THE INITIATION OF ALCOHOLIC AND/OR MALOLACTIC FERMENTATIONS:

THEIR EFFECT ON MICROBES CONDUCTING CHARDONNAY WINE

FERMENTATION AND ON THE RESULTING SENSORIAL WINE PROFILE

by

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THE INITIATION OF ALCOHOLIC AND/OR MALOLACTIC FERMENTATIONS: THEIR EFFECT ON MICROBES CONDUCTING CHARDONNAY WINE FERMENTATION AND ON THE RESULTING SENSORIAL WINE PROFILE

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Abstract

Alcoholic and malolactic fermentations are two important conversions that are involved in wine fermentation. Changes in wine microorganisms from these inoculations can modify the final wine characteristics, thus monitoring changes in microorganisms during these fermentations is necessary. Next-generation sequencing (NGS) is a technology that uses a culture independent technique to identify different species of fungi and bacteria from a sample. At present, when using this technique, it is difficult to distinguish between live and dead cells. The chemical, PMA, has been shown to bind DNA of dead cells, which prevents it from being amplified. In Chapter 2, the use of PMA were optimized, as a precursor to NGS, for accurate identification and quantification of yeast and bacterial species. In Chapter 3, the living microbial community were monitored in fermentations that differed in their yeast (*S. cerevisiae*) inoculation method (inoculated versus spontaneous) and their timing of bacterial (*Oenococcus oeni*) inoculation (inoculated during the same time of *S. cerevisiae* inoculation, post alcoholic fermentation, and uninoculated, which resulted in a spontaneous inoculation). Using this 2 x 3 factorial design, the effect of these factors on the strain and species relative abundance, diversity, and composition of yeast and bacteria were explored . A successful implantation (>80% relative abundance) of the *S. cerevisiae* inoculum were found, which resulted in changes in relative abundance for both yeast and bacterial populations and community composition as compared with spontaneous alcoholic fermentation (AF). The inoculation of the *O. oeni* MBR31 strain affected both bacterial communities and *O. oeni* strain composition with no apparent effect on yeast strain and species relative abundance, diversity and composition. An interactive effect was found, where the bacteria in spontaneous alcoholic fermentations (AF) as compared with those in inoculated AF were more easily influenced by *O. oeni* inoculations. Sensorial profiles indicated that inoculation of both *O. oeni* and *S. cerevisiae* changed final wine sensorial attributes. Different timing of *O. oeni* inoculation treatments resulted in a smaller change in the bacterial community and wine

attributes compared with adding or not adding *S. cerevisiae*. Results suggest that fermentations without an addition of yeast inoculum had higher yeast species and strain diversity, which correlated with higher positive sensorial attributes of the wine, such as body, long finish, tropical fruit flavors as well as butter and vanilla aromas. Different timing of *O. oeni* inoculation affect *O. oeni* strain composition and bacterial species diversity and composition with no apparent correlation between microbial changes and the final wine sensorial profiles.

Preface

I was responsible for implementing wine fermentations according to the experimental design as well as collecting all the samples from Quails' Gate Estate Winery during the 2014 vintage for chemical and sensorial analysis. Most of the microbial identification and quantification were performed in the molecular lab at the University of British Columbia's Okanagan campus.

Sensory data collection, with ethics approval from the UBC Research Ethics Board and Agriculture Canada, was collected at the Summerland Research and Development Center (SRDC) under the general supervision of Kareen Stanich and Dr. Margaret Cliff.

I was responsible for data collection in its entirety, including the interpretation of statistical analysis and writing the thesis, under the supervision of my supervisor Dr. Daniel Durall. My supervisory committee members Dr. Miranda Hart, and Dr. Vladimir Jiranek have also reviewed this thesis.

All microbial microsatellite DNA fingerprints were included in the manuscript entitled 'Composition of *Saccharomyces cerevisiae* strains in spontaneous fermentations of Pinot Noir and Chardonnay' (Scholl et al. 2016) in which I was a co-author. The use of propidium monoazide from Chapter 2 was published in the journal IJFM and was entitled 'The use of propidium monoazide in conjunction with qPCR and Illumina sequencing to identify and quantify live yeasts and bacteria' (Tantikachornkiat 2016). All *O. oeni* DNA fingerprint data, as well as, yeast and bacterial identifications from NGS are included in Chapter 3, which will be published as a manuscript entitled 'The effect of initiating alcoholic and/or malolactic fermentations on microbes of wine and its sensorial profile'.

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List of Abbreviations

ADB   Active dry bacteria

ADY   Active dry yeast

AF   Alcoholic fermentation

ANOVA   Analysis of variance

BLAST   Basic local alignment search tool

BRAES   Biodiversity resilience and ecosystem services

BSA   Bovine serum albumin

CFU   Colony-forming unit

DNA   Deoxyribonucleic acid

dNTPs   Deoxynucleotide triphosphate

FLASh   Fast length adjustment of short reads

ITS   Internal transcribed spacer

LAB   Lactic acid bacteria

MLF   Malolactic fermentation

NGS   Next-Generation Sequencing

Oe   *Oenococcus oeni*

PCoA   Principal Coordinate Analysis

PCR	Polymerase chain reaction
PMA	Propidium monoazide
QGEW	Quails' Gate Estate Winery
Sc	*Saccharomyces cerevisiae*
TA	Titratable acidity
UBCO	University of British Columbia Okanagan
VA	Volatile acidity
YEPD	Yeast extract peptone dextrose

Acknowledgements

Dedication

      I officially dedicate this thesis to my mentors, family, and all my friends for nursing me with encouragement and love. Thank you for all the support along the way. I couldn't have done this without all of you.

Chapter 1 – Introduction

Wine fermentation, the conversion of grape juice to wine, involves complex interactions of both yeast and bacteria. There are two important conversions that are involved in wine fermentation. One of these is alcoholic fermentation (AF), where *Saccharomyces cerevisiae* is the main organism responsible for converting sugars to ethanol and CO_2 (Fleet, 1993; Fugelsang, 1997). The other major process is malolactic fermentation (MLF), which involves the decarboxylation of L-malate to L-lactic acid. It is beneficial to most red and some white wine varietals and it is associated with lactic acid bacteria (LAB) due to their higher expression of malate associated enzymes (Edwards 1992). The bacterium, *Oenococcus oeni*, is the most commonly used wine organism associated with this process. Monitoring microbes and their interactions in the wine industry is crucial because they can influence wine characteristics, not only through the production of alcohol and lactic acid, but also by producing aroma and flavor compounds through other pathways. The vineyard and winery are the two major sources of organisms found in fermenting wine. The microbial community composition of the vineyard and its associated soil can depend on grape clonal type, climate, pesticide use (Setati, 2012), as well as factors associated with the soil (Zarraonaindia, 2015). But in the winery, factors such as air-flow, moisture, sanitation, access to visitors and personnel, and use of yeast inoculants can determine the diversity and composition of these communities (Ocon, 2013 & 2014).

1.1   Origin of *S. cerevisiae* yeasts

Yeasts originating from the vineyard are generally thought to be limited to non-*Saccharomyces* because *S. cerevisiae* can rarely be found on undamaged grape surfaces (Torok et al. 1996). Populations of *S. cerevisiae* on damaged grapes, on the other hand, are more

abundant (Mortimer & Polsinelli 1999). In contrast to the vineyard, *S. cerevisiae* can be commonly found in the winery; they reside in many places including, air, equipment, barrels, cellar surfaces, and pumps (Martini, 1993). In the winery, *S. cerevisiae* were found to be able to occupy winery surfaces, allowing them to survive and take part in fermentations conducted in subsequent years (Constanti et al. 1997). In commercial wineries, both spontaneous and inoculated fermentation are used. In spontaneous alcoholic fermentations, a commercial *S. cerevisiae* strain is not added, whereas it is added in an inoculated fermentation. In wineries that use both inoculated and spontaneous fermentations, spontaneous fermentations are usually dominated by commercial *S. cerevisiae* strains (Hall et al. 2011). Nevertheless, the strain composition between different wineries is often unique due to the unique selection of commercial yeasts from one winery to the next (Scholl et al. 2016). The source of *S. cerevisiae* in these fermentations is likely due to a constant introduction of active dry yeast (ADY) that were brought into the winery for each vintage. Relatively high numbers of *S. cerevisiae* in the winery environment and its air were found when AF was in progress but yeast numbers declined when fermentations in the winery were curtailed (Garijo et al. 2008; Bokulich et al. 2013). Strains isolated in the winery seem to be similar from one year to the next but the use of new strains in the winery can influence the strain composition in subsequent years (Beltran et al., 2002). Researchers have found that ascospore production by yeasts can enable them to survive for a long periods in the winery; it is very difficult for them to be eliminated (Constanti et al. 1997; Garijo et al. 2008). Following fermentation, deposition of dead yeast cells, lees, are often transported to the vineyard as organic fertilizer in which a small portion of cells may survive in the vineyard environment (Valero et al. 2007). The yeast cells are subsequently bought back to the winery during harvest (Blanco et al, 2011; Ocón et al, 2010). Nevertheless, the major sources

of *S. cerevisiae* are still considered to be equivocal. Studies by Ocón et al. (2014) and Garijo et al. (2008, 2009) have shown evidence that air can be a source of mold and many microorganism contaminations. In nature, the presence of *S. cerevisiae* appears to be extremely low in soils and on healthy undamaged berries (Frezier and Dubourdieu 1992; Martini et al. 1996), which has led to the idea that damaged grapes are able to harbor high amounts of *S. cerevisiae* (Mortimer and Polsinelli 1999). Researchers have provided some evidence that *S. cerevisiae* detected in the damaged grape (Valero et al. 2007) are responsible for carrying out spontaneous fermentation (Mortimer & Polsinelli 1999). However, some research demonstrates that the occurrence of *S. cerevisiae* is not related to damaged or undamaged grapes in the natural environment (Comitini and Ciani, 2006).

1.2   Origin of Non-*Saccharomyces* yeasts

The origin of non-*Saccharomyces* in both inoculated and spontaneous fermentation is mainly from grapes. The genera of non-*Saccharomyces* yeasts on ripened grapes typically include *Metschnikowia, Pichia, Candida, Hanseniaspora,* and *Torulaspora.* But in the unripe grape, predominant species may include *Rhodotorula, Cryptococcus, Candida*, and *Aureobasidium pullulans*; they are often found on the mature/ripe grapes (Fleet et al., 2002). Some non-*Saccharomyces* species in grape must seem to be very sensitive to temperature during the cold maceration. A study by Zott et al. (2008) showed that at lower temperature (4-10 °C), *Candida* spp. were usually the dominant yeast species but once temperatures reached around 15 °C, *Hanseniaspora* spp. were favored. Non-*Saccharomyces* seem to be dependent on temperature and sugar level, they tend to dominate during coldsettling and often decline during alcoholic fermentation due to their low tolerance to ethanol.

1.3   Non-*Saccharomyces* in vineyards and wine fermentations

The contribution of non-*Saccharomyces* in both inoculated and spontaneous fermentations cannot be ignored. They are usually the first to be present in the vineyard and in grape must; they play a major role in affecting final wine characteristics through changing wine composition before AF, and they subsequently affect *S. cerevisiae* strain compositions (Lema et al. 1996). Yeast population dynamics involve a complex interaction and transition between non-*Saccharomyces* and *Saccharomyces.* Spontaneous fermentations as compared with inoculated fermentation tend to have a higher diversity of non-*Saccharomyces*, which may result in a wine that is described as more complex.

The reduction of non-*Saccharomyces* following coldsoak may also be due to the addition of sulfur dioxide between coldsoak (pre-fermentation) and the early stages of fermentation, which results in a major reduction of yeast and bacterial counts (Ocon et al. 2010). Besides the addition of SO_2, a decrease in oxygen availability and an increase in both temperature and ethanol concentration can greatly diminish non-*Saccharomyces* (Goddard 2008). The relationship between non-*Saccharomyces* yeasts, hyphal forming fungi, and bacteria could be very complex due to the potential mutualistic relationships between them (Guilloux-Benatier et al. 2006). Furthermore, the disruption of fungal and bacterial microbiota could result in the dominance of *S. cerevisiae*. Interspecific competition, therefore, tends to be more prominent in coldsoak and the early stages than in the later stages of fermentation. Wineries that never use commercial ADY strains were found to have more non-*Saccharomyces* yeasts in the mid and end stages of fermentation (Torija et al. 2001), possibly due to reduced dominance and competition from aggressive ADY strains.

1.4   Alcoholic fermentation

The process of wine making is a complex interaction between numerous bacteria and yeast species. Complete control of these populations is hard to achieve. Wine-making begins by the crushing and pressing of grapes, which results in a grape must (juice). Usually there are 4 stages that follow pressing (coldsettling/coldsoak, early, mid, and late). Prior to AF, the must is often cold settled in a stage called coldsettling. Subsequently, there are at least three stages during AF (early, mid, and late) that can be defined by sugar content levels (°Brix). During the coldsoak and early stages, non-*Saccharomyces* yeasts dominate and have a minor contribution to AF. Nevertheless, they can produce secondary metabolites that can influence the sensory attributes of the final wine product (Fleet, 1993). In contrast, *S. cerevisiae* is the main yeast species responsible for AF of grape must. Due to its relatively high ethanol resistance, *S. cerevisiae* tends to dominate over non-*Saccharomyces* yeasts in the mid and final stages of fermentation (Frezier & Dubourdieu 1992; Martini et al. 1996). *S. cerevisiae* contributes to the production of major aroma compounds such as esters (Saerens et al., 2008), higher alcohols (Zambonelli, 1990), aldehydes and fatty acids (Trotter, 2001). Specifically, ethanol and fatty acids are the major compounds that favor *S. cerevisiae* survival because they create a condition that is toxic to most other yeast and bacterial species in the wine. Thus, by producing a high ethanol environment, *S. cerevisiae* appears to benefit its own growth during fermentation (Goddard, 2008). Properties other than alcohol that tend to inhibit or kill non-*Saccharomyces* during AF include: SO_2, anaerobic conditions and high temperature (Torija et al. 2001).

1.5   Spontaneous fermentation

Traditionally, wine AF relies on yeast species present in the winery and vineyard environment to initiate fermentation in the grape juice (Ciani et al. 2004; Maro et al. 2007). This method is known as spontaneous fermentation and it is still in practice today. Environmental factors such as climate and soil can directly affect vine health and microbial species, which indirectly contribute to both the quality and aroma of the wine. Microbes in the winery can reside almost anywhere including winery walls, equipment, oak barrels, drainage, and also can be brought in by human or insect vectors such as fruit flies (*Drosophila melanogaster*). Spontaneous fermentations, as compared with inoculated fermentations, were found to have higher involvement of non-*Saccharomyces* (usually *Candida, Metschnikowia, Torulaspora, Hanseniaspora,* and *Pichia*) fermenting the wine, especially in the early stages of fermentation. Spontaneous fermentation comes with both benefits and risks. For example, non-*Saccharomyces* can provide a perceived increase in quality by introducing complexity or they can produce haze and off-flavours in the final product. Spontaneous fermentations tend to encourage the involvement of indigenous strains of *S. cerevisiae*, which can produce unique wine with a relatively high complexity (Vilanova et al. 2005, Fleet 2007). This is true for regions where indigenous strains participate as major contributors in the wine fermentation (Vigentini et al., 2014). However, in wineries that use inoculated fermentations, commercial strains are often the dominant *S. cerevisiae* strains in spontaneous fermentations (Hall et al. 2011, Scholl et al. 2016). In wineries that do not use inoculated fermentations, indigenous strains are reported to outcompete commercial strains, resulting in a population that is stable from year to year (Frezier & Dubourdieu 1992).

1.6   Inoculated fermentation

Most modern wineries rely on inoculated fermentations in which commercially available active dry yeast or bacteria (usually a single strain) are added to the grape must in order to initiate fermentation. It is assumed that the added strain will dominate and complete the fermentation, which is not always the case (Lange et al, 2014). Nevertheless, the use of commercial *S. cerevisiae* active dry yeast (ADY) has allowed winemakers to better control fermentation. Successful implantation of ADY promotes a quick and uniform fermentation, reduces the chances of stuck fermentation (Valero et al. 2007), decreases the lag phase, and promotes a reproducible product (Beltran et al. 2002).

Currently, Lallemand Inc. (one of the largest Canadian companies that produces and markets yeasts and bacteria) offer over 200 yeast and bacterial strains for producing alcoholic products, each with their own unique characteristics. For example, an ADY yeast strain may have the ability to ferment rapidly at low temperatures, tolerate high ethanol and high temperatures, as well as, produce low levels of hydrogen sulfide (Barrajón et al. 2009). Some commercial ADY strains have been found to express a killer phenotype by excretion of toxic glycoproteins, which kill off other microbiota, resulting in a pure strain culture (Zargoc et al. 2001). There can be disadvantages to using an ADY strain as a starter, as overuse of ADY strains may result in the reduction of indigenous *S. cerevisiae* strains at a given winery (Beltran et al. 2002). Wineries that use the same ADY strain each year may encounter a situation where complexities arising from indigenous yeasts are reduced, which may affect the overall distinctive aromatic qualities of the wine unique to their location (Vilanova et al. 2005; Fleet 2007). There are many factors that should be considered when choosing a commercial *S. cerevisiae* yeast. For example, the rate at which they ferment, their ability to complete fermentation to dryness, and

their tolerance to high temperature, SO_2, and alcohol. Choice of the use of inoculum may give the winemaker some control, but it could be detrimental to the normal flora that already resides in the winery. Research from the Okanagan region has shown that *S. cerevisiae strains* from spontaneous fermentations are typically commercial yeast strains, which were previously used in the winery with little or no involvement of indigenous *Saccharomyces* yeast (Hall et al. 2011, Scholl et al. 2016). In addition, when inoculated fermentations are not fully implanted, the strains that are isolated, other than the inoculum, are usually commercial *S. cerevisiae* strains (Lange et al. 2014). The choice of a commercial *S. cerevisiae* strain may also depend on how it interacts with microbes residing within the winery; it should be determined whether it interacts positively with lactic acid bacteria (LAB) and other beneficial organisms found in the fermentation (Arnink and Henick-Kling, 2005). For example, yeast metabolites, such as SO_2 (Osborne 2006) and fatty acids, were found to have inhibitory effects toward *Oenococcus oeni* (Capucho and San Romão, 1994). In-depth studies on the interaction between *O. oeni* and other microbes, including *S. cerevisiae*, is required in order for a winemaker to understand how to properly manage MLF.

