AN ABSTRACT OF THE THESIS OF

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The yeast *Brettanomyces bruxellensis* is the most important wines spoilage yeast encountered during winemaking as it can survive in wine for long periods, requires minimal nutrients for growth, and can be difficult to control. *Brettanomyces* produces two major spoilage products, the volatile phenols 4-ethylphenol (Band-Aid, medicinal smell) and 4-ethylguaiacol (smoke, clove smell) by decarboxylation and subsequent reductions of the hydroxycinnamic acids *p*-coumaric acid and ferulic acid. Hydroxycinnamic acids are naturally present in grapes and wine and are also often present in the form of tartaric acid-hydroxycinnamic acid esters which *B. bruxellensis* cannot utilize. The first objective of this study was therefore to investigate the ability of other wine microorganisms to hydrolyze tartaric acid bound hydroxycinnamic acids and the impact this may have on volatile phenol production by *Brettanomyces.* Of the thirty five strains of wine microorganisms tested only one, the commercial strain *O. oeni* VFO, hydrolyzed tartaric acid bound hydroxycinnamic acids in wine and increased the concentrations of the free hydroxycinnamic acids *p*-coumaric acid and ferulic acid. Because of this, *B. bruxellensis* produced significantly higher concentrations of 4-ethylphenol and 4-ethylguiaciol when growing in wine where VFO had

conducted the malolactic fermentation. In contrast, wines that underwent MLF with *O. oeni* Alpha or VP41 contained similar 4-EP and 4-EG concentrations to the control.

A subsequent study investigated interactions between the wine spoilage yeast *Brettanomyces bruxellensis* and wine lactic acid bacteria (LAB) and the impact on *Brettanomyces* growth and volatile phenol production. Studies in acidic grape juice (AGJ) broth (pH 3.50, 5% ethanol) demonstrated that growth of *O. oeni* could inhibit *Brettanomyces* growth in a strain dependent manner. Production of 4-ethyphenol was also delayed and reduced in a strain dependent manner. For example, in the control a maximum of 19 mg/L 4-EP was produced by *B. bruxellensis* UCD-2049 while during growth in media where VP41 had previously grown only 7.9 mg/L 4-EP was produced. When *B. bruxellensis* was inoculated into Pinot noir wine where malolactic fermentation (MLF) had been performed by *O. oeni* VFO, populations of *B. bruxellensis* quickly decreased below detectable levels and did not recover during the course of the experiment (50 days). In contrast, when *B. bruxellensis* UCD-2049 was inoculated into Pinot noir wine where *Pediococcus damnosus* OW2 had previously grown populations did not decrease and growth occurred in a similar manner to the control. The reason for this decrease in population was further explored by inoculating *B. bruxellensis* into AGJ broth (pH 3.50) where there had been previous growth of *O. oeni* Alpha or VP41. In half the treatments the *O. oeni* were removed by sterile filtration prior to *Brettanomyces* inoculation. Although there was delayed growth of *B. bruxellensis* in media where *O. oeni* had previously grown there was no significant difference between growth in sterile filtered and non-sterile filtered treatments. The relief of inhibition by sterile filtration suggests that neither nutrient depletion nor the production of an extracellular inhibitory compound by *O. oeni*

were the causes of *Brettanomyces* inhibition, as sterile filtration would not have impacted these.

Future work in this field should include screening a larger and more diverse group of wine microorganisms for the ability to hydrolyze tartaric ester bound hydroxycinnamic acids. This should include non-*Saccharomyces* yeast such as *Kloeckera* that are known to be present during early fermentation as well as spoilage bacteria such as *Lactobacillus*. In addition, the inhibition of *B. bruxellensis* should be explored further by testing *O. oeni* against a larger number of *B. bruxellensis* strains and additional mechanisms of inhibition should be investigated.

Effect of Lactic Acid Bacteria Growth on *Brettanomyces bruxellensis* Growth and Production of Ethylphenols

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Effect of Lactic Acid Bacteria Growth on *Brettanomyces bruxellensis* Growth and Production of Ethylphenols

LITERATURE REVIEW

Winemaking Process

While there is no single standard way to make wine, most wine production follows a standard procedure with minor adjustments based on type and desired wine style. Grapes are harvested at a given ripeness level and delivered to the winery. Here they can be destemmed and, in the case of most white wines, pressed to remove the juice from the berry. For red wines no pressing is performed as the skins and seeds remain present during the alcoholic fermentation. Fermentation begins either through use of yeast present on the grapes or in the winery, or inoculation with a purified commercially produced yeast strain. The yeast utilized, *Saccharomyces cerevisiae;* converts sugar, primarily glucose and fructose, into ethanol and carbon dioxide (Jackson 2008). Additional secondary metabolites that can contribute to aroma, flavor, and mouthfeel are also produced by the yeast. S. *cerevisiae* dominates other yeast present during the alcoholic fermentation due to its tolerance to the acidic, high sugar, anaerobic environment present during fermentation of grapes and to its relatively ethanol tolerance (Delfini and Formica 2001).

After alcoholic fermentation, wine is typically separated from the fermentation lees and stored for later clarification, filtration, and bottling. For red wines, the wine is separated

from the grape skins and seeds by pressing. At this point many red wines undergo a secondary fermentation called the malolactic fermentation (MLF). During this process lactic acid bacteria (LAB), predominately *Oenoccous oeni,* convert malic acid to lactic acid. Malic acid is perceived as more tart than lactic acid, which is a weaker acid. Thus MLF causes an increase in pH and a loss of perceived acidity(Boulton et al. 1996). Wine can then be left to age in tank, barrel and/or bottle. It is during this point in the wine's life that it is most susceptible to microbial spoilage. Nutrient levels, temperature, and lack of antimicrobial compounds allow the growth of certain unwanted bacteria and yeast.

Winemakers typically try and prevent spoilage by maintaining anaerobic conditions, conducting sound and proper sanitation, and adding appropriate levels of the antimicrobial and antioxidant agent sulfur dioxide (SO_2). However, despite the vigilance of the winemaker, microbial spoilage can still occur and have an adverse and irreversible effect on wine quality. The major wine spoilage micoorganisms are the LAB *Pediococcus, and Lactobacillus*; the acetic acid bacteria *Acetobacter*; and oxidative film yeasts such as *Candida* and *Pichia* (Fugelsang and Edwards 2007). The wine spoilage organism that is of most concern to winemakers, however is the yeast *Brettanomyces bruxellensis* (Puig et al. 2011).

Brettanomyces bruxellensis

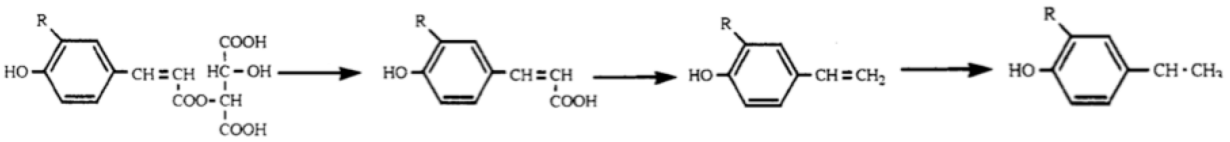
Brettanomyces bruxellensis is the most important spoilage microbe in wine, and it is present in a significant portion of commercial wines, causing economic losses (Martorell et al. 2006; Puig et al. 2011) *B. bruxellensis* can survive in finished wine and produce diverse aromatic compounds (Joseph et al. 2013). However, "Brettanomyces taint" is typically

associated with the presence of volatile ethylphenols such as 4-ethyphenol and 4-ethylguiacol. These compounds are derived from free hydroxycinnamic acids naturally present in grapes (Chatonnet 1992). Production of volatile phenols from hydroxycinnamic acids is a two-step enzymatic process. During the first step, hydroxycinnamic acids (coumaric acid, ferulic acid, and caffeic acid) are decarboxylated by hydroxycinnamic acid decarboxylase to create vinylphenols (4-vinylphenol, 4-vinylguaicol, and 4-vinylcatechol, respectively). During the second step, vinylphenols are reduced by vinylphenol reductase to produce ethylphenols (4-ethylphenol, 4-ethylguaicon and 4-ethylcatechol, respectively) (Figure 1.1). Many wine microorganisms can perform the initial step of this process, producing vinylphenols, including *S. cerevisiae, Lactobacillus,* and *Pediococcus* (Chatonnet et al. 1993; Calvin et al. 1994; Couto et al. 2006). However, *B. bruxellensis* is the only known microorganism that is capable of producing significant quantities of ethyphenols in wine.

B. bruxellensis is of particular concern to the wine industry because it can be difficult to control. Traditional methods of control have moderate efficiency on *B. bruxellensis* but there is some strain variability with regards to sensitivity to SO_2 (Agnolucci et al. 2010). *Brettanomyces* can also survive in nutrient poor environments due its ability to utilize a wide range of carbon sources, such as ethanol (Dias et al. 2003) and cellobiose present in barrels (Conterno et al. 2006). Modern methods to control *B bruxellensis* such as dimethyl dicarbonate (DMDC) have shown greater efficiency than traditional methods, but have met with resistance by industry (Wedral, Shewfelt, and Frank 2010; Zuehlke, Petrova, and Edwards 2013).

The source of *Brettanomyces* in the winery has, and continues to be, a disputed and controversial topic. While some winemakers and wine writers believe that *Brettanomyces* enters the winery from yeast present on the grapes, these claims are not confirmed by peer reviewed research. *B. bruxellensis* has been detected in the vineyard/grapes thanks to new methods of isolation, but the populations are typically very low (Vincent Renouf and Lonvaud-Funel 2007). While the vineyard is a possible source of *Brettanomyces,* the much more common source of infection is the winery. Here, infected barrels and infected wines are the main carriers of this yeast and once present in a winery *Brettanomyces* is very difficult to completely eliminate (Boulton et al. 1996). *B. bruxellensis* does not contribute to spoilage during alcoholic fermentation, as it is a poor competitor with *S. cerevisiae*. However, its ability to growth in the low nutrient, high acid, high alcohol environment of wine allows it to grow after alcoholic fermentation (Renouf et al. 2006). Renouf et al. 2006 reported that during the initial stages of alcoholic fermentation the ratio of *S. cerevisiae: B. bruxellensis* was greater than 1000:1. However, after several weeks, *S. cerevisiae* population was surpassed by *B. bruxellensis.*

Brettanomyces can exist in oak barrels and once *Brettanomyces* contaminates an oak barrel, it can be very to remove. This is because *B. bruxellensis* can be found 8mm deep in oak staves, preventing easy sanitation (Malfeito-Ferreira 2005). *Brettanomyces* can also utilize cellobiose present in the barrels and survive in this semi-anaerobic environment. Only small amounts of sugars are required for large population increases and wine spoilage. Furthermore, if the winemaker is unaware that the wine is contaminated, then



Tartaric Acid-Hydroxycinnamic acid esters:		Hydroxycinnamic acids:		Vinylphenols:		Ethylphenols:	
Coutaric acid:	R=H	Coumaric acid:	R=H	4-vinylphenol:	R=H	4-ethylphenol:	R=H
Fetaric acid:	$R=OCH_3$	Ferulic acid:	$R=OCH_3$	4-vinylguaiacol:	$R=OCH_3$	4-ethylguaicol:	$R=OCH_3$
Caftaric acid:	R=OH	Caffeic acid:	R=OH	4-vinylcatechol:	R=OH	4-ethylcatechol:	R=OH

Figure 1.1- Structure and breakdown of tartaric acid-hydroxycinnamic acid esters to ethylphenols. Adapted from Dugelay et al. 1993

other wines may be cross-contaminated through use of the infected wine for topping or blending.

Monitoring and quantification *B. bruxellensis* during winemaking can be difficult as it can exist in a viable but not culturable state (VBNC) (Serpaggi et al. 2012). When in the VBNC state *B. bruxellensis* will not grow in media, preventing quantification through traditional plating on agar media. Other methods of detection such as molecular and microscopy methods of detection and quantification can detect *B. bruxellensis* in the VBNC state, but these are often expensive and time-consuming methods. Questions regarding the appropriate methods of quantification are still up for debate, as microscopy methods fail to differentiate between live and dead cells and PCR methods of quantification may include DNA from dead cells (Tessonnière et al. 2009). The VBNC state for *B. bruxellensis* can be induced by the addition of SO_2, though the actual mechanism of the induction is still being explored. *B. bruxellensis* can still produce ethylphenols when in the VBNC state (Serpaggi et al. 2012).

Volatile phenols

The major cause of wine spoilage by *Brettanomyces* is the production of volatile phenols. Volatile phenols present in wine include ethylphenols and vinyl phenols. While other volatile phenols can exist in wine, 4-ethylphenols (4-EP) and 4-ethylguical (4-EG) are the compounds produced by *B. bruxellensis* (Pascal Chatonnet 1992; Etievant 1981) Each ethylphenol and vinylphenol is an aroma active compound. Sensory thresholds differ, and median detection thresholds for 4-ethylphenol, 4-ethlyguaicol and 4-ethlycatechol have

been measured at 195 µg/L, 84 µg/L and 385 µg/L, respectively (Botha 2010). However, sensory thresholds are difficult to evaluate as they change depending on media and potential synergistic effects. For example, a lighter bodied wine such as Pinot noir may have a sensory detection threshold for 4-EP of 250 µg/L, while in a heavy style red wine such as a Cabernet sauvignon the detection threshold may be above 500 µg/L. In addition, the volatile phenol 4-ethylcatechol (4-EC), which is rarely present above sensory thresholds in wine, may enhance the perception of 4-EP in a wine (Chatonnet 1992; Chatonnet, Dubourdieu, and Boidron 1995; Botha 2010; Buron et al. 2011; Petrozziello et al. 2014). The ratio of 4-EP: 4-EG is also thought to impact how these compounds are sensorially perceived.

A number of factors affect the production of volatile phenols by *B. bruxellensis.* Strain variation is one of the most dominant factors; some strains of *B. bruxellensis* produce very high amounts of 4-EP and 4-EG while other strains produce lower amounts (Conterno et al. 2006; Curtin et al. 2007; Conterno et al. 2006; Brock et al. 2006). The conversion rate of hydroxycinnamic acids to ethylphenols by *B. bruxellensis* is also dependent on the carbon source (Dias et al. 2003). The production of ethylphenol is also limited on the availability of the precursor compounds, the hydroxycinnamic acids. *B. bruxellensis* does not produce ethylphenols in the absence of hydroxycinnamic acids (Joseph et al. 2013)

Hydroxycinnamic Acids

Hydroxycinnamic acids are monophenolic organic acids that exist in plant material (Harbone and Corner 1961). In wine grapes three significant hydroxycinnamic acids are present; Ferulic acid, coumaric acid and caffeic acid. Hydroxycinnamic acids generally exist

in plants in a bound form. In grapes they are predominately ester bound to tartaric acid, (Figure 1.1), known as fetaric acid, coutaric acid and caftaric acid, respectively (Winter and Herrmann 1986; Ong and Nagel 1978).

The free forms of hydroxycinnamic acids show antimicrobial properties against lactic acid bacteria (Salih, Quere, and Drilleau 2000). In other work the free forms of hydroxycinnamic acids also exhibit antimicrobial properties against *B. bruxellensis* (Harris et al. 2010) with a synergistic effect being noted. For example, a blend of ferulic acid and p-coumaric acid inhibited growth of *B. bruxellensis* at lower levels than ferulic acid alone, despite ferulic acid having stronger anti-microbial properties than *p*-coumaric acid. However, these studies were performed with significantly higher concentrations of hydroxycinnamic acids than those found in wines (Ong and Nagel 1978; Salih, Quere, and Drilleau 2000; Harris et al. 2010). Nevertheless, the antimicrobial ability of hydroxycinnamic acids has been posited as a rationale for the ability of microorganisms to hydrolyze them (Harris et al. 2010). The ester bound forms of hydroxycinnamic acids, though, do not show antimicrobial properties against lactic acid bacteria (Salih, Quere, and Drilleau 2000).

The concentration of hydroxycinnamic acid in grapes and wines can differ significantly depending on many different viticulture and winemaking practices. Grape ripeness, cultivar, site, and the degree of sun exposure can impact both the concentration of hydroxycinnamic acids as well as the ratio of free to bound forms (Romeyer et al. 1983; Ong and Nagel 1978; Bubola et al. 2012; Pérez-Magariño and González-San José 2005). For example, Nagel et al. 1979 reported that of the cultivars from the Pacific Northwest

evaluated that Pinot noir had the highest level of total tartaric bound hydroxycinnamic acids in grape musts out of the cultivars tested. Significant variations existed between grape cultivars and viticultural regions, however, with concentrations ranging from 33.4 to 143.2 mg/L tartaric bound hydroxycinnamic acids within Pinot noir samples and between 27.6 and 380.8 mg/L across all cultivars sampled.

Winemaking and Hydroxycinnamic acids

Winemaking practices can also impact the concentration of free and bound hydroxycinnamic acids. Some authors reported that the concentration of caftaric increased during extended post-fermentation maceration, however, other data show no significant effect of on caftaric or coutaric acid due to extended maceration (Mulero et al. 2011; Auw et al. 1996). The use of the fining agent polyvinyl polypyrrolidone (PVPP) as a clarification agent may also affect the level of caftaric and coutaric acid in wine (Gómez-Plaza et al. 2000). During fermentation, levels of tartaric acid bound hydroxycinnamic acid have been reported to decrease in some cultivars such as Cabernet Sauvignon and Merlot. Levels in Noble and Chambourcin wines rise during fermentation. (Ong and Nagel 1978; Auw et al. 1996).

The change in free and bound hydroxycinnamics acids during fermentation is likely due to one of two mechanisms. The first is acid hydrolysis of the tartaric ester bond (Nagel et al. 1979), resulting in an increase in the free form. The second mechanism is enzymatic hydrolysis of the tartaric ester bond. Some early commercial enzymatic preparations prepared from *Aspergillus niger* (pectinases) that are often added to grapes to increase juice yields were noted to have cinnamic esterase activities resulting in an increase of free

hyroxycinnamic acids (Dugelay et al. 1993; Wightman et al. 1997; He et al. 2010; Mulero et al. 2011). Because *Brettanomyces* is not able to utilize the bound from of the hydroxycinnamic acids, this increase in free hydroxycinnamic acids was of concern to winemakers as a higher concentration of pre-cursors for *Brettanomyces* spoilage were now present in their wines. Because of this, commercial enzymes for use during winemaking are now typically produced in a manner such that side activities such as cinnamic esterase activity are minimized or eliminated.

