AN ABSTRACT OF THE THESIS OF

Matthew T. Strickland for the degree of Master of Science in Food Science and Technology presented on September 18, 2012.
Title: Effects of *Pediococcus* spp. on Oregon Pinot noir

Abstract approved:

James P. Osborne

This research investigated the effects of *Pediococcus* spp. on Oregon Pinot noir wines. *Pediococcus* (*P. parvulus* (7), *P. damnosus* (1), *P. inopinatus* (1)) isolated from Oregon and Washington state wines demonstrated differences in their susceptibility to SO_2 with some isolates growing well in model media at 0.4 mg/L molecular SO_2. All isolates were all able to degrade *p*-coumaric acid to 4-vinyl phenol. The conversion of *p*-coumaric acid to 4-VP by pediococci resulted in accelerated production of 4-EP by *B. bruxellensis* in a model system. Growth of the pediococci isolates in Pinot noir wine resulted in a number of chemical and sensory changes occurring compared to the control. Very low concentrations of biogenic amines were measured in the wines with only wine inoculated with *P. inopinatus* OW-8 having greater than 5 mg/L. D-lactic acid production varied between isolates with OW-7 producing the highest concentration (264 mg/L). Diacetyl content of the wines also varied greatly. Some wines contained very low levels of diacetyl (< 0.5 mg/L) while others contained very high concentrations (> 15 mg/L) that were well

above sensory threshold. Despite suggestions to the contrary in the literature, glycerol was not degraded by any of the isolates in this study. Color and polymeric pigment content of the wines also varied with wine inoculated with OW-7 containing 30% less polymeric pigment than the control. This may be related to acetaldehyde as a number of *Pediococcus* isolates, including OW-7, reduced the acetaldehyde content of the wine. Sensory analysis revealed differences in the aroma and mouthfeel of the wines compared to each other and to the control. In particular growth of some isolates produced wines with higher intensities of butter, plastic, and vegetal aromas while other also had lower perceived astringency.

Effects of *Pediococcus* spp. on Oregon Pinot noir

by
Matthew T. Strickland

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented September 18, 2012
Commencement June 2013

Master of Science thesis of Matthew T. Strickland presented on September 18, 2012.

APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

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

Matthew T. Strickland, Author

ACKNOWLEDGEMENTS

The author sincerely expresses his appreciation for the guidance and support given by Dr. James Osborne. The author also wishes to acknowledge the Northwest Center for Small Fruits Research for funding. The author would also like to thank all friends and family who provided support and encouragement throughout the completion of this work. Last but certainly not least the author would like to thank Kerri Welsh who's love and friendship have been unwavering and invaluable.

CONTRIBUTION OF AUTHORS

Dr. Michael Qian and Qin Zhou of OSU collaborated on the project and performed the diacetyl analysis. Stuart Chescheir performed volatile phenol analysis and monitored for hydroxycinnamic acid degradation. Victor Algazzali performed the sensory statistical analysis and provided guidance in interpreting their results. Dr. Charles Edwards of Washington State University provided guidance and Washington State pediococci isolates for this research. Dr. Rich DeScenzo of ETS Laboratories collaborated and provided biogenic amine analysis for this project. David Philbin also lent assistance on several aspects of this work.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

Chapter 1

Literature Review

Overview of the Winemaking Process

In a sense red wine has been made the same way for thousands of years with occasional technological innovations making improvements in efficiency and quality control. In red wine production grapes are harvested, destemmed, and may also be crushed. The grapes are left relatively intact for fermentation to occur with the juice having contact with the grape skins. Alcoholic fermentation is carried out by yeasts either naturally present on the grapes or through the addition of cultured yeasts by the winemaker. The yeast that most commonly performs the alcoholic fermentation is *Saccharomyces cerevisiae* as it has a high tolerance to ethanol and sulfur dioxide and can reliably complete fermentation where grape sugars are converted to ethanol and carbon dioxide.

After primary alcoholic fermentation is complete the grapes are pressed and the grape skins removed. The wine is then usually transferred into barrels for aging. During this aging period malolactic fermentation often occurs where Lactic Acid Bacteria (LAB) convert malic acid into lactic acid and CO_2, reducing the wine's perceived acidity. After malolactic fermentation is complete most red wines are left to age for a time until the winemaker decides the wine is ready for bottling. During this period the wine may be susceptible to spoilage organisms in the form of oxidative yeasts and lactic acid bacteria from a variety of sources, potentially compromising final wine

quality. To prevent this, the winemaker will make additions of the antimicrobial agent sulfur dioxide.

Lactic Acid Bacteria

Oenococcus oenii

O. oeni is perhaps the most important bacteria found in wine. Formerly labeled *Leuconostoc oenos*, after phylogenetic studies it was realized that *L. oenos* was actually a distinct subline separate from other *Leuconostoc* spp. and was subsequently placed in its own genus (Dicks et al. 1995). *O. oeni* are Gram-positive, coccoidal, facultatively anaerobic, and often found in chains (Fugelsang and Edwards 2007; Dicks and Endo 2009). They are heterofementative bacteria producing equal amounts of lactate, CO_2, and ethanol or acetate from the metabolism of glucose. Lactic acid is produced from glucose primarily in the D form and mannitol is formed from fructose metabolism (Fugelsang and Edwards 2007).

The main role of *O. oeni* in wine is the malolactic fermention (MLF) where L-malic acid is decarboxylated to L-lactic acid (Lonvaud-Funel 1999; Liu 2002; Bartowsky and Henschke 2004) reducing the perceived acidity of the wine. This reaction results in a proton motive force across the cell membrane and yields ATP from membrane bound ATPases (Olsen et al. 1991; Versari et al. 1999). This process is commonly performed in red wines from cooler climates and chardonnay.

O. oeni is found naturally on the grapes, in the winery, or may be inoculated into the wine by the addition of a starter. In addition to the conversion of malic acid to lactic acid, strains may also produce various secondary metabolites such as diacetyl, acetoin, and tetrahydropyridines, further altering flavor and aroma of the finished wine (Bartowsky and Henschke 2004; Bartowsky 2009). Although commonly thought of as a beneficial organism in red wine production, in white wines it may be viewed as a spoilage organism, lowering desired acidity and producing aroma compounds that may be objectionable in white wines.

The Genus *Lactobacillus*

Aside from *O. oeni* the other LAB that may be present in wine are *Lactobacillus* spp. and *Pediococcus* spp. The genus *Lactobacillus* is composed of numerous species deemed important to wine though they are almost universally viewed as spoilage organisms. They are Gram-positive, rod shaped bacteria that are either homofermentative or heterofermentative depending on species. They are often found as single cells though they can be found in chains. The metabolism of lactobacilli is fermentative where at least half of the resulting metabolites is composed of lactic acid.

With regards to other metabolites formed, species and strain variation exist. Lactobacilli have been found to produce acetic acid, formic acid, carbon dioxide, ethanol and diacetyl (Dicks and Endo 2009). Some strains are capable of producing acrolein from glycerol metabolism. Additionally it has been found that some strains

are capable of producing tetrahyrapyridines which are responsible for "mousy taint" in wine (Costello and Henschke 2002; Bartowsky 2009). *Lactobacillus* spp. have been implicated in vinyl and ethyl phenol production in wine though they are unable to produce concentrations of these compounds to the same extent as *Brettanomyces* spp. (Chatonnet et al. 1995; Dias et al. 2003; Couto et al. 2006; Rodríguez et al. 2008). Some strains of *Lactobacillus* spp. are also known to produce large amounts of biogenic amines such as tyramine and histamine (Moreno-Arribas et al. 2000; Landete et al. 2005; Coton et al. 2010)

***Pediococcus* spp.**

Morphology

Pediococci are coccoidal or ovoid, Gram-positive, and homofermentative. They are non-motile, catalase negative, and anaerobic to microaerophilic (Fugelsang and Edwards 2007). The cells are usually found in pairs or tetrads though single cells are also common. Chain formation has not been found. DL-Lactic acid is formed from the metabolism of glucose via the Embden-Meyerhof pathway while carbon dioxide and ethanol are not produced. The genus contains eight species though only a few have been observed in wine (Dicks and Endo 2009).

Species

P. damnosus is perhaps the most well-known of wine-related pediococci. Optimum pH is 5.5 and they cannot grow above 35°C. They are incapable of hydrolyzing

arginine. *P. damnosus* is closely related to *P. parvulus* and *P. inopinatus* (Dicks and Endo 2009).

P. damnosus is capable of producing numerous metabolites that may affect wine quality. In addition to lactate, diacetyl and acetoin are also produced by the species. It has also been implicated in the formation of long chain polysaccharides composed of beta-glucan often referred to as "ropiness" and cause increased wine viscosity (Dicks and Endo 2009).

Other wine-related *Pediococcus* species include *P. parvulus* which shows optimal growth at pH 6 and are unable to ferment pentoses. *P. inopinatus* grow best at 30°C, are genetically very similar to *P. parvulus,* and can be distinguished by comparing 16S RNA sequences (Dicks and Endo 2009). *P. pentosaceus* is distinguishable from *P. damnosus* and *P. parvulus* in that they can produce ammonia from arginine and can ferment arabinose. Cells of *P. pentosaceus* can grow well either aerobically or anaerobically at 30°C (Dicks and Endo 2009).

Some strains of *P. parvulus* have shown similar behavior to *P. damnosus* with regards to polysaccharide production and have also been shown to be responsible for histamine formation in wine (Dicks and Endo 2009). Some strains of *P. pentosaceus* have the ability to ferment mannitol and xylose (Fugelsang and Edwards 2007).

Spoilage by *Pediococcus* spp.

Polysaccharide Production

While *Pediococcus* has the potential to alter wine in a number of different ways, perhaps the most problematic of these alterations is that of polysaccharide production. Some species of *Pediococcus* have the ability to synthesize β-glucans in wine. These β-glucans tend to be homopolymers of repeating units consisting of D-glucose (Dueñas-Chasco et al. 1997). The resulting compounds may increase wine viscosity, create problems during filtration, and give the wine a "ropy" appearance. β-glucan contents of 20-30mg/L are enough to visibly alter wine texture (Walling et al. 2005a). This type of spoilage does not appear to affect wine flavor or aroma, but the texture is negatively affected to such a degree that the wine is often no longer commercially viable. This can cause severe economic losses to the winemaker as the wine quality is often unsalvageable (Delaherche et al. 2004; Walling et al. 2005a; Walling et al. 2005b; Dols-Lafargue et al. 2008).

The precise cause of polysaccharide production by *Pediococcus* remains unclear though it is believed that a plasmid based gene identified in many ropy strains may be the genetic source. The plasmid, pF8801contains a glucosyltransferase gene named *dps* (Walling et al. 2005a). The gene is believed to cause production of a transmembrane glucosyltransferase (Gtf) which causes the polymerization of UDP-glucose into β-glucan (Dols-Lafargue et al. 2008). The plasmid itself seems to be lost over successive transfers in non-alcoholic media (Walling et al. 2005b).

The conditions favoring polysaccharide synthesis are not well understood. It has been suggested that *Pediococcus* β-glucan production is linked to bacterial growth in

the wine and is due to a metabolic leak as UDP-glucose consumed for biomass during exponential growth phase gets diverted to glucan production when growth slows down (Walling et al. 2005a). It was observed that greater polysaccharide production took place in the presence of stress factors such as ethanol or SO_2 (Manca de Nadra and Strasser de Saad 1995). Others have argued that glucan production is enhanced by increased levels of glucose and available nitrogen, especially at lower temperatures when growth is slowed (Dueñas et al. 2003).

Diacetyl

Diacetyl is the compound responsible for the buttery aroma and flavor in some wines. The primary function of its production by *Pediococcus* spp. appears to be the regeneration of NADP (Ramos and Santos 1996). Aroma and flavor thresholds for diacetyl vary depending on wine type. Generally speaking, amounts between 1-4 ppm contribute a desirable butterscotch character whereas amounts above 5-7 ppm are often associated with spoilage (Bartowsky and Henschke 2004).

While wine yeasts may also produce diacetyl the pathway to its production is somewhat different than that of lactic acid bacteria. For *Pediococcus* spp. the formation of diacetyl comes from the oxidative decarboxylation of α-acetolactate in the pathway where pyruvate is converted to 2,3-butanediol (Ramos and Santos 1996). There are numerous factors that may encourage or discourage diacetyl production. It has been shown that glucose may stimulate diacetyl production, however this point is in contention as some have observed a decrease in diacetyl with residual fermentable

sugars (Escamilla et al. 2000; Bartowsky and Henschke 2004; Fugelsang and Edwards 2007). It has been shown that higher citric acid contents, higher storage temperatures, and lower pH possibly increase its production. Diacetyl can also be formed non-enzymatically by reduction of α-acetolactate when the wine has ample contact with air. Also, SO_2 will bind to diacetyl rendering it sensorially inactive, however the reaction has been shown to be transitory and diacetyl levels may rise again after several weeks of storage (Bartowsky and Henschke 2004; Fugelsang and Edwards 2007).

Some strains of *Pediococcus* can further reduce diacetyl to the compounds acetoin and 2,3-butanediol, both of which have higher aroma thresholds in wine and are less likely to have a sensory impact on the finished wine. Yeast will also perform this process. Wines that are left in contact with high yeast cell counts tend to have lower final diacetyl levels (Martineau and Henick-Kling 1996). *Pediococcus* spp. can cause spoilage levels of diacetyl because these bacteria tend to occur in wine at points after alcoholic fermentation has finished and yeast cell counts are low (Bartowsky and Henschke 2004; Fugelsang and Edwards 2007). However, to our knowledge little work has been done to elucidate *Pediococcus* spp. contributions to diacetyl contents in red wine.

Acrolein

Some lactic acid bacteria have been shown to produce acrolein precursors in wine and often *Pediococcus* spp. are considered among the organisms responsible. Acrolein is

a compound responsible for bitterness in wine. It is formed through the enzymatic degradation of glycerol to 3-hydroxypropionaldehyde (3-HPA) which in turn is spontaneously dehydrated to acrolein (Garai-Ibabe et al. 2008; Bauer et al. 2010a; Bauer et al. 2010b). Acrolein itself is not a bitter compound, but is thought to interact with as yet undetermined phenolic compounds in wine to contribute bitterness at concentrations as low as 10 ppm (Bauer et al. 2010b).

Glycerol is a major constituent of wine, being second in production by yeast after ethanol. Lactic acid bacteria form 3-HPA from glycerol by a reductive pathway. Glycerol is converted by B_{12}-dependent glycerol/diol dehydratases into 3-HPA. It can then be reduced to 1,3-propanediol or oxidized to 3-hydroxypropionic acid (Garai-Ibabe et al. 2008). If 3-HPA is not enzymatically reduced or oxidized however, it may be dehydrated to acrolein. Due to its high level of reactivity arcrolein is difficult to detect even though it may still affect wine sensory properties (Bauer et al. 2010b).

The conditions favoring the production of acrolein are poorly understood. There seems to be large amounts of strain variation, but there is evidence that winemaking practices favoring glycerol production by yeast such as high must-sugar concentrations and high fermentation temperatures might increase its production. High pH also seems to have a positive effect on acrolein production (Fugelsang and Edwards 2007). Higher cell concentrations may also increase levels of 3-HPA to a point, but it was observed that at higher cell concentrations its accumulation was

inhibited, presumably due to quorum sensing which is a mechanism that allows bacteria to express or unexpress various genes depending on surrounding cell density. It was also noted that acrolein can easily be bound by sulfite additions forming stable disulfonate adducts (Bauer et al. 2010a). While numerous studies have shown the capacity of *Lactobacillus* spp. to produce 3-HPA, we have not encountered any studies providing evidence for the capacity of *Pediococcus* spp. to produce 3-HPA.

It has been observed that some strains of *Pediococcus* can utilize glycerol as the sole carbon source. Glycerol was converted to D-lactate, acetate, and diacetyl through the glycerol kinase pathway (Pasteris and Stasser de Saad 2004; Pinto et al. 2004). Glycerol uptake appears to produce a proton motive force coupled with ATP synthesis (Pasteris and Strasser de Saad 2008). The extent to which *Pediococcus* opts for metabolizing glycerol through the glycerol kinase pathway versus reduction to 3-HPA has not been well documented.

Volatile Phenols

Volatile phenols are compounds responsible for wine aromas of barnyard, medicinal, and "horse sweat" (Chatonnet et al. 1995; Couto et al. 2006). The hydroxycinnamic acids *p*-coumaric acid and ferulic acid are the primary precursors for these compounds. Volatile phenols are formed in a two-step process where the precursor cinnamic acid is decarboxylated to a vinyl derivative and then reduced to an ethyl compound (Couto et al. 2006). The ethyl forms are usually associated with the presence of *Brettanomyces* spp. however, a few strains of *Lactobacillus* spp. have

been found capable of the final reducing step (Chatonnet et al. 1995; Dias et al. 2003; Couto et al. 2006; Rodríguez et al. 2008).

Volatile phenols have low aroma thresholds depending on the wine variety. A marked barnyard aroma can appear in wines with a 1:10 ratio of 4-ethylguaiacol/4-ethylphenol at levels of less than 500µg/L and vinylphenols, while not as aromatic as their ethyl derivatives, can affect wines at levels of 800µg/L (Chatonnet et al. 1995).

The ability of *Pediococcus* spp. to produce vinylphenols is very much strain specific. Genes encoding an inducible phenolic acid decarboxylase (PDC) have shown to be necessary for lactic acid bacteria vinylphenol production (Barthelmebs et al. 2000; Van Beek and Priest 2000; de las Rivas et al. 2009). There seems to be a preference among *Pediococcus* spp. with regards to cinnamic acids. It was found that *Pediococcus* spp. had molar conversions up to 84% of *p*-coumaric acid in 50:50 MRS/Tomato-Juice broth when 500mg/L of *p*-coumaric acid was added to the system giving a maximum 4-vinylphenol yield of 293.5 ppm, however the strains under study were able to only convert a high of 18% of ferulic acid with a maximum 4-vinylguaiacol level of 64.7 ppm (Couto et al. 2006). It should be noted that these concentrations far exceed what is commonly found in wine.

It has also been shown that hydroxycinnamic acids have an inhibitory effect on lactic acid bacteria in high concentrations (García-Ruiz et al. 2008; García-Ruiz et al. 2009). Ferulic acid appears to be less inhibitory to the growth of *Pediococcus* spp. than *p*-coumaric acid (Campos et al. 2003; García-Ruiz et al. 2008; Campos et al.

2009; García-Ruiz et al. 2011a). Interestingly *p*-coumaric acid seems less inhibitory than ferulic acid with respect to *Brettanomyces* growth (Harris et al. 2009). To our knowledge very little work has been done regarding vinyl phenol production by *Pediococcus* spp. or potential interactions with *Brettanomyces* yeasts and ethyl phenol production during wine aging.

Biogenic Amines

Of particular interest to winemakers and enologists in recent years is the production of biogenic amines in wines by microorganisms. Biogenic amines are small molecular weight compounds naturally present in a variety of foods and fermented beverages. They can have numerous health implications if their presence is found in high enough concentrations such as vomiting, headache, asthma, hypotension, and cardiac palpitation (Santos 1996; Gloria et al. 1998; Maintz and Novak 2007; Anli and Bayram 2009). Typically red wines contain higher levels of biogenic amines than white wines. A survey of commercial Oregon Pinot noirs revealed that histamine levels may reach concentrations of 24 mg l^{-1} and putrescine as high as 203 mg l^{-1} (Gloria et al. 1998) however it is possible for concentrations to be considerably higher (Maintz and Novak 2007).

Biogenic amines are formed through the decarboxylation of amino acids by microorganisms with enzymes capable of performing the decarboxylation step (Lonvaud-Funel 2001). For example, histidine and tyrosine are decarboxylated to histamine and tyramine, respectively. The formation of biogenic amines may be an

energy production mechanism or a way for the bacterium to deacidify its envirionment (Lucas et al. 2005).