1.7   Malolactic fermentation

The malolactic fermentation (MLF), conducted by *Oenococcus oeni*, can reduce available nutrients, which may reduce spoilage; it can provide a less harsh mouth-feel to the wine through deacidification, and it can increase wine flavour and aroma (Bartowsky 2005; Henick-Kling 1993; Kunkee 1991). Specifically, MLF involves the decarboxylation of malic acid to carbon dioxide and lactic acid (Boulton et al., 1996; Capucho and San Romão, 1994). The most common MLF-inducing genera include *Oenococcus, Lactobacillus,* or *Pediococcus* (Fugelsang et al., 2010). Nevertheless, *O. oeni* is most commonly encountered in wine and is stable due its

acidophilic and alcohol tolerant nature (Van Vuuren and Dicks, 1993). Some wines undergo

malolactic fermentation (MLF) as a secondary fermentation, which usually takes place during

AF or at the end of it. MLF can be detrimental or beneficial depending on the type of wine; it is

typically performed on most red and some white varietals aged in oak barrels. In some wine

varietals, MLF is not desirable because acidity reduction and MLF derived flavours, such as

buttery tones, are not desirable. Bacteria, which perform MLF, are able to reduce the number of

other bacteria by competing for nutrients and producing bateriocins (Lonvaud-Funel and Joyeux,

1993). It is important for MLF to occur before bottling. When it occurs in the bottle, cloudiness,

a shift in pH, and off-odors/flavours may result (Bisson, 2004). These properties are usually a

result of increased bacterial cell growth. The consumer often perceives these characteristics as

wine spoilage. Overall, MLF results in changes in the organoleptic profile and the quality of the

wine through secondary metabolic reactions, which result in increasing levels of acetaldehyde,

acetic acid, acetoin, diacetyl, 2,3-butanediol, ethyl lactate and higher alcohols (Bartowsky,

2004).

Oenococcus oeni are considered the most common and well accepted LAB species for

MLF. Interestingly, however, O. oeni is hypermutable due to the lack of mutation mismatch

repair systems, specifically MutS and MutL genes (Marcobal et al., 2008. This results in a rapid

emergence of new strains, which is probably one reason for the variability found associated with

the timing and efficiency of MLF in wine (Beelman et al., 1980; Semon et al., 2001). One of the

main flavor compounds produced during MLF by LAB is diacetyl, which produces a buttery

mouth-feel and butterscotch aromas. Surprisingly, S. cerevisiae produce small amounts of

diacetyl during AF, but they are usually not implicated in the production of these flavors and

aromas. O. oeni is usually the main organism responsible for producing diacetyl and its resulting

sensory characteristics during MLF (Mink et al., 2014). Diacetyl is an intermediate in the citric acid metabolism and can be reduced to acetoin and 2,3-butanediol, depending on physical and chemical factors of the wine (Ramos, 1995). Acetoin and 2,3-butanediol (neutral sensorial quality) have a very high sensorial threshold (150 mg/liter) and usually do not contribute to aroma and flavor of the wine (Gonzalez et al., 2001; Styger et al., 2011). Overall, factors that can influence the level of diacetyl include the *O. oeni* strain, wine type, length of lees contact, pH, temperature, oxygen, citrate level, and SO_2 concentrations (Bartowsky and Henschke, 2004).

There are several factors that reduce *O. oeni* fermentative capabilities, including high SO_2, low pH, fatty acids, and high ethanol level (Henick-Kling, 1993; Lonvaud-Funel et al., 1988). Certain yeast strains can also influence LAB growth (Osborne & Edwards, 2006 & 2007). The major factor that seems to influence MLF is SO_2, which is added before AF or generated from yeast metabolism. Reguant et al. (2005) found a relationship between high levels of added SO_2 and a timing delay of MLF. For example, *O. oeni* initiated MLF 20 days earlier in 40 ppm as compared with 100 ppm SO_2 treatment. In one instance, a particular *S. cerevisiae* strain, in combination with relatively high SO_2 resulted in total inhibition of MLF (Carrete et al., 2002). A few researches have also found inhibitory antimicrobial peptide produced by *S. cerevisiae* during AF, which is active against both yeast and bacteria including the yeast *Dekkera bruxellensis* and the bacterium *O. oeni* (Branco et al., 2014; Comitini et al., 2005). Furthermore, different *S. cerevisiae* strains were also found to differ in the level of SO_2 production, therefore SO_2 should be used with consideration of the above factors.

1.8   Microbial identification during fermentations

1.8.1   Culture dependent

1.8.1.i *S. cerevisiae* strain differentiation via microsatellites

Several methods have been developed to differentiate between strains of a specific species; restriction fragment length polymorphism (RFLP), delta-PCR, random amplified polymorphic DNA (RAPD), DNA hybridization, and PCR-based assays are examples (Perez et al. 2001). However, a common way to distinguish strains of *S. cerevisiae* is by using hyper-variable microsatellites or simple sequence repeats (SSRs), which are dispersed throughout the genome (Legras et al., 2004). Using multiple loci, one can obtain a unique fingerprint for a single strain. A database displaying these fingerprints for a large number of commercial *S. cerevisiae* strains has been published by Richards et al. (2009). In studies at UBC Okanagan, based on a study from Legras et al. (2004), eight hypervariable microsatellite loci were selected for use (Scholl et al. 2016) to distinguish between indigenous and commercial strains at multiple wineries.

1.8.1.ii *O. oeni* strain differentiation via VNTRs

Multiplex variable number tandem repeats (VNTR) have been developed as a reliable method to differentiate between different strains. Five tandem repeats (TR 1 to 5) were developed by Claisse et al. (2012, 2014) for *O. oeni*, which flanked 5 different loci of different lengths and repeats. Similarly to microsatellites, lengths of VNTRs vary due to DNA slippage during replication.

1.8.2 Culture independent identification of yeast and bacteria

In the past, detection of yeasts and bacteria was made possible by using culture dependent techniques such as growing organisms on culture media. This method is time consuming and relatively inaccurate due to its inability to detect many viable but non-culturable (VBNC) (Cocolin and Mills 2003). Species identification from the culture dependent method usually relies on Sanger DNA sequencing, which requires purified DNA from one species. In contrast, the culture independent method, which involves coupling with high-throughput sequencing is by far the most thorough way to determine all organisms in a given sample. In the past 20 years, many methods to achieve this have been developed including Denaturing Gradient Gel Electrophoresis (DGGE), microarray, and fluorescence in situ hybridization (FISH). Current NGS methods are able to sequence up to 20 million sequences per run. For example, Illumina Miseq amplicon-sequencing can accurately determine the relative abundance for both bacteria and yeast species in wine samples (San Miguel 2011, Bokulich et al. 2013). This method can be used for the detection of microbial species in nature. Nevertheless, there are still many challenges including: 1) distinction between live and dead cells (Tantikachornkiat et al., 2016); 2) targeting more than one copy of a gene (Medinger et al., 2010); and 3) PCR amplification bias (Kanagawa, 2003). NGS has been used to determine the relative abundance of species (Bokulich et al., 2013), but it hasn't been used in a way that differentiates between viable and dead cells of wine samples.

1.9 PMA

Propidium monoazide (PMA) is a photo-reactive DNA binding dye, which through photolysis, causes permanent DNA modification of dead cells and renders it un-amplifiable by polymerase chain reaction (PCR) (Nocker et al., 2006). Previous research by Andorra et al.

(2010) successfully optimized the combination of PMA and qPCR to determine number viable wine yeast. Because both Illumina sequencing and qPCR utilize the same fundamental technique of PCR, the use of PMA with Illumina sequencing should allow for the quantification of the different yeast species in a wine sample and allow for the determination of their relative abundance.

1.10   Sensorial attributes

The production of high quality wine depends on picking the highest quality grapes in the vineyard. Perceived quality by an individual can depend upon both physiological and psychological/emotional factors (Ferrarini et al., 2010). For example, for one reason or another, one person's threshold level of detection of a specific compound might be heightened or lowered compared with others. Human detectable volatile compounds are those that have low boiling points and are able to escape through aeration. Currently, over 680 volatile compounds have been identified from wine, indicating the complexity of wine aroma (Rapp 1998, Guth and Sies 2002). Sensorial attributes are greatly dependent on the chemical composition of the wine, which can be influenced by many factors including terrior, type of wine, grape variety, viticulture practices, wine making techniques, technologies used in the winery, and microorganisms present during the fermentation (Bakker and Clarke, 2011; Swiegers et al., 2005). The term "terrior" or "the sense of place", is defined as a group of vineyards (or even vines) that share similar soil, topography, climate, and weather conditions, which results in a unique wine characteristic.

The wine microbiome, which can be monitored and controlled during wine making, can have a major impact on the chemicals found in wine. Bacteria, such as *O. oeni*, which provides

deacidification during malolactic fermentation are also found to enhance the wine flavor profile (Henick-Kling 1993; Bartowsky et al. 2002). Additionally, *S. cerevisiae* (a major yeast responsible for AF) does not only convert sugars to ethanol and carbon dioxide; it also is responsible for a production of a wide range of important volatile metabolites (Lambrechts and Pretorius 2000, Romano et al. 2003). Non-*Saccharomyces* yeasts, including genera such as *Hanseniaspora, Candida, Metschnikowia*, *Torulaspora,* and *Pichia*, which are commonly found on grapes, are typically found to be important in the development of wine flavor and aroma (Fleet, 2003; Romano et al., 2003). For example, the presence of *Hanseniaspora uvarum* during coldsoak can produce higher alcohols, acetate, ethyl esters and medium-chain fatty acids (Andorra et al., 2010), thus in moderation (proper length of coldsoak), it can help with an increase in complexity and color extraction. Nevertheless, it can act as a spoilage yeast when it produces an abundance (over 150-200 ppm) of ethyl acetate (formed by esterification of ethanol with acetic acid), where it imparts a nail polish remover odor (Rojas et al., 2001). Other potential spoilage yeasts include *Brettanomyces, Kluyveromyces, Schizosaccharomyces*, and *Zygosaccharomyces*. These yeasts are commonly found to contribute to undesired wine attributes by producing chemicals associated with off-odors (Egli & Henick-Kling 2001; Loureiro & Malfeito-Ferreira, 2003; Peinado et al., 2007). For example, *Brettanomyces bruxellensis* is a spoilage organism that produces 4-ethylphenol, 4-ethylguaiacol, and isovaleric acid, which have aromas of barnyard, sweaty gym socks, and rancid yeast (Oelofse et al., 2008)

1.11   Background of this study

Chardonnay is a common white wine variety that is fermented in an oak barrel for both alcoholic and malolactic fermentation. Wine-making begins by the crushing and pressing of

grapes, which results in a grape juice. Subsequently, AF begins either by inoculation (addition of commercially available active dry yeast) or spontaneously (initiated by resident grape + winery yeast) (Ribereau-Gayon et al., 2006; Mercado et al., 2007). In AF, yeasts are responsible for the conversion of sugar into ethanol and CO_2 (Kunkee, 1984). The most dominant yeast species found during AF is *Saccharomyces cerevisiae*, which is tolerant to high ethanol concentrations.

In addition to AF, some wines undergo malolactic fermentation (MLF), which is beneficial to Chardonnay wine, aiding in wine stabilization by deacidifying and reducing nutrient residues. It converts the tart malic acid to a much rounder mouth-feel of lactic acid (Capucho and San Romão, 1994). MLF is performed by lactic acid bacteria (LAB), of which *Oenococccus oen*i is the most commonly used MLF-inducing bacterium. Commercial strains of *O. oeni* are available on the market for the initiation of MLF (Ruiz et al., 2010). To our knowledge, different timings of *O. oeni* inoculation (before or after AF) and their effect on the microbial community during wine production have not been tested in a winery setting. A detailed study of the changes in microbial community structure during commercial wine fermentation and its effect on the wine's sensory attributes will help in the understanding of how to consistently make a high quality wine.

Culture-independent methods of microbial identification have been developed, which allow for DNA extraction directly from environmental samples without subjecting microbes to growth on nutrient media. Despite the benefits of extracting all DNA from the sample, results may be compromised by amplifying DNA from dead cells (Lebeis, 2014; Lundberg et al., 2012). To address this short-coming, propidium monoazide (PMA) has been used to deactivate DNA in non-viable cells (Nocker et al., 2006). The use of PMA in conjunction with NGS has not been explored under a variety of conditions. The research, using NGS technology, aims to use

propidium monoazide (PMA) to distinguish between living and dead microbes. Future research, which utilizes the above techniques, can therefore accurately monitor living microbial communities in any given sample.

1.12   Research objectives and hypotheses

Objective 1: To optimize the PMA method for both yeast and bacteria when used in combination with NGS. Specifically, to determine the effect of different PMA concentrations and different cell densities (at all PMA concentrations) on DNA amplification from both dead and viable bacteria and yeast cells.

Prediction 1-1: The optimum concentration of PMA concentrations will be 6 µM.

> A previous study, using PMA coupled with qPCR to quantify wine yeast, indicated that 6 µM was the optimum PMA concentration (Andorra et al., 2010).

Prediction 1-2: High concentrations of PMA will show inhibitory effects on viable yeast and bacterial cells.

> Research by Andorra et al (2010) showed that low PMA concentration result in unreliable detection of live cells, while high PMA concentrations were toxic to viable cells.

Prediction 1-3: The optimum concentration of PMA will be dependent on cell density (Xiao et al., 2015)

> This is proposed because a relatively high cell density will result in higher turbidity of the sample, which in turn, can prevent light from contacting DNA that is bound by PMA.

Overall, this will result in a relatively low efficiency of DNA inactivation in dead cells, which will result in an under-estimation of dead cells. We are not aware of any study that has specifically tested the effect of cell density on PMA efficiency.

Objective 2: To determine the effect of *S. cerevisiae* inoculation on species/strain diversity and species/strain composition of live yeast and bacteria in Chardonnay wine fermentations.

Prediction 2-1: The +Sc AF treatments will be composed of > 80% of the inoculum at the late stage of fermentation, which indicates a successful yeast inoculation.

> It is commonly found that in the successful inoculation of *S. cerevisiae*, the implantation is usually >80% during the late stage of fermentation (Medina et al. 2013). This tends to lower the involvement of non-*Saccharomyces* yeasts during AF (Torija et al. 2001).

Prediction 2-2: The diversity of *S. cerevisiae* strains will be higher in the spontaneous alcoholic fermentations (-Sc) than in the inoculated AF treatments (+Sc).

> Typically, in inoculated fermentations, the inoculated *S. cerevisiae* strain will dominate and exclude or suppress all other *S. cerevisiae* strains; however, there have been situations reported where the inoculated strain does not fully implant (Lange et al. 2014).

Prediction 2-3: Higher species diversity of non-*Saccharomyces* species will be found in spontaneous AF (-Sc) than inoculated AF (+Sc).

> Spontaneous as opposed to *S. cerevisiae* inoculated fermentations have shown higher numbers of non-*Saccharomyces* species, such as *Candida stellata* and *Hanseniaspora uvarum*, during the fermentation (Raspor et al. 2002).

Prediction 2-4: *Oenococcus oeni* strain and bacterial species diversity will be lower in the spontaneous AF treatments (-Sc) than in the inoculated AF treatments (+Sc).

> *S. cerevisiae* strains can interact either negatively or positively with *O. oeni* strains during wine fermentation. In the commercial setting, the bacterial community and *O. oeni* strains can be heterogeneous and complex, thus inoculation of a single *S. cerevisiae* strain can have a negative or positive effect toward certain bacterial strains or species, resulting in higher diversity of bacterial species and *O. oeni* strains (Henschke, 1993). For example, *S. cerevisiae* strains can produce proteinaceous compounds, which can be active against *O. oeni* and might be the cause for low *O. oeni* counts and an unsuccessful MLF (Comitini et al. 2005). Depending on which *O. oeni* strains they affect, they can have either an increasing or decreasing effect on diversity.

Prediction 2-5: *O oeni* strain and bacterial species composition will differ between inoculated (+Sc) and spontaneous (-Sc) AF treatments.

> As mentioned previously, *S. cerevisiae* interaction with bacteria is known (Henschke, 1993). *S. cerevisiae* strains in either spontaneous or inoculated fermentation will be one of the major factors that shapes bacterial communities and *O. oeni* populations responsible for conducting MLF.

Objective 3: To determine the effect of *O. oeni* inoculation and the timing of *O. oeni* inoculation on MLF commencement, species/strain diversity, and species/strain composition of live yeast and bacteria in chardonnay wine fermentations.

Prediction 3-1: When *O. oeni* is added prior to fermentation, MLF will commence sooner in the inoculated AF (+Sc) than in the spontaneous AF (-Sc) treatments.