Aside from *Aspergillus niger*, the ability to hydrolyze ester bound hydroxycinnamic acids is shared by a number of different microbes. Hydroxycinnamic acids have antimicrobial properties when in the free form, and are more likely to be in the free form during some stages of *Botrytis cinera* infection, suggesting a potential role in immune response in grape vines (Geny et al. 2003). *Lactobaccilus* spp. and other LAB have shown the ability to hydrolyze quinic acid ester bound hydroxycinnamic acids in media, which has a similar structure as tartaric acid ester bound hydroxycinnamic acids (Guglielmetti et al. 2008). *B. bruxellensis* has been shown to hydrolyze hydroxycinnamic acid ethyl-esters and convert them to ethylphenols (Hixson et al. 2012), but to date has not been shown to be able to hydrolyze tartaric ester bound hydroxycinnamic acids (Schopp et al. 2013).

Oenoccocus oeni

An additional microorganism that may change the concentration of free and bound hydroxycinnamic acids in wine is *Oenococcus oeni*. Aside from *S. cerevisiae*, this is the most commonly found microorganism during winemaking. *Oenococcus oeni* is a heterofermentative gram positive LAB. It is a facultative anaerobe, cocciodad, and can be

found in chains, especially in growing cultures (Fugelsang and Edwards 2007). Until 1995 it was known as *Leuconostoc oenos,* at which point it was placed in its own genus due to phylogenetic differences (Dicks, Dellaglio, and Collins 1995). *O. oeni* is the primary bacteria used to perform the malolactic fermentation (MLF). The MLF is not an actual fermentation but rather an enzyme mediated decarboxylation of malic acid to lactic acid. The decarboxylation step generates a proton motive forces which generates ATP via proton pumping ATPases (Olsen, Russell, and Henick-Kling 1991).

O. oeni is the dominant cultured lactic acid bacteria that performs MLF in wine, and represents 97.5% of organisms isolated from wine undergoing spontaneous MLF. 90% of spontaneous MLF contain multiple strains of *O. oeni* (López et al. 2007). *O. oeni* is not competitive with *Saccharomyces cerevisiae* and as such, even if present in must during primary fermentation, it typically does not grow or perform MLF until primary fermentation is nearing completion (Abrahamse and Bartowsky 2012). *O. oeni* is sensitive to sulfur-dioxide as such SO_2 cannot be added to a wine until MLF is completed. Thus during MLF wine can be particularly vulnerable to microbial spoilage if MLF is delayed or becomes difficult to finish (Bauer and Dicks 2004).

O. oeni selection is based on ability to finish decarboxylation of malic acid, the ability to grow in high alcohol and low pH environment, and low production of diacetyl and amines (Solieri et al. 2010). Research has implicated *O. oeni* in the release of aroma compound precursors by enzyme mediate hydrolysis of glycosylated terpenoids (Ugliano, Genovese, and Moio 2003). Species of LAB have demonstrated the ability to produce vinylphenols,

and in some cases small amounts of ethylphenols, though *O. oeni* has not shown the ability to produce either (Couto et al. 2006; Silva et al. 2011).

Despite having a wide range of enzymatic activities, *O. oeni* has not been documented to have cinnamic esterase activity. A number of studies have reported an increase in free hydroxycinnamic acids after the completion of MLF (Burns and Osborne 2013; Hernández et al. 2007; Cabrita et al. 2008). For example, Burns and Osborne (2013) noted that after MLF with *O. oeni* VFO there was an increase from approximately 1 mg/L *p*-coumaric acid to over 4 mg/L *p*-coumaric acid. 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. Hernández et al. (2006) demonstrated significant hydrolysis of caftaric acid and coutaric acid, as well as increases in caffeic acid and coumaric acid in wine that had undergone MLF. However, the MLF occurred naturally (no inoculation) so the identity of the wine LAB responsible was unknown. This wine also showed an increase in ferulic acid relative to the other treatments, but it is unclear it this was significant. Hernández et al. (2007) also reported a strain of *Lactobacillus plantarum* showed limited hydrolysis of bound hydroxycinnamic acids and an increase in free hydroxycinnamic acids. Cabrita et al. (2008) also reported an increase in free hydroxycinnamic acids post-MLF, but used a mixed culture of LAB, so the specific bacteria performing this transformation was unknown. Clearly, growth of wine LAB during the MLF can have an effect on free and bound hydroxycinnamic acid content. To date this has not been clearly and systematically shown and the consequences of this action on volatile phenol production by *Brettanomyces* have not been demonstrated.

While the growth of *O. oeni* may impact *Brettanomyces* through hydrolysis of bound hydroxycinnamic acids, its growth may also have other effects on *Brettanomyces.* In one of the few reports regarding interactions between *Brettanomyces* and *O. oeni*, Gerbaux et al. (2009) noted that *Brettanomyces* produced significantly lower amounts of volatile phenols in wines that had undergone MLF. However, little information regarding populations of *O. oeni* or *Brettanomyces* was provided, making it difficult to identify the cause of the reduced volatile phenols. Gerbaux, Vincent, and Bertrand (2002) also reported inhibition of *B. bruxellensis* in wine that had previous completed MLF. An initial drop in population of *B. bruxellensis* was reported when inoculated into wine that had undergone MLF, while no drop in population occurred in wine that had not undergone MLF. However, wines in this study were not sterilized before inoculation and no information was given regarding the bacteria used to perform the MLF. Recent work in our laboratory has shown that some inhibition of *Brettanomyces* growth can occur if inoculated into wine post-MLF when there is still a large population of viable *O. oeni* cells (J. P. Osborne, Strickland, and Edwards 2012).

A better understanding of interactions between *O. oeni* and *Brettanomyces* may help reduce the risk of wine spoilage if *Brettanomyces* infection occurs. For example, the use of malolactic bacteria strains that increase the amount of volatile phenol precursor compounds could be avoided. On the other hand, if *O. oeni* strains show inhibitory action towards *Brettanomyces* then use of these strains could be an additional 'hurdle' that *Brettanomyces* must overcome. Therefore, the objectives of this study were to:

1. Determine the ability of wine microorganisms to hydrolyze tartaric acid ester bound hydroxycinnamic acids

2. Evaluate the effect of hydrolysis of tartaric acid ester bound hydroxycinnamic acids on volatile phenol production by *Brettanomyces bruxellensis*
3. Investigate interactions between *Brettanomyces bruxellensis* and *O. oeni*

Microbial Hydrolysis of Tartaric Acid-Hydroxycinnamic Acid Esters and the Effect on *Brettanomyces bruxellensis* Production of Volatile Phenols

Stuart C. Chescheir, James P. Osborne

Abstract

Brettanomyces bruxellensis is a major cause of wine spoilage due to its production of volatile phenols from free hydroxycinnamic acid. Hydroxycinnamic acids are naturally present in grapes and wine and are often present in the form of tartaric acid-hydroxycinnamic acid esters which *B. bruxellensis* cannot utilize. The purpose of this study was to investigate the ability of other wine microorganisms to hydrolyze tartaric acid bound hydroxycinnamic acids and the impact this had on volatile phenol production by *Brettanomyces.* Thirty five wine microorganisms including *B. bruxellensis, Pediococcus,* and *Oenococcus oeni* isolates were screened for the ability to hydrolyze tartaric acid bound hydroxycinnamic acids. Only one isolate, *O. oeni* strain VFO, was shown to be able to hydrolyze bound hydroxycinnamic acids during growth in Pinot Noir wine. Hydrolysis occurred during logarithmic growth and at a similar time to the decarboxylation of malic acid during the malolactic fermentation (MLF). The hydrolysis of the tartaric acid bound hydroxycinnamic acids caftaric, coutaric, and fetaric acid, resulted in an increase in their free forms; caffeic, *p*-coumaric, and ferulic acid respectively. The impact of this increase in free hydroxycinnamic acids on *Brettanomyces* volatile production was investigated by inoculating *B. bruxellensis* UCD-2049 into Pinot noir wine after the completion of MLF conducted by either *O. oeni* VFO, Alpha or VP41. Growth was tracked by plating, free and bound hydroxycinnamic acids were analyzed by HPLC, while 4-ethyphenol (4-EP) and 4-ethylguiacol (4-EG) were assessed by GC-MS. During growth in the wine *B. bruxellensis* metabolized *p*-coumaric acid but was unable to metabolize coutaric acid. Wine where VFO had conducted MLF contained significantly higher amounts of *p*-coumaric and ferulic acid

resulting in significantly higher production of 4-EP and 4-EG in these wines by *B. bruxellensis*. In contrast, wines that underwent MLF with *O. oeni* Alpha or VP41 contained similar 4-EP and 4-EG concentrations to the control.

Introduction

During winemaking, the growth of certain yeast can cause the spoilage of wine due to the production of undesirable aromas and flavors. The yeast *Brettanomyces bruxellensis* is the most important spoilage yeast as it: can survive in wine for long periods, requires minimal nutrients for growth, can be difficult to control, and can be a cause of significant financial loss (Loureiro and Malfeito-Ferreira 2005; Puig et al. 2011). *B. bruxellensis* can cause wine spoilage through the production of many different volatile compounds with a diverse set of sensory effects (Heresztyn 1986; A. Romano et al. 2008; Joseph et al. 2013). However, *Brettanomyces* spoilage is generally attributed to the production of the ethylphenols 4-ethyl phenol (4-EP), 4-ethyl guiacol (4-EG) and 4-ethylcatechol (4-EC) (Pascal Chatonnet 1992). *B. bruxellensis* produces these ethylphenols from the hydroxycinnamic acids (HCA) coumaric acid, ferulic acid and caffeic acid. These are converted to 4-EP, 4-EG, and 4-EC respectively, through a two-part enzymatic process (Pascal Chatonnet 1992). Firstly, the hydroxycinnamic acid is converted to a vinylphenol by a hydroxycinnamic decarboxylase. The vinylphenol is then converted to an ethylphenol by a vinylphenol reductase.

Ethylphenols have been described as having a “Band Aid”, “barnyard”, “horse sweat”, or “medicinal” aromas while vinylphenols tend to be described as “clove-like”, and “spicy” (Andrea Romano et al. 2009). Both vinylphenols and ethylphenols can exist in wine above sensory threshold, though exact thresholds depend on wine and potential synergistic

effects (Pascal Chatonnet et al. 1993). In addition, the sensory threshold for ethylphenols is much lower than that of vinylphenols. Although a number of wine microorganisms, such as some wine lactic acid bacteria (LAB) and *Saccharomyces cerevisiae*, have shown the ability to decarboxylate hydroxycinnamic acid, forming vinylphenols (Couto et al. 2006; Pascal Chatonnet et al. 1993; Cavin et al. 1993), only *Brettanomyces* are able to produce high concentrations of ethylphenols during winemaking (P. Chatonnet, Dubourdieu, and Boidron 1995; Cavin et al. 1993).

While growth of *Brettanomyces* in wine is the primary factor impacting the production of ethylphenols in wine, the second factor to consider is the concentration of the precursor compounds, the HCA, in the wine. HCA are a group of aromatic organic acids found in plants and in grapes and are located in the vacuoles of the skin and pulp cells (Conde et al. 2007). In grape juice and wine HCA can be present in a free or bound form with the bound form being the predominate form in grape juice. The bound form of HCAs are ester bound to tartaric acid, forming a hydroxycinnamic tartaric acid ester (bound HCAs). HCAs found in grape juice include coumaric, ferulic, and caffeic acid and their respective bound forms are coutaric, fetaric, and caftaric acid. The concentration of total HCAs, relative amounts of free and bound HCAs, and the amount of specific HCA depend on multiple factors including: cultivar, climate and enological practices (Ong and Nagel 1978; Nagel and Wulf 1979; Nagel et al. 1979).

To date, *B. bruxellensis* has not demonstrated the ability to utilize the bound form of the HCAs (Schopp et al. 2013). Because of this the bound hydroxycinnamic acids represent a potential source of spoilage aroma precursors in wine. If other wine microorganisms can

hydrolyze tartaric bound hydroxycinnamic acids then this may result in the release of free HCAs and higher production of volatile phenols by *Brettanomyces.* Previous work shows that enzymatic preparations isolated from *Aspergillus niger* are able to free bound HCAs (Dugelay et al. 1993). Also some examples of increases in free HCAs following malolactic fermentation (MLF) by commercial cultures and spontaneous MLF have been reported (Hernández et al. 2007; Burns and Osborne 2013). However, in the study by Hernandez et al. (2006) the MLF occurred naturally (no inoculation) so the identity of the wine LAB responsible was unknown. In addition, while Burns and Osborne noted that one *O. oeni* strain was able to hydrolyze bound HCAs, only a small number of *O. oeni* strains were used in the study and the effect on volatile phenol production by *Brettanomyces* was not reported. Therefore, the purpose of this work was to screen a large number of wine LAB and *B. bruxellensis* strains for the ability to hydrolyze tartaric acid bound hydroxycinnamic acids and investigate the impact this had on 4-ethylphenol production by *B. bruxellensis.*

Material and Methods

Microorganisms

Yeast and bacteria isolates were collected from a number of different sources (Table 2.1). These included commercial companies (Chr. Hansen, Lallemend), University culture collections (Oregon State University , Washington State University, University of California Davis), private company's culture collections (E & J Gallo) and bacteria isolated previously by our own lab (Strickland 2012).

LAB isolates were initially streaked for isolation on MRS media (20 g/L Tryptone, 5 g/L Peptone, 5 g/L Yeast Extract, 5 g/L Glucose, 1 mL/L 5% Tween solution, 20g/L Agar 200 mL/ L Apple Juice, pH 4.5) before single colonies were grown in MRS broth. After seven days growth at 25°C the cultures were stored in glycerol at -80°C for future use as described by Strickland (2012).

Brettanomyces bruxellensis isolates were streaked for isolation on YPD media (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L dextrose, 20 g/L Agar, pH 6.5) before single colonies were grown in YPD broth. After seven days growth at 25°C the cultures were stored in glycerol at -80°C for future use as described by Strickland (2012).

Commercial *O. oeni* cultures came as freeze dried preparations. A loop-full of this preparation was dissolved in 0.1% Peptone solution (1g/L Peptone) and streaked for isolation on MRS media before a single colony was grown in MRS broth. After seven days growth at 25°C the cultures were stored in glycerol at -80°C for future use as described by Strickland (2012).

Table 2.1 Strains and sources of microorganisms screened for their ability to hydrolyze tartaric acid bound hydroxycinnamic acids.

Strain Name	Species	Source
493	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection (Modesto, CA, USA)
495	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
496	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
497	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
607	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
608	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection

Table 2.1 (continued)

613	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
614	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
615	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
616	*Brettanomyces bruxellensis*	EJ Gallo Culture Collection
VFO	*Oenococcus oeni*	Chr. Hansen (Horsholm, Denmark)
Alpha	*Oenococcus oeni*	Lallemand (Montreal, Canada)
VP41	*Oenococcus oeni*	Lallemand
CH35	*Oenococcus oeni*	Chr. Hansen
CH16	*Oenococcus oeni*	Chr. Hansen
Elios	*Oenococcus oeni*	Lallemand
OW1	*Pediococcus parvulus*	Oregon Pinot Noir
OW2	*Pediococcus damnosus*	Oregon Pinot Noir
OW3	*Pediococcus parvulus*	Oregon Pinot Noir
OW4	*Pediococcus parvulus*	Oregon Pinot Noir
OW5	*Pediococcus parvulus*	Oregon Pinot Noir
OW6	*Pediococcus parvulus*	Oregon Pinot Noir
OW7	*Pediococcus parvulus*	Oregon Pinot Noir
OW8	*Pediococcus inopinatus*	Oregon Pinot Noir
OW9	*Pediococcus inopinatus*	Oregon Pinot Noir
WW1	*Pediococcus parvulus*	Washington Syrah
WS9	*Pediococcus parvulus*	Charles Edwards Collection- Washington State University (Pullman, WA, USA)
ATCC 43013	*Pediococcus damnosus*	ATTC collection (Manassas, VA, USA)
CM12	*Brettanomyces bruxellensis*	Alan Bakalinsky Collection- Oregon State University (Corvallis, OR, USA)
SL15	*Brettanomyces bruxellensis*	Alan Bakalinsky Collection- Oregon State University
UCD-2049	*Brettanomyces bruxellensis*	University California Davis Culture Collection (Davis, CA, USA)
AWRI 1499	*Brettanomyces bruxellensis*	Australian Wine Research Institute (Adelaide, Austalia)
AWRI 1608	*Brettanomyces bruxellensis*	Australian Wine Research Institute

Wine making

Pinot Noir wine was produced at the Oregon State University Research Winery from grapes harvested from Woodhall Vineyard (Alpine, Oregon, USA) in 2011 and 2012. Harvest was determined by soluble solid levels and perceived fruit ripeness by the managing team at the vineyard. Grapes were stored at 4 °C for 48 hours and then hand sorted and destemmed using a Velo DPC 40 destemmer/crusher (Altivole, Italy). Grapes were divided into 100 L stainless steel tanks each containing approximately 60 L of must. 50 mg/L of SO_2 (in the form of potassium metabisulfite) was added to each tank and the yeast nutrient Fermaid K® (Lallemand, Montreal, Canada) was added to a concentration of 0.125 g/L. Each tank was inoculated with the commercial strain of *Saccharomyces cerevisiae* RC-212 (Lallemand) at a rate of 0.25 g/L of must. Yeast was hydrated according to manufacturer's specification prior to inoculation. Fermentations were performed in a temperature-controlled room held at 27°C. Cap management was done through punch downs twice a day and temperature and °Brix were measured with an Anton-Paar DMA 35N Density Meter (Graz, Austria). Fermentation continued until sugar levels fell below 0.2g/100mL confirmed by Bayer Clinitest tablets (Morristown, New Jersey, USA).

Following fermentation, the wine was pressed using a Willmes model 6048 pneumatic bladder press (Lorsch, Germany). Pressed wine was put in 100 L stainless steel tanks and stored at 4 °C. Following settling for 72-hours wine was filtered through a plate and frame filter fitted with Beco K-1 2.0 µm nominal filter sheets (Langenlonsheim, Germany). Wine was then homogenized and filtered through 1.0 µm nylon cartridge and a 0.45 µm polyethersulfone sterile filter (G.W. Kent, Ypsilanti, Michigan, USA) in succession. Filtered

wine was dispensed into sterile carboys and stored at 4 °C for future use. Basic wine parameters for the 2012 Pinot noir were 13.2% (v/v) ethanol, pH 3.53 and 0 .67 g/100 mL tartaric acid equivalents of Titratable Acidity. Final 2011 Pinot Noir was 12.6% ethanol by volume, pH 3.44 and 0.68 g/100 mL tartaric acid equivalents of Titratable Acidity.

