THE INTERACTION OF TWO SACCHAROMYCES CEREVISIAE STRAINS
(LALVIN® RC212 AND LALVIN® ICV-D254) DURING WINE FERMENTATION
AND ITS EFFECT ON FERMENTATION-DERIVED BY-PRODUCTS IN WINE

by

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Abstract

Little is known about the direct effect *Saccharomyces cerevisiae* strains have on operational wine production during fermentation. Previous research has shown two strains, Lalvin® RC212 (RC212) and Lalvin® ICV-D254 (D254), co-fermenting and occupying > 50% of the yeast population at the end of operational level fermentations. These findings suggest that D254 is competitive in tanks inoculated with RC212. The aims of this study were: (1) to follow the interactions between RC212 and D254 under in-lab and operational fermentations; and (2) to compare the fermentation-derived by-products between in-lab and operational fermentations. Five inoculation ratios of RC212 to D254 were analyzed for in-lab controlled fermentations: a) 1:0; b) 9:1; c) 3:1; d) 1:1; and e) 0:1. During the in-lab fermentations, the strain abundance was analyzed with two methods: 1) microsatellite analysis and 2) a novel strain tracking method using fluorescent quantum dot particles. Operational fermentations at Quails’ Gate Estate Winery, both inoculated and spontaneous, consisted of varying, but defined ratios obtained at the end of fermentation. Strain populations at the operational level were sampled and analyzed solely by microsatellite analysis. Wine samples were analyzed for the following fermentation-derived by-products: acetaldehyde, ethyl acetate, methanol, propanol, isobutanol, active amyl alcohol, isoamyl alcohol, ethyl butyrate, isoamyl acetate, hexanol, and 2-phenylethanol. Regression analysis was used to determine whether these compounds were associated with a specific yeast strain. During the in-lab study, both tracking methods revealed the absence of competition between the yeast
strains and populations remained at the inoculated ratio throughout the fermentation. In the operational fermentations, the strain RC212 dominated inoculated fermentations, whereas D254 dominated spontaneous fermentations. During the in-lab study, acetaldehyde, propanol, isobutanol, and isoamyl alcohol significantly increased in concentration as RC212 abundance increased. Meanwhile, ethyl acetate and active amyl alcohol significantly increased as D254 increased; however, in the operational samples, only isobutanol and isoamyl alcohol were shown to be strain specific. Additionally, hierarchical cluster analysis showed that the inoculation treatment of 1:1 most closely resembled the pure D254 treatment, suggesting that D254 has a greater effect on the chemical profile than does RC212 under in-lab conditions.
Preface

Chapter 2 is based on work conducted in Dr. Daniel Durall’s lab at The University of British Columbia Okanagan. I was responsible for conducting, sampling, and analyzing the controlled fermentations. The operational samples used in this study were collected from Quails’ Gate Estate Winery and the population dynamics were analyzed by Erin Faasse (BSc with Honours, UBCO) for an Honour’s project. I was responsible for analyzing the Quails’ Gate Estate Winery Samples for fermentation-derived compounds. With guidance from my supervisors, Dr. Daniel Durall and Dr. Vladimir Jiranek, I was responsible for developing the experimental design, collecting experimental data, and writing the thesis.

The quantum dot experiment presented in chapter 2 is a collaborative project with Dr. Matthew Whiteside. Dr. Whiteside and I developed the concept, the design, performed the experiments, and analyzed the data. Dr. Daniel Durall and Dr. Vladimir Jiranek supervised the collaboration. My supervisory committee; Dr. Joyce Boon, Dr. Daniel Durall, Dr. Vladimir Jiranek, and Dr. Susan Murch reviewed this thesis.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADY</td>
<td>Active dry yeast</td>
</tr>
<tr>
<td>AMH</td>
<td>Enoferm® Assmanshauser</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BIVB</td>
<td>Burgundy wine board</td>
</tr>
<tr>
<td>CCEW</td>
<td>Cedar Creek Estate Winery</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CV</td>
<td>Canonical variable</td>
</tr>
<tr>
<td>CVA</td>
<td>Canonical variate analysis</td>
</tr>
<tr>
<td>D254</td>
<td>Lalvin® ICV-D254</td>
</tr>
<tr>
<td>DMDC</td>
<td>Dimethyldicarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FADSS</td>
<td>Fragment Analysis and DNA Sequencing Service</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography flame ionization detector</td>
</tr>
<tr>
<td>GCL</td>
<td>Glutamate cysteine ligase</td>
</tr>
<tr>
<td>GGT</td>
<td>$\gamma$-glutamyltranspeptidase</td>
</tr>
<tr>
<td>GS</td>
<td>Glutathione synthetase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HSD</td>
<td>Honest Significant Difference</td>
</tr>
<tr>
<td>ICV</td>
<td>Institut Coopératif du Vin</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>L2TD</td>
<td>Level2 <em>Torulaspora delbrueckii</em></td>
</tr>
<tr>
<td>MCFA</td>
<td>Medium chained fatty acids</td>
</tr>
<tr>
<td>MLF</td>
<td>Malolactic fermentation</td>
</tr>
<tr>
<td>OAV</td>
<td>Odour activity value</td>
</tr>
<tr>
<td>OIV</td>
<td>International Organization of Vine and Wine</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dot</td>
</tr>
<tr>
<td>QD-GSH</td>
<td>Quantum dot conjugated to glutathione</td>
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<tr>
<td>QGEW</td>
<td>Quails' Gate Estate Winery</td>
</tr>
<tr>
<td>RC212</td>
<td>Lalvin® RC212</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>TLS</td>
<td>Tantalus Vineyards</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
</tr>
<tr>
<td>YEPD</td>
<td>Yeast extract peptone dextrose</td>
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</table>
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Chapter 1  Introduction