> Based on Arnink and Henick-Kling (2005) findings, there is a high probability that one (or more) *S. cerevisiae* strains in spontaneous fermentation is capable of producing MLF inhibiting compounds.

Prediction 3-2: Pre-AF inoculation of *O. oeni* (+-Oe) will result in an earlier commencement of MLF compared with post-AF inoculation of *O. oeni* (-+Oe) or spontaneous inoculation of O. oeni (--Oe).

> Previous research comparing sequential and co-inoculation of *S. cerevisiae*/*O. oeni* in a Chardonnay fermentation found a higher rate of malic acid consumption (MLF) and slightly higher volatile acidity in co-inoculation (Semon et al., 2001). Thus, co-inoculation presents a potential strategy for a successful MLF.

Prediction 3-3: *O. oeni* strain and bacterial species diversity will increase in the following order of treatments: inoculation of *O. oeni* strain MBR31 after AF (-+Oe), pre-AF inoculation of *O. oeni* (+-Oe), and spontaneous (uninoculated) (--Oe).

> As with *S. cerevisiae*, inoculation of an *O. oeni* strain for MLF of cider (depending on the strains) has shown that the inoculation of *O. oeni* would dominate and thus exclude or suppress all other *O. oeni* strains (Sánche et al, 2014). There are reports that discourage

the inoculation of *O. oeni* prior to AF because LAB will be inhibited by fermenting yeasts (high yeast activity during AF) (Nehme et al., 2008; Arnink and Henick-Kling, 2005). In addition, the presence of SO_2 (from SO_2 addition or yeast SO_2 production) (Osborne, 2005) can inhibit the inoculated *O. oeni*, which results in a bacterial diversity similar to that of the spontaneous treatment. The addition of *O. oeni* after AF might be the best time to inoculate in terms of encouraging inoculum survival and high implantation (lowest bacterial diversity). But ultimately, spontaneous treatments should result in highest bacterial diversity because they don't have an influence from inoculation and they allow for the widest range of bacteria to thrive.

Prediction 3-4: *O. oeni* strain and bacterial species composition will differ between the three different treatments (+-Oe, -+Oe , and --Oe).

As mentioned previously, *O. oeni* inoculation will likely result in the dominance of the inoculated strain (Sánche et al, 2014). However, inoculation before AF can result in lower implantation because of AF activity. Thus, each treatment should result in their own unique bacterial profiles.

Prediction 3-5: There will be no differences in *S. cerevisiae* strain and yeast species composition among the three different treatments (+-Oe, -+Oe , and --Oe).

It is common that LAB were found to be inhibited by yeasts from AF (Osborne & Edwards, 2007), but an inhibition of yeast by *O. oeni* is not common. Alcoholic fermenting yeast is typically not affected by simultaneous inoculation of *O. oeni* and *S.*

cerevisiae and thus co-inoculation could be a good strategy for induction of MLF (Semon et al., 2001).

Objective 4: To determine whether microbial differences found between treatments will correlate with changes in the sensory profiles of the final wine product.

Prediction 4-1: Microbial differences found between treatments will correlate with changes in the sensory profiles of the final wine product.

Compounds produced by yeast and bacteria during fermentation have a major impact on wine aroma and flavor (Fleet, 2003; Romano et al., 2003; Jolly et al., 2013). In addition, the interaction of both yeast and bacteria, as well as, the conditions in which they are interacting can influence the type and amount of microbial derived compounds (Bisson, 2004). Thus, it would be reasonable to expect a correlation between different microbial treatments and wine sensory attributes.

Chapter 2 – The use of propidium monoazide in conjunction with qPCR and Illumina sequencing to identify and quantify live yeasts and bacteria

2.1 Synopsis

Culture-dependent microbe enumeration methods rely on the culturing of microorganisms onto media, are relatively time consuming, and may not detect slow growing or fastidious microorganisms (Treco and Winston, 2008). These short-comings may lead to underestimates of species diversity, including both species richness and relative abundance. Recently, culture-independent methods of microbial identification and enumeration have been developed, which allow for DNA extraction directly from environmental samples without subjecting microbes to growth on nutrient media. These methods often involve next generation DNA sequencing (NGS) for identifying microbes and qPCR for quantifying them. Despite these advantages, culture-independent methods still face many challenges. One of these challenges includes the ability to determine whether the DNA or RNA was extracted from living or dead cells (Lebeis, 2014; Lundberg et al., 2012). Quiros et al. (2009), studying wine fermentations, found that the quantity of VBNC and non-viable cells in the harsh environment of wine can be over 60% and can change rapidly throughout the fermentation. Thus, it is imperative to be able to distinguish live from dead cells. The use of propidium monoazide (PMA) may provide a remedy to this problem. Nevertheless, its optimization has not been fully explored under a variety of conditions. An overall objective was to optimize the PMA method, when used as a precursor to NGS, for both yeasts and bacteria. Specifically, the experiment determined the effect different PMA concentrations and different cell densities (at all PMA concentrations) had on DNA amplification from both dead and viable bacteria and yeasts. Based on the literature (Andorra et al., 2010), we predicted that there would be an optimum concentration at a specific cell

concentration, which will provide a relatively accurate description of our mock communities for both bacteria and yeasts. This prediction is based on the idea that relatively high densities of cells will hinder the binding of PMA with DNA, which will in turn reduce the effectiveness of DNA photolysis within dead cells. The 'optimum concentration' was achieved when 90% of the DNA in dead cells was eliminated but the amount of DNA associated with live cells was not compromised.

2.2   Materials and Methods

2.2.1   Yeast and bacterial species preparation

Our selection of yeast species was based on the criteria that they are all typically found in wine fermentations and they have similar generation times (80-100 min). Our selection of bacterial species, however, was based on similar growth rates (generation time between 17-40 min), but varying cell structure. Acetic acid bacteria (generation time between 8-10 h) and lactic acid bacteria, including *Oenococcus oeni* (generation time between 18-28 h), were not used because they have relatively slow growth rates and they require very specific nutrient conditions. Organisms with similar growth rates were used to minimize variation during culturing and to ensure that most cells were alive prior to the experiment. Yeast species selected were *Hanseniaspora uvarum* (QGEW Chardonnay 2014- wild)*, Saccharomyces cerevisiae* (Fermol® Chardonnay)*,* and *Torulaspora delbrueckii* (Lallemand® Level2 TD). Bacterial species selected were *Escherichia coli* (DH10B)*, Bacillus subtilis* (ATCC# 6633), and *Micrococcus luteus* (ATCC# 533). All yeast species were grown in yeast extract peptone dextrose (YEPD) media. They were incubated at 28°C-30°C for 1 (liquid media) or 2 days (agar/solid media). All bacterial species were grown on tryptic soy (TS) media at 32°C for 1 (liquid media) or 2 days (agar/solid media).

2.2.2   Experimental design – Bacterial and yeast communities

For yeast, a mixture of live *S. cerevisiae*, *T. delbrueckii,* and *H. uvarum* was used as a live mock community. For comparison of the efficacy of the PMA treatment, a second community containing live *T. delbrueckii*, heat killed *S. cerevisiae* and heat killed *H. uvarum* was prepared. All yeast mock communities contained $10.0 \pm 2.6\%$ *S. cerevisiae*, $83.6 \pm 2.1\%$ *H. uvarum*, and $6.5 \pm 0.5\%$ *T. delbrueckii.* For bacteria, a mixture of live *E. coli*, *M. luteus*, and *B. subtilis* was used as a live mock community. For comparison, a second community containing live *E. coli*, heat-killed *M. luteus* and heat killed *B. subtilis* was prepared. All bacterial mock communities contained $50.8 \pm 2.8\%$ *E. coli*, $17.7 \pm 0.7\%$ *B. subtilis*, and $31.4 \pm 2.0\%$ *M. luteus.* Heat-killing was accomplished by heating the cells in DNA grade water at 85°C for 10 minutes. Each species of yeast or bacteria were separately cultured in liquid yeast extract peptone dextrose medium and each of the two communities was made from the same pure cultures (Figure 2.1). Heat-killed and live cell viability was confirmed by plating on agar media and incubating as stated above.

Our main goal with both the bacterial and yeast proportions was to achieve $\geq$50% dead cells in the sample to accurately test the PMA method. The proportion of species were obtained from serial dilution and plate count methods. The yeast community containing live *T. delbrueckii*, dead *H. uvarum* and *S. cerevisiae* were made to mimic post alcoholic fermentation. Following fermentation, *S. cerevisiae* and ethanol intolerant yeasts are usually dead, but *T. delbrueckii* may survive and obtain a relatively high abundance. At mid-fermentation, the use of PMA is expected to detect no major differences between PMA treated and non-treated samples because live *S. cerevisiae* at this stage is usually relatively high in abundance. *Torulaspora delbrueckii* was chosen as the only viable species to challenge the detection of viable non-

Saccharomyces species in the midst of other dead yeasts, including *S. cerevisiae*. For bacteria, *E. coli* was chosen as a representative of a gram negative rod, *M. luteus* as a gram positive coccus, and *B. subtilis* as a gram positive rod. Parallel experiments were run on yeast and bacterial species. An experiment that contained a mixture of yeast and bacterial species was not performed so as to minimize the interaction between them.
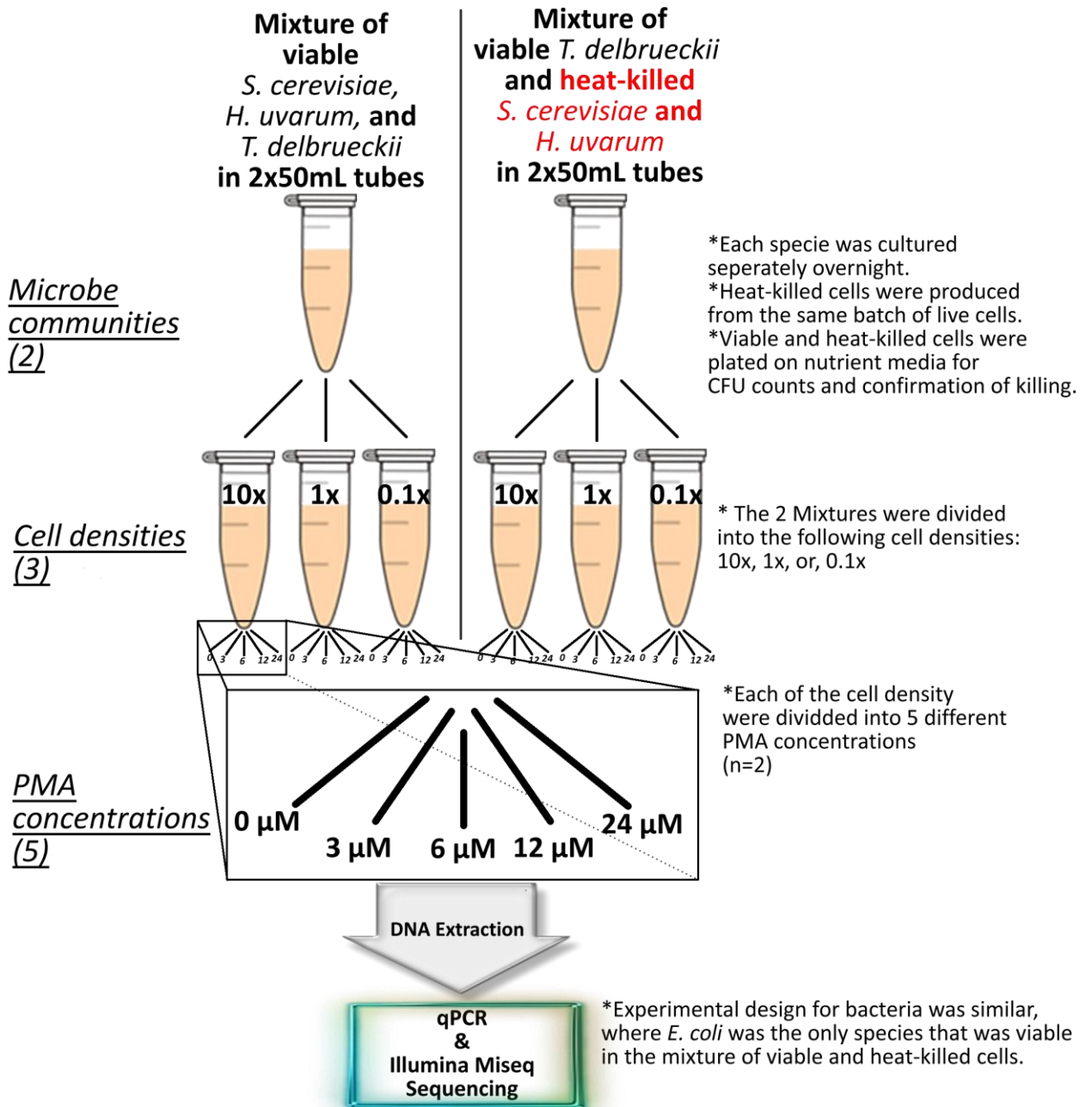
Figure 2.1 Flow diagram of the nested experimental design to explore the effect of cell densities and PMA concentrations on microbial detection and quantification of the community, which contained either viable and heat-killed species or only viable species.

2.2.3 Cell densities and PMA concentrations

Two yeast and two bacterial communities were divided into subgroups of three different cell densities: 10X (Yeast: ~10^8 CFU/mL; Bacteria: ~10^9 CFU/mL), 1X (Yeast: ~10^7 CFU/mL; Bacteria: ~10^8 CFU/mL), and 0.1X (Yeast: ~10^6 CFU/mL; Bacteria: ~10^7 CFU/mL). A cell density of 10X was achieved by mixing equal volumes of the three separately cultured species. The mixed volumes were concentrated via centrifugation (4650 x g for 10 min) and then pellets were suspended in 1/10 the starting volume. A cell density of 1X and 0.1X was achieved by diluting the 10X cell density accordingly. Each cell density was subjected to five different PMA concentrations: 0, 3, 6, 12, and 24 µM, which were duplicated (Figure 2.1).

2.2.4 PMA treatment

PMA (Biotium Inc., CA, USA) was added at all the above mentioned concentrations and mixed thoroughly before incubating on ice for 10 minutes in the dark. Photolysis of PMA-bound DNA was performed by directing a 550 Watt halogen lamp toward the samples on ice for 8 minutes on a moderately shaking platform (100 rpm). Cells were centrifuged and the pellet was washed twice with DNA grade water prior to DNA extraction.

2.2.5 DNA extraction

Yeast and bacterial DNA extractions were performed using OMEGA Stool Kits (Omega Biotek, Norcross, USA). In addition to extracting DNA, these kits were useful because they removed phenolic compounds, which are often found in wine samples and are known to interfere with DNA amplification. Extraction was performed following the manufacturer's protocol with the slight modification of homogenizing cells with 0.1 mm glass beads in extraction buffer for 30 minutes. Purified DNA was stored at -20 °C before further processing.

2.2.6   qPCR quantification of yeasts and bacteria

The universal yeast primers YEASTF and YEASTR (Hierro et al. 2006), targeting within the D1/D2 domains of the 26S rRNA gene, were used to quantify yeasts. The universal primers Uni334F and Uni514R (A.3), which targeted the V3 regions of the 16S rRNA gene, were used to quantify bacteria. The yeast and bacteria qPCR reactions contained 10 μL of SsoFast™ EvaGreen® Supermixes (Biorad, Hercules, USA), 4.0 pmol of each of the forward and reverse primers, and 2.0 μL of purified DNA. The final volume was adjusted to 12.5 μL using sterilized milli-Q H_2O. Amplification conditions were 95 °C for 2.5 minutes, followed by 40 cycles of 95°C for 15 seconds, 61 °C (bacteria) / 60 °C (yeast) for 1 minute, with a final extension at 72 °C for 30 seconds. Three technical replicates of each sample were amplified using the CFX96 Touch™ Real-Time PCR Detection System (Biorad).

The CFU/mL concentrations of each sample were determined by comparing the threshold value (Cq) with the Cq value of a standard curve of known microbe concentrations. Yeast standard curves of Cq vs CFU/mL were made from known colony densities of *S. cerevisiae, T. delbrueckii*, and *H. uvarum*. Bacterial standard curves of Cq vs CFU/mL were made from known colony densities of *E. coli, B. subtilis,* and *M. luteus*. Within the acceptable PCR efficiency range of 80% to 120%, reactions were designed and performed according to the guidelines for minimum information for publication of quantitative real-time PCR experiments (MIQE) (Bustin et al. 2009).

2.2.7 Illumina MiSeq library preparation

Sample preparations were conducted via a 2-step PCR amplification consisting of "amplicon" and "index" PCR reactions. Firstly, extracted DNA was amplified with primer pairs CS1-F515 and CS2-R806 for bacteria (Caporaso et al. 2011) or CS1-BITS & CS2-B58S3 for yeasts (Bokulich et al. 2013) (A.1). These primers are specific for the 16S rRNA gene in bacteria and the ITS1 region in yeast species, respectively. In addition, they contain the overhanging linker sequences (CS1/CS2) that are crucial for the secondary index PCR reaction. The purpose of the index PCR is to attach Illumina Miseq adapter sequence and unique barcodes on each sample.

