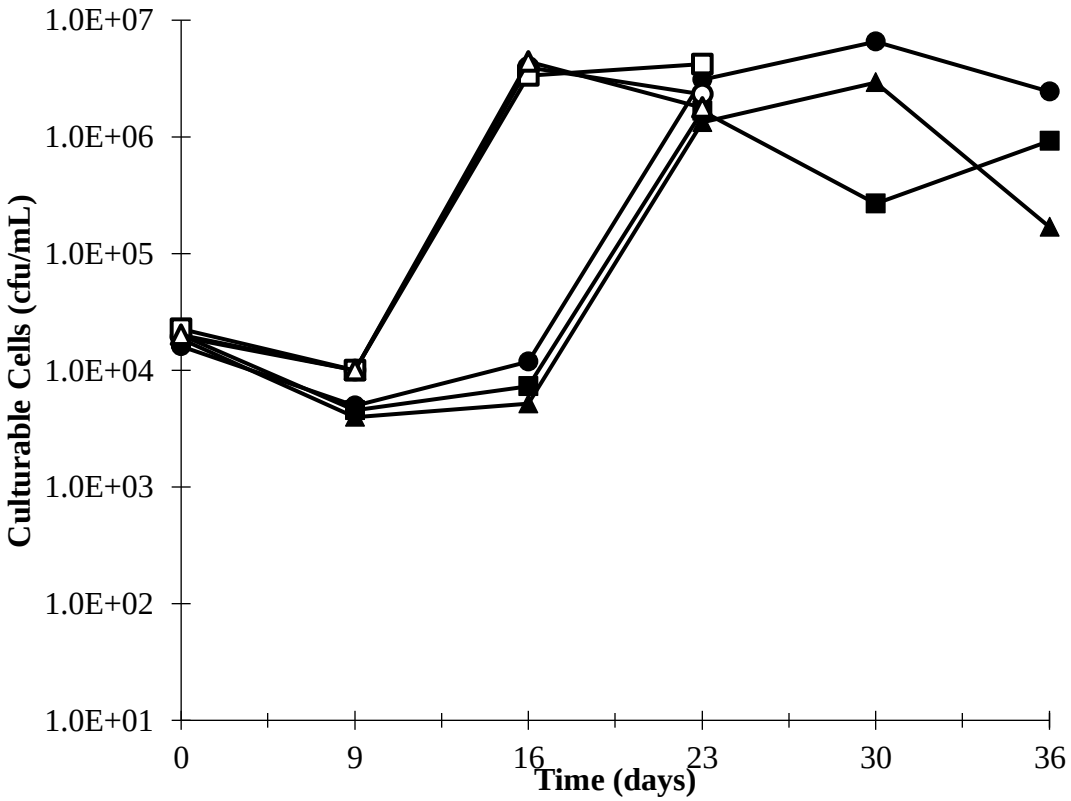

Figure 3.4 Culturable cells of *B. bruxellensis* strain B1b (●○), E1 (■□), and AWRI-1499 (▲Δ) after inoculation into Pinot noir wine that has not undergone MLF (open symbols) or that underwent MLF conducted by *O. oeni* VP41 (closed symbols). Data points represent mean of replicates, n=3.