The production of biogenic amines in wines has several requirements. There must be a pool of free amino acids (Herbert et al. 2006). Second, there must be microorganisms capable of decarboxylating the amino acids and lastly the conditions must be conducive for growth of these microorganisms (Anli and Bayram 2009). There are a myriad of factors that alter how these requirements affect the final biogenic amine content in wines. Viticulture region and grape cultivar have both been shown to have an effect on final amine contents in wine (Herbert et al. 2005; Marques et al. 2008). Nitrogen additions to the must prior to alcoholic fermentation have been shown to increase histamine levels (Bach et al. 2011). Lees aging and extended maceration also seem to increase biogenic amine levels (Pedro et al. 2006). Potentially compounds that are inhibitory to biogenic amine producing organisms should reduce final wine amine content, however Polo et al. showed that moderate additions of SO_2 and lysozyme did not reduce biogenic amine contents (Polo et al. 2011). Similarly Landete et al. showed that there was no difference in histamine production by various bacterial strains between SO_2 treatments and that ethanol actually increased production (Landete et al. 2008). Also there may be additive effects due to multiple organisms in the wine as one study found that *Lactobacillus hilgardii* increased histamine production by 34% when in the presence of *O. oeni* compared to single culture conditions (Pedro et al. 2010).

Pediococcus spp. have been implicated in the formation of biogenic amines such as histamine and tyramine (Izquierdo-Pulido et al. 2000; Landete et al. 2005; Landete et al. 2008; Nanneli et al. 2008; Coton et al. 2010). While *O. oeni* is considered the most prevalent biogenic amine producer in wine systems, *Pediococcus* spp. seem capable of considerably higher levels of production (Landete et al. 2005; Landete et al. 2008). In beer systems *Pediococcus* spp. were able to form tyramine above 20mg/L and formation was largely correlated with bacterial growth (Izquierdo-Pulido et al. 2000). Landete et al. in 2005 found that *P. parvalus* incubated in H-MDBmod media could produce histamine at levels well above 300mg/L (Landete et al. 2005). In 2008 Landete et al. showed that while histidine decarboxylase activity in *P. parvalus* was significantly reduced at wine pH, the bacteria were still able to produce 2.5 times more histamine than *O. oeni* in similar conditions (Landete et al. 2008). Interestingly it has been shown that some strains of *Pediococcus* spp. have the ability to degrade tyramine and histamine and may have the ability to lower final biogenic amine contents in wine (García-Ruiz et al. 2011b).

Much of what has been reported regarding wine spoilage by lactic acid bacteria has focused on *Lactobacillus* spp. and *O. oeni*. *Pediococcus* spp. have received relatively little attention. For instance, in a review of wine spoilage compounds by Bartowsky (2009) *Pediococcus* spp. were included in the group of acrolein producers, but to our knowledge there have not been any actual studies showing this behavior in the genera (Bartowsky 2009). A similar situation was found with regards to biogenic amine production. In an extensive study on biogenic amine producers by Coton et al.

(2010), 223 strains of *Lactobacillus* and 113 of *O. oeni* were studied while only 48 strains of *Pediococcus* spp. were analyzed (Coton et al. 2010). While diacetyl production by *Pediococcus* spp. has been documented, specifics regarding concentrations produced and differences between species and strains have not. In addition to the lack of information on production of specific spoilage compounds there is also very little information in the literature regarding the sensory impact of *Pediococcus.* Anecdotally, a number of winemakers in Oregon and Washington have commented on the variability they have observed regarding *Pediococcus* spoilage of wines. For example, at times the presence of high populations of *Pediococcus* resulted in overt spoilage of the wine while at other times the bacteria caused little to no changes to the sensory properties of the wine. Because of this reported variability and the lack of information regarding the specific spoilage caused by different *Pediococcus* species and strains the aim of this research was to determine the various effects that *Pediococcus* species may have on Pinot noir wine. The specific objectives of the study were:

1. Isolate and identify *Pediococcus* spp. from Oregon and Washington wine
2. Investigate the impact of pediococci isolated from Oregon and Washington state wines on the chemical and sensory properties of Pinot noir wine
3. Determine the SO_2 tolerance of different *Pediococcus* species and strains

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF *PEDIOCOCCUS* SPP. AND THEIR EFFECTS ON THE CHEMICAL COMPOSITION OF OREGON PINOT NOIR

ABSTRACT

Pediococcus have been isolated from wines worldwide and are generally regarded as being wine spoilage organisms. However, little is known concerning the occurrence of these organisms in Washington and Oregon state wines or their impact, if any, on quality. *Pediococcus* were isolated from Oregon and Washington state wines and seven isolates were identified as *P. parvulus*, one was identified as *P. damnosus*, while one was identified as *P. inopinatus*. All isolates degraded *p*-coumaric acid to 4-vinyl phenol with this conversion resulting in accelerated production of 4-ethy phenol by *B. bruxellensis* in a model system. Growth of the pediococci isolates in Pinot noir wine resulted in a number of chemical and sensory changes occurring compared to the control. Very low concentrations of biogenic amines were measured in the wines with only wine inoculated with *P. inopinatus* OW-8 containing greater than 5 mg/L. D-lactic acid production varied between isolates with OW-7 producing the highest concentration (264 mg/L). Diacetyl content of the wines also varied greatly. Some wines contained very low levels of diacetyl (< 0.5 mg/L) while others contained very high concentrations (> 15 mg/L) that were well above sensory threshold. Color and polymeric pigment content of the wines also varied with wine inoculated with OW-7 containing 30% less polymeric pigment than the control. This may have been related

to acetaldehyde concentration as a number of *Pediococcus* isolates, including OW-7, reduced the acetaldehyde content of the wine.

INTRODUCTION

During the red winemaking process wine can be spoiled at many different points by a number of wine microorganisms. However, spoilage most often occurs in red wine during the maturation process while the wine is barrel and when levels of SO_2 are low. During this time microorganisms such as *Brettanomyces*, acetic acid bacteria, film yeast, and lactic acid bacteria can grow and produce many different compounds causing undesirable sensory changes to the wine. The major lactic acid bacteria that can cause spoilage are the lactobacilli and pediococci. While lactobacilli have received considerable attention (van Beek and Priest 2000; Costello and Henschke 2002; Moreno-Arribas and Polo 2008; Bartowsky 2009) little work has been performed investigating the impact of *Pediococcus* growth on red wine quality despite these bacteria having been isolated from red wines all over the world (Manca de Nadra and Strasser de Saad 1995, Mesas et al. 2011, Pilone et al. 1966).

Pediococci are generally thought of as wine spoilage organisms although the specifics of how they can spoil wine and whether there are species and strain differences are not well understood. Much of the information in the literature concerning pediococci in wine has focused primarily on β-glucan production (Dueñas-Chasco et al. 1997, Walling et al. 2005ab). These polysaccharides are thought to be primarily produced by *P. damnosus* in wine although Manca de Nadra and Strasser de Saad (1995)

recently isolated two strains of *P. pentosaceus* from 'ropy' Argentinean wines (Manca de Nadra and Strasser de Saad, 1995; Lonvaud-Funel, 1999). However, discussions with Northwest winemakers suggest that this phenomenon is rarely seen and not of primary concern with regards to wine spoilage by *Pediococcus*.

An additional spoilage issue associated with *Pediococcus* that may be a concern for winemakers is the production of biogenic amines. These low molecular weight organic bases may have undesirable physiological effects on humans when absorbed at too high a concentration (Silla Santos, 1996; Arena and Manca de Nadra, 2001; Lonvaud-Funel, 2001). Lactic acid bacteria are often implicated in their production from corresponding amino acids in fermented products and there are a number of reports regarding *Pediococcus* production of biogenic amines in wine (Izquierdo-Pulido et al. 2000; Landete et al. 2005; Landete et al. 2008; Nanneli et al. 2008; Coton et al. 2010). However, the majority of these studies have focused on the genetic basis for biogenic amine production while few have focused on conditions that lead to production of biogenic amines as well as species and strain differences. In addition, although biogenic amines are thought to cause health issues they have also been reported to give undesirable aromas and tastes in wines at elevated concentrations (Edwards and Fugelsang, 2007). The concentrations of biogenic amines that cause detrimental sensory effects is not clear however, as is whether the levels produced by *Pediococcus* would contribute to this sensory defect.

Other aspects of wine spoilage often attributed to pediococci are often ill-defined in the literature and mentioned briefly in review articles rather than research studies. For example, Bartowsky (2009) reported in a review article that *Pediococcus* is involved in the production of the bitter compound acrolein. However, to our knowledge this type of spoilage by *Pediococcus* has not been demonstrated. Instead, some species of pediococci have been reported to degrade glycerol (Pasteris and Strasser de Saad 2008) producing a precursor involved in the formation of acrolein but the actual formation of acrolein has not been shown. In addition, while production of spoilage compounds such as diacetyl has been reported (Edwards et al. 1994) differences between strains and species as well as relative concentrations produced is poorly reported.

An area of spoilage associated with pediococci that has not been well reported is their ability to impact the concentration of volatile phenols in red wines. This may occur either through the direct production of these compounds of through the interaction of pedioccoci with other wine microorganisms capable of producing volatile phenols.

Of particular concern to the wine industry is the production of 4-ethyl phenol and 4-ethyl guaicol, compounds responsible for bandaid and smoke aromas in wine respectively (Chatonnet et al. 1995; Couto et al. 2006). Volatile phenols are formed from the decarboxylation of hydroxycinnamic acids to a vinyl phenol and the vinyl phenol may then be reduced to an ethyl phenol (Couto et al. 2006). In wine there are a number of microorganisms capable of forming vinyl phenols, including *Pediococcus*,

while *Brettanomyces* is the only microorganism capable of producing significant ethyl phenols. Whether the production of vinyl phenols by *Pediococcus* is advantageous to *Brettanomyces* or not is unknown and requires investigation. Because of this and the fact that the spoilage of red wine by *Pediococcus* is poorly defined in the literature, the goal of this study was to investigate the impact of a number of pediococci isolated from Northwest wines for their ability to produce spoilage compounds such as diacetyl, biogenic amines, and acrolein, as well as their impact on 4-ethyl phenol production by *Brettanomyces*.

MATERIALS AND METHODS

Isolation and Identification of *Pediococcus* spp.

Isolates of *Pediococcus* spp. were taken from Oregon and Washington state commercial wines. Wine samples were plated onto MRS media pH 4.5 (20g/L Tryptone, 5g/L glucose, 5g/L yeast extract, 5g/L peptone, 20% v/v preservative-free apple juice) containing 100 mg/L cycloheximide. Plates were incubated in an anaerobic jar (BD Diagnostics, New Jersey, USA) for 10 days at 25°C. Colonies were then streaked onto MRS media for single colonies and examined microscopically for tetrad formation. Colonies showing tetrads were tentatively labeled as *Pediococcus*. Further identification at species level was performed as per Edwards and Jensen (1992). Isolates that did not grow at 35°C or in the presence of 5.5% w/v NaCl were identitified as *P. damnosus*. Isolates capable of producing lactate from lactose were identified as *P. inopinatus*. Isolates capable of growing at

pH 8.0 were labeled as *P. pentosaceus*. All other isolates were tentatively identified as *P. parvalus*. Confirmation of isolate identity was performed by ETS Laboratories (St. Helena, CA) by Scorpion^{TM} analysis.

SO_2 tolerance experiment

A growth media based on Hood (1983) that contains very low quantities of SO_2 binding compounds was used to measure the impact of SO_2 on *Pediococcus* growth. The media (pH 3.5, 3g/L yeast extract, 3g/L Casamino acids, 2g/L fructose, 6g/L tartaric acid, 2g/L L-malic acid, 2g/L K2HPO4, 1g/L MgSO4 7H2O, 0.02g/L MnSO4 H2O, 0.02g/L CaCl2, 0.50g/L FeCl3, 1ml/L Tween 80) was adjusted to pH 3.50, sterile filtered, and 10 ml dispensed into 20 ml screw-cap test tubes. Various concentrations of SO_2 were added to the media to give 0, 0.1, 0.2, 0.4, and 0.8 mg/L molecular SO_2 and after 24 hrs free and bound SO_2 concentrations were measured by the aeration oxidation method to confirm these concentrations. After 7 days growth in MR broth (pH 4.5) at 25°C *Pediococcus* isolates were harvested by centrifugation (15 min @ 4500 rpm), washed twice with sterile phosphate buffer (pH 6.0) then inoculated into the Hood media at approximately 1 x 10^5 CFU/mL. Tubes were incubated at 25°C and growth was followed by measuring changes in optical density at 550 nm using a visible light spectrophotometer. All treatments were performed in triplicate except for the control which was performed in duplicate.

Pinot noir wine production

Grapes

Pinot noir grapes were harvested on October 16th, 2010, from Oregon State University's Woodhall Vineyard (Alpine, Oregon, USA). Harvest was determined by sugar levels and fruit ripeness according to the specifications of the vineyard manager. Upon harvest, grapes were stored for forty-eight hours at 4°C (39.2°F) before being hand-sorted and destemmed with a Velo DPC 40 destemmer/crusher (Altivole, Italy). The grapes were then pooled and divided into 100L stainless steel tanks containing approximately sixty liters of grape must each. An addition of 50mg/L SO_2 (in the form of potassium metabisulfite) was added to each tank and the yeast nutrient Fermaid K® (Lallemand, Montreal, Canada) was added at a rate of 0.125g/L. Basic juice parameters of the Pinot noir must after processing were pH 3.35, 25.2 °Brix, and 0.683g/100mL titratable acidity (grams tartaric acid).

Alcoholic Fermentation

Grape must was inoculated with *Saccharomyces cerevisae* yeast strain VQ-15 (Lallemand) at a rate of 0.25 grams dried yeast per liter of must (approximately $1x10^6$ CFU/mL). Yeast was hydrated according to manufacturer's specifications prior to inoculation. Fermentations were conducted in a temperature-controlled room held at 26.6°C (80°F). Cap punch downs were performed uniformly twice daily and temperature and °Brix were measured with an Anton-Paar DMA 35N Density Meter (Graz, Austria). Completion of alcoholic fermentation (reducing sugar concentration below 0.2g/100mL) was confirmed by testing with Bayer Clinitest tablets

(Morristown, New Jersey, USA). Alcoholic fermentations proceeded quickly and were completed in nine days (< 0.5g/L reducing sugars).

Upon completion of alcoholic fermentation, wines were pressed with a Willmes model 6048 pneumatic bladder press (Lorsch, Germany). Wines were pressed at 0.5 bar for one minute before the cake was manually broken up and pressed again at 1.0 bar for two minutes. All wine was then pooled and mixed after pressing. Wines were placed in a cold room at 3°C for forty-eight hours.

Filtration

Following cold settling, wines were racked and then pad filtered (Beco K-1 3.0μm nominal filter sheets (Langenlonsheim, Germany)). The wines were then adjusted to pH 3.75 (addition of NaOH) before being filtered through 1.0μm and 0.45μm polyethersulfone cartridges (G.W. Kent, Ypsilanti, Michigan, 40 USA) in succession. Filtered wine was dispensed into sterile one gallon carboys and utilized in the following trials.

***Pediococcus* inoculation**

Pediococci isolates were grown in MRS media pH 4.5 (20g/L Tryptone, 5g/L glucose, 5g/L yeast extract, 5g/L peptone, 20% v/v preservative-free apple juice) for 7 days at 25°C prior to inoculation. Pediococci were then centrifuged (15 min @ 4500 rpm) and washed with sterile phosphate buffer (pH 7.0) prior to inoculation. Pediococci isolates WW1, WS9, OW1, OW2, OW4, OW5, OW6, OW7, and OW8

were inoculated into sterile filtered Pinot noir wine at a rate of approximately 1.0 x 10^5 CFU/mL. An uninoculated control was also prepared. Samples (50mL) were taken for analysis every 10 days and stored at -80°C while growth was monitored by plating on MRS media after appropriate dilutions in sterile peptone blanks.

Bottling

After all treatments went into population decline (approximately 8 weeks), wines received a sulfite addition equal to 25mg/L SO_2 before being sterile filtered (0.45µm PES, GW Kent) and bottled in 350mL glass bottles (crown capped) and 750mL wine bottles (screw capped closure). Wines were stored at 13°C until required for analysis.

Chemical Analysis

Determination of diacetyl with headspace sampling-gas chromatography-Electron Capture Detector

Diacetyl was measured by gas chromatography using an Electron Capture Detector. Chemicals used were 2,3-Butanedione (Alfa Aesar, 99%), 2,3-Hexanedione as an Internal Standard (Alfa Aesar, 94%), pure ethanol (Koptec, 200 proof), ammonium sulfate (AR grade), and Mili-Q water. Samples were prepared by weighing 3.5g of ammonium sulfate into a 20-mL autosampler vial. 2.5ml of Mili-Q water and 2.5ml of wine were pipetted into the vial. Finally, 50µl of internal standard solution (2,3-Hexanedione,5mg/L) and a PTFE resin-coated magnetic stir bar were added in. The vial was tightly capped with Teflon-faced silicone septa and placed in an automatic

headspace sampling system for analysis. The samples were kept in a cooling tray at 8°C when they were not analyzed. Samples were incubated with constant stirring at 35°C for 40 minutes. After incubation, the headspace syringe was inserted and sampled 250µl of the headspace for injection.

A HP6890 gas chromatograph equipped with an electron capture detector (ECD) system (Agilent Technologies, Palo Alto, CA) and fitted with a RTX-1 capillary column, 30mm X 0.32mm X1.5 µm (J&W Scientific, Folsom, CA) was used for analysis. Helium was used as the carrier gas at a flow rate of 1.5mL/min. The inlet temperature was 120°C. The injection was performed in split mode with split ratio of 10:1. The detector was 120°C and the combined column and make-up gas were kept constant at 60ml/min. The oven temperature program used was 35°C for 2 min, followed by an increase of 5°C/min to 100°C, holding for 2 minutes. The total run time was 17 minutes. For the calibration purposes, a 9-point calibration curve using an internal standard method for diactyl was measured. The calibration range was approximately 10ppb-20ppm. The concentration of diacetyl was determined by comparing the peak area ratio of diacetyl with a standard curve.

Color Analysis

Color analysis was performed two months after bottling. All wine samples were adjusted to pH 3.60 prior to testing by addition of 2N NaOH or 25% H_3PO_4. Color was determined by spectrophotometric analysis (Shimadzu UV-3101PC, Kyoto, Japan) at 420nm and 520nm in a quartz 1mm path length cuvette. Polymeric pigment

was measured by spectrophotometric analysis (Thermo Scientific Genesys, Madison, Wisconsin, USA) according to Levengood and Boulton (2004).

Anthocyanin, Hydroxycinnamic acids, Volatile Phenol Analysis

Monomeric anthocyanins, *p*-coumaric acid, 4-vinylphenol, and 4-ethyphenol were analyzed by high-performance liquid chromatography (HPLC) based on Benito et al. (2009) using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA) equipped with HP ChemStation software and photodiode-array detector (DAD). The HPLC was fitted with a LiChroSpher reverse-phase C18 column (4 x 250mm, 5mm particle size) (Merck, Darmstadt, Germany) held at 30°C. All chromatographic solvents were HPLC grade (98% Formic acid (EMD Chemicals, Darmstadt, Germany), 99.8% Methanol (EMD Chemicals). Gradients of solvent A (water/formic acid, 90:10, v/v) and solvent B (methanol) were applied as follows: 5 to 35% B linear (1.0 mL/min) from 0 to 15 min, static at 35% B (1.0mL/min) from 15 to 20 min, 35 to 80% B linear (1.0mL/min) from 20 to 25 min, then 5% B (1.0mL/min)from 25 to 32 min to re-equilibrate the column to initial conditions. Quantification of anthocyanins was determined from a standard curve for malvidin-3-glucoside (Sigma) at 520nm and expressed as mg/L of malvidin-3-glucoside equivalents. Quantification of *p*-coumaric acid, 4-ethylphenol and 4-vinylphenol was performed at 320nm, 280nm, and 260nm respectively and determined by standard curves of individual compounds (Sigma).

Biogenic Amine Analysis

Biogenic amines were analyzed by ETS Laboratories using HPLC with fluorimetric detection based on the method of Costantini et al. (2006).

Additional Analysis

Acetaldehyde, D-Lactic Acid, Glycerol, and L- Malic Acid concentrations were measured utilizing enzymatic assay (R-Biopharm, Darmstadt, Germany). Tannin levels were measured according to protein precipitation assay (Adams and Harbertson 1999).