All PCR amplifications were performed in an Applied Biosystems Veriti Thermal Cycler (Foster City, CA, USA). Bacterial amplicons were created using primers CS1-F515 and CS2-R806 following these parameters: one cycle at 94 °C for 3 minutes, followed by 20 cycles at 94 °C for 40 seconds, 50 °C for 1 minute, 72 °C for 1.5 minutes, with a final extension at 72 °C for 10 minutes. Yeast amplicons were created using primers CS1-BITS and CS2-B58S3 following these parameters: 95 °C for 2 minutes, followed by 25 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1.5 minutes, with a final extension at 72 °C for 10 minutes (1 cycle). A Gel Logic 400 Imaging System (Mandel, Rochester, USA) was used to visualize and confirm PCR amplification in a 1.5% agarose gel containing 1μL SYBR® Safe DNA gel stain (Life Technologies, Carlsbad, USA).

The amplicon products were diluted five to ten-fold with DNA grade water depending on the band strength visualized in the agarose gel. The diluted products were then subjected to a second amplification for sequencing and indexing using the Illumina MiSeq platform by primers that contained the CS1/CS2 linker sequence, unique 8 nucleotide barcode sequences and the

Illumina adapter sequences P5 and P7 that hybridize into the MiSeq® instrument flow cell (A.1).

Index PCR amplification was performed in an Applied Biosystems Veriti Thermal Cycler by

using the following these parameters: 95 °C for 1 minute (1 cycle), 95 °C for 30 seconds, 62 °C

for 30 seconds, 68 °C for 1.5 minutes (12 cycles), and 68 °C for 5 minutes (1 cycle). The PCR

products were again visualized in 1.5% agarose gel containing 1µL SYBR® Safe DNA gel stain.

Attachment of the barcode and the adapter sequences were deemed successful when the

amplicons length were extended by 69bp (Miseq adapter sequence length + barcode) compared

to the 1st PCR amplicon length. The bacterial 1st PCR amplicon lengths were between 300-400

bp and yeast 1st PCR amplicon lengths were between 250-350 bp.

Samples were submitted to The IBEST Genomics Resources Core facility at the

University of Idaho, USA, for quantification, normalization, pooling, and sequencing. The 300

bp paired-end sequencing was performed using a MiSeq Desktop Sequencer (Illumina Inc., San

Diego, USA).

2.2.8   Illumina Miseq data processing

Illumina Miseq 300 bp-pair-ended sequencing of forward and reverse reads were merged

using the software Fast Length Adjustment of Short reads (FLASh). Assembled sequences were

trimmed at both ends, removing low quality, and contaminated reads using the extended

randomized numerical aligner – filter (erne-filter). Any short (<240 bp) sequences,

barcode/primer errors, and sequences with a Phred score of less than 20 (Q<20) were considered

to be of low quality and were removed from further analysis. The open-source bioinformatics

pipeline, Quantitative Insights Into Microbial Ecology (QIIME), and appropriate command

scripts were used to demultiplex, assign similar sequences (97% or higher) to operational

taxonomic units (OTUs), and classify OTUs into taxonomic groups. OTU classification was

performed by comparing them with the databases UNITE v. 7.0 for yeasts or Green Genes v. 13.8 for bacteria.

2.2.9   Statistical data analysis

2.2.9.i qPCR quantification

Yeast and bacterial qPCR data was transformed by the Box-Cox transformation to optimize and to improve the linearity and the homogeneity of variance before performing a nested-analysis of variance (nested-ANOVA). A 3-stage nested fixed-effect ANOVA was then performed to investigate the differences within the communities (live only and live and non-viable), cell density (10X, 1X, 0.1X), and PMA concentrations (0, 3, 6, 12, 24 µM). A post-hoc pairwise comparison was performed using the Tukey honest significant difference (HSD) test on any significant data (n = 2).

2.2.9.ii Illumina Miseq sequencing analysis

Data obtained through Illumina MiSeq sequencing was transformed to reflect the species composition and the relative abundance of the three yeasts or bacterial species present in the samples. Distances between samples were measured using the Bray-Curtis dissimilarity index. A nested PERMANOVA was performed using Primer 7+PERMANOVA (PRIMER-E Ltd., Devon, UK) to investigate the statistical difference in the 3-stages design (the two communities, the cell density, and the PMA concentrations treatments). A post-hoc pairwise test was performed to detect differences between pairs. Monte Carlo (MC) sampling with 999 permutations was performed to make a pairwise statistical test possible.

2.3   Results and Discussion

2.3.1   Yeast

As expected, the PMA treated community, which contained both viable and non-viable yeasts, had lower total CFU/mL than the community containing only viable cells for each of the cell densities (F = 42.55, p<0.001; Figure 2.2). Also in the mock community containing both viable and non-viable cells, treatments with PMA significantly decreased PCR amplification of DNA from dead cells in the 0.1X and 1X treatments (Figure 2.2A). These results support previous non-NGS studies that PMA binds to DNA in non-viable yeasts, preventing it from being amplified (Andorra et al., 2010). This also supports the notion that PMA readily enters non-viable and dead cells through their ruptured cell membranes, and any DNA bound by PMA is permanently modified through photolysis, which prevents the DNA from being amplified. In the mock community containing both viable and non-viable cells, killed *S. cerevisiae* was detected in a much lower relative abundance than killed *H. uvarum* (Figure 2.2A). This result may occur because *S. cerevisiae* could be more heat sensitive than *H. uvarum*. There could be some cell components or compounds found in *H. uvarum*, which are absent in *S. cerevisiae* that lead to better stability at higher temperatures.

In the mock community, all three yeast species were present but the relative abundance of *Hansenispora uvarum* was greatest (Figure 2.2B). This is to be expected because *H. uvarum* was the most abundant species when originally added to the mock community (10.0 ± 2.6% *S. cerevisiae*, 83.6 ± 2.1% *H. uvarum*, and 6.5 ± 0.5% *T. delbrueckii*). The 1X yeast cell density and 24 μM PMA effectively inhibited the amplification of dead cells, but qPCR data demonstrated inconsistencies among the 2 replicates, suggesting a lack of reliability (Figure 2.2A). At this density, there were no major differences between 3, 6, and 12 μM of PMA. At

0.1X cell density, 3 and 6 µM were sufficient in eliminating the detection of dead species, but 6

µM had less variability among the replicates, indicating a greater amplification efficiency.

Overall, DNA extracted from viable yeasts amplified most abundantly when the PMA

concentration was at 6 µM and when yeast densities ranged between 10^6 to 10^7 CFU/mL. Thus,

our data supports our original prediction that relatively high densities of cells would interfere

with PCR amplification and that there will be an optimum PMA concentration. Our findings are

in agreement with those of Andorra et al. (2010) who found that 6 µM is the optimum PMA

concentration and that higher concentrations of PMA will begin to inhibit the DNA amplification

of the viable cells. In contrast, Vendrame et al. (2014) were able to quantify a target spoilage

yeast species by using 100 µM PMA, which is over 16 times greater than our optimum

concentration. The discrepancy in optimum concentration between the two studies may be due to

the difference in total cell density (~10^8 CFU/mL), length of PMA incubations, incubation

temperature, light exposure time (Nkuipou-Kenfack et al., 2013), photo-crosslinking equipment,

and the organisms used. Vendrame et al. (2014) used PMA-qPCR to determine the quantity of

the live *B. bruxellensis* in a wide range of densities (10^1 - 10^8 CFU/mL) when mixed with the

same species of dead yeasts at the PMA concentration of 100 µM. Nevertheless, their experiment

followed only one species via the qPCR enumeration method. When multiple species are present

in the sample, the data supports the approach of using a combination of Miseq sequencing and

qPCR, which provides a more accurate measurement of yeast abundance.
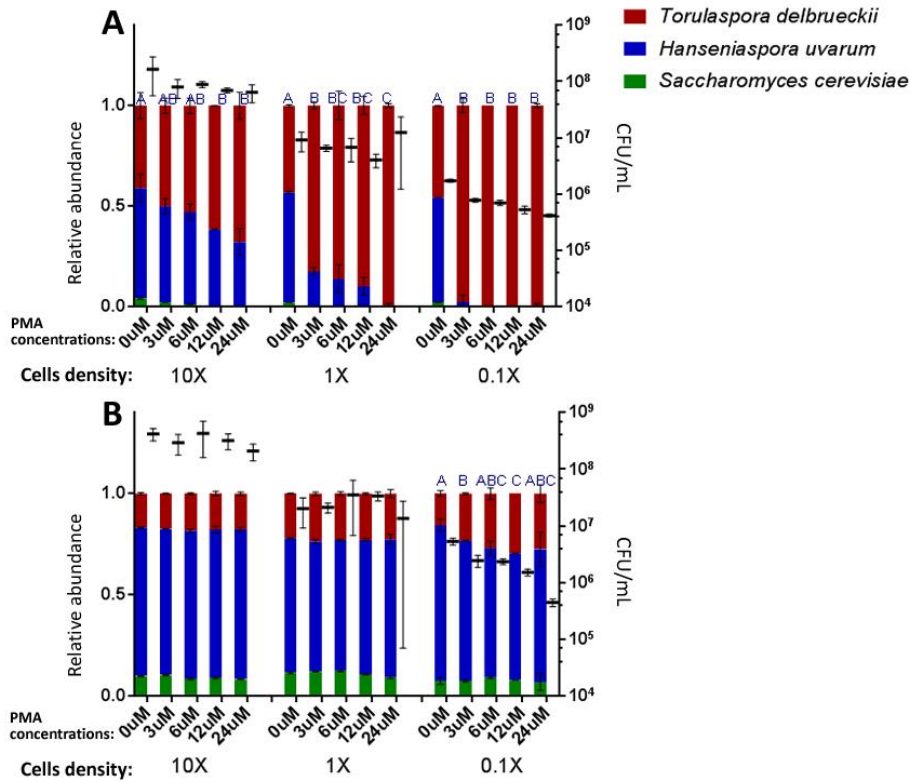
Figure 2.2 Relative abundance of yeast species detected by Illumina Miseq sequencing (left axis) and the total yeast CFU/mL as quantified by qPCR (right axis). (A) Viable *T. delbrueckii* with heat-killed *H. uvarum* and *S. cerevisiae* mixtures and (B) viable *T. delbrueckii*, *H. uvarum*, and *S. cerevisiae* mixtures. Of these mixtures, three different cell densities were subjected to five different PMA concentrations. Differences in letters above columns indicate significant differences in species composition between PMA concentrations at each cell density (n = 2). Each black bar indicates the mean of CFU/mL with vertically extending whiskers showing variability as standard deviation.

2.3.2 Bacteria

The PMA treated community, with both viable and non-viable bacteria, had a
significantly greater relative abundance of viable *E. coli* than the community containing only
viable cells for each of the cell densities (Pseudo-F = 193.2, p <0.001; Figure 2.3). Additionally,
in the mock community, with both viable and non-viable cells, the treatment void of PMA (0
µM) had a significantly greater relative abundance of killed *B. subtilis* and *M. luteus* than the
PMA treated treatments at all densities (Figure 2.3A). These results support the idea that PMA
can bind and permanently modify exposed DNA of the non-viable cells more readily than that of
the intact viable cells (Bae & Wuertz, 2009; Nocker et al., 2006; Sánchez et al., 2013; Nam et al.,
2011). However, the PMA treated community, with both viable and heat-killed species, was not
significantly different in relative abundance from the pure viable community (F = 0.02, p =
0.895). Even though this result is contradictory to our Illumina results, qPCR quantifications
were found to have high variability among replicates and it may not be sensitive enough to detect
differences. The heat-killed bacterial community contained 50% living *E. coli* when the yeast
community contained 6% living *T. delbrueckii.* The amount of initial viable cells could have an
impact on the non-significant results of qPCR; however, many factors including different primer
sets and robustness of quantification are possible explanations. Nevertheless, qPCR
quantification was able to estimate CFU/mL accurately between different cell densities.

I also found major changes in relative abundance and relatively low qPCR values with 12
and 24 µM of PMA, as compared with 0, 3 and 6 µM of PMA, in the viable mock community at
0.1X (10^7 CFU/mL) cell density. This strongly suggested that some bacterial species are more
susceptible to PMA modification than others (Figure 2.3B). *Bacillus subtilis* appeared to be the
most susceptible to high concentrations of PMA as they were the first to disappear at 12µM of

PMA followed by *M. luteus* at 24μM of PMA (Figure 2.3B). *E. coli* appeared to be the most resistant to the entry of PMA in live cells. This may be due to its Gram negative cell morphology; *B. subtilis* (rod shape) and *M. luteus* (cocci shape) are Gram positive bacteria. From our results, it appeared that Gram positive bacteria are more susceptible to high concentrations of PMA than Gram negative cells. Cell morphology such as bacilli shape, which have more surface area, also appeared to be more susceptible to PMA than cocci. It could also be that the morphology of *M. luteus*, which often shows tetrad arrangement, reduces surface exposure to the environment. Our results are based on only 3 bacterial species, thus further research is necessary to determine whether our findings are the same for mixtures of other bacterial species.

Overall, the result that DNA extracted from viable bacteria amplified most abundantly when the PMA concentration was at 6 μM and when bacterial densities ranged between 10^7 to 10^8 CFU/mL. Our conclusion is supported by results (1X and 0.1X densities in the community containing both viable and non-viable cells) showing that the relative abundance of dead *M. luteus* and dead *B. subtilis* decreased significantly up to 6 μM PMA when cell density ranged between 10^7 to 10^8 CFU/mL (Figure 2.3A). Our findings support our original prediction that relatively high densities of cells would interfere with PCR amplification and that there is a consistently optimum PMA concentration. Our findings are in agreement with those of Nkuipou-Kenfack et al. (2013) who found that 10 μM PMA concentrations were enough to be very effective in modifying dead cell DNA without interfering with living cells. In contrast, Xiao et al. (2015) used 100μM of PMA in an effort to reduce 100% of the qPCR signal from 10^2-10^6 CFU/mL dead cells. This discrepancy may be because it was done only on dead cells without controlling for viable cells under the influence of PMA. Similar research by Vendrame et al. (2013) used PMA-qPCR to determine the quantity of the live *O. oeni* in a wide range of densities

(10^1 - 10^8 CFU/mL) when mixed with the same species of dead bacteria at a PMA concentration of 100 µM. The discrepancy in the optimum PMA concentration found between our study and others may be due to the difference in length of the PMA incubations, temperature exposure time (Nkuipou-Kenfack et al., 2013), variation in cell concentration, photo-crosslinking equipment, and the organisms used in the study. Our results suggest that the combination of Miseq sequencing and qPCR provides an accurate measurement of bacterial abundance when mixtures of bacterial species are present and cell densities vary.
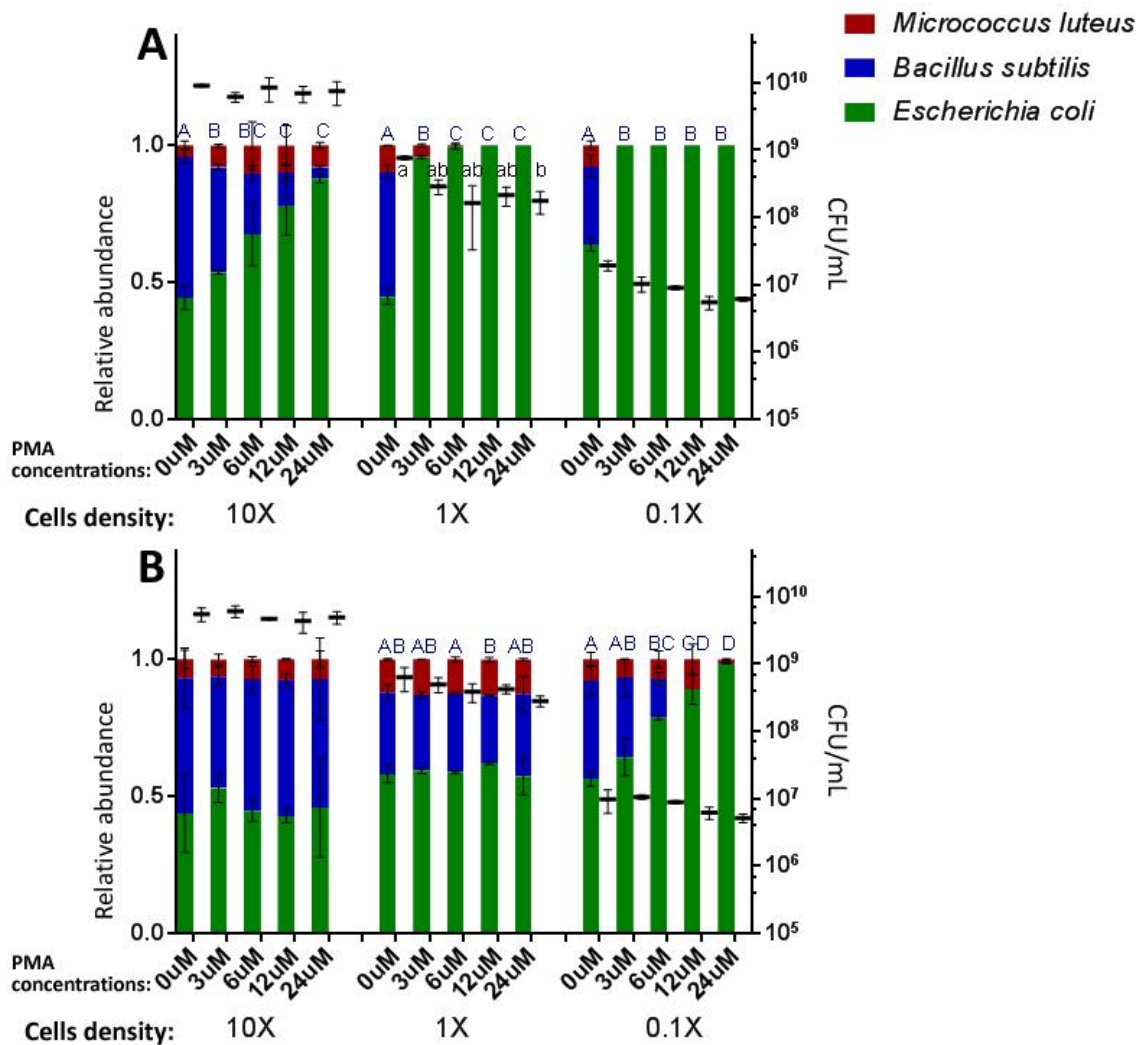
Figure 2.3 Relative abundance of each bacterial species detected by Illumina Miseq sequencing (left axis) and total bacterial CFU/mL as quantified by qPCR (right axis). (A) Viable *E. coli*, non-viable *B. subtilis* and *M. luteus* mixtures and (B) viable *E.coli*, *B. subtilis* and *M. luteus* mixtures were created at different cell densities and subjected to five different PMA concentrations. Differences in lower-case letters indicate significant differences in Tukey HSD in CFU/mL quantification between PMA concentrations at each cell density. Differences in upper-case letters indicate significant differences in species composition between PMA treatments at each cell density (n = 2). Each black bar indicates the mean of CFU/mL ± standard deviation.

