AN ABSTRACT OF THE THESIS OF

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Title: The Impact of Malolactic Fermentation Conducted by *Oenococcus oeni* on *Brettanomyces bruxellensis* Growth and Volatile Phenol Production

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Wine is particularly vulnerable to infection by *Brettanomyces bruxellensis* during or shortly after malolactic fermentation (MLF). While different methods and techniques enable winemakers to prevent wine spoilage due to this organism, no single intervention is universally sufficient. Moreover application of sulfur dioxide, the most commonly used of these methods, is unavailable to winemakers during MLF due to the sensitivity of *Oenococcus oeni* to that substance. It is therefore generally suggested that winemakers carry out a rapid and healthy MLF in order to reduce the time a wine is without the protection of sulfur dioxide. In recent years however, some studies have suggested that MLF may also act to inhibit wine spoilage by *Brettanomyces bruxellensis*. This study investigated this additional benefit of MLF by studying the influence of MLF on the growth and volatile phenol production of *Brettanomyces bruxellensis*. Additionally, the impacts of microbial strain and wine ethanol concentration were also investigated with respect to that influence. Potential causes for the inhibitory interactions between *O. oeni* and *B. bruxellensis* were also explored. Experiments were conducted in Pinot noir wine produced in the 2018 and 2019 winegrowing seasons. A strain of *Brettanomyces*

bruxellensis that had previously shown potential sensitivity to MLF, strain UCD-2049, was inoculated into wine at the end of a 14 day MLF conducted by ten different strains of *Oenococcus oeni*. UCD-2049 populations declined after inoculation after MLF for all strains of *O. oeni* tested. When inoculated into a control wine that had not undergone MLF with *O. oeni*, no suppression of growth was observed. Significantly higher concentrations of 4-ethyl phenol and 4-ethyl guaiacol were measured in wine that had not undergone MLF when compared to wine that had undergone MLF using any of the ten strains tested.

To determine possible mechanisms of inhibition an experiment was conducted where *B. bruxellensis* was inoculated into wine that had just completed MLF but *O. oeni* cells were separated from *B. bruxellensis* by a dialysis membrane. The dialysis membrane allowed physical separation of the microorganisms but free flow of wine. While *B. bruxellensis* populations declined rapidly and remained repressed for many weeks when in direct contact with *O. oeni*, populations only declined slightly and quickly recovered if *O. oeni* was separated from *B. bruxellensis* by a dialysis membrane. This finding suggests that inhibition by *O. oeni* is related to cell to cell contact rather than depletion of nutrients or production of an inhibitory compound.

Strain variation in *Brettanomyces bruxellensis* was tested by selecting a highly "suppressive" strain of *Oenococcus oeni,* strain Alpha, and determining the effect of MLF by this strain on the growth and volatile phenol production of a number of strains of *Brettanomyces bruxellensis*. The impact of *Oenococcus oeni* strain Alpha was tested by

inoculating each different *Brettanomyces bruxellensis* strain into a wine at the end of a 14 day MLF. *B. bruxellensis* populations in both the control and MLF treated wines recovered to a similar level by the end of the experiment. The possibility that ethanol tolerance differences between the *B. bruxellensis* strains contributed to their variable susceptibility to inhibition by *O. oeni* was investigated. *B. bruxellensis* strains were inoculated into wines that had been adjusted to a high or low ethanol content and that had or had not just completed MLF. While *B. bruxellensis* populations behaved similarly to the control groups in the low ethanol wines, in the high ethanol wines two of the three strains of *B. bruxellensis* tested were inhibited in the MLF treated wines with respect to the control. For the third *B. bruxellensis* strain, the reverse was true. *B. bruxellensis* strain was a major factor in volatile phenol production, though some suppression of volatile phenol production by MLF was observed in cases where there was no suppression of growth.

This study demonstrated that *Brettanomyces bruxellensis* response to MLF is dependent on *Brettanomyces bruxellensis* strain, and that this response may be impacted by the ethanol concentration of the wine. It was also determined that sensitivity to MLF with respect to culturable cell growth is likely related to cell-cell contact between *Brettanomyces bruxellensis* and *Oenococcus oeni*.

The Impact of Malolactic Fermentation Conducted by *Oenococcus oeni* on
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Dr. Michael Qian and Dr. Yanping Qian conducted the wine volatile phenol

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Red Winemaking Overview

The process of making wine is rich and varied; nevertheless, certain steps and procedures have become standardized over the years: Winemaking begins in the vineyard with the production of grapes suitable for wine. The vineyard manager determines the ripeness of the grapes with respect to color, flavor, sugar, and acid content. When the fruit is determined to be at peak ripeness, it is harvested and transported to the winery. Some wineries elect to sort incoming fruit either manually or mechanically. Sorting serves to remove any extraneous material from grapes stems and clusters, as well as to remove clusters that have been infected with mold (Clary et al., 1990.). Removing damaged or infected fruit also serves to reduce the microbial load in the must, and can aid in sanitation efforts as grapes with damaged skins have been found to harbor more spoilage organisms (Du Toit et al., 2005).

For red winemaking, grape skins are not removed until after the completion of alcoholic fermentation. Fermentation is allowed to progress on the skins in order to extract phenolic compounds such as anthocyanins and tannins that give color and mouthfeel to a red wine (Bautista-Ortín et al., 2004; Canals et al., 2005; Ivanova et al., 2012). To allow yeast to access the sugary pulp of the fruit red wine grapes are usually crushed and de-stemmed before initiation of alcoholic fermentation. Grapes at harvest can host a number

of bacteria and yeast species (Renouf et al., 2006). If alcoholic fermentation is not initiated in a timely manner, some of these microbes may begin to grow and produce undesirable metabolites (Du Toit and Pretorius, 2000; Fugelsang and Edwards, 2006; Renouf et al., 2006). If incoming fruit cannot be processed immediately, it is common to apply an amount of sulfur dioxide (SO_2) to the fruit to protect it from oxidation and microbial infections before initiating alcoholic fermentation (Henick-Kling and Park, 1994). Aside from keeping the fruit at low temperatures, the acts of sorting out fungal infections and the addition of SO_2 to incoming grapes are usually the only microbial controls applied to incoming fruit in a winery.

Alcoholic fermentation in wine production is a microbially mediated process whereby the grape sugars, glucose and fructose, are converted to ethanol and CO_2 under anaerobic conditions. This process results in the production of ATP and regeneration of NAD+ from NADH. Fermentation of sugars can also result in the production of a number of flavor active compounds such as esters that contribute to a wines aroma and flavor (Lambrechts & Pretorius, 2000; Lilly et al., 2006; Swiegers et al., 2007; Swiegers et al., 2009). Alcoholic fermentation is generally performed by the yeast *Saccharomyces cerevisiae*. Winemakers may choose to inoculate a pure culture of a commercial *Saccharomyces cerevisiae* strains to their wines for fermentation, or they may choose to allow *S. cerevisiae* naturally present on the grapes and/or winery to perform the fermentation. This is often referred to as "wild" or "natural" fermentation strain (Fleet & Ribéreau-Gayon, 1984; Heard & Fleet 1985; Pardo, García, Zúñiga, & Uruburu 1989;

Torija et al. 2001). While some "wild" strains of *S. cerevisiae* may be unique to a specific winery or vineyard (Drumonde-Neves et al., 2016; Schuller et al., 2005; Török et al., 1996; Varela et al., 2009), recent studies have demonstrated that often what is thought to be a "wild" strain is in fact a commercial wine yeast strain that is resident in the winery.

After alcoholic fermentation is complete, red wines are pressed to remove the skins and seeds of the fruit from a wine. This process removes many of the insoluble solids in a red wine, but will not remove suspended yeast cells. These cells will eventually flocculate and settle into a cake called the lees which settles at the bottom of the vessel. After pressing, winemakers may elect to remove this residual yeast by clarification and filtration before proceeding to malolactic fermentation. This is often done in order to enhance the clarity of the final product (Zamora, 2003). Another reason winemakers may not want their wine to age on the lees is that doing so may result in unpleasant odors and flavors such as reduction occurring in the final wine (Fornairon-Bonnefond, Camarasa, Moutounet, & Salmon, 2002; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 1999; Zamora, 2002). After pressing and racking, many red wines undergo bacterially-mediated malolactic fermentation (MLF). Here, wine lactic acid bacteria (LAB) such as *Lactobacillus, Pediococcus,* and most commonly, *Oenococcus oeni*, convert malic acid to lactic acid. This process results in the reduction of acidity and is particularly important for high acid red wines.

Microbial Ecology of Red Wine

Saccharomyces cerevisiae and *Oenococcus oeni* are two key microorganisms for winemaking but they are not the only microorganisms present during the wine production process. A wide range of microorganisms can be present on the grapes or on winery surfaces. Unlike other foods and beverages, the presence of pathogenic microorganisms in wine production is not generally a concern due to the specific chemistry of grapes and wine. Before fermentation the acidic environment of grape must can be as low as 3.0. Since pathogenic organisms begin to grow in food or beverage systems at a pH of 4.6 or above, wine's low pH provides significant protection. Acid is just the first layer of defense a wine has against pathogens: After fermentation, a wine has the advantage of being both acidic and alcoholic. The ethanol concentration of a finished wine can exceed 13% (v/v) (Arroyo-López et al., 2010). The combined effects of low pH and ethanol content effectively prevents the survival and growth of pathogenic microorganisms in wine (Jeon et al., 2015; Møretrø and Daeschel, 2006; Sugita-Konishi et al., 2001).

Since wine has natural resistance to pathogenic organisms, winemakers do not have to pasteurize the grapes or wines to prevent food safety issues as occurs in other food and beverages such as milk and cheese. In practice, this lack of a pasteurization step means that there can be quite a high and diverse population of microorganisms present at the beginning of alcoholic fermentation. While the winemaker may not have to be concerned with pathogenic microorganisms, they must consider the potential of microbial spoilage due to a number of yeast and bacteria that can survive the inhospitable environment of

grape juice and/or wine. These microorganisms can affect adverse quality changes due to the production of an array of flavor and aroma active compounds.

A range of microorganisms are present during wine production that originate from the grapes and/or winery surfaces. Certain microorganisms will dominate at various stages of wine as the physiochemical and biological parameters of a wine evolve over time. For example, at the start of winemaking non-*Saccharmoyces* yeast species tend to dominate as they are the most abundant species found on the surface of grapes, (Barata et al., 2008a; 2012; Bisson & Joseph, 2009). As alcoholic fermentation proceeds non-*Saccharomyces* yeast populations decrease due to the creation of an anaerobic environment and production of ethanol by *S. cerevisiae*. Bacterial species such as *Acetobacter* and *Lactobacillus* are also present on grapes and in must at this time, but they typically do not proliferate to high numbers at this stage of wine production. After the initiation of alcoholic fermentation, *S. cerevisiae* quickly dominates the fermentation, outcompeting all of the other microbial species present. At the completion of alcoholic is typically when MLF will occur. *Oenococcus oeni* tend to be the most common wine LAB that performs MLF due to its addition as a starter culture and its dominance at low pH. During or shortly after MLF other microorganisms such as *Lactobacillus* species or *Brettanomyces bruxellensis* may grow depending on the wine conditions. Film producing yeasts such as *Candida* and *Pichia* species along with aerobic bacteria such as *Acetobacter* can also proliferate during aging if wine tanks and barrels are not topped, thus providing a more aerobic environment. At every stage of wine production,

winemakers seek to manage the populations of microorganism by encouraging the growth of beneficial microbes (*S. cerevisiae* and *O. onei*) and creating an environment that will inhibit spoilage microbes. Complete elimination of microbes is not possible during wine production except just prior to bottling when wines can be sterile filtered.

Beneficial Microorganisms: *Saccharomyces Cerevisiae* and Alcoholic Fermentation

As noted earlier, alcoholic fermentation is the process by which microorganisms such as *S. cerevisiae* convert the fermentable sugars (fructose, glucose) into carbon dioxide and ethanol in order to generate ATP anaerobically through substrate level phosphorylization (Strathern, Jones, Broach, 1982). This process begins with glycolysis, which consumes one of these 6 carbon sugars as well as 2 NAD+ molecules to produce 2 ATP, 2 pyruvate molecules, and 2 NADH molecules (Herskowitz, 1988). Because the process requires NAD+, the NADH produced during glycolysis must be oxidized for re-use. In order to do this, *S. cerevisiae* can decarboxylate pyruvate producing a carbon dioxide molecule, after which NADH can be used to reduce the resulting two carbon molecule which regenerates the needed NAD+ and also produces a molecule of ethanol (Herskowitz, 1988). While it can metabolize both aerobically and anaerobically *S. cerevisiae* preferentially ferments glucose anaerobically. This is due to the Crabtree effect. This effect causes suppression in aerobic glucose respiration in environments with high glucose concentrations (De Deken, 1966). *S. cerevisiae* is one of the most ethanol tolerant microorganisms, but eventually they will die from exposure to high ethanol concentrations (Strathern, Jones, Broach, 1982).

S. cerevisiae is particularly well suited to winemaking. It can survive the otherwise hostile environment of a wine undergoing alcoholic fermentation as it has a specific tolerance to low pH and high ethanol environments (Arroyo-López et al., 2010). *S.cerevisiae* is also able to tolerate high levels of SO_2 (Henick-Kling and Park, 1994; Martini, 1993) enabling winemakers to selectively suppress the growth of other wine tolerant organisms by adding SO_2 without inhibiting the alcoholic fermentation. Wine yeast also produces many secondary metabolites such as esters and higher alcohols that can positively affect the aroma, taste, and mouthfeel of a wine (Lilly et al., 2006; Swiegers et al., 2007; Swiegers et al., 2009).

Beneficial Microorganisms: *Oenococcus Oeni* and Malolactic fermentation

MLF is the enzymatic conversion of malic acid to lactic acid and is an important winemaking tool (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985; Kunkee, 1968; Lonvaud-Funel, 1999; Van Vuuren, & Dicks, 1993; Wibowo, Eschenbruch, Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985). MLF is carried out by microorganisms in the order Lactobacillales, commonly called lactic acid bacteria (LAB). Lactic acid bacteria that commonly inhabit wine include *O. oeni*, as well as strains of *Lactobacillus* and *Pediococcus* species (Edwards and Jensen, 1992). While all of these species can carry out malolactic fermentation, the species *Pediococcus* and *Lactobacillus* are generally considered to be spoilage organisms (Osborne & Edwards, 2005; Sponholz, 1993). As with yeast, winemakers can elect to inoculate a pure or mixed culture of lactic acid

bacteria to carry out MLF, or they may allow bacteria already present in the wine to

multiply and undergo the fermentation. To ensure a more predictable MLF winemakers

may choose to inoculate a commercial bacterial starter culture (Henick-Kling, 1993;

Krieger et al., 1993; Nielsen et al., 1996; Pilone, 1995).

Winemakers who choose to inoculate their wine with *O. oeni*, generally pick a strain that

is associated with metabolic or enzymatic activities that produce or enhance positive

flavors and aromas. Some *O. oeni* strains are associated with enhanced buttery flavors

due to the production of diacetyl from primarily citric acid (Bartowsky and Henschke,

2004; Bloem et al., 2008; Costello and Henschke, 2002; Malherbe et al., 2013;

Michlmayr et al., 2012; Ugliano et al., 2003; Ugliano and Moio, 2005). Malolactic

fermentation also raises the pH of a wine which can lead to a decreased perception of

acidity (Costantini et al., 2009; Liu, 2002). For these reasons, *O. oeni* is the species most

commonly inoculated into wine due to its positive organoleptic characteristics (Guzzo et

al., 1994; Krieger et al., 1993; Liu, 2002; Nielsen et al., 1996; Wibowo et al., 1985).

O. oeni is a facultatively anaerobic, Gram-positive, non-motile species with cocci shaped

cells usually arranged as pairs or chains (Dicks et al., 1995; Garvie, 1967). *O. oeni* is

almost exclusively associated with wine production and wine MLF is considered its main

ecological niche (Terrade and Mira de Orduna, 2009). The physicochemical parameters

of a wine are among the most significant factors in determining the health and vitality of

O. oeni during malolactic fermentation. *O. oeni* is both acid and ethanol tolerant, but the

optimal conditions for malolactic fermentation exist below 15% alcohol, and at a pH of 3.0 or higher (Terrade and Mira de Orduna, 2009). Optimal malolactic fermentation takes place at temperatures between 14 and 22°C (Terrade and Mira de Orduna, 2009). Like all lactic acid bacteria, *O. oeni* is sensitive to sulfur dioxide with optimal free SO_2 levels for malolactic fermentation below 12 ppm (Terrade and Mira de Orduna, 2009). *O. oeni* may also liberate SO_2 bound as carbonyl sulfonates through its own enzymatic activity (Wells and Osborne, 2011). Due to this sensitivity, winemakers who wish to encourage MLF will not treat a wine with SO_2 until MLF is completed, therefore leaving the wine vulnerable to other forms of microbial infection.

O. oeni is also nutritionally fastidious and requires a diverse range of nutrients for growth. *O. oeni* strains display variable phenotypes regarding the carbohydrate they can use as single growth substrate (Wibowo et al. 1985; Henick-Kling 1993; Fugelsang 1997; Terrade and Mira de Orduna, 2009). Metabolism of glucose and ribose is a typical trait in the species but different strains have various abilities to metabolize fructose, galactose, mannose, arabinose, xylose, trehalose, sucrose, lactose, maltose and melibiose (Boukhemis et al., 2009). Besides a carbon source, these bacteria require an organic source of nitrogen including amino acids and peptides; although unlike wine yeasts, *O. oeni* is unable to use inorganic nitrogen such as ammonium provided by diammonium phosphate (DAP), a nitrogen supplement commonly used by winemakers to adjust the nitrogen available to yeasts (Henick-Kling 1993). Lactic acid bacteria also require vitamins, especially B-group and pantothenic acid vitamins, as well as trace elements

such as magnesium, potassium and manganese (Fugelsang, 1997). In the harsh environment of wine, a deficiency in any one of the nutritional requirements can impair the ability of *O. oeni* to grow and conduct malolactic conversion.

Malolactic fermentation does not involve the metabolism of carbohydrates or organic acids into ethanol or lactic acid and is therefore not a true fermentation. Instead, it is an enzymatic conversion of malic acid to lactic acid (Costantini et al., 2009; Fugelsang and Edwards, 2006). This conversion is carried out by lactic acid bacteria in order to generate ATP9 (Cox and Henick-Kling, 1989). The conversion of malic acid to lactic acid consumes an acidic proton and produces a molecule of CO_2 (Korkes et al., 1950; Pilone and Kunkee, 1970, 1972; Wibowo et al., 1985). By reducing the proton concentration inside the cell, the energy of the proton gradient is increased. Lactic acid bacteria is able to then allow protons from the acidic environment to enter the cell, harnessing that proton motive force to create ATP through chemiosmosis (Fugelsang and Edwards, 2006; Salema et. al., 1994).

Oenococcus oeni is also a potential spoiler of red wine, and physiochemical changes made to wine during MLF may lead to spoilage from other organisms as well. Alongside the conversion of malic acid to lactic acid during MLF, *O. oeni* may also produce diacetyl (Collins, 1972; Fornachon & Lloyd, 1965; Martineau & Henick-Kling, 1995ab; Nielsen & Richelieu, 1999; Rodriguez et al., 1990). Diacetyl is described as having a buttery, butterscotch aroma and is indicative of wines which have undergone MLF

(Bartowsky et al., 2002; Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985; Martineau and Henick-Kling, 1995a; Martineau, Henick-Kling, & Acree, 1995). The perception of these flavors is related both to concentration of diacetyl in wine as well as to wine matrix effects such as the concentration of SO_2 (Bartowsky et al., 2002). In concentrations below 4 mg/L, diacetyl is often viewed positively, but at higher concentrations can become pungent and unpleasant (Bartowsky et al., 2002; Malherbe et al., 2013; Ramos et al., 1995; Rankine et al., 1969). While most commercial strains of *O. oeni* do not produce excessive amounts of diacetyl, tolerance of this compound depends largely on wine style and acceptance by the consumer. *O. oeni* and other lactic acid bacteria are also capable of producing ethyl carbamate as a byproduct of arginine metabolism (Mira de Orduna et al., 2000; Liu et al., 1994). This process requires the deamination of arginine, yielding citruline which is then converted to carbamyl phosphate. Carbamyl phosphate is then used to convert ADP to ATP yielding ammonia and CO2. However if carbamyl phosphate is excreted from the cell during this process, it can react with ethanol to produce ethyl carbamate (urethane) (Ough et al., 1988). Ethyl carbamate is a carcinogen and thus, very undesirable. Regardless, levels in wine generally do not exceed a few ppb (Arena et al., 1999). Finally, the rise in pH that occurs in wine during MLF may allow certain spoilage yeasts and bacteria to grow more easily as many wine spoilage microbes prefer pH values > 3.50 (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985; Du Toit and Pretorius, 2000).

Spoilage Microorganisms: *Pediococcus* and *Lactobacillus*

In addition to *O. oeni,* wine LAB species belonging to the genus *Pediococcus* and *Lactobacillus* are also often present on grapes and in the winery. Spoilage by these bacteria are most likely to occur when the wine is warm, when the wine lacks sufficient SO_2, or above pH 3.5. Unlike *O. oeni*, these bacteria are always considered to be spoilage microorganisms due to their potential to produce harsh or unpleasant aroma compounds such as acetic acid, n-heterocyclic volatile bases (associated with mousy taint), or bitter tasting compounds like acrolein (Bauer, Cowan, & Crouch, 2010; Davis, Wibowo, Fleet, & Lee, 1988; Du Toit, & Pretorius, 2000). *Pediococcus* species can also produce exopolysaccharide compounds that can result in ropy or mucosal textures (Costello and Henschke, 2002; Francis and Newton, 2005; König et al., 2009; Swiegers et al., 2005; Walling et al., 2005; Wisselinka et al., 2002). Finally, growth in wine of some species of *Lactobacillus* and *Pediococcus* has resulted in elevated concentrations of biogenic amines (Silla Santos, 1996; Arena and Manca de Nadra, 2001). These compounds can cause wine spoilage at high concentrations and are have health implications (Silla Santos, 1996; Arena and Manca de Nadra, 2001).

Spoilage Microorganisms: Acetic Acid Bacteria

Acetic acid bacteria (AAB) are both ubiquitus in and well adapted to winemaking environments. AAB belong to the family Acetobacteraceae, which is subdevided into the genera *Acetobacter*, *Acidomonas, Gluconobacter*, and *Gluconacetobacter* (Holt et al., 1994; Ruiz et al., 2000). The most commonly isolated genus of AAB found in wine are *Gluconobacter* and *Acetobacter* (Joyeux et al., 1984a; Drysdale and Fleet 1984). Unlike

S. cerevesiae and LABs, AAB require an aerobic environment, and are most likely to spoil wines if they are exposed to oxygen (Joyeux et al., 1984a). While AAB are commonly found on grapes they usually do not proliferate during early fermentation due to the anaerobic conditions resulting from fermentation, the high acidity of must and wine, and the presence of SO_2 that can arise from yeast fermentation or through additions made by the winemaker. (Drysdale and Fleet 1984; Joyeux et al., 1984a; Joyeux et al., 1984b). Though Drysdale and Fleet (1988) showed that spoilage by AAB can happen during many stages of winemaking, not all species of AABs affect wine at all stages. The more ethanol sensitive *Gluconobacter* species are commonly isolated from grapes, and musts, but die off due to lack of oxygen and pressure from ethanol as alcoholic fermentation progresses (Joyeux et al., 1984a). The more ethanol tolerant *Acetobacter* species can survive the fermentation process, and are most commonly isolated later in the process during aging in tanks and/or barrels (Drysdale and Fleet, 1984, 1988; Joyeux et al., 1984a).

AAB spoil wine by converting ethanol to acetic acid through acetaldehyde (Fleet, 1993; Sponholz, 1993; Lonvaud-Funel, 1996). Acetic acid bacteria metabolize ethanol to acetic acid through the catalytic action of two membrane bound enzymes. The enzyme alcohol dehydrogenase first converts ethanol to acetaldehyde which is then converted to acetic acid by acetaldehyde dehydrogenase (Adachi et al., 1978; Adachi et al., 1980; Ameyama et al., 1981; Adachi et al., 1987; Fukaya et al., 1989; Tayama et al., 1989). Acetic acid is considered undesireable in wine at concentrations above 0.4 to 0.5 g/L, which is still around three times lower than the legal limit of 1.2 to 1.4 g/L (Davis, Wibowo,

Eschenbruch, Lee, & Fleet, 1985; Drysdale and Fleet, 1989a; Eglinton and Henschke 1999a; Eglinton and Henschke 1999b; Sponholz, 1993). The presence of acetic acid in wine can be discerned through its contribution of sour flavors and vinegar-like aromas (Peynaud, 1984). Acetaldehyde produced by AAB can also contribute to spoilage through the addition of sherry, nutty, and bruised apple aromas (Francis and Newton 2005). Another avenue of spoilage by AAB is through the production of ethyl acetate, which is the ethyl ester of acetic acid. This compound has a solvent-like aroma that can be described as similar to nail polish remover (Francis and Newton 2005). The presence of AAB in wine can also reduce the effectiveness of SO_2 through the production of acetaldehyde that binds with SO_2, reducing its effectiveness as an antimicrobial and antioxidant (Fornachon, 1963; Hood, 1983; Romano and Suzzi, 1993). Similarly, gluconic acid, produced by *Gluconobacter* species, can also bind to SO_2, decreasing antimicrobial and antioxidant capabilities (Joyeux et al., 1984b; Barbe et al., 2000; Barbe et al., 2002; Sponholz et al., 2004).