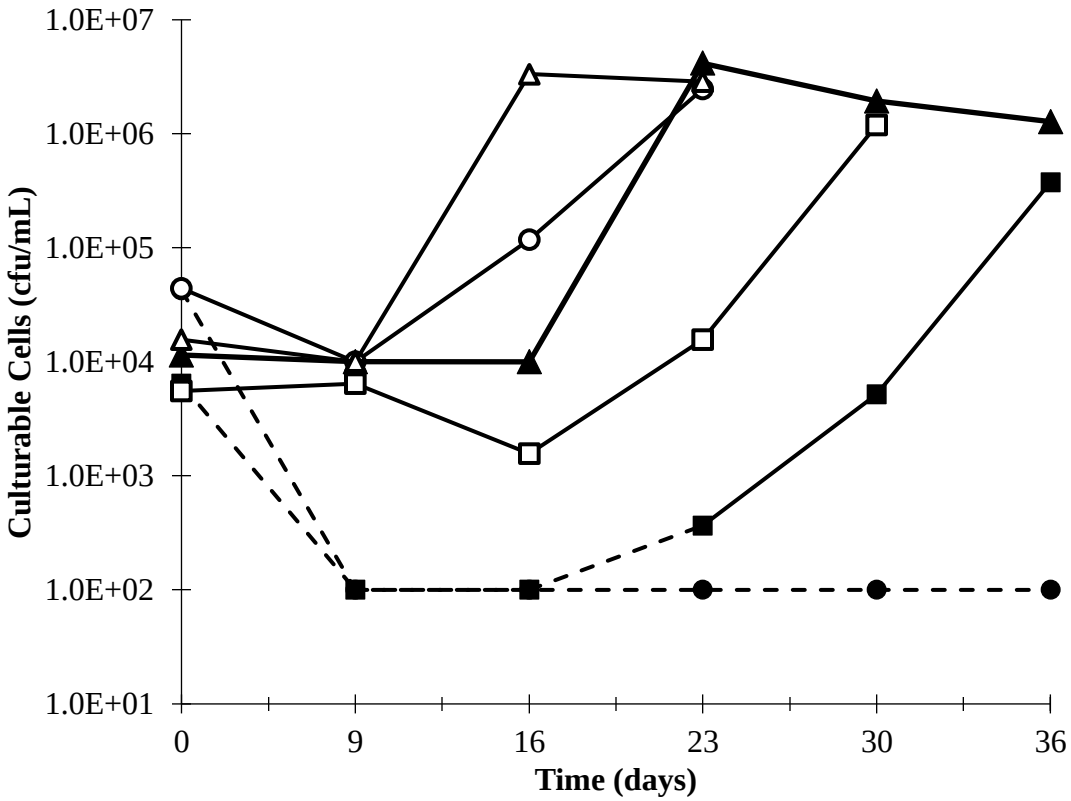

Figure 3.5 Culturable cells of *B. bruxellensis* strain UCD-2049 (●○), Copper Mt (■□), and 643 (▲Δ) after inoculation into Pinot noir wine that has not undergone MLF (open symbols) or that underwent MLF conducted by *O. oeni* VP41 (closed symbols). Data points represent mean of replicates, n=3.

Discussion

Interactions between *O. oeni* and *B. bruxellensis* have not been well-studied despite these microorganisms often being present together in wine (Renouf et al., 2007). Recent studies have noted that during MLF growth of certain *O. oeni* strains can result in higher concentrations of *p*-coumaric acid in wine (Chescheir et al., 2015; Burns and Osborne, 2013) such that if a *B. bruxellensis* infection occurs, significantly higher concentrations of volatile phenols are produced (Chescheir et al., 2015). In this study, an additional impact of MLF and *O. oeni* has been demonstrated in that the growth and volatile phenol production of *B. bruxellensis* was reduced when inoculated into wines that had undergone MLF compared to wines that did not undergo MLF. This finding is in support of Gerbaux et al. (2009) in which the authors noted that *B. bruxellensis* produced significantly lower amounts of volatile phenols in wines that had undergone MLF. However, unlike Gerbaux et al. (2009), this study reports on the populations of *B. bruxellensis* and *O. oeni* in addition to the final volatile phenol content of the wines. As such, the present study demonstrates the sharp decline in *B. bruxellensis* after inoculation into wine that had undergone MLF while strong growth occurred in control wines that did not undergo MLF.