Chapter 3 – The effect of initiating alcoholic and/or malolactic fermentations on microbes of wine and on the resulting wine sensorial profile

3.1   Synopsis

Wine fermentation, the conversion of grape juice to wine, involves complex interactions of both yeast and bacteria. There are two important conversions that are involved in wine fermentation. One of these is alcoholic fermentation (AF), where *Saccharomyces cerevisiae* is the main organism responsible for converting sugars to ethanol and CO_2 (Fleet, 1993; Fugelsang, 1997). The other major process is malolactic fermentation (MLF), which involves the decarboxylation of L-malic acid to L-lactic acid. It is beneficial to most red and some white wine varietals and is associated mostly with lactic acid bacteria (LAB) due to their higher expression of malate specific enzymes (Masque, 1996; Edwards 1992). *Oenococcus oeni*, is the most commonly found wine bacterium associated with this process. In the wine making process, it is a common practice to inoculate grape must or juice with *Saccharomyces cerevisiae* for AF and/or *Oenococcus oeni* for malolactic fermentation (MLF). Therefore, it is crucial to monitor wine microbes because they influence wine characteristics, not only through the production of alcohol and lactic acid, but also by producing aroma and flavor enhancing molecules through secondary metabolites. Several methods have been developed to differentiate between strains of a specific species; however, a common way to distinguish strains of *S. cerevisiae* is by using hyper-variable microsatellites or simple sequence repeats (SSRs), which are dispersed throughout the genome (Legras et al., 2004). The use of VNTRs, in multiplex reaction, is a fast and reliable method that is used to distinguish different *O. oeni* strains (Claisse et al. 2012, 2014).

To study wine microbes, an accurate culture independent method for quantifying and identifying bacterial and yeast species is important (Bokulich and Mills 2012). This method allows for DNA extraction directly from environmental samples without subjecting microbes to growth on nutrient media and it involves next generation DNA sequencing (NGS) for identifying microbes and qPCR for quantifying them. In the previous chapter, the optimization of PMA was described (Tantikachornkiat et al., 2016), which allows for an accurate detection of viable cells in a wine sample. The overall objectives in this chapter were to determine the effect of *S. cerevisiae* QA23 inoculation and the timing of *O. oeni* MBR31 inoculation on living bacteria and yeasts during wine fermentation. The experiment also examined the effect of these treatments on the resulting flavor and aroma of the final wine product. It is known that the interaction between *S. cerevisiae* and bacteria can result in a positive or negative outcome for either the yeast or bacterium (Henschke, 1993; Nehme et al., 2010). The inoculation of *S. cerevisiae* (QA23) was predicted to change yeast and bacterial composition at both the species and strain levels. However, *O. oeni* (MBR31) inoculation was predicted to only change bacterial species and strain composition and not the species and strain composition of yeasts. Additionally, we predicted, when *O. oeni* is added prior to fermentation, that it will result in an earlier commencement of MLF than post-AF inoculation of *O. oeni* and spontaneous inoculation, especially when co-inoculated with a *S. cerevisiae* strain. Lastly, we also predicted that these changes would correlate with the changes in the resulting sensorial wine profiles (Fleet, 2003).

3.2   Materials and Methods

3.2.1   Study Site & Sampling

The experiment was conducted at Quail's Gate Estate Winery in British Columbia, Canada. All samples were taken in 2014 from the fermenting must of the *Vitis vinifera* grape

varietal Chardonnay. Samples were collected in 50 mL conical tubes, where they were used to detect strain composition of *S. cerevisiae* and *O. oeni* as well as species composition of, yeast and bacteria during fermentation. Samples for *S. cerevisiae* strain identification were collected during the late/end stage of AF (<2 °Brix). Samples for *O. oeni* strain identification were collected at malic acid levels between 0-1.4 g/L. Lastly, yeast and bacterial species were identified using Illumina sequencing (with propidium monoazide) at six time points during fermentation, specifically: 1) during the late stage of AF (<2 °Brix); 2) 10 days after AF; 3) 19 days after AF; 4) 33 days after AF; 5) during MLF (0-1.4 g/L malic acid; 65 days after AF); and 6) 96 days after AF. Prior to AF, the must underwent coldsettling in a stainless steel tank before its distribution into 18 separate experimental units as described in Section 3.2.2. All oak barrels were 3 years-old, where they were used previously during white wine fermentation. They were cleaned thoroughly by hot water prior to exposure of the experimental wines. Three year-old barrels were used instead of new barrels so as to prevent the oak aroma from overshadowing other important wine attributes.

3.2.2 Experimental Design

The experimental design consisted of 6 treatments that were replicated in triplicate for a total of 18 experimental units or barrels. The design was a 2 x 3 factorial, where the two factors were yeast and bacterial inoculation. Yeast inoculation consisted of 2 levels, where the must was either inoculated with a commercial yeast strain (Sc^+) or was uninoculated and spontaneously fermented (Sc^-). Bacterial inoculation consisted of 3 levels, where the must/wine was inoculated with *O. oeni*, which was added at the same time as *S. cerevisiae* (three days after coldsettling)(+-Oe), or after AF (-+Oe), or left uninoculated (--Oe). (Figure 3.1, Table 1). All replicates were treated with 50 ppm SO_2 between coldsettling and the early stage of fermentation before

distribute them into separate experimental units. Strain Lalvin QA23 (Lallemand®, Montreal, Canada) was used as the *S. cerevisiae* inoculant and strain Lalvin MBR31 was used as the *O. oeni* inoculant. Rehydration of bacteria was performed by adding commercial active dry bacteria (ADB)(2.25 g ADB/225 L barrel) into a preparation of Leucofood® (Beverage Supply Group, Napa, USA) (10.125 g/225 L barrel) and water (20°C) for 15 – 20 min. Yeast inoculum was made by adding commercial active dry yeast (ADY)(56.25 g of ADY/225 L barrel) into a preparation of DYNASTART® (Laffort, Petaluma , USA) (67.5 g/225 L barrel) and water (37-40°C) 15-20 min. Prior to the addition of any inoculum, a small amount of grape must was gradually added to the inoculum until the temperature reached 10°C. Depending on the experimental designs (Figure 3.1), Pre-AF inoculation of *S. cerevisiae* and/or *O .oeni* were inoculate in to the barrels (+Sc, +-Oe treatments) three days after coldsettling. Post-AF inoculation of *O. oeni* (-+ Oe treatments) was performed 18 days after coldsettling (7 days after AF). Chardonnay samples were collected at ~0.5 m depth from the top of the barrel using a sterile pipette. Three sub-samples per barrel were dispensed and were subsequently transported on ice to the lab for molecular analysis.
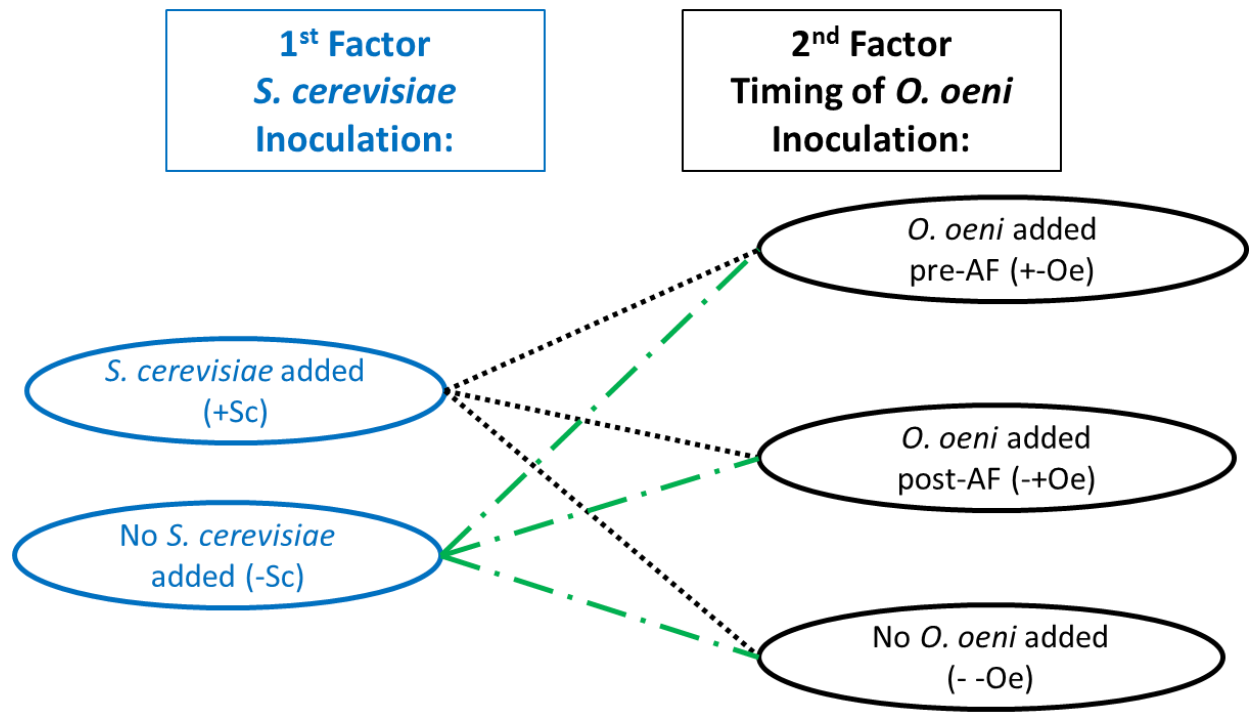
Figure 3.1 Different scenarios of treatments generated from yeast and bacteria inoculation factors.

3.2.3 *S. cerevisiae* and *O. oeni* strain isolation

Wine samples for *S. cerevisiae* isolation were collected during the late stage of fermentation (<0 °Brix). For *O. oeni* isolation, samples were collected from must that had malic acid less than 0.9 g/L. Prior to plating the sample onto a growth media, samples were diluted appropriately and distributed evenly into either Yeast Extract Peptone Dextrose (YEPD) agar plates for *S. cerevisiae* isolation or MRS medium (containing cycloheximide and tomato juice at pH 4.5) for *O. oeni* isolation. *S. cerevisiae* samples were incubated for 1.5-2 day at 28°C, whereas *O. oeni* samples were incubated anaerobically for 14-20 days at 28 °C. The number of colony forming units per mL (CFU/mL) was calculated for plates containing 30-300 colonies. Twenty-four colonies per sub-sample were selected randomly and were subsequently transferred

to a 24-well culture plate. Culture plates were stored at 4°C for further molecular investigation and long term storage.

3.2.4 *S. cerevisiae* strain identification

Identification of *S. cerevisiae* strains was performed using a multiplex PCR reaction that targets eight different hyper-variable microsatellite loci: C3, C4, C8, C11, SCY, YML, YPL and YLR. The primers used to target the loci (Appendix 1) were modified to have 5' fluorescent labelled dye at the forward primer. Polymerase Chain Reaction (PCR) reaction for multiplex PCR (8-plex) contained: 1 X colorless GoTaq® reaction buffer (Promega, Madison, USA), 9.0 nmol of each dNTP, 0.9 pmol of C3 forward and reverse primer, 12.7 pmol of C4forward and reverse primer, 4.3 pmol of C8 forward and reverse primer, 2.2 pmol of C11 forward and reverse primer, 1.2 pmol of SCY forward and reverse primer, 7.8 pmol of YML forward an reverse primer, 1.5 pmol of YPL forward and reverse primer, 1.5 pM of YLR forward and reverse primer, 10.0 μg BSA (bovine serum albumin), 35 nmol $MgCl_2$, 1.25 U of GoTaq® DNA Polymerase (Promega, Madison, USA), and 5 ng – 50 μg genomic yeast DNA. The final volume was adjusted to 15.0 μL using sterilized MilliQ-H_2O. Amplification was performed in an Applied Biosystems Veriti Thermal Cycler, by using the following parameters: 94 °C for 3 min (1 cycle), 94 °C for 30 sec, 55 °C for 35 sec, 72 °C for 45 sec (36 cycles), 72 °C for 10 min (1 cycle). Amplification was confirmed by visualizing a 1.5% agarose gel containing 1x SYBR® Safe DNA gel stain using a Gel Logic 400 Imaging System (Mandel, Rochester, USA). DNA fragment lengths of the amplified products were determined using an ABI 3130 XL Genetic Analyzer (Applied Biosystems). Results of fragment analysis were analyzed using Gene Mapper 4.0 software (Applied Biosystems). Each *S. cerevisiae* strain generated a unique microsatellite fragments pattern that could be classified as a commercial or an indigenous *S. cerevisiae* strain

by comparing them to commercial strains databases of Richards et al. (2009), and Hall et al. (2011).

3.2.5 *Oenococcus oeni* strain identification

Screening and identification of *O. oeni* strains was performed by using 5 sets of *O. oeni* species-specific primers that target 5 different VNTR loci. These primers (Appendix2) were modified to have 5' fluorescent labelled dye at the forward primer (Applied Biosystems, Foster City, USA). Polymerase Chain Reaction (PCR) reaction for multiplex PCR (5-plex) was similar as described above (Section 3.2.6) except that the primer sets TR 1, 2, 3, 4, and 5, at the amount of 0.9 pM, was used. The final volume was adjusted to 15.0 µL using sterilized MilliQ-H$_2$O. Applied Biosystems Veriti Thermal Cycler (Foster City, CA, USA) was used to perform PCR, by using the following parameters: 94 °C for 3 min (1 cycle), 94 °C for 30 sec, 55 °C for 35 sec, 72 °C for 45 sec (36 cycles), 72 °C for 10 min (1 cycle). Amplification, detection, and analysis of the PCR product were performed as described above for *S. cerevisiae* identification (Section 3.2.4). Each *O. oeni* strain generated a unique VNTR fragment pattern, which could be classified as a previously used commercial strain or a unique *O. oeni* strain by comparing them to the commercial active dry bacteria (ADB) strains database at UBCO. This database encompassed all ADB strains previously used at QGEW.

3.2.6 Microbial genus/species quantification and identification (culture independent)

PMA treatment, DNA extraction, qPCR quantification, and taxonomic identification of yeast and bacteria were performed as described above in section 2.2.4 to 2.2.8, except that the bacterial primers CS1-F341 and CS2-R805 (Herlemann et al. 2011) were used in Miseq NGS instead of the CS1-F515 and CS2-R806 primers.

3.2.7   Sensorial Assessment

Sensory evaluation of the wines was conducted 3-4 months after bottling with 2 replicates. Only four out of six treatments, +Sc+-Oe, +Sc-+Oe, -Sc+-Oe, and -Sc--Oe, were selected for the evaluation to ensure better judges accuracy in sensorial attributes detection. This is because tasting all 12 wines (6 treatments with duplicates) would result in a higher chance of judging error. The sensory evaluation, spanning over 2 sessions, consisted of 11 judges evaluating 8 wines (4 treatments in duplicate) for their intensity of flavor, aroma, and mouthfeel. Each tasting session was conducted with a maximum of 6 judges in a completely randomized design, where judges evaluated all 8 wines. All wines were served in coded, clear wine glasses, which contained a plastic lid so as to contain aroma. Wine evaluations were conducted in individual booths equipped with illuminated red lights and a computer with an installed Compusense five-sensory-evaluation software (Compusense Inc., Guelph, ON). Training sessions were conducted prior to evaluation to familiarize judges with the expected sensory characteristics and scoring sheet (A.4). All judges have been involved in the winemaking industry and have extensive experience in tasting wine

3.2.8   Statistical data analysis

3.2.8.i *S. cerevisiae* and *O. oeni* strain composition

Data obtained through culturing, and strain identification via microsatellite (*S. cerevisiae*) or VNTRs (*O. oeni*) were transformed to reflect the strains composition and the relative abundance of the strains present in the samples. Distances between samples were measured using the Bray-Curtis dissimilarity index. A 2 factor PERMANOVA was performed using Primer 7+PERMANOVA (PRIMER-E Ltd., Devon, UK) to investigate the statistical difference in the 2-factors (yeast and bacteria inoculation). If significance was found among the three inoculation

methods to initiate MLF, a post-hoc pairwise test was performed to detect differences between pairs. Principal Coordinate Analysis (PCoA) was used to observe the effect size and the distribution of samples in a two-dimensional space. Additionally, Similarity Percentage analysis (SIMPER) was used to find out which species/strains contribute most to the dissimilarity between 2 groups.