Spoilage Microorganisms: *Brettanomyces bruxellensis*

Among the most prolific spoilers of wine is *Brettanomyces bruxellensis*. This yeast has been isolated from wine, cider, beer, sake, kimchi, dairy, and bioethanol fermentations as well as soft drinks and other foods (Andrews & Gilliland, 1952; Gilliland, 1961; Kolfschoten & Yarrow, 1970; Peynaud & Domercq 1956; Steensels et al., 2015; van der Walt & van Kerken 1961). *B. bruxellensis* can cause significant financial losses due to its ability to produce undesirable flavor and aroma compounds in wines (Crauwels et al.,

2014; Fugelsang et al., 1993; Sponholz 1993; Heresztyn, 1986). These compounds are often associated with the descriptors 'Band-Aid', mousy, barnyard, fecal, medicinal, smoke, and clove. (Chatonnet et al., 1995; Licker et al., 1998). Defining a standard spoilage character is difficult as *Brettanomyces bruxellensis* is a diverse species with significant inter-strain variability (Borneman et al., 2014; Crauwels et al., 2014; Curtin et al., 2012a) that can lead to the production of a range of spoilage compounds at various concentrations.

Brettanomyces bruxellensis is often found in oak barrels in the winery but can also be present on a range of winery surfaces if sanitation is poor. While rare, there have also been reports of *B. bruxellensis* being present on grapes. Once in the winery, *B. bruxellensis* can be difficult to eradicate due its ability to survive in wine for long periods, its' relative resistance to SO_2, and its' minimal nutrient requirements (Joseph et al., 2007; Oelofse et al., 2008; Tristezza et al., 2010). Even minor wine infections of this yeast can be problematic. All it takes is few cells to survive for extended periods of time in wine for them to multiply and begin to create characteristic off-flavors. Key to the tenacity of this organism is the production of biofilms and the ability to metabolize cellobiose (Blomqvist, 2012; Joseph et al., 2007). Biofilm formation allows *B. bruxellensis* to survive winery sanitation practices, while the ability to metabolize cellobiose as a carbon source enables it to survive within the structure of oak barrels (Oelofse et al., 2008). Due to these factors, winemakers invest considerable time and effort attempting to avoid or mitigate the effects of *B. bruxellensis* spoilage.

Various factors such as temperature, pH, and ethanol concentration can increase or decrease the likelihood that *Brettanomyces* will proliferate in an infected wine. *Brettanomyces* grows best at temperatures between 25 and 32°C (Brandam et al. 2008), is relatively ethanol tolerant being able to survive in concentrations up to 15-16%, (Barata et al., 2008a; Dias et al., 2003) and grows well at wine pH with growth favored above pH 3.60 (Blomqvist et al., 2012). *Brettanomyces* is less nutritionally fastidious than other wine microorganisms like wine lactic acid bacteria. Much of carbon metabolism in *B. bruxellensis* is strain dependent, however most strains can utilize glucose, fructose, sucrose, maltose, trehalose, and cellobiose (Crauwels et al., 2015). Besides the carbon sources listed, various strains of *B. bruxellensis* can utilize a vast array of secondary carbon sources. Strains of *B. bruxellensis* can utilize galactose, raffinose, arabinose, and lactose as additional carbon sources. Some strains can even use ethanol as a sole carbon source given adequate access to oxygen (Crauwels et al., 2015; Fugelsang and Edwards, 2006; Silva et al., 2004). Due to these robust metabolic strategies, *B. bruxellensis* is commonly able to grow and produce volatile phenols above sensory threshold concentrations even in fully dry wines (Barata et al., 2008b). Like *S. cerevisiae, B. bruxellensis* can ferment sugars to alcohol. Unlike *S. cerevisiae* however, *B. bruxellensis* exhibits the Custer effect where the typical alcohol metabolic pathway is inhibited under anaerobic conditions (Van Dijken et al., 1986; Vigentini et al., 2008). Conversely, the Custer effect can also cause *B. bruxellensis* to produce acetic acid from sugar when growing in an oxygen-rich environment (Ciani & Ferraro, 1997; Freer et al., 2003).

Aside from being able to utilize a range of carbon sources, *B. bruxellensis* can also utilize a diverse array of nitrogen sources found in wine. As with many wine microbes, *B. bruxellensis* can use organic nitrogen in the form of free amino acids, and like *S. cerevisiae*, *Brettanomyces bruxellensis* can use ammonium as an inorganic nitrogen source (Uscanga et al. 2000). Unique to some strains of *B. bruxellensis* however, is the ability to utilize nitrate as a sole nitrogen source (Crauwels et al., 2015). The majority of strains tested have demonstrated the ability to utilize this nitrogen source, and because of this, *B. bruxellensis* can have a competitive advantage over other microbes in a post fermentation environment where many of the nutrients have been scavenged and removed.

B. bruxellensis causes wine spoilage by producing multiple undesirable flavor and aroma compounds. In studies focused on consumer response, clear evidence exists tying presence of *B. bruxellensis* character to dislike of that wine by consumers. As with many other fermenting organisms, *B. bruxellensis* produces small amounts of acetic acid at the end of glucose metabolism (Ciani & Ferraro 1997; Freer et al., 2003). Concentrations of acetic acid above 1 g/L result in undesirable sharp or vinegar aromas (Fugelsang and Edwards, 2006; Licker et al., 1998). Isovaleric acid (rancid, gym socks, goat) is formed by *B. bruxellensis* through metabolism of the amino acid L-leucine (Vigentini et al., 2013; Licker et al., 1998). The compounds most indicative of *Brettanomyces* wine spoilage are the volatile phenols 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG), and 4-ethylcatechol (4-EC) (Oelofse et al., 2008; Hesford et al., 2004). These volatile phenols, are associated with 'Band-Aid', mousy, barnyard, fecal, medicinal, smoke, and clove

descriptors, and are also known to suppress the perception of positive aroma qualities in a wine, especially fruity and floral characteristics (Chatonnet et al., 1995; Gerbaux and Vincent, 2002; Licker et al., 1998). Small amounts of *B. bruxellensis* are able to alter the visual appeal of a wine as well, as less than 102 cells/mL have been shown to develop a distinct haziness in wines (Edelenyi, 1966).

Control of *Brettanomyces*

While wine is at risk for infection by *B. bruxellensis* throughout its' production, it is particularly vunerable post-alcoholic fermentation and during aging in barrels. There are a number of steps a winemaker can take to reduce the risk of wine infection and spoilage by *B. bruxellensis* and/or manage *Brettanomyces* in already infected wine. Control of *Brettanomyces* begins once the grapes are harvested. Clean disease-free grapes will reduce the overall microbial load entering the winery which can reduce the risks of microbial spoilage (Du Toit et al., 2005). Besides reduction of microbial load from incoming grapes, another key step in reducing the risk of infection is to maintain a robust cleaning and sanitation regime. Regular cleaning of winery surfaces and equipment removes organic material and helps reduce mold and biofilm buildup (Du Toit et al., 2005). Removal of organic matter through cleaning will also make sanitizing chemicals more effective.

Not all surfaces in a winery can be easily cleaned. Such is the case with barrels, especially those received secondhand. Barrels are commonly home to *Brettanomyces* and

can be the source of constant re-infection of wines (Malfeito-Ferreira, 2011).

Winemakers don't just worry about used barrels; new barrels may also be particularly

susceptible to contamination by *Brettanomyces* due to both their cellobiose contents and

the quicker evaporation of SO_2 (Wedral et al., 2010). Once infected, barrels are often

difficult to sanitize. The porosity of wood barrels offers *Brettanomyces* safe harbor from

cleaning efforts as cells can survive deep in the vascular matrix of the wood, and

culturable *B. bruxellensis* cells have been recovered from as deep as 8 mm in infected oak

from barrels (Cartwright et al., 2016; Malfeito-Ferreira et al., 2004). In order to eliminate

Brettanomyces from infected barrels, winemakers use techniques including washing with

hot water, steam, and even ozone treated water (Alejandra Aguilar Solis, 2014; Malfeito-

Ferreira, 2004; Wilker et al., 1997). More experimental techniques also include

microwave radiation, high power ultrasonics, and dry ice (Costantini et al., 2016;

González-Arenzana et al., 2013; Jiranek et al., 2008; Schmid et al., 2011).

Cleaning and sanitation are key steps in reducing the risk of microbial spoilage.

However, given that there will always be some level of background microorganisms

present in the wine and winery, proper use of antimicrobial additives to inhibit microbial

growth is required. While usage of these chemical additives can be effective, consumers

are often wary of their presence in wine (Du Toit and Pretorius, 2000) and winemakers

often seek to reduce their usage. The most prominent of these chemical additions is sulfur

dioxide (SO_2) (Agnolucci et al., 2010; von Cosmos et al., 2016; Zuehlke and Edwards,

2013). Sulfur dioxide (SO_2) is naturally occurring in wine. Between 12 mg/L and 64

mg/L of SO_2 can accumulate from yeast metabolic activities alone (Larue et al., 1985). Winemakers commonly add external SO_2 to wine as well, as an anti-oxidant and antimicrobial agent (Divol et al., 2012). Winemakers have been documented using SO_2 as a preservative since at least the late 1700s CE (McGovern, 2003). Today it remains one of the most widely used antimicrobial agents, and chief among the tools used by winemakers in the control of *B. bruxellensis* (Coulter et al. 2003; Suarez et al. 2007; Wedrel et al. 2010).

The effectiveness of SO_2 as an antimicrobial deterrent against *B. bruxellensis* has been well documented (Agnolucci et al., 2014). In winemaking practice, SO_2 is generally added to musts and wines in the form of potassium metabisulfite with concentrations ranging from 25 mg/L to 150 mg/L (Ribereau-Gayon et al., 2006). Winemakers are most likely to add SO_2 to wine during times when it would be most vulnerable to oxidative or microbial stress. Wines often receive their first dose of SO_2 during processing of fruit or must before fermentation in order to reduce the populations of naturally occurring microorganisms, with subsequent doses occurring after alcoholic or malolactic fermentation to protect the wine from spoilage during ageing (Jackson, 2020). The last dose of SO_2 generally occurs as a preparation for bottling in order to protect the wine from oxidation during the bottling process (Waters et al., 1996).

SO_2 in wine can be bound to other wine compounds, or be present as free SO_2 in the form of molecular SO_2, ionic bisulfate and/or sulfate forms (Burroughs, 1975). Ripper (1892)

showed that of forms of SO_2, free SO_2 is the form which inhibits microbial growth. Free SO_2 is not a single chemical state however. The chemical equilibrium between the different forms of free SO_2 is dependent on the pH of the solution it is in. Molecular SO_2 is the most prevalent species at pH 0-2, while the bisulfite anion dominates from pH 2 to 7, and from pH 7 to 10 the sulfite anion is the most prevalent (Divol et al. 2012). At typical wine pH, bisulfate is the most abundant form of free SO_2, with molecular SO_2 consisting of less than 5% of free SO_2 (Waterhouse et al. 2016). This form of SO_2 is 100 to 500 times more antimicrobially active than the bisulfate form (Rehm and Wittmann 1962; Rehm and Wittmann 1963). The neutral molecular form of free SO_2 is the most antimicrobial as it can freely diffuse across cell membranes (Divol et al. 2012). Once a molecule of SO_2 diffuses into the less acidic interior of a cell it rapidly dissociates into bisulfite and sulfite anions, thus decreasing the molecular SO_2 concentration inside the cell and driving further diffusion of the molecule across the membrane.

Inside the cell, SO_2 derives its antimicrobial activity from its strong reactive nature and its ability to interfere with the enzymatic functions of the organism. SO_2 inhibits the key glyosidic enzyme glyceraldehyde-3-phospate dehydrogenase (Hinze and Holzer 1986). In addition to disrupting and inhibiting cellular enzymatic activity, SO_2 preferentially binds to coenzymes such as NAD, and co-factors such as thiamine as well as to proteins, cutting disulfide bridges in the process (Carmack et al. 1950). SO_2 can also bind to metabolites such as glucose, preventing use by cellular metabolic pathways (Rankine and Pocock, 1969). Finally, SO_2 is a mild mutagen capable of disrupting DNA by causing

adenosine/tyrosine and cysteine/guanine mutations (Mukai et al. 1970; Pagano and Zeiger 1987; Pagano et al. 1990; Meng and Zhang 1992).

While SO_2 has been used historically to reduce the activity of *S. cerevisiae* and *B. bruxellensis*, these yeasts show respectively strong and varied tolerance to its effects. Wine yeast (*S. cerevisiae*) is able to mitigate the toxicity of SO_2 by rapidly excreting it through a membrane bound SO_2 export protein encoded by the SSU1 gene (Park & Bakalinsky, 2000). Tolerance of SO_2 by *Brettanomyces* can vary widely, with studies showing similar tolerance to *S. cerevisiae*, and other studies showing that comparatively minor amounts of SO_2 can affect its viability (Beech et al., 1979; du Toit et al., 2005). SO_2 tolerance by *B. bruxellensis* is a heritable feature (Beech and Thomas, 1985; Warth, 1985; Pilkington and Rose, 1988; Divol et al., 2006; Ventre, 1934) that varies by degrees depending on genotype (Curtin et al. 2012a, Curtin et al., 2012b; Vigentini et al., 2013). Curtin et al. (2012a) found that strain AWRI 1499 could tolerate up to 0.55 mg/L of molecular SO_2 before suffering a > 4 log reduction in culturability. It is not surprising that *B. bruxellensis* should express such a highly varying resistance to SO_2 given the genetic diversity displayed between strains and the varied resilience to the unfavorable conditions found in wine (Curtin et al., 2007; Agnolucci et al., 2009; Hellborg & Piskur, 2009; Vigentini et al., 2012). Such genetic variability and resistance can be explained in terms of a co-evolution of *B. bruxellensis* and *S. cerevisiae* in fermentation ecosystems (Rozpedowska et al., 2011).

Aside from SO_2, another chemical that is used to control *Brettanomyces bruxellenisis* in wine is dimethyl dicarbonate (DMDC). This chemical, commercially known as Velcorin™, inhibits *B. bruxellensis* growth in by inhibiting glycolytic enzymes (Temple & Ough, 1978). DMDC however, is very limited in its ability to hinder the growth of bacterial species (Costa et al., 2008). Its efficiency depends on the strain, initial cell concentration, temperature, ethanol content and pH (Daudt & Ough, 1980; Ough et al., 1978, 1988; Porter & Ough, 1982; Threlfall & Morris, 2002). Unlike SO_2, DMDC is not used as a microbial deterrent, but as a measure against wine that has already been infected (Costa et al., 2008). This is because it quickly reacts in wine and has no residual activity. Following application of DMDC, antimicrobial effects are immediate. The compound quickly decomposes within a few hours into insignificant amounts of carbon dioxide and methanol which have no sensory impacts on the wine (Zuehlke et al., 2013). To this end, DMDC has proven to be effective as a one-off treatment to treat a microbial contamination in wines (Zuehlke et al., 2013). It is most commonly used just prior to bottling to prevent microbial spoilage occurring in bottle (Zuehlke et al., 2015).

Winemakers can also treat wine with a fungally derived compound called chitosan. Chitosan is a chitinous polysaccharide isolated from *Aspergillus niger*. The substance has proven to be effective at preventing the growth of *Brettanomyces* (Ferreira et al., 2013). Chitosan interferes with *Brettanomyces bruxellensis* cells by interacting with anionic groups on the cell surface and limiting the diffusion of solutes such as sugars and heavy metal cations (Brady, Stoll, Starke, & Duncan, 1994; Roller & Covill, 1999). The effects

of chitosan are dependent on concentration: Chitosan added to wines in concentrations of

as low as 1mg/ml seem to suppress *Brettanomyces* growth, but not reduce the overall

Brettanomyces population in infected wines (Gómez-Rivas et al., 2004).

In addition to the use of antimicrobial chemicals, a winemaker has at their disposal the

inhibitory characteristics of the wine to aid in the prevention of growth of spoilage

microorganisms. For example, low pH, high ethanol, and high and low temperature are

all factors that will restrict the growth of a spoilage microbe such as *B. bruxellensis*.

Although *B. bruxellensis* is somewhat tolerant to these individual inhibitory factors, the

combined effect of these inhibitory factors can effect greater inhibition than their

individual magnitudes. This concept, that combinations of effects can be more inhibitory

than their constituent parts, is commonly called the 'hurdle' concept in microbiology

(McMeekin et al. 2000). This approach was first explored by Leistner (1978) in the

context of food safety. It has since been found that such synergistic relationships are a

viable means to reducing microbial populations in foods without resorting to novel

techniques (Leistner, 1992). For wine, the most common example of this 'hurdle effect'

is the synergistic effect of low pH and SO_2. As noted earlier, at low pH a higher

proportion of the SO_2 will be in the antimicrobial molecular SO_2 state. In practice, this

means less SO_2 is needed to control spoilage microbes in a low pH wine than a high pH

wine. The synergistic effect of various inhibitors in wine has been explored in the context

of controlling *B. bruxellensis* growth. Well known 'hurdles' that affect *B. bruxellensis*

growth include temperature, ethanol concentration, pH, and SO_2 (Smith, 2011; Sturm et

al., 2014; Zuehlke and Edwards, 2013). While strain variable tolerance exists, synergistic

interactions between these factors can reduce overall *B. bruxellensis* populations in wine (Edwards et al., 2015; Ramirez et al., 2014).

Sturm et al (2014) studied the interactions between pH, ethanol, and free SO_2, and developed a mathematical model that is predictive of growth over a period of time. An additional study found interactive effects existed between ethanol, SO_2, and residual sugar, resulting in repressed growth and ethylphenol production in an artificial media (Chandra et al. 2014). Finally, Edwards and Oswald (2017) found that even without added SO_2, interactive effects between temperature and ethanol concentration in wine are capable of extending lag time and reducing volatile acidity and ethylphenol concentrations in certain strains of *B. bruxellensis*.

An additional factor that could be considered a "hurdle" to control spoilage microorganisms is antagonistic interactions with other microorganisms present during wine production. For example, the production of ethanol by *S. cerevisiae* during alcoholic fermentation results in the suppression of ethanol sensitive microbes such as many non-*Saccharomyces* species (Fleet et al., 1984; Heard & Fleet, 1985; Heard & Fleet, 1988; Pina et al., 2004). With regards to *Brettanomyces*, it has been observed that when *B. bruxellensis* and *S. cerevisiae* are co-inoculated in grape juice, *S. cerevisiae* will dominate the fermentation with *B. bruxellensis* growth occurring mainly after the completion of alcoholic fermentation when *S. cerevisiae* populations are in decline (Renouf et al., 2006). As such, it is a common winemaking practice to inoculate a vigorous strain of *Saccharomyces* that can outcompete spoilage organisms (Albergaria &

Arneborg, 2016; Beltran et al., 2002; Fleet & Heard, 1993; Torija et al. 2001; Xufre et al., 2006). By contrast, if an alcoholic fermentation becomes sluggish or stuck, it can become infected with non-*Saccharomyces* yeasts (Agnolucci et al., 2009) and/or spoilage bacteria such as *Lactobacillus*. Alcoholic fermentation also leads to decreased oxygen levels in must, which can lead to the suppression of organisms with oxidative or weakly fermentative metabolisms while promoting the growth of fermentative organisms such as *S. cerevisiae* (Holm Hansen et al., 2001). In addition to ethanol, *S. cerevisiae* can produce other metabolic byproducts with known antimicrobial properties such as SO_2 (Eschenbruch 1974, Dott et al. 1976, Eschenbruch and Bonish 1976, Suzzi et al. 1985, Romano and Suzzi 1993, Henick-Kling and Park 1994, Carrete et al. 2002, Osborne and Edwards 2006), medium chain fatty acids (Edwards and Beelman 1987, Lonvaud-Funel et al. 1988, Edwards et al. 1990, Capucho and San Ramao 1994), and antimicrobial peptides (Dick et al. 1992, Comitini et al. 2005).

An additional instance of inhibitory interactions between wine microbes is the killer phenomenon Bevan and Makover (1963) first discovered in which certain yeast strains kill other strains by secreting certain proteins and glycoproteins. These killer proteins are well described in the literature (van Vuuren & Jacobs, 1992; Shimizu, 1993; Musmanno et al., 1999; Gutierrez et al., 2001). While killer proteins generally only affect organisms of the same species, there are forms that are known to be active against other yeasts, filamentous fungi, and even bacteria (Magliani et al., 1997). Some non-*Saccharomyces* yeasts have gotten attention recently for the production of killer proteins that have shown activity against *B. bruxellensis*. *Torulaspora delbrueckii*, *Pichia anomala*, and

Kluyveromycer wickerhamii, have all been found to produce compounds that can limit *B. bruxellensis* growth (Comitini et al., 2004). The toxin PMKT2 produced by *Pichia membranifaciens*, described by Santos et al. (2009) is active against *B. bruxellensis*, as are the killer factors CpKT1, and CpKt2 which are produced by *Candida pyralidae*. These last two are notable, as they seem to be active against *B. bruxellensis*, but not against *S. cerevisiae* or lactic acid bacteria (Mehlomakulu et al., 2014). It is important to note however that due to the lower overall ethanol tolerance of non-*Saccharomyces* yeasts, their potential to suppress the growth of *B. bruxellensis* will diminish as alcoholic fermentation progresses, leaving wine at the end of alcoholic fermentation unprotected and vulnerable to infection.

Examples of interactions between *B. bruxellensis* and *O. oeni* have also been reported in literature. For example, Cheschier et al (2015) determined that certain *O. oeni* strains had cinnamic esterase activity which resulted in wine with higher concentrations of ρ-coumaric acid at the end of MLF due to degradation of tartaric acid bound ρ-coumaric acid (coutaric acid). This led to significantly higher production of 4-EP by *B. bruxellensis* in these wines compared to wines that underwent MLF with an *O. oeni* strain that did not have cinnamic esterase activity. Antagonistic interactions between *O. oeni* and *B. bruxellensis* have also been reported in literature. Renouf & Murat (2008) noted that malolactic fermentation may restrict *B. bruxellensis* development given a high *O. oeni* density. In a similar investigation, Gerbaux et al. (2009) showed that wines which had undergone MLF had considerably less volatile phenol concentrations than wines which had not undergone MLF. While some of the findings of these studies may indicate that

there is a connection between MLF and suppression of *B. bruxellensis*, the authors did not report on microbial populations, focusing instead on volatile phenol data. This lack of data was filled in partially when Chescheir (2014) investigated whether the reduced volatile phenol content reported by Gerbaux et al. (2009) was due to interactions between *O. oeni* and *B. bruxellensis*. Chescheir (2014) observed that *B. bruxellensis* populations would immediately decline when inoculated into wine that had recently undergone MLF. However, only a small number of *O. oeni* strains were investigated and only one *B. bruxellensis* strain was used.

Objectives

Given that *B. bruxellensis* is a key wine spoilage microorganism that can be difficult to control using current winemaking practices, this study investigated the interactions between *O. oeni* and *B. bruxellensis* with the goal of determining if MLF could be used as an additional tool to prevent *B. bruxellensis* growth in wine. Wine is particularly vulnerable to *B. bruxellensis* infection during or shortly after MLF as the wine will contain sufficient nutrients for *B. bruxellensis* growth, it may still be relatively warm due to heat generated from alcoholic fermentation, and no additions of SO_2 can be made until MLF is complete. Therefore, it has been suggested that the conduction of a rapid MLF initiated by inoculation of a commercial *O. oeni* strain is a useful strategy to prevent *B. bruxellensis* spoilage as this minimizes the length of time the wine is not protected by SO_2. This study investigated an additional benefit to the conduction of a rapid successful MLF: the inhibition of *B. bruxellensis* growth and volatile phenol production. Initial experiments tested a large number of commercial *O. oeni* strains for their ability to

inhibit *B. bruxellensis* growth when inoculated at the end of MLF. The susceptibility of a number of *B. bruxellensis* strains to *O. oeni* was then tested and experiments were also conducted to try and better understand the mechanism by which *O. oeni* inhibits *B. bruxellensis* growth. Finally, the effect of ethanol concentration in combination with MLF was considered. The specific objectives of the study were to:

1) Investigate the ability of *Oenococcus oeni* to inhibit *Brettanomyces bruxellensis* growth and volatile phenol production and determine if strain variability exists

2) Determine mechanism by which *Brettanomyces bruxellensis* is inhibited by *Oenococcus oeni*

Chapter 2
Inhibition of *Brettanomyces bruxellensis* UCD-2049 growth and volatile
phenol production in wine by *Oenococcus oeni*

Abstract

The effect of malolactic fermentation (MLF) on *B. bruxellensis* strain UCD-2049 growth

and volatile phenol production was investigated. The impact of ten commercial

Oenococcus oeni strains on *B. bruxellensis* strain UCD-2049 growth was tested by

inoculating *B. bruxellensis* into wine at the end of a 14 day MLF conducted by different

O. oeni strains. *B. bruxellensis* strain UCD-2049 populations declined rapidly after

inoculation after MLF regardless of *O. oeni* strain. *B. bruxellensis* populations did not

recover during the course of the experiment for over half of the *O. oeni* strains tested.

When *B. bruxellensis* was inoculated into the wine that had not undergone MLF with *O.

oeni*, an initial decline in population was observed followed by increased growth to

populations $> 1 \times 10^6$ CFU/mL by the end of the experiment. Significantly higher

concentrations of 4-ethyl phenol and 4-ethyl guaiacol were measured in wine that had not

undergone MLF when compared to the MLF treated wines. To determine possible

mechanisms of inhibition an experiment was conducted where *B. bruxellensis* was

inoculated into wine that had just completed MLF but *O. oeni* cells were separated from

B. bruxellensis by a dialysis membrane. While *B. bruxellensis* populations declined

rapidly and remained repressed for many weeks when in direct contact with *O. oeni,*

populations only declined slightly and quickly recovered if *O. oeni* was separated from *B.

bruxellensis* by a dialysis membrane.

Introduction

Among the most prolific microbial spoilers of wine is the yeast *Brettanomyces bruxellensis*. *B. bruxellensis* can cause significant financial losses due to its ability to produce undesirable flavor and aroma compounds in wines (Crauwels et al., 2014; Fugelsang et al., 1993; Sponholz 1993; Heresztyn, 1986). The compounds most indicative of *Brettanomyces* wine spoilage are the volatile phenols 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG), and 4-ethylcatechol (4-EC) (Oelofse et al., 2008; Hesford et al., 2004). These volatile phenols, are associated with 'Band-Aid', mousy, barnyard, fecal, medicinal, smoke, and clove aroma descriptors, and are also known to suppress the perception of positive aroma qualities in a wine such as fruity and floral (Chatonnet et al., 1995; Gerbaux and Vincent, 2002; Licker et al., 1998).