Screening experiment

Strains of *O. oeni, Pedioccocus* , and *B. bruxellensis* were screened for the ability to hydrolyze hydroxycinnamic acid-tartaric acid esters. Microorganisms were inoculated into Acidic Grape Juice Broth (250ml/L white grape juice, 5 g/L yeast extract. 0.125 g/L magnesium sulfate, 0.0025 g/L manganese sulfate, 1 mL/L 5% Tween solution, pH 3.5) and incubated at 25°C until stationary phase was reached. Cultures were centrifuged (15 min @ 4500 RPM) and washed with sterile phosphate buffer (27.8 g/L $NaH_2PO_4 * H_2O$, 28.38 g/L Na_2HPO_4, pH 7.0) prior to inoculation into 20 mL of 2012 Oregon Pinot Noir in test tubes at a rate of approximately 10^6 CFU/mL in triplicate and incubated a 25°C. Growth was confirmed through visual confirmation of pellet formation. In addition, decarboxylation of malic acid by *O. oeni,* and *Pedioccocus* isolates was followed by measuring malic acid using an enzymatic test kit(R-Biopharm, Darmstadt, Germany). Upon completion of growth and/or completion of malic acid decarboxylation, a 1 mL sample was taken and stored at -80°C for later hydroxycinnamic acid analysis.

Time Course experiment

O. oeni strains VFO, VP41 and Alpha were taken as frozen glycerol cultures, inoculated into Acidic Grape Juice Broth and incubated at 25°C until stationary phase was reached.

Cultures were centrifuged (15 min @ 4500 ROM) and washed with sterile phosphate buffer prior to inoculation into 500 ml of 2012 Pinot Noir in 500 mL Schott Bottles at a rate of approximately 10^6 CFU/mL in triplicate. Growth was followed through plating on MRS agar plates at the day of inoculation and semi-weekly throughout the study. A 1 mL sample of wine was taken at each sample date and frozen at -80°C for hydroxycinnamic acid analysis via HPLC. Wine was sampled until MLF was complete, confirmed via malic acid loss measured by enzymatic kit (R-Biopharm).

Volatile phenol production by B. bruxellensis

O. oeni strains VFO, VP-41 and Alpha were prepared as outlined previously. Bacteria were then inoculated into 500 ml of 2011 Pinot Noir in 500 mL Schott Bottles at a rate of approximately 10^6 CFU/mL in triplicate along with an un-inoculated control. Samples were incubated at 25°C until MLF was complete, as confirmed via enzymatic kit (R-Biopharm). At this point the wines were inoculated with *B. bruxellensis* strain UCD-2049 at a rate of approximatley10^4 CFU/mL. UCD-2049 was prepared from frozen cultures by inoculation into Acidic Grape Juice Broth and incubation at 25°C until stationary phase was reached. Cultures were centrifuged (15 min @ 4500 ROM) and washed with sterile phosphate buffer prior to inoculation into samples. Growth of *B. bruxellensis* was followed through plating on YPD media weekly, with concurrent samples taken and stored at -80°C for hydroxycinnamic acid analysis by HPLC. Tracking of samples continued until full conversion of *p*-coumaric acid by *B. bruxellensis* was observed. At this point a 50 mL sample was taken and assessed for volatile phenol content. This was conducted as outlined by (Jensen et al. 2009). Briefly, 4-EP and 4-EG were analyzed using a headspace-solid phase

microextraction method with an 85 μm polyacrylate fiber (Supelco, Bellefonte, PA). The fiber was thermally desorbed at 280°C for 3 min by the injection port of a GC–MS/MS (Varian model 4000, Walnut Creek, CA).Separation was achieved using a DB-5MS capillary column (0.18 mm ID 20 m) with 0.18 mm film thickness obtained from J&W/Agilent Technologies (Wilmington, DE). The carrier gas, helium, was held at a constant flow of 0.8 mL/min. The temperature program consisted of: 40°C held for 2.0 min, increased 20°C/min to 160°C and held for 0.0 min, and then increased 50°C/min to 300°C and held for 0.2 min. The volatile phenols were identified by retention times as well as fragmentation patterns compared to chemical standards.

Hydroxycinnamic acid analysis

Reversed-phase high-performance liquid chromatography (HPLC) was performed using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA) equipped with HP ChemStation software and photodiode-array detector (DAD). The HPLC was fitted with a LiChroSpher reversed-phase C18 column (4 x 250 mm, 5 mm particle size) (Merck, Darmstadt, Germany) held at 30°C. HPLC grade 98% formic acid and 99.8% methanol were obtained from EMD Chemicals (Darmstadt, Ger- many) while caffeic acid, and *trans-p*- coumaric acid were obtained from Sigma Aldrich (St. Louis, MO). Gradients of solvent A (water/formic acid, 90:10, v/v) and solvent B (methanol) were applied as follows: 5 to 35% B linear (1.0 mL/min) from 0 to 15 min, static at 35% B (1.0 mL/min) from 15 to 20 min, 35 to 80% B linear (1.0 mL/min) from 20 to 25 min, and 5% B (1.0 mL/min) from 25 to 32 min to re-equilibrate the column to initial conditions. Prior to HPLC analysis, wine samples were centrifuged using an Allegra X-22 instrument (Beckman Coulter, Brea, CA) at 12,000 rpm

for 10 min. Wines were sampled in 20 μL aliquots and hydroxycinnamic acids were detected by scanning from 190 to 700 nm. Identification and quantification of hydroxycinnamic acids were performed at 320 nm based on caffeic and *trans-p*-coumaric acid standard curves, with caftaric acid expressed as caffeic acid equivalents.

Statistics

Statistical analysis was performed using The R Project for Statistical Computing (Auckland, New Zealand). Statistical significance was evaluated by Tukey's honest significance difference test for $p>.05$.

Results

Twenty-nine of wine microorganisms were screened for their ability to hydrolyze tartaric acid bound hydroxycinnamic acid esters. Of the thirteen *B. bruxellensis* strains, six *O. oeni* strains, and twelve *Pediococcus* isolates screened only the *O. oeni* strain VFO significantly decreased the concentration of tartaric ester bound HCAs caftaric acid and coutaric acid (Table 2.2). *O. oeni* VFO was also the only microorganism that caused an increase in the corresponding free HCAs, caffeic acid and coumaric acid during growth in wine (Table 2.2). Ferulic acid concentrations also increased in wines where *O. oeni* VFO grew although the corresponding bound form of this HCA, fetaric acid, was unable to be measured by this method due to interference at dilution time.

A time course study was performed to determine when during growth in wine *O. oeni* VFO hydrolyze bound HCAs. After an initial drop in population following inoculation into wine,

O. oeni VFO grew well reaching a maximum population of over 1 x 10^7 CFU/mL (Figure 2.1). During the logarithmic portion of this growth (days 15-30) malic acid was decarboxylated and coutaric acid was hydrolyzed(Figure 2.1). Malic acid decarboxylation was complete a few days prior to the full hydrolysis of coutaric acid. As coutaric acid concentration decreased a corresponding increase in coumaric acid was observed (Figure 2.1). In contrast, when Pinot noir wine was inoculated with *O. oeni* Alpha there was no decrease in coutaric acid or an increase in coumaric acid even though Alpha grew well and fully decarboxylated the malic acid present in the wine (Figure 2.2).

Table 2.2 Ability of various wine microorganisms to hydrolyze tartaric ester bound hydroxycinnamic acids and increase free hydroxycinnamic acids

		Degradation of:		Increase in:		
Microorganisms	Strain	Caftaric	Coutaric	Caffeic	Coumaric	Ferulic
B. bruxellensis	493	-	-	-	-	-
	495	-	-	-	-	-
	496	-	-	-	-	-
	497	-	-	-	-	-
	607	-	-	-	-	-
	608	-	-	-	-	-
	613	-	-	-	-	-
	614	-	-	-	-	-
	615	-	-	-	-	-
	616	-	-	-	-	-
	CM12	-	-	-	-	-
	SL15	-	-	-	-	-
	UCD-2049	-	-	-	-	-
	AWRI 1499	-	-	-	-	-
	AWRI 1608	-	-	-	-	-
O. oeni	VFO	+	+	+	+	+
	VP41	-	-	-	-	-
	VP31	-	-	-	-	-

	Alpha	-	-	-	-	-
	Beta	-	-	-	-	-
	Elios	-	-	-	-	-
P. parvulus	OW1	-	-	-	-	-
	OW3	-	-	-	-	-
	OW4	-	-	-	-	-
	OW5	-	-	-	-	-
	OW6	-	-	-	-	-
	OW7	-	-	-	-	-
	WW1	-	-	-	-	-
	WS9	-	-	-	-	-
P. inopinatus	OW8	-	-	-	-	-
	OW9	-	-	-	-	-
P. damnosus	OW2	-	-	-	-	-
	ATCC 43013	-	-	-	-	-

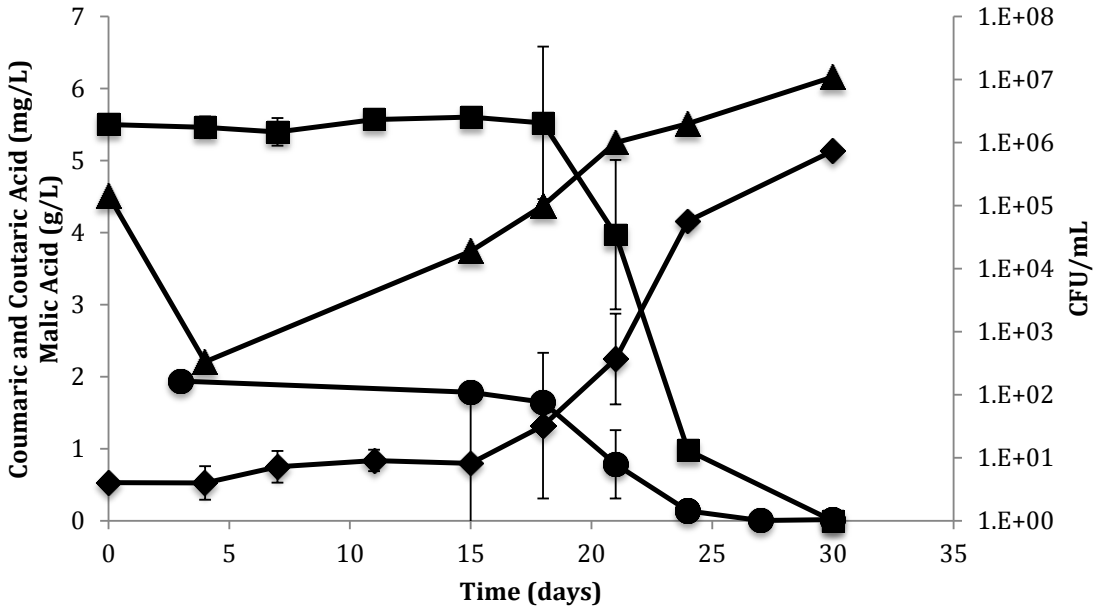


Figure 2.1 Concentration of coumaric acid (◆), coutaric acid (■), and malic acid (●) during growth (▲) of *O. oeni* VFO in Pinot noir wine.

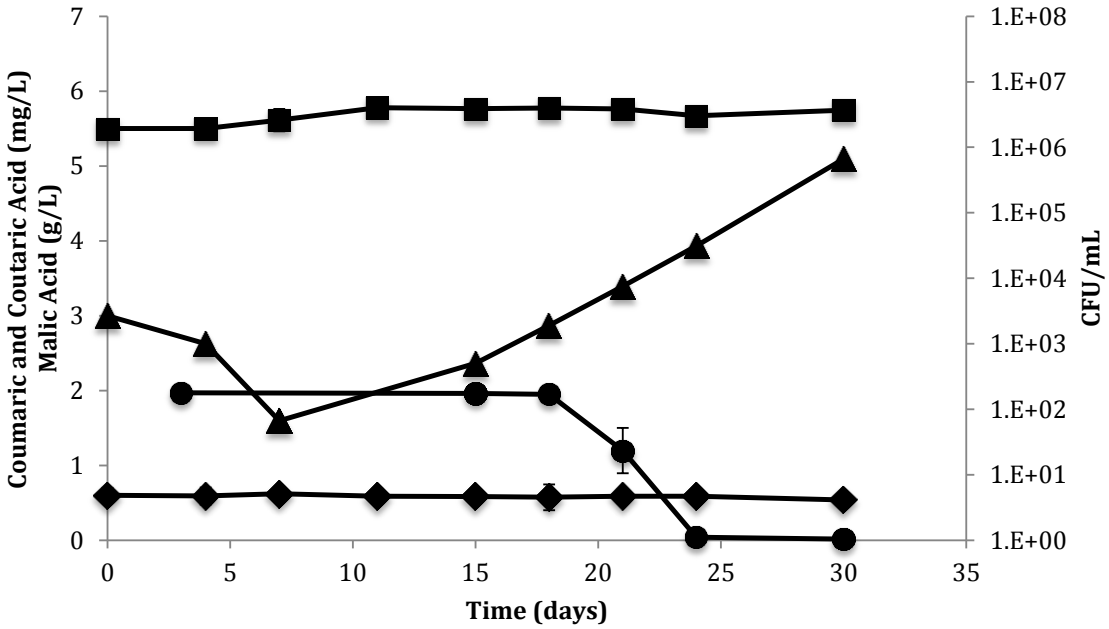


Figure 2.2 Concentration of coumaric acid (◆), coutaric acid (■), and malic acid (●) during growth (▲) of *O. oeni* Alpha in Pinot noir wine.

The impact of *O. oeni* on volatile phenol production by *B. bruxellensis* was investigated by inoculating *B. bruxellensis* UCD-2049 into Pinot noir wine where MLF had been conducted by either *O. oeni* VFO, Alpha, or VP41. At the completion of MLF and prior to inoculation of *B. bruxellensis* wines were assessed for HCA content (Table 2.3). Wine that underwent MLF with *O. oeni* strain VFO had significantly lower concentrations of caftaric acid and coutaric acid and higher concentrations of caffeic acid and coumaric acid compared to the control which did not undergo MLF and wines that underwent MLF with either Alpha or VP41 (Table 2.3). For example, at the end of MLF with *O. oeni* VFO the wine contained 5 mg/L of *p*-coumaric acid while the other wines contained between .4 and 1.3 mg/L. When *B. bruxellensis* UCD-2049 was inoculated into these wines it grew well in all treatments including an uninoculated control that did not undergo MLF (Figure 1.3). After 40 days of growth the wines were assessed for 4-EP and 4-EG content. In wine that had undergone

MLF with *O. oeni* VFO there was significantly higher amounts of both 4-EP and 4-EG (Table 2.3). For example, the control wine contained 0.26 g/L 4-EP while the VFO inoculated wine contained 1.58 g/L. There was no significant difference between the 4-EP and 4-EG content of any of the other wines (Table 2.3).

Table 2.3 Concentration (mg/L) of free and bound hydroxycinnamic acids after completion of MLF (Initial) and concentration (mg/L) of free and bound hydroxycinnamic acids and 4-ethyphenol and 4-ethyguiacol after 40 days growth of *B. bruxellensis* UCD-2049 (Final)

	Initial				Final					
	Caftaric	Caffeic	Coutaric	Coumaric	Caftaric	Caffeic	Coutaric	Coumaric	4-EP	4-EG
Control	29.5[a]	2.5[a]	9.2[a]	1.3[a]	27.8[a]	1.9[a]	8.9[a]	0.0[a]	.26[a]	.08[a]
VFO	11.1[b]	18.4[b]	3.7[b]	5.0[b]	5.3[b]	15.2[b]	1.7[b]	1.9[b]	1.58[b]	.27[b]
Alpha	31.6[a]	3.3[a]	10.0[a]	0.8[a]	30.1[a]	2.9[a]	9.6[a]	0.1[a]	.18[a]	.07[a]
VP-41	31.3[a]	2.8[a]	9.9[a]	0.4[a]	29.5[a]	2.7[a]	9.6[a]	0.1[a]	.22[a]	.08[a]

[a-b]Values sharing the same letter within a column are not significantly different at $p < 0.05$, n=3

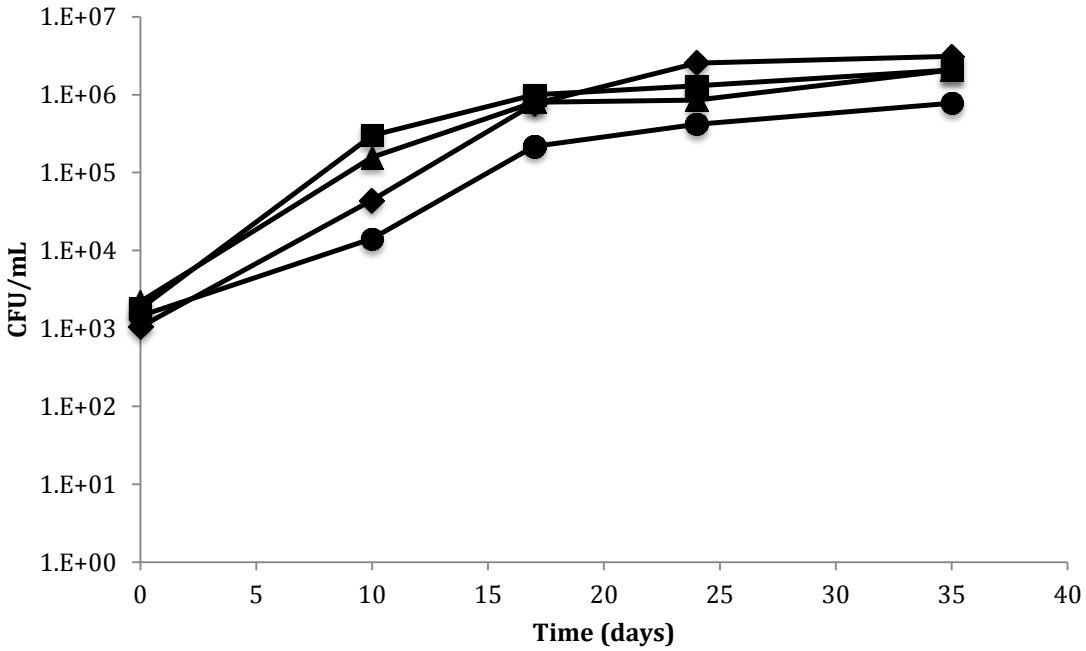


Figure 2.3 Growth of *B. bruxellensis* UCD-2049 in Pinot noir wine that underwent MLF with *O. oeni* strains VFO (■), Alpha (●), and VP-41 (▲) and an un-inoculated control (◆), during incubation, following inoculation with *B. bruxellensis.*

Discussion

Brettanomyces can cause wine spoilage through the conversion of hydroxycinnamic acids to volatile phenols (Pascal Chatonnet 1992). However, a large portion of the HCAs in a wine may be inaccessible to *Brettanomyces* due to its inability to hydrolyze tartaric ester bound HCAs as demonstrated in the current study as well as (Schopp et al. 2013). Therefore, the identification of an *O. oeni* strain capable of hydrolyzing tartaric ester bound HCAs is significant as additional substrate for *Brettanomyces* production of volatile phenols. While other works have reported an increase in free HCAs post MLF specific strains of *O. oeni* were not identified due to the MLF being conducted spontaneously (Hernández et al. 2007) or with a mixed culture of wine LAB (Cabrita et al. 2008). In the current study a commonly used commercial strain, *O. oeni* VFO, was identified as being capable of hydrolyzing tartaric ester bound HCAs resulting in a significant increase in free HCAs in the wine.