3.2.8.ii *S. cerevisiae* and *O. oeni* strain diversity

Diversity of *S. cerevisiae* and *O. oeni* strains were measured using the Simpson's index of diversity (1-D) for all replicate fermentation vessels (n=3). A 2-factor ANOVA was performed to investigate differences in strain diversity of *S. cerevisiae* and *O. oeni* within each factor.

If the differences were found in the timing of *O. oeni* inoculation factor, a post-hoc pairwise comparison was performed using the Tukey honest significant difference (HSD) test to find out differences in strains diversity between the 3 levels of *O. oeni* addition (Pre, post and non-added).

3.2.8.iii Microbial composition

Data obtained through Illumina MiSeq sequencing was transformed to reflect the composition and the relative abundance of the yeasts or bacterial composition by converting the number of sequence reads to proportions (decimals).

Distances between six different time points in each sample was measured using the Bray-Curtis similarity index to generate resemblance matrices for all of the samples. Each matrix represents a sample with the repeated measure from six sampling time point. A 2STAGE

analysis was used to measure distances between samples (multiple matrices) via weighted

Spearman rank correlation method (Clarke et al., 2006). A 2 factor PERMANOVA was

performed using Primer 7+PERMANOVA (PRIMER-E Ltd., Devon, UK) to investigate the

statistical difference between the 2-factors. Additionally, if differences were found among the

three bacteria inoculation treatments (+-Oe, -+Oe, --Oe), a post-hoc pairwise test was performed

to detect differences between pairs.

As mentioned in Section 3.2.8.ii, PCoA and SIMPER were used to visualize and identify

the unique features and components of the composition between treatments.

3.2.8.iv Microbial diversity

Diversity of yeast and bacteria species was measured using the Simpson's index of

diversity (1-D) for all replicate fermentation vessels (n=3). A 2-factor ANOVA with repeated

measures was performed to investigate differences in species diversity of yeast and bacteria

within each factor. If the differences were found in the timing of *O. oeni* inoculation factor, a

Tukey honest significant difference (HSD) test was performed to find out differences between

pre *O. oeni* addition, post *O. oeni* addition and non- *O. oeni* addition levels.

3.2.9   Sensory Assessment

A three-factor ANOVA was performed on each of the sensory attributes. The main

effects were 'judge', 'replicate', and 'wine'. Two-factor interactions (judge x wine [panel

agreement], judge x rep [judge reliability], rep x wine [presentation errors]) were assessed using

a fixed effect. Principle component analysis (PCA) was performed only on the significant

attributes using their average intensity score. Judge inconsistencies (significant judge x wine

effects) were not found on any of the significant wine sensory attributes, indicating a similar pattern of response among judges.

3.3 Results and Discussion

3.3.1 Inoculation and species/strain diversity

In support of prediction 2-1, Lalvin QA23 comprised 95% of *S. cerevisiae* strains for all inoculated AF treatments, which indicated a successful implantation (Medina et al. 2013; Lange et al., 2014). In contrast, spontaneous AF treatments were composed of 3 dominant commercial strains, including Vitilevure 3001, Zymaflore FX 10, and Lalvin QA23, as well as, several unknown (potentially indigenous) *S. cerevisiae* strains, with an overall relative abundance of 10% (Figure 3.5A). As predicted in prediction 2-2, the spontaneous AF treatments (-Sc +-Oe; -Sc -+Oe; -Sc - -Oe) had a higher strain diversity than the inoculated AF treatments (+Sc +-Oe; +Sc -+Oe; +Sc - -Oe) (F=142.37, p<0.001; Figure 3.5B). The additional Vitilevure 3001 and Zymaflore FX10 were the main *S. cerevisiae* strains in the spontaneous AF treatments. These were likely responsible for the difference in strain diversity between the inoculated and spontaneous AF treatments (Lalvin QA23= 48.11%, Vitilevure 3001 = 27.48%, and Zymaflore FX10 = 10.41%; 85% cumulative).

Torulaspora delbrueckii, a non-*Saccharomyces*, had a higher relative abundance in spontaneous AF treatments as compared with the inoculated AF treatments. Specifically, *S. cerevisiae* and *T. delbrueckii* contributed to 97.1% of the species present in the spontaneous AF treatment, whereas *S. cerevisiae* alone comprised over 95% of the yeast species present during the late stage of inoculated AF treatments. Spontaneous treatments appeared to be able to harbor a higher abundance of many commonly found non-*Saccharomyces* wine species, including *H. osmophila* and *Pichia spp*. (Figure 3.5A). Non-*Saccharomyces* species diversity was also found

to be significantly higher in spontaneous AF treatments compared with inoculated treatments (F=4384.29, p<0.001). These results support the prediction 2-3 that a successful inoculation of an *S. cerevisiae* strain results in a lower involvement of non-*Saccharomyces* species (Raspor et al. 2002).

In contrast to our prediction 2-4, both bacterial species diversity and *O. oeni* strain diversity were not affected by the addition of yeast Lalvin QA23 (F=0.83, p=0.379; Figure 3.5C). Even though it is commonly found that yeast and bacteria interaction can be positive or negative, we found no evidence of Lalvin QA23 affecting bacterial diversity. This may be due to high bacterial and *O. oeni* strains composition variation. Supporting this notion, we found that one of the three replicates within the treatment, –Sc –Oe, had a very different *O. oeni* strain composition than the other 2 replicates, in addition, bacterial species composition was often found to vary greatly among the replicates (see section 3.3.2). It is unlikely that this is due to PCR bias because previous research focusing on pooling many PCR replicates showed no detectable differences in sequence diversity between a single replicate and multiple pooled PCR replicates (Smith and Peay 2014).

3.3.2 Species and Strain Composition

The results support prediction 2-5 that the composition of both bacterial species (F=6.9175, p=0.003; Figure 3.5A & E) and *O. oeni* strains (Pseudo-F= 4.8127, p < 0.008; Figure 3.1) significantly differed between inoculated (+Sc) and spontaneous AF treatments (-Sc). Specifically, a major reduction in the relative abundance of *O. oeni* (relative to other bacterial species) occurred in the spontaneous AF treatment (Figure 1A; between days 12 and 22; -Sc +-

Oe) as compared with the inoculated AF treatment, (+Sc +-Oe). As previously mentioned, the spontaneous AF treatments contained a higher relative abundance of non-*Saccharomyces* (especially *T. delbrueckii*) than the inoculated AF treatments. It may be that *T. delbrueckii* is the cause for the changes in the bacterial species and *O. oeni* strains. However, the results also suggest that certain *S. cerevisiae* strains in spontaneous AF treatments may have negatively impacted the inoculated *O. oeni* MBR31 strains in the -Sc +-Oe and -Sc -+Oe treatments. In the +Sc+-Oe treatment, *O. oeni* MBR31 maintained a relatively high abundance throughout both fermentations as compared with the –Sc +-Oe treatment, which contained a diversity of *S. cerevisiae* strains. In the past, researchers have found that bacteria were affected by the presence of specific yeast species (Wibowo et al., 1988 and Gilis et al., 1996) and *S. cerevisiae* strains (Arnink and Henick-Kling, 2005, and Nehme et al., 2008). Under operational conditions, our findings suggest that yeast inoculation can result in a chain reaction that ultimately changes both yeast and bacterial composition.
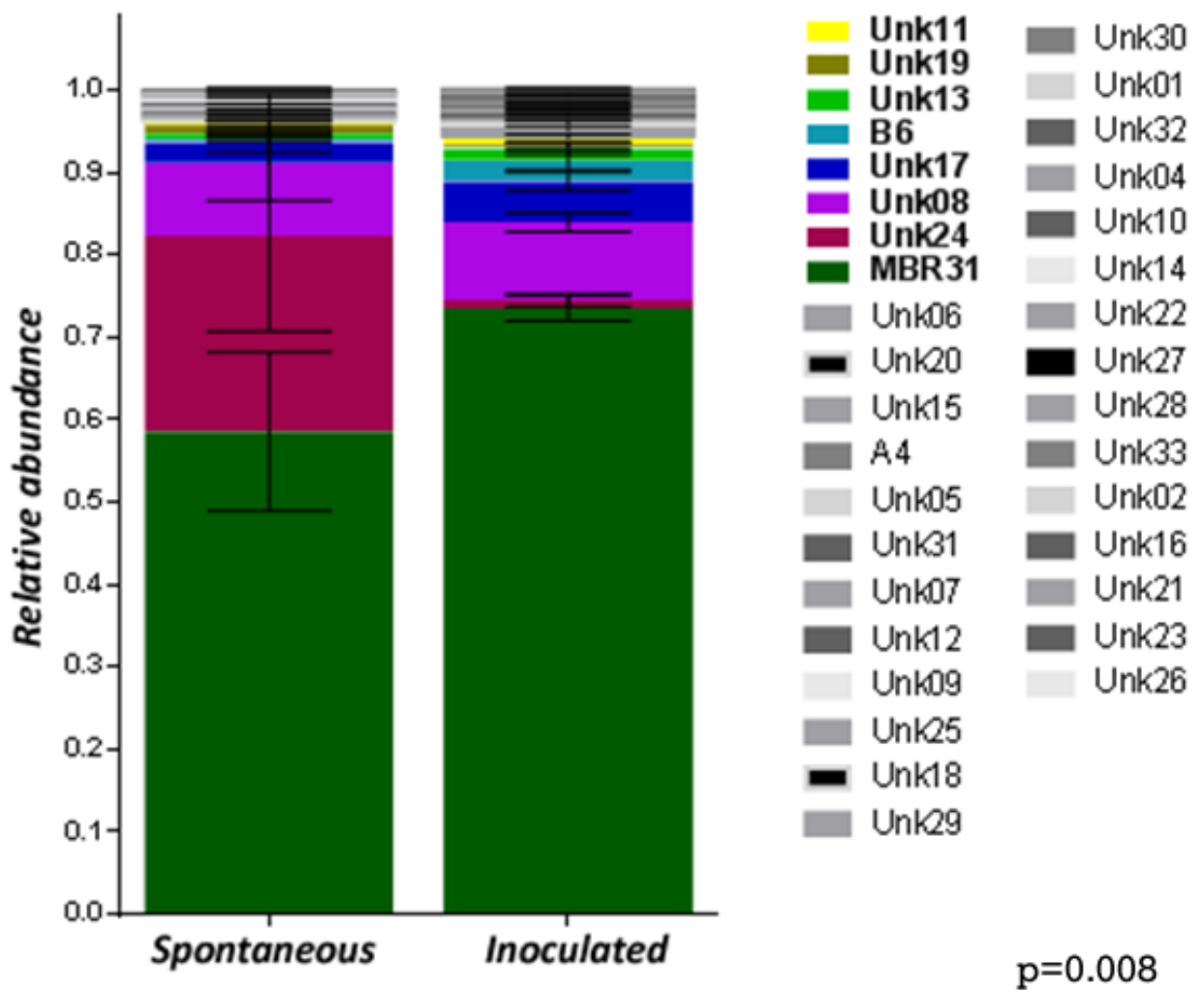
Figure 3.2 The relative abundance of *O. oeni* strains found in spontaneous AF (-Sc) and QA23 inoculated (+Sc) treatments. *O. oeni* strains MBR31 and Unk24 seem to be the prominent strains contributing to the difference in strain composition (n = 3).

In support of our prediction 3-1, the +Sc +-Oe treatment was found to start malolactic fermentation significantly earlier than the other treatments, including -Sc +-Oe treatment (Figure 3.3). However, it is also important to notice that malic level measured at day 44 appeared to be higher than day 30. This is not possible and the error is most likely due to some flaw in the

measurement of the malic acid that was performed in batches of samples, which were collected on the same day. Thus, the samples are only comparable within each measuring time point (day). Overall, samples taken at both 30 and 44 days showed that -Sc +-Oe treatment contained distinctively lower malic acid (indicating earlier MF) than the rest of the treatments. This result agrees with our bacterial composition data that *O. oeni* abundance remained relatively high when the commercial *S. cerevisiae* strain was added (+Sc) than when it was not added (-Sc). Because *O. oeni* abundance was higher in only the +-Oe treatment and not in the - -Oe or -+Oe treatments, it indicates that malic acid depletion by bacteria is dependent on both the *S. cerevisiae* strains present and the timing at which *O. oeni* is added. At a commercial winery, exposure of *O. oeni* to many different strains of *S. cerevisiae* strains is inevitable, especially in spontaneous fermentation, where there is a relatively high *S. cerevisiae* strain diversity. These results suggest that a specific yeast strain may be responsible in delaying MLF. Our findings agree with those of a previous in-lab study that malic acid depletion is different between co-inoculation (*O. oeni* added before AF) and sequential inoculation (*O. oeni* added after AF) and that malic acid degradation efficiency differs between different *O. oeni* strains (Nehme et al., 2010).
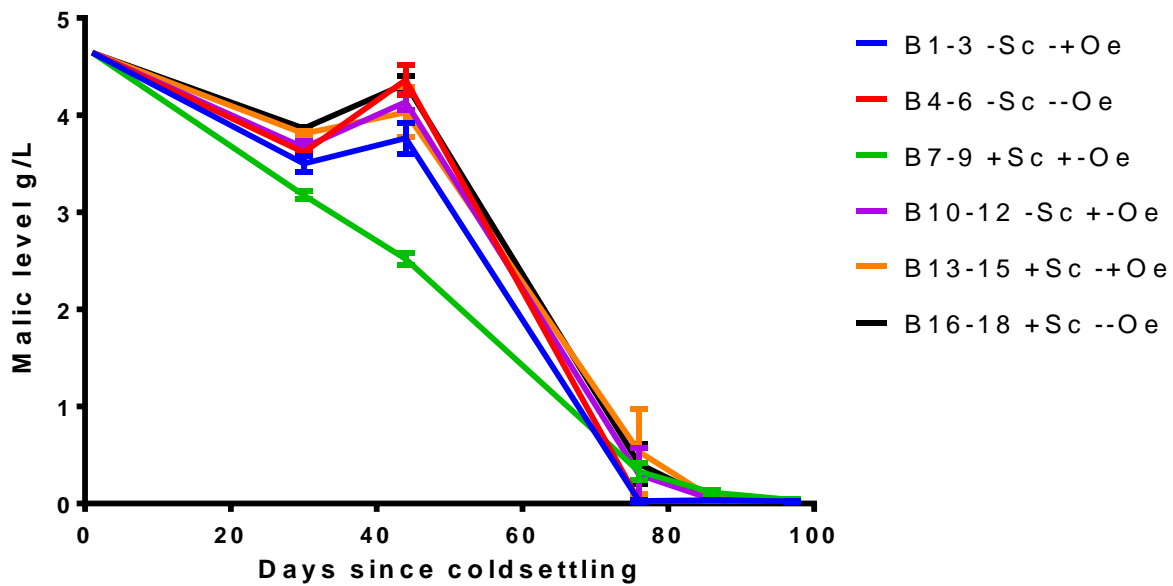
Figure 3.3 Changes in malic acid level at 6 different time points after coldsettling (Day 0). Samples for *O. oeni* strain typing were collected at 72 days after coldsettling (n = 3).

3.3.3 Timing of *O. oeni* inoculation

Except for the +Sc +-Oe treatment, where MLF appeared to begin as early as 30 days after coldsettling, most treatments started MLF about 44 days following coldsettling (Figure3.3). In prediction 3-2, we predicted that both the +Sc and –Sc AF treatments with the +-Oe treatment would commence MLF sooner than the other treatments. This was partially supported because the +Sc +-Oe treatment started MLF the earliest, while the -Sc +-Oe commenced MLF at the same time as all the other treatments. As previously discussed, high *S. cerevisiae* strain diversity may have a negative impact on *O. oeni* (Arnink and Henick-Kling, 2005, and Nehme et al., 2008). These results suggest that both the timing of *O. oeni* inoculation and the *S. cerevisiae* strain and non-*Saccharomyces* species composition present during AF can be important for an

early initiation of MLF, particularly when inoculated AF and early inoculation of *O. oeni* are performed. Previous research agrees with our findings that co-inoculated fermentations are the first to initiate MLF and reduce fermentation time (Abrahamse & Bartowsky, 2012; Knoll et al. 2012). However, different timing of inoculation was found to result in changes in volatile compound composition, but overall, co-inoculation may provide a better alternative if an early MLF is desired (Knoll et al. 2012). Interestingly, *O. oeni* strain abundance usually declined after inoculation with *O. oeni*, regardless of the timing of inoculation (Figure 3.5A). This is in agreement with the findings of Semon et al. (2001) who also found that the density of all three *O. oeni* strains declined after inoculation, regardless of the timing of inoculation. Previous studies have suggested that interactions between *S. cerevisiae* and *O. oeni* strains could affect MLF efficiency (Nehme et al. 2008; Henschke, 1993; Henick-Kling and Park, 1994). In a negative interaction, *S. cerevisiae* strains inhibited certain *O. oeni* strains by producing strain-specific metabolites during fermentation (Reguant et al., 2005; Comitini et al. 2005). Our findings are in agreement with others that success in co-inoculation is highly dependent on the choice of yeast and bacterial inoculants (Semon et al., 2001; Alexandre et al. 2004)

In contrast to our prediction 3-3, no differences in *O. oeni* strain diversity were found among the three different *O. oeni* inoculation treatments (F = 0.80, p = 0.472). Nevertheless, the +-Oe treatment had lower bacterial diversity than the treatments -+Oe and --Oe (F = 14.50, p = 0.001). This finding is more likely due to differences in AF conditions and winemaking techniques used during AF than to differences between inoculation timing of *O. oeni.* This conclusion is supported by the finding that the Simpson's diversity index among the three treatments varied slightly (between 0.54 and 0.62).