While wine is at risk for infection by *B. bruxellensis* throughout its' production, it is particularly vulnerable post-alcoholic fermentation and during aging in barrels. There are a number of steps a winemaker can take to reduce the risk of wine infection and spoilage by *B. bruxellensis* and/or manage *Brettanomyces* in already infected wine. Regular cleaning and sanitation of winery surfaces and equipment removes organic material and helps reduce microbial buildup (Du Toit et al., 2005). Most commonly, winemakers also use sulfur dioxide (SO_2) as an antimicrobial agent against infection (Agnolucci et al., 2010; von Cosmos et al., 2016; Zuehlke and Edwards, 2013). Apart from these steps, winemakers are generally limited in strategies to prevent the growth of *B. bruxellensis* in

wine. Moreover, consumers are often wary of the usage of chemical additives such as SO_2 in wine, so winemakers often seek to reduce their usage. (Du Toit and Pretorius, 2000)

Wine is particularly vulnerable to *B. bruxellensis* infection during or shortly after the malolactic fermentation (MLF). At this point the wine will contain sufficient nutrients for *B. bruxellensis* growth, and competition from *S. cerevisiae* will be reduced. Furthermore, no additions of SO_2 can be made until this process is complete, due to the inhibitory effect SO_2 has on malolactic bacteria. For this reason it has been suggested that the conduction of a rapid MLF initiated by inoculation of a commercial *Oenococcus oeni* strain is a useful strategy to prevent *B. bruxellensis* spoilage as this minimizes the length of time the wine is not protected by SO_2 (Gerbaux et al. 2009).

An additional benefit to the conduction of a rapid successful MLF was noted by Renouf & Murat (2008) and Gerbaux et al. (2009). Renouf & Murat (2008) noted that malolactic fermentation may restrict *B. bruxellensis* development given a high *O. oeni* density. In a similar investigation, Gerbaux et al. (2009) showed that wines which had undergone MLF had considerably less volatile phenol concentrations than wines which had not undergone MLF. While this study suggested that there is a connection between MLF and suppression of *B. bruxellensis,* the authors did not report on microbial populations, focusing instead on volatile phenol data. This lack of data was filled in partially when Chescheir (2014) investigated whether the reduced volatile phenol content reported by Gerbaux et al. (2009) was due to interactions between *O. oeni* and *B. bruxellensis*.

Chescheir (2014) observed that *B. bruxellensis* populations would immediately decline when inoculated into wine that had recently undergone MLF. However, only a small number of *O. oeni* strains were investigated and only one *B. bruxellensis* strain was used.

Given that *B. bruxellensis* is a key wine spoilage microorganism that can be difficult to control using current winemaking practices, this study investigated interactions between *O. oeni* and *B. bruxellensis* with the goal of determining if MLF could be used as an additional tool to prevent *B. bruxellensis* growth in wine. A large number of commercial *O. oeni* strains were tested for their ability to inhibit *B. bruxellensis* growth and volatile phenol production. Additional experiments were also conducted to determine the possible mechanism(s) by which growth inhibition of *B. bruxellensis* is caused by *O. oeni*.

Materials 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), and University culture collections (Oregon State University, University of California Davis). 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 (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 was 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).

When needed, microorganisms were prepared from frozen cultures by streaking onto YPD agar (*B. bruxellensis*) or MRS agar (*O. oeni*) and grown at 25°C for one week. Single colonies were picked from the agar plates for inoculation into acidic grape juice broth (AGJ) (2.5 mg/L manganese sulfate, 125 mg/L magnesium sulfate, 5g/L yeast extract, 1 mL/L 5% (w/w) Tween 80, 250 mL/L white grape juice, pH 3.5) and grown at 25°C for eight (*B. bruxellensis*) or twelve (*O. oeni*) days. Cells were harvested by centrifugation (4,000 x g for 20 minutes) and re-suspended in sterile peptone solution (0.1%) prior to inoculation. *S. cerevisiae* RC212 (Lallemand) for winemaking was used as freeze-dried culture direct from the manufacturer.

Table 2.1 Strains and Sources of Microorganisms used in Screening Experiment

Strain Name	Species	Source
350	*Oenococcus oeni*	Laffort (St Helena, CA)
Alpha	*Oenococcus oeni*	Lallemand (Montreal, Canada)
Beta	*Oenococcus oeni*	Lallemand
Omega	*Oenococcus oeni*	Lallemand
VP41	*Oenococcus oeni*	Lallemand
CH11	*Oenococcus oeni*	Chr. Hansen (Horsholm, Denmark)
CH16	*Oenococcus oeni*	Chr. Hansen
CH35	*Oenococcus oeni*	Chr. Hansen
VFO 2.0	*Oenococcus oeni*	Chr. Hansen
PN4	*Oenococcus oeni*	Lallemand
31	*Oenococcus oeni*	Lallemand
UCD-2049	*Brettanomyces bruxellensis*	University California Davis Culture Collection (Davis, CA, USA)

Wine production

Pinot Noir wine was produced at the Oregon State University Research Winery from grapes harvested from Woodhall Vineyard (Alpine, Oregon, USA) in 2018 and 2019 following the same basic protocol. 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 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. 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.

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, 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. Basic wine parameters for the 2018

Pinot noir were 15% (v/v) ethanol, pH 3.61, 1.41 g/L malic acid and 11.2 mg/L total SO_2.

Basic wine parameters for the 2019 Pinot Noir were 13.7% ethanol by volume, pH 3.48,

1.27 g/L malic acid and 16.0 mg/L total SO_2.

Oenococcus oeni Screening

Sterile filtered 2018 Pinot noir wine was diluted with deionized water to bring the final

ethanol concentration to 13% by volume and spiked with 5 mg/L coumaric acid (Sigma-

Aldrich). The wine was then filtered through Nalgene™ single use bottle top filters using

a 0.2 μm polyethersulfone membrane as 100 mL aliquots into sterilized 100 mL Schott

bottles. The wine was brought to room temperature before inoculation in triplicate with

one of each of the 10 commercial strains of *O. oeni* prepared as previously noted. *O. oeni*

was inoculated at approximately $1x10^7$ CFU/mL. Three bottles were left inoculated as a

control. All bottles were blanketed with filtered argon gas and placed at 21°C. Malolactic

fermentation was monitored by analysis of malic acid by enzymatic test kit L

(Vintessential Enzymatic L-Malic Acid Test Kit; Victoria, Australia) until completed (<

50 mg/L). MLF was completed for all *O. oeni* strains within 14 days. All three bottles

from each *O. oeni* treatment and all three control bottles were inoculated in a laminar

flow hood under sterile conditions with *B. bruxellensis* UCD-2049 at approximately

$1x10^4$ CFU/mL. These represent the 'Day 0' treatment. All wines were then blanketed

with filtered argon gas and stored at 21°C.

B. bruxellensis populations were monitored periodically for 51 days post-inoculation by plating on YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar, pH 6.50) after appropriate dilution (0.1% peptone). Plates were incubated for 7 days at 25°C before being counted. After sampling, bottles were topped with filtered argon gas. On the final day of sampling for each treatment, 50 mL samples were pulled for volatile phenol analysis and stored frozen at -20°C until needed.

Cell separation experiment

To determine the potential mechanism by which *B. bruxellensis* may be inhibited by *O. oeni,* an experiment was conducted where *B. bruxellensis* is inoculated into Pinot noir wine that recently underwent MLF but *B. bruxellensis* cells will be physically separated from *O. oeni* cells using a dialysis membrane. The experimental design set-up is shown in figure Y1. In brief, wine is aliquoted into sterile 24 ounce Whirl-Pak® bags (Whirl-Pak, WI, USA) into which a wine-filled Slide-A-Lyzer™ 10 kDa sterile dialysis cassette (Thermo-Scientific, MA, USA) is placed. The packs are stored in such a way that the wines in the bag and the in the cassette are separated by the dialysis membrane. In the cell contact treatment, both *Oenococcus oeni* and *Brettanomyces bruxellensis* were inoculated into the Whirlpak® bags. In the cell separation treatment, *Oenococcus oeni* was inoculated into the cassettes, and *Brettanomyces bruxellensis* was inoculated into the Whirlpak® bags, in the control treatment *Brettanomyces bruxellensis* was inoculated into the Whirlpak® bags.
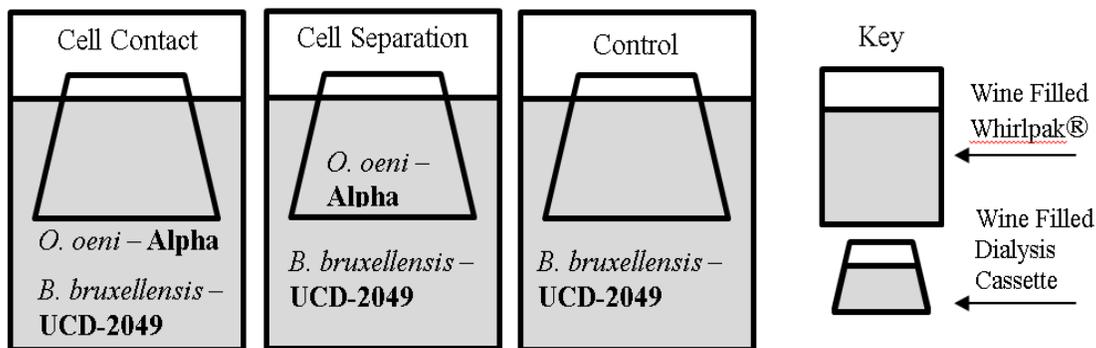
Figure 2.1 Cassette apparatus and experimental design for cell separation with dialysis cassettes

The seventy mL dialysis membrane cassettes (10 kDa, Thermo-Scientific) came treated in a number of preservatives including SO_2, so a soaking procedure was required to remove these preservatives before they could be used. Aside from the step to remove SO_2, the washing steps were outlined by the manufacturer. The reagents EDTA (1% w/w), sodium sulfite (0.3% w/v), sulfuric acid (0.2% v/v) and hydrogen peroxide (0.3% v/v) were prepared and sterile filtered through Nalgene™ single use bottle top filters using a 0.2 μm polyethersulfone membrane into sterile 1000 mL Schott bottles. Deionized water was sterilized by autoclave at 250°F for 15 minutes at 15 psi. The rinsing procedure took place under a laminar flow hood in sterilized glass containers. Cassettes were first soaked in EDTA solution for 2 minutes, followed by a 2 minute soak in hot deionized water, followed by a 2 minute soak in sodium sulfite solution, followed by a 2 minute soak in hot deionized water, followed by a 2 minute soak in sulfuric acid solution, followed by a 10 minute soak in hot deionized water, followed by a 10 minute soak in hydrogen peroxide, followed by a 10 minute soak in hot deionized water followed by a final overnight soak in room temperature deionized water.

2019 Pinot noir wine produced as previously described was used for this experiment. When needed, the wine was filtered through Nalgene™ single use bottle top filters using a 0.2 μm polyethersulfone membrane into sterilized 1000 mL Schott bottles. A total of 170 mL of sterile filtered wine was then added to each cassette/whirlpak combination. Seventy mLs of wine was added to the cassette and 100 mLs of wine was added to the whirlpak. The cassettes were then soaked in the wine at room temperature for 24 hours. The removal of SO_2 was checked by aeration-oxidation analysis of the wines after the 24 hr soak. *O. oeni* strain Alpha (Lallemand) was inoculated in triplicate into the cell contact and cell separation treatments at approximately $1x10^6$ CFU/mL. *O. oeni* was prepared as previously noted. All treatments, including the uninoculated control, were incubated at 21°C. Malolactic fermentation was allowed to progress for 14 days for all treatments after which time, malic acid in the cell contact and cell separation treatments was confirmed to be less than 50 mg/L (Vintessential Enzymatic L-Malic Acid Test Kit; Victoria, Australia). All treatments were then inoculated with *B. bruxellensis* UCD-2049 as per the experimental design (Figure 2.1). *B. bruxellensis* was inoculated at approximately $1x10^5$ CFU/mL after preparation as previously noted. These represent the 'Day 0' treatment. All wines were incubated at 21°C and *B. bruxellensis* populations were monitored periodically for 70 days post-inoculation by plating on YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar, pH 6.50) after appropriate dilution (0.1% peptone). Plates were incubated for up to 7 days at 25°C before counting.

Impact of MLF timing on growth of *B. bruxellensis*

2019 Pinot noir wine was adjusted to 13% v/v ethanol by addition of deionized. Wine

was then filtered through Nalgene™ single use bottle top filters using a 0.2 μm

polyethersulfone membrane as 100 mL aliquots into sterilized 100 mL Schott bottles.

Three bottles were randomly assigned to be the "beginning MLF" treatment, three were

assigned as "partial MLF" treatment, and three were assigned as "post MLF" treatment.

For each treatment, three bottles were also assigned as controls. The "beginning MLF",

"partial MLF", and "post MLF" treatments were all inoculated with *O. oeni* strain Alpha

at approx. $1x10^7$ CFU/mL after being prepared as previously described. The "beginning

MLF" treatments was also inoculated with *B. bruxellensis* UCD-2049 at approx. $1x10^4$

CFU/mL after being prepared as previously described. A control set of bottles was also

inoculated with *B. bruxellensis* UCD-2049 at approx. $1x10^4$ CFU/mL. All bottles were

topped with filtered argon gas and placed at 21°C. MLF was monitored by malic acid

degradation (Vintessential Enzymatic L-Malic Acid Test Kit; Victoria, Australia). Three

days post inoculation of *O. oeni* Alpha, the malic acid in the wines of the partial MLF

treatment had been degraded to approximately 100 mg/L, and these three wines were

inoculated with *B. bruxellensis* UCD-2049 at approx. 1×10^4 CUF/mL prepared as

previously described. A control set of wines (no MLF) was also inoculated with *B.

bruxellensis* UCD-2049 at approx. 1×10^4 CUF/mL. At the completion of MLF (approx.

14 days after *O. oeni* inoculation) the "post-MLF" treatment wines were inoculated with

B. bruxellensis UCD-2049 at approx. 1×10^4 CUF/mL prepared as previously described.

A control set of wines (no MLF) was also inoculated with *B. bruxellensis* UCD-2049 at

approx. 1×10^4 CUF/mL. For all treatments, *O. oeni* and *B. bruxellensis* populations were

monitored by plating on de Man, Rogosa, and Sharpe (MRS) media (pH 4.5) containing

15 g/L pimaricin (Sigma-Alrich) or YPD media respectively. Plates were incubated for 7 days at 25°C before being counted. After sampling, bottles were topped with filtered argon gas.

<u>Wine volatile phenol analysis</u>

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

<u>Statistical analysis:</u>

Statistical analysis was conducted using R-studio (Boston, MA, USA) at a significance level (α) of 0.05. Statistical differences among treatments were determined by ANOVA followed by Tukey's honestly significant difference (HSD) if appropriate.

Results

The impact of a number of commercial *O. oeni* strains on *B. bruxellensis* was tested by inoculating *B. bruxellensis* strain UCD-2049 into wine that had just completed MLF by *O. oeni* and comparing growth to a control where *B. bruxellensis* UCD-2049 was inoculated into wine that had not undergone MLF. A total of ten *O. oeni* strains were used. When inoculated into wine *B. bruxellensis* UCD-2049 populations initially declined in all treatments including the control (Figure 2.2, Figure 2.3). The populations of the control group (no MLF) soon recovered and entered exponential growth by day 5, reaching 6.3×10^4 CFU/mL. *B. bruxellensis* UCD-2049 reached a maximum population

of 8.9 x 10^6 CFU/mL by day 16 before entering a slow and steady population decline

over the next 35 days, settling to a population of 6.3 x 10^5 by day 51 (Figure 2.2, Figure

2.3).
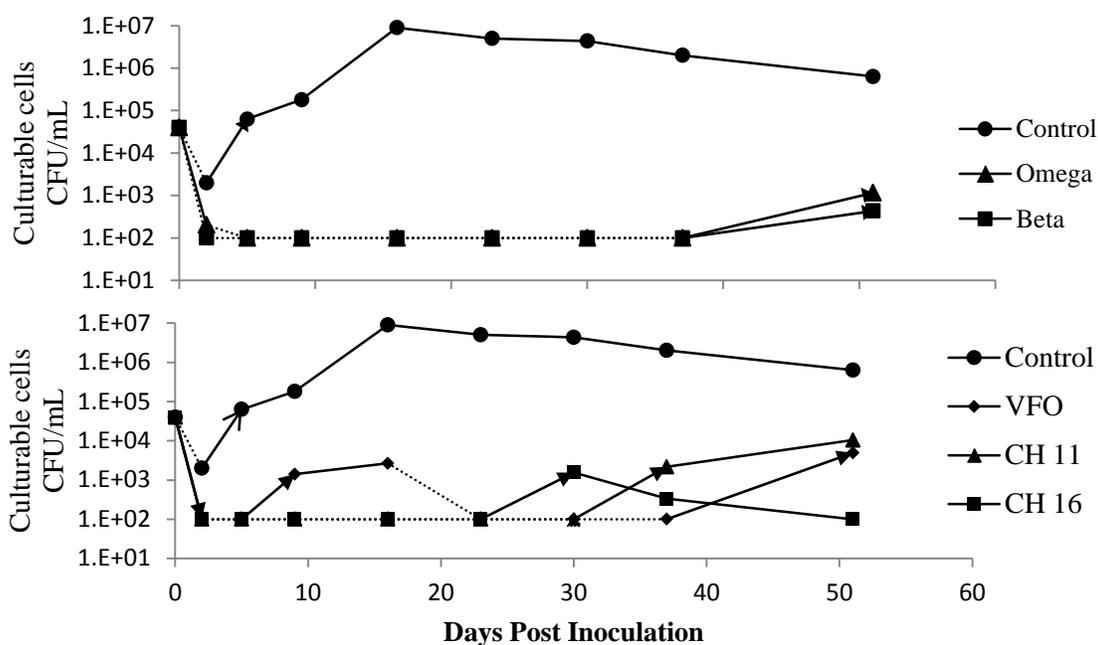
Figure 2.2 Culturable *B. bruxellensis* cells in Pinot noir wine that did not undergo MLF (control) and wine that underwent MLF with *O. oeni* strains Omega, Beta, VFO, CH 11. Data points represent mean of replicates, n=3.
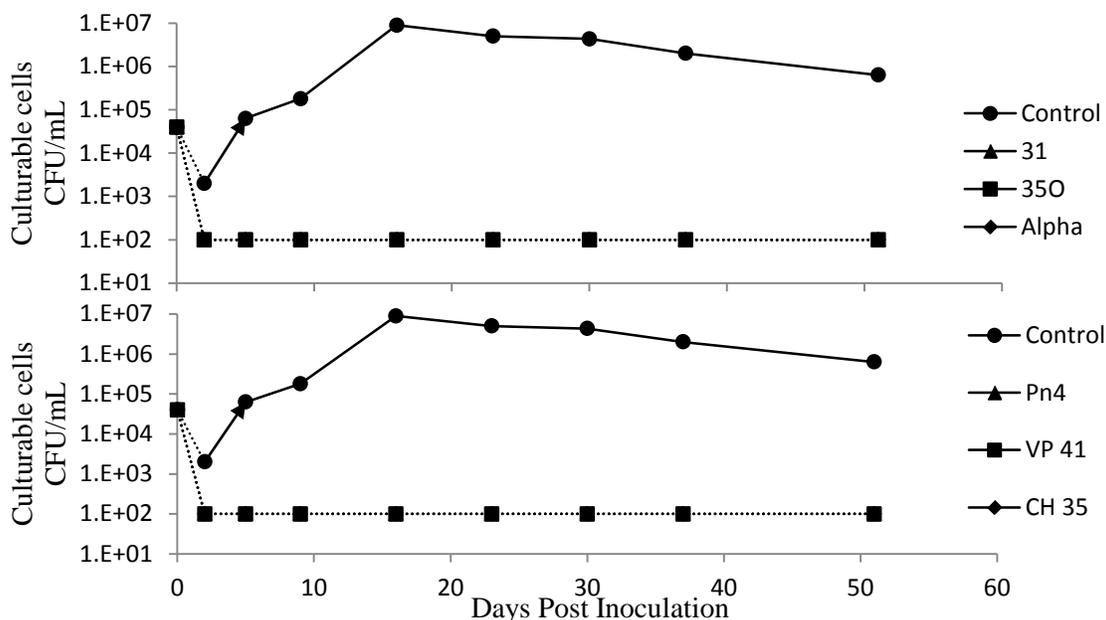
Figure 2.3 Culturable *B. bruxellensis* cells in Pinot noir wine that did not undergo MLF (control) and wine that underwent MLF with *O. oeni* strains 31, 350, Alpha, PN4, VP41, CH 35. Data points represent mean of replicates, n=3.

B. bruxellensis populations in all wines that had undergone a 14 day MLF experienced a greater initial population decline than the control by day 2 (Figure 2.1, Figure 2.2). All of these treatments declined to below the detection threshold by day 2, except for the *O. oeni* strain Omega treatment, which had declined to below detection threshold by day 5 (Figure 2.1). *B. bruxellensis* populations did not recover until after 51 days for over half of the *O. oeni* strains tested (Figure 2.1, Figure 2.2). *B. bruxellensis* populations did recover slightly by day 51 in in the *O. oeni* Omega and Beta treatments (Figure 2.1), reaching 1.2×10^3, and 4.3×10^2 CFU/mL respectively. *B. bruxellensis* populations in the *O. oeni* strain CH 11 treatment recovered slightly earlier than the Omega and Beta treatments, reaching 2.2×10^3 CFU/mL, and 1.0×10^4 CFU/mL by days 37 and 51 respectively. Unlike all the other treatments, *B. bruxellensis* populations in the *O. oeni* strain VFO treatment experienced sporadic recovery. *B. bruxellensis* populations in this treatment recovered earlier than any of the other treatments, reaching 1.4×10^3 CFU/mL by day 9 and 2.7×10^3 CFU/mL by day 16, however by day 23, *B. bruxellensis* populations had declined below detection threshold again and did not recover until day 51 (Figure 2.1).

Wine samples were taken 51 days after *B. bruxellensis* inoculation from all treatments and assessed for 4-ethyl phenol (4-EP) and 4-ethyl guaiacol (4-EG) by GC-MS. Wines that had not undergone MLF had significantly higher concentrations of 4-EP and 4-EG than wines that went through MLF regardless of what *O. oeni* strain was used (Table 2.2). Though the averages varied somewhat, 4-EP and 4-EG concentrations in the MLF treated wines were not significantly different between *O. oeni* strains. The control wine had a

significantly higher concentration of 4-EP than all of the MLF treated samples with the

average concentration being 934.1±162 µg/L. For many of the MLF treatments the 4-EP

concentrations was below detectible thresholds (<10 µg/L) (Table 2.2). The 4-EP

concentration averages for the strain 31, Beta, Omega, CH11, CH16, and VFO2.0

treatments ranged from 37.0±11.6 µg/L, to 262.6±432 µg/L but no significant differences

were noted. A similar trend was seen for 4-EG where the control sample had a

significantly higher concentration of 4-EG than all of the MLF treated wines (Table 2.2).

4-EG concentrations for MLF treated wines ranged from 8.7±0.2 µg/L to 59.4±55 µg/L

(Table 2.2).

Table 2.2 Concentration (µg/L) of 4-ethyl phenol and 4-ethyl guaiacol 51 days after
inoculation of *B. bruxellensis* UCD-2049 into 2018 Pinot noir wine that had or had not
previously undergone MLF with various *O. oeni* strains.