The increased free HCAs content in wines that underwent MLF with *O. oeni* VFO resulted in a large increase in the production of 4-EP and 4-EG by *B. bruxellensis*. Because of the relatively low amount of free HCAs in the original wines a relatively low amount of volatile phenols were produced in the control wine and wines where MLF was conducted by *O. oeni* Alpha or VP41. In fact, the concentrations of volatile phenols in these wines were at or below the reported sensory threshold of 4-EP and 4-EG in Pinot noir. For example, Chattonet et al. (1990) reports sensory threshold for 4-EP and 4-EG in Pinot noir wine at 0.23 mg/L and 0.047 mg/L respectively. Chattonet et al. (1992) also reported a combined detection threshold of 0.426 mg/L when at a ratio of 10:1 4-EP:4-EG. However trained judges have been shown to not be able to identify “Brett characters” until past combined

ethylphenol concentrations of 0.668 mg/L (Goode 2005). The concentration of 4-ethylphenol and 4-ethylguaicol found in wines that did not undergo malolactic fermentation, or underwent malolactic fermentation with strains of *O. oeni* unable to hydrolyze tartaric acid-hydroxycinnamic acid esters was around sensory threshold, and below the level identifiable by judges (Table 2.3). In contrast, *B. bruxellensis* UCD-2049 produced volatile phenol levels more than five time higher, and surpassed the average for highly tainted wine (Andrea Romano et al. 2009). This would likely have a significant negative impact the sensory quality of the wine.

The significance of tartaric ester bound HCAs hydrolysis on wine spoilage may depend on the levels of free HCAs present in the wine before MLF. The 2011 Oregon Pinot Noir used in this study had only 1.3 mg/L of coumaric acid while the 2012 Oregon Pinot Noir contained only 0.6 mg/L of coumaric acid. These levels are low compared to other reported values for Pinot Noir from the Pacific Northwest which are closer to 2.4 mg/L on average (Goldberg et al. 1998). However the same paper noted significant differences between Pinot noir wines based on geographical origin. Some cultivars contained *p*-coumaric acid concentrations in excess of that measured in the 2011 Oregon Pinot Noir even after complete hydrolysis of coutaric acid by *O. oeni* VFO. As such, wine from some cultivars and regions would likely produce levels of ethylphenols significant beyond sensory thresholds if spoiled by *B. bruxellensis* regardless of whether or not the tartaric ester bound HCAs were hydrolyzed during MLF.

The initiation of hydroxycinnamic acid-tartaric acid hydrolysis by *O. oeni* strain VFO coincided with malic acid decarboxylation, but was completed after malic acid was

depleted. This can be observed not only during the time course study, but also indicated during the ethylphenol production study. The levels of caftaric acid and coutaric acid decreased significantly in samples that underwent MLF with *O. oeni* strain VFO from after completion of MLF and after growth of *B. bruxellensis.* This means that it would be very difficult to prevent the hydrolysis of bound HCAs from occurring even if you were closely monitoring malic acid decarboxylation and adding SO_2 to kill *O. oeni* immediately after depletion of malic acid. A winemaker's only option to prevent hydrolysis of bound HCAs therefore is to use a strain of *O. oeni* to conduct the MLF that cannot hydrolyze bound HCAs such as those identified in this study. In addition, performing spontaneous MLFs may also increase the risk of wine spoilage by *Brettanomyces* as you do not know the identity of the wine LAB that is conducting the MLF and you do not know whether or not it can hydrolyze bound HCAs.

The current study screened a number of *Brettanomyces* , *Pediococcus*, and *Oenococcus* strains for their ability to hydrolyze bound HCAs. A larger number of organisms should be evaluated in the future to help determine how widespread and/or common this trait is in wine microorganisms. For example, no *Lactobacillus* species were evaluated in this study. Interestingly, Guglielmetti et al. (2008) reported that some *Lactobaccilus* sp. have the ability to hydrolyze quinic acid-hydroxycinnamic acid ester so the potential for the ability to hydrolyze tartaric acid hydroxycinnamic acid esters should be explored. In addition, a larger number of *Brettanomyces* should be evaluated as these yeast are known to have large genetic and physiological diversity for other traits (Conterno et al. 2006). The selection of Brettanomyvces strains AWRI 1499 and 1608 was of particular interest

because they demonstrated hydrolysis of hydroxycinnamic acid ethyl esters in previous work (Hixson et al. 2012). Finally, identification of the genes encoding the *O. oeni* tartaric acid-hydroxycinnamic acid esterase would allow for more efficient screening of wine microorganisms. This would assist in both the selection of strains for use as commercial cultures, and also for characterization of resident strains in wineries that perform spontaneous MLF.

Conclusion

Of the thirty five strains of wine microorganisms tested only one, the commercial strain *O. oeni* VFO, hydrolyzed tartaric acid bound hydroxycinnamic acids in wine. The hydrolysis initiated approximately at the same time as malic acid decarboxylation during MLF, but finished several days after completion of MLF. The decrease in bound hydroxycinnamic acids was mirrored by an increase of free hydroxycinnamic acids. This increase in free hydroxycinnamic acids continued following conversion to ethylphenols by *B. bruxellensis.* 4-EP and 4-EG were seven and three times higher respectively following growth of *B. bruxellensis* in wine where VFO had conducted the MLF. While wineries must continue to use sound winemaking practices to prevent the growth of *Brettanomyces* in their wines, this research has shown that minimizing the amount of free coumaric and ferulic acid present in wine will also reduce the risk of wine spoilage. The use of an *O.oeni* strain that cannot hydrolyze bound coumaric and ferulic acid is a simple practical strategy to achieve this.

Inhibition of *Brettanomyces bruxellensis* Growth by *Oenococcus oeni* in Media and Oregon Pinot Noir

Stuart C. Chescheir, James P. Osborne

Abstract

This study investigated interactions between the wine spoilage yeast *Brettanomyces bruxellensis* and wine lactic acid bacteria (LAB) and the impact on *Brettanomyces* growth and volatile phenol production. Studies in acidic grape juice (AGJ) broth (pH 3.50, 5% ethanol) demonstrated that growth of *O. oeni* could inhibit *Brettanomyces* growth in a strain dependent manner. Production of 4-ethylphenol (4-EP) was also delayed and reduced in a strain dependent manner. For example, in the control a maximum of 19 mg/L 4-EP was produced by *B. bruxellensis* UCD-2049 while during growth in media where VP41 had previously grown only 7.9 mg/L 4-EP was produced. When *B. bruxellensis* was inoculated into Pinot noir wine where malolactic fermentation (MLF) had been performed by *O. oeni* VFO populations quickly decreased below detectable levels and did not recover during the course of the experiment (50 days). In contrast, when *B. bruxellensis* UCD-2049 was inoculated into Pinot noir wine where *Pediococcus damnosus* OW2 had previously grown populations did not decrease and growth occurred in a similar manner to the control. The reason for this decrease in population was further explored by inoculating *B. bruxellensis* into AGJ broth (pH 3.50) where there had been previous growth of *O. oeni* Alpha or VP41. In half the treatments the *O. oeni* were removed by sterile filtration prior to *Brettanomyces* inoculation. Although there was delayed growth of *B. bruxellensis* in media where *O. oeni* had previously grown there was no significant difference between growth in sterile filtered and non-sterile filtered treatments. The relief of inhibition by sterile filtration suggests that neither nutrient depletion nor the production of an extracellular inhibitory compound by *O. oeni* were the causes of *Brettanomyces* inhibition as sterile filtration would not have impacted these. Additional research is required to determine the

mechanism by which *O. oeni* inhibits *B. bruxellensis* as the use of *O. oeni* strains that have this inhibitory effect would be useful in reducing the risk of *Brettanomyces* spoilage of wine.

Introduction

B. bruxellensis is the most important wine spoilage microbe encountered during winemaking and causes significant financial losses annually (Loureiro and Malfeito-Ferreira 2005; Puig et al. 2011). Spoilage by *B. bruxellensis* is usually caused by production of ethylphenols that smell medicinal (4-ethylphenol) or smokey/clovey (4-ethylguiacol). *Brettanomyces* growth typically occurs following alcoholic fermentation (Suárez et al. 2007) and during the storage of the wine in barrels. Winemakers typically try and prevent the infection of *Brettanomyces* in their wine through robust cleaning and sanitation programs and yet *Brettanomyces* spoilage of wine still frequently occurs (Puig et al. 2011). If *Brettanomyces* infects a wine one of the winemakers only tools to prevent growth of this yeast is the antimicrobial sulfur dioxide (SO_2). However, controlling the growth of *B. bruxellensis* with SO_2 can also be challenging as there is a wide range of strain sensitivity to SO_2 with some strains of *Brettanomyces* demonstrating resistance to this antimicrobial agent (Zuehlke, Petrova, and Edwards 2013). Therefore, additional means to prevent or minimize the growth of *Brettanomyces* in wine are required.

An additional approach to the control of *Brettanomyces* growth in wine is to explore and exploit possible inhibitory relationships between microorganisms present during the winemaking process. Many different species of microorganisms may be present simultaneously during winemaking and their interactions are not always neutral. For

example, a number of studies have shown that the fermentative yeast *Saccharomyces cerevisiae* can be inhibitory to growth of the malolactic bacteria *Oenococcus oeni* (Beelman et al. 1982; Cannon and Pilone 1993; Henick-Kling and Park 1994; Osborne and Edwards 2007; Osborne and Edwards 2006) The antagonism of *O. oeni* by yeast is thought to be due to the production of toxic metabolites during the alcoholic fermentation) or through the depletion of nutrients essential for the growth of the bacteria. Huang et al. (1996) also reported that rapid growth of certain *Lactobacillus* species early during the alcoholic fermentation could result in inhibition of *S. cerevisiae* due to the production of large amounts of acetic acid by the bacteria.

Despite this evidence that interactions between wine microorganisms can be inhibitory in nature, little is known about the interactions between *B. bruxellensis* and other wine microorganisms. *B. bruxellensis* is reported to be a poor competitor compared to *S. cerevisiae,* demonstrated by a study performed by Renouf et al. (2006). Renouf reported that when both of these yeast were inoculated into a grape juice *S. cerevisiae* dominated the fermentation while *B. bruxellensis* growth occurred mainly after the completion of the alcoholic fermentation when *S. cerevisiae* populations were in decline. One of the few reports regarding interactions between *Brettanomyces* and *O. oeni*, (Gerbaux et al. 2009) noted that *Brettanomyces* produced significantly lower amounts of volatile phenols in wines that had undergone MLF. However, little information regarding populations of *O. oeni* or *Brettanomyces* was provided making it difficult to conclude what the cause of the reduced volatile phenols was. Recent work in our laboratory has shown that growth of *O. oeni* may have an inhibitory effect on *B. bruxellensis* (Strickland 2012). However, this work was

preliminary in its nature and requires additional research to confirm whether the growth of *O. oeni* can cause inhibition of *B. bruxellensis* and by what mechanism. Therefore, the purpose of this study was to investigate the impact of *O. oeni* on *B. bruxellensis* growth and volatile phenol production.

Material and Methods

Microorganisms

Brettanomyces bruxellensis strain UCD-2049 was sourced from the UC Davis culture collection. UCD-2049 was streaked for isolation onto YPD media (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L dextrose, 20 g/L Agar, pH 6.5) then grown in YPD broth at 25°C until stationary phase was reached. Aliquots were then stored in glycerol at -80°C for future use as described by Strickland (2012).

The strains of *Oenococcus oeni* used for the study were Viniflora Oenos (VFO) (Chr. Hansen, Hørsholm, Denmark), CH35 (Chr. Hansen), VP41 (Lallemand, Montreal, Canada) Elios (Lallemand) and Enoferm® Alpha (Lallemand). After rehydration in sterile peptone solution (0.1%) the cultures were streaked for isolation onto MRS media (20 g/L Tryptone, 5 g/L Peptone, 5 g/L Yeast Extract, 5 g/L Glucose, 1 mL/L 5% Tween solution, 20g/L Agar 200 mL/ L Apple Juice, pH 4.5) then grown in MRS broth at 25°C until stationary phase was reached. Aliquots were then stored in glycerol at -80°C for future use as described by Strickland (2012). *Pediococcus damnosus* OW-2 was initially isolated from Oregon Pinot noir wine as outlined by Strickland (2012) and stored at -80°C.

When needed, *O. oeni* and *B. bruxellensis* cultures taken from frozen glycerol samples were inoculated into Acidic Grape Juice (AGJ) broth (250ml/L Grape Juice, 5 g/L Yeast Extract. .125 g/L Magnesium Sulfate, .0025 g/L Manganese Sulfater, 1 mL/L 5% Tween solution, pH 3.5) and incubated at 25°C until stationary phase was reached. Cultures were centrifuged (15 min @ 4500 RPM) and washed with sterile phosphate buffer (27.8 g/L $NaH_2PO_4 * H_2O$, 28.38 g/L Na_2HPO_4, pH 7.0) prior to inoculation.

Winemaking

Pinot Noir wine was produced at the Oregon State University Research Winery from grapes harvested from Woodhall Vineyard (Alpine, Oregon, USA) in 2011 and 2012. Harvest was determined by soluble solid levels and perceived fruit ripeness by the managing team at the vineyard. Grapes were stored at 4 °C for 48 hours and then hand sorted and destemmed using a Velo DPC 40 destemmer/crusher (Altivole, Italy). Grapes were divided into 100 L stainless steel tanks containing approximately 60 L of must each. 50 mg/L of SO_2 (in the form of potassium metabisulfite) was added to each tank and the yeast nutrient Fermaid K® (Lallemand, Montreal, Canada) was added to a concentration of 0.125 g/L. Each tank was inoculated with the commercial strain of *Saccharomyces cerevisiae* RC-212 (Lallemand) at a rate of 0.25 g/L of must. Yeast was hydrated according to manufacturer's specification prior to inoculation. Fermentations were performed in a temperature-controlled room held at 27°C. Cap management was done through punch downs twice a day and temperature and °Brix were measured with an Anton-Paar DMA 35N Density Meter (Graz, Austria). Fermentation continued until sugar levels fell below 0.2g/100mL confirmed by Bayer Clinitest tablets (Morristown, New Jersey, USA).

Following fermentation the wine was pressed using a Willmes model 6048 pneumatic bladder press (Lorsch, Germany). Pressed wine was put in 100 L stainless steel tanks and stored at 4°C. Following settling for 72-hours wine was filtered through a plate and frame filter fitted with Beco K-1 2.0 µm nominal filter sheets (Langenlonsheim, Germany). Wine was then homogenized and filtered through 1.0 µm nylon cartridge and a 0.45 µm polyethersulfone sterile filter (G.W. Kent, Ypsilanti, Michigan, USA) in succession. Filtered wine was dispensed into sterile carboys and stored at 4 °C for future use. Basic wine parameters for the 2012 Pinot Noir wine was 13.2% ethanol by volume, pH 3.53 and had 0.67 g/100 mL tartaric acid equivalents of Titratable Acidity. Final 2011 Pinot Noir was 12.6% ethanol by volume, pH 3.44 and 0.68 g/100 mL tartaric acid equivalents of Titratable Acidity.

Media growth studies

The impact of *O. oeni* on the growth and volatile phenol production of *Brettanomyces* was initially studied in a media system at wine pH. *O. oeni* strains VFO, CH35, Elios, VP41, and Alpha were prepared from frozen samples as previously described. These strains were inoculated at approximately 1 x10^6 CFU/mL into 100 mL of AGJ broth (pH 3.50) containing 30 mg/L *p*-coumaric acid and 5% (v/v) ethanol. All treatments, including a non-inoculated control, were prepared in triplicate. Initial populations were checked by plating on MRS plates and all treatments were incubated at 25°C until stationary phase was observed. At this point *B. bruxellensis* UCD-2049 was inoculated into all treatments at approximately 1 x10^3 CFU/mL. *B. bruxellensis* UCD-2049 was prepared from a frozen sample as previously described. Initial *B. bruxellensis* populations were checked by plating on YPD plates while

ongoing growth of *B. bruxellensis* was followed through plating on YPD plates every 48 hours. One mL samples were also taken and stored at -80°C for later analysis of hydroxycinnamic acids and 4-ethylphenol.

In a second experiment, the impact of the presence of *O. oeni* cells on *Brettanomyces* growth and volatile phenol production was assessed by removal of *O. oeni* cells by sterile filtration prior to inoculation by *B. bruxellensis*. *O. oeni* cultures VFO, VP41, and Alpha were prepared as previously described and inoculated at approximately 1 x10^6 CFU/mL into 100 mL of AGJ broth (pH 3.50) containing 30 mg/L *p*-coumaric acid. A non-inoculated control was also prepared. All treatments were incubated at 25°C. The inoculation scheme is outlined in Figure 3.1. Initial *O. oeni* populations were checked by plating on MRS plates (20 g/L Tryptone, 5 g/L Peptone, 5 g/L Yeast Extract, 5 g/L Glucose, 1 mL/L 5% Tween solution, 20g/L Agar 200 mL/ L Apple Juice, pH 4.5) and populations were checked after stationary phase was observed. At this point half of the treatments were sterile filtered (0.45 μm PES filter) to remove *O. oeni* cells. *B. bruxellensis* UCD-2049 was prepared as previously described and inoculated at approximately 1 x10^3 CFU/mL into all treatments. Initial *B. bruxellensis* populations were checked by plating on YPD plates while ongoing growth of *B. bruxellensis* was followed through plating on YPD plates every 48 hours. One mL samples were also taken and stored at -80°C for later analysis of *p*-coumaric acid and 4-ethylphenol.