In support of Prediction 3-4, bacterial species composition was also different depending on when *O. oeni* MBR31 was added (F=17.073, p<0.001; Figure 3.6-D2), which, in turn, appeared to change the overall dynamics of the bacterial community throughout both AF and MLF fermentations. These findings are expected because addition of bacteria directly changes bacterial composition. Also, in support of this prediction, strain composition of the - - Oe was significantly different from the -+Oe treatment (Pseudo-F= 3.1779, p = 0.031). It appears that timing of the bacterial inoculation is crucial in determining the resulting composition of *O. oeni* strains because there was no significant difference in strain composition between +-Oe and - -Oe treatments but there was a significant difference between -+Oe and the other two treatments. As previously discussed, this may be because the *O. oeni* viable cell density were often found to decline rapidly after inoculation depending on the inoculation timing, the method of rehydration, and as well as the *S. cerevisiae-O. oeni* strain compatibility (Semon et al., 2001). There is a paucity of information on *O. oeni* strain genotypic diversity in different regions (Bridier et al., 2010; Wang et al., 2015). We are not aware of any studies that have monitored the changes in strain composition in response to different timing of inoculation in both inoculated and non-inoculated AF. One of the reasons for the limited research may be the hyper mutability of *O. oeni* genes (Marcobal et al., 2008). The results of this study, which discovered 30 unique DNA fingerprint generated via VNTR, supports the idea of hyper mutability of *O. oeni* genes. It also supports the general opinion that the database for *O. oeni* is still in a developmental stage, especially when considering there are much fewer commercial *O. oeni* strains compared with commercial *S. cerevisiae* strains.

An interactive effect in the bacterial composition were found between yeast inoculation and bacterial inoculation treatments (F=6.37, p=0.003). This interaction suggests that there is

less change in the bacterial species composition when the wine is inoculated with *S. cerevisiae* QA23 than when it is not inoculated (spontaneous). This might have to do with either *S. cerevisiae* strain diversity or non-*Saccharomyces* yeast, such as *T. delbrueckii*, which were found to be much higher in spontaneous AF treatments. This, in turn, may allow for a spontaneous fermentation, as compared with an inoculated fermentation, to be more influenced by the inoculation of an *O. oeni* strain. In support of this interaction, the PCoA analysis showed 3 separate groupings for the *O. oeni* inoculation treatments (Figure 3.6-D2) and two separate groupings for the *S. cerevisiae* inoculation treatments. Overall, the results suggest that higher species and strain diversity of yeast during fermentation may allow for a greater change in the bacterial community.

Finally, in support of prediction 3-5, *S. cerevisiae* strain composition did not differ among any of the *O. oeni* treatments (F = 1.26, p = 0.294). Thus, yeast species composition did not change when *O. oeni* inoculation occurred at different times (F = 0.90, p = 0.507). In previous work, addition of *O. oeni* prior to AF didn't affect the abundance of *S. cerevisiae* in AF; however, elevated numbers of bacteria (especially LAB and acetic acid bacteria), prior to AF, have been found to result in sluggish fermentation (Bisson, 1999). Our results are in contrast to these findings, possibly because our experiment was conducted with short coldsettling (3 days) and a 50 ppm SO_2 addition in an effort to control bacterial density. Furthermore, research by Ludovico et al. (2001) indicated that high acetic acid concentration can severely inhibit *S. cerevisiae* growth. In our fermentations, it is unlikely we had this situation because our NGS data indicated a lack of acetic acid bacteria. It is known that the long-term use of commercial *S. cerevisiae* strains in the winery may favor *S. cerevisiae* over indigenous strains in spontaneous fermentation (Beltran et al. 2002, Hall et al. 2011). But currently, it is still unknown whether the

use of commercial *O. oeni* selects for commercial *S. cerevisiae* strains that are compatible with the inoculated *O. oeni*. Since our data only detected commercial *S. cerevisiae* strains that reside during spontaneous AF, more research is required on this matter. Overall, no change in the *S. cerevisiae* population, yeast community or sluggishness of AF could be attributed to the inoculation or lack of inoculation of *O. oeni*. This may because most of the conditions for AF were favourable and it only took approximately 8 days for AF to finish.

3.3.2   Sensorial attributes

When inoculated and spontaneous AF treatments were compared, the sensorial profile moved from acidity (acid-tasting sour) to tropical fruit/full body, whereas when *O. oeni* treatments were compared, the profile changed variably; perhaps it was overshadowed by the addition of *S. cerevisiae*. Inoculation with *Oenococcus oeni* changed the overall bacterial species and *O. oeni* strain compositions. But the inoculation of *S. cerevisiae*, as compared with the inoculation of *O. oeni*, was found to have a higher impact on both yeast and bacterial communities and ultimately a negative impact on the wine sensorial profiles (Figure 3.4). In support of prediction 4-1, spontaneous AF treatments had higher tropical fruit and higher body attributes as compared with inoculated AF treatments (Figure 3.4), which is likely due to differences in their microbial populations and communities. In support of this conclusion, differences in yeast and bacterial species, as well as, *S. cerevisiae* and *O. oeni* strain composition differed substantially between uninoculated and inoculated AF treatments, whereas these microbial characteristics differed minimally between the *O. oeni* uninoculated and inoculated treatments. The magnitude of changes between the two pairs of treatments was found to correlate with changes in sensorial profile of the wine (Figure 3.4). Even though, correlation doesn't

always mean causation, many correlations were found between changes in tropical fruitful flavor and microbes detected during fermentation (Table 3.1). Higher diversity of yeast species and *S. cerevisiae* strains are most likely the driving force for the differences in sensory attributes rather than the presence of *T. delbrueckii*. This is because, even though a higher proportion of *T. delbrueckii* correlates with tropical fruitful flavor, it doesn't mean that a fermentation containing only *T. delbrueckii* would result in even higher tropical fruitful flavor. *T. delbrueckii*, as a single species, is not capable of finishing AF (Bely et al., 2008). The same logic goes with the higher involvement of *S. cerevisiae* strains (Vitilevure 3001, Zymaflore FX10, and QA23) in spontaneous fermentation. Each individual strain is most likely to behave differently if it is used as a monoculture. All other wine attributes (body, long finish, tropical fruit, buttery aroma, and vanilla aromas and flavor) were detected with only a slight correlation with tropical fruit flavor and acidity, which suggests that the unique microbial composition and high diversity in spontaneous AF treatments appeared to contribute to wine complexity and higher sensorial attributes of the wine (Figure 3.4). Our results agreed with other recent studies ~~findings~~ comparing co-fermentations of *T. delbrueckii* and *S. cerevisiae* with their monoculture fermentations. These studies showed an increase in wine aromatic intensity and complexity (Renault et al., 2015; Azzolini et al. 2014) in the co-fermentations as compared with the monocultures. Renault et al. (2015) suggest that a higher production of ethyl propanoate, ethyl isobutanoate, ethyl dihydrocinnamate and isobutyl acetate may be the reason for the flavor enhancement but in this study there were no differences in pH, TA, and residual sugar between treatments (Appendix 5). These results support the findings of Azzolini et al. (2012, 2014) who found that differences in acid compounds (citric, acetic, tartaric, lactic acid), which result in a sour taste, cannot be explained by pH and titratable acidity alone (Pangborn, 1963).

Table 3.1 Positive and negative correlations between tropical fruit flavor and microbial

population found during fermentation.

Wine attribute	Positive correlation	Negative correlation
Tropical Fruit Flavor	Higher yeast species diversity	Higher proportion of *S. cerevisiae* species
	Higher *S. cerevisiae* strains diversity	Higher proportion of *O. oeni* strains Unk 08
	Higher proportion of *T. delbrueckii*	Higher proportion of *S. cerevisiae* strains QA23
	Higher proportion of *S. cerevisiae* strains Vitilevure 3001	
	Higher proportion of *S. cerevisiae* strains Zymaflore FX10	
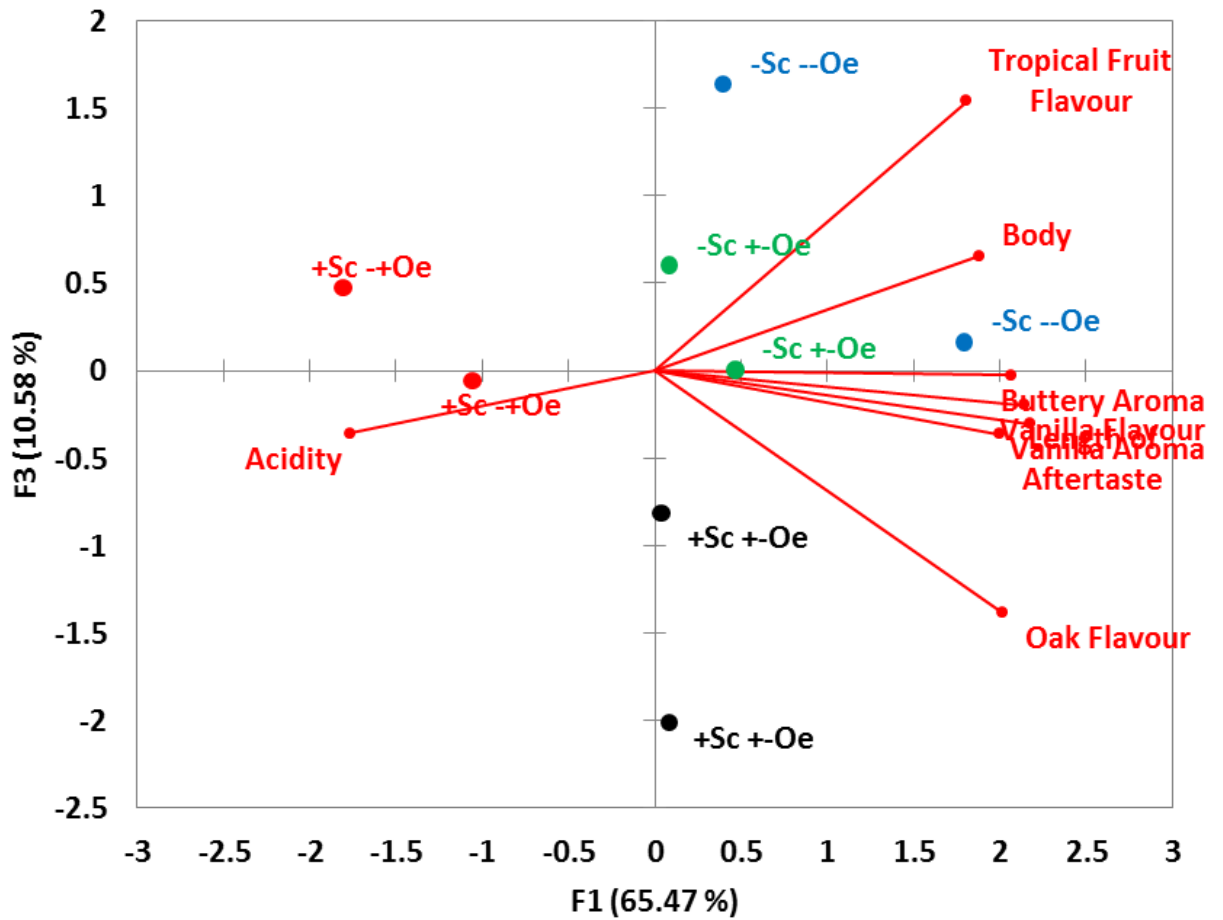
Figure 3.4 Plot of the 2 component of a PCA (accounting for 76.05% of total variance) model, showing the relationship of the 4 wine treatments data in relation to sensorial attributes detected. Sensorial analysis was ran in duplicate as coded by color.

A

Yeast | Bacteria | *S. cerevisiae strains* | *O. oeni strains*

-Sc --Oe

-Sc +-Oe

-Sc -+Oe

+Sc --Oe

+Sc +-Oe

+Sc -+Oe

12 22 31 45 77 107

Days since coldsettling

12 22 31 45 77 107

Days since coldsettling

Late AF (<2 Brix)

Late MLF <0.9 g/L Malic acid

B

Simpson_1-D

Spontaneous

Inoculated

-Sc --Oe   -Sc +-Oe   -Sc -+Oe   +Sc --Oe   +Sc +-Oe   +Sc -+Oe

Treatments

C

Simpson_1-D

-Sc --Oe   -Sc +-Oe   -Sc -+Oe   +Sc --Oe   +Sc +-Oe   +Sc -+Oe

Treatments

D

Simpson_1-D

Spontaneous:
— -Sc +-Oe
— -Sc -+Oe
— -Sc --Oe

Inoculated:
— +Sc +-Oe
— +Sc -+Oe
— +Sc --Oe

Days since coldsettling

E

Simpson_1-D

— -Sc -+Oe
— -Sc --Oe
— +Sc +-Oe
— -Sc +-Oe
— +Sc -+Oe
— +Sc --Oe

Days since coldsettling

Yeast
Torulaspora delbrueckii
Saccharomyces uvarum
Saccharomyces cerevisiae
Pichia spp.
Hanseniaspora uvarum
Hanseniaspora osmophila
Fungi (Others)

Bacteria
Micrococcus
Sphingomonas
Methylobacterium
f__Bacillaceae
f__Enterobacteriaceae
Sediminibacterium
Thermus
Bradyrhizobium
Bacillus
Anoxybacillus
Geobacillus
Acinetobacter
Oenococcus

S. cerevisiae strains
Unk1
Lallemand_DV10
F_Arome_Plus
Lalvin_ICV_D254
Lalvin_RC212
Zymaflore_FX10
Vitilevure_3001
Lalvin QA23
Unk2   Unk10
Unk3   Unk11
Unk4   Unk12
Unk5   Unk13
Unk6   Unk14
Unk7   Unk15
Unk8   Unk16
Unk9

O. oeni strains
Unk11   Unk30
Unk19   Unk01
Unk13   Unk32
B6   Unk04
Unk17   Unk10
Unk08   Unk14
Unk24   Unk22
MBR31   Unk27
Unk06   Unk28
Unk20   Unk33
Unk15   Unk02
A4   Unk16
Unk05   Unk21
Unk31   Unk23
Unk07   Unk26
Unk12
Unk09
Unk25
Unk18
Unk29

63

Figure 3.5 A) Yeast & bacteria communities and relative abundance of *S. cerevisiae* & *O. oeni* strains. B) *S. cerevisiae* Simpson's diversity Index C) *O. oeni* Simpson's diversity Index D) Yeast species Simpson's diversity changes over time E) Bacteria Simpson's diversity Index changes over time (n = 3).
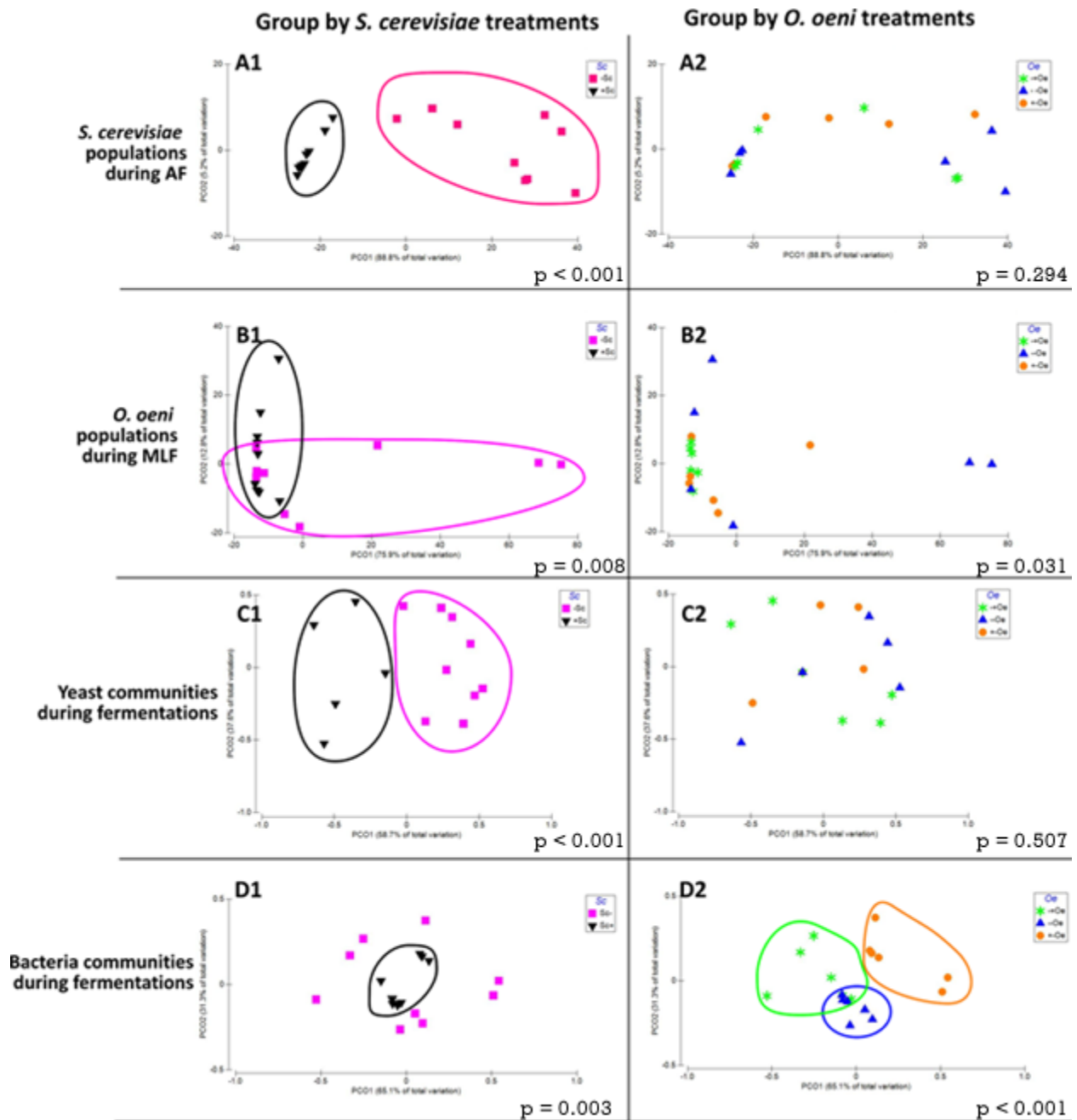
Figure 3.6 PCoA, each point represents an experimental unit (n=3), separated by 1) *S. cerevisiae* Lalvin QA23 addition treatments 2) *O .oeni* MBR31 addiction treatment; A) *S. cerevisiae* strain composition during late AF (<2 °Brix). B) *O. oeni* strain composition during late MLF (<0.9 g/L). C) Yeast community during wine fermentation. D) Bacterial community during wine fermentation. Each point represents an experimental unit (n = 3).