Volatile phenol production

The ability of pediococci to degrade *p*-coumaric acid and produce volatile phenols as well as the impact of *Pediococcus* on *Brettanomyces* was assessed using an acidic grape juice broth pH 3.50 (25% preservative-free grape juice, 5g/L yeast extract, 0.125g/L $MgSO_4$, 0.0025g/L $MnSO_4$). Ethanol (5%) and 30mg/L *p*-coumaric acid were added to the broth prior to sterile filtration (0.45μm). Treatments were inoculated at a rate of $1.0x10^5$ CFU/mL with *Pediococcus* isolates WS9 and OW2 previously grown in MR broth (pH 4.50) and harvested by centrifugation. An uninoculated control was also prepared. Pediococci were grown for 14 days at 25°C with growth being monitored by plating on MR plates. After 14 days growth samples were taken and analyzed for *p*-coumaric acid and 4-vinylphenol by HPLC-DAD. At this point *B. bruxellensis* was also inoculated into the broth at approximately 1×10^3 CFU/mL. *B. bruxellensis* was also inoculated into AGJ broth in which no bacteria had previously been grown. *B. bruxellensis* was previously grown in YPD broth (pH 5.50)

for 48 hrs and harvested by centrifugation prior to inoculation. Samples were taken daily and *B. bruxellensis* growth was assessed by plating on YPD plates. Samples were analyzed for *p*-coumaric acid, 4-vinylphenol, and 4-ethyphenol by HPLC-DAD (Benito et al. 2009) as described previously.

Statistical Analysis

A univariate Analysis of Variance (ANOVA) was used to determine differences between wine treatments. ANOVA was performed using Microsoft Excel (Version 14.1.3) and XL Stat (Version 2011.4.02). Fisher's LSD multiple comparison was performed to test least squares means of treatment effects at the 0.05% significance level.

RESULTS

Isolation and Identification of pediococci

Nine *Pediococcus* isolates were isolated from Oregon and Washington wines. Species identification of the isolates was performed as per Edwards and Jensen (1992). One isolate was identified as *P. inopinatus* due to its ability produce lactate from lactose (Table 1) while *P. damnosus* was identified due to growth in 5.5% (w/v) NaCl solution (Table 1). No isolates were able to grow at pH 8.0 indicating that none of the isolates were *P. pentosaceus*. All other isolates that grew at 35°C, and could not grow at pH 8.0 or produce lactate from lactose were tentatively identified as *P.*

parvalus (Table 1). The identity of the pediococci isolates was confirmed by Scorpion™ analysis conducted by ETS laboratories.

Table 1. Identification of pediococci isolates based on growth at 35°C, acid production from lactose, growth at pH 8.0, and growth in 5.5% (w/v) NaCl

Isolate	Growth at 35°C	Acid from lactose	Growth pH 8.0	5.5% (w/v) NaCl	ID
WW1	+	-	-	+	*P. parvulus*
WS-9	+	-	-	+	*P. parvulus*
OW1	+	-	-	+	*P. parvulus*
OW2	-	-	-	-	*P. damnosus*
OW4	+	-	-	+	*P. parvulus*
OW5	+	-	-	+	*P. parvulus*
OW6	+	-	-	+	*P. parvulus*
OW7	+	-	-	+	*P. parvulus*
OW8	+	+	-	+	*P. inopinatus*

SO_2 Tolerance

The tolerance to SO_2 of the pediococci isolates was studied by observing changes in optical density in Hood media containing 0, 0.1, 0.2, 0.4, and 0.8mg/L molecular SO_2. Isolates of *Pediococcus* showed a wide array of tolerance to SO_2. OW6 had the lowest tolerance growing well only in media containing 0.1 mg/L molecular SO_2 (Figure 6). WS9, OW1, OW2, OW7, and OW8 showed moderate tolerance to SO_2 (Figures 1, 2, 3, 7, 8) growing well at 0.2 mg/L molecular SO_2. OW4 and OW5 had the highest level tolerance to SO_2 growing well at concentrations of 0.4 mg/L molecular SO_2 (Figures 4 & 5).

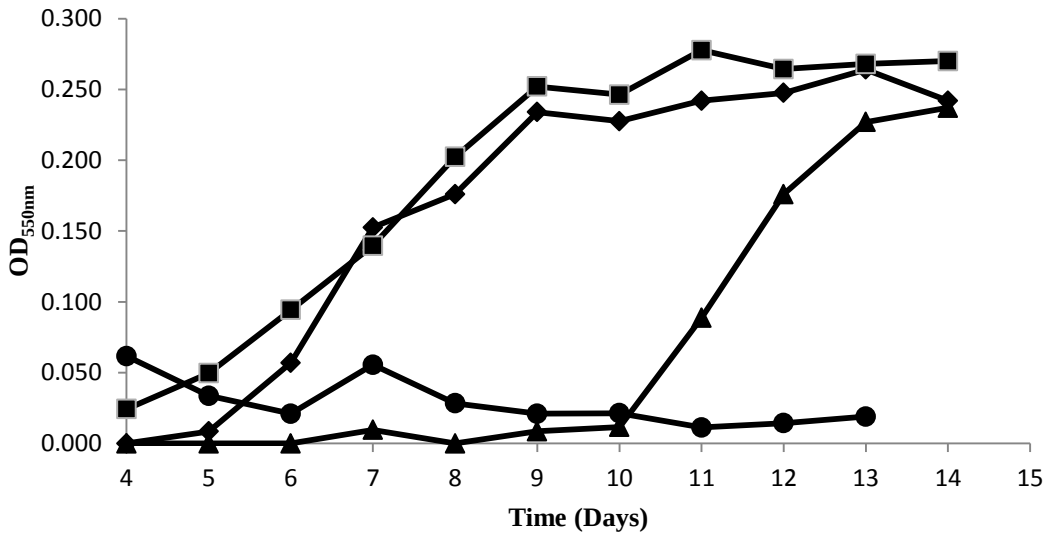


Figure 1. Growth of *Pediococcus parvulus* WS9 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

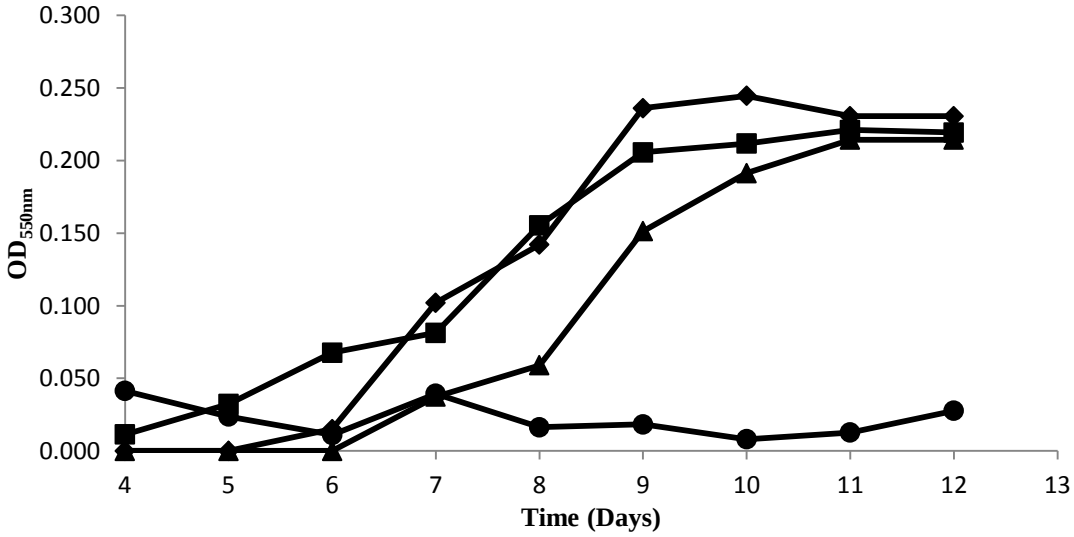


Figure 2. Growth of *Pediococcus parvulus* OW1 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

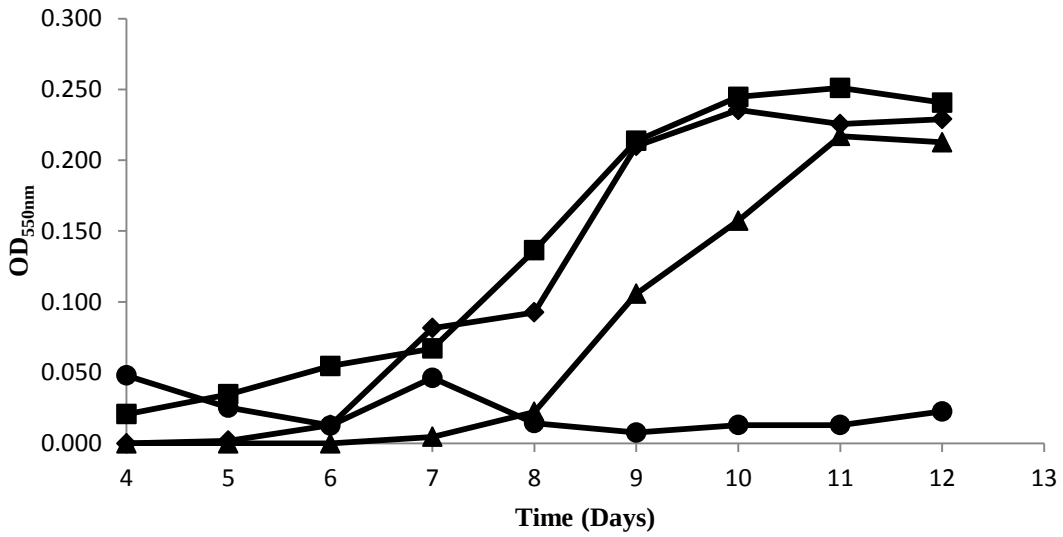


Figure 3. Growth of *Pediococcus damnosus* OW2 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

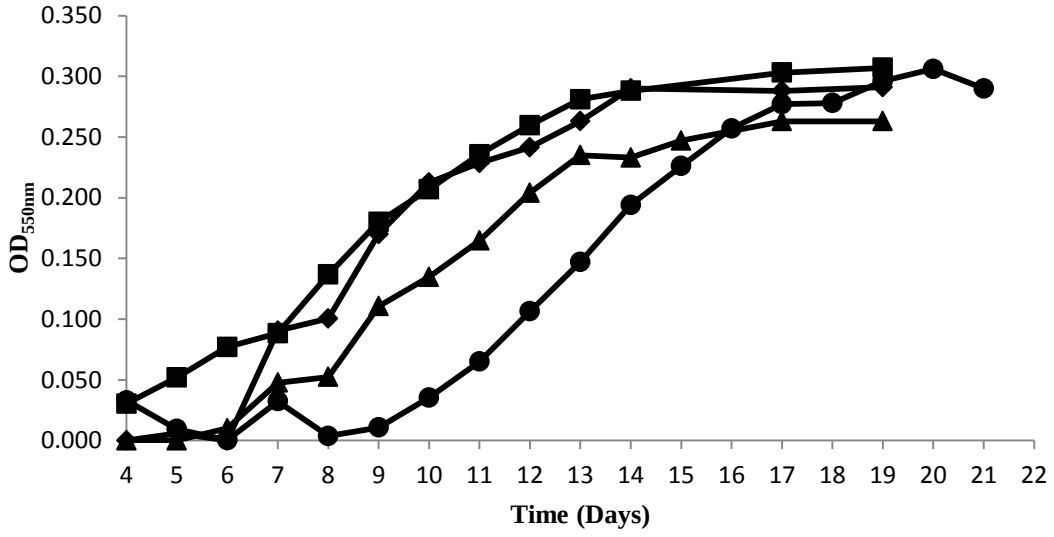


Figure 4. Growth of *Pediococcus parvulus* OW4 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

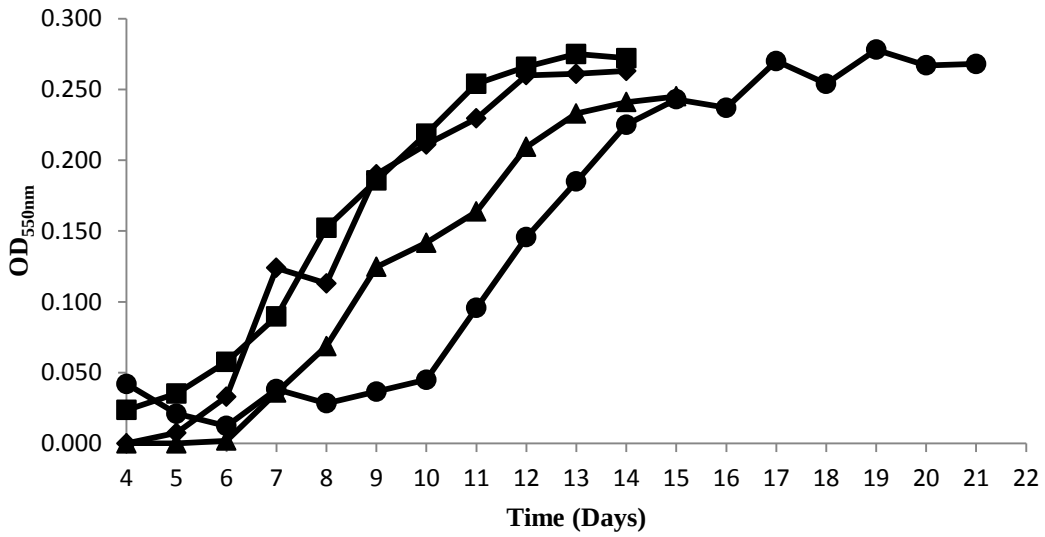


Figure 5. Growth of *Pediococcus parvulus* OW5 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

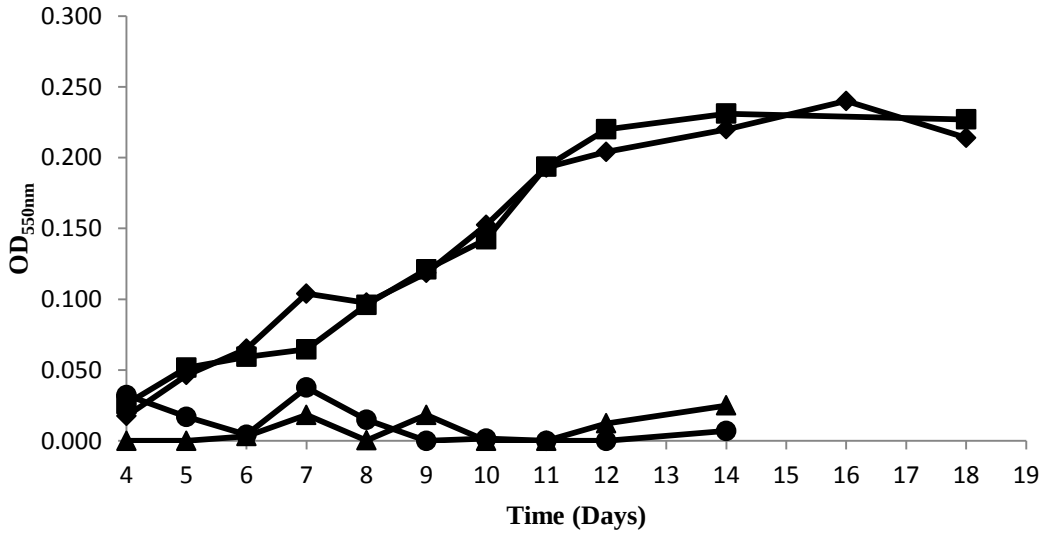


Figure 6. Growth of *Pediococcus parvulus* OW6 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

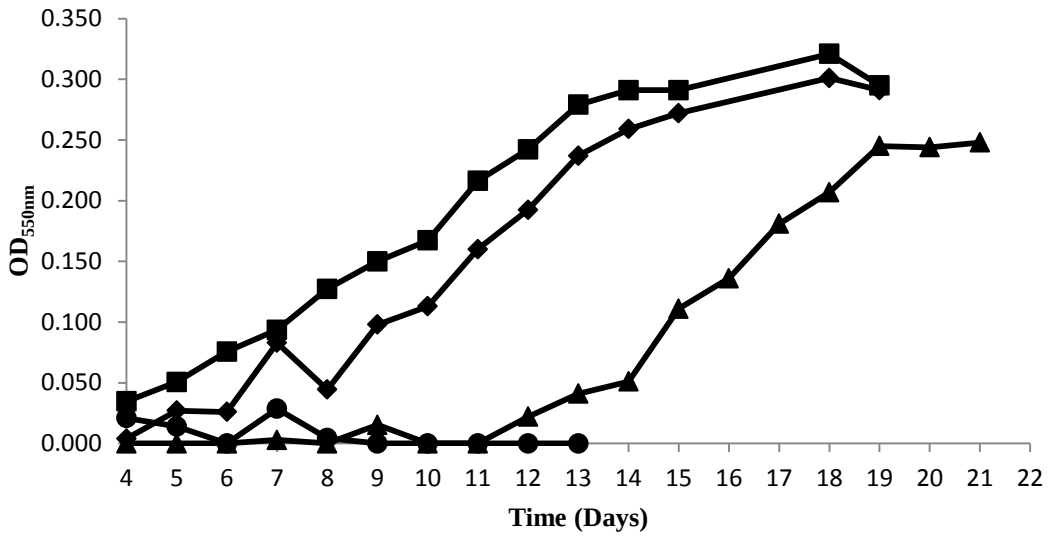


Figure 7. Growth of *Pediococcus parvulus* OW7 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

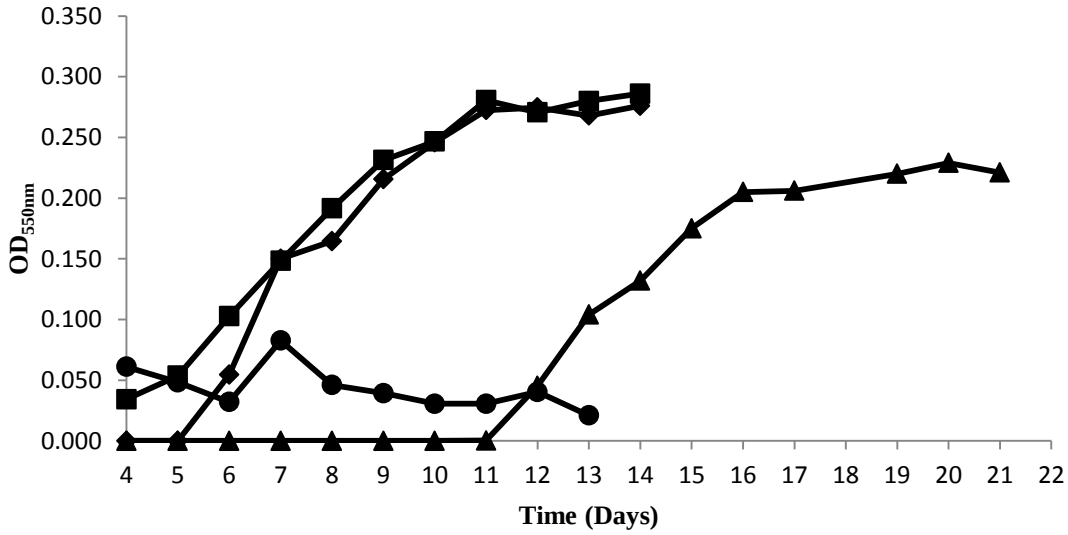


Figure 8. Growth of *Pediococcus inopinatus* OW8 in Hood media (pH 3.5) containing (♦) 0, (■) 0.1, (▲) 0.2, (●) 0.4mg/L molecular SO_2

Volatile Phenol Trial

P. parvulus WS-9 and *P. damnosus* OW-2 degraded *p*-coumaric acid to below 0.5mg/L resulting in the production of 4-VP. This resulted in the media containing approximately 28mg/L of 4-VP prior to inoculation with *B. bruxellensis* (Figures 10B & 10C) while the control contained undetectable concentrations of 4-VP (Figure 10A). After inoculation *B. bruxellensis* grew well in the acidic grape juice broth containing *p*-coumaric acid as well as in acidic grape juice broth in which *P. parvulus* WS-9, and *P. damnosus* OW-2 had previously grown in (Figure 9). In media in which *P. parvulus* WS-9, and *P. damnosus* OW-2 had previously grown *B. bruxellensis* degraded all of the 4-VP after 3 days (Figures 10B & 10C). This coincided with 4-EP production where maximum 4-EP production occurred 3 days after inoculation (Figure 11). In media in which no bacteria had previously grown, *B. bruxellensis* degraded *p*-coumaric acid and maximum 4-EP production occurred 5 days after inoculation (Figure 11).