O. oeni Strain	4-Ethyl Phenol	4-Ethyl Guaiacol
Control - No MLF	934.1±162[a]	322.4±4.8[a]
31	14.6±3.8[b]	8.9±0.4[b]
Beta	130.8±195[b]	35.7±45[b]
Omega	159.4±194[b]	59.4±55[b]
PN4	<10[b]	8.9±0.01[b]
CH11	111.0±192[b]	44.2±59[b]
CH16	262.6±432[b]	44.4±56[b]
CH35	<10[b]	11.1±1.8[b]
VFO2.0	37.0±11.6[b]	19.3±4.2[b]
VP41	<10[b]	10.9±2.1[b]
350	<10[b]	9.6±0.08[b]
Alpha	<10[b]	8.7±0.2[b]
Effect significance		
Malolactic Fermentation	<0.0001	<0.0001

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

To investigate the mechanism of inhibition resulting from MLF, *B. bruxellensis* strain

UCD-2049 was inoculated into wine that had or had not undergone MLF using *O. oeni*

strain Alpha, a strain determined to be strongly inhibitory in the previous screening experiment. Using a dialysis membrane the two microorganisms were able to be physically separated to help determine whether physical contact was required for inhibition to occur. In all treatments, *B. bruxellensis* populations rapidly decreased after inoculation (Figure 2.4). The control sample that was not inoculated with *O. oeni* fell below detection threshold by day 10, and entered exponential growth by day 18, reaching a population cap of approximately 8×10^5 by day 56 (Figure 2.4). Populations remained stationary until day 63, before undergoing an approximately tenfold decline by day 70 (Figure 2.4). In the cell separation treatment, wherein *B. bruxellensis* and *O. oeni* cells were separated by a 10 KDa membrane, *B. bruxellensis* populations did not fall below detection threshold. The populations in these treatments fell to approximately 5×10^2 CFU/mL before entering exponential growth and reaching a population maximum of approximately 1×10^6 by day 28, which was 28 days earlier than the control. These populations remained stationary until day 33, before entering a slow population decline, reaching approximately 3×10^4 CFU/mL by day 70 (Figure 2.4). In the cell separation treatment, wherein *B. bruxellensis* and *O. oeni* cells were not separated by a membrane, *B. bruxellensis* populations declined after inoculation to approximately 5×10^2 CFU/mL by day 4. From this point, the populations continued to decline and fell below detection threshold by day 28. *B. bruxellensis* populations did not rise above detection threshold until Day 56 reaching 1.6×10^6 CFU/mL by day 70 (Figure 2.4).
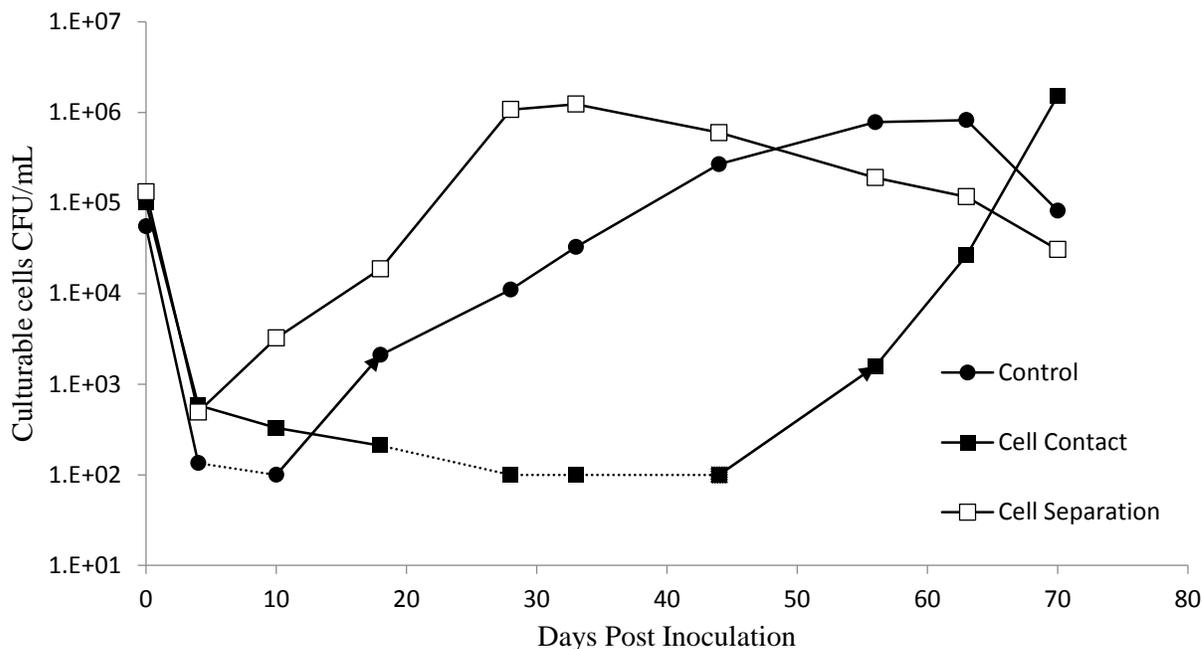
Figure 2.4 Culturable cells of *B. bruxellensis* UCD-2049 in Pinot noir wine that did not undergo MLF (control) and wine that underwent a MLF with *O. oeni* Alpha. *B. bruxellensis* and *O. oeni* cells were separated by a 10 kDa membrane (cell separation) or were not separated (cell contact). Data represent mean of replicates, n=3.

To further investigate the nature of the inhibition resulting from MLF, *B. bruxellensis* strain UCD-2049 was inoculated into wine at different time points during MLF being conducted by *O. oeni* strain Alpha. *B. bruxellensis* populations in treatments that were co-inoculated with *O. oeni* behaved similarly to their controls, declining rapidly to below detection threshold by day 7, and not returning again by the end of the experiment on day 42 (Figure 2.5). *B. bruxellensis* populations in treatments that were inoculated after a partial MLF declined slightly faster than their control, with both control and treatment populations falling below detection threshold by day 11. The control recovered slightly by day 21, reaching a population of 2.3×10^2 CFU/mL, and continued to recover sluggishly before reaching a population of approximately 7×10^2 CFU/mL by day 39. *B. bruxellensis* populations in the partial MLF treatment wine did not recover until day 39,

reaching a population of approximately $3x10^2$ CFU/mL (Figure 2.5). *B. bruxellensis*

populations in treatments that were inoculated after a 14 day MLF responded differently

than their controls. *B. bruxellensis* in the control wine for this treatment had reached a

population of approximately 9×10^4 CFU/mL by day 21, and had multiplied to

approximately 4×10^6 CFU/mL by day 35. *B. bruxellensis* populations in the MLF

treated wine declined to below detection thresholds by day 21, and had not recovered by

day 35 (Figure 2.5).
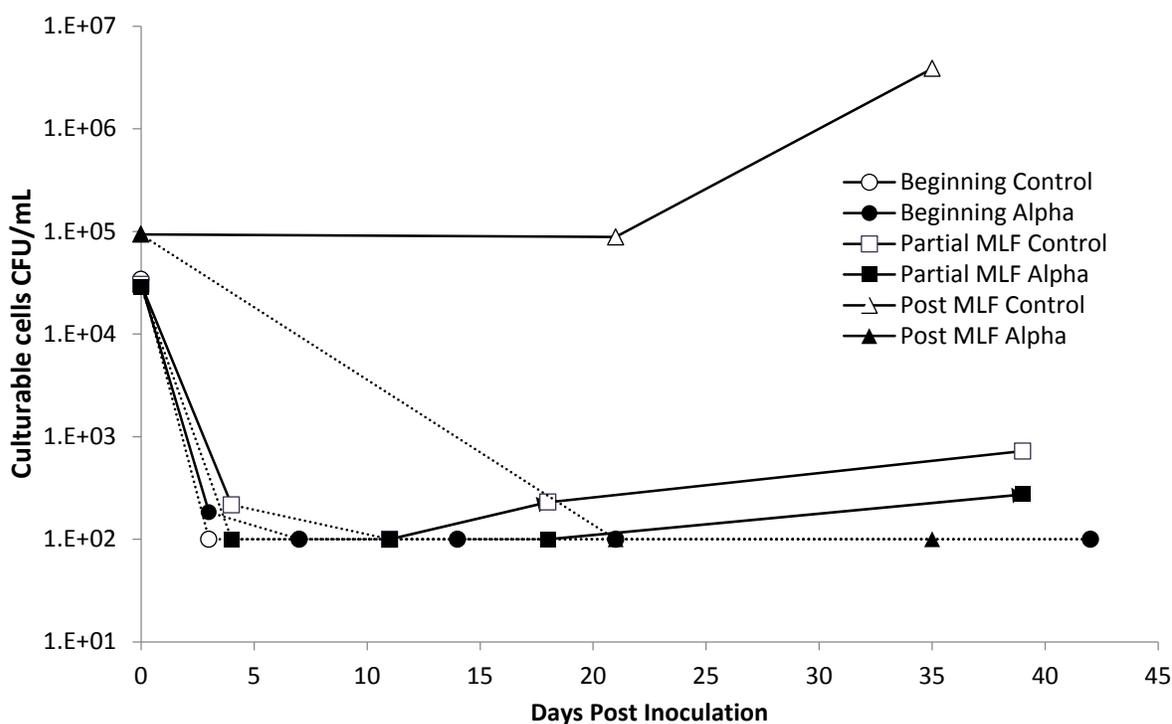
Figure 2.5 Culturable *B. bruxellensis* strain UCD-2049 cells in Pinot noir wine that did not undergo MLF (control) and wine that underwent MLF with *O. oeni* strain Alpha. *B. bruxellensis cells* were either co-inoculated (beginning), inoculated after partial MLF (partial), or inoculated at the end of MLF (post). Data points represent mean of replicates, n=3.

Discussion

B. bruxellensis is a prodigious spoiler of wine. Though methods for the control of *B. bruxellensis* exist, no method is comprehensive and the species is highly adapted to survival in a wine environment. Wine is particularly susceptible to *Brettanomyces* spoilage during and shortly after MLF wine since sulfur dioxide (SO_2), the main tool used to prevent *Brettanomyces* growth, cannot be added until MLF is complete. Because of this, it has been recommended to conduct a rapid MLF with inoculated cultures so that the time that the wine is without SO_2 protection is minimized (Gerbaux et al. 2009). An additional reason why performing a rapid MLF may aid in preventing *Brettanomyces* spoilage was investigated in the present study. Namely, whether MLF conducted by *O. oeni* could inhibit the growth of *B. bruxellensis*.

The inhibition of *B. bruxellensis* growth by MLF had been suggested previously by Gerbaux et al. (2009) who noted that *B. bruxellensis* produced less 4-EP and 4-EG when inoculated into wines that had undergone a MLF using commercial *O. oeni* strains. However, *B. bruxellensis* populations were not enumerated in the Gerbaux study, leaving open the question as to the effect of MLF on *B. bruxellensis* growth. In the present study, *B. bruxellensis* strain UCD-2049 growth was suppressed when inoculated into wine that recently completed MLF by a commercial *O. oeni* strain. Though all *O. oeni* strains tested were associated with a decrease in culturable *B. bruxellensis* cells, there did exist some variation. One strain (Omega) was associated both with a slower initial drop in *B. bruxellensis* populations, and with a partial recovery of *B. bruxellensis* by the end of the experiment. Partial recoveries of *B. bruxellensis* were also observed in the Beta, VFO 2.0 and CH 11 treatments. The concentrations of volatile phenols in the wines paralleled

what was seen with *B. bruxellensis* culturable cells where the control (no-MLF) had significantly higher 4-EP and 4-EG than the MLF treated wines. Indeed over half of the MLF treated samples were found to have 4-EP concentrations below detectible thresholds (<10 μg/L). These results support the findings of Gerbaux (2009) where it was noted that wines that underwent MLF contained lower concentrations of volatile phenols. The results from this study demonstrate that this reduced concentration of volatile phenol was due to reduced growth of *B. bruxellensis*.

While suppression of *B. bruxellensis* strain UCD-2049 was observed by all commercial *O. oeni* strains tested when *B. bruxellensis* was inoculated at the completion of MLF, would the same suppression be seen if *B. bruxellensis* was inoculated at the start of MLF (co-inoculated with *O. oeni*) of in the middle of MLF? As was seen previously, *B. bruxellensis* growth was suppressed when inoculated into wine at the end of MLF. *B. bruxellensis* growth was also suppressed when co-inoculated with *O. oeni* or part way through of MLF. However, poor growth in the controls for the partial and co-inoculation treatments do not allow for comparison. The reason for the reduced growth in the control wine sis not known and the experiment could not be extended to see if populations recovered due to COVID-19 restrictions. This experiment should be repeated in a future study and allowed to run for an extended period of time in order to understand whether the timing of *B. bruxellensis* timing during MLF impacts the suppression of *B. bruxellensis* growth by MLF.

Given the broad suppression of UCD-2049 observed during the *O. oeni* screening experiment, the question remains as to the possible mechanisms of this effect. To investigate the mechanism, *O. oeni* and *B. bruxellensis* cells were separated using a dialysis membrane which allowed for fluids and solutes smaller than 10 kDa to flow freely across. When *B. bruxellensis* and *O. oeni* were separated *B. bruxellensis* grew well and in a similar manner to the control. However, when *B. bruxellensis* and *O. oeni* were not separated by the dialysis membrane, suppressed growth of *B. bruxellensis* was observed. The following explanations for these results were considered:

1. Sequestration of nutrients by *O. oeni*

2. Inhibitory effects on wine chemistry changes by *O. oeni*

3. Production of inhibitory compound by *O. oeni*

4. Effects of quorum sensing

5. Cell-Cell contact

Sequestration of nutrients by *O. oeni*

O. oeni cells require nutrients from a wine to survive. Such nutrients include a carbon source, a nitrogen source, and vitamins such as B-group and pantothenic acid vitamins, as well as trace elements such as magnesium, potassium and manganese (Fugelsang, 1997). It is conceivable that during growth, *O. oeni* cells may sequester some essential nutrients needed by *B. bruxellensis*, thereby suppressing its growth. However, this solution is unlikely, as it would be difficult for *O. oeni* to sequester enough nutrients during MLF to suppress the growth of *B. bruxellensis* given that this species is known to grow in very low nutrient environments (Fugelsang and Edwards, 2006). Furthermore, findings from

the dialysis cassette experiment indicate that nutrient-based suppression is unlikely. The

dialysis cassette experiment allowed for exchange of solutes between the membrane-

separated environments. If the observed suppression was caused by a depletion of

nutrients, we would expect the separation and contact treatments to have behaved the

same. It was observed however, that suppression occurred only when *B. bruxellensis* cells

were in contact with *O. oeni*.

Inhibitory effects on wine chemistry by *O. oeni*

Certain organisms can affect wine chemistry such that it becomes less hospitable to

spoilage organisms like *B. bruxellensis*. MLF is known to alter wine chemistry in ways

that may harm or benefit *B. bruxellensis* growth. One method by which *O. oeni* may

affect wine chemistry to become less hospitable to *B. bruxellensis* is through the

enzymatic liberation of SO_2 bound as carbonyl sulfonates (Wells and Osborne, 2011).

While liberation of SO_2 would certainly affect the wine chemistry, it is unlikely that this

was the cause of the suppression observed in this study. The wine used in this study was

not treated with SO_2 during production, and had low total SO_2 before initiation of MLF,

so a large change in the amount of free SO_2 in this wine due to the enzymatic action of *O.*

oeni is not expected. As with the question of nutrient sequestration above, the findings of

the dialysis cassette experiment also indicate that liberation of SO_2 is an unlikely cause

for the observed suppression of *B. bruxellensis*, as liberated SO_2 would have crossed the

membrane and suppressed the growth of the separated cells. An additional change in

wine chemistry that MLF is known to cause may actually benefit *B. bruxellensis* growth.

MLF results in a raise in pH due to the conversion of malic acid to lactic acid (Costantini

et al., 2009; Liu, 2002). Higher pH will favor *B. bruxellensis* growth and may explain

why *B. bruxellensis* populations in the treatment that was membrane-separated from *O.*

oeni grew faster than *B. bruxellensis* cells in the wine that had not undergone MLF.

Production of inhibitory compound by *O. oeni*

Some microbial species can produce targeted anti-microbial proteins that act against

other microbes in the environment. Such proteins are often called 'killer factors' when

referring to compounds of eukaryotic origin. While killer factors generally affect

organisms of the same species, forms exist that are known to be active against bacteria as

well (Magliani et al., 1997). Some non-*Saccharomyces* yeasts are known to produce

killer proteins that have shown activity against *B. bruxellensis*. *Pichia anomala*, and

Kluyveromycer wickerhamii, have both been found to produce the killer compounds

PMKT2 and Kwkt respectively that can limit *B. bruxellensis* growth (Santos et al. 2009).

Candida pyralidae was also found to produce the killer factors CpKT1, and CpKt2 which

are active against *B. bruxellensis* (Mehlomakulu et al., 2014). As stated above, killer

factors are produced by eukaryotic species, and are generally active against closely

related species, so it is unlikely that a lactic acid bacterium such as *O. oeni* would

produce such a compound.

Bacterial species are known to produce their own anti-microbial compounds. These are

called bacteriocins, and like killer factors are generally active against closely related

species (Yang et al. 2014). Lactic acid bacteria produced bacteriocins such as nisin (3-5

kDa), are commonly used in food preservation schemes to protect from bacterial spoilage

(Ghrairi et al., 2012). *O. oeni* is itself susceptible to suppression by the *Pediococcus* produced pediocin PD-1 (Bauer et al., 2003). A 2008 study (Knoll et al., 2008) found that a number of *O. oeni* strains did produce bacteria-inhibiting compounds. A further study in 2018 described a number of bacteria-inhibiting compounds also produced by *O. oeni* (Lasik-Kurdyś and Sip, 2019). However, these studies did not test *O. oeni* produced anti-bacterial compounds against eukaryotic organisms, such as *B. bruxellensis*. If *B. bruxellensis* was inhibited by a bacteriocin produced by *O. oeni* it would have to have been larger than 10kDa as *B. bruxellensis* that were separated from *O. oeni* by the 10 kDa dialysis membrane were not suppressed during the experiment. The gram positive lactic acid bacteria *Lactobacillus casei, Enterococcus faecalis*, and *Lactobacillus helveticus* can produce class III bacteriocins, which are larger than 10 kDa in size (Yang et al. 2014). However, the majority of gram positive bacteriocins described in the literature are smaller than 10 kDa. In addition, if a bacteriocin larger than 10kDa was produced by *O. oeni*, it would have to be active against the eukaryote *B. bruxellensis,* something that has not been reported in literature to date. Another possibility is that the production of an inhibitory compound only occurred in response to cell-cell contact between *O. oeni* and *B. bruxellensis*. Again, this type of response has not been reported in literature. An additional experiment that could explore this possibility would be to repeat the previously described dialysis cassette experiment with *B. bruxellensis* cells on both sides of the membrane. If cell-cell contact with *B. bruxellensis* induces the production of a killer factor that could cross the membrane, then we would expect to see suppression of *B. bruxellensis* on both sides of the membrane.

Quorum Sensing

Many bacterial species regulate themselves via a cell-cell system of communication called quorum sensing. This system is regulated through gene expression and involves the release of small soluble, cell density-dependent signal molecules such as homo-serine lactones and peptide pheromones (Rutherford and Bassler, 2012). Quorum sensing-like regulation has been described in yeasts, using signal molecules such as ammonia, acetaldehyde, bicarbonate, and farnesol (Ohkuni et al., 1998; Palková et al., 1997; Sprague and Winans, 2006; Hornby et al., 2001). Could the observed suppression of *B. bruxellensis* be a result of the yeast reacting to *O. oeni* produced quorum sensing molecules? Again, the results from the dialysis cassette experiment indicate that this is not the case. The quorum sensing compounds described above are small enough to pass through a 10 kDa membrane, and quorum sensing compounds are generally small in comparison to the dialysis membrane size used in the experiment. If quorum sensing was the cause of *B. bruxellensis* growth repression then we should have seen growth repression in the treatment where *B. bruxellensis* and *O. oeni* were separated by the dialysis membrane as well as when they were together.

The results of the dialysis cassette experiment provide strong evidence that the observed inhibition of *B. bruxellensis* when inoculated into wine that has recently undergone MLF is due to cell-cell contact with *O. oeni*. While a cell-cell contact inhibitory mechanism has not been described for *B. bruxellensis*, it has been described in the literature for other organisms. Cell-cell contact inhibition is known in mammalian (Nelson and Chen, 2002), yeast (Honigberg, 2011; Li and Palecek, 2008), and bacteria cells (Donlan, 2002). In a

study by Nissen et al. (2003) inhibition of *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii* by *Saccharomyces cerevisiae* was determined to be caused by cell-cell contact by live *S. cerevisiae* cells. Renault et al. (2013) also noted significant inhibition of *T. delbrueckii* due to cell contact with live *S. cerevisiae* cells. A cell-cell contact mechanism also supports previous experiments from the Osborne enology lab that observed reduced suppression of *B. bruxellensis* growth in wine that had undergone MLF if *O. oeni* was removed by sterile filtration (0.45 µm) prior to *B. bruxellensis* being inoculated (Chescheir 2015).

Additional work is needed to fully determine the exact mechanism of inhibition. This should include assessments of using non-culture based methods to detect potential viable but non-culturable populations of *B. bruxellensis* that may be present in these wines as the yeast has been reported to enter this metabolic state (Willenburg et al., 2012; Serpaggi et al., 2012; Zuehlke et al., 2013). Furthermore, additional *B. bruxellensis* strains should be assessed to determine if the growth repression of strain UCD-2049 by *O. oeni* noted in this study is strain specific or not.

Conclusions

B. bruxellensis UCD-2049 growth and volatile phenol production was inhibited when inoculated into wine that had recently undergone MLF by *O. oeni* with all *O. oeni* strains having a similar effect on *B. bruxellensis*. Both the microbial growth and volatile phenol production of *B. bruxellensis* was suppressed in wine that had recently undergone MLF. If *B. bruxellensis* was physically separated from *O. oeni* then repression of growth was

not observed. While this evidence suggests that the mechanism of growth suppression is related to cell-cell contact between *O. oeni* cells and *B. bruxellensis* cells, further research is needed to confirm this. The results from this study indicate that conducting a robust MLF may provide additional protection against *B. bruxellensis* spoilage aside from the ability to stabilize with SO_2 quickly. Additional research should focus on whether the inhibition of *B. bruxellensis* UCD-2049 by *O. oeni* observed in this study will also occur if different *B. bruxellensis* strains are used as well as the extent and longevity of this inhibition.

Chapter 3

Impact of *Oenococcus oeni* strain Alpha on growth and volatile phenol
production of various *Brettanomyces bruxellensis* strains under different
ethanol conditions.

Abstract

The effect of malolactic fermentation (MLF) by *Oenococcus oeni* strain Alpha on the growth and volatile phenol production of a number of *B. bruxellensis* strains sourced from different geographical locations under differing ethanol concentrations was investigated. Initially eight different strains were inoculated into 13% (v/v) ethanol Pinot noir wine that had or had not recently undergone MLF with *O. oeni* strain Alpha. While the populations of many *B. bruxellensis* strains initially declined after inoculation, *B. bruxellensis* populations in both the control and MLF treated wines recovered to a similar level by the end of the experiment. No significant differences in volatile phenol concentrations were noted between the treatments. The impact of ethanol concentration on *B. bruxellensis* inhibition by *O. oeni* was investigated by inoculating strains of *B. bruxellensis* into wines of either high or low ethanol content that had or had not just completed MLF. While *B. bruxellensis* populations for all strains behaved similarly to the control groups in the low ethanol wines, in the high ethanol wines two of the three strains of *B. bruxellensis* tested were inhibited in the MLF treated wines with respect to the control. For *B. bruxellensis* strain Copper Mountain the reverse was true. *B. bruxellensis* strain and ethanol concentration was a significant major for volatile phenol production,

though some reduction in volatile phenol production was observed in wines that had

undergone MLF despite no observable suppression of *B. bruxellensis* growth.

Introduction

Among the most prolific spoilers of wine is *Brettanomyces bruxellensis*. This yeast can

cause significant financial losses due to its ability to produce undesirable flavor and

aroma compounds in wines (Crauwels et al., 2014; Fugelsang et al., 1993; Heresztyn,

1986; Sponholz 1993). The compounds most indicative of *Brettanomyces* wine spoilage

are the volatile phenols 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG), and 4-

ethylcatechol (4-EC) (Oelofse et al., 2008; Hesford et al., 2004). These volatile phenols,

are associated with 'Band-Aid', mousy, barnyard, fecal, medicinal, smoke, and clove

descriptors, and are also known to suppress the perception of positive aroma qualities in a

wine, especially fruity and floral characteristics (Chatonnet et al., 1995; Gerbaux and

Vincent, 2002; Licker et al., 1998).

While wine is at risk for infection by *B. bruxellensis* throughout its' production, it is

particularly vulnerable post-alcoholic fermentation and during aging in barrels. There are

a number of steps a winemaker can take to reduce the risk of wine infection and spoilage

by *B. bruxellensis* and/or manage *Brettanomyces* in already infected wine. Regular

cleaning of winery surfaces and equipment removes organic material and helps reduce

microbial buildup (Du Toit et al., 2005). Winemakers can also use sulfur dioxide (SO_2) as

an antimicrobial agent against infection (Agnolucci et al., 2010; von Cosmos et al., 2016;

Zuehlke and Edwards, 2013). However, consumers are often wary of the usage of

chemical additives such as SO_2 in wine, so winemakers often seek to reduce their usage

(Du Toit and Pretorius, 2000). In addition, *B. bruxellensis* exhibits a highly varying

resistance to SO_2 and other antimicrobial agents due to a great genetic variety between

strains (Curtin et al., 2007; Agnolucci et al., 2009; Hellborg & Piskur, 2009; Vigentini et

al., 2012).

In addition to the use of antimicrobial chemicals, a winemaker has at their disposal the

inhibitory characteristics of the wine to aid in the prevention of growth of spoilage

microorganisms. For example, low pH, high ethanol, and low temperature are all factors

that will restrict the growth of a spoilage microbe such as *B. bruxellensis*. Such factors

have been described as 'hurdles' (McMeekin et al. 2000). Although *B. bruxellensis* may

be somewhat tolerant to individual hurdles, the combined effect of these inhibitory

factors can affect greater inhibition than their individual magnitudes. These synergistic

relationships can be a viable means to reducing microbial populations in foods without

resorting to novel techniques or excessive use of one particular control method (Leistner,

1992). Well known hurdles in wine that affect *B. bruxellensis* growth include

temperature, ethanol concentration, pH, and SO_2 (Smith, 2011; Sturm et al., 2014;

Zuehlke and Edwards, 2013). While strain variable tolerance exists, synergistic

interactions between these factors can reduce overall *B. bruxellensis* populations in wine

(Edwards et al., 2015; Ramirez et al., 2014). Sturm et al (2014) studied the interactions

between pH, ethanol, and free SO_2, and developed a mathematical model that is

predictive of growth over a period of time. Edwards and Oswald (2017) found that even

without added SO_2, interactive effects between temperature and ethanol concentration in

wine are capable of extending lag time and reducing volatile acidity and ethylphenol concentrations in certain strains of *B. bruxellensis*.

A potential additional hurdle that may impact *B. bruxellensis* growth in wine is antagonistic interactions with other wine microorganisms. A well-known example of such an interaction would be the inhibitory effect *S. cerevisiae* can have on *B. bruxellensis* growth when co-inoculated in grape juice (Renouf et al., 2006). Recent studies have also noted that growth and metabolism of certain *O. oeni* strains can affect spoilage of wine by *B. bruxellensis* (Chescheir et al. 2015, Burns and Osborne, 2013). Chescheir et al. (2015) reported that cinnamoyl esterase activity of *O. oeni* resulted in higher concentrations of ρ-coumaric acid in wine post-MLF and that subsequent *B. bruxellensis* growth resulted in significantly higher volatile phenols in these wines. In contrast to these reports of increased volatile phenol production due to MLF, Renouf & Murat (2008) and Gerbaux et al. (2009) noted that MLF may result in reduced volatile phenols in wine. Furthermore, Chescheir (2014) observed that *B. bruxellensis* populations declined when inoculated into wine that had recently undergone MLF while grew well in wine that had not undergone MLF. Finally, the investigations laid out in Chapter 2 demonstrated that growth of *B. bruxellensis* strain UCD-2049 was suppressed in wines that had undergone MLF using any of the eleven strains of *O. oeni* tested, with the effect being strongly observed in a few specific strains. However, only one *B. bruxellensis* strain was used and the impact of different wine conditions was not considered.