Chapter 4 – Conclusion

4.1 Summary

The objective of work detailed in Chapter 2 was to optimize the PMA method for concentration and cell culture size for both yeasts and bacteria so that only live cells will be detected by subsequent NGS analysis. The result showed the use of PMA was effective in eliminating DNA associated with dead yeast and bacterial cells for all cell concentrations used. Nevertheless, DNA extracted from viable yeast and bacterial cells amplified most abundantly at 6 µM PMA with yeast densities between 10^6 to 10^7 CFU/mL and bacterial densities at approximately 10^8 CFU/mL. The result suggested that the optimum concentration of PMA may be dependent on cell density because higher cell density required higher concentrations of PMA to effectively eliminate amplification from DNA of dead cells. Based on the results, it was speculated that in bacteria, some species may be more susceptible to relatively high PMA concentrations than others, resulting in their elimination. The experiment showed that without the PMA treatment, non-viable cells were still detected. Therefore, the results suggest that when using an NGS method, viable yeasts and bacteria in environmental samples can be accurately quantified by using PMA at our recommended cell densities and PMA concentrations.

In work described in Chapter 3, the objective, using Chardonnay wine fermentations, was to determine the effect of *S. cerevisiae* inoculation and timing of *O. oeni* inoculation on: 1) yeast and bacterial species and strain diversity; 2) yeast and bacterial composition; and 3) the sensory attributes of the final wine product. The experiment quantified and monitored 6 different inoculation treatments associated with AF and MLF. The result from a 2 x 3 factorial analysis showed that inoculated AF treatments significantly impacted both *S. cerevisiae* strain and yeast species composition and diversity, which later caused changes in *O. oeni* strain and bacterial

species composition. The difference in bacterial community and wine attributes between adding or not adding *S. cerevisiae* was larger than when adding or not adding *O. oeni*. We have also correlated the results from each treatment with the sensorial attributes of the final wine. Sensorial profiles indicated that both *O. oeni* and *S. cerevisiae* inoculation influenced final wine sensorial attributes. Additionally, an interactive effect was found; when AF was conducted spontaneously, the bacterial community composition responded more dramatically to *O. oeni* inoculation as compared to no-*O.oeni* inoculated treatment. These results suggest that spontaneous AF treatments had higher yeast species and strain diversity, which correlated with positive sensorial attributes of the wine, such as greater body and longer finish, tropical fruit, vanilla flavors, as well as, buttery and vanilla aromas.

4.2 Novelty of the research

The work described in Chapter 2 is novel because dead cell DNA was successfully eliminated from detection when using downstream applications that require PCR. Specifically, The results have shown an optimized use of PMA in combination with the latest NGS technology to only identify living microbes in a sample. Furthermore, this method were used to study wine fermentation, that monitored both yeast and bacterial changes over time during fermentation, as well as, monitored *S. cerevisiae* and *O. oeni* strain populations during AF and MLF, respectively. The experimental design involved 2 factors: 1) adding or not adding *S. cerevisiae* strains QA23; 2) co-inoculation / sequential inoculation or not adding *O. oeni* strains. We were able to determine the effect of *S. cerevisiae* strains or the timing of *O. oeni* addition on the microbial profiles of the wine during fermentation. The results showed that timing of inoculation of *O. oeni* such as co-inoculation or post AF inoculation change both bacterial species and *O. oeni* strains, which later affected the final wine product. In addition, the 2 factor

design allowed for determining the interactive effects between the factors; We found that if AF was conducted spontaneously, the bacterial community composition responded more dramatically to *O. oeni* inoculation treatments.

In addition, We confirmed previously reported findings from lab-based or small-scale operational experiments. Our research is novel because we monitored, at a commercial scale, microbial response to treatments at 2 levels of taxonomic classification: 1) species, and 2) *S. cerevisiae* and *O. oeni* strains.

From an industrial perspective, sequential inoculation (*S. cerevisiae* co-inoculation with *O. oeni*) resulted in a faster initiation of MLF than the other treatments tested. We also found that sequential and co-inoculation was relatively lower in many of the positive attributes of the wine compared with the uninoculated fermentation (-Sc - - Oe). However, fermentation without the use of yeast or bacterial inoculum in newly-established wineries or in regions that have had a history of stuck fermentations might pose risks that allow for unwanted microorganisms to thrive during fermentation. It is also important to note that Quails' Gate Estate Winery, has introduced many commercial microbial strains into the winery in the past 20-years, which might result in the establishment of previously used yeast or bacterial strains that out-compete spoilage organisms. Nevertheless, in our study, we did not detect by NGS analysis any of the typical wine spoilage microbes. In the non-microbial added fermentation, A completely different bacterial composition profile were found, as well as, higher involvement of non-*Saccharomyces* (*T. delbrueckii*) and different strains of *S. cerevisiae* conducting AF. The resulting wines were found to have many of the positive attributes that are correlated with the treatments that have elevated yeast species and strain diversity.

4.3 Suggestions for further research

A recently published paper by Scholz et al. (2016) has presented an algorithm to differentiate and identify between strains from shotgun sequencing data. It might be of interest in the future to look into whole genome sequencing for strain identification, preferably in combination with PMA to identify live bacteria and yeast populations.

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Appendix A: Molecular primers information and wine fermentation parameters

A.1 Characteristics of the eight primer pairs used to generate microsatellite fingerprints. (Legras et al. 2004; Field and Willis, 1998; Perez et al. 2001).

Locus name	Motif	ORF[a] or coordinates	Primers-Forward	Primers-Reverse	Dye	Estimate Size range (bp)
C3	CAA	YGL139w	GTGTCTCTTTTTATTTACGAGCGGGCCAT	AAATCTCATGCCTGTGAGGGGTAT	6-FAM*	94 - 135
C4	TAA+TAG	XV-110701/110935; YOL109W	AGGAGAAAAATGCTGTTTATTCTGACC	TTTTCCTCCGGGACGTGAAATA	NED*	242 - 361
C8	TAA	YGL014c	GTGTCTCAGGTCGTTCTAACGTTGGTAAAATG	GCTGTTGCTGTTGGTAGCATTACTGT	NED*	128 - 157
C11	GT	YJR044C	TTCCATCATAACCGTCTGGGATT	TGCCTTTTTCTTAGATGGGCTTTC	PET*	187-239
SCY	GTT	YOR267c	TACTAACGTCAACACTGCTGCCAA	GGATCTACTTGCAGTATACGGG	PET*	280-348
YLR	CAG	YLR177W	CTTAAACAACAGCTCCCAAA	ATGAATCAGCGCATCAGAAAT	VIC*	80-150
YML	AAT	YML091C	GTGTCTAAGCCTCTTCAAGCATGAC	GTGTCTGGACAATTTTGCCACCTTA	6-FAM*	243 - 327
YPL009c	CTT	YPL009c	AACCCATTGACCTCGTTACTATCGT	TTCGATGGCTCTGATAACTCCATTC	VIC*	393 - 497

*6-carboxyfluorescein (6-FAM) (blue), 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) (green), 2′-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED) (yellow), and PET (red) (Applied Biosystem, Foster City, USA)

[a]Open Reading Frame (ORF)

A.2. Characteristics of the five primer pairs used to generate VNTRs fingerprints. (Claisse et al. 2012, 2014)

Locus name	Location**	Genome ID**	Primers-Forward	Primers-Revese	Dye
TR1	114127–114352	OEOE_0123	GGTAAGGGAAAAGTTATCCTCG	GTTTTACCTTCGGTCGAGC	NED*
TR2	1136035–1136540	OEOE_1200	CATAATAGAATTCACTTCGCTTACC	GTAGCTGGTACGAGCTCTTC	6-FAM*
TR3	942050–942641	OEOE_1007	CTAATTCTTCCTCGCCCTTTG	GGACTGACTGTACTTATTTGAGG	VIC*
TR4	260077–260109	OEOE_0270	GTGACCGACCAAAGCATAAC	AAAAACGCTCCAAGAAAGGT	PET*
TR5	1024906–1024924	OEOE_1095	AAATCCTGGTTTTGTCCGTA	GGCTTCCTATCCATTTTGGT	6-FAM*

*6-carboxyfluorescein (6-FAM) (blue), 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) (green), 2′-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED) (yellow), and PET (red) (Applied Biosystem, Foster City, USA)

**Based on the *O. oeni* PSU-1 genome sequence, GenBank accession No. NC_008528.

A.3. Primer pairs used to amplify yeasts and bacterial samples by qPCR or Illumina Miseq

qPCR	**Primer name**	**Sequence**		**Fragment size**	
Universal for yeasts	YEASTf*	5'-GAGTCGAGTTGTTTGGGAATGC-3'			D1/D2 domains (26S rRNA gene sequences)
	YEASTr*	5'-TCTCTTTCCAAAGTTCTTTTCATCTT-3′		124 bp	
Universal for bacteria	Uni334F**	5′-ACTCCTACGGGAGGCAGCAGT-3′			16S rRNA position 334-514 (V3 regions)
	Uni514R**	5'-ATTACCGCGGCTGCTGGC-3'		180 bp	
Amplicon PCR	**Primer name**	**Overhanging linker sequence CS1/CS2**		**Spacer**	**Universal yeast or bacteria specific primer**
Bacteria V4 domain	CS1-F515***	CS1: 5' ACA CTG ACG ACA TGG TTC TAC A		GT	GTG CCA GCM GCC GCG GTA A '3
of 16S region	CS2-R806***	CS2: 5' TAC GGT AGC AGA GAC TTG GTC T		-	GG ACT ACH VGG GTW TCT AAT '3
Yeast ITS1 region	CS1-BITS****	CS1: 5' ACA CTG ACG ACA TGG TTC TAC A		CT	ACC TGC GGA RGG ATC A '3
	CS2-B58S3****	CS2: 5' TAC GGT AGC AGA GAC TTG GTC T		-	GA GAT CCR TTG YTR AAA GTT '3
Index PCR		**Miseq adapter sequence**		**8nt barcode**	**CS1/CS2 linker binding site**
	P5	5' AATGATACGGCGACCACCGAGATCTACAC		**NNNNNNNN**	CS1: ACA CTG ACG ACA TGG TTC TAC A '3
	P7	5' CAAGCAGAAGACGGCATACGAGAT		**NNNNNNNN**	CS2: TAC GGT AGC AGA GAC TTG GTC T '3

* Hierro et al., 2006; **Hartman et al., 2009; ***Caporaso et al., 2011 (modified); ****Bokulich et al., 2013 (modified)

A.4 Roundtable scorecard sample.

<div align="center"><u>**Instructions**</u></div>

Please assess the wines in the order presented. First, **SMELL** the wine using 'small rabbit sniffs' and rate the intensity of the **AROMA** attributes. Second, **TASTE** the wine and rate the intensity of the **FLAVOUR AND MOUTHFEEL** attributes. Mark the scale with a vertical line and write the sample number above. Please expectorate samples into the spittoon provided. A tray of standards is provided for reference. Have a bite of Melba toast and a sip of water between samples.

AROMA ATTRIBUTES

Citrus Aroma: All citrus notes (lemon, grapefruit, lime, orange) detected by nose

Pome Fruit Aroma: All apple and pear notes detected by nose

Tropical Fruit Aroma: All tropical notes (pineapple, mango, passion fruit) detected by nose

Vanilla Aroma: Vanilla notes detected by nose

Oak Aroma: All toasted oak notes (toasty, smoky, woody, coconut) detected by nose

Floral: All floral notes (jasmine, honeysuckle) detected by nose

Buttery: Buttery notes detected by nose

FLAVOUR AND MOUTHFEEL ATTRIBUTES

Citrus Flavour: All citrus notes (lemon, grapefruit, lime, orange) detected by mouth

Pome Fruit Flavour: All apple and pear notes detected by mouth

Tropical Fruit Flavour: All tropical notes (pineapple, mango, passion fruit) detected by mouth

Vanilla Flavour: Vanilla notes detected by mouth

Oak Flavour: All toasted oak notes (toasty, smoky, woody, coconut) detected by mouth

Buttery: Buttery notes detected by mouth

Acidity: The taste stimulated by acids

Body: The feeling of fullness or viscosity in the mouth

Length of Aftertaste: The length of time that flavours persist in the mouth after expectoration

AROMA ATTRIBUTES

Citrus Aroma

Low   High

Pome Fruit Aroma

Low   High

Tropical Fruit Aroma

Low   High

Vanilla Aroma

Low   High

Oak Aroma

Low   High

Floral Aroma

Low   High

Buttery Aroma

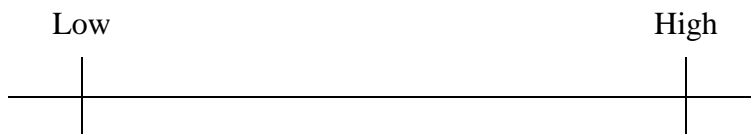Low   High

FLAVOUR AND MOUTHFEEL ATTRIBUTES

Citrus Flavour

Low   High

Pome Fruit Flavour

Low   High

Tropical Fruit Flavour

Low   High

Vanilla Flavour

Low   High

Oak Flavour

Low   High

Buttery Flavour

Low   High

Acidity

Low   High

Body

Low   High

Length of Aftertaste

Short   Long
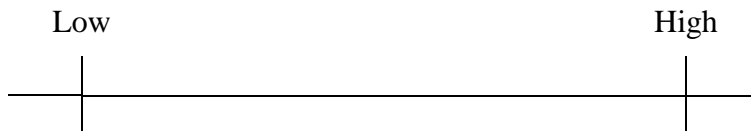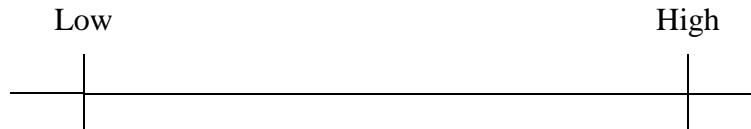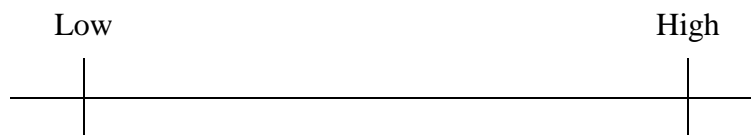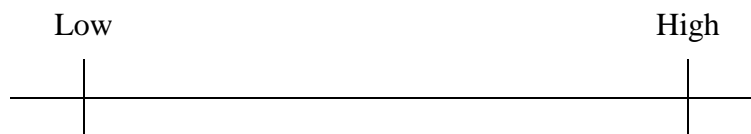
Comments:

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A.5 Standard measurement of the final wine product.

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18
		-Sc -+Oe		-Sc --Oe			+Sc +-Oe			-Sc +-Oe			+Sc -+Oe			+Sc --Oe		
Ethanol Content (%)	-	12.1	12.7	12.2	12.9	-	-	12.3	12.7	12.8	-	12.9	12.8	12.5	-	12.9	12.5	-
Volatile Acidity (g/L)	-	0.41	0.42	0.42	0.36	-	-	0.38	0.35	0.43	-	0.45	0.35	0.37	-	0.32	0.38	-
pH	-	3.66	3.67	3.64	3.69	-	-	3.64	3.64	3.66	-	3.63	3.69	3.64	-	3.68	3.65	-
T.A. (g/L)	-	4.8	4.8	4.7	4.9	-	-	5.1	5.3	5	-	5.3	5.2	5.1	-	5.3	5.2	-
Residual Sugar (g/L)	-	0.25	0.36	0.48	0.43	-	-	0.58	0.44	0.35	-	0.24	0.31	0.57	-	0.41	0.42	-
Malic Acid (g/L)	-	0	0	0	0	-	-	0	0	0	-	0	0	0	-	0	0	-
Glucose (g/L)	-	0.1	0.1	0.2	0.1	-	-	0.1	0	0	-	0	0.1	0	-	0	0	-
Fructose (g/L)	-	1.1	1.1	1.1	1.2	-	-	1	1.1	1	-	0.9	1.2	1	-	1	1.1	-
Lactic Acid (g/L)	-	1.8	1.9	1.8	1.8	-	-	2.1	2.1	1.9	-	2	2	2.1	-	2	2	-