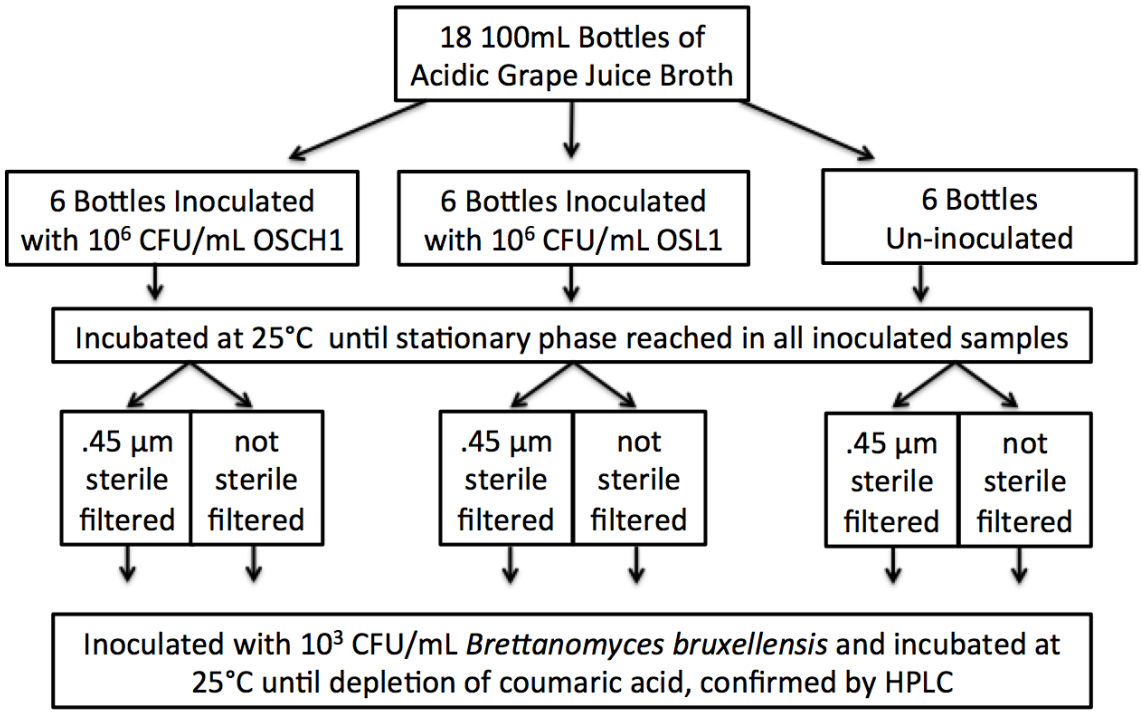


Figure 3.1 Experimental design for sterile filtration study in Acidic Grape Juice Broth

Pinot noir wine growth studies

An initial experiment to investigate the impact of *O. oeni* on *B. bruxellensis* growth in wine was undertaken where *O. oeni* VFO or *P. damnosus* OW2 were inoculated into a 2011 Pinot noir prepared as previously described. VFO and OW2 were prepared from frozen cultures and inoculated into the wine at approximately 1 x 10^6 CFU/mL. All treatments were prepared in triplicate. Initial populations were checked by plating on MRS plates. The malolactic fermentation was monitored by measuring malic acid using an enzymatic kit (R-Biopharm, Darmstadt, Germany). At the completion of the MLF *B. bruxellensis* UCD-2049 was inoculated into all treatments at approximately 1 x10^3 CFU/mL. *B. bruxellensis* UCD-

2049 was prepared from a frozen sample as previously described. Initial *B. bruxellensis* populations were checked by plating on YPD plates while ongoing growth of *B. bruxellensis* was followed through plating on YPD plates every 48 hours.

A second set of experiments were prepared to investigate the effect of removal of *O. oeni* cultures by sterile filtration prior to *B. bruxellensis* inoculation in wine. *O. oeni* cultures VFO, VP41, and Alpha were prepared as previously described and inoculated at approximately 1 $x10^6$ CFU/mL into 500 mL of 2012 Pinot noir wine (prepared as previously described). A non-inoculated control was also prepared and all treatments were prepared in triplicate. The inoculation scheme is outlined in Figure 3.2. Initial populations were checked by plating on MRS plates while the malolactic fermentation was monitored by measuring malic acid using an enzymatic kit (R-Biopharm, Darmstadt, Germany). All treatments were incubated at 25°C until MLF was completed. At this point half of the treatments were sterile filtered (0.45 μm PES filter) to remove *O. oeni* cells. *B. bruxellensis* UCD-2049 was prepared as previously described and inoculated at approximately 1 $x10^3$ CFU/mL into all treatments. Initial *B. bruxellensis* populations were checked by plating on YPD plates while ongoing growth of *B. bruxellensis* was followed through plating on YPD plates every 48 hours. One mL samples were also taken and stored at -80°C for later analysis of hydroxycinnamic acids.

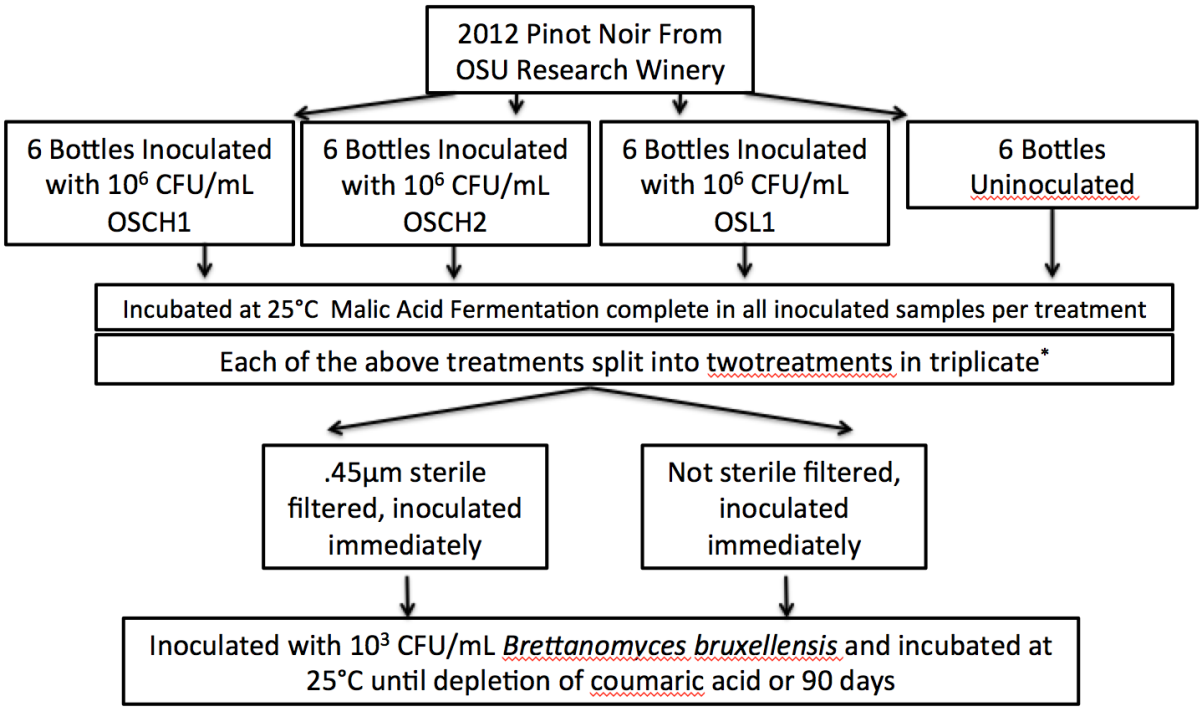


Figure 3.2 Experimental design for sterile filtered study in Oregon Pinot Noir

Hydroxycinnamic acid and 4-ethyphenol analysis

Reversed-phase high-performance liquid chromatography (HPLC) was performed using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA) equipped with HP ChemStation software and photodiode-array detector (DAD). The HPLC was fitted with a LiChroSpher reversed-phase C18 column (4 x 250 mm, 5 mm particle size) (Merck, Darmstadt, Germany) held at 30°C. HPLC grade 98% formic acid and 99.8% methanol were obtained from EMD Chemicals (Darmstadt, Ger- many) while caffeic acid, and *trans-p*- coumaric acid were obtained from Sigma Aldrich (St. Louis, MO). Gradients of solvent A (water/formic acid, 90:10, v/v) and solvent B (methanol) were applied as follows: 5 to 35% B linear (1.0

mL/min) from 0 to 15 min, static at 35% B (1.0 mL/min) from 15 to 20 min, 35 to 80% B linear (1.0 mL/min) from 20 to 25 min, and 5% B (1.0 mL/min) from 25 to 32 min to re-equilibrate the column to initial conditions. Prior to HPLC analysis, wine samples were centrifuged using an Allegra X-22 instrument (Beckman Coulter, Brea, CA) at 12,000 rpm for 10 min. Wines were sampled in 20 µL aliquots and hydroxycinnamic acids and 4-ethyphenol were detected by scanning from 190 to 700 nm. Identification and quantification of hydroxycinnamic acids were performed at 320 nm based on caffeic and *trans-p*-coumaric acid standard curves, with hydroxycinnamic acid tartaric acid esters expressed as their respective free hydroxycinnamic acid equivalents. Identification and quantification of 4-ethyphenol was performed at 280 nm based on 4-ethylphenol standard curves.

Results

The impact of *O. oeni* growth on *B. bruxellensis* growth and 4-EP production was initially investigated in acidic grape juice broth containing 5% ethanol and *p*-coumaric acid. After inoculation into media where *O. oeni* had previously grown there was a drop in population of *B.bruxellensis*, a lengthened lag phase and a lower maximum population compared to the control (Figure 3.1). The drop in population was less so in media where *O. oeni* Alpha had grown (Figure 3.1). This reduction in growth correlated with delayed decrease of *p*-coumaric acid where depletion occurred only in the control and in media where *O. oeni* Alpha had grown (Figure 3.2). Delayed and reduced production of 4-EP by *B. bruxellensis* was also noted (Figure 3.3) with the final amount of 4-EP being produced differing depending on which strain of *O. oeni* had grown in the media. For example, in the control a

maximum of 19 mg/L 4-EP was produced by *B. bruxellensis* while during growth in media where VP41 had previously grown only 7.9 mg/L 4-EP was produced (Figure 3.3).

B. bruxellensis was inoculated into Pinot noir wine where *O. oeni* VFO or *P. damnosus* OW-2 had previously grown. Compared to the control there was a small delay in entering logarithmic growth for *B. bruxellensis* when inoculated into wine where there had been previous growth of *P. damnosus* OW-2 (Figure 3.4). However, a similar max population was eventually reached. In contrast, *B. bruxellensis* populations fell below detectable levels after the initial sampling point, and were not detectable at any point throughout the study (Figure 3.4).

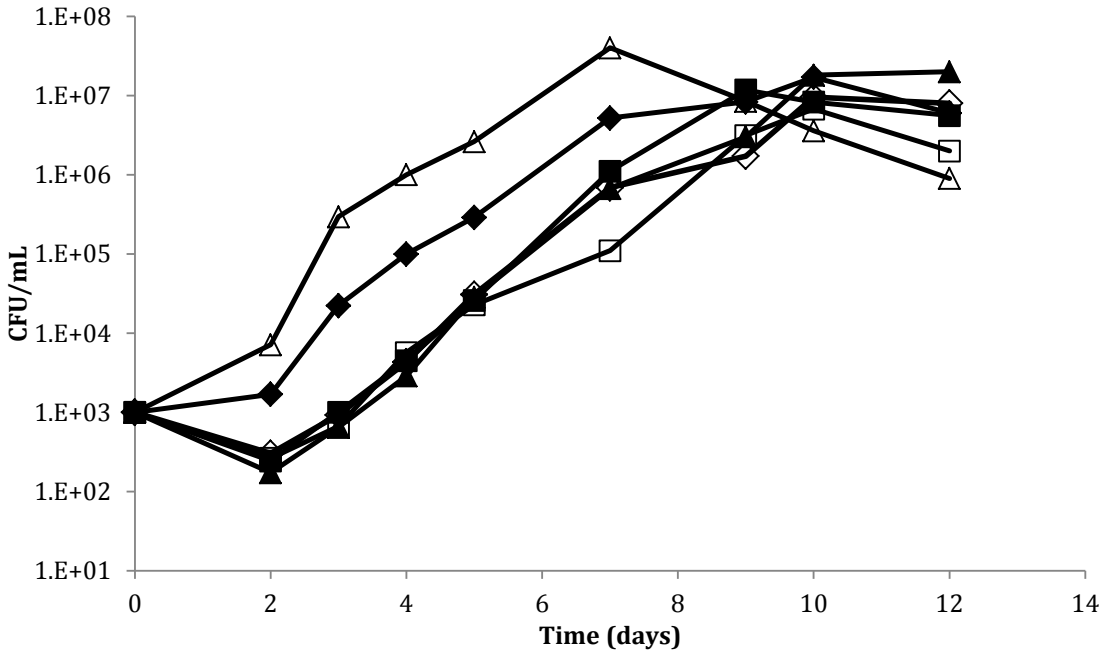


Figure 3.3 Growth of *B. bruxellensis* UCD-2049 in AGJ broth containing 5% ethanol and 30 mg/L *p*-coumaric acid after prior growth of *O. oeni* Alpha (◆), CH35 (◊), Elios (■), VFO (□) and VP41 (▲) and a control with no prior growth of *O. oeni* (Δ).

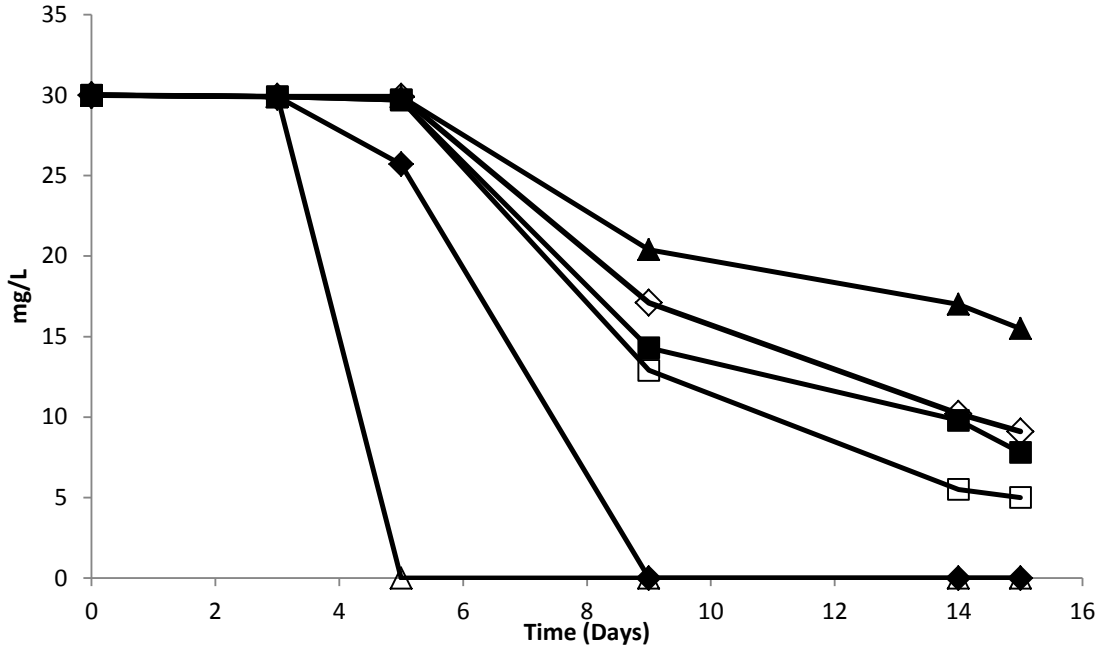


Figure 3.4 Concentration of p-coumaric acid during growth of *B. bruxellensis* UCD-2049 in AGJ broth containing 5% ethanol and 30 mg/L *p*-coumaric acid after prior growth of *O. oeni* Alpha (◆), CH35 (◊), Elios (■), VFO (□) and VP41 (▲) and a control with no prior growth of *O. oeni* (Δ).

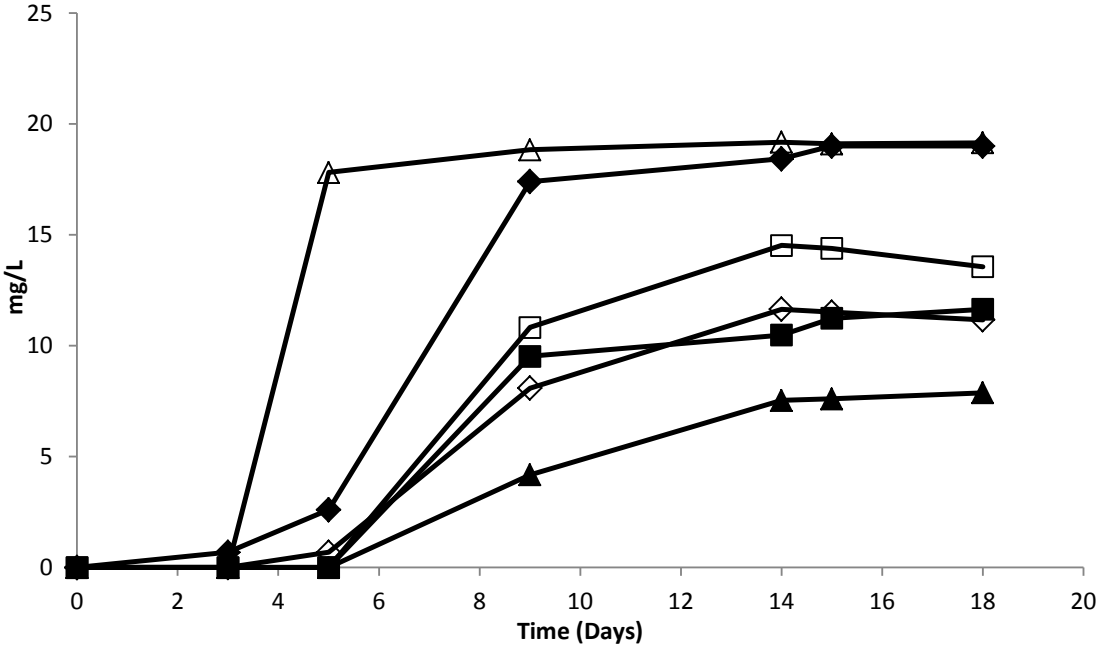


Figure 3.5 Concentration of 4-ethylphenol during growth of *B. bruxellensis* UCD-2049 in AGJ broth containing 5% ethanol and 30 mg/L *p*-coumaric acid after prior growth of *O. oeni* Alpha (◆), CH35 (◊), Elios (■), VFO (□) and VP41 (▲) and a control with no prior growth of *O. oeni* (Δ).