The findings of this study also support Chescheir (2014) where it was reported that in both broth and Pinot noir wine *B. bruxellensis* UCD-2049 populations declined after inoculation if *O. oeni* had previously grown in the media or wine. In contrast, Madsen et al. (2016) monitored the growth of *B. bruxellensis* and *O. oeni* simultaneously

inoculated into wine post-alcoholic fermentation and concluded that there were no clear growth interactions between the two microorganisms. Madsen et al. (2016) stated that *B. bruxellensis* populations did not reach 4.5 logs cfu/mL until 114 day after being inoculated, however no other *B. bruxellensis* growth data was provided and *B. bruxellensis* was inoculated into the wines at very low populations (10^2 cfu/mL). In addition, Gerbauex et al. (2009) noted that the inoculation of *O. oeni* and *B. bruxellensis* together in wine did not impact the length of time for MLF to be conducted. However, Gerbauex et al. (2009) did not report on *B. bruxellensis* populations during MLF so any inhibition when inoculated together would not have been detected. Furthermore, it is possible that the timing of infection during MLF may play a role in any growth inhibition. In the current study, *B. bruxellensis* was only inoculated into wines that had completed MLF and was not inoculated at the start or mid-point of MLF. It may be interesting to investigate this to determine if wine is protected from *B. bruxellensis* growth during MLF as well as after MLF as demonstrated in the present study.

Apart from the control at day 0, growth of *B. bruxellensis* in the control wines entered stationary phase by 34 days post-MLF. The cause for the lack of culturable growth noted in the control at the day 0-time point remains unknown. However, recent work repeating this experiment with wine produced in 2016 has shown *B. bruxellensis* growth in the controls (No MLF) at 0, 30, and 90 day post-MLF (in the treatments) as expected. One difference between the 2015 and 2016 Pinot noir wines is that the 2015

wines contained higher alcohol (14%) than the 2016 wines (12.5%). *B. bruxellensis* is sensitive to high alcohol (Oswald and Edwards, 2017) and so this may have played a role. Nonetheless, aside from Day 0, *B. bruxellensis* grew well in the control wines that did not undergo MLF. The length of inhibition of *B. bruxellensis* post-MLF was dependent on the *O. oeni* strain used to conduct MLF. For example, if MLF was conducted by strain VP41 then *B. bruxellensis* was inhibited after 0, 34, and 112 days post-MLF. In contrast, *B. bruxellensis* growth occurred after 34 and 112 days post-MLF when *O. oeni* strain Beta conducted MLF. These differences were reflected in the populations of culturable *O. oeni* present in the wine when *B. bruxellensis* was inoculated. After the completion of MLF culturable cells of *O. oeni* Beta declined rapidly while populations remained high for strains Alpha and VP41 after 34 days and only declined below detectable populations 84 days post-MLF. These findings support what was reported by Chescheir (2014) where the presence of culturable *O. oeni* in the wine was necessary for the most growth inhibition of *B. bruxellensis*. If *O. oeni* cells were removed from the wines by sterile filtration after MLF then *B. bruxellensis* growth was similar to growth in a control wine that had not undergone MLF. This present study, along with previous work by Chescheir (2014), provides evidence that the mechanism of inhibition likely involves cell contact with culturable *O. oeni* cells.

Alternative inhibitory mechanisms would include the production of an inhibitory compound that breaks down during aging. Therefore, as the wine ages post-MLF the

concentration of the antimicrobial compounds declines allowing *B. bruxellensis* growth. However, to date there are no reports of any antimicrobial compounds being produced by *O. oeni*. Furthermore, Chescheir (2014) argued that the sterile filtration used to remove *O. oeni* cells was unlikely to impact the concentration of any inhibitory compounds as low protein binding sterile filters were used (polyethersulfone membrane). Competition for nutrients is also not likely as if it were the case then sterile filtration would not have relieved the inhibition in the case of Chescheir (2014). In the present study, it is difficult to argue how time post-MLF would have impacted the nutritional status of the wine but it is known that *B. bruxellensis* can grow in very low nutrient environments (Fugelsang and Edwards, 2007). Additional work is needed to fully determine the exact mechanism of inhibition. This should include assessments of using non-culture based methods to detect potential viable but non-culturable populations of *B. bruxellensis* that may be present in these wines as the yeast has been reported to enter this metabolic state (Willenburg et al., 2012; Serpaggi et al., 2012; Zuehlke et al., 2013).