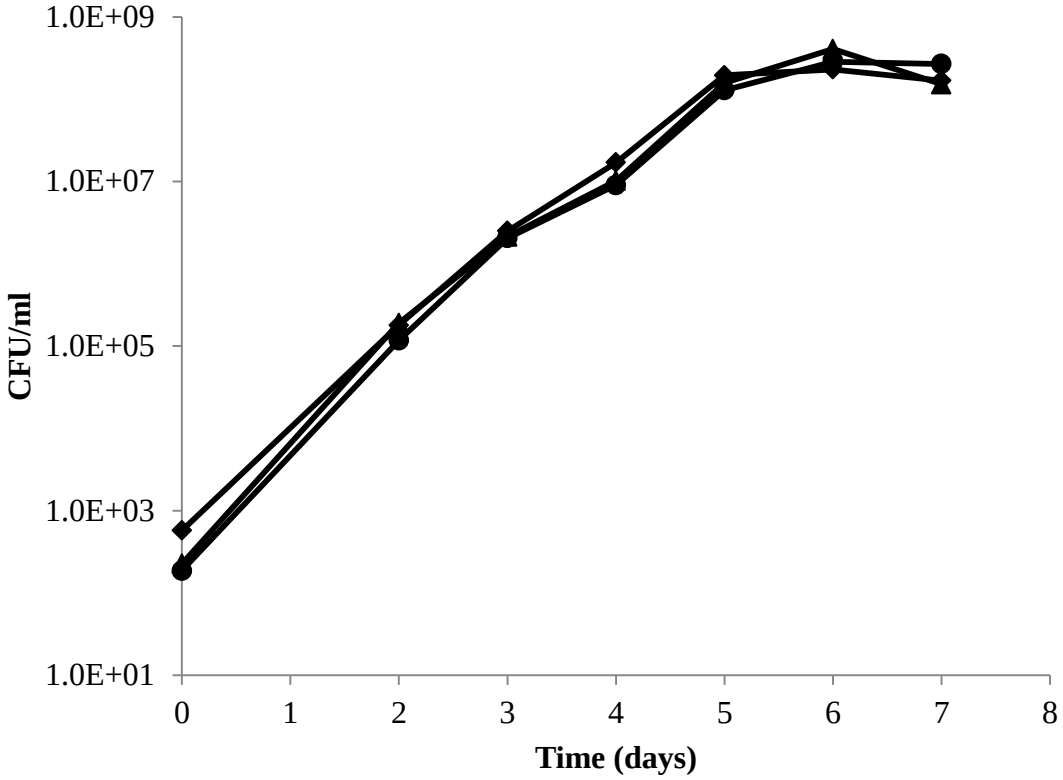


Figure 9. Growth of *Brettanomyces bruxellensis* in AGJ broth (pH 3.5, 5% ETOH, 30 mg/L *p*-coumaric acid) previously inoculated with *Pediococcus* spp. (●) Control (no *Pediococcus*), (▲) *P. parvalus* WS9, (♦) *P. damnosus* OW2.

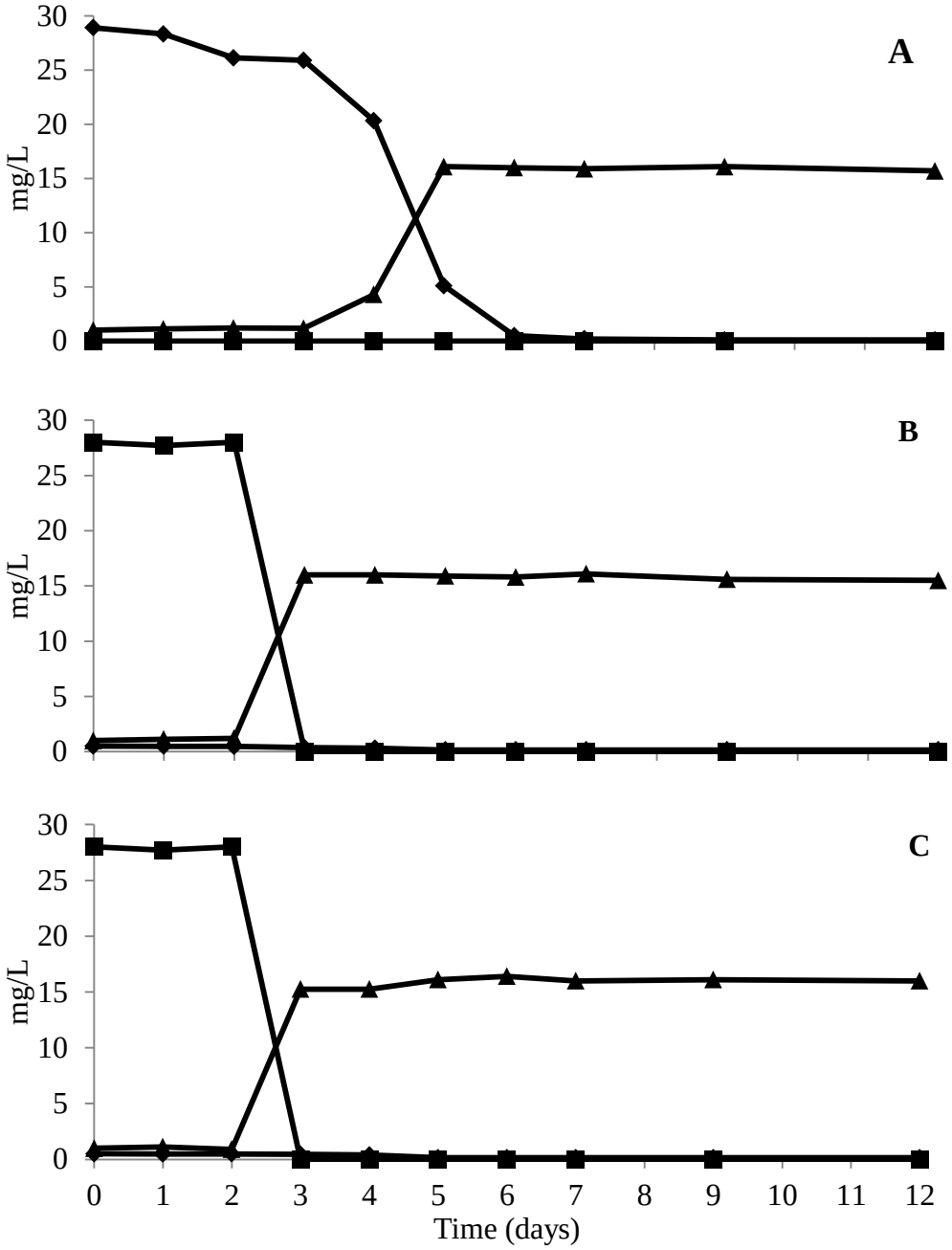


Figure 10. Concentration of (♦) *p*-coumaric acid, (■) 4-VP, and (▲) 4-EP during growth of *B. bruxellensis* in acidic grape juice broth (A) or in acidic grape juice broth previously inoculated with *P. parvulus* WS-9 (B) or *P. damnosus* OW-2 (C).

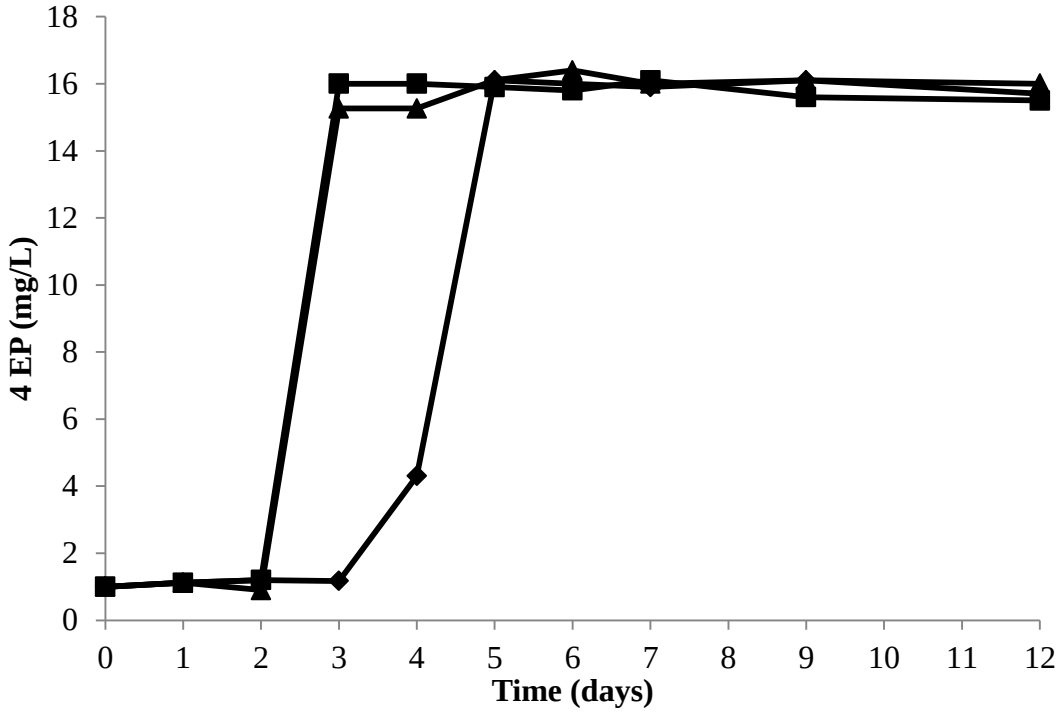


Figure 11. 4-EP production by *Brettanomyces bruxellensis* in AGJ broth (pH 3.5, 5% EtOH, 30 mg/L *p*-coumaric acid) previously inoculated with (♦) no *Pediococcus*, (■) *P. parvalus* WS9, and (▲) *P. damnosus* OW2

Fermentation

After alcoholic fermentation, pressing and filtering, Pinot noir wines were inoculated with pediococci isolates. All pediococci isolates grew to $1.0x10^6$ CFU/ml or greater by the end of 60 days while isolates OW1, OW2, OW5, and OW7 grew to $1.0x10^7$ CFU/ml or greater. An example of this growth is seen in Figure 12. Populations peaked for nearly all isolates approximately 30 days after inoculation.

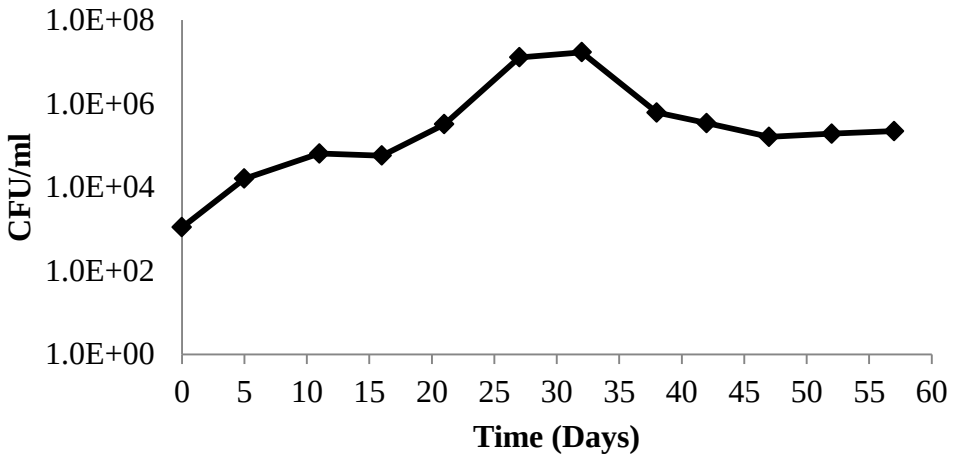


Figure 12. Growth of *Pediococcus parvalus* (WS9) in Pinot noir wine

Chemical Analysis of Pinot noir Inoculated with *Pediococcus* spp.

Wines in which pediococci isolates had grown were analyzed for various compounds thought to contribute to wine spoilage by *Pediococcus*. D-lactic acid was formed in significant amounts by only one isolate, OW7 (Table 2). However, all isolates were capable of degrading at least a partial amount of the total malic acid content. OW-2 and OW-7 completely degraded the malic acid while less than 0.5g/L malic acid remained in wine inoculated with OW-1 (Table 2). The other isolates degraded varying amounts of malic acid although more than 1g/L remained in all of these wines (Table 2). Production of diacetyl also varied greatly between isolates. OW1, OW2, OW5, and OW8 produced greater than 10mg/L of diacetyl while wines inoculated with OW7 contained lower diacetyl concentrations than the control (Table 2). Glycerol levels were also analyzed to determine the potential ability of *Pediococcus* spp. to form acrolein or to use glycerol as a carbon source. However, none of the pediococci isolates reduced the level of glycerol in the wine compared to the control (Table 2).

Table 2. Concentrations of D-lactic acid, L-malic acid, diacetyl, and glycerol in Pinot noir wines inoculated with various *Pediococcus* isolates

Treatment	D-Lactic Acid (mg/L)	L-Malic Acid (g/L)	Diacetyl (mg/L)	Glycerol (g/L)
Control	$115.2^{b} \pm 3.6$[1]	$3.02^{a} \pm 0.17$	$3.20^{cd} \pm 0.84$	$2.79^{a} \pm 0.01$
WW1	$121.7^{b} \pm 6.3$	$2.38^{b} \pm 0.18$	$3.01^{cd} \pm 0.57$	$2.60^{a} \pm 0.38$
WS9	$114.4^{b} \pm 19.2$	$2.25^{b} \pm 0.16$	$1.83^{cd} \pm 0.79$	$2.85^{a} \pm 0.01$
OW1	$111.6^{b} \pm 16.3$	$0.29^{f} \pm 0.16$	$15.06^{a} \pm 4.10$	$2.88^{a} \pm 0.00$
OW2	$123.5^{b} \pm 1.1$	$0.01^{f} \pm 0.00$	$13.07^{ab} \pm 1.05$	$2.88^{a} \pm 0.01$
OW4	$124.9^{b} \pm 22.4$	$1.52^{cd} \pm 0.12$	$4.23^{c} \pm 1.98$	$2.86^{a} \pm 0.02$
OW5	$136.1^{b} \pm 25.5$	$1.34^{de} \pm 0.01$	$10.98^{b} \pm 2.92$	$2.90^{a} \pm 0.01$
OW6	$113.8^{b} \pm 4.2$	$1.73^{c} \pm 0.01$	$5.20^{c} \pm 2.87$	$2.86^{a} \pm 0.01$
OW7	$264.4^{a} \pm 23.1$	$0.02^{f} \pm 0.00$	$0.33^{d} \pm 0.01$	$2.87^{a} \pm 0.00$
OW8	$124.3^{b} \pm 4.7$	$1.13^{e} \pm 0.25$	$15.53^{a} \pm 1.81$	$3.02^{a} \pm 0.24$

[1]values are means of three replicates ± one standard deviation

[a-e] Mean values with different superscript letters within a row are significantly different at $p < 0.05$, Fisher's LSD, n=3

Based on previous work in our lab on the effects of *Oenococcus oeni* on Pinot noir color (Burns 2011), color was measured in all treatment wines. Compared to the control, OW7 reduced color @ 520 nm by 15% and polymeric pigment content by 29% (Table 3). Because acetaldehyde concentration can impact polymeric pigment content acetaldehyde was also measured in all treatment wines. Compared to the control OW2 and OW7 both significantly decreased acetaldehyde concentrations with OW7 reducing acetaldehyde by 74% compared to the control (Table 3). While pediococci isolates impacted color they did not impact total tannins measured as catechin equivalents (Table 3).

Table 3. Color @ 520nm, polymeric pigment, acetaldehyde, and total tannin (Catechin equivalents) in Pinot noir wines inoculated with various *Pediococcus* isolates.

Treatment	A_{520}	Polymeric pigment (A_{520})	Acetaldehyde (mg/L)	Total tannin (mg/L)
Control	$3.36^{a} \pm 0.004$[1]	$0.900^{a} \pm 0.007$	$10.3^{abc} \pm 0.3$	$221.5^{a} \pm 3.2$
WW1	$3.37^{a} \pm 0.011$	$0.887^{ab} \pm 0.008$	$9.4^{abc} \pm 2.7$	$211.6^{a} \pm 10.5$
WS9	$3.48^{a} \pm 0.009$	$0.910^{a} \pm 0.022$	$12.0^{a} \pm 3.3$	$224.0^{a} \pm 3.5$
OW1	$3.34^{ab} \pm 0.030$	$0.856^{ab} \pm 0.077$	$7.4^{abc} \pm 2.4$	$221.6^{a} \pm 9.3$
OW2	$3.31^{abc} \pm 0.015$	$0.828^{bc} \pm 0.071$	$3.6^{cd} \pm 2.2$	$219.1^{a} \pm 13.2$
OW4	$3.12^{cd} \pm 0.014$	$0.765^{cd} \pm 0.024$	$8.7^{abc} \pm 1.3$	$221.1^{a} \pm 2.7$
OW5	$2.99^{de} \pm 0.009$	$0.723^{d} \pm 0.028$	$6.5^{bcd} \pm 4.4$	$216.2^{a} \pm 4.1$
OW6	$3.04^{de} \pm 0.007$	$0.737^{d} \pm 0.021$	$11.2^{ab} \pm 0.1$	$218.9^{a} \pm 2.4$
OW7	$2.86^{e} \pm 0.002$	$0.637^{e} \pm 0.003$	$2.7^{d} \pm 0.2$	$212.0^{a} \pm 12.4$
OW8	$3.14^{bcd} \pm 0.004$	$0.784^{cd} \pm 0.013$	$12.5^{a} \pm 0.4$	$225.1^{a} \pm 10.5$

[1]values are means of three replicates ± one standard deviation

[a-e] Mean values with different superscript letters within a row are significantly different at $p < 0.05$, Fisher's LSD, n=3

The Biogenic amine content of all the treatment wines was very low (Table 4). No isolate produced total concentrations of biogenic amines greater than7 mg/L. *P. inopinatus* (OW8) produced the highest amount of histamine while all other isolates produced undetectable amounts of this biogenic amine. All wines, including the uninoculated control, contained small amounts of putrescine (Table 4) while no wines contained detectable tyramine.

Table 4. Biogenic amine content (mg/L) in Pinot noir wines inoculated with various *Pediococcus* isolates

Treatment	Histamine	Tyramine	Putrescine	Cadaverine
Control	<1	<1	3	2
WW1	<1	<1	2.3	1
WS-9	<1	<1	2	<1
OW1	<1	<1	3	1
OW2	<1	<1	2.3	1
OW4	<1	<1	2.3	1
OW5	<1	<1	2	<1
OW6	<1	<1	2	<1
OW7	<1	<1	2	<1
OW8	3.8	<1	2	1

DISCUSSION

Although generally thought of as wine spoilage organisms little is known about the specific effects of *Pediococcus* on red wine quality and in particular what effects different species and strains may have. In an effort to better characterize the impact of this bacteria on red wine, pediococci were isolated from Oregon and Washington State commercial wines and utilized in a number of experiments. The pediococci isolated were almost all *P. parvulus* while no isolates were identified as *P. pentosaceus*. Given the small sample size little can be extrapolated from this finding. However the fact that three different species were isolated from a total of nine wines does suggest that there is some species diversity within the Oregon wine industry. Future efforts in this area should focus on collecting a much larger number of samples from Oregon wineries to better represent what species of *Pediococcus* are present and their frequency.

The impact of *Pediococcus* on wine quality is often ill-defined and simplified. For example review papers most often make reference to *Pediococcus* capacity for polysaccharide production, diacetyl formation, and tyramine production, but little else (Bartowsky and Henschke 2004; Bartowsky 2009; Landete et al. 2005; Landete et al. 2008). There are also references in papers to acrolein production by *Pediococcus*, but little evidence to support it (Bartowsky 2009). Furthermore, spoilage caused by pediococci is often not defined at a species or strain level with the assumption that growth of any pediococci strain or species would result in similar wine spoilage (Lonvaud-Funel 1999; Bartowsky 2009). Results from the present study demonstrate that this is not the case. In particular, there was large variability in the production of certain spoilage products between pediococci isolates of the same species. For example, *P. parvalus* OW7 produced high amounts of D-lactic acid compared to the other *P. parvulus* isolates and other pediococci species tested. As D-lactic acid production is a result of glucose metabolism this finding was surprising as the initial residual glucose was the same in all the wines. The reasons for this are unclear although Grimaldi (2005) observed that some wine lactic acid bacteria including strains of *Pediococcus* may possess glycosidase activity, thereby potentially allowing them to metabolize bound glucose molecules (Grimaldi 2005).