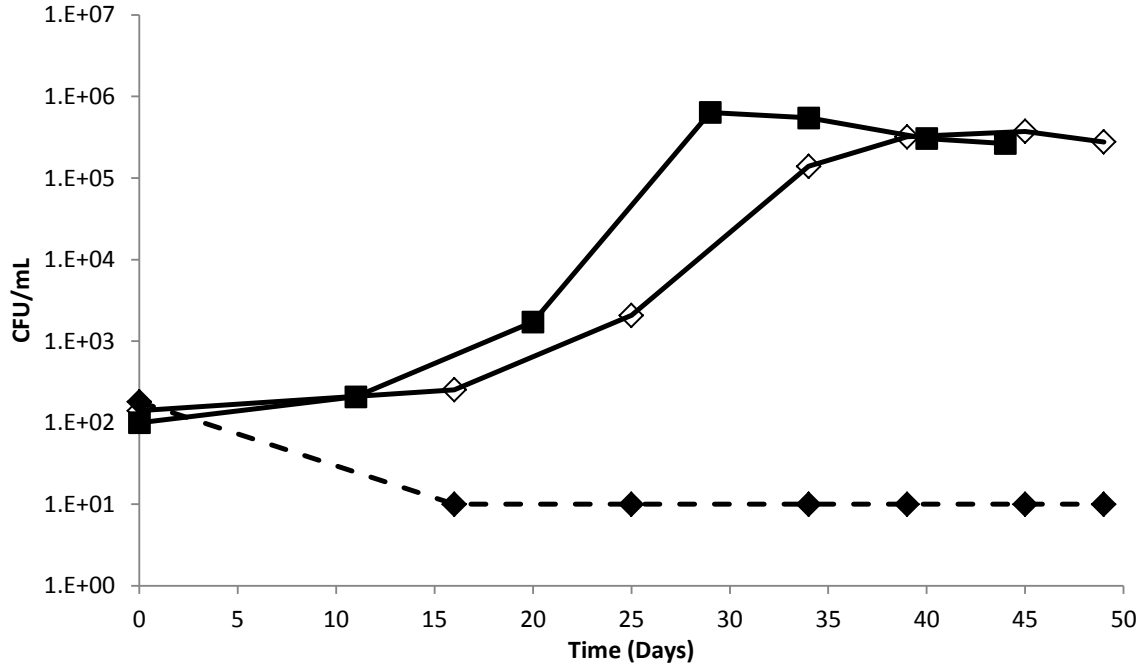


Figure 3.6 Population of *B. bruxellensis* UCD-2049 after inoculation into a Pinot noir wine where there had been previous growth of *O. oeni* VFO (◆) or *P. damnosus* OW2(◊) or where there had been no previous growth of bacteria (■).

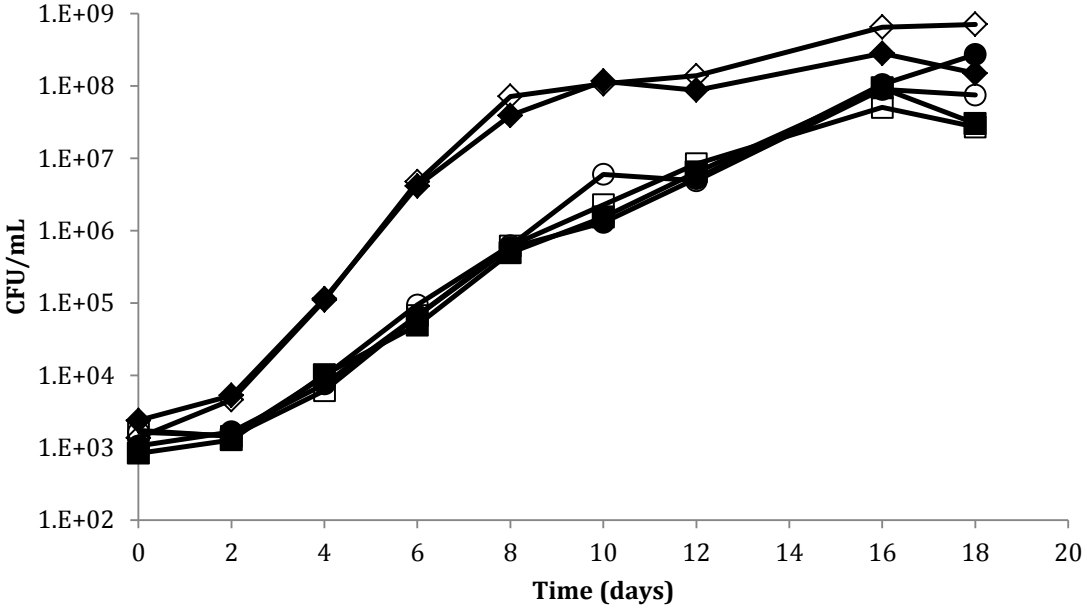


Figure 3.7 Growth of *B. bruxellensis* UCD-2049 in AGJ broth containing 30 mg/L *p*-coumaric acid after prior growth of *O. oeni* strains VP41 (■), Alpha (●) or an un-inoculated control (◆). Corresponding open symbols indicate growth in broth where sterile filtration occurred to remove the *O. oeni* cells prior to inoculation with *B. bruxellensis*.

Growth of *B. bruxellensis* UCD-2049 was monitored after inoculation into AGJ broth where either *O. oeni* VP41 or Alpha had previously grown. In addition, growth was assessed in media where the *O. oeni* had been removed by sterile filtration prior to inoculation with *B. bruxellensis*. In all treatments *B. bruxellensis* grew well although there was a delay in growth (increased lag phase) in media where *O. oeni* had previously grown (Figure 3.5). There was no significant difference in growth between sterile filtered and non-sterile filtered treatments, or between different strains of *O. oeni* (Figure 3.5)

Depletion of *p*-coumaric acid (Figure 3.6) and production of 4-EP (Figure 3.7) occurred significantly sooner in treatments where there had been no previous growth of *O. oeni*. The depletion of *p*-coumaric acid (Figure 3.6) and production of 4-EP production (Figure 3.7) by *B. bruxellensis* began earlier in the sterile filtered treatments. There was a significant difference between sterile filtered treatments and their respective non-sterile filtered treatments for the inoculated treatments in both *p*-coumaric acid and 4-EP levels on day 15 ($p < 0.05$).

When the study was repeated in Pinot noir the impact of the sterile filtration treatment was more pronounced. In all treatments there was a rapid decrease in *B. bruxellesis* populations soon after inoculation (Figure 3.8, 3.9, 3.10). However, in the control and in wines which were sterile filtered prior to *B. bruxellensis* inoculation occurred, growth was detected again after a lengthy lag phase. In wine where VP41 had been grown *B. bruxellesis* growth occurred approx. 15 days after inoculation reaching a max. population of almost 1×10^6 CFU/mL. For Alpha this growth occurred after 21 days and for wine where VFO had

previously grown this growth was detected 42 days after *B. bruxellesis* inoculation (Figure 3.8, 3.9, 3.10). In contrast, in wine where *O. oeni* Alpha or VFO had grown but were not removed by sterile filtration *B. bruxellensis* populations remained below detectable levels throughout the course of the experiment (Figure 3.8, 3.9, 3.10). For wines where VP41 had grown but were not removed by sterile filtration, *B. bruxellensis* growth was only detected 55 days after inoculation (Figure 3.8).

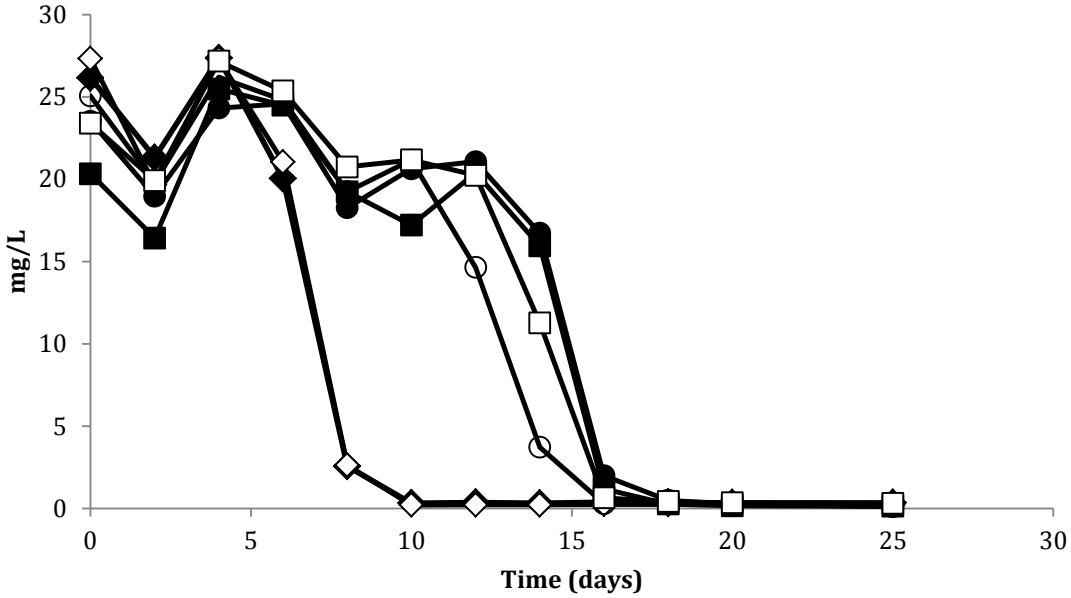


Figure 3.8 Concentration of *p*-coumaric acid in AGJ broth during growth of *B. bruxellensis* UCD-2049 after prior growth of *O. oeni* strains VP41 (■) or Alpha (●) or an un-inoculated control (◆). Corresponding open symbols indicate growth in broth where sterile filtration occurred to remove the *O. oeni* cells prior to inoculation with *B. bruxellensis*.

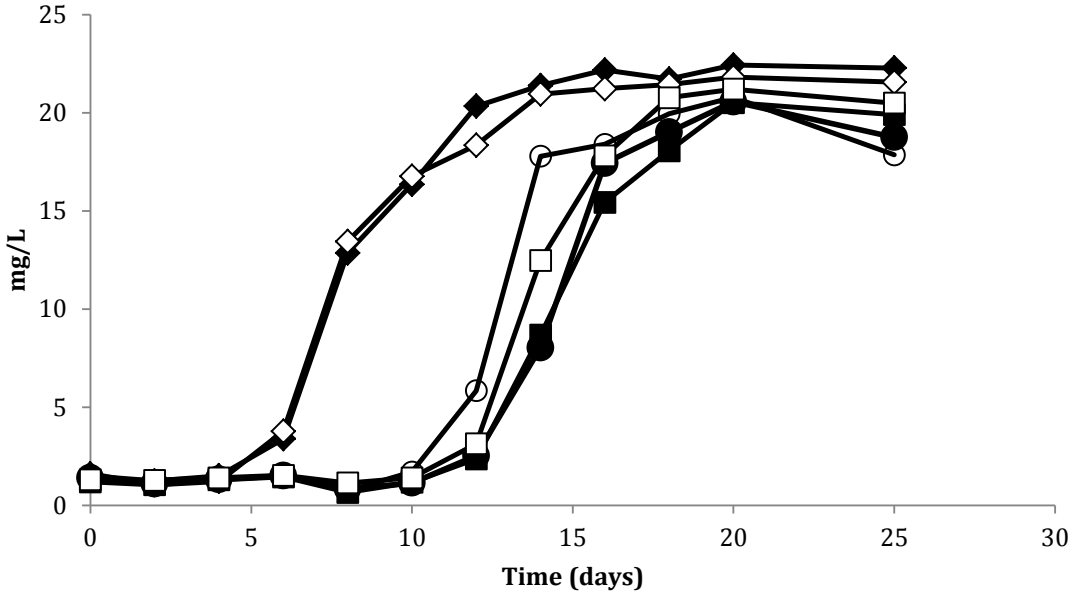


Figure 3.9 Concentration of 4-ethyphenol in AGJ broth during growth of *B. bruxellensis* UCD-2049 after prior growth of *O. oeni* strains VP41 (■) or Alpha (●) or an un-inoculated control (◆). Corresponding open symbols indicate growth in broth where sterile filtration occurred to remove the *O. oeni* cells prior to inoculation with *B. bruxellensis*.

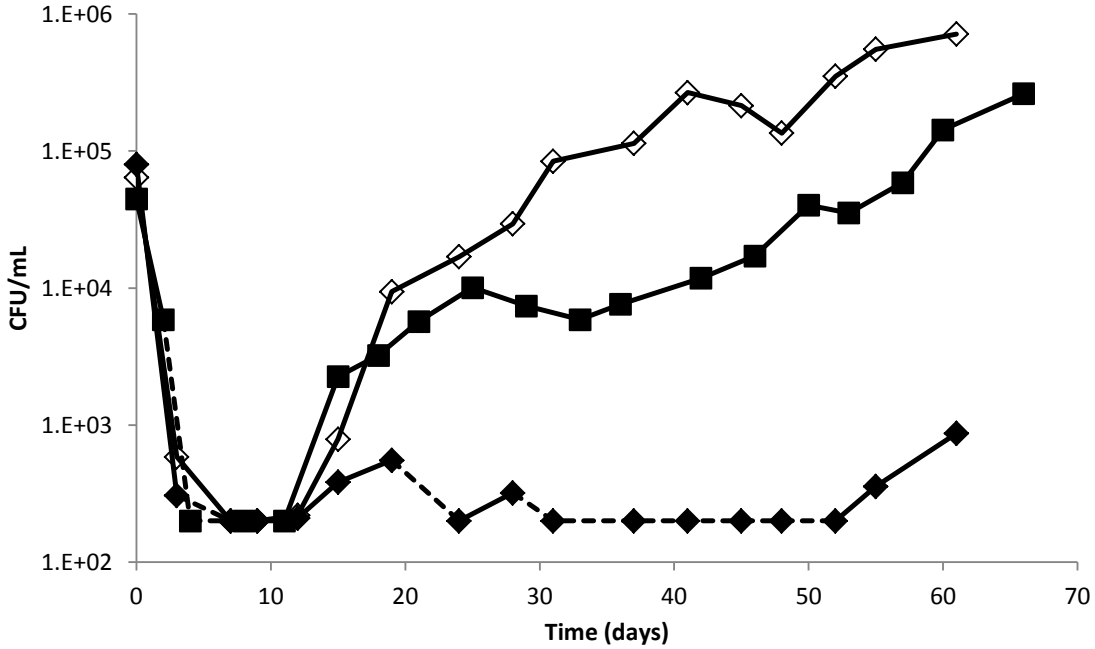


Figure 3.10 Growth of *B. bruxellensis* UCD-2049 after inoculation into Pinot noir wine where MLF was previously conducted by *O. oeni* VP41 followed by sterile filtration (◊) or no sterile filtration (◆) or a control (■) where there was no prior growth of *O. oeni* VP41.

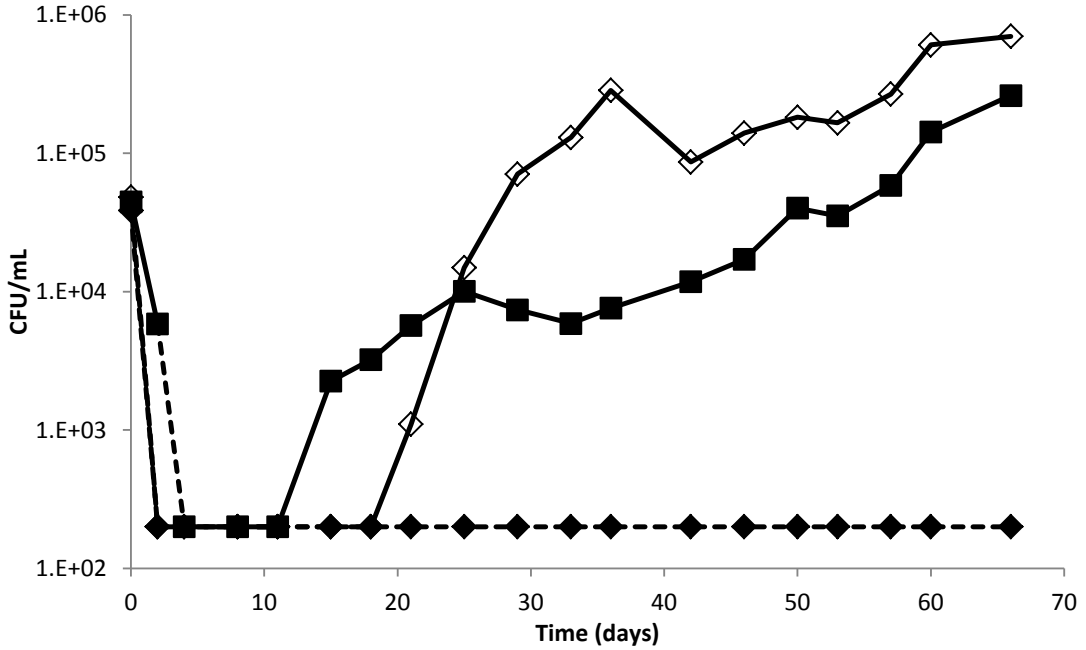


Figure 3.11 Growth of *B. bruxellensis* UCD-2049 after inoculation into Pinot noir wine where MLF was previously conducted by *O. oeni* Alpha followed by sterile filtration (◊) or no sterile filtration (◆) or a control (■) where there was no prior growth of *O. oeni* Alpha.