1.1  Microbes in a wine fermentation

The conversion of grape juice into wine is a complex biochemical process that is dependent on the kinetics of growth and metabolism of a dynamic combination of microorganisms. Yeasts are responsible for primary (alcoholic) fermentation and lactic acid bacteria are responsible for secondary, or malolactic fermentation (MLF); these two microbial groups are essential for proper vinification. The yeast *Saccharomyces* is used to ferment wine, brew beer, and leaven bread. *Saccharomyces cerevisiae* is the preferred yeast for winemaking because of its ability to ferment consistently at higher ethanol concentrations than other yeasts (Boulton 1996). The microbial ecology has a major influence on the quality of the wine produced because of the metabolic products and by-products of each microbe; this includes the microbial flora prior to grape harvest, and continuing through to the packaging of the final product (Romano et al. 2003, Vilanova et al. 2005, Fleet 2008). Therefore, the quality of the wine may be enhanced or spoiled by different yeast-yeast, yeast-bacteria, or bacteria-bacteria interactions (Nissen and Arneborg 2003, Fleet 2008).

Yeasts are diverse and the population dynamics within a wine fermentation, even if inoculated, can be complex (Hall et al. 2011, Barata 2012). The successional changes seen in yeast population dynamics are in part a response to an increase in ethanol concentration and a decrease in sugar availability. The population dynamics are also affected by factors such as the climate during harvest and vineyard treatments, as well as the age, size and geographic location of the vineyard (Barata 2012). A typical succession of microbes during vinification occurs as follows: initially the must is populated by non-*Saccharomyces* yeasts including *Rhodotorula, Pichia, Candida, Metschnikowia, Kluyveromyces, Torulopsis* and *Kloeckera*
(Hanseniaspora); minor numbers of *Saccharomyces* spp. and low levels of lactic acid bacteria including *Pediococcus*, *Leuconostoc* and *Lactobacillus* spp. (Romano et al. 2003, Howell et al. 2005, Zott et al. 2010). As vinification progresses, non-*Saccharomyces* and *Saccharomyces* yeasts are prevalent and the bacteria largely perish (Fleet et al. 1984). At this time, non-*Saccharomyces* yeasts can reach cell densities of $10^6$-$10^7$ cells/mL, which is comparable to the *S. cerevisiae* populations at later stages of fermentation (Fleet 2003, Ocón et al. 2010). During the mid and late stages of fermentation, the conditions change and *S. cerevisiae* predominates (Le Jeune et al. 2006). The succession of species and strains during fermentation is dependent on nutrient availability, inhibitors present, and cell density (Nissen and Arneborg 2003). Originally, it was thought that the increase in the alcohol concentration was the cause of the succession of species (Gao and Fleet 1988, Fleet 2003, Pina et al. 2004). However, some species that are rarely found in the later stages of fermentation, such as *Metschnikowia* spp. and *Candida* spp., can tolerate ethanol levels between 7% (v/v) and up to 12% (v/v), respectively (Fleet 2003). *Hanseniaspora* spp. have also been shown to tolerate high ethanol concentration, but are rarely found in the later stages of fermentation (Capece et al. 2011). Cooler fermentation temperatures and aerobic conditions can result in non-*Saccharomyces* species