While a cell-cell contact inhibitory mechanism has not been described for *B. bruxellensis,* it has been well characterized in mammalian (Nelson and Chen, 2002; Matsuda et al., 2012), yeast (Honigberg, 2011; Li and Palecek, 2008), and bacteria cells (Nickel et al., 1994; Donlan, 2002). Many species of bacteria are widely known to participate in cell-cell communication called quorum sensing, which is regulated through gene expression and the release of signal molecules (Miller and Bassler,

2001; Rutherford and Bassler, 2012). Recent research has also shown the yeast *C. albicans* is capable of cell density-induced quorum sensing-like behavior (Sprague and Winans, 2006; Hornby et al., 2001; Hogan, 2006). However, little research has demonstrated these types of interactions within a wine environment. In 2003, Nissen et al. investigated inhibition of *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii* by *Saccharomyces cerevisiae*. The researchers determined that the mechanism of inhibition was induced by cell-cell contact, specifically with live *S. cerevisiae*. Renault et al. (2013) also noted significant inhibition of *T. delbrueckii* due to cell contact with live *S. cerevisiae*. It is possible similar contact-induced growth inhibition may be the mechanism observed in this study where live *O. oeni* cells inhibited *B. bruxellensis*.

Inhibition of *B. bruxellensis* by *O. oeni* occurred in a strain-dependent manner. For example, while some *B. bruxellensis* strains such as B1b, E1, and AWRI-1499 were only minimally impacted when growing in wine that had undergone MLF with VP41, strain UCD-2049 populations declined below detectable numbers rapidly after inoculation. Several studies have noted large genetic and physiological strain variability for *B. bruxellensis* (Conterno et al., 2006; Hellborg and Piškur, 2009; Curtin et al., 2012b; Curtin and Pretorius, 2014). The genetic differences amongst *B. bruxellensis* strains have been implicated in effects on SO_2 resistance (Zuelkhe et al., 2013), carbon source utilization (Crauwels et al., 2015), and volatile phenol

production (Licker et al., 1998, Schopp et al., 2013). The strain differences noted in this study may be related to the strain variability for this yeast.

While the growth of some of the *B. bruxellensis* strains were largely unaffected when growing in wine where VP41 had conducted MLF, there was a reduction in the amount of volatile phenols in all the wines where MLF had been conducted. This was despite the fact that strains B1b, E1, AWRI-1499, and 643 all reached populations around 10^6 cfu/mL in these wines. Furthermore, in the timing experiment *B. bruxellensis* eventually grew when inoculated at 112 days post-MLF to populations around 10^5 cfu/mL in the wines containing *O. oeni* Beta but volatile phenol concentrations only reached 3.56 µg/L compared to 102.99 µg/L in the control. Strong growth of culturable *B. bruxellensis* cells has been linked to volatile phenol production. A study by Coulon et al. (2010) compared volatile phenol production by *B. bruxellensis* in bottled wines, specifically looking at differences in production between culturable cells and cells deemed viable but non-culturable (VBNC). Significantly higher amounts of volatile phenols were produced in wines where culturable *B. bruxellensis* cells represented a larger portion of the total viable population. In addition, Gerbaux et al. (2002) noted that wines which had undergone MLF prior to *Brettanomyces* infection had lower overall volatile phenols even though *Brettanomyces* populations had all reached over 10^6 cfu/mL. These observations suggest implications beyond growth inhibition of *B. bruxellensis* by the presence of

O. oeni; where metabolic changes, particularly in hydroxycinnamic acid utilization, may occur even when growth is relatively unaffected.

Conclusions

The presence of culturable *Oenococcus oeni* at the end of MLF results in the inhibition of *Brettanomyces bruxellensis*. Strain differences between *O. oeni* were most likely due to how long culturable cells of the different strains persisted in the wine. *B. bruxellensis* strain differences also occurred but the reason why the growth of some *B. bruxellensis* strains were impacted more by culturable *O. oeni* cells than others is unknown and deserves additional investigation. Interestingly, while the growth of some *B. bruxellensis* strains was not largely affected by *O. oeni*, in all cases there were lower concentrations of volatile phenols in wines that underwent MLF. While winemakers must continue to use sound winemaking practices to prevent the growth of *Brettanomyces* in their wines, this study has shown that the presence of high populations of *O. oeni* at the end of MLF may offer added some degree of protection for the wine until sulfur dioxide can be added.