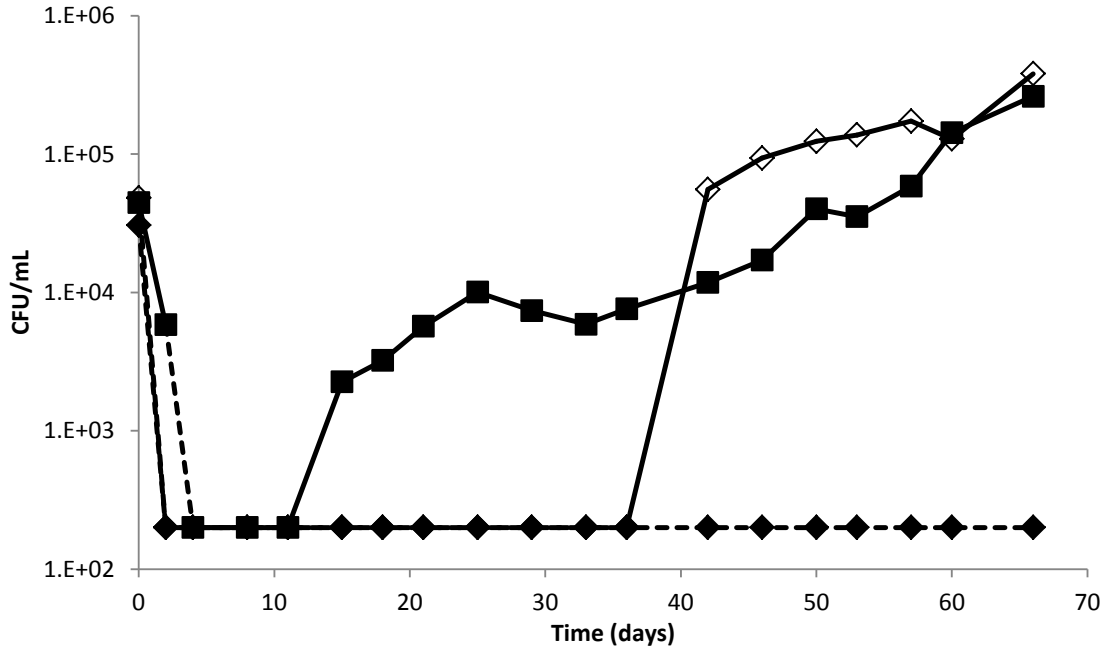


Figure 3.12 Growth of *B. bruxellensis* UCD-2049 after inoculation into Pinot noir wine where MLF was previously conducted by *O. oeni* VFO followed by sterile filtration (◊) or no sterile filtration (◆) or a control (■) where there was no prior growth of *O. oeni* VFO.

Discussion

Interactions between *O. oeni* and *B. bruxellensis* have not been extensively studied despite evidence that *O. oeni* has the potential to impact *B. bruxellensis* growth and production of volatile phenols by changing the concentrations of hydroxycinnamic acids in wine (Burns and Osborne 2013; Cabrita et al. 2008; Hernández et al. 2007; Gerbaux, Vincent, and Bertrand 2002). In this study the impact of *O. oeni* on *B. bruxellesis* growth and 4-EP production was studied in both a broth based system and in Pinot noir wine. Initial studies in broth demonstrated that in media with prior growth of *O. oeni B. bruxellensis* population dropped following initial inoculation and there was a delay and reduction in the production of 4-EP. The reduction of 4-EP production by *Brettanomyces* by prior growth of LAB has previously been reported (Gerbaux et al. 2009) although no growth data regarding *Brettanomyces* or *O. oeni* was provided. The reduction in 4-EP by *Brettanomyces* was strain dependent with growth of *O. oeni* VP41 causing the largest reduction in 4-EP production b*y B. bruxellesis*. However, this strain dependent impact on *B. bruxellensis* growth was not observed in a subsequent study in AGJ broth. This may have been due to the presence of 5% ethanol in the initial experiment while subsequent studies in media did not include ethanol. Because of the potential practical significance of this finding (selecting commercial *O. oeni* cultures that have an inhibitory effect against *Brettanomyces*) determining whether there are certain *O. oeni* strains that are more antagonistic towards *Brettanomyces* than others should be a high priority for future research. Furthermore, no strain dependent effects have previously been reported for this phenomenon.

The results from the present study confirmed previous work in our lab where growth of *O. oeni* VFO had caused delayed growth of *B. bruxellensis* in broth (J. P. Osborne, Strickland, and Edwards 2012). This work had also shown that the wine lactic acid bacteria *P. damnosus* did not cause any inhibition of *B. bruxellensis* growth. This finding was confirmed in Pinot noir wine in the present study where *B. bruxellesis* grew well in wine where *P. damnosus* had grown but growth *B. bruxellensis* fell below detectable levels in wine with prior growth of *O. oeni*. This result provides some evidence as to a possible mechanism by which *O. oeni* affects *Brettanomyces.* This is because *Pedicoccus* sp. and *O. oeni* (as a former member of genus *Leuconostoc*) have very similar growth requirements and are part of the so called LLPW (*Lactobacillus, Leuconsotoc, Pediococcus, Weisella*) physiological group (Holzapfel et al. 2009). This suggests that the depletion of nutrients by *O. oeni* is unlikely to be the cause of delayed *B. bruxellensis* growth as no significant reduction in growth was observed after growth of *P. damnosus.* However, there is still the potential for differences between nutrient demands of a particular *Pediococcus* and *O. oeni* strain. Specific vitamin requirements between *Pediococcus* spp. and *O. oeni* are similar, though one differences is that *Pediococcus* spp. requires biotin, while *O. oeni* does not. (Garvie 1986; Terrade and Mira de Orduña 2009). Further investigation into whether nutrient depletion by *O. oeni* is the cause of differences in *Brettanomyces* growth is required.

The possibility of nutrient depletion being the cause of *Brettanomyces* inhibition is also not supported by the results from the sterile filtration experiments. Although in media there was not a significant difference in growth rate between sterile filtered and non-sterile

filtered treatments, the differences in p-coumaric acid and 4-EP levels on day 14 demonstrate a delayed ability of this activity in non-sterile filtered treatments. The lack of a difference in growth rate may be to do with the lower stress nature of the media (nutrient rich, no ethanol). In contrast, large differences were seen between sterile filtered and non-sterile filtered treatments in the harsher environment of Pinot noir wine (lower nutrients, high ethanol). In wine, sterile filtration after growth of *O. oeni* relieved the inhibition of *Brettanomyces* growth compared to the non-filtered treatments. Sterile filtration should have no increase the availability of nutrients, and in fact could potentially reduce available nutrients, and so should not relieve inhibition if nutrient depletion by *O. oeni* was the cause. Sterile filtration is also unlikely to impact the concentration of any inhibitory compounds that *O. oeni* may have produced during growth. This suggests that the production of an inhibitory compound by *O. oeni* is also unlikely to be the mechanism of inhibition. However, other studies have reported a reduction of yeast growth in media where there had been previous growth of *O. oeni*, and antimicrobial action of other lactic acid bacteria such as *Lactobacillus* suggesting that this may be a possibility (Huang et al. 1996; Edwards et al. 1999). Again, more thorough and focused experiments should be conducted to confirm this finding.

An additional mechanism of inhibition that has shown to occur when populations of different microorganisms are present together is quorum sensing (Hogan, Vik, and Kolter 2004). Quorum sensing is a phenomenon where the accumulation of signaling molecules enables a single organism to sense the number of surrounding organisms based on the concentration of signaling molecules. The organism may regulate gene expression based on

this and increase or decrease growth rate. Past research has shown the ability of bacteria to affect morphology of yeast through quorum sensing (Hogan, Vik, and Kolter 2004). *B. bruxellensis* is also known to be a poor competitor compared to *Saccharomyces cerevisiae* (Renouf et al. 2006). If co-inoculated with *S. cerevisiae* its population usually declines and does not increase until *S. cerevisiae* populations decline. It is not known whether this is due to quorum sensing or to direct competition for nutrients. However, it is possible that *B. bruxellensis* UCD-2049 reacted in a similar manner when inoculated into a high population of *O. oeni* cells. The relief of growth inhibition in wine by removal of *O. oeni* suggests that the presence of the bacteria may have caused the inhibition. However, the presence of *P. damnosus* did not have the same effect on *Brettanomyecs* so if quorum sensing is involved it must occur in the presence of *O. oeni* but not *P. damnosus*. Further work in this area is required.

It is unclear if the reduced growth of *B. bruxellensis* in media and wine that had prior growth of *O. oeni* is due to cell death or to *Brettanomyces* entering a viable but non-culturable state (VBNC). *Brettanomyces* is known to enter this state in wine under stressful conditions (Millet and Lonvaud-Funel 2000) such as high SO_2 and recover when conditions are more favorable for growth (Serpaggi et al. 2012). The method of quantification of *B. bruxellensis* in this work did not quantify VBNC cells so we cannot determine whether this occurred. Additional studies utilizing both traditional plating and PCR based methods would help determine whether *O. oeni* induced *B. bruxellensis* to enter a VBNC state much like occurs if SO_2 is added to a wine. Furthermore, additional strains of *B. bruxellensis*

should be tested as the current work used only a signal strain of *B. bruxellensis* and it is unknown whether certain strains are more or less sensitive to growth of *O. oeni*.

Conclusion

The growth of *O. oeni* was inhibitory to *B. bruxellensis* UCD-2049 growth in both a media system and Pinot noir wine. In some cases the inhibition appeared to be dependent on the *O. oeni* strain used but in others the strain did not play a role. Removal of *O. oeni* cells by sterile filtration prior to *B. bruxellensis* inoculation did not significantly change the growth of *B. bruxellensis* in media. In contrast, sterile filtration of wine after growth of *O. oeni* did relieve the growth inhibition of *B. bruxellensis* UCD-2049 observed in non-sterile filtered wines. Differences between sterile filtered and non-sterile filtered treatments in wine indicate that the inhibitory effect of *O. oeni* is likely not caused by nutrient differences alone. Instead, unknown inhibitory mechanisms must be involved. These findings lead to major questions with regards to the method of inhibition of *B. bruxellensis* by *O. oeni* and how it could be used to control *B. bruxellensis* in wine.

SUMMARY

B. bruxellensis is a major cause of wine spoilage through the production of ethylphenols that can give a wine an aroma of "band-aids" "fecal" and "barnyard". This study investigated the impact of wine LAB on the growth and ethylphenol production by *B. bruxellensis* with a focus on the ability of wine LAB to impact the amount of ethylphenol pre-cursor compounds present in a wine. Of the thirty five strains of wine microorganisms tested only one, the commercial strain *O. oeni* VFO, impacted the concentration of hydroxycinnamic acids through the hydrolysis of tartaric acid bound hydroxycinnamic acids in wine. There was no evidence *Pediococcus* spp. or *Brettanomyces* are able to perform this hydrolysis. The release of free hydroxycinnamic acids was shown to increase ethylphenol production by *B. bruxellensis* significantly, with the potential to greatly increase the degree of spoilage.

Aside from changing the concentration of the hydroxycinnamic acids, growth of *O. oeni* could also be inhibitory to *B. bruxellensis* UCD-2049 growth as shown in both a broth system and in Pinot noir wine. In some cases the inhibition appeared to be dependent on the *O. oeni* strain used but in others the strain did not play a role. Removal of *O. oeni* cells by sterile filtration prior to *B. bruxellensis* inoculation did not significantly change the growth of *B. bruxellensis* in media. In contrast, sterile filtration of wine after growth of *O. oeni* did relieve the growth inhibition of *B. bruxellensis* UCD-2049 observed in non-sterile filtered wines. Differences between sterile filtered and non-sterile filtered treatments in wine indicate that the inhibitory effect of *O. oeni* is likely not caused by nutrient differences alone. Instead, unknown inhibitory mechanisms must be involved.

Future work in this field should be to extend the screening for the hydrolysis of tartaric acid-hydroxycinnamic esters by wine microbes. This should include additional *Brettanomyces bruxellensis* strains as well as *Lactobacillus* as previous work has demonstrated some strains of *Lactobacillus* spp. may be capable of hydrolysis of ester bound hydroxycinnamic acids (Guglielmettis 2008). In addition, the inhibition of *B. bruxellensis* should be explored further by testing *O. oeni* against a larger number of *B. bruxellensis* strains and additional mechanisms of inhibition should be investigated. If certain strains of *O. oeni* are more inhibitory to *B. bruxellensis* than others then these strains should be utilized as an additional tool against *Brettanomyces* spoilage.

BIBLIOGRAPHY

Abrahamse, CarolineE, and EvelineJ Bartowsky. 2012. "Timing of Malolactic Fermentation Inoculation in Shiraz Grape Must and Wine: Influence on Chemical Composition." *World Journal of Microbiology and Biotechnology* 28 (1): 255–65.

Agnolucci, Monica, Francesco Rea, Cristiana Sbrana, Caterina Cristani, Daniela Fracassetti, Antonio Tirelli, and Marco Nuti. 2010. "Sulphur Dioxide Affects Culturability and Volatile Phenol Production by Brettanomyces/Dekkera Bruxellensis." *International Journal of Food Microbiology* 143 (1-2): 76–80. doi:10.1016/j.ijfoodmicro.2010.07.022.

Auw, J. M., V. Blanco, S. F. O'keefe, and Charles A. Sims. 1996. "Effect of Processing on the Phenolics and Color of Cabernet Sauvignon, Chambourcin, and Noble Wines and Juices." *American Journal of Enology and Viticulture* 47 (3): 279–86.

Bauer, R., and L. M. T. Dicks. 2004. "Control of Malolactic Fermentation in Wine. A Review." *S. Afr. J. Enol. Vitic* 25 (2): 74–88.

Beelman, R. B., R. M. Keen, M.J. Banner, and S. W. King. 1982. "Interactions between Wine Yeast and Malolaction Bacteria under Wine Conditions [Saccharomyces Cerevisiase, Leuconostoc Oenos]." *Developments in Industrial Microbiology*.

Botha, Janita J. 2010. "Sensory, Chemical and Consumer Analysis of Brettanomyces Spoilage in South African Wines". Stellenbosch: University of Stellenbosch. https://ir1.sun.ac.za/handle/10019.1/4141.

Boulton, Roger, Vernon Singleton, Linda Bisson, and Ralph Kunkee. 1996. *Principles and Practices of Winemaking*. London, England: Chapman & Hall.

Brock, PE, Lorenza Conterno, T Acree, and T. Henick-Kling. 2006. "Identification of Aroma Compounds from Different Strains of Brettanomyces Bruxellensis in Model Wines." *American Journal of Enology and Viticulture* 57: 529–529.

Bubola, Marijan, \DJordano Peršurić, Karin Kovačević Ganić, Marko Karoglan, and Bernard Kozina. 2012. "Effects of Fruit Zone Leaf Removal on the Concentrations of Phenolic and Organic Acids in Istrian Malvasia Grape Juice and Wine." *Food Technology and Biotechnology* 50 (2): 159–66.

Burns, T. R., and J. P. Osborne. 2013. "Impact of Malolactic Fermentation on the Color and Color Stability of Pinot Noir and Merlot Wine." *American Journal of Enology and Viticulture* 64 (3): 370–77. doi:10.5344/ajev.2013.13001.

Buron, Nicolas, Hugues Guichard, Emmanuel Coton, Jérôme Ledauphin, and Daniel Barillier. 2011. "Evidence of 4-Ethylcatechol as One of the Main Phenolic off-Flavour Markers in French Ciders." *Food Chemistry* 125 (2): 542–48. doi:10.1016/j.foodchem.2010.09.046.

Cabrita, M.J., M. Torres, V. Palma, E. Alves, R. Patão, and A.M. Costa Freitas. 2008. "Impact of Malolactic Fermentation on Low Molecular Weight Phenolic Compounds." *Talanta* 74 (5): 1281–86. doi:10.1016/j.talanta.2007.08.045.

Cannon, M. C., and G. J. Pilone. 1993. "Interactions between Commercial Wine Yeast and Malolactic Bacteria." *New Zealand Grape and Wine Symposium* 8.

Cavin, J. F., V. Andioc, P. X. Etievant, and C. Divies. 1993. "Ability of Wine Lactic Acid Bacteria to Metabolize Phenol Carboxylic Acids." *American Journal of Enology and Viticulture* 44 (1): 76–80.

Chatonnet, P., D. Dubourdieu, and J. N. Boidron. 1995. "The Influence of Brettanomyces/Dekkera Sp. Yeasts and Lactic Acid Bacteria on the Ethylphenol Content of Red Wines." *American Journal of Enology and Viticulture* 46 (4): 463–68.

Chatonnet, Pascal. 1992. "The Origin of Ethylphenols in Wines." *Journal of the Science of Food and Agriculture* 60 (2): 165–78.

Chatonnet, Pascal, Denis Dubourdieu, Jean-noël Boidron, and ValéRie Lavigne. 1993. "Synthesis of Volatile Phenols by Saccharomyces Cerevisiae in Wines." *Journal of the Science of Food and Agriculture* 62 (2): 191–202. doi:10.1002/jsfa.2740620213.

Conde, Carlos, Paulo Silva, Natacha Fontes, Alberto Carlos Pires Dias, Rui M. Tavares, Maria João Sousa, Alice Agasse, Serge Delrot, and Hernâni Gerós. 2007. "Biochemical Changes throughout Grape Berry Development and Fruit and Wine Quality." http://repositorium.sdum.uminho.pt/handle/1822/6820.

Conterno, Lorenza, CM Lucy Joseph, Torey J. Arvik, Thomas Henick-Kling, and Linda F. Bisson. 2006. "Genetic and Physiological Characterization of Brettanomyces Bruxellensis Strains Isolated from Wines." *American Journal of Enology and Viticulture* 57 (2): 139–47.

Couto, José A., Francisco M. Campos, Ana R. Figueiredo, and Tim A. Hogg. 2006. "Ability of Lactic Acid Bacteria to Produce Volatile Phenols." *American Journal of Enology and Viticulture* 57 (2): 166–71.

Curtin, Chris D., Jennifer R. Bellon, Paul A. Henschke, Peter W. Godden, and Miguel A. de Barros Lopes. 2007. "Genetic Diversity of *Dekkera Bruxellensis* Yeasts Isolated from Australian Wineries." *FEMS Yeast Research* 7 (3): 471–81. doi:10.1111/j.1567-1364.2006.00183.x.

Delfini, Claudio, and Joseph V. Formica. 2001. *Wine Microbiology: Science and Technology*. CRC Press.

Dias, L., S. Pereira-da-Silva, M. Tavares, M. Malfeito-Ferreira, and V. Loureiro. 2003. "Factors Affecting the Production of 4-Ethylphenol by the Yeast Dekkera Bruxellensis in

Enological Conditions." *Food Microbiology* 20 (4): 377–84. doi:10.1016/S0740-0020(03)00023-6.