Of all the spoilage compounds measured in this study the one that varied the most between isolates, and perhaps of most importance from a spoilage perspective, was diacetyl. With a reported sensory threshold as low as 0.9 mg/L in Pinot noir (Marineau et al. 1995) diacetyl may potentially have a large impact on the sensory

properties of this wine. While others have reported that LAB species can have significant impacts on diacetyl contents of wine, little work has shown these effects with respect to production by pediococci. For example, *O. oeni* under experimental conditions can produce levels of 13mg/L and perhaps levels considerably higher if citric acid is added to the system (Nielsen and Richelieu 1999). In this study OW1, OW2, and OW8 produced high levels of diacetyl. Diacetyl production did not appear to be a species related as OW1 was a *P. parvulus* isolate, OW2 was a *P. damnosus* isolate, and OW8 was a *P. inopinatus* isolate. In contrast, OW7 reduced the level of diacetyl in the wine suggesting it was capable of reducing diacetyl to acetoin or perhaps further to 2,3-butanediol. However, because neither acetoin or 2,3-butanediol were measured in this study it is not possible to confirm this.

Aside from production of spoilage products, pediococci isolates also impacted the quality of the wine by decreasing red color and polymeric pigment content. A number of isolates reduced color @ 520 nm by greater than 10% while polymeric pigment content was reduced by almost 30% in wines in which *P. parvulus* OW-7 had grown. This reduction in color could be detrimental to the quality of lightly pigmented wines such as Pinot noir. In addition, the loss of the more stable color compounds, polymeric pigments, may impact the long term color of the wine. Although others have reported color loss due to *S. cerevisiae* (Medina et al 2005) and *Oenococcus oeni* (Abrahamse and Bartowsky 2012; Burns 2011), to our knowledge this is the first time that loss of color due to *Pediococcus* has been reported.

The reduction of color, and in particular the reduction of polymeric pigment, may have been due to the degradation of acetaldehyde by a number of pediococci isolates. For example, OW-7 reduced acetaldehyde by nearly 75% and also caused a reduction of almost 30% in polymeric pigment content. Previously researchers had reported that *Pediococcus* could not degrade acetaldehyde (Osborne et al 2000). However, the authors only tested two pediococci. In contrast, Wells and Osborne (2012) reported that a *P. parvulus* and *P. damnosus* were able to degrade acetaldehyde in a model system. These findings suggest the ability to degrade acetaldehyde is strain and species specific, something observed in the present study where some pediococci isolates degraded large amounts of acetaldehyde while others did not. While the degradation of acetaldehyde by some pediococci may have impacted polymeric pigment formation this was not always the case. For example, OW2 did not significantly affect polymeric pigment levels but did degrade acetaldehyde while OW8 significantly reduced polymeric pigment content but did not degrade acetaldehyde. Therefore, mechanisms other than acetaldehyde reduction may be responsible for some of the observed polymeric pigment loss. Further work in this area is required to fully understand the potential for *Pediococcus* to affect color, polymeric pigment, and acetaldehyde.

One area related to *Pediococcus* spoilage that has received a lot of attention in the last few years is their ability to produce biogenic amines (Santos 1996; Gloria et al. 1998; Maintz and Novak 2007; Anli and Bayram 2009). Biogenic amines are small molecular weight compounds that are naturally present in a variety of fermented

foods and beverages. They can have numerous health implications if their presence is found in high enough concentrations such as vomiting, headache, asthma, hypotension, and cardiac palpitation (Santos 1996; Gloria et al. 1998; Maintz and Novak 2007; Anli and Bayram 2009). In the present study none of the pediococci produced large amounts of biogenic amines during growth in Pinot noir wine. For example, *P. inopinatus* OW8 was the only isolate that produced measurable levels of histamine (3.3mg/L). This concentration is slightly higher than proposed limits of 2mg/L however considering the necessary histamine blood plasma levels generally required to illicit negative health responses, this figure may be conservative (Maintz and Novak 2007). This finding was surprising given the number of reports of biogenic amine production by *Pediococcus* (Landete et al. 2005, Landete et al. 2008). However, aside from the growth of biogenic amine producing bacteria, a number of other factors may impact biogenic amine production in wine. These include substrate (amino acids), the presence of other biogenic amine producing bacteria, lees aging, extended maceration, and the presence of sugars (Aredes Fernández et al. 2010, Moreno-Arribas et al. 2000, Martin-Álvarez et al. 2005). It is possible that the amino acid concentration was low after alcoholic fermentation and provided insufficient substrate for biogenic amine production. Because the amino acid composition of the wine is unknown it is not possible to make this conclusion however. More research is required to understand these isolates' capacity for biogenic amine formation and possible factors that may increase their production.

Bitterness in red wine is sometimes attributed to acrolein production by *Pediococcus* (Bartowsky 2009). Because of this, glycerol, one of the precursor compounds for acrolein production was measured. In this study none of the pediococci isolates could degrade glycerol in contrast to the findings of Pasteris and Stasser de Saad (2004) who reported that certain strains of *P. pentosaceus* were capable of utilizing glycerol. However, Pasteris and Stasser de Saad (2004) reported that the pediococci could utilize glycerol as a carbon source under aerobic conditions rather than the anaerobic conditions of the present study. In addition, although there has been mention of acrolein production by *Pediococcus* (Bartowsky 2009) to our knowledge there is no evidence in the literature to support assumptions that *Pediococcus* is capable of forming the acrolein precursor, 3-HPA. Further screening of *Pediococcus* species and strains is required to determine if *Pediococccus* is capable of causing bitterness in red wine.

While growth of *Pediococcus* can directly impact wine quality through production of spoilage compounds their growth in wine may also impact spoilage of the wine by other wine microorganisms. In this study *Brettanomyces bruxellensis* produced 4-ethyl phenol at an accelerated rate when growing in media in which *Pediococcus* had previously grown. The pediococci were able to produce 4-vinyl phenol from *p*-coumaric acid while *B. bruxellensis* further converted this compound to 4-ethyl phenol. The production of 4-VP by lactic acid bacteria (LAB) has been reported by others (Chatonnet et al. 1995). However, what has not been reported before is the conversion of the LAB produced 4-VP by *Brettanomyces* into 4-EP. The accelerated

production of 4-EP by *B. bruxellensis* is likely due to the fact that the production of 4-EP from *p*-coumaric acid is an energy dependent two-step process involving an initial decarboxylation of the hydroxycinnamic acids catalyzed by cinnamate decarboxylase and the reduction of the vinyl phenol intermediates by vinyl phenol reductase. If LAB perform the first step then this may provide a benefit to *Brettanomyces.* While these results were from a study performed in a model system they demonstrate a mechanism by which *Pediococcus* growth may be beneficial to *Brettanomyces*. Future studies should repeat these experiments in wine focusing on growth and 4-EP production of *Brettanomyces*.

Currently, the best winemaking tool to deal with pediococci is the antimicrobial agent SO_2. However, the sensitivity of pediococci to SO_2 is not well understood with conflicting reports in the literature. For example, Davis et al. (1988) indicated that strains of *L. oenos* (*O. oeni*) were less tolerant to sulfur dioxide than strains of *P. parvulus* while Hood (1983) reported that pediococci were less tolerant to SO_2 than lactobacilli or oenococci. In this study there were differences in SO_2 sensitivity between pediococci species as reported by Wells and Osborne (2012). In addition, there were also differences between isolates of the same species with *P. parvulus* isolates differing in their sensitivity to SO_2. Interestingly, SO_2 showed a bacteriostatic nature in this study, by inhibiting growth or lengthening lag phase in a number of cases. This was also observed by Wells and Osborne (2012) where acetaldehyde bound SO_2 also inhibited *P. damnosus* and *P. parvulus* causing delayed lag phase and lower final populations. Future studies should focus on repeating these

experiments in wine where both bound and free SO_2 will be present in case the degradation of SO_2 bound acetaldehyde impacts the inhibition of certain pediococci through the release of free SO_2. An additional aspect of SO_2 that should be explored is whether SO_2 causes pediococci to enter a viable but non-culturable state (VBNC). A number of other wine microorganisms have been demonstrated to enter this state in response to stress conditions such as osmotic pressure, temperature, oxygen concentration or SO_2 (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005; Du Toit et al., 2005; Oliver, 2005). However it is currently not known if pediococci can enter this state and this may impact their detection and control in wine.

CONCLUSIONS

Growth of various pediococci isolates (*P. parvulus*, *P. damnosus* and *P. inopinatus*) in Pinot noir wine resulted in significant production of various wine spoilage compounds. Production of certain spoilage product varied greatly between the different isolates. In particular, there was large disparity in the amount of diacetyl produced although this was not species specific. Pediococci also impacted the color and polymeric content of the wine with some wines containing 30% less polymeric pigment than the control. This may have been related to the degradation of acetaldehyde by some of the isolates, a property that *Pediococcus* was thought not to possess. Although pediococci are often linked to the production of biogenic amines in wine none of the isolates in this study produced appreciable amounts of any biogenic

amine. Finally, pedioccci also impacted the production of 4-ethyl phenol by *Brettanomyces bruxellensis* as in a model system all isolates degraded *p*-coumaric acid to 4-VP resulting in accelerated production of 4-EP by *B. bruxellensis*. This finding and the interactions between *Pediococcus* and *Brettanomyces* should be explored further given the significance of *Brettanomyces* spoilage to the wine industry.

CHAPTER 3

IMPACTS OF *PEDIOCOCCUS* SPP. ON THE SENSORY CHARACTERISTICS OF PINOT NOIR WINES.

ABSTRACT

Pediococcus are lactic acid bacteria that have been isolated from red wines all over the world however little is known of their impacts, if any, on the organoleptic properties of red wine. Pinot noir wines were inoculated with various *Pediococcus* isolates and presented for sensory analysis to a trained panel. The panel consisted of 12 panelists from Oregon State University and the local winemaking community. Panelists were trained for several weeks on general Pinot noir sensory characteristics as well as common spoilage traits. Once a list of descriptors was generated and agreed upon by panelists, wines were then assessed and scored for a number of aromas, flavors, and tastes on a 16 point intensity scale. Panelists found differences between pediococci treatments as well as the uninoculated control wine with regards to floral, overall fruit, red fruit, buttery, sour, and astringency. Characters such as buttery did not correlate well with previous chemical analyses. Results from this study aid in the directions for future sensory and chemical analyses of wines inoculated with *Pediococcus* spp.

INTRODUCTION

The microbial spoilage of wine can cause major economic losses due to loss of quality. Spoiled wines may need to be used as blending wines in lower quality products or the wines may be unusable. The major spoilage microorganisms of concern to the wine industry are *Brettanomyces bruxellensis*, *Acetobacter*, and the lactic acid bacteria species *Lactobacillus* and *Pediococcus*. While a number of studies have reported on the chemical and sensory changes caused by *Brettanomyces* and *Lactobacillus* (Chatonnet et al. 1995; Fugelsang and Zoecklein 2003), few studies have reported on the chemical changes caused by *Pedioococcus* while few if any have commented on wine sensory changes caused by growth of *Pediococcus*. In particular, differences between *Pediococcus* species and strains have not been investigated with spoilage caused by *Pediococcus* often generalized as "lactic taint" (Du Toit and Pretorious, 2000).

Pediococcus are known to produce compounds in wine that potentially could impact wine sensory properties. For example, pediococci have been reported to produce excess diacetyl (Edwards et al. 1994). This compound can contribute slight buttery or butterscotch aromas to red wine at low levels (1-4 mg/L) but at higher concentrations these aromas may take on an unpleasant buttered popcorn character (Bartowsky and Henschke 2004). Degradation of malic acid to lactic acid by some species of pediococci has also been reported (Edwards and Jensen 1992; Edwards et al. 1994) resulting in a reduction in the acidity of the wine. This process, the malolactic fermentation, is considered beneficial when conducted by *Oenococcus oeni*, but may be considered undesirable when conducted by pediococci or when malolactic

fermentation is undesired. In addition, pediococci can also form DL-lactic acid from the metabolism of glucose further affecting the acid content of the wine matrix (Dicks and Endo 2009). *Pediococcus* have also been shown to produce biogenic amines (Izquierdo-Pulido et al. 2000; Landete et al. 2005; Landete et al. 2008; Nanneli et al. 2008; Coton et al. 2010). While biogenic amines are thought to cause health issues they have also been reported to give undesirable aromas and tastes in wines at elevated concentrations (Edwards and Fugelsang, 2007). However, evidence of this sensory impact and the concentrations required to cause it have not been reported.

Despite evidence that pediococci can produce numerous compounds that can impact the sensory properties of wine few if any studies have reported on the sensory impact of *Pediococcus* spoilage of wine and whether spoilage is species or strain specific.

Therefore, the goal of this study was to investigate the impact of a number of pediococci isolates on the sensory properties of an Oregon Pinot noir. Three different species of *Pediococcus* were investigated including numerous isolates of *P. parvulus*.

MATERIALS AND METHODS

Pinot noir wine production

Pinot noir wine was produced as described previously (Chapter 2, Materials and Methods). In brief, Pinot noir grapes were harvested from Oregon State University's Woodhall Vineyard (Alpine, Oregon, USA), destemmed, and fermented in 100L tanks. Fermentations were conducted by *S. cerevisiae* RC212 and were complete in

less than 14 days. After pressing, wines were pooled and settled for 48 hrs at 4°C prior to filtration. Wines were pad filtered (Beco K-1 3.0μm nominal filter sheets (Langenlonsheim, Germany)), pH adjusted to pH 3.75 (addition of NaOH) and then filtered through a 1.0μm cartridge and a sterile 0.45μm polyethersulfone cartridge (G.W. Kent, Ypsilanti, Michigan, 40 USA). Filtered wine was dispensed into sterile one gallon carboys and inoculated with various pediococci isolates as outlined previously (Chapter 2, Materials and Methods). Pediococci isolates used were *P. parvulus* WW1, WS9, OW1, OW4, OW5, OW6, OW7, *P. damnosus* OW2, and *P. inopinatus* OW8. After significant growth of all isolates had occurred (bacteria entered stationary phase after approximately 6-8 weeks) all wines including an uninoculated control were sterile filtered and bottled (750 mL screw capped bottles) after an addition of 30mg/L SO_2. Wines were stored at 13°C until required for analysis.

Wine sensory analysis

Sensory panelists and training

Twelve panelists were recruited from the Oregon State University Food Science and Technology Department, from the Corvallis, OR, community, and local wineries. The panel contained six men and six women and all were experienced with wine sensory evaluation or trained panel sensory analysis. Eight training sessions were conducted where panelists tasted several Pinot noir wines of similar age to the experimental wines as well as the actual experimental wines and created a list of

descriptors describing the wine aroma and flavor. During training the list was amended until all panelists agreed on a final list of descriptors and their definitions. References were created based on Noble et al. (1987) and Guinard and Cliff (1987) to help panelists understand and define descriptors (Noble et al. 1987; Guinard and Cliff 1987). Thirteen aroma descriptors (overall intensity, floral, overall fruit, red fruit, dark fruit, jammy, spicy, earthy, herbaceous/vegetal, aged aromas, reduced, buttery, and plastic), four retronasal aroma/tactile (flavor) descriptors (overall flavor intensity, fruit flavor, spicy flavor, and aged flavor), and three taste/mouthfeel descriptors (sour, bitter, astringent) were chosen. A sixteen point intensity scale was utilized and intensity standards were created to aid panelists in the rating of intensities. At the beginning of each training and evaluation session, panelists were encouraged to re-familiarize themselves with a warm-up wine as well as the standards.

Sensory evaluation

Based on preliminary sensory evaluation of the wines by the investigators, six wines that had been inoculated with different *Pediococcus* isolates were chosen for evaluation by the panel as well as the control wine (no inoculation of *Pediococcus*). Wines were kept at 13°C until being assessed by the sensory panel twelve months post-bottling. All wines were allowed to equilibrate to room temperature and poured 30 minutes before evaluation. 30mL samples were served in 240mL INOVA tulip glasses (St. George Crystal Ltd., Jeannette, PA, USA) and covered with a plastic lid. Glasses were coded and evaluated in a completely randomized order with panelists

tasting each wine treatment three times. Panelists rated the samples based on the list of descriptors and rated each descriptor using a sixteen-point intensity scale. Panelists were allowed five minutes to evaluate each wine and were given a mandatory one-minute break between samples. Evaluation of the wines took place over three separate one hour sessions.

Statistical Analysis

A univariate Analysis of Variance (ANOVA) was used to determine differences between the wine samples for mean scores of each descriptor. The ANOVA was performed using Microsoft Excel (Version 14.1.3) and XL Stat (Version 2011.4.02). The ANOVA model comprised two main effects (Panelist (PAN) and Wine (WINE), a nested effect (replication which was nested in PAN or (REP(PAN)), and a two-way interaction effect between PAN and WINE (WINE*PAN). The PAN, REP(PAN) and (WINE*PAN) were treated as random effects and WINE was treated as a fixed effect. Type III Sums of Squares were used in the testing of main effects and interactions. Significant differences detected by ANOVA were subjected to post-hoc Tukey HSD multiple comparison to test least squares means of WINE (means) at the 0.05% significance level. The means with the same letter are not significantly different at the 0.05% level.

RESULTS

As previously detailed (Chapter 2) all pediococci isolates grew well in Pinot noir wine reaching populations greater than 1×10^6 CFU/mL. These wines were

subsequently evaluated by a trained sensory panel. Results of the ANOVA indicated panelists were a significant source of variation for all the attributes (Table 3.1) while there were significant ($p<0.05$) differences between wines for floral aroma, overall fruit aroma, red fruit aroma, buttery aroma, sour, and astringency (Table 3.1). No significant differences were found between wines for the other aroma, flavor, and taste/mouthfeel attributes assessed. Despite the ANOVA not finding statistical significance for dark fruit, earthy, and plastic aromas, Tukey's HSD considered them significantly different between treatments. A significant treatment x panelist interaction was found for overall aroma and spicy flavor indicating that these terms were not being used consistently by all panelists (Table 3.1).

When mean wine sensory descriptor intensities were compared significant differences between wines inoculated with different pediococci were found (Table 3.2). Compared to the control wine, a number of wines inoculated with *Pediococcus* isolates had higher floral intensity with OW7 having a mean intensity value of 4.5 compared to the 2.9 for the control. A similar trend was seen for overall fruit aroma with OW1, OW2, and OW5 having higher intensity values than the control while OW8 had the lowest. Some of the differences in overall fruit aroma were reflected in red fruit and dark fruit aroma intensities with OW8 having the lowest red fruit intensity while OW1 and OW5 had the highest red fruit aroma intensities. OW6 and OW7 wines had significantly lower buttery aroma intensities than the other wines although the intensity values were all very low. In addition to differences in aroma some wines differed in the taste and mouthfeel descriptors, sour and astringency. For

sour, the control had the highest intensity value while OW5 and OW8 had significantly lower sour intensity values (Table 3.2). For astringency, OW8 wines had significantly lower intensity values than OW7 while the other wines were statistically the same.

Principle Component Analysis

PCA was used to reduce the number of variables and to illustrate the relationships between the wine aroma descriptors and wines inoculated with different pediococci isolates. For aroma (Figure 3.1A & B) PC1 accounted for 36.4% of the variation and was characterized by herbaceous, reduced, spicy, and earthy aromas against dark fruit, jammy, and overall fruit aromas. PC2 accounted for 29.4% of the variation and was characterized by aged and buttery aromas against floral aromas. PC3 accounts for 15.9% of the variation and was characterized by plastic aromas. For flavor, taste, and mouthfeel (Figure 3.2 A & B) PC1 accounted for 53.2% of the variation and was characterized by overall fruit and spicy flavor against aged flavor. PC2 accounted for 28.5% of the variation and was characterized by sour taste while PC3 only accounted for 9.7% of the variation.

Table 5: Analysis of variance F-ratio attribute ratings for wines inoculated with *Pediococcus* spp. (*) indicates significance at the $p<0.05$ level, (**) indicates significance at the $p<0.01$ level; (***) indicates significance at the $p<0.001$ level.

Descriptors	Treatment	Panelist	Rep (Panelist)	Panelist x Treatment
Overall Aroma	0.96	15.30***	2.02**	1.43*
Floral	2.86*	10.32***	1.15	1.29
Overall Fruit	2.67*	22.45***	1.11	1.24
Red Fruit	3.01**	13.26***	1.27	1.15
Dark Fruit	1.52	14.96***	1.79**	1.56
Jammy	1.37	7.87***	1.23	0.86
Spicy	1.62	7.45***	1.32	1.22
Earthy	1.56	8.15***	1.80*	1.28
Herbaceous	1.31	4.18***	1.62	1.23
Aged Aromas	0.86	11.76***	2.06**	1.01
Reduced	1.43	6.50***	2.17**	1.24
Buttery	2.26*	9.32***	2.19**	1.19
Plastic	1.41	13.95***	1.24	1.35
Overall Flavor	0.33	18.45***	2.50**	0.95
Fruit Flavor	1.00	25.77***	1.38	0.87
Spicy Flavor	1.40	17.39***	2.68***	1.56**
Aged Flavor	1.33	18.50***	2.24**	0.93
Sour	2.87*	15.99***	2.83***	1.07
Bitter	1.37	12.28***	1.76*	0.92
Astringent	2.89*	19.62***	2.42**	0.88
Degrees of Freedom	6	12	21	72

Table 6: Mean wine sensory descriptor intensities for wines inoculated with *Pediococcus* isolates.