This study follows up on the findings from Chapter 2 by investigating whether other *B. bruxellensis* strains are also susceptible to inhibition by *O. oeni*. In addition, experiments were undertaken in wine adjusted to different ethanol concentrations to determine if ethanol tolerance differences between *B. bruxellensis* strains impacts their inhibition by *O. oeni*. The overall goal was to determine if MLF could be considered a hurdle that could prevent *B. bruxellensis* growth in wine when combined with pressure from increased ethanol concentrations, and whether variability exists within *B. bruxellensis* strains. Specifically we investigated the ability of *Oenococcus oeni* strain Alpha to inhibit growth and volatile phenol production of a number of *B. bruxellensis* strains, and to determine if ethanol concentration impacted how *Brettanomyces bruxellensis* may be inhibited by *O. oeni*.

Materials and Methods

Microorganisms

Brettanomyces bruxellensis isolates were collected from a number of culture collections as noted in Table 3.1. *Oenococcus oeni* Alpha was sourced from Lallamend (Montreal, Canada). *B. 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). *O. oeni* Alpha 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).

When needed, microorganisms were prepared from frozen cultures by streaking onto YPD agar (*B.bruxellensis*) or MRS agar (*O. oeni*) and grown at 25°C for one week. Single colonies were picked from the agar plates for inoculation into acidic grape juice broth (AGJ) (2.5 mg/L manganese sulfate, 125 mg/L magnesium sulfate, 5g/L yeast extract, 1 mL/L 5% (w/w) Tween 80, 250 mL/L white grape juice, pH 3.5) and grown at 25°C for eight (*B. bruxellensis*) or twelve (*O. oeni*) days. Cells were harvested by centrifugation (4,000 x g for 20 minutes) and re-suspended in sterile peptone solution (0.1%) prior to inoculation. *S. cerevisiae* (RC212) for winemaking was used as freeze-dried culture direct from the manufacturer.

Table 3.1 Strains and Sources of Microorganisms used in Screening

Strain Name	Microorganism	Source
Alpha	*Oenococcus oeni*	Lallemand (Montreal, Canada)
UCD-2049	*Brettanomyces bruxellensis*	University California Davis Culture Collection (Davis, CA, USA)
UCD-73B	*Brettanomyces bruxellensis*	University California Davis Culture Collection
AWRI-1499	*Brettanomyces bruxellensis*	Australian Wine Research Institute (Adelaide, Australia)
I1A	*Brettanomyces bruxellensis*	Washington State University Culture Collection (Pullman, WA, USA)
F3	*Brettanomyces bruxellensis*	Washington State University Culture Collection
Y16	*Brettanomyces bruxellensis*	Christopher Curtin Collection – Oregon State University (Corvallis OR, USA)
Y18	*Brettanomyces bruxellensis*	Christopher Curtin Collection – Oregon State University
Copper Mt.	*Brettanomyces bruxellensis*	Alan Bakalinsky Culture Collection- Oregon State University (Corvallis, OR, USA)

Wine production

Pinot Noir wine was produced at the Oregon State University Research Winery from

grapes harvested from Woodhall Vineyard (Alpine, Oregon, USA) in 2018 and 2019

following the same basic protocol. 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 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. Yeast nutrient Fermaid K® (Lallemand, Montreal, Canada) was added at a concentration of 0.125 g/L. Each tank was inoculated with the commercial yeast *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 0.5g/L.

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, 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. Basic wine parameters for the 2018 Pinot noir were 15% (v/v) ethanol, pH 3.61, 1.41 g/L malic acid and 11.2 mg/L total SO_2. Basic wine parameters for the 2019 Pinot Noir were 13.7% (v/v) ethanol, pH 3.48, 1.27 g/L malic acid and 16.0 mg/L total SO_2.

B. bruxellensis Screening

Pinot noir wine produced in 2018 as previously described was used for this experiment.
The wine was diluted with deionized water to reduce the final ethanol concentration to
13% by volume and spiked with 5 mg/L ρ-coumaric acid. The wine was then filtered
through Nalgene™ single use bottle top sterile filters using a 0.2 μm polyethersulfone
membrane as 100 mL aliquots into sterilized 100 mL Schott bottles. 45 of the bottles
were brought to room temperature and divided into three groups: 21 to undergo MLF, 21
to not receive MLF and 3 to serve as a sterile control. The 21 MLF wines were inoculated
with *O. oeni* strain Alpha at approximately $1x10^7$ CFU/mL. All bottles were topped with
filtered argon gas and placed at 21°C. Malolactic fermentation was complete in all
treatments after 14 days (<50 mg/L malic acid as measured by enzymatic assay)
(Vintessential Enzymatic L-Malic Acid Test Kit; Victoria, Australia). The 21 bottles that
had undergone MLF and the 21 bottles that had not undergone MLF were then randomly
divided into 7 treatments, and each treatment was inoculated with a different strain of *B.*
bruxellensis in triplicate at approximately $1 x10^3$ CFU/mL. All wines were topped with
filtered argon gas and incubated at 21°C.

B. bruxellensis populations were monitored periodically by plating on YPD agar (10 g/L
yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar, pH 6.50) after suitable
dilution (0.1% peptone). Plates were incubated for 10 days at 25°C before being counted.
After sampling, bottles were topped with filtered argon gas. On the final day of sampling

for each treatment, 50 mL samples were pulled and stored at -20°C until needed for volatile phenol analysis.

O. oeni and ethanol experiment

Experimental design is illustrated in Figure 3.1. A portion of sterile filtered Pinot noir wine produced in 2019 as previously described was adjusted to either 12.5% v/v EtOH or 14.0% v/v EtOH with sterile deionized water or 95% ethanol (Everclear). Ethanol concentrations were checked by ebulliometer. The EtOH adjusted wine was stored in argon topped sterile media bottles at 21°C until needed. Additional 2019 Pinot noir wine was filtered through Nalgene™ single use bottle top filters using a 0.2 μm polyethersulfone membrane and 100 mL aliquots were dispensed into sterilized 100 mL Schott bottles. The wine was brought to room temperature before inoculation in triplicate with *O. oeni* strain Alpha at approximately $1x10^6$ CFU/mL after preparation as previously described. Bottles were topped with filtered argon gas and placed at 21°C. Malolactic fermentation was complete in all treatments after 14 days (<50 mg/L malic acid as measured by enzymatic assay) (Vintessential Enzymatic L-Malic Acid Test Kit; Victoria, Australia).

At the completion of MLF, a small sample was aseptically taken from a number of random bottles, combined, and measured for pH. Previously EtOH adjusted wine that had not undergone MLF was then pH adjusted (1N NaOH) to match the pH of the wine that had undergone MLF. After pH adjustment, the ethanol adjusted wine was filtered through Nalgene™ single use bottle top sterile filters using a 0.2 μm polyethersulfone membrane

as 100 mL aliquots into sterilized 100 mL Schott bottles. Wines that had undergone MLF were randomly separated into two groups and adjusted to either 12.5% v/v EtOH or 14.0% v/v EtOH with sterile deionized water or 95% ethanol (Everclear) with additions based on volumes used previously for ethanol adjustments. Wines were then randomly divided into groups of 12, each group containing 3 each of 12.5% EtOH + MLF, 12.5% EtOH no MLF, 14% EtOH MLF, and 14% EtOH no MLF. All wines were then inoculated with one of three *B. bruxellensis* strains UCD-2049, AWRI-1499, or Copper Mountain at approximately $1X10^4$ CFU/mL prepared as previously described. These represent the 'Day 0' treatment. All wines were topped with filtered argon gas and incubated at 21°C. *B. bruxellensis* populations were monitored periodically by plating on YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar, pH 6.50) after appropriate dilutions (0.1%) peptone. Plates were incubated for 10 days at 25°C before being counted. After sampling, bottles were topped with filtered argon gas. On the final day of sampling for each treatment, 50 mL samples were pulled and stored at -20°C until needed for volatile phenol analysis.
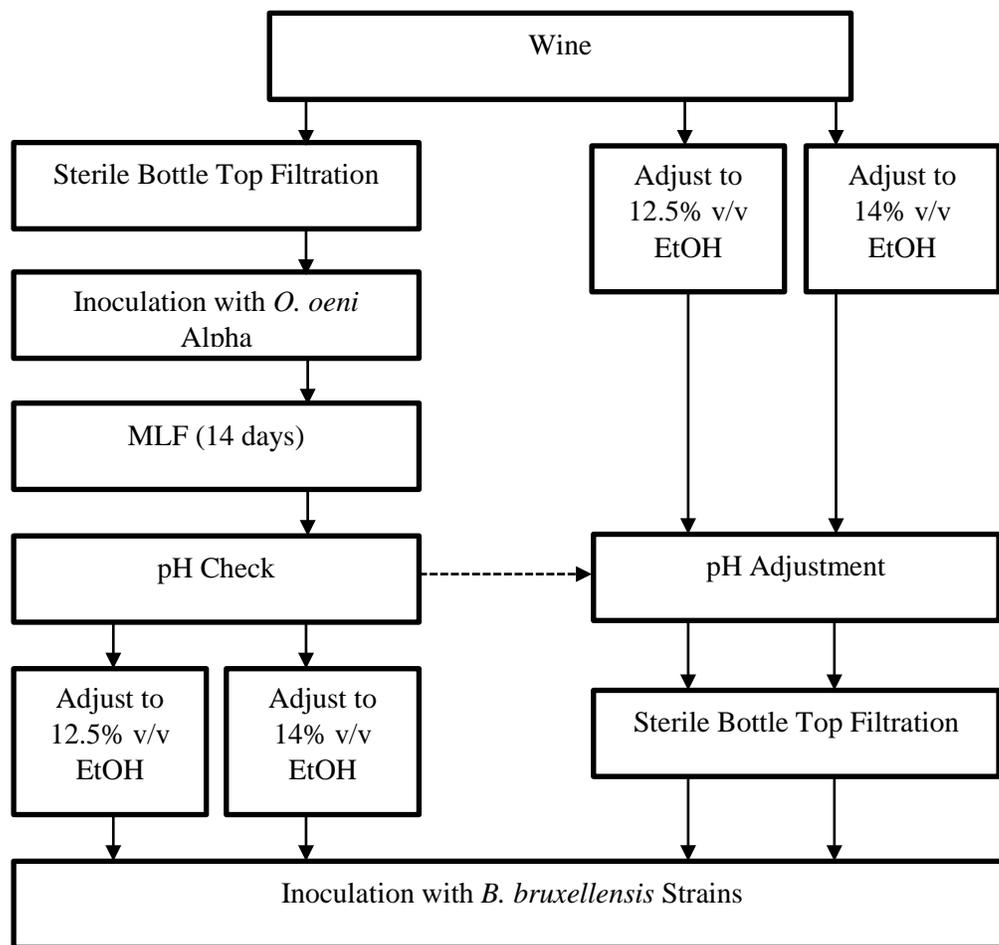
Figure 3.1 Experimental design for impact of *O. oeni* strain Alpha on a variety of *B. bruxellensis* strains in high and low ethanol environments

Wine volatile phenol analysis

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

Statistical analysis

Statistical analysis was conducted using R-studio (Boston, MA, USA) at a significance level (α) of 0.05. The effect of malolactic fermentation by *O. oeni* strain Alpha on

volatile phenol production by various *B. bruxellensis* strains was determined by two-way ANOVA. The effect of ethanol and malolactic fermentation by *O. oeni* strain Alpha on volatile phenol production by various *B. bruxellensis* strains was determined by three-way ANOVA. Significant differences between treatments, where an interactive effect was found, were determined by Tukey's HSD.

Results

The impact of a number of *O. oeni* strain Alpha on seven strains of *B. bruxellensis* was tested by inoculating each *B. bruxellensis* strain into wine that had just completed MLF by *O. oeni* Alpha and comparing growth when inoculated into wine that had not undergone MLF. While many of the *B. bruxellensis* strains tested experienced an initial decline in population by day 7 of testing, all of the strains entered exponential growth by day 21 (Figure 3.2). All *B. bruxellensis* strains tested reached population maximums of approximately $1x10^6$ CFU/mL by day 30, before entering a gradual decline by day 37 (Figure 3.2). While some early variability between MLF treated wines and their controls did exist for a number of *B. bruxellensis* strains, by day 30 no variability between growth in wine that had or had not undergone MLF was apparent (Figure 3.2). Higher *B. bruxellensis* cell counts were observed on days 7 and 21 in MLF treated wines for three of the strains tested, though all differences between treatments diminished by day 30 (Figure 3.2). For the *B. bruxellensis* strain Copper Mountain, approximately $5x10^3$ CFU/mL *B.* bruxellensis culturable cells were detected in the MLF treated wine on day 7, while culturable *B. bruxellensis* populations in the control were below detectible levels.

However, by day 21, the culturable *B. bruxellensis* populations in the control had reached

similar population numbers to those of the MLF treated wine (Figure 3.2).
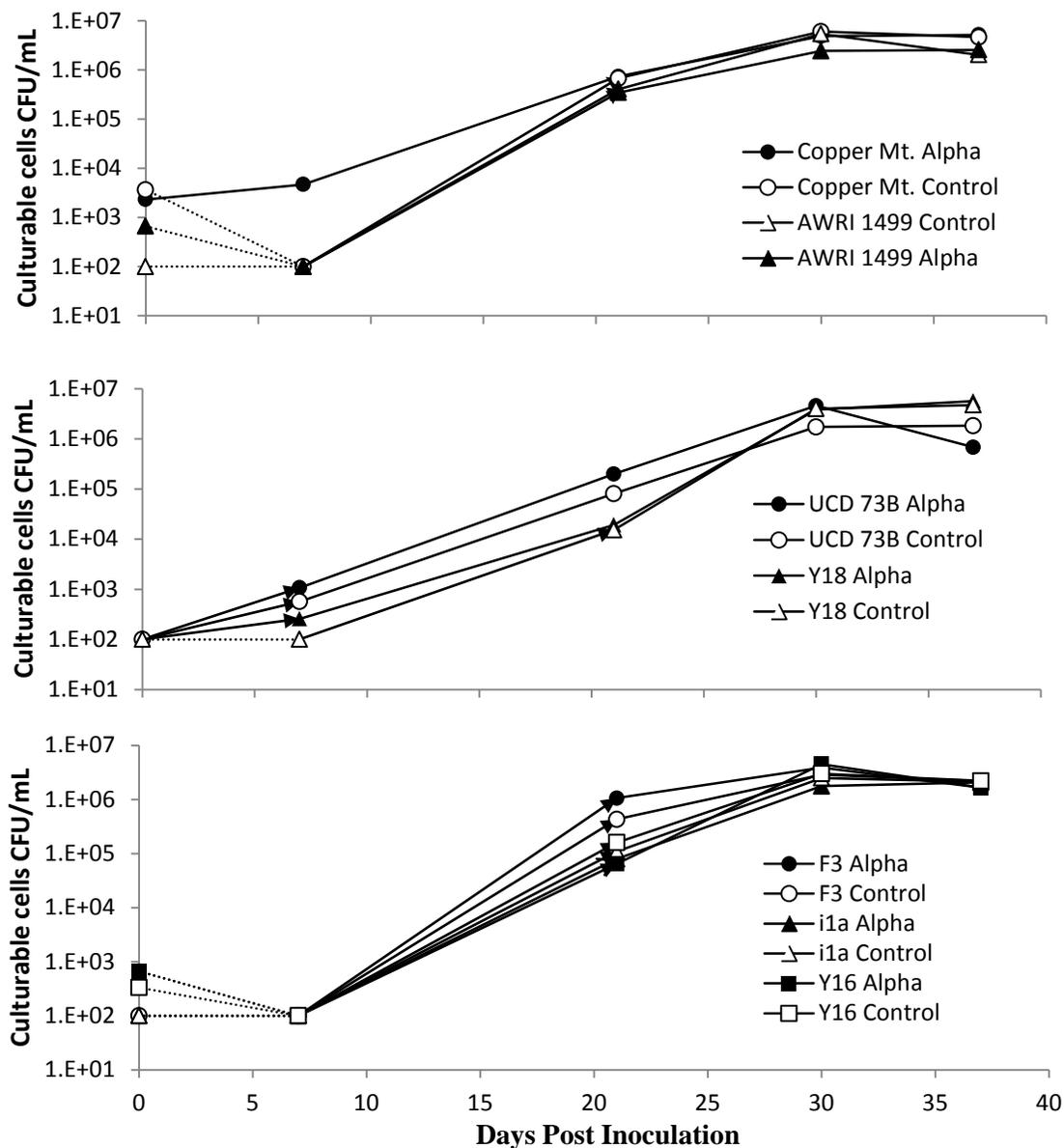
Figure 3.2 Culturable *B. bruxellensis* cells in Pinot noir wine that did not undergo MLF and wine that underwent MLF with *O. oeni* strain Alpha. Data points represent mean of replicates, n=3.

At the end of the 37 day screening, 50 mL samples were taken from all samples and analyzed for 4-Ethyl Phenol (4-EP) and 4-Ethyl Guaiacol (4-EG) concentrations. Within *B. bruxellensis* strains, no significant differences were found in 4-EP or 4-EG concentrations between wines that underwent MLF with *O. oeni* strain Alpha and those that did not (Table 3.2). Significant differences between *B. bruxellensis* strains with respect to production of both 4-EP and 4-EG was noted. For example, significantly higher concentrations of 4-EP were measured in wines inoculated with *B. bruxellensis* strain i1a compared to wines inoculated with AWRI-1499 or Y18 wines regardless of whether the wines had undergone MLF or not (Table 3.2).

Table 3.2 Concentration (µg/L) of 4-ethyl phenol and 4-ethyl guaiacol 37 days after inoculation of various *B. bruxellensis* strains into 2018 Pinot noir wine that had or had not previously undergone MLF with *O. oeni* Alpha.

Treatment		4-Ethyl Phenol	4-Ethyl Guaiacol
Strain	MLF		
AWRI-1499	Yes	366.0±3.4[bc]	191.7±7.8[b]
AWRI-1499	No	355.1±14.5[c]	190.8±18.4[b]
Copper Mt	Yes	426.0±3.3[abc]	250.2±10.1[a]
Copper Mt.	No	391.3±15.8[abc]	221.2±11.3[ab]
i1a + Alpha	Yes	462.4±69.7[a]	212.1±34.4[ab]
i1a	No	463.9±18.4[a]	247.0±9.7[a]
UCD-73B	Yes	435.6±15.6[ab]	230.8±18.7[ab]
UCD-73B	No	415.2±8.2[abc]	220.7±17.3[ab]
Y18	Yes	374.7±33.6[bc]	210.5±10.4[ab]
Y18	No	374.7±23.0[bc]	210.5±3.7[b]
AWRI-1499	Yes	366.0±3.4[bc]	191.7±7.8[b]
AWRI-1499	No	355.1±14.5[c]	190.8±18.4[b]
Effect significance			
B. bruxellensis strain		<0.0001	<0.001
MLF		ns	ns
B. bruxellensis strain X MLF		<0.0001	<0.0001

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

To investigate interactive effects between ethanol (EtOH) concentration and MLF, *B. bruxellensis* strains UCD-2049, Copper Mountain, and AWRI-1499 were inoculated into wines of low and high EtOH concentrations (12.5 and 14.0% EtOH v/v respectively) that had or had not undergone MLF with *O. oeni* strain Alpha. Wines were pH adjusted after MLF to minimize the impact this variable could have on *B. bruxellensis* growth in wines that had or had not undergone MLF. In the low EtOH wines, *B. bruxellensis* strain UCD-2049 experienced an initial decline in populations over the first 7 days with no difference existing between MLF wines and the control (Figure 3.3). By day 11, populations had entered exponential growth and reached population maximum of approximately 1×10^6 CFU/mL by day 25 with minimal differences between MLF treated wines and the controls. After day 25, *B. bruxellensis* populations in the MLF treated wine began to decline, reaching approximately 1×10^5 CFU/mL by day 67, while *B. bruxellensis* populations in the control wine dipped slightly after day 25, but recovered to approximately 1×10^6 CFU/mL by day 67 (Figure 3.3). In the high EtOH treatment, *B. bruxellensis* strain UCD-2049 populations declined by day 4 in both the MLF treated wine and in the control, with populations falling to 1×10^2 CFU/mL in the control and below detection threshold in the MLF treatment. *B. bruxellensis* populations in the control and MLF treated wines were below detection threshold by day 7, with populations in the MLF treated wines not recovering at any time during the 67 days of the experiment (Figure 3.3). *B. bruxellensis* populations in the control wine were detectable on day 25, but declined below detection threshold again by day 32. *B. bruxellensis*

populations in the control wine began to recover near the end of the experiment with 2×10^3 CFU/mL being detected on day 67 (Figure 3.3).

B. bruxellensis strain Copper Mountain populations did not decrease after inoculation in the low EtOH control wines and reached a population maximum of approximately 6×10^6 CFU/mL by day 16 and remained stable until the end of the experiment (Figure 3.3). *B. bruxellensis* Copper Mountain populations in the low EtOH MLF wine decreased to approximately 1×10^3 CFU/mL by day 4, but entered exponential growth afterwards, reaching a similar population maximum to the control by day 16 (Figure 3.3). In high EtOH wine, *B. bruxellensis* Copper Mountain populations in the control decreased slightly by day 4 and then declined to approximately 2×10^2 CFU/mL by day 9. *B. bruxellensis* Copper Mountain populations did not enter exponential growth, only recovering to approximately 5×10^3 CFU/mL by day 67. In high EtOH wines that underwent MLF, *B. bruxellensis* populations decreased to approximately 3×10^2 CFU/mL by day 4, and to below detection threshold by day 9. Populations recovered to a maximum of approximately 3×10^6 CFU/mL by day 49, remaining stable until the end of the experiment (Figure 3.3).

Brettanomyces bruxellensis strain AWRI-1499 populations in low EtOH wine for both the control and MLF treatments behaved similarly. For both treatments, *B. bruxellensis* AWRI-1499 entered exponential growth by day 9, reaching a population maximum by day 48 (Figure 3.3). Differences between *B. bruxellensis* populations between the control and MLF wines was however observed in the high EtOH wine (Figure 3.3). *B.*

bruxellensis populations in the control wines declined to approximately 4 x 10^3 CFU/mL by day 4 but had entered exponential growth by day 9, reaching a population maximum of 5 x 10^6 CFU/mL by day 49. In high EtOH wine that had undergone MLF with *O. oeni* Alpha, *B. bruxellensis* populations declined to approximately 4 x 10^2 CFU/mL by day 9, and recovered slower than the populations in the control. For example, 16 days after inoculation *B. bruxellensis* populations in the control had reached approx. 2 x 10^5 CFU/mL while in wine that had undergone MLF the population was only approx. 1 x 10^3 CFU/mL (Figure 3.3). *B. bruxellensis* populations in wine that underwent MLF did not reach similar populations to the control until day 49.
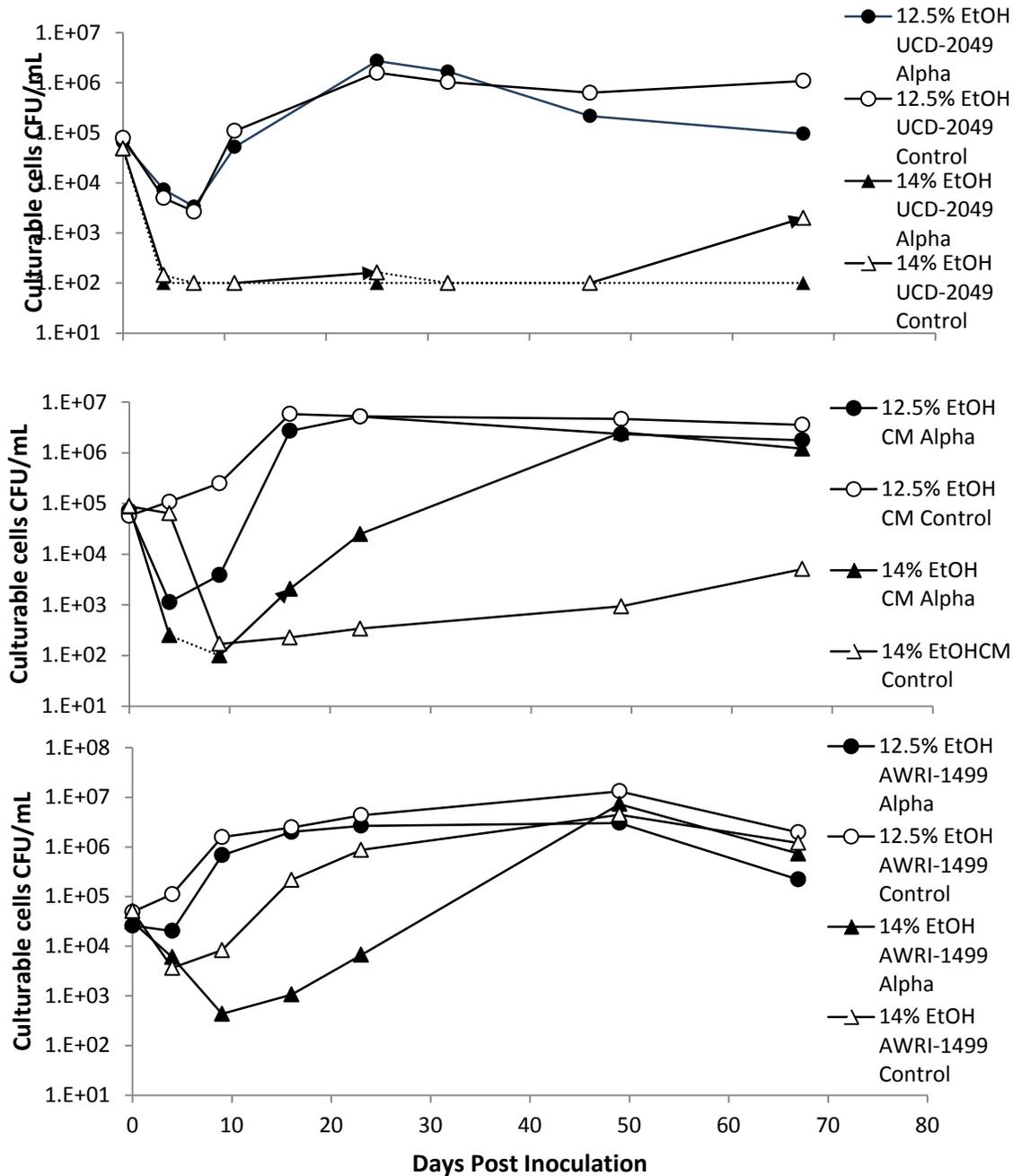
Figure 3.3 Growth of *B. bruxellensis* strain UCD-2049, Copper Mountain (CM), or AWRI-1499 (C) in Pinot noir wine adjusted to 12.5% EtOH v/v or to 14% EtOH v/v that has (Alpha) or has not (Control) undergone MLF with *O. oeni* strain Alpha. Data points represent mean of replicates, n=3.