Dicks, L. M. T., F. Dellaglio, and M. D. Collins. 1995. "Proposal To Reclassify Leuconostoc Oenos as Oenococcus Oeni [corrig.] Gen. Nov., Comb. Nov." *International Journal of Systematic Bacteriology* 45 (2): 395–97.

Dugelay, Isabelle, Ziya Gunata, Jean Claude Sapis, Raymond Baumes, and Claude Bayonove. 1993. "Role of Cinnamoyl Esterase Activities from Enzyme Preparations on the Formation of Volatile Phenols during Winemaking." *Journal of Agricultural and Food Chemistry* 41 (11): 2092–96.

Edwards, C. G., A. G. Reynolds, A. V. Rodriguez, M. J. Semon, and J. M. Mills. 1999. "Implication of Acetic Acid in the Induction of Slow/stuck Grape Juice Fermentations and Inhibition of Yeast by Lactobacillus Sp." *American Journal of Enology and Viticulture* 50 (2): 204–10.

Etievant, Patrick X. 1981. "Volatile Phenol Determination in Wine." *Journal of Agricultural and Food Chemistry* 29 (1): 65–67.

Fugelsang, K., and Charles G. Edwards. 2007. *Wine Microbiology: Practical Applications and Procedures*. New York: Springer.

Garvie, E.I. 1986. "Genus Pediococus." In *Bergey's Manual of Systematic Baceteriology*, edited by P. H. A. Sneath, N.S. Mair, M.E. Sharpe, and Holt. MD: Williams & Wilkins.

Geny, Laurence, Cedric Saucier, Sandrine Bracco, Freddy Daviaud, and Yves Glories. 2003. "Composition and Cellular Localization of Tannins in Grape Seeds during Maturation." *Journal of Agricultural and Food Chemistry* 51 (27): 8051–54. doi:10.1021/jf030418r.

Gerbaux, Vincent, Carole Briffox, Ann Dumont, and Sibylle Krieger. 2009. "Influence of Inoculation with Malolactic Bacteria on Volatile Phenols in Wines." *American Journal of Enology and Viticulture* 60 (2): 233–35.

Gerbaux, Vincent, Béatrice Vincent, and Alain Bertrand. 2002. "Influence of Maceration Temperature and Enzymes on the Content of Volatile Phenols in Pinot Noir Wines." *American Journal of Enology and Viticulture* 53 (2): 131–37.

Goldberg, David M., Elisa Tsang, Alex Karumanchiri, and George J. Soleas. 1998. "Quercetin and P-Coumaric Acid Concentrations in Commercial Wines." *American Journal of Enology and Viticulture* 49 (2): 142–51.

Gómez-Plaza, E., R. Gil-Muñoz, J. M. López-Roca, and A. Martínez. 2000. "Color and Phenolic Compounds of a Young Red Wine. Influence of Wine-Making Techniques, Storage Temperature, and Length of Storage Time." *Journal of Agricultural and Food Chemistry* 48 (3): 736–41. doi:10.1021/jf9902548.

Goode, Jaime. 2005. *The Science of Wine: From Vien to Glass*. University of California Press.

Guglielmetti, S., I. De Noni, F. Caracciolo, F. Molinari, C. Parini, and D. Mora. 2008. "Bacterial Cinnamoyl Esterase Activity Screening for the Production of a Novel Functional Food Product." *Applied and Environmental Microbiology* 74 (4): 1284–88. doi:10.1128/AEM.02093-07.

Harbone, J., and J. Corner. 1961. "Plant Polyphenols. 4. Hydroxycinnamic Acid-Sugar Derivatives." *Biochem J.* 81 (2): 242–50.

Harris, Victoria, Vladimir Jiranek, Christopher M. Ford, and Paul R. Grbin. 2010. "Inhibitory Effect of Hydroxycinnamic Acids on Dekkera Spp." *Applied Microbiology and Biotechnology* 86 (2): 721–29. doi:10.1007/s00253-009-2352-6.

He, Jian-Jun, Fu-Liang Han, Qing-Quan Yu, Qiu-Hong Pan, Chang-Qing Duan, and Guo-Li Cheng. 2010. "Effects of the Maceration Enzymes on Evolution of Pyranoanthocyanins and Cinnamic Acids during the Cabernet Gernischet (Vitis Vinifera L. Cv.) Red Wine Making." *Food Science and Biotechnology* 19 (3): 603–10.

Henick-Kling, Thomas, and Yun Hee Park. 1994. "Considerations for the Use of Yeast and Bacterial Starter Cultures: SO2 and Timing of Inoculation." *American Journal of Enology and Viticulture* 45 (4): 464–69.

Heresztyn, Tamila. 1986. "Formation of Substituted Tetrahydropyridines by Species of Brettanomyces and Lactobacillus Isolated from Mousy Wines." *American Journal of Enology and Viticulture* 37 (2): 127–32.

Hernández, T., I. Estrella, M. Pérez-Gordo, E. G. Alegría, C. Tenorio, and M. V. Moreno-Arribas. 2007. "Contribution of Malolactic Fermentation by *Oenococcus Oeni* and *Lactobacillus Plantarum* to the Changes in the Nonanthocyanin Polyphenolic Composition of Red Wine." *Journal of Agricultural and Food Chemistry* 55 (13): 5260–66. doi:10.1021/jf063638o.

Hixson, JL, NR Sleep, GM Capone, CD Curtin, MA Sefton, and DK Taylor. 2012. "Hydroxycinnamic Acid Ethyl Esters as Precursors to Ethylphenols in Wine." *Journal of Agricultural and Food Chemistry* 60 (7): 2293–98.

Hogan, Deborah A., Åshild Vik, and Roberto Kolter. 2004. "A Pseudomonas Aeruginosa Quorum-Sensing Molecule Influences Candida Albicans Morphology: Pseudomonas Inhibition of C. Albicans Filamentation." *Molecular Microbiology* 54 (5): 1212–23. doi:10.1111/j.1365-2958.2004.04349.x.

Holzapfel, Wilhelm H., Charles M.A.P. Franz, Wolfgang Ludwig, and Leon M. T. Dicks. 2009. "Genus II. Pediococcus Claussen 1903, 68." In *Bergy' Manual of Systematic Bacteriology*, by P. Vos, G. Garrity, D. Jones, N. R. Krieg, F. A. Rainey, K.-H. Schleifer, W. Whitman, and Wolfgang Ludwig, 2nd Edition, Volume 3: The Firmicutes:513–20.

Huang, Y.-C., C. G. Edwards, J. C. Peterson, and K. M. Haag. 1996. "Relationship between Sluggish Fermentations and the Antagonism of Yeast by Lactic Acid Bacteria." *American Journal of Enology and Viticulture* 47 (1): 1–10.

J. P. Osborne, Matthew T. Strickland, and Charles G. Edwards. 2012. "Impact of Pediococcus Spp. on the Quality of Oregon and Washington Wines." presented at the American Society of Enology and Viticulture Annual Meeting, Portland Oregon, June.

Jackson, Ronald S. 2008. *Wine Science: Principles and Applications*. Academic Press.

Jensen, Susanne L., Nicole L. Umiker, Nils Arneborg, and Charles G. Edwards. 2009. "Identification and Characterization of Dekkera Bruxellensis, Candida Pararugosa, and Pichia Guilliermondii Isolated from Commercial Red Wines." *Food Microbiology* 26 (8): 915–21. doi:10.1016/j.fm.2009.06.010.

Joseph, C. M. L., L. W. Gorton, S. E. Ebeler, and L. F. Bisson. 2013. "Production of Volatile Compounds by Wine Strains of Brettanomyces Bruxellensis Grown in the Presence of Different Precursor Substrates." *American Journal of Enology and Viticulture* 64 (2): 231–40. doi:10.5344/ajev.2013.12095.

López, Isabel, Carmen Tenorio, Myriam Zarazaga, Marta Dizy, Carmen Torres, and Fernanda Ruiz-Larrea. 2007. "Evidence of Mixed Wild Populations of Oenococcus Oeni Strains during Wine Spontaneous Malolactic Fermentations." *European Food Research and Technology* 226 (1-2): 215–23. doi:10.1007/s00217-006-0529-0.

Loureiro, Virgilio, and Manuel Malfeito-Ferreira. 2005. "Dekkera/Brettanomyces Spp." In *Food Spoilage Microorganisms*, edited by C. W. Blackburn, 354–98. Boca Raton, FL: CRC Press.

Malfeito-Ferreira, Manuel. 2005. "Avances Recientes En El Control de Brettanomyces/Dekkera Bruxellenesis En Vinos." *Enotour 2005 Agrovin*, June, 28–30.

Martorell, Patricia, André Barata, Manuel Malfeito-Ferreira, M. Teresa Fernández-Espinar, Virgilio Loureiro, and Amparo Querol. 2006. "Molecular Typing of the Yeast Species Dekkera Bruxellensis and Pichia Guilliermondii Recovered from Wine Related Sources." *International Journal of Food Microbiology* 106 (1): 79–84. doi:10.1016/j.ijfoodmicro.2005.05.014.

Millet, V., and A. Lonvaud-Funel. 2000. "The Viable but Non-Culturable State of Wine Micro-Organisms during Storage." *Letters in Applied Microbiology* 30 (2): 136–41.

Mulero, Juana, Pilar Zafrilla, Jose M. Cayuela, Adela Martínez-Cachá, and Francisco Pardo. 2011. "Antioxidant Activity and Phenolic Compounds in Organic Red Wine Using Different Winemaking Techniques." *Journal of Food Science* 76 (3): C436–C440. doi:10.1111/j.1750-3841.2011.02104.x.

Nagel, C. W., J. D. Baranowski, L. W. Wulf, and J. R. Powers. 1979. "The Hydroxycinnamic Acid Tartaric Acid Ester Content of Musts and Grape Varieties Grown in the Pacific Northwest." *American Journal of Enology and Viticulture* 30 (3): 198–201.

Nagel, C. W., and L. W. Wulf. 1979. "Changes in the Anthocyanins, Flavonoids and Hydroxycinnamic Acid Esters during Fermentation and Aging of Merlot and Cabernet Sauvignon." *American Journal of Enology and Viticulture* 30 (2): 111–16.

Olsen, Erik Bernt, J. B. Russell, and T. Henick-Kling. 1991. "Electrogenic L-Malate Transport by Lactobacillus Plantarum: A Basis for Energy Derivation from Malolactic Fermentation." *Journal of Bacteriology* 173 (19): 6199–6206.

Ong, B. Y., and C. W. Nagel. 1978. "Hydroxycinnamic Acid-Tartaric Acid Ester Content in Mature Grapes and during the Maturation of White Riesling Grapes." *American Journal of Enology and Viticulture* 29 (4): 277–81.

Osborne, James P., and Charles G. Edwards. 2006. "Inhibition of Malolactic Fermentation by Saccharomyces during Alcoholic Fermentation under Low-and High-Nitrogen Conditions: A Study in Synthetic Media." *Australian Journal of Grape and Wine Research* 12 (1): 69–78.

Osborne, James P., and Charles G. Edwards. 2007. "Inhibition of Malolactic Fermentation by a Peptide Produced by Saccharomyces Cerevisiae during Alcoholic Fermentation." *International Journal of Food Microbiology* 118 (1): 27–34. doi:10.1016/j.ijfoodmicro.2007.05.007.

Pérez-Magariño, Silvia, and M Luisa González-San José. 2005. "Effect of Ripening Stage of Grapes on the Low Molecular Weight Phenolic Compounds of Red Wines." *European Food Research and Technology* 220 (5-6): 597–606. doi:10.1007/s00217-004-1106-z.

Petrozziello, Maurizio, Andriani Asproudi, Massimo Guaita, Daniela Borsa, Silvia Motta, Loretta Panero, and Antonella Bosso. 2014. "Influence of the Matrix Composition on the Volatility and Sensory Perception of 4-Ethylphenol and 4-Ethylguaiacol in Model Wine Solutions." *Food Chemistry* 149 (April): 197–202. doi:10.1016/j.foodchem.2013.10.098.

Puig, Anna, Eva Bertran, Rosó Franquet, Joan García, and Santiago Mínguez. 2011. "Brettanomyces Bruxellensis Prevalence in Wines Produced and Marketed in Spain." *Annals of Microbiology* 61 (1): 145–51.

Renouf, V., M. Falcou, C. Miot-Sertier, M. C. Perello, G. De Revel, and A. Lonvaud-Funel. 2006. "Interactions between Brettanomyces Bruxellensis and Other Yeast Species during the Initial Stages of Winemaking." *Journal of Applied Microbiology* 100 (6): 1208–19. doi:10.1111/j.1365-2672.2006.02959.x.

Renouf, Vincent, and Aline Lonvaud-Funel. 2007. "Development of an Enrichment Medium to Detect Dekkera/Brettanomyces Bruxellensis, a Spoilage Wine Yeast, on the Surface of Grape Berries." *Microbiological Research* 162 (2): 154–67. doi:10.1016/j.micres.2006.02.006.

Romano, A., M.C. Perello, G. de Revel, and A. Lonvaud-Funel. 2008. "Growth and Volatile Compound Production by Brettanomyces/Dekkera Bruxellensis in Red Wine." *Journal of Applied Microbiology* 104 (6): 1577–85. doi:10.1111/j.1365-2672.2007.03693.x.

Romano, Andrea, Marie Claire Perello, Aline Lonvaud-Funel, Gilles Sicard, and Gilles de Revel. 2009. "Sensory and Analytical Re-Evaluation of 'Brett Character.'" *Food Chemistry* 114 (1): 15–19. doi:10.1016/j.foodchem.2008.09.006.

Romeyer, Francoise M., Jean J. Macheix, Jean J. Goiffon, Claude C. Reminiac, and Jean C. Sapis. 1983. "Browning Capacity of Grapes. 3. Changes and Importance of Hydroxycinnamic Acid-Tartaric Acid Esters during Development and Maturation of the Fruit." *Journal of Agricultural and Food Chemistry* 31 (2): 346–49.

Salih, A.G., J.-M. Quere, and J.-F. Drilleau. 2000. "Action Des Acides Hydroxycinnamiques Libres et Estérifiés Sur La Croissance Des Bactéries Lactiques." *Science Des Aliments* 20 (6): 537–60.

Schopp, Lauren M., Jungmin Lee, James P. Osborne, Stuart C. Chescheir, and Charles G. Edwards. 2013. "Metabolism of Nonesterified and Esterified Hydroxycinnamic Acids in Red Wines by *Brettanomyces Bruxellensis*." *Journal of Agricultural and Food Chemistry* 61 (47): 11610–17. doi:10.1021/jf403440k.

Serpaggi, Virginie, Fabienne Remize, Ghislaine Recorbet, Eliane Gaudot-Dumas, Anabelle Sequeira-Le Grand, and Hervé Alexandre. 2012. "Characterization of the 'viable but Nonculturable' (VBNC) State in the Wine Spoilage Yeast Brettanomyces." *Food Microbiology* 30 (2): 438–47. doi:10.1016/j.fm.2011.12.020.

Silva, I., F.M. Campos, T. Hogg, and J.A. Couto. 2011. "Wine Phenolic Compounds Influence the Production of Volatile Phenols by Wine-Related Lactic Acid Bacteria: Production of Volatile Phenols by LAB." *Journal of Applied Microbiology* 111 (2): 360–70. doi:10.1111/j.1365-2672.2011.05052.x.

Solieri, L., F. Genova, M. De Paola, and P. Giudici. 2010. "Characterization and Technological Properties of Oenococcus Oeni Strains from Wine Spontaneous Malolactic Fermentations: A Framework for Selection of New Starter Cultures: Selection of New O.Â Oeni Starter Cultures." *Journal of Applied Microbiology* 108 (1): 285–98. doi:10.1111/j.1365-2672.2009.04428.x.

Strickland, Matthew T. 2012. "Effects of Pediococcus Spp. on Oregon Pinot Noir." http://ir.library.oregonstate.edu/xmlui/handle/1957/33835.

Suárez, R., J.A. Suárez-Lepe, A. Morata, and F. Calderón. 2007. "The Production of Ethylphenols in Wine by Yeasts of the Genera Brettanomyces and Dekkera: A Review." *Food Chemistry* 102 (1): 10–21. doi:10.1016/j.foodchem.2006.03.030.

Terrade, Nicolas, and Ramón Mira de Orduña. 2009. "Determination of the Essential Nutrient Requirements of Wine-Related Bacteria from the Genera Oenococcus and Lactobacillus." *International Journal of Food Microbiology* 133 (1-2): 8–13. doi:10.1016/j.ijfoodmicro.2009.03.020.

Tessonnière, H., S. Vidal, L. Barnavon, H. Alexandre, and F. Remize. 2009. "Design and Performance Testing of a Real-Time PCR Assay for Sensitive and Reliable Direct Quantification of Brettanomyces in Wine." *International Journal of Food Microbiology* 129 (3): 237–43. doi:10.1016/j.ijfoodmicro.2008.11.027.

Ugliano, Maurizio, Alessandro Genovese, and Luigi Moio. 2003. "Hydrolysis of Wine Aroma Precursors during Malolactic Fermentation with Four Commercial Starter Cultures of *Oenococcus Oeni*." *Journal of Agricultural and Food Chemistry* 51 (17): 5073–78. doi:10.1021/jf0342019.

Wedral, Danielle, Robert Shewfelt, and Joseph Frank. 2010. "The Challenege of Brettanomyces in Wine." *LWT-Food Science and Technology* 43 (10): 1474–79.

Wightman, Jolynne D., Steven F. Price, Barney T. Watson, and Ronald E. Wrolstad. 1997. "Some Effects of Processing Enzymes on Anthocyanins and Phenolics in Pinot Noir and Cabernet Sauvignon Wines." *American Journal of Enology and Viticulture* 48 (1): 39–48.

Winter, Michael, and Karl Herrmann. 1986. "Esters and Glucosides of Hydroxycinnamic Acids in Vegetables." *Journal of Agricultural and Food Chemistry* 34 (4): 616–20. doi:10.1021/jf00070a007.

Zuehlke, J.M., B. Petrova, and C.G. Edwards. 2013. "Advances in the Control of Wine Spoilage by *Zygosaccharomyces* and *Dekkera/Brettanomyces*." *Annual Review of Food Science and Technology* 4 (1): 57–78. doi:10.1146/annurev-food-030212-182533.