Descriptors	CON	OW1	OW2	OW5	OW6	OW7	OW8
Ov. Aroma^{NS}	8.1^{a}	7.9^{a}	7.9^{a}	7.9^{a}	7.7^{a}	8.0^{a}	7.7^{a}
Floral	2.9^{c}	3.9^{abc}	4.0^{ab}	3.5^{abc}	3.3^{bc}	4.5^{a}	3.2^{bc}
Overall Fruit	5.8^{b}	6.6^{a}	6.5^{a}	6.5^{a}	6.1^{ab}	6.0^{ab}	5.5^{b}
Red Fruit	4.1^{ab}	5.0^{a}	5.0^{a}	4.3^{ab}	4.7^{ab}	4.7^{ab}	3.7^{b}
Dark Fruit^{NS}	3.9^{bc}	4.6^{a}	4.2^{abc}	4.6^{ab}	4.1^{abc}	3.7^{c}	4.0^{abc}
Jammy^{NS}	2.8^{a}	3.2^{a}	2.8^{a}	3.2^{a}	2.8^{a}	2.2^{a}	2.6^{a}
Spicy^{NS}	3.9^{a}	3.6^{a}	3.9^{a}	3.8^{a}	3.3^{a}	4.1^{a}	3.6^{a}
Earthy^{NS}	2.6^{a}	1.9^{b}	2.3^{ab}	2.4^{ab}	1.8^{b}	2.0^{ab}	2.2^{ab}
Herbaceous^{NS}	1.7^{a}	1.1^{a}	1.6^{a}	2.0^{a}	1.2^{a}	1.6^{a}	1.7^{a}
Aged Aromas^{NS}	3.0^{a}	2.9^{a}	3.2^{a}	2.8^{a}	2.9^{a}	2.5^{a}	3.3^{a}
Reduced^{NS}	1.4^{a}	0.7^{a}	1.3^{a}	0.5^{a}	1.1^{a}	1.3^{a}	1.2^{a}
Buttery	1.2^{ab}	1.1^{ab}	1.0^{ab}	1.2^{ab}	0.9^{b}	0.6^{b}	1.9^{a}
Plastic^{NS}	0.7^{b}	1.0^{ab}	1.6^{a}	0.8^{ab}	0.9^{ab}	0.9^{ab}	1.2^{ab}
Ov. Flavor^{NS}	6.5^{a}	6.6^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.4^{a}
Fruit Flavor^{NS}	5.1^{a}	5.4^{a}	5.3^{a}	5.7^{a}	5.2^{a}	5.3^{a}	4.9^{a}
Spicy Flavor^{NS}	3.7^{a}	4.1^{a}	4.1^{a}	4.0^{a}	3.9^{a}	4.2^{a}	3.9^{a}
Aged Flavor^{NS}	2.8^{a}	2.4^{a}	2.2^{a}	2.4^{a}	2.2^{a}	2.3^{a}	2.6^{a}
Sour	5.3^{a}	4.7^{ab}	4.4^{ab}	4.3^{b}	4.5^{ab}	4.9^{ab}	4.2^{b}
Bitter^{NS}	3.6^{a}	4.4^{a}	4.1^{a}	4.3^{a}	4.1^{a}	4.3^{a}	3.9^{a}
Astringent	4.6^{ab}	4.7^{ab}	4.2^{ab}	4.5^{ab}	4.4^{ab}	5.0^{a}	4.1^{b}

$^{a-c}$ Mean values with different superscript letters within a row are significantly different at $p < 0.05$ level, Tukey's HSD, $n = 3$. CON, Control.

The control wine was characterized by herbaceous, earthy, and reduced aromas as well as sour taste and aged aroma. OW1 and OW5 showed similar trends to each other being characterized by fruit aroma and flavor descriptors while OW7 trended more towards spicy aromas and higher astringency while OW2 was the only treatment characterized by plastic aroma. In general OW8 was characterized as having low intensities for all flavor, taste, and mouthfeel attributes although it was more associated with buttery aroma.

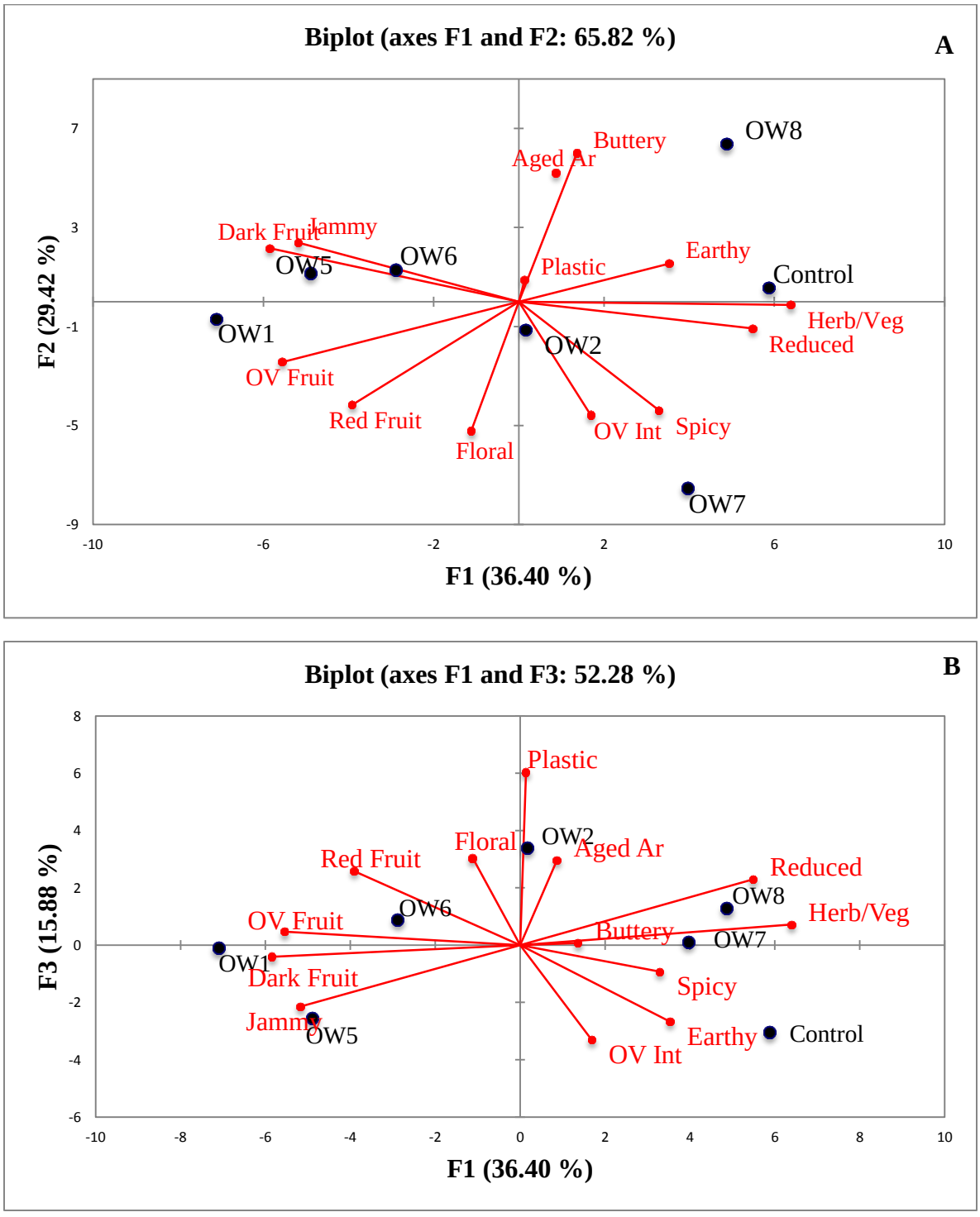


Fig 13. Principle Component Analysis of mean sensory aroma data for wines inoculated with different pedioccoci isolates.

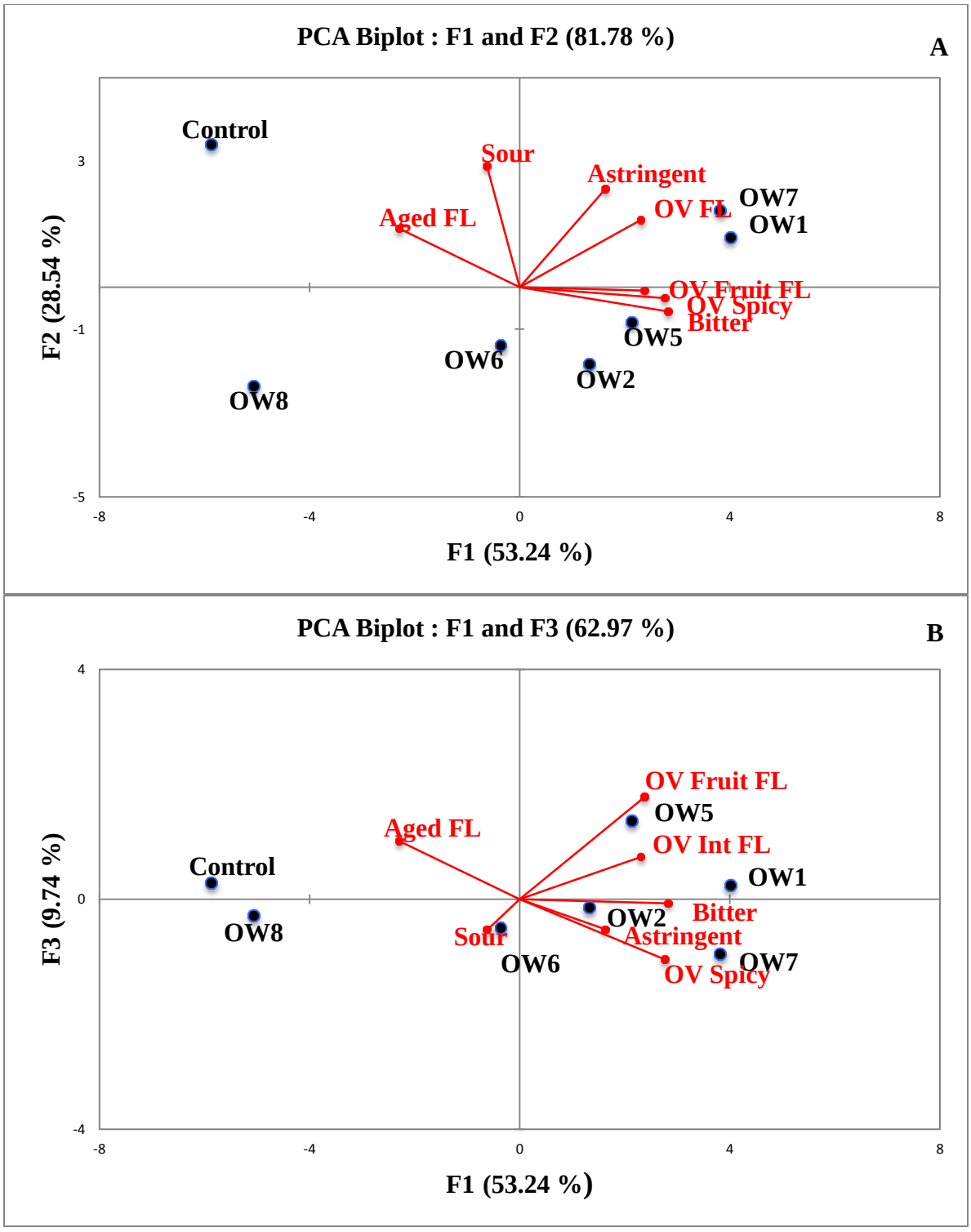


Figure 14. Principle Component Analysis for mean sensory data for flavor, taste, and mouthfeel descriptors of wines inoculated with different pedioccoci isolates.

DISCUSSION

Although commonly thought of as a wine spoilage microorganism, the sensory impact of *Pediococcus* on red wine is relatively unreported. This study has shown that *Pediococcus* spp. may affect numerous sensory aspects of wine and that these changes may range from a general depression or muting of aroma intensity to changes in specific attributes such as decreased red and dark fruit or increased butter aromas. For example, OW7 wines had elevated floral aromas, but lower dark fruit aromas while OW1 had elevated dark fruit aromas, but also depressed earthy aromas. While the sensory changes caused by *Pediococcus* ranged in their intensity they also were not species specific. In this study four *P. parvulus* isolates were used as well as a *P. damnosus* and *P. inopinatus* isolate. The sensory impacts varied between the *P. parvulus* isolates as well as between the different species. In addition, no clear trends or groupings were noted with the PCA. These findings suggest that wine spoilage by pediococci can cause a wide range of sensory effects that will differ depending on the species and strain causing the spoilage.

Some of the results from this study align with previous chemical analyses conducted in Chapter 2. For example, OW8 wine was rated highest for buttery by panelists and also contained very high concentrations of diacetyl (15mg/L). The Control wine was rated lower for buttery and contained only 3.2mg/L of diacetyl. Similarly OW7 contained the lowest level of diacetyl at only 0.33mg/L and was considered by panelists to have low buttery intensity. Interestingly OW1, OW2, and OW5 all had

comparable diacetyl contents to OW8, but were not scored by panelists accordingly. This was surprising given that the sensory detection level of diacetyl is considered to be approximately 1mg/L with a spoilage level of 5-7mg/L in delicate wines such as Pinot noir (Bartowsky and Henschke 2004). It is also interesting to note that the mean scores for buttery are relatively low. OW8 had a mean score of 1.9 while the Control had a mean score of 1.2. It would be expected then that OW8, which contained diacetyl levels of 15mg/L, would be scored higher than 1.9 on a 16 point scale. Although the panel was trained on wines where diacetyl had been added these findings suggest that additional training focused on detecting diacetyl in red wine may be required when evaluating wines spoiled by *Pediococcus.*

As expected, the Control wine was considered to be the most sour by panelists with a mean score of 5.3. The control did not undergo malolactic fermentation (MLF) and so still contained a high concentration of malic acid. The malolactic fermentation lowers the perceived acidity of the wine by converting malic acid to lactic acid (Lonvaud-Funel 1999; Liu 2002; Bartowsky and Henschke 2004). However, the sour intensity of wines inoculated with pediococci isolates did not always correlate well with whether they had undergone ML. For example, all pediococci isolates used in this study were able to at least partially perform the malolactic fermentation with OW1, OW2, and OW7 reducing malic acid levels to below 100mg/L. However, panelists indicated OW1, OW2, and OW7 were not significantly less sour than the Control. Again, difficulties in defining sourness and sour intensity may have resulted in the sensory data not always correlating with the chemical analysis.

The results from this sensory analysis indicate directions for future chemical analyses. For example, a number of panelists commented on a plastic aroma from some of the wines. When the sensory data was analyzed Tukey's HSD found differences amongst treatments for plastic aromas, while the ANOVA did not find any significant differences between treatments (Table 3.2). This may be due to higher standard deviations. However, it is clear from the principle component analysis that panelists considered OW2 more strongly associated with this aroma, though at low levels with a mean of 1.6. Plastic aroma may be due to indole or indole-derivative production by *Pediococcus* isolates. Indole has been shown to be the source of plastic aromas and has an exceedingly low sensory detection threshold of only 23µg/L in white wine (Arevalo-Villena et al. 2010). This compound was not analyzed in this study but may be an additional compound to evaluate when investigating *Pediococcus* spoilage of red wines.

An additional group of compounds that should be assessed in pediococci spoiled wines would be the volatile phenols. When panelists were given a chance to write down additional descriptors for the wines a number noted aromas of bandaid and medicinal, descriptors often associated with 4-ethyphenol (Chatonnet et al. 1995). While *Pediococcus* spp. have not been reported to produce 4-ethylphenol they produce the vinyl form 4-vinyl phenol. These vinylphenols can have relatively low sensory thresholds of 800µg/L (Chatonnet et al. 1995) and so could contribute to the sensory qualities of a wine.

Aside from aroma differences, panelists also noted a decreased astringency is some of the wines. However, tannin analysis performed previously showed that there were no significant differences between the tannin content of any of the wines. While tannin content is one of the major influences on red wine astringency there are other factors that impact this sensory attribute. They include acidity, sugar content, and tannin composition (versus concentration) (Kennedy et al. 2007). This makes relating perceived astringency to tannin composition very challenging. Panel training for astringency in wine is also very difficult due to the lack of standards and the wide range of descriptors used for astringency. If future studies pursue the impact of *Pediococcus* on red wine astringency then greater sensory training and more in depth analysis of the phenolic composition of the wines will be required.

The majority of mean scores given by panelists for the various attributes in this study were generally in the low to medium range of the 16-point scale. Descriptors for which it was found there to be significant differences often were significant based on mean differences of only 1 or 2 points. While these differences might cause statistical significance, it is important to consider what the practical significance of these scores might be. For instance, while panelists scored OW7 at 5.0 for Astringency versus OW8 at 4.1 would a winemaker be concerned with this little difference in astringency? The low intensity ratings given by the panelists for many of the descriptors may be related to the fact that Pinot noir is typically not an intensely aromatic or astringent wine. It is also possible that panelists felt uncomfortable with the 16-point scale or the breadth of intensities that it may imply.

Performing sensory analysis on wines in which *Pediococcus* had grown presented a number of challenges. Firstly, with so little available information in the literature on spoilage characteristics of wine infected with *Pediococcus* it was difficult to develop the training necessary for the trained panel. Additionally, another difficulty arose when discussing the actual nature of the panel. Panelists were not told that this was a study on wine spoilage for fear of introducing panelist bias. Therefore, panelists were trained using a standard Pinot noir as well as exposed to spoiled wines but were not explicitly trained to focus only on spoilage characteristics. This may have resulted in the spoilage descriptors used for the final ballot not being as varied or diverse as would have been necessary to completely describe *Pediococcus* spoilage. Future works will include more trained sensory panels with a larger focus on spoilage descriptors. Also, efforts should be made to perform a broader spectrum of chemical analyses in an effort to correlate chemical spoilage with sensory data. Also, using a different varietal of wine may alter the extent to which pediococci may impact the sensory characters of wine.

CONCLUSIONS

Pediococcus isolates affected numerous sensory aspects of a Pinot noir wine with changes ranging from a general depression or muting of aroma intensity to changes in specific attributes. Spoilage character was not species specific with spoilage by pediococci largely depends on individual isolates. While some of the spoilage characteristics of the wine correlated well with chemical analysis a number of

characteristics did not. While there were significant differences between treatments with regards to various aromas, flavors, and tastes, many of these differences were relatively small as panelists overall rated the wines low for most descriptors. Future work should focus on wine spoilage specific training coupled with additional chemical analysis based on the sensory characteristics outlined in the present study. In addition, different varieties of wine should also be assessed.

OVERALL SUMMARY AND FUTURE WORK

Pediococcus spp. have been isolated from red wines all over the world. However, little is known about the potential impacts they may have on red wine quality. Instead research regarding red wine spoilage has focused heavily on other members of the lactic acid bacteria group as well as *Brettanomyces* yeasts. This lack of information on the spoilage characteristics of *Pediococcus* poses considerable problems for modern winemakers as they develop strategies to control microbial spoilage of their wines. This study attempted to ascertain the potential effects that pediococci may have on Oregon Pinot noir wines.

Pediococci isolates from Oregon and Washington state wines produced a number of potential spoilage compounds in various concentrations during growth in Pinot noir wine. Despite utilizing isolates in this study with the capacity for biogenic amine formation, very low concentrations of biogenic amines were measured in the wines with only wine inoculated with *P. inopinatus* OW-8 containing greater than 5mg/L. D-lactic acid production varied between isolates with OW-7 producing the highest concentration (264mg/L). Diacetyl content of the wines also varied greatly. Some wines contained very low levels of diacetyl (< 0.5mg/L) while others contained very high concentrations (> 15mg/L) that were well above sensory threshold. Color and polymeric pigment content of the wines also varied with wine inoculated with OW-7 containing 30% less polymeric pigment than the control. This may have been related to acetaldehyde concentration as a number of *Pediococcus* isolates, including OW-7,

reduced the acetaldehyde content of the wine. To our knowledge this is the first time that it has been shown that pediococci can have significant impacts on red wine color and polymeric pigment.