At the end of the experiment, 50 mL samples were taken from all samples and analyzed for 4-EP and 4-EG concentrations. Both *B. bruxellensis* strain and ethanol concentration had a significant effect on 4-EG and 4-EP production while MLF did not (Table 3.3). For strain AWRI-1499 there were no significant differences in 4-EP or 4-EG concentrations between any of the treatments (Table 3.3). For the Copper Mountain strain, there were no significant differences in 4-EG or 4-EP between MLF treated and control wines in 12.5% EtOH wine. However, lower 4-EG and 4-EP was measured in 14% EtOH wines that had undergone MLF compared to wines that had not (Table 3.3). In the 14% EtOH wines for the Copper Mountain treatments, there was significantly lower 4-EG concentrations in the control wines than in all the AWRI-1499 treated wines except for the 14% EtOH MLF treatment. There was also significantly lower 4-EG concentrations in the 14% EtOH wines for the Copper Mt. treatments than in the 12.5% EtOH UCD-2049 treated wines. Similarly, the 14% EtOH Copper Mt. treatments had significantly lower 4-EP than all other treatments except for the 14% EtOH UCD-2049 treated wines (Table 3.3).

In the 12.5% EtOH wines inoculated with UCD-2049, there was significantly higher 4-EP and 4-EG concentrations in the control wine as opposed to wine that had undergone MLF (Table 3.3). The higher ethanol wine (14% v/v) contained significantly lower 4-EP and 4-EG than the low ethanol wine no matter whether the wine had undergone MLF or not (Table 3.3). In fact, there was no detectable 4-EP in the 14% EtOH wines that had undergone MLF prior to being inoculated with *B. bruxellensis* UCD-2049 (Table 3.3).

Table 3.3 Concentration (µg/L) of 4-ethyl phenol and 4-ethyl guaiacol 67 days after inoculation of various *B. bruxellensis* strains into 2019 Pinot noir wine that had or had not previously undergone MLF with *O. oeni* Alpha and was adjusted to either 12.5 or 14.0% ethanol.

Treatment			4-Ethyl Phenol	4-Ethyl Guaiacol
Strain	MLF	Ethanol % (v/v)		
AWRI-1499	Yes	12.5	312.4 ± 3.8^b	312.5 ± 1.1^{ab}
AWRI-1499	No	12.5	318.5 ± 9.5^{ab}	313.5 ± 12.3^{ab}
AWRI-1499	Yes	14.0	307.6 ± 12.2^b	286.7 ± 9.8^{bc}
AWRI-1499	No	14.0	318.5 ± 4.5^b	313.5 ± 4.2^{ab}
Copper Mt.	Yes	12.5	304.8 ± 15.2^b	331.1 ± 16.3^{ab}
Copper Mt.	No	12.5	313.8 ± 15.5^b	330.1 ± 10.2^{ab}
Copper Mt.	Yes	14.0	260 ± 15.8^b	300.3 ± 12.2^{bc}
Copper Mt.	No	14.0	113.6 ± 98.3^c	211.4 ± 185.2^{cd}
UCD-2049	Yes	12.5	261.9 ± 34.3^b	314.9 ± 36.4^{ab}
UCD-2049	No	12.5	461.8 ± 11.9^a	449.8 ± 5.4^a
UCD-2049	Yes	14.0	$<10^c$	12.1 ± 3.1^d
UCD-2049	No	14.0	82.3 ± 120.5^c	87.2 ± 121.9^d
Effect significance				
B. bruxellensis strain			<0.0001	<0.001
MLF			ns	ns
Ethanol			<0.0001	<0.0001
B. bruxellensis strain X MLF			<0.0001	<0.001
B. bruxellensis strain X Ethanol			<0.0001	<0.0001
MLF X Ethanol			<0.05	ns
B. bruxellensis strain X MLF X Ethanol			ns	ns

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

Discussion

The effectiveness of *O. oeni* to inhibit *B. bruxellensis* growth and volatile phenol

production in wine was investigated. The inhibition of *B. bruxellensis* growth by MLF

had been suggested previously by Gerbaux et al. (2009) who noted that *B. bruxellensis*

produced less 4-EP and 4-EG when inoculated into wines that had undergone a MLF using commercial *O. oeni* strains. Previous work reported in Chpt. 2 showed that *B. bruxellensis* strain UCD-2049 growth and volatile phenol production was inhibited when inoculated into wine that had recently undergone MLF. While a wide range of *O. oeni* were previously tested, only one *B. bruxellensis* strain was used. In the present study, an additional seven *B. bruxellensis* strains originally sourced from different geographical regions were used. As was done previously, *B. bruxellensis* was inoculated into wine that had recently completed MLF. A single strain of *O. oeni,* Alpha, was used as this had previously been shown to strongly inhibit *B. bruxellensis* UCD-2049. In contrast to the previous findings for *B. bruxellensis* strain UCD-2049, no differences in *B. bruxellensis* growth was noted for any of the seven strains in wines that had recently undergone MLF when compared to growth in a control wine. Indeed all strains tested followed similar growth curves, reaching similar population maximums by the end of the experiment. This finding suggests that the effect of *O. oeni* on *B. bruxellensis* growth is variable between *B. bruxellensis* strains. The lack of growth differences was also reflected in the volatile phenols measured in the wines. While there were some differences in 4-EP and 4-EG concentrations between *B. bruxellensis* strains, whether the wines had or had not undergone MLF was not a significant factor. The differences between strains is not surprising given the previously reported differences in volatile phenol production between *B. bruxellensis* strains (Conterno et al. 2006).

Variation between strains of *B. bruxellensis* for a number of wine conditions have been previously reported and *B. bruxellensis* displays great genetic variety between strains

(Curtin et al., 2007; Agnolucci et al., 2009; Hellborg & Piskur, 2009; Vigentini et al., 2012). Tolerance of SO_2 can vary widely for example, with some studies reporting similar tolerance to *S. cerevisiae*, while other studies show that a comparatively minor amounts of SO_2 can affect *B. bruxellensis* viability (Beech et al., 1979; du Toit et al., 2005). Curtin et al. (2012a) also found that *B. bruxellensis* strain AWRI-1499 was particularly tolerant to SO_2. Furthermore, Cibrario et al. (2020) recently reported that some AWRI-1499-like strains of *B. bruxellensis* are tolerant of both high ethanol concentration and low pH. There arises a question as to whether strain differences to wine factors such as ethanol and pH could modulate the *B. bruxellensis* response to any stress resulting from being inoculated into wine at the end of MLF. While MLF may not universally affect *B. bruxellensis* strains equally, could it be an additional hurdle when coupled with additional stressors such as high ethanol? Others have noted that known 'hurdles' such as temperature, ethanol concentration, pH, and SO_2 (Smith, 2011; Sturm et al., 2014; Zuehlke and Edwards, 2013) can act in a synergistic manner to suppress *B. bruxellensis* growth (Edwards et al., 2015; Ramirez et al., 2014). Edwards and Oswald (2017) found that even without added SO_2, interactive effects between temperature and ethanol concentration in wine are capable of extending lag time and reducing volatile acidity and ethylphenol concentrations in certain strains of *B. bruxellensis*.

In the present study, ethanol was investigated as an additional hurdle that may affect *B. bruxellensis* inhibition by *O. oeni*. The previous work using *B. bruxellensis* UCD-2049 was in Pinot noir wine adjusted to 13% (v/v) ethanol as was the wine used for the *Brettanomyces* screening experiment in the current study. Therefore, an ethanol

concentration lower (12.5%) and higher (14%) was used to test various strains of *B. bruxellensis* in order to determine if any combinatorial effects existed between a known inhibitory factor (i.e. ethanol concentration) and MLF. *B. bruxellensis* strain UCD-2049 was included in this experiment, as it has been previously observed to be sensitive to MLF in 13% (v/v) ethanol wine. *B. bruxellensis* strain Copper Mountain was included due to its strong growth in the screening experiment, and *B. bruxellensis* strain AWRI-1499 was included due to this strains reported high tolerance to SO_2 (Curtin et al., 2012a) and potentially high ethanol concentration (Cibrario et al. 2020).

If we just consider ethanol concentration, differences in growth between *B. bruxellensis* strains were observed with UCD2049 growing well in 12.5% but not 14% (v/v) ethanol wine while strain AWRI-1499 grew well in both the 12.5% and 14% (v/v) ethanol wines. This is consistent with what was reported by Cibrario et al. (2020) where higher ethanol tolerance was noted for an 'AWRI-1499 like' strain. These differences were also reflected in the production of volatile phenols. For these compounds low concentrations were present in high ethanol wines inoculated with UCD-2049 while significantly higher concentrations were present in wines inoculated with either AWRI-1499 or Copper Mountain. Whether the wines had undergone MLF or not did not impact growth of any of the three *B. bruxellensis* strains in the 12.5% (v/v) ethanol wine. This was a surprising finding for strain UCD-2049 as in a previous study this strain was strongly inhibited when inoculated into wine that had just completed MLF. However, the wine used in that study had been adjusted to 13% (v/v) ethanol rather than 12.5% (v/v) as was used here. Interestingly, 4-EP production by UCD-2049 in 12.5% (v/v) ethanol was significantly

lower in wine that had undergone MLF, even though the growth was not affected. These findings, in combination with the noted lower ethanol tolerance of UCD-2049, suggests that the suppression of *B. bruxellensis* UCD-2049 growth and volatile phenol production is at least partially augmented by ethanol concentration. Additional research in this area should utilize a wider range of ethanol concentrations to confirm this interactive effect.

The effect of ethanol on inhibition of *B. bruxellensis* by *O. oeni* was less clear for the other two *B. bruxellensis* strains tested. While the growth of *B. bruxellensis* strain AWRI-1499 was similar between the MLF treated wines and the controls in the low ethanol wines, growth in higher ethanol wine that had undergone MLF was initially slower compared to the control. However, AWRI-1499 populations in both treatments reached similar maximum populations and no differences in the production of volatile phenols were measured. Interestingly enough, *B. bruxellensis* strain Copper Mountain actually grew better in high ethanol wine that had undergone MLF compared to the control. Although the experimental method sought to minimize any pH differences between wines that had or had not undergone MLF, the pH may still have been higher in the MLF treatments due to the metabolic conversion of malic acid to lactic acid by *O. oeni.* If the pH of the MLF treated wines was in fact higher, this may explain the observed difference in growth, with a higher pH being more favorable for growth of *B. bruxellensi* (Blomqvist et al., 2012). Aside from pH changes, it is also possible that the growth of *O. oeni* modified the wine in such a way to make it more hospitable for growth of *B. bruxellensis* Copper Mountain. How this may have occurred is unknown at this point.

Conclusion

Whether a wine had undergone MLF or not did not impact the growth of seven strains of *B. bruxellensis,* in contrast to what was observed previously with *B. bruxellensis* stran UCD-2049. The concentration of ethanol in the wine had a variable effect on *B. bruxellensis* strain response to MLF. For the ethanol tolerant strain AWRI-1499, MLF did not affect growth or volatile phenol production with respect to controls in either low or high ethanol conditions. While the growth and 4-ethylphenol production of *B. bruxellensis* strain Copper Mountain improved in high ethanol wine if it had undergone MLF. In the case of *B. bruxellensis* strain UCD-2049; volatile phenol production was reduced in low-ethanol wine if it had undergone MLF although no difference in *B. bruxellensis* growth was observed. This finding, when considered alongside findings from Chpt. 2, implies that ethanol tolerance of *B. bruxellensis* strains may play a role in how susceptible they are to inhibition by *O. oeni*. Additional research in this area should utilize a wider range of ethanol concentrations as well as additional *B. bruxellensis* strains that have been identified as ethanol tolerant or sensitive. Additional wine stressors such as pH should also be investigated.

General Summary and Conclusions

Conducting a vigorous and quick MLF has been reported as a good practice to reduce the risk of *Brettanomyces* spoilage as the antimicrobial sulfur dioxide cannot be added to a wine until MLF has been completed. This study reports on an additional benefit of MLF with regards to preventing *Brettanomyces* wine spoilage. Namely, the inhibition of *B. bruxellensis* growth and volatile phenol production by *O. oeni*. Initial experiments demonstrated that both the growth and volatile phenol production of *B. bruxellensis* strain UCD-2049 were suppressed when inoculated into a wine that had recently undergone MLF by *O. oeni*. This response was consistent for all eleven strains of *O. oeni* tested. The cause of *B. bruxellensis* inhibition was not likely due to depletion of nutrients by *O. oeni* or production of an inhibitory compound(s) by *O. oeni*. Instead, cell to cell contact was observed to be necessary for inhibition as dialysis membrane separation of *O. oeni* and *B. bruxellensis* cells resulted in no suppression of *B. bruxellensis* growth.

B. bruxellensis strain-variable tolerance to MLF was also tested using a number of *B. bruxellensis* strains sourced from diverse geographic locations. In contrast to what was observed for *B. bruxellensis* strain UCD-2049, the growth and volatile phenol production of the other *B. bruxellensis* strains screened in this study was not impacted by MLF when compared to a control. The differences between the strains may have been due to ethanol tolerance as the strain susceptible to inhibition by MLF, UCD-2049, was the most ethanol sensitive of the three strains tested while a more ethanol tolerant strain, AWRI-1499, was less impacted by MLF. Further research using a variety of ethanol concentrations and as

well as additional *B. bruxellensis* strains that have been identified as ethanol tolerant or sensitive is needed to confirm any interactive effects.

The results of this study show that MLF may offer some protection from spoilage by *B. bruxellensis*, though any protection conferred by MLF is likely to vary depending on *B. bruxellensis* strain and wine chemistry. Overall, the effects of MLF on the growth and volatile phenol production of *B. bruxellensis* are not such that they could be considered a standalone spoilage prevention strategy by winemakers. Instead, MLF could be considered as an additional hurdle that could be used to lower the risk of wine spoilage by *B. bruxellensis,* particularly during the period of time between the end of MLF and the addition of SO_2 by the winemaker.

Literature Cited

Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K., & Ameyama, M. (1980). Purification and characterization of membrane-bound aldehyde dehydrogenase from Gluconobacter suboxydans. *Agricultural and Biological Chemistry*, *44*(3), 503-515.

Adachi, O., Miyagawa, E., Shinagawa, E., Matsushita, K., & Ameyama, M. (1978). Purification and properties of particulate alcohol dehydrogenase from Acetobacter aceti. *Agricultural and Biological Chemistry*, *42*(12), 2331-2340.

Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K., & Ameyama, M. (1978). Purification and characterization of particulate alcohol dehydrogenase from Gluconobacter suboxydans. *Agricultural and Biological Chemistry*, *42*(11), 2045-2056.

Agnolucci, M., Cristani, C., Maggini, S., Rea, F., Cossu, A., Tirelli, A., & Nuti, M. (2014). Impact of sulphur dioxide on the viability, culturability, and volatile phenol production of Dekkera bruxellensis in wine. *Annals of Microbiology*, *64*(2), 653-659.

Agnolucci, M., Rea, F., Sbrana, C., Cristani, C., Fracassetti, D., Tirelli, A. & Nuti, M. (2010). Sulphur dioxide affects culturability and volatile phenol production by Brettanomyces/Dekkera bruxellensis. *International journal of food microbiology*, **143**, 76–80. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.07.022

Agnolucci, M., Vigentini, I., Capurso, G., Merico, A., Tirelli, A., Compagno, C., Foschino, R. & Nuti, M. (2009). Genetic diversity and physiological traits of Brettanomyces bruxellensis strains isolated from Tuscan Sangiovese wines. *International journal of food microbiology*, **130**(3), 238–244. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.01.025

Albergaria, H., & Arneborg, N. (2016). Dominance of Saccharomyces cerevisiae in alcoholic fermentation processes: role of physiological fitness and microbial interactions. *Applied microbiology and biotechnology*, *100*(5), 2035-2046.

Alejandra Aguilar Solis, M. (2014). Evaluation of common and novel sanitizers against spoilage yeasts found in wine environments. Thesis. *Cornell University.* http://dx.doi.org/10.13140/RG.2.1.1353.4966

Ameyama, M., Osada, K., Shinagawa, E., Matsushita, K., & Adachi, O. (1981). Purification and characterization of aldehyde dehydrogenase of Acetobacter aceti. *Agricultural and Biological Chemistry*, *45*(8), 1889-1890.

Andrews, B. J., & Gilliland, R. B. (1952). Super-attenuation of beer: a study of three organisms capable of causing abnormal attenuations. *Journal of the Institute of Brewing*, *58*(3), 189-196.

Arena, M. E., & Manca de Nadra, M. C. (2001). Biogenic amine production by Lactobacillus. *Journal of Applied Microbiology*, *90*(2), 158-162.

Arena, M. E., Saguir, F. M., & De Nadra, M. M. (1999). Arginine, citrulline and ornithine metabolism by lactic acid bacteria from wine. *International Journal of Food Microbiology*, *52*(3), 155-161.

Arroyo-lópez, F., Salvadó, Z., Tronchoni, J., Guillamón, J., Barrio, E. & Querol, A. (2010). Susceptibility and resistance to ethanol in Saccharomyces strains isolated from wild and fermentative environments. *Yeast*. http://dx.doi.org/10.1002/yea.1809

Barata, A., Caldeira, J., Botelheiro, R., Pagliara, D., Malfeito-Ferreira, M., & Loureiro, V. (2008a). Survival patterns of Dekkera bruxellensis in wines and inhibitory effect of sulphur dioxide. *International journal of food microbiology*, *121*(2), 201-207.

Barata, A., Pagliara, D., Piccininno, T., Tarantino, F., Ciardulli, W., Malfeito-Ferreira, M., & Loureiro, V. (2008b). The effect of sugar concentration and temperature on growth and volatile phenol production byDekkera bruxellensisin wine. *FEMS Yeast Research, 8*(7), 1097-1102. doi:10.1111/j.1567-1364.2008.00415.x

Barata, A., Malfeito-Ferreira, M. & Loureiro, V. (2012). The microbial ecology of wine grape berries. *International journal of food microbiology*, **153**, 243–259.

Barbe, J. C., de Revel, G., & Bertrand, A. (2002). Gluconic acid, its lactones, and SO_2 binding phenomena in musts from botrytized grapes. *Journal of agricultural and food chemistry*, *50*(22), 6408-6412.

Barbe, J. C., de Revel, G., Joyeux, A., Lonvaud-Funel, A., & Bertrand, A. (2000). Role of carbonyl compounds in SO_2 binding phenomena in musts and wines from botrytized grapes. *Journal of agricultural and food chemistry*, *48*(8), 3413-3419.

Bartowsky, E.J. & Henschke, P.A. (2004). The "buttery" attribute of wine— diacetyl— desirability, spoilage and beyond. *International journal of food microbiology*, **96**, 235–252. https://doi.org/10.1016/j.ijfoodmicro.2004.05.013

Bartowsky, E.J., Francis, I.L., Bellon, J.R. & Henschke, P.A. (2002). Is buttery aroma perception in wines predictable from the diacetyl concentration? *Australian journal of grape and wine research*, **8**, 180–185. http://dx.doi.org/10.1111/j.1755-0238.2002.tb00254.x

Bauer, R., Cowan, D.A. and Crouch, A. (2010) Acrolein in wine: importance of 3-hydroxypropionaldehyde and derivatives in production and detection. Journal of Agricultural and Food Chemistry 58, 3243–3250.

Bauer, R., Nel, H. A., & Dicks, L. M. (2003). Pediocin PD-1 as a method to control growth of Oenococcus oeni in wine. *American journal of enology and viticulture*, *54*(2), 86-91.

Bautista-Ortín, A.B., Fernández-Fernández, J.I., López-Roca, J.M. & Gómez-Plaza, E. (2004). Wine-making of High Coloured Wines: Extended Pomace Contact and Run-off of Juice Prior to Fermentation. *Revista de Agaroquimica y Tecnologia de Alimentos*, **10**, 287–295. http://dx.doi.org/10.1177/1082013204047565

Beech, F. W., & Thomas, S. (1985). Action antimicrobienne de l'anhydride sulfureux. *Bull OIV*, *58*, 564-581.

Beech, R.W., Burroughs, L.F., Timberlake, C.F. and Whiting, G.C. (1979). Progres recents sur l'aspect chimique et l'actopm amtomicrobienne de l'anhydride sulfureux. *Bull OIV* 186, 1001–1022.

Beltran, G., Torija, M. J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J. M., ... & Mas, A. (2002). Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Systematic and Applied Microbiology*, *25*(2), 287-293.

Bevan, E. A. (1963). The physiological basis of the killer character in yeast. *Proc. 11th Int. Cong. Gene*, *1*, 202-203.

Bisson, L.F. and Joseph, C.M.L. (2009). Fungi of grapes. In : Biology of Microorganisms on Grapes, in Must and in Wine, pp. 47-60. König, H., Unden, G., and Fröhlich, J., Eds., Springer-Verlag, Berlin, Heidelberg.

Bloem, A., Lonvaud-Funel, A. & Revel, G. de. (2008). Hydrolysis of glycosidically bound flavour compounds from oak wood by Oenococcus oeni. *Food microbiology*, **25**, 99–104. http://dx.doi.org/10.1016/j.fm.2007.07.009

Blomqvist, J., Nogué, V.S., Gorwa-Grauslund, M. & Passoth, V. (2012). Physiological requirements for growth and competitiveness of Dekkera bruxellensis under oxygen-limited or anaerobic conditions. *Yeast*, **29**(7), 265–274. http://dx.doi.org/10.1002/yea.2904

Borneman, A. R., Zeppel, R., Chambers, P. J., & Curtin, C. D. (2014). Insights into the Dekkera bruxellensis genomic landscape: comparative genomics reveals variations in ploidy and nutrient utilisation potential amongst wine isolates. *PLoS Genet*, **10**(2), e1004161.

Boukhemis, M., Djeghri-Hocine, B., Tahar, A., & Amrane, A. (2009). Phenotypic characterization of Lactobacillus strains isolated from different biotopes. *African Journal of Biotechnology*, *8*(19).

Brady, D., Stoll, A. D., Starke, L., & Duncan, J. R. (1994). Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of Saccharomyces cerevisiae. *Biotechnology and Bioengineering*, **44**, 297–302.

Brandam, C., Castro-Martínez, C., Délia, M.-L., Ramón-Portugal, F. & Strehaiano, P. (2008). Effect of temperature on Brettanomyces bruxellensis: metabolic and kinetic aspects. *Canadian journal of microbiology*, **54**(1), 11–18. http://dx.doi.org/10.1139/w07-126a

Burns, T. R., & Osborne, J. P. (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), 370-377.

Burroughs, L. F. (1975). Determining free sulfur dioxide in red wine. *American journal of Enology and Viticulture*, *26*(1), 25-29.

Canals, R., Llaudy, M.C., Valls, J., Canals, J.M. & Zamora, F. (2005). Influence of ethanol concentration on the extraction of color and phenolic compounds from the skin and seeds of Tempranillo grapes at different stages of ripening. *Journal of agricultural and food chemistry*, **53**, 4019–4025. http://dx.doi.org/10.1021/jf047872v

Capucho, I., & San Romao, M. V. (1994). Effect of ethanol and fatty acids on malolactic activity ofLeuconostoc oenos. *Applied Microbiology and Biotechnology*, *42*(2-3), 391-395.

Carmack, M., Moore, M. B., & Balis, M. E. (1950). The structure of the antihemorrhagic sodium bisulfite addition product of 2-methyl-1, 4-naphthoquinone (menadione). *Journal of the American Chemical Society*, *72*(2), 844-847.

Carreté, R., Vidal, M. T., Bordons, A., & Constantí, M. (2002). Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of Oenococcus oeni. *FEMS microbiology letters*, *211*(2), 155-159.

Cartwright, Z.M., Glawe, D. & Edwards, C.G. (2016). Analysis of Brettanomyces bruxellensis in Different Types of Oak Staves. Poster presentation. *American society of enology and viticulture. Alimentos*, **10**, 287–295. http://dx.doi.org/10.1177/1082013204047565

Chandra, M., Barata, A., Ferreira-Dias, S., Malfeito-Ferreira, M., & Loureiro, V. (2014). A Response Surface Methodology study on the role of factors affecting growth and volatile phenol production by Brettanomyces bruxellensis ISA 2211 in wine. *Food Microbiology, 42*, 40-46. doi:10.1016/j.fm.2014.03.002

Chatonnet , P., Dubourdieu, D. & Boidron, J.N. (1995.). Yeasts and lactic acid bacteria on the ethylphenol content of red wines. *American journal of enology and viticulture*, **46**,(4), 463-468. http://www.ajevonline.org/content/46/4/463.full-text.pdf

Chescheir, S., Philbin, D. & Osborne, J.P. (2015). Impact of Oenococcus oeni on Wine Hydroxycinnamic Acids and Volatile Phenol Production by Brettanomyces bruxellensis. *American journal of enology and viticulture*, **66**(3), 357–362. http://dx.doi.org/10.5344/ajev.2015.14108

Chescheir, S. (2014). Effect of Lactic Acid Bacteria Growth on Brettanomyces bruxellensis Growth and Production of Ethylphenols. Thesis. *Oregon State University.*

Ciani, M., & Ferraro, L. (1997). Role of oxygen on acetic acid production by Brettanomyces/Dekkera in winemaking. *Journal of the Science of Food and Agriculture*, *75*(4), 489-495.