General Summary and Conclusions

Brettanomyces bruxellensis is a major spoilage yeast encountered during the winemaking process. Unfortunately, winemakers have limited tools at their disposal to control the growth of this yeast and so additional strategies are needed. *B. bruxellensis* growth and production of the spoilage compounds 4-EP and 4-EG can be impacted by several factors during wine production. These factors may offer avenues to minimize wine spoilage. Simultaneous fermentation using a *S. cerevisiae* strain that can degrade *p*-coumaric acid and a *O. oeni* strain that can degrade tartaric acid-bound *p*-coumaric acid resulted in the largest reduction of these volatile phenol precursors. In contrast, when these same strains were utilized in sequential fermentations, it resulted in the largest number of precursors in the form most readily usable by *B. bruxellensis*. When MLF is conducted after the completion of alcoholic fermentation it should be conducted using an *O. oeni* strain that cannot degrade tartaric acid-bound *p*-coumaric acid. The choice of *O. oeni* strain to conduct MLF will have a larger impact on the concentration of free *p*-coumaric acid in the wine than acid hydrolysis of coutaric acid during aging. In this study, no major changes in the concentration of *p*-coumaric and coutaric acid were noted during 180 days of aging under a number of different storage conditions. Although most *O. oeni* strains evaluated in the present study could not degrade tartaric acid-bound hydroxycinnamic acids, two commercial strains as well as one non-commercial strain demonstrated this degradation ability. This finding provides further evidence for the use of well-

characterized *O. oeni* strains because if a winemaker conducts a non-inoculated MLF there is little real control over the strain performing MLF. As such, there are increased risks of MLF being performed by a strain that can degrade tartaric acid-bound hydroxycinnamic acids, thereby potentially leading to an increase in volatile phenols if *Brettanomyces* infection occurs.

The presence of culturable *Oenococcus oeni* at the end of MLF may offer some protection for the wine depending on how long culturable cells of *O. oeni* remain in the wine. Some differences between *O. oeni* strains were observed and appeared to relate to how long the strains remained culturable in wine. Under different wine conditions (pH, ethanol, phenolic content) different strains may survive for longer and this should be an area for additional research. *B. bruxellensis* strain differences also occur but the reason why the growth of some *B. bruxellensis* strains were impacted more by culturable *O. oeni* cells than others is unknown. It is also unknown why reduced volatile phenols were noted in wines inoculated with *B. bruxellensis* if MLF had previously occurred.

To conclude, interactions between *Oenococcus oeni* and *Brettanomyces bruxellensis* can have significant impacts on wine sensory quality. While it appears that some *O. oeni* can provide a degree of protection against *B. bruxellensis* growth and volatile phenol production, the most effective measures still include maintaining effective SO_2 levels and practicing regular, thorough cleaning and sanitation of all winery

surfaces. However, winemakers should pay special attention to microorganisms used for fermentations. The practice of native alcoholic and malolactic fermentations increases the potential for proliferation of microbes which can induce conditions more favorable to volatile phenol production if infected with *B. bruxellensis*. Therefore, it is suggested that when producing wines more favorable to *B. bruxellensis* (e.g. barrel-aged reds, wines with residual sugars, etc.), winemakers should consider the use of inoculated alcoholic fermentation as well as malolactic fermentation with a cinnamoyl esterase (-) strain to ensure that hydroxycinnamic acid levels remain low and so that SO_2 may be added to the wine sooner.

Future work in this area should focus on screening additional commercial and non-commercial *O. oeni* for cinnamoyl esterase activity. Additionally, characterization of more *B. bruxellensis* strains and the impact *O. oeni* has on these strains should be performed. Focusing on *B. bruxellensis* strains which have been sequenced and better classified from an -omics point of view would possibly provide better insight behind inhibition variation as well as inhibition mechanisms.

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