A trained sensory panel also assessed the experimental wines for various descriptors related to aroma, flavor, and taste. The panel found differences in the aroma and mouthfeel of the wines compared to each other and to the control. In particular, growth of some isolates produced wines with higher intensities of butter, plastic, and vegetal aromas while other also had lower perceived astringency.

Future works should include collecting a larger number of pediococci isolates to further ascertain species distribution and occurrence in the Oregon wine industry. Efforts should also be made to study the effects of sulfur dioxide on pediococci in wine systems as opposed to model media with an emphasis on determining whether pediococci can enter a viable but non-culturable state. While Pinot noir is an important varietal in the Oregon wine industry, efforts should be made to utilize different varietals especially wines with typically higher phenolic content. While this study did not show a large increase in biogenic amine content from the presence of pediococci isolates, these compounds still remain a considerable concern to winemakers and consumers due to the health implications they impose. Future works attempting to answer questions surrounding their production by examining model systems containing different sources and levels of substrate for the formation of biogenic amines. Future sensory work should include greater focus on spoilage

compounds coupled with more extensive chemical analyses to better correlate the total effects that pediococci may have on wine.

.

BIBLIOGRAPHY

Abrahamse, C. E. and Bartowsky, E.J. (2012). "Timing of malolactic fermentation inoculation in Shiraz grape must and wine: influence on chemical composition". World Journal of Microbiology and Biotecnology. 28: 255-265.

Adams, Douglas O., and J. F. Harbertson. (1999). "Use of Alkaline Phosphatase for the Analysis of Tannins in Grapes and Red Wines." American Journal of Enology and Viticulture. 50 (3): 247-252.

Anli, R.E. and M. Bayram. (2009). "Biogenic amines in wines". Food Reviews International. 25: 86-102

Arevalo-Villena, M., Bartowsky, E.J., Capone, D., and M.A. Sefton. (2010). "Production of indole by wine-associated microorganisms under oenological conditions." Food Microbiology. 27: 685-690

Bach, B., Colas, S., Massini, L., Barnavon, L., and P. Vuchot. (2011). "Effect of nitrogen addition during alcoholic fermentation on the final content of biogenic amines in wine". Annals of Microbiology. 61: 185-190

Barthelmebs, L., LeComte, B., Divies, C., and C. Jean-Francois. (2000). "Inducible Metabolism of Phenolic Acids in *Pediococcus pentosaceus* Is Encoded by an Autoregulated Operon Which Involves a New Class of Negative Transcriptional Regulator". Journal of Bacteriology. 182(23): 6724-6731

Bartowsky, Evaline J. (2009). "Bacterial Spoilage of Wine and Approaches to Minimize It". Letters in Applied Microbiology. 48: 149-156

Bartowsky, Evaline J., and P.A. Henschke. (2004). "The 'Buttery' Attribute of Wine Diacetyl-Desirability, Spoilage and Beyond". International Journal of Food Microbiology. 96: 235-252

Bauer, R., du Toit, M., and J. Kossmann. (2010a). "Influence of environmental parameters on production of the acrolein precursor 3-hydroxypropionaldehyde by *Lactobacillus reuteri* DSMZ 20016 and its accumulation by wine lactobacilli". International Journal of Food Microbiology. 137: 28-31

Bauer, R., Cowan, D.A., and A. Crouch. (2010b). "Acrolein in Wine: Importance of 3 Hydroxypropionaldehyde and Derivatives in Production and Detection". Journal of Agricultural and Food Chemistry. 58: 3243-3250

Benito, S., A. Palmoero, C. Uthurry, and J.A. Suarez. (2009). "Minimization of ethylphenol precursors in red wines via the formation of pyranoanthocyanins

by selected yeasts". International Journal of Food Microbiology. 132: 145-152.

Burns, T.R. (2011). Impact of malolactic fermentation on red wine color and color stability. MS thesis, Oregon State University.

Campos, F.M., Couto, J.A., and T.A. Hogg. (2003). "Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*". Journal of Applied Microbiolgy. 94: 167-174

Campos, F.M., Couto, J.A., Figueiredo, A.R., Tóth, I.V., Rangel, A.O.S.S., and T.A. Hogg. (2009). "Cell membrane damage induced by phenolic acids on wine lactic acid bacteria". International Journal of Food Microbiology. 135: 144-151

Chatonnet, P., Dubourdieu, D., and J.N. Boidron. (1995). "The Influence of *Brettanomycs/Dekkera* sp. Yeasts and Lactic Acid Bacteria on the Ethylphenol Content of Red Wines". American Journal of Enology and Viticulture. 46 (4): 463-468

Constantini, C., Cersosimó, M., Del Prete, V., and E. Garcia-Maruno. (2006). Production of biogenic amines by lactic acid bacteria: Screening by PCR, thin-layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. Journal of Food Protection. 69: 391-396

Costello, P.J. and P.A. Henschke. (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

Coton, M., Romano, A., Spano, G., Ziegler, K., Vetrana, C., Desmarais, C., Lonvaud Funel, A., Lucas, P., and E. Coton. (2010). "Occurrence of biogenic amine forming lactic acid bacteria in wine and cider". Food Microbiology. 27: 1078-1085

Couto, J.A., Campos, F.M., Figueiredo, A.R., and T.A. Hogg. (2006). "Ability of Lactic Acid Bacteria to Produce Volatile Phenols". American Journal of Enology and Viticulture. 57 (2): 166-171

De las Rivas, B., Rodríguez, H., Curiel, J.A., Landete, J.M., and R. Muñoz. (2009). "Molecular Screening of Wine Lactic Acid Bacteria Degrading Hydroxycinnamic Acids". Journal of Agricultural and Food Chemistry. 57: 490-494

Delaherche, A., Claisse, O., and A. Lonvaud-Funel. (2004). "Detection and quantification of *Brettanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction". Journal of Applied Microbiology. 97: 910-915

Dias, L., Pereira-da-Silva, S., Tavares, M., Malfeito-Ferreira, M., and V. Loureiro. (2003). "Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions". Food Microbiology. 20: 377-384

Davis, C.R., Wibowo, D.J. and G.H. Fleet. (1986). "Growth and Metabolism of Lactic Acid Bacteria during and after Malolactic Fermentation of Wines at Different pH." Applied and Environmental Microbiology. 51 (3): 539-545

Dicks, L.M.T., Dellaglio, F., and M.D. Collins. (1995). "Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni*". International Journal of Systemic Bacteriology. 45: 395-397

Dicks, L.M.T. and A. Endo. (2009). "Taxonomic Status of Lactic Acid Bacteria in Wine and Key Characteristics to Differentiate Species". South African Journal Of Enology and Viticulture. 30 (1): 72-90

Dols-Lafargue, M., Lee, H.Y., Le Marrec, C., Heyraud, A., Chambat, G., and A. Lonvaud-Funel. (2008). "Characterization of gtf, a glucosyltransferase gene in the genomes of *Pediococcus parvulus* and *Oenococcus oeni*, two bacterial species commonly found in wine". Applied and Environmental Microbiology. 74 (13): 4079-4090

Du Toit, M., and I.S. Pretorius. (2000). "Microbial spoilage and preservation of wine: using weapons from nature's own arsenal-a review". South African Journal of Enology and Viticulture. 91: 19-29

Dueñas-Chasco, M.T., Rodríguez-Carvajal, M.A., Mateo, P.T., Franco-Rodríguez, G., Espartero, J.L., Irastorza-Iribas, A., and A.M. Gil-Serrano. (1997). "Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus* 2.6". Carbohydrate Research. 303: 453-458

Dueñas, M., Munduate, A., Perea, A., and A. Irastorza. "2003". "Exopolysaccharide production by *Pediococcus damnosus* 2.6 in a semidefined medium under different growth conditions". International Journal of Food Microbiology. 87: 113-120

Edwards, C.G., J.C. Peterson, T.D. Boylston, and T.D. Vasile. (1994). Interactions

between *Leuconostoc oenos* and *Pediococcus* spp. during vinification of red wines. American Journal of Enology and Viticulture. 45: 49-55

Edwards, C.G. and K.A. Jensen. (1992). "Occurrence and characterization of lactic acid bacteria from Washington state wines: *Pediococcus* spp". American Journal of Enology Viticulture. 43: 233-238

Escamilla, M.L., Valdés, S.E., Soriano, J., and A. Tomosini. (2000). "Effect of some nutritional and environmental parameters on the production of diacetyl and on starch consumption by *Pediococcus pentosaceus* and *Lactobacillus acidophilus* in submerged cultures". Journal of Applied Microbiology. 88 (1): 142-153

Fugelsang, K.C. and C.G. Edwards. (2007). *Wine Microbiology: Practical Applications and Procedures* 2nd ed. Springer Science. New York, NY

Fugelsang, K.C. and B.W. Zoecklein. (2003). "Population dynamics and effects of Brettanomyces bruxellensis strains on Pinot noir (Vitis vinifera L.) wines". American Journal of Enology and Viticulture. 54: 294-300

Garai-Ibabe, G., Ibarburu, I., Berregi, I., Claisse, O., Lonvaud-Funel, A., Irastorza, A., and M.T. Dueñas. (2008). "Glycerol metabolism and bitterness producing lactic acid bacteria in cidermaking". International Journal of Food Microbiology. 121: 253-261

García-Ruiz, A., Bartolomé, B., Martínez-Rodríguez, A.J., Pueyo, E., Martin Álvarez, P.J., and M.V. Moreno-Arribas. (2008). "Potential of phenolic compounds for controlling lactic acid bacteria growth in wine". Food Control. 19: 835-841

García-Ruiz, A., Bartolomé, B., Cueva, C., Martín-Álvarez, P.J., and M.V. Moreno Arribas. (2009). "Inactivation of oenological lactic acid bacteria (*Lactobacillus hilgardii* and *Pediococcus pentosaceus*) by wine phenolic compounds". Journal of Applied Microbiology. 107: 1042-1053

García-Ruiz, A., Moreno-Arribas, M.V., Martin-Álvarez, P.J., and B. Bartolomé. (2011a). "Comparative study of the inhibitory effects of wine polyphenols on the growth of enological lactic acid bacteria". International Journal of Food Microbiology. 145: 426-431

García-Ruiz, A., González-Rompinelli, E.M., Bartolomé, B., and M.V.Moreno Arribas. (2011b). "Potential of wine-associated lactic acid bacteria to degrade biogenic amines". International Journal of Food Microbiology. 148: 115–120

Garvie, E.I. (1986). Genus *Pediococcus*. In: *Bergey's Manual of Systematic Bacteriology*. P.H.A. Sneath, N.S. Mair, M.E. Sharpe, and J.G. Holt (Eds.), pp. 1075-1079. The Williams and Wilkins Co., Baltimore, MD.

Gloria, M.B.A., Watson, B.T., Simon-Sarkadi, L. and M.A. Daeschel. (1998). "A Survey of Biogenic Amines in Oregon Pinot noir and Cabernet Sauvignon Wines". American Journal of Enology and Viticulture. 49: 279-282

Grimaldi, A., Bartowsky, E., and V. Jiranek. (2005). "Screening of Lactobacillus spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology". Journal of Applied Microbiology. 99: 1061-1069

Guinard, J. and Cliff, M. (1987). "Descriptive analysis of Pinot Noir wines from Carneros, Napa, and Sonoma". American Journal of Enology and Viticulture. 38: 211-215

Harris, V., Jiranek, V., Ford, C.M., and P.R. Grbin. (2009). "Inhibitory effect of hydroxycinnamic acids on *Dekkera* spp.". Applied Microbial and Cell Physiology. 86: 721-729

Herbert, P., Cabrita, M.J., Ratola, N., Laureano, O., and A. Alves. (2005). "Free amino acids and biogenic amines in wines and musts from the Alentejo region: Evolution of amines during alcoholic fermentation and relationship with variety, sub-region and vintage". Journal of Food Engineering. 66: 315–322

Herbert, P., Cabrita, M.J., Ratola, R., Laureano, O., and A. Alves. (2006). "Relationship Between Biogenic Amines and Free Amino Acid Contents of Wines and Musts from Alentejo (Portugal)". Journal of Environmental Science and Health. 41: 1171–1186

Izquierdo-Pulido, M., Mariné-Font, A., and M.C. Vidal-Carou. (2000). "Effect of tyrosine on tyramine formation during beer fermentation". Food Chemistry. 70: 329-332

Kennedy JA, Robinson S, and M. Walker. (2007). Grape and wine tannins: Production, perfection and perception. Practical Winery and Vineyard. May/June: 57-67.

Landete, J.M., Ferrer, S., and I. Pardo. (2005). "Which lactic acid bacteria are responsible for histamine production in wine?". Journal of Applied Microbiology. 99: 580–586

Landete, J.M., Pardo, I., and S. Ferrer. (2008). "Regulation of hdc expression and HDC activity by enological factors in lactic acid bacteria". Journal of Applied Microbiology. 105: 1544-1551

Levengood Joanne, and R. Boulton. (2004). "The Variation in the Color Due to Copigmentation in Young Cabernet Sauvignon Wines". In *Red Wine Color*, 886:35-52. ACS Symposium Series. American Chemical Society

Liu, S.Q. (2002). "Malolactic fermentation in wine-beyond deacidification". Journal Of Applied Microbiology. 92 (4): 589-601

Lonvaud-Funel, A. (1999). "Lactic acid bacteria in the quality improvement and depreciation of wine". Antonie van Leeuwenhoek. 76: 317–331

Lonvaud-Funel, A. (2001). "Biogenic amines in wine: role of lactic acid bacteria". FEMS Microbiology Letters. 199: 9-13

Lucas, P.M., Wolken, W.A.M., Claisse, O., Lolkema, J.S., and A. Lonvaud-Funel. (2005). "Histamine-Producing Pathway Encoded on an Unstable Plasmid in *Lactobacillus hilgardii* 0006". Applied and Environmental Microbiology. 71 (3): 1417-1424

Maintz, L. and N. Novak. (2007). "Histamine and Histamine Intolerance". American Journal of Clinical Nutrition. 85: 1185-1196

Manca de Nadra, M.C. and A.M. Strasser de Saad. (1995). "Polysaccharide production by *Pediococcus pentosaceus* from wine". International Journal of Food Microbiology. 27: 101-106

Marques, A.P., Leitão, M.C., and M.V. San Romão. (2008). "Biogenic amines in wines: Influence of oenological factors". Food Chemistry. 107: 853-860

Martineau, B. and T. Henick-Kling. (1996). "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: 442-448

Martineau, B., Acree, T.E., and T. Henick-Kling. (1995). "Effect of wine type on the detection threshold for diacetyl". Food Research International. 28: 139-143

Mesas, J.M., Rodríguez, M.C., and M.T. Alegre. (2011). "Characterization of lactic acid bacteria from musts and wines of three consecutive vintages of Ribeira Sacra". Letters in Applied Microbiology. 52: 258-268

Moreno-Arribas, V., Torlois, S., Joyeux, A., Bertrand, A., and A. Lonvaud-Funel. (2000). "Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine". Journal of Applied Microbiology. 88: 584-593

Nannelli, F., Claisse, O., Gindreau, E., de Revel1, G., Lonvaud-Funel, A., and P.M. Lucas. (2008). "Determination of lactic acid bacteria producing biogenic amines in wine by quantitative PCR methods". Letters in Applied Microbiology. 47: 594–599

Nielsen, J.C. and M. Richelieu. (1999). "Control and flavor development in wine during and after malolactic fermentation with *Oenococcus oeni"*. Applied and Environmental Microbiology. 65: 740-745

Noble, A., Arnold, R.A., Buechsenstein, J. Leach, E.J. Schmidt, J.O. and Stern, P.M. (1987). "Modification of a standardized system of wine aroma terminology". American Journal of Enology and Viticulture. 38: 143-146

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

O'Neil, A., and Osborne J.P. (2012). "Impact of acetaldehyde and pyruvic acid bound sulfur dioxide on wine lactic acid bacteria". Letters in Applied Microbiology. 54**:** 187-194

Osborne, J.P., Mira de Orduña, R., Pilone, G.J., and S.-Q. Liu. (2000). "Acetaldehyde metabolism by wine lactic acid bacteria". FEMS Microbiology Letters. 191: 51-55

Pasteris, S.E. and A.M. Strasser de Saad. (2004). "Aerobic glycerol catabolism by *Pediococcus pentosaceus* isolated from wine". Food Microbiology. 22: 399-407

Pasteris, S.E. and A.M. Strasser de Saad. (2008). "Transport of glycerol by *Pediococcus pentosaceus* isolated from wine". Food Microbiology. 25: 545-549

Pedro, J., Álvarez, M., Marcobal, Á., Polo, C., and M.V. Moreno-Arribas. (2006). "Influence of technological practices on biogenic amine contents in red wines". European Food Research and Technology. 222: 420–424

Pedro, A., Fernández, A., Farías, M.E., and M. C. Manca de Nadra. (2010).

"Interaction between *Oenococcus oeni* and *Lactobacillus hilgardii* isolated from wine: Modification of available nitrogen and biogenic amine production". Biotechnology Letters. 32:1095–1102

Pinto, M.G.V., Pasteris, S.E., and A.M. Strasser de Saad. (2004). "Glycerol catabolism by Pediococcus pentosaceus isolated from beer". Food Microbiology. 21: 111-118

Polo, L., Ferrer, S., Peña-Gallego, A., Hernández-Orte, P., and I. Pardo. (2011). "Biogenic amine synthesis in high quality Tempranillo wines: Relationship with lactic acid bacteria and vinification conditions". Annals of Microbiology. 61: 191-198

Rodríguez, H., Landete, J.M., de las Rivas, B., and R. Muñoz. (2008). "Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748^{T}". Food Chemistry. 107: 1393-1398

Ramos, A., and H. Santos. (1996). "Citrate and Sugar Cofermentation in *Leuconostoc oenos*, a ^{13}C Nuclear Magnetic Resonance Study". Applied and Environmental Microbiology. 62 (7): 2577–2585

Santos, M.H.S. (1996). Biogenic amines: their importance in foods. Food Microbiology. 29: 213-231

Van Beek, S. and F.G. Priest. (2000). "Decarboxylation of Substituted Cinnamic Acids by Lactic Acid Bacteria Isolated during Malt Whisky Fermentation". Applied and Environmental Microbiology. 66 (12): 5322-5328

Versari, A., Parpinello, G.P., and M. Catteneo. (1999). *"Leuconostoc oenos* and malolactic fermentation in wine: a review". Journal of Industrial Microbiology and Biotechnology. 23: 447-455

Walling, E., Dols-Lafargue, M., and Lonvaud-Funel, A. (2005a). "Glucose fermentation kinetics and exopolysaccharide production by ropy *Pediococcus damnosus* IOEB8801". Food Microbiology. 22: 71-78

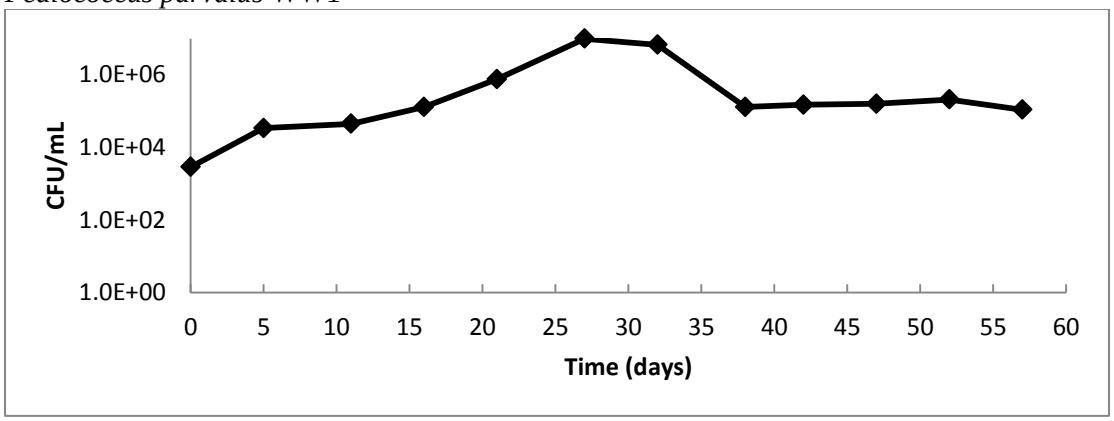
Walling, E., Gindreau, E., and A. Lonvaud-Funel. (2005b). "A putative glucan synthase gene dps detected in exopolysaccharide-producing *Pediococcus damnosus* and *Oenococcus oeni* strains isolated from wine and cider". International Journal of Food Microbiology. 98: 53-62