Cibrario, A., Miot-Sertier, C., Paulin, M., Bullier, B., Riquier, L., Perello, M. C., ... & Dols-Lafargue, M. (2020). Brettanomyces bruxellensis phenotypic diversity, tolerance to wine stress and wine spoilage ability. *Food Microbiology*, *87*, 103379.

Clary, C. D., Steinhauer, R. E., Frisinger, J. E., & Peffer, T. E. (1990). Evaluation of machine-vs. hand-harvested Chardonnay. *American journal of enology and viticulture*, *41*(2), 176-181.

Collins, E. B. (1972). Biosynthesis of flavor compounds by microorganisms. *Journal of dairy science*, *55*(7), 1022-1028.

Comitini, F., Ferretti, R., Clementi, F., Mannazzu, I., & Ciani, M. (2005). Interactions between Saccharomyces cerevisiae and malolactic bacteria: preliminary characterization of a yeast proteinaceous compound (s) active against Oenococcus oeni. *Journal of applied microbiology*, *99*(1), 105-111.

Comitini, F., Ingeniis De, J., Pepe, L., Mannazzu, I., & Ciani, M. (2004). Pichia anomala and Kluyveromyces wickerhamii killer toxins as new tools against Dekkera/Brettanomyces spoilage yeasts. *FEMS Microbiology letters*, *238*(1), 235-240.

Conterno, L., Joseph, C. L., Arvik, T. J., Henick-Kling, T., & Bisson, L. F. (2006). Genetic and physiological characterization of Brettanomyces bruxellensis strains isolated from wines. *American Journal of Enology and Viticulture*, *57*(2), 139-147.

Costa, A., Barata, A., Malfeito-Ferreira, M., & Loureiro, V. (2008). Evaluation of the inhibitory effect of dimethyl dicarbonate (DMDC) against wine microorganisms. *Food microbiology*, *25*(2), 422-427.

Costantini, A., Vaudano, E., Cravero, M., Petrozziello, M., Piano, F., Bernasconi, A. & Garcia-Moruno, E. (2016). Dry ice blasting, a new tool for barrel regeneration treatment. *European food research and technology,* **242**(10), 1673-1683. https://doi.org/10.1007/s00217-016-2667-3

Costantini, A., García-Moruno, E. & Victoria Moreno-Arribas, M. (2009). Biochemical Transformations Produced by Malolactic Fermentation. In: *Wine Chemistry and Biochemistry*. Pp. 27–57. Springer, New York, NY. http://dx.doi.org/10.1007/978-0-387-74118-5_2 *in applied microbiology*, **55**(1), 56–61. http://dx.doi.org/10.1111/j.1472-765X.2012.03257.x

Costello, P.J. & Henschke, P.A. (2002). Mousy off-flavor of wine: precursors and biosynthesis of the causative N-heterocycles 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine, and 2-acetyl-1-pyrroline by Lactobacillus hilgardii DSM 20176. *Journal of agricultural and food chemistry*, **50**, 7079–7087. http://dx.doi.org/10.1021/jf020341r

Coulter, A., Robinson, E., Cowey, G., Francis, I.L., Lattey, K., Capone, D., Gishen, M. and Godden, P. (2003). *Dekkera / Brettanomyces* yeast – an overview of recent AWRI investigations and some recommendations for its control. In *Proceedings of a seminar organised by the Australian Society for Viticulture and Oenology – Grapegrowing at the edge, managing the wine business, impacts on wine flavour. eds.* Bell, S.M., de Garis, K.A., Dundon, C.G., Hamilton, R.P., Partridge, S.J. and Wall, G.S. pp. 41–50. Tanunda, SA: Australian Society for Viticulture and Oenology.

Coutard, F., Pommepuy, M., Loaec, S., & Hervio-Heath, D. (2005). mRNA detection by reverse transcription–PCR for monitoring viability and potential virulence in a pathogenic strain of Vibrio parahaemolyticus in viable but nonculturable state. *Journal of applied microbiology*, *98*(4), 951-961.

Cox, D. J., & Henick-Kling, T. (1989). Chemiosmotic energy from malolactic fermentation. *Journal of bacteriology*, *171*(10), 5750-5752.

Crauwels, S., Van Assche, A., Jonge, R. de, Borneman, A.R., Verreth, C., Troels, P., De Samblanx, G., Marchal, K., Van de Peer, Y., Willems, K.A., Verstrepen, K.J., Curtin, C.D. & Lievens, B. (2015). Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different Brettanomyces bruxellensis strains. *Applied microbiology and biotechnology*, **99**(21), 9123–9134. http://dx.doi.org/10.1007/s00253-015-6769-9

Crauwels, S., Zhu, B., Steensels, J., Busschaert, P., De Samblanx, G., Marchal, K., Willems, K.A., Verstrepen, K.J. & Lievens, B. (2014). Assessing genetic diversity among Brettanomyces yeasts by DNA fingerprinting and whole-genome sequencing. *Applied and environmental microbiology*, **80**(14), 4398–4413. http://dx.doi.org/10.1128/AEM.00601-14

Curtin, C.D. & Pretorius, I.S. (2014). Genomic insights into the evolution of industrial yeast species Brettanomyces bruxellensis. *FEMS yeast research*, **14**(7), 997–1005. http://dx.doi.org/10.1111/1567-1364.12198

Curtin, C. D., Borneman, A. R., Chambers, P. J., & Pretorius, I. S. (2012). De-novo assembly and analysis of the heterozygous triploid genome of the wine spoilage yeast Dekkera bruxellensis AWRI1499. *PLOS one*, *7*(3), e33840.

Curtin, C., Kennedy, E., & Henschke, P. A. (2012). Genotype-dependent sulphite tolerance of Australian Dekkera (Brettanomyces) bruxellensis wine isolates. *Letters in applied microbiology*, *55*(1), 56-61.

Curtin, C. D., Bellon, J. R., Henschke, P. A., Godden, P. W., & de Barros Lopes, M. A. (2007). Genetic diversity of Dekkera bruxellensis yeasts isolated from Australian wineries. *FEMS Yeast Research*, *7*(3), 471-481.

Daudt, C. E., & Ough, C. S. (1980). Action of dimethyldicarbonate on various yeasts. *American Journal of Enology and Viticulture*, *31*(1), 21-23.

Davis, C.R., Wibowo, D., Fleet, G.H., and Lee, T.H. (1988). Properties of wine lactic acid bacteria: Their potential enological significance. *American journal of enology and viticulture*, **39**, 137–142.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H. (1985). Practical Implications of Malolactic Fermentation: A Review. *American journal of enology and viticulture*, **36**, 290–301.

De Deken, R. H. (1966). The Crabtree effect: a regulatory system in yeast. Microbiology, 44(2), 149-156.

Dias, L., Pereira-da-Silva, S., Tavares, M., Malfeito-Ferreira, M. & Loureiro, V. (2003). Factors affecting the production of 4-ethylphenol by the yeast Dekkera bruxellensis in enological conditions. *Food microbiology*, **20**(4), 377–384. http://dx.doi.org/10.1016/S0740-0020(03)00023-6

Dick, K. J., Molan, P. C., & Eschenbruch, R. (1992). The isolation from Saccharomyces cerevisiae of two antibacterial cationic proteins that inhibit malolactic bacteria. *Vitis*, *31*(2), 105-116.

Dicks, L. M. T., Dellaglio, F., & Collins, M. D. (1995). Proposal To Reclassify Leuconostoc oenos as Oenococcus oeni [corrig.] gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *45*(2), 395-397.

Divol, B., du Toit, M., & Duckitt, E. (2012). Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. *Applied microbiology and biotechnology*, *95*(3), 601-613.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*, *15*(2), 167-193.

Dott, W., & Trüper, H. G. (1976). Sulfite formation by wine yeasts. *Archives of Microbiology*, *108*(1), 99-104.

Drumonde-Neves, J., Franco-Duarte, R., Lima, T., Schuller, D. & Pais, C. (2016). Yeast Biodiversity in Vineyard Environments Is Increased by Human Intervention. *PloS one*, **11**, e0160579. http://dx.doi.org/10.1371/journal.pone.0160579

Drysdale, G.S. and Fleet, G.H. (1989). The eVect of acetic acid bacteria upon the growth and metabolism of yeasts during the fermentation of grape juice. J. *Appl. Bacteriol*. 67, 471–481.

Drysdale, G. S., & Fleet, G. H. (1988). Acetic acid bacteria in winemaking: a review. *American Journal of Enology and Viticulture*, *39*(2), 143-154.

Drysdale, G.S. and Fleet, G.H. (1984). Acetic acid bacteria in some Australian wines. *Food Technol. Aust*. 37, 17–20.

Du Toit, W., Pretorius, I.S. & Lonvaud-Funel, A. (2005). The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of Acetobacter pasteurianus and a strain of Brettanomyces bruxellensis isolated from wine. *Journal of applied microbiology*, **98**(4), 862–871. http://dx.doi.org/10.1111/j.1365- 2672.2004.02549.x

Du Toit, M. & Pretorius, I.S. (2000). Microbial Spoilage and Preservation of Wine: Using Weapons from Nature's Own Arsenal- A Review. *S. Afr. J. Enol. Vitic.*, **21**.

Edelényi, M. (1966). Study on the stabilization of sparkling wines. *Borgazdaság*, *12*, 30-32.

Edwards, C. G. Childs, B. C., & Bohlscheid, J. C. (2015). Impact of available nitrogen and sugar concentration in musts on alcoholic fermentation and subsequent wine spoilage by Brettanomyces bruxellensis. *Food Microbiology, 46*, 604-609. doi:10.1016/j.fm.2014.10.006

Edwards, C. and Jensen, K. (1992). Occurrence and characterization of lactic acid bacteria from Washington state wines: Pediococcus spp. *American journal of enology and viticulture, 43*(3), 233-238.

Edwards, C. G., Beelman, R. B., Bartley, C. E., & McConnell, A. L. (1990). Production of decanoic acid and other volatile compounds and the growth of yeast and malolactic bacteria during vinification. *American Journal of Enology and Viticulture*, *41*(1), 48-56.

Edwards, C. G., & Beelman, R. B. (1987). Inhibition of the malolactic bacterium, Leuconostoc oenos (PSU-1), by decanoic acid and subsequent removal of the inhibition by yeast ghosts. *American journal of enology and viticulture*, *38*(3), 239-242.

Eglinton, J. M., & Henschke, P. A. (1999a). The occurrence of volatile acidity in Australian wines. *Australian Grapegrower and Winemaker*, 7-14.

Eglinton, J., Henschke, P., (1999b). Restarting incomplete fermentations: the effect of high concentrations of acetic acid. Australian Journal of Grape and Wine Research 5, 71–78.

Eschenbruch, R. (1974). Sulfite and sulfide formation during winemaking--a review. *American journal of Enology and Viticulture*, *25*(3), 157-161.

Eschenbruch, R., & Bonish, P. (1976). Production of sulphite and sulphide by low-and high-sulphite forming wine yeasts. *Archives of Microbiology*, *107*(3), 299-302.

Ferreira, D., Moreira, D., Costa, E.M., Silva, S., Pintado, M.M. & Couto, J.A. (2013). The Antimicrobial Action of Chitosan Against the Wine Spoilage Yeast Brettanomyces/Dekkera. *Journal of Chitin and Chitosan Science*, **1**, 240–245. https://doi.org/10.1166/jcc.2013.1037

Fleet GH, Heard GM (1993). Yeast growth during fermentation. In: Fleet GH (ed) Wine microbiology and biotechnology. *Harwood Academic Publishers, Switzerland*, pp 27–54

Fleet, G. (1993). Wine Microbiology and Biotechnology. *CRC Press.* Fleet, G.H. (2003). Yeast interactions and wine flavour. *International journal of food microbiology*, **86**, 11–22. http://dx.doi.org/10.1016/S0168-1605(03)00245-9

Fleet, G. H., Lafon-Lafourcade, S., & Ribéreau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology*, *48*(5), 1034-1038.

Fornachon, J. C. M. (1963). Inhibition of certain lactic acid bacteria by free and bound sulphur dioxide. *Journal of the Science of Food and Agriculture*, *14*(12), 857-862.

Fornachon, J. C. M., & Lloyd, B. (1965). Bacterial production of diacetyl and actoin in wine. *Journal of the Science of Food and Agriculture*, *16*(12), 710-716.

Fornairon-Bonnefond C., Camarasa C., Moutounet M. and Salmon J.M. (2002). New trends on yeast autolysis and wine ageing on lees: a bibliographical review. *Journal International des Sciences de la Vigne et du Vin* **36**: 49–69.

Francis, I.L. & Newton, J.L. (2005). Determining wine aroma from compositional data. *Australian journal of grape and wine research*, **11**, 114–126. http://dx.doi.org/10.1111/j.1755-0238.2005.tb00283.x

Françoise, Larue., Park, M. K., & Caruana, C. (1985). Quelques observations sur les conditions de la formation d'anhydride sulfureux en vinification. *OENO One*, *19*(4), 241-248.

Freer, S.N., Dien, B. & Matsuda, S. (2003). Production of acetic acid by Dekkera/Brettanomyces yeasts under conditions of constant pH. *World journal of microbiology & biotechnology*, **19**, 101–105. https://doi.org/10.1023/A:1022592810405

Fugelsang, K.C. & Edwards, C.G. (2006). *Wine Microbiology: Practical Applications and Procedures*. Springer Science & Business Media. http://www.dx.doi.org/10.1007/978-0-387-33349-6

Fugelsang, K. C. (1997). Yeasts and molds. In *Wine microbiology* (pp. 68-116). Springer, Boston, MA.

Fugelsang, K. C., Osborn, M. M., & Muller, C. J. (1993). Brettanomyces and Dekkera: Implications in wine making.

Fukaya, M., Tayama, K., Okumura, H., Kawamura, Y., Beppu, T., 1989. Purification and characterization of membrane-bound aldehyde dehydrogenase from Acetobacter polyoxogenes sp. nov. *Agricultural and Biological Chemistry* 32, 176–180.

Garvie, E. I. (1967). Leuconostoc oenos sp. nov. *Microbiology*, *48*(3), 431-438.

Gerbaux, V., Briffox, C., Dumont, A. & Krieger, S. (2009). Influence of Inoculation with Malolactic Bacteria on Volatile Phenols in Wines. *American journal of enology and viticulture,* **60**(2), 234-235.

Gerbaux, V., Vincent, B. & Bertrand, A. (2002). Influence of maceration temperature and enzymes on the content of volatile phenols in Pinot noir wines. *American journal of enology and viticulture*, **53**, 131-137.

Gilliland, R. B. (1961). Brettanomyces. I. Occurrence, characteristics, and effects on beer flavour. *Journal of the Institute of Brewing*, *67*(3), 257-261.

Gómez-Rivas, L., Escudero-Abarca, B., Aguilar-Uscanga, M., Hayward-Jones, P., Mendoza, P. & Ramirez, M. (2004). *Journal ofindustrial microbiology and biotechnology,* **31**(1), 16-22. https://doi.org/10.1007/s10295-004-0112-2

González-Arenzana, L., Santamaría, P., López, R., Garijo, P., Gutiérrez, A., Garde-Cerdán, T. & López-Alfaro, L. (2013). Microwave technology as a new tool to improve microbiological control of oak barrels: a preliminary study. *Food control*, **30**(2), 536-539. https://doi.org/10.1016/j.foodcont.2012.08.008

Gutiérrez, A. R., Epifanio, S., Garijo, P., López, R., & Santamaría, P. (2001). Killer yeasts: incidence in the ecology of spontaneous fermentation. *American Journal of Enology and Viticulture*, *52*(4), 352-356.

Guzzo, J., Cavin, J. F., & Divies, C. (1994). Induction of stress proteins inLeuconostoc oenos to perform direct inoculation of wine. *Biotechnology letters*, *16*(11), 1189-1194.

Guzzon, R., Larcher, R., Guarcello, R., Francesca, N., Settanni, L., & Moschetti, G. (2018). Spoilage potential of brettanomyces bruxellensis strains isolated from Italian wines. *Food Research International, 105*, 668-677. doi:10.1016/j.foodres.2017.11.078

Heard, G. M., & Fleet, G. H. (1988). The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *Journal of Applied Bacteriology*, *65*(1), 23-28.

Heard, G. M., & Fleet, G. H. (1985). Growth of natural yeast flora during the fermentation of inoculated wines. *Applied and Environmental Microbiology*, *50*(3), 727-728.

Hellborg, L., & Piškur, J. (2009). Complex nature of the genome in a wine spoilage yeast, Dekkera bruxellensis. *Eukaryotic cell*, *8*(11), 1739-1749.

Henick-Kling, T. & Park, Y.H. (1994). Considerations for the Use of Yeast and Bacterial Starter Cultures: SO$_2$ and Timing of Inoculation. *Am. J. Enol. Vitic.*, **45**.

Henick-Kling, T. 1993. Malolactic Fermentation. In ''Wine Microbiology and Biotechnology'' (G.H. Fleet, ed.), pp. 286–326. Harwood Academic Publishers, Switzerland.

Heresztyn, T. (1986). Metabolism of volatile phenolic compounds from hydroxycinnamic acids byBrettanomyces yeast. *Archives of Microbiology*, *146*(1), 96-98.

Herskowitz, I. (1988). Life cycle of the budding yeast Saccharomyces cerevisiae. *Microbiological reviews*, **52**, 536–553.

Hesford F., Schneider K., Porret N. A. & Gafner J. (2004). Identification and analysis of 4-ethyl catechol in wine tainted by Brettanomyces off-flavor. Abstract. *American journal of enology and viticulture,* **55**(3):304A.

Hinze, H., & Holzer, H. (1986). Analysis of the energy metabolism after incubation of Saccharomyces cerevisiae with sulfite or nitrite. *Archives of microbiology*, *145*(1), 27-31.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. (1994). Genus Acetobacter and Gluconobacter. In ''Bergey's Manual of Determinative Bacteriology'' (J.G. Holt, ed.), pp. 71–84. Williams & Wilkins, MD.

Honigberg, S. M. (2011). Cell signals, cell contacts, and the organization of yeast communities. *Eukaryotic cell*, *10*(4), 466-473.

Hood, A. (1983). Inhibition of growth of wine lactic-acid bacteria by acetaldehyde-bound sulphur dioxide. *Aust. Grapegrow. Winemaker*, *232*, 34-43.

Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., ... & Nickerson, K. W. (2001). Quorum sensing in the dimorphic fungusCandida albicans is mediated by farnesol. *Applied and environmental microbiology*, *67*(7), 2982-2992.

Ivanova, V., Vojnoski, B. & Stefova, M. (2012). Effect of winemaking treatment and wine aging on phenolic content in Vranec wines. *Journal of food science and technology*, **49**, 161–172. http://dx.doi.org/10.1007/s13197-011-0279-2

Jackson, R. S. (2020). *Wine science: Principles and applications*. Cambridge: Elsevier.

Jeon, S., Kim, N., Shim, M., Jeon, Y., Ahn, J., Lee, S., Hwang, I. & Rhee, M. (2015). Microbiological Diversity and Prevalence of Spoilage and Pathogenic Bacteria in Commercial Fermented Alcoholic Beverages (Beer, Fruit Wine, Refined Rice Wine, and Yakju). *Journal of food protection*, **78**(4), 812–818. http://dx.doi.org/10.4315/0362-028X.JFP-14-431

Jiranek, V., Grbin, P., Yap, A., Barnes, M. & Bates, D. (2008). High power ultrasonics as a novel tool offering new opportunities for managing wine microbiology. *Biotechnology letters*, **30**(1), 1-6. https://doi.org/10.1007/s10529-007- 9518-z

Joseph, C. L., Kumar, G., Su, E., & Bisson, L. F. (2007). Adhesion and biofilm production by wine isolates of Brettanomyces bruxellensis. *American journal of enology and viticulture*, *58*(3), 373-378.

Joyeux, A., Lafon-Lafourcade, S., and Ribe´reau-Gayon, P. (1984a). Evolution of acetic acid bacteria during fermentation and storage of wine. *Appl. Environ. Microbiol*. 48, 153–156

Joyeux, A., Lafon-Lafourcade, S., and Ribe´reau-Gayon, P. (1984b). Metabolism of acetic acid bacteria in grape must. Consequences on alcoholic and malolactic fermentation. *Sci. Aliments* 4, 247–255

Knoll, C., Divol, B., & Du Toit, M. (2008). Genetic screening of lactic acid bacteria of oenological origin for bacteriocin-encoding genes. *Food microbiology*, *25*(8), 983-991.

Kolfschoten, G.A., Yarrow, D. (1970). *Brettanomyces naardenensis*, a new yeast from soft drinks. *Antonie van Leeuwenhoek* **36,** 458–460. https://doi.org/10.1007/BF02069047

König, H., Unden, G. & Fröhlich, J. (2009). *Biology of Microorganisms on Grapes, in Must and in Wine*. 1st edn. Springer-Verlag Berlin Heidelberg. http://dx.doi.org/10.1007/978-3-540-85463-0

Korkes, S., Campillo, A. D., & Ochoa, S. (1950). Biosynthesis of dicarboxylic acids by carbon dioxide fixation. IV. Isolation and properties of an adaptive" malic" enzyme from Lactobacillus arabinosus. *Journal of Biological Chemistry*, *187*(2), 891-905.

Krieger, S. A., Hammes, W. P., & Henick-Kling, T. (1993). How to use malolactic starter cultures in the winery. *Australian and New Zealand Wine Industry Journal*, *8*, 153-153.

Kunkee, R. E. (1968). Malo-lactic fermentation. In *Advances in Applied Microbiology* (Vol. 9, pp. 235-279). Academic Press.

Lambrechts, M. G., & Pretorius, I. S. (2000). Yeast and its importance to wine aroma-a review. *South African Journal of Enology and Viticulture*, *21*(1), 97-129.

Lasik-Kurdyś, M., & Sip, A. (2019). Evaluation of the antimicrobial activity of bacteriocin-like inhibitory substances of enological importance produced by Oenococcus oeni isolated from wine. *European Food Research and Technology*, *245*(2), 375-382.

Leistner, L. (1992). Food preservation by combined methods. *Food Research International, 25*(2), 151-158. doi:10.1016/0963-9969(92)90158-2

Leistner, X. (1978). Hurdle effect and energy saving. WK Downey (ed.) Food Quality and Nutrition.

Li, F., & Palecek, S. P. (2008). Distinct domains of the Candida albicans adhesin Eap1p mediate cell–cell and cell–substrate interactions. *Microbiology*, *154*(4), 1193-1203.

Licker, J.L., Acree, T.E. & Henick-Kling, T. (1998). What Is "Brett" Brettanomyces Flavor?: A Preliminary Investigation. In: *Chemistry of Wine Flavor*, ACS Symposium Series. p. 96–115. American Chemical Society. http://dx.doi.org/10.1021/bk-1998-0714.ch008

Lilly, M., Bauer, F.F., Styger, G., Lambrechts, M.G. & Pretorius, I.S. (2006). The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. FEMS yeast research. http://dx.doi.org/10.1111/j.1567-1364.2006.00057.x

Liu, S.-Q. (2002). A review: malolactic fermentation in wine -- beyond deacidification. *Journal of applied microbiology*, **92**(4), 589–601. https://doi.org/10.1046/j.1365-2672.2002.01589

Liu, S.-Q., Pritchard, G.G., Hardman, M.J., and Pilone, G.J. (1994). Citrulline production and ethyl carbamate (urethane) precursor formation from arginine degradation by wine lactic acid bacteria Leuconostoc oenos and Lactobacillus buchneri. *American journal of enology and viticulture*. 45, 235–242

Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. In *Lactic acid bacteria: Genetics, metabolism and applications* (pp. 317-331

Lonvaud-Funel, A. (1996). Microorganisms of winemaking. *Cerevisia*, *21*(1), 55-58.

Lonvaud-Funel, A., Joyeux, A., & Desens, C. (1988). Inhibition of malolactic fermentation of wines by products of yeast metabolism. *Journal of the Science of Food and Agriculture*, *44*(2), 183-191.

Magliani, W., Conti, S., Gerloni, M., Bertolotti, D., & Polonelli, L. (1997). Yeast killer systems. *Clinical microbiology reviews*, *10*(3), 369-400.

Malfeito-Ferreira, M. (2011). Yeasts and wine off-flavours: a technological perspective. *Annals of microbiology*, **61**, 95–102. http://dx.doi.org/10.1007/s13213- 010-0098-0

Malfeito-Ferreira M, Laureano P, Barata A, D'antuono I, Stender H, Loureiro V. (2004). Effect of different barrique sanitation procedures on yeasts isolated from the inner layers of wood. Abstract. *American journal of enology and viticulture*, **55**, 304A.

Malherbe, S., Menichelli, E., Toit, M. du, Tredoux, A., Muller, N., Naes, T. & Nieuwoudt, H. (2013). The relationships between consumer liking, sensory and chemical attributes of Vitis vinifera L. cv. Pinotage wines elaborated with different Oenococcus oeni starter cultures. *Journal of the science of food and agriculture*, **93**, 2829–2840. http://dx.doi.org/10.1002/jsfa.6115

Martineau, B., & Henick-Kling, T. (1995a). Formation and degradation of diacetyl in wine during alcoholic fermentation with Saccharomyces cerevisiae strain EC1118 and malolactic fermentation with Leuconostoc oenos strain MCW. *American Journal of Enology and Viticulture*, *46*(4), 442-448.

Martineau, B., & Henick-Kling, T. (1995b). Performance and diacetyl production of commercial strains of malolactic bacteria in wine. *Journal of applied bacteriology*, *78*(5), 526-536.