Wells, A. and J.P. Osborne. (2012). "Impact of acetaldehyde- and pyruvic acid-bound sulphur dioxide on wine lactic acid bacteria". Letters in Applied Microbiology. 54: 187-194

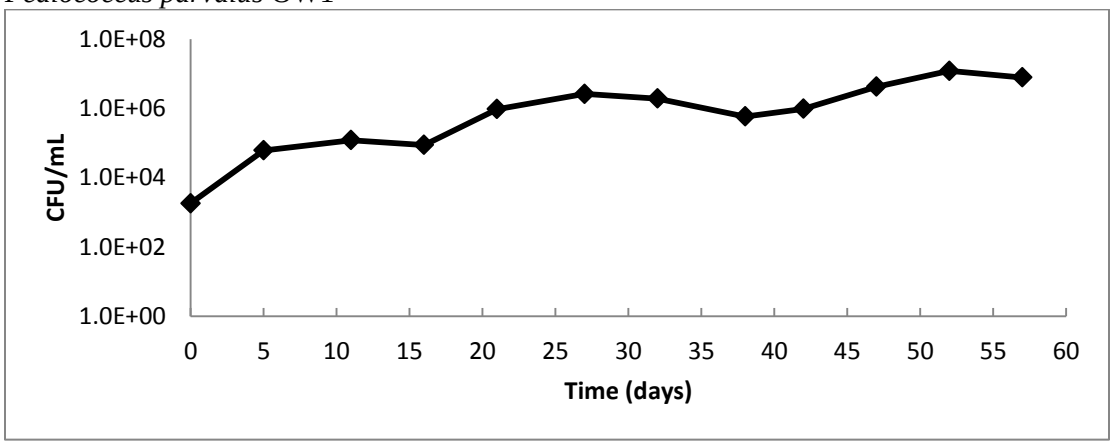
APPENDICES

APPENDIX A
Growth of pediococci isolates in Pinot noir wine

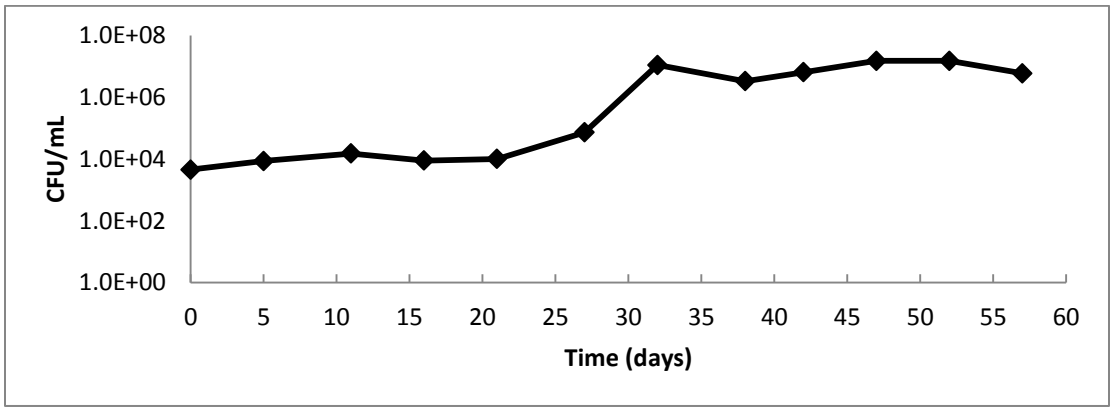
Pediococcus parvalus WW1



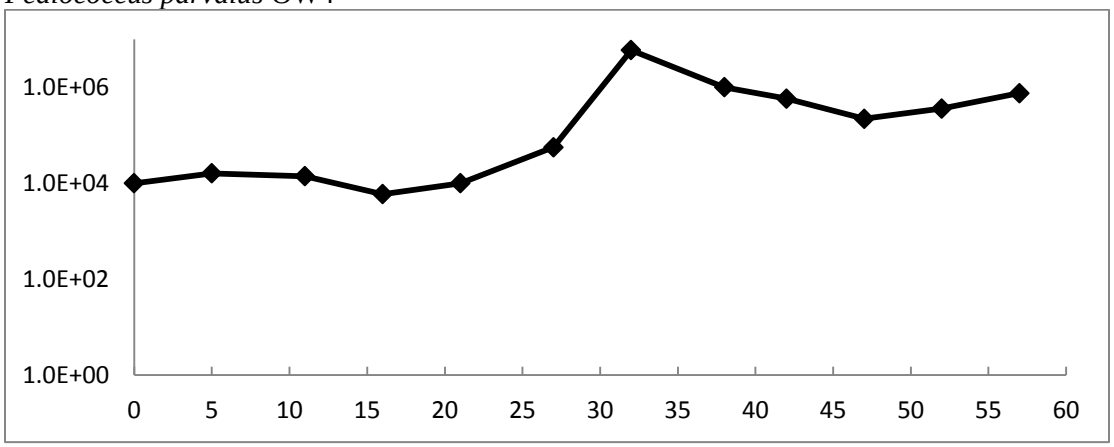
Pediococcus parvalus OW1



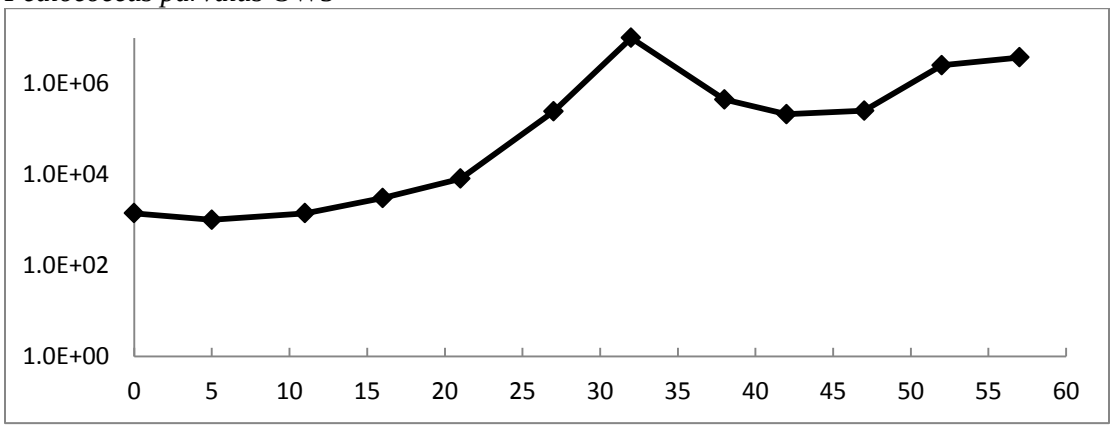
Pediococcus damnosus OW2



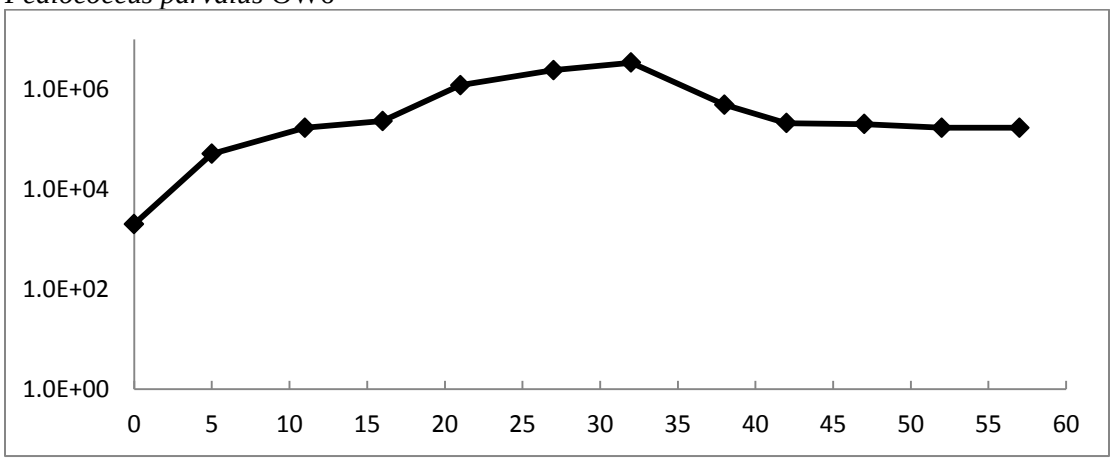
Pediococcus parvalus OW4



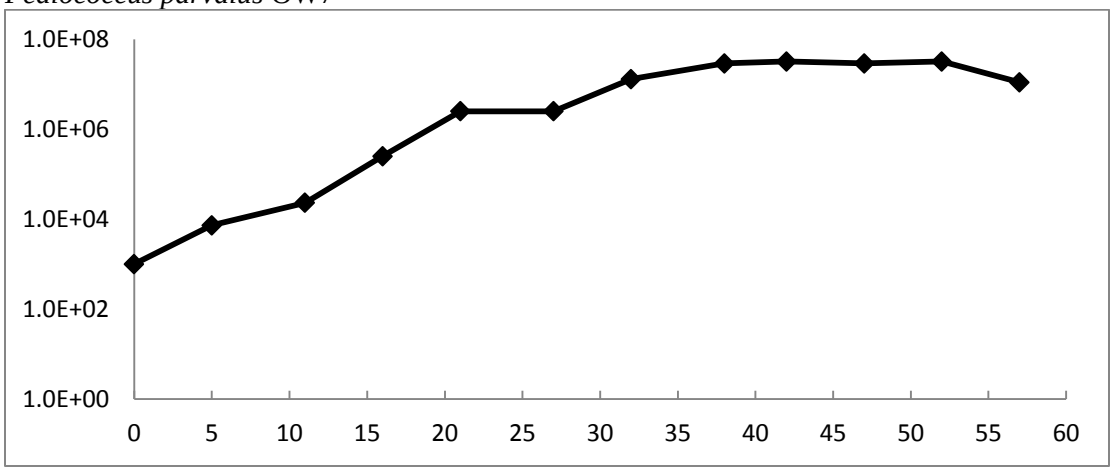
Pediococcus parvalus OW5



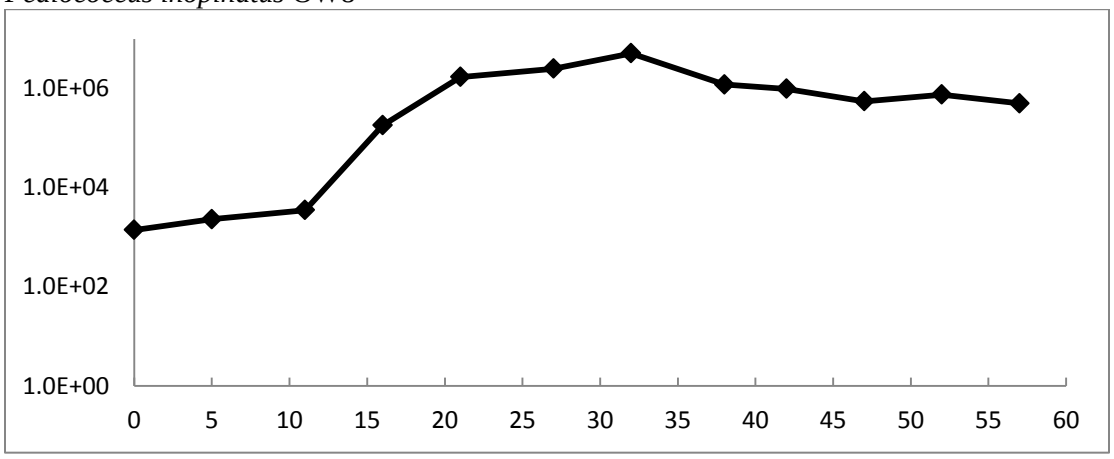
Pediococcus parvalus OW6



Pediococcus parvalus OW7



Pediococcus inopinatus OW8



APPENDIX B

Trained Panel Informed Consent Document

Project Title: Effects of Pediococcus spp. on Oregon and Washington Wines
Principal Investigator: James Osborne, Ph.D
Co-Investigator(s): Matt Strickland
Product: Oregon Pinot Noir and Washington Cabernet Franc
Ingredient: Water, Sulfur Dioxide and Ethanol

WHAT IS THE PURPOSE OF THIS STUDY?

You are being invited to take part in a research study designed to determine specific characteristics associated with *Pediococcus* spp. spoilage of Oregon Pinot Noir. The research will take place in the Sensory Science Lab at Oregon State University. The sensory lab engages in product testing and evaluation research. As a voluntary participant in such research, you may be offered the opportunity to sample small quantities of wine. The results from this test will benefit the wine industry by providing specific sensory information on spoilage characteristics of Pediococcus spp. in Oregon Pinot Noir and Washington Cabernet Franc.

WHAT IS THE PURPOSE OF THIS FORM?

This consent form gives you the information you will need to help you decide whether to be in the study or not. Please read the form carefully. You may ask any questions about the research, the possible risks and benefits, your rights as a volunteer, and anything else that is not clear. When all of your questions have been answered, you can decide if you want to be in this study or not.

WHY AM I BEING INVITED TO TAKE PART IN THIS STUDY?

You are being invited to take part in this study because you are 21 years or older, you are willing to consume wine, and you do not have allergies to sulfites or ethanol.

WHAT WILL HAPPEN DURING THIS STUDY AND HOW LONG WILL IT TAKE?

Procedures for Trained Panel

There will be approximately eight training sessions that will occur two times a week for four weeks beginning the first week of February. Each session will take no more than one and one-half hours. The sessions will take place at a round table consisting of twelve people with a maximum of sixteen. Training will be concluded once the panelists demonstrate the ability to distinguish wine samples reproducibly. The experimental testing sessions will take place after training is completed and will consist of three panelist replications of five sets of Pinot Noir and Cabernet Franc samples.

1. Trained Panel Profiling

- For any training or test session, you will be given a maximum of six 30-ml samples of wine. You will be asked to expectorate (spit your sample into a lidded expectoration cup provided) or swallow. The amount of wine you consume will be less than the amount specified by the ASTM E-18 guidelines for sampling products containing alcohol (5).
- You will be given a total of up to six samples, served one at a time. For each sample, you will be asked to rate the intensity of each descriptor using a 16-point intensity scale (provided on your ballot). You will evaluate aroma, flavor, mouth feel, and aftertaste characteristics. You will take a one to two minute break between samples.

- You should expect to finish within one to one and one-half hours maximum. Total training and testing sessions will not exceed 20 sessions. Panel completion is expected by the end of March, 2012.
- As part of your training exercises for the descriptive panel, you will work with the following food grade compounds that have bitter and astringent flavors: red wine, quinine sulfate, alum, grape-derived tannin, and oak-derived tannin, caffeine, citric acid, and alum in water. Other food grade flavor chemicals may be used to aid in panel training to aid in identifying wine descriptors. Theses compounds will be used in minute levels (less than one ounce at very low concentrations) in distilled water or wine and be similar to concentrations normally encountered in food and beverage products.

WHAT ARE THE RISKS OF THIS STUDY?

The possible risks and/or discomforts associated with the procedures described in this study include: 1) allergies wine and/or 2) effects of alcohol.

The Sensory Science Lab considers the health and safety of research participants and the public to be of utmost importance. Therefore, you should refrain from sampling any alcoholic beverage offered as part of this research if you have been advised by your doctor or if you have any medical reason to refrain from consuming alcoholic beverages (beer, wine or distilled spirits.). You should also refrain from sampling any alcoholic beverage on a given day if:

- You have consumed any beer, wine or distilled spirits on that day.
- You are taking any prescription or over-the-counter (non-prescription) medication and your doctor or the label has advised you, or instructions state that you should refrain from consuming alcoholic beverages while taking the medication.

Interactions with medications: Alcohol may interact harmfully with more than 100 medications, including some sold over the counter (2). The effects of alcohol are especially augmented by medications that depress the function of the central nervous system, such as sedatives, sleeping pills, anticonvulsants, antidepressants, anti-anxiety drugs, and certain painkillers. There is a consequent increased danger of driving an automobile after even moderate drinking if such medications are taken (3). In advanced heart failure, alcohol may not only worsen the disease, but also interfere with the function of medications to treat the disease (4).

Motor vehicle crashes

While there is some evidence to suggest that low blood alcohol concentrations (BACs) bear little relationship to road crashes, impairment of driving-related skills by alcohol has been found to begin at 0.05 percent BAC or lower, with rapidly progressing deterioration as the BAC rises (1). A man weighing 140 pounds might attain a BAC of 0.05 percent after two drinks. Food and drink will be provided to each panelist after the test is complete. You may be asked by the person conducting the research in which you are participating or by other facility personnel to remain at this facility for a period of time after your last sampling of an alcoholic beverage. Moreover, if you appear to be impaired at the end of such time period, you will be provided with an alternative means of transportation to your home and arrangements will be made for you to return at a later date for your car, at the Sponsor's expense.

In addition, Federal Law requires that alcoholic beverage labels contain the following statement:

GOVERNMENT WARNING:
1. According to the Surgeon General, women should not drink alcoholic beverages during pregnancy because of the risk of birth defects.
2. Consumption of alcoholic beverages impairs your ability to drive a car or operate machinery and may cause health problems.

Furthermore, you should follow your doctor's advice if you are pregnant, attempting to become pregnant or nursing.

WHAT ARE THE BENEFITS OF THIS STUDY?

A potential benefit to you may be gaining experience in descriptive profiling of wine using descriptive analysis techniques and learning about *Pediococcus* spp. spoilage of Pinot noir.

WILL I BE PAID FOR PARTICIPATING?

Food incentives (cookies, candy bars, snacks, fruit) (up to $3 per person) will be given out after every training or testing session. In addition, you will receive an $8 gift certificate (from Fred Meyer) per session awarded at the end of the panel.

WHO WILL SEE THE INFORMATION I GIVE?

The information you provide during this research study will be kept confidential to the extent permitted by law. To help protect your confidentiality, you will be assigned a subject number in order to keep your identity confidential. If the results of this project are published your identity will not be made public.

DO I HAVE A CHOICE TO BE IN THE STUDY?

Participation is voluntary and as a participant, you may withdraw from this study at any time without penalty. You are never required to sample such products, nor are you required to finish any product you elect to sample. Indeed, the decision as to whether to sample any product offered during the research is yours alone, and you alone should determine how much of the sample you wish to consume.

You will not be treated differently if you decide to stop taking part in the study. If you choose to withdraw from this project before it ends, the researchers may keep information collected about you and this information may be included in study reports.

WHAT IF I HAVE QUESTIONS?

If you have any questions about this research project, please contact: **Dr**. **James Osborne at 541-737-6494 (james.osborne@oregonstate.edu)**

If you have questions about your rights as a participant, please contact the Oregon State University Institutional Review Board (IRB) Human Protections Administrator, at (541) 737-4933 or by email at IRB@oregonstate.edu.

Your signature indicates that this research study has been explained to you, that your questions have been answered, and that you agree to take part in this study. You will receive a copy of this form.

Participant's Name (printed):

_____

_____

(Signature of Participant) (Date)

REFERENCES:

(1) Council on Scientific Affairs. Alcohol and the driver. Journal of the American Medical Association 255(4):522-527, 1986.

(2) SHINN, A.F., & Shrewsbury, R.P., eds. Evaluations of Drug Interactions. New York: Macmillan, 1988.

(3) GILMAN, A.G.; Rall, T.W.; Nies, A.S.; & Taylor, P., eds. Goodman and Gilman's The Pharmacological Basis of Therapeutics. New York: Pergamon Press, 1990.

(4) THOMAS, B.A., & Regan, T.J. Interactions between alcohol and cardiovascular medications. Alcohol Health & Research World 14(4):333-339, 1990.

(5)ASTM Committee E-18, 1997. "Sensory Evaluation of beverages containing alcohol", E-1879, American Society for Testing Materials, West Conshohocken, PA.

APPENDIX C
Panelist Descriptor Ballot

Descriptors	Sample #	Sample #	Sample #	Sample #
Aroma				
Overall Intensity				
Floral				
Overall Fruit				
Red Fruit				
Dark Fruit				
Jammy				
Spicy				
Earthy				
Herbacous/Vegetal				
Aged Aromas				
Oxidized				
Reduced				
Buttery				
Plastic				
Flavor				
Overall Intensity				
Overall Fruit				
Overall Spicy				
Aged Flavors				
Mouthfeel				
Sour				
Bitter				
Astringent				