Martineau, B., Henick-Kling, T. and Acree, T. (1995). Reassessment of the influence of malolactic fermentation on the concentration of diacetyl in wines. *American Journal of Enology and Viticulture* **46**, 385–388.

Martini, A. (1993). Origin and domestication of the wine yeast Saccharomyces cerevisiae. *Journal of wine research / the Institute of Masters of Wine*, **4**, 165–176. http://dx.doi.org/10.1080/09571269308717966

McGovernMacGovern, P. E. (2003). *Ancient wine: The search for the origins of viniculture*. Princeton: Princeton Unaiversity Press.

Mcmeekin, T. A., Presser, K., Ratkowsky, D., Ross, T., Salter, M., & Tienungoon, S. (2000). Quantifying the hurdle concept by modelling the bacterial growth/no growth interface. *International Journal of Food Microbiology, 55*(1-3), 93-98. doi:10.1016/s0168-1605(00)00182-3

Mehlomakulu, N. N., Setati, M. E., & Divol, B. (2014). Characterization of novel killer toxins secreted by wine-related non-Saccharomyces yeasts and their action on Brettanomyces spp. *International journal of food microbiology*, *188*, 83-91.

Meng, Z., & Zhang, L. (1992). Cytogenetic damage induced in human lymphocytes by sodium bisulfite. *Mutation Research/Genetic Toxicology*, *298*(2), 63-69.

Michlmayr, H., Nauer, S., Brandes, W., Schümann, C., Kulbe, K.D., Hierro, A.M. del & Eder, R. (2012). Release of wine monoterpenes from natural precursors by glycosidases from Oenococcus oeni. *Food chemistry*, **135**, 80–87. http://dx.doi.org/10.1016/j.foodchem.2012.04.099

Mira de Orduña, R., Liu, S. Q., Patchett, M. L., & Pilone, G. J. (2000). Ethyl carbamate precursor citrulline formation from arginine degradation by malolactic wine lactic acid bacteria. *FEMS microbiology Letters*, *183*(1), 31-35.

Møretrø, T. & Daeschel, M.A. (2006). Wine is Bactericidal to Foodborne Pathogens. *Journal of food science*, **69**, M251–M257. http://dx.doi.org/10.1111/j.1365-2621.2004.tb09938.x

Mukai, F., Hawryluk, I., & Shapiro, R. (1970). The mutagenic specificity of sodium bisulfite. *Biochemical and biophysical research communications*, *39*(5), 983-988.

Musmanno, R. A., Di Maggio, T., & Coratza, G. (1999). Studies on strong and weak killer phenotypes of wine yeasts: production, activity of toxin in must, and its effect in mixed culture fermentation. Journal of applied microbiology, 87(6), 932-938.

Nelson, C. M., & Chen, C. S. (2002). Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal. *FEBS letters*, *514*(2-3), 238-242.

Nielsen, J. C., & Richelieu, M. (1999). Control of flavor development in wine during and after malolactic fermentation by Oenococcus oeni. *Applied and environmental microbiology*, *65*(2), 740-745.

Nielsen, J. C., Prahl, C., & Lonvaud-Funel, A. (1996). Malolactic fermentation in wine by direct inoculation with freeze-dried Leuconostoc oenos cultures. *American Journal of Enology and Viticulture*, *47*(1), 42-48.

Nissen, P., Nielsen, D., & Arneborg, N. (2003). Viable Saccharomyces cerevisiae cells at high concentrations cause early growth arrest of non-Saccharomyces yeasts in mixed cultures by a cell–cell contact-mediated mechanism. *Yeast*, *20*(4), 331-341.

Oelofse, A., Pretorius, I.S. & Toit, M. du. (2008). Significance of Brettanomyces and Dekkera during Winemaking: A Synoptic Review. *South African Journal of Enology and Viticulture*, **29**(2), 128-144. https://doi.org/10.21548/29-2-1445

Ohkuni, K., Hayashi, M., & Yamashita, I. (1998). Bicarbonate-mediated social communication stimulates meiosis and sporulation of Saccharomyces cerevisiae. *Yeast*, *14*(7), 623-631.

Osborne, J. P., & Edwards, C. G. (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, J.P. & Edwards, C.G. (2005). Bacteria Important during Winemaking. *Advances in food and nutrition research*, **50**, 139–177. http://dx.doi.org/10.1016/S1043-4526(05)50005-6

Oswald, T. A., & Edwards, C. G. (2017). Interactions between Storage Temperature and Ethanol that Affect Growth of Brettanomyces bruxellensis in Merlot Wine. *American Journal of Enology and Viticulture, 68*(2), 188-194. doi:10.5344/ajev.2017.16102

Ough, C. S., Crowell, E. A., & Gutlove, B. R. (1988). Carbamyl compound reactions with ethanol. *American Journal of Enology and Viticulture*, *39*(3), 239-242.

Ough, C. S., Kunkee, R. E., Vilas, M. R., Bordeu, E., & Huang, M. C. (1988). The interaction of sulfur dioxide, pH, and dimethyl dicarbonate on the growth of Saccharomyces cerevisiae Montrachet and Leuconostoc oenos MCW. *American journal of enology and viticulture*, *39*(4), 279-282.

Ough, C. S., Langbehn, L. L., & Stafford, P. A. (1978). Influence of pH and ethanol on the effectiveness of dimethyl dicarbonate in controlling yeast growth in model wine systems. *American Journal of Enology and Viticulture*, *29*(1), 60-62.

Pagano, D. A., Zeiger, E., & Stark, A. A. (1990). Autoxidation and mutagenicity of sodium bisulfite. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *228*(1), 89-96.

Pagano, D. A., & Zeiger, E. (1987). Conditions affecting the mutagenicity of sodium bisulfite in Salmonella typhimurium. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *179*(2), 159-166.

Palkova, Z., Janderová, B., Gabriel, J., Zikánová, B., Pospíŝek, M., & Forstová, J. (1997). Ammonia mediates communication between yeast colonies. *Nature*, *390*(6659), 532-536.

Pardo, I., García, M. J., Zúñiga, M., & Uruburu, F. (1989). Dynamics of microbial populations during fermentation of wines from the Utiel-Requena region of Spain. *Applied and environmental microbiology*, *55*(2), 539-541.

Park, H., & Bakalinsky, A. T. (2000). SSU1 mediates sulphite efflux in Saccharomyces cerevisiae. *Yeast*, *16*(10), 881-888.

Peynaud, E., 1984. *Knowing and making wine*. John Wiley & Sons

Peynaud, E., & Domercq, S. (1956). Sur les Brettanomyces isolés de raisins et de vins. *Archiv für Mikrobiologie*, *24*(3), 266-280.

Pilkington, B. J., & Rose, A. H. (1988). Reactions of Saccharomyces cerevisiae and Zygosaccharomyces bailii to sulphite. *Microbiology*, *134*(10), 2823-2830.

Pilone, G. J. (1995). A New Zealand experience in direct-vat inoculation for malolactic fermentation. *Australian and New Zealand Wine Industry Journal*, *10*, 169-169.

Pilone, G. J., & Kunkee, R. E. (1972). Characterization and energetics of Leuconostoc oenos ML 34. *American Journal of Enology and Viticulture*, *23*(2), 61-70.

Pilone, G. J., & Kunkee, R. E. (1970). Carbonic acid from decarboxylation by "malic" enzyme in lactic acid bacteria. *Journal of bacteriology*, *103*(2), 404-409.

Pina, C., Santos, C., Couto, J. A., & Hogg, T. (2004). Ethanol tolerance of five non-Saccharomyces wine yeasts in comparison with a strain of Saccharomyces cerevisiae—influence of different culture conditions. *Food Microbiology*, *21*(4), 439-447.

Porter, L. J., & Ough, C. S. (1982). The effects of ethanol, temperature, and dimethyl dicarbonate on viability of Saccharomyces cerevisiae Montrachet No. 522 in wine. *American Journal of Enology and Viticulture*, *33*(4), 222-225.

Ramirez, M., Arroyo-López, F., Garrido-Fernández, A., Querol, A., Mercado, L., Sturm, M., & Combina, M. (2014). Probabilistic model for the spoilage wine yeast Dekkera bruxellensis as a function of pH, ethanol and free SO_2 using time as a dummy variable. *International Journal of Food Microbiology, 170*, 83-90. doi:10.1016/j.ijfoodmicro.2013.10.019

Ramos, A., Lolkema, J.S., Konings, W.N. & Santos, H. (1995). Enzyme Basis for pH Regulation of Citrate and Pyruvate Metabolism by Leuconostoc oenos. *Applied and environmental microbiology*, **61**(4), 1303–1310.

Rankine, B. C., & Pocock, K. F. (1969). Influence of yeast strain on binding of sulphur dioxide in wines, and on its formation during fermentation. *Journal of the Science of Food and Agriculture*, *20*(2), 104-109.

Rankine, B.C., Fornachon, J.C.M., and Bridson, D.A. 1969. Diacetyl in Australian dry red wines and its significance in wine quality. *Vitis* **8**, 129–134.

Rehm, HJ, & Wittmann, H. (1963). Contribution to the knowledge of the antimicrobial effect of sulphurous acid. *Journal of Food Study and Research* , *120* (6), 465-478.Stratford M, Rose IM (1986) Transport of sulphur dioxide by *Saccharomyces cerevisiae*. *J Gen Microbiol* 132:1–6

Rehm, HJ, & Wittmann, H. (1962). Contribution to the knowledge of the antimicrobial effect of sulphurous acid I. Communication Overview of influencing factors on the antimicrobial effect of sulphurous acid. *Journal of Food Study and Research* , *118* (5), 413-429.

Renault, P. E., Albertin, W., & Bely, M. (2013). An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Applied microbiology and biotechnology*, *97*(9), 4105-4119.

Renouf, V., & Murat, M. L. (2008). L'utilisation de levains malolactiques pour une meilleure maîtrise du risque Brettanomyces. *Revue des oenologues et des techniques vitivinicoles et oenologiques: magazine trimestriel d'information professionnelle*, *35*(126), 11-14.

Renouf, V., Falcou, M., Miot-Sertier, C., Perello, M.C., De Revel, G. & Lonvaud- Funel, A. (2006). Interactions between Brettanomyces bruxellensis and other yeast species during the initial stages of winemaking. *Journal of applied microbiology*, **100**, 1208–1219. http://dx.doi.org/10.1111/j.1365-2672.2006.02959.x

Ribéreau-Gayon, P., Dubourdieu, D., Doneche, B., & Lonvaud, A. (2006). The use of sulfur dioxide in must and wine treatment. *Handbook of Enology. The Microbiology of Wine and Vinifications,*, 193-197.

Ribéreau-Gayon, P., Glories, Y., Maujean, A., & Dubourdieu, D. (Eds.). (2006). *Handbook of Enology, Volume 2: The Chemistry of Wine-Stabilization and Treatments* (Vols. 1 & 2). John Wiley & Sons.

Ripper, M. (1892). The sulphurous acid in wine and its determination (information from Schmitt's laboratory, [investigation office, chemical test station and hygienic institute] in Wiesbaden). *Journal of Practical Chemistry* , *46* (1), 428-473.

Rodriguez, S. B., Amberg, E., Thornton, R. J., & McLellan, M. R. (1990). Malolactic fermentation in Chardonnay: growth and sensory effects of commercial strains of Leuconostoc oenos. *Journal of Applied Bacteriology*, *68*(2), 139-144.

Roller, S., & Covill, N. (1999). The antifungal properties of chitosan in laboratory media and apple juice. *International journal of food microbiology*, *47*(1-2), 67-77.

Romano, P. and Suzzi, G. (1993). Sulphur dioxide and wine microorganisms. In ''Wine Microbiology and Biotechnology'' (G.H. Fleet, ed.), pp. 373–393. Harwood Academic Publishers, Switzerland.

Rozpedowska, E., Hellborg, L., Ishchuk, O.P., Orhan, F., Galafassi, S., Merico, A., Woolfit, M., Compagno, C. et al. (2011). Parallel evolution of the make-accumulate-consume strategy in Saccharomyces and Dekkera yeasts. *Nat Comms*, 2, 302. doi:10.1038/ncomms1305.

Ruiz, A., Poblet, M., Mas, A., & Guillamón, J. M. (2000). Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *International Journal of Systematic and Evolutionary Microbiology*, *50*(6), 1981-1987.

Rutherford, S. T., and Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* 2, 1–25. doi: 10.1101/cshperspect.a012427

Salema, M., Poolman, B., Lolkema, J. S., Dias, M. C. L., & Konings, W. N. (1994). Uniport of monoanionic L-malate in membrane vesicles from Leuconostoc oenos. *European journal of biochemistry*, *225*(1), 289-295.

Santos, A., San Mauro, M., Bravo, E., & Marquina, D. (2009). PMKT2, a new killer toxin from Pichia membranifaciens, and its promising biotechnological properties for control of the spoilage yeast Brettanomyces bruxellensis. *Microbiology*, *155*(2), 624-634.

Santos, M. S. (1996). Biogenic amines: their importance in foods. *International journal of food microbiology*, *29*(2-3), 213-231.

Schmid, F., Grbin, P., Yap, A. & Jiranek, V. (2011). Relative efficacy of high- pressure hot water and high-power ultrasonics for wine oak barrel sanitization. *American journal of enology and viticulture,* **62**(4), 519-526. http://dx.doi.org/10.5344/ajev.2011.11014

Schuller, D., Alves, H., Dequin, S. & Casal, M. (2005). Ecological survey of Saccharomyces cerevisiae strains from vineyards in the Vinho Verde Region of Portugal. *FEMS microbiology ecology*, **51**, 167–177. http://dx.doi.org/10.1016/j.femsec.2004.08.003

Serpaggi, V., Remize, F., Recorbet, G., Gaudot-Dumas, E., Sequeira-Le Grand, A., & Alexandre, H. (2012). Characterization of the "viable but nonculturable"(VBNC) state in the wine spoilage yeast Brettanomyces. *Food microbiology*, *30*(2), 438-447.

Shimizu, K. (1993). Killer yeasts. *Wine Microbiology and Biotechnology*, 243-264.

Silva, P., Cardoso, H. & Gerós, H. (2004). Studies on the Wine Spoilage Capacity of Brettanomyces/Dekkera spp. *American journal of enology and viticulture,* **55**(1), 65- 72.

Smith, M. (2011). Brettanomyces Kufferath & van Laer. *The Yeasts,* 983-986. doi:10.1016/b978-044481312-1/50068-x

Sponholz, W. R. (1993). Wine spoilage by microorganisms. *Wine microbiology and biotechnology*, 395-420.

Sponholz, W.R. 1993. Wine spoilage by microorganisms. In ''Wine Microbiology and Biotechnology'' (G.H. Fleet, ed.), pp. 395–420. Harwood Academic Publishers, Switzerland.

Sponholz, WR, & Brendel, M. (2004). Formation of sugar acids by acetic acid bacteria on grapes and in wine. *Messages Klosterneuburg vine and wine* , *54* , 77-85.

Sprague, G. F., & Winans, S. C. (2006). Eukaryotes learn how to count: quorum sensing by yeast. *Genes & development*, *20*(9), 1045-1049.

Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H. & Verstrepen, K. (2015). Brettanomyces yeasts – from spoilage organisms to valuable contributors to industrial fermentations. *International journal of food microbiology,* **206**, 24-38. https://doi.org/10.1016/j.ijfoodmicro.2015.04.005

Strathern, J. N., Jones, E. W., & Broach, J. R. (1982). *Molecular biology of the yeast Saccharomyces*. Cold Spring Harbor Laboratory.

Strickland, M. T. (2012). Effects of Pediococcus spp. on Oregon Pinot noir.

Sturm, M., Arroyo-López, F., Garrido-Fernández, A., Querol, A., Mercado, L., Ramirez, M., & Combina, M. (2014). Probabilistic model for the spoilage wine yeast Dekkera bruxellensis as a function of pH, ethanol and free SO_2 using time as a dummy variable. *International Journal of Food Microbiology, 170*, 83-90. doi:10.1016/j.ijfoodmicro.2013.10.019

Suarez, R., Sua´rez-Lepe, J.A., Morata, A. and Calderon, F. (2007). The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. *Food Chem* 102, 10–21.

Sugita-Konishi, Y., Hara-Kudo, Y., Iwamoto, T. & Kondo, K. (2001). Wine has activity against entero-pathogenic bacteria in vitro but not in vivo. *Bioscience, biotechnology, and biochemistry*, **65**, 954–957. http://dx.doi.org/10.1271/bbb.65.954

Suzzi, G., Romano, P., & Zambonelli, C. (1985). Saccharomyces strain selection in minimizing SO_2 requirement during vinification. *American journal of enology and viticulture*, *36*(3), 199-202.

Swiegers, J.H., Kievit, R.L., Siebert, T., Lattey, K.A., Bramley, B.R., Francis, I.L., King, E.S. & Pretorius, I.S. (2009). The influence of yeast on the aroma of Sauvignon Blanc wine. *Food microbiology*. http://dx.doi.org/10.1016/j.fm.2008.08.004

Swiegers, J.H., Capone, D.L., Pardon, K.H., Elsey, G.M., Sefton, M.A., Francis, I.L. & Pretorius, I.S. (2007). Engineering volatile thiol release in Saccharomyces cerevisiae for improved wine aroma. *Yeast*. http://dx.doi.org/10.1002/yea.1493

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A. & Pretorius, I.S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian journal of grape and wine research*, **11**, 139–173. http://dx.doi.org/10.1111/j.1755-0238.2005.tb00285.x

Temple, D. D. (1978). *The inactivation of yeast alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase by dimethyldicarbonate*. U. of Calif., Davis.

Terrade, N. & Mira de Orduña, R. (2009). Determination of the essential nutrient requirements of wine-related bacteria from the genera Oenococcus and Lactobacillus. *International journal of food microbiology*, **133**, 8–13. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.03.020

Threlfall, R. T., & Morris, J. R. (2002). Using dimethyldicarbonate to minimize sulfur dioxide for prevention of fermentation from excessive yeast contamination in juice and semi-sweet wine. *Journal of food science*, *67*(7), 2758-2762.

Torija, M. J., Rozes, N., Poblet, M., Guillamón, J. M., & Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Antonie Van Leeuwenhoek*, *79*(3-4), 345-352.

Török, T., Mortimer, R.K., Romano a, P., Suzzi, G. & Polsinelli, M. (1996). Quest for wine yeasts--an old story revisited. *Journal of industrial microbiology*, **17**, 303–313. https://doi.org/10.1007/BF01574705

Tristezza, M., Lourenço, A., Barata, A., Brito, L., Malfeito-Ferreira, M. & Loureiro, V. (2010). Susceptibility of wine spoilage yeasts and bacteria in the planktonic state and in biofilms to disinfectants. *Annals of microbiology*, **60**(3), 549–556. http://dx.doi.org/10.1007/s13213-010-0085-5

Ugliano, M. & Moio, L. (2005). Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of Oenococcus oeni. *Journal of agricultural and food chemistry*, **53**(26), 10134–10139. http://dx.doi.org/10.1021/jf0514672

Ugliano, M., Genovese, A. & Moio, L. (2003). Hydrolysis of wine aroma precursors during malolactic fermentation with four commercial starter cultures of Oenococcus oeni. *Journal of agricultural and food chemistry*, **51**, 5073–5078. http://dx.doi.org/10.1021/jf0342019

Uscanga, M. G. A., Delia, M. L., & Strehaiano, P. (2000). Nutritional requirements of Brettanomyces bruxellensis: growth and physiology in batch and chemostat cultures. *Canadian journal of microbiology*, *46*(11), 1046-1050.

van der Walt, J., & van Kerken, A. (1961). The wine yeasts of the cape. V. Studies on the occurrence of Brettanomyces intermedius and Brettanomyces schanderlii. *Antonie van Leeuwenhoek*, *27*, 81–90. https://doi.org/10.1007/BF02538426

Van Dijken, J. & Scheffers, W. (1986). Redox balances in the metabolism of sugars by yeasts. *FEMS microbiology letters,* **32**, 199-224. http://dx.doi.org/10.1111/j.1574-6968.1986.tb01194.x

Van Vuuren, H. J. J., & Dicks, L. M. T. (1993). Leuconostoc oenos: a review. *American Journal of Enology and Viticulture*, *44*(1), 99-112.

Van Vuuren, H. J. J., & Jacobs, C. J. (1992). Killer yeasts in the wine industry: a review. American Journal of Enology and Viticulture, 43(2), 119-128.

Varela, C., Siebert, T., Cozzolino, D., Rose, L., McLean, H. & Henschke, P.A. (2009). Discovering a chemical basis for differentiating wines made by fermentation with "wild" indigenous and inoculated yeasts: Role of yeast volatile compounds. *Australian journal of grape and wine research*. http://dx.doi.org/10.1111/j.1567- 1364.2006.00161.x

Ventre, J. (1934). *Les levures en vinification*. Bureaux de la Revue de Viticulture.

Vigentini, I., Joseph, C.M., Picozzi, C., Foschino, R. & Bisson, L.F. (2013). Assessment of the Brettanomyces bruxellensis metabolome during sulphur dioxide exposure. *FEMS yeast research*, **13**(7), 597–608. http://dx.doi.org/10.1111/1567- 1364.12060

Vigentini I, De Lorenzis G, Picozzi C, Imazio S, Merico A, Galafassi S, Piskur J & Foschino R (2012). Intraspecific variations of *Dekkera/Brettanomyces bruxellensis* genome studied using capillary electrophoresis separation of the Intron Splice Site profiles. *Int J Food Microbiol* 157: 6–15

Vigentini, I., Romano, A., Compagno, C., Merico, A., Molinari, F., Tirelli, A., Foschino, R. & Volonterio, G. (2008). Physiological and oenological traits of different Dekkera/Brettanomyces bruxellensis strains under wine-model conditions. *FEMS yeast research*, **8**(7), 1087–1096. http://dx.doi.org/10.1111/j.1567- 1364.2008.00395.x

von Cosmos, N. & Edwards, C.G. (2016). Use of Nutritional Requirements for Brettanomyces bruxellensis to Limit Infections in Wine. *Fermentation*, **2**(3), 17. http://dx.doi.org/10.3390/fermentation2030017

Walling, E., Dols-Lafargue, M. & Lonvaud-Funel, A. (2005). Glucose fermentation kinetics and exopolysaccharide production by ropy Pediococcus damnosus IOEB8801. *Food microbiology*, **22**, 71–78. http://dx.doi.org/10.1016/j.fm.2004.04.003

WARTH, A. D. (1985). Resistance of yeast species to benzoic and sorbic acids and to sulfur dioxide. *Journal of Food Protection*, *48*(7), 564-569.

Waterhouse, A. L., Sacks, G. L., & Jeffery, D. W. (2016). *Understanding wine chemistry*. John Wiley & Sons.

Waters, E. J., Peng, Z., Pocock, K. F., & Williams, P. J. (1996). The role of corks in oxidative spoilage of white wines. *Australian Journal of Grape and Wine Research*, *2*(3), 191-197.

Wedral, D., Shewfelt, R. & Frank, J. (2010). The challenge of Brettanomyces in wine. *LWT - Food Science and Technology*, **43**(10), 1474–1479. http://dx.doi.org/10.1016/j.lwt.2010.06.010

Wells, A., Osborne, J.P. (2011). Production of SO_2 binding compounds and SO_2 by Saccharomyces during alcoholic fermentation and the impact on malolactic fermentation. S. Afr. J. Enol. Vitic. 32, 267e279.

Wibowo, D., Eschenbruch, R., Davis, C. R., Fleet, G. H., & Lee, T. H. (1985). Occurrence and growth of lactic acid bacteria in wine: a review. *American Journal of Enology and Viticulture*, *36*(4), 302-313.

Wilker KL, Dharmadhikari MR (1997) Treatment of barrel wood infected with acetic acid bacteria. *American journal of enology and viticulture,* **48**, 516–520. http://www.ajevonline.org/content/48/4/516.full-text.pdf

Willenburg, E., & Divol, B. (2012). Quantitative PCR: an appropriate tool to detect viable but not culturable Brettanomyces bruxellensis in wine. *International journal of food microbiology*, *160*(2), 131-136.

Wisselinka, H.W., Weusthuisa, R.A., Egginka, G., Hugenholtza, J. & Grobbena, G.J. (2002). Mannitol production by lactic acid bacteria: a review. *International Dairy Journal*. http://dx.doi.org/10.1016/S0958-6946(01)00153-4

Xufre, A., Albergaria, H., Inácio, J., Spencer-Martins, I., & Gírio, F. (2006). Application of fluorescence in situ hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *International Journal of Food Microbiology*, *108*(3), 376-384.

Yang, S. C., Lin, C. H., Sung, C. T., & Fang, J. Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in microbiology*, *5*, 241.

Zamora F. (2003). Elaboración y crianza del vino tinto. Aspectos científicos y prácticos. Madrid: Editorial Mundi-Prensa)

Zamora F. (2002). La crianza del vino tinto sobre lías. Una nueva tendencia. Enólogos 19: 24–29.

Zhou, Q., Qian, Y. & Qian, M. (2015). Analysis of volatile phenols in alcoholic beverage by ethylene glycol-polydimethylsiloxane based stir bar sorptive extraction and gas chromatography-mass spectrometry. *Journal of chromatography a*, **1390**, 22-27. https://doi.org/10.1016/j.chroma.2015.02.064

Zuehlke, J., Glawe, D. & Edwards, C. (2015). Efficacy of dimethyl dicarbonate against yeasts associated with Washing state grapes and wines. *Journal of food processing and preservation*, **39**(6), 1016-1026. http://doi.org/10.1111/jfpp.12315

Zuehlke, J.M. & Edwards, C.G. (2013). Impact of sulfur dioxide and temperature on culturability and viability of Brettanomyces bruxellensis in Wine. *Journal of food protection*, **76**(12), 2024–2030. http://dx.doi.org/10.4315/0362-028X.JFP-13-243R

Zuehlke, J.M., Petrova, B. & Edwards, C.G. (2013). Advances in the control of wine spoilage by Zygosaccharomyces and Dekkera/Brettanomyces. *Annual review of food science and technology*, **4**, 57–78. http://dx.doi.org/10.1146/annurev-food-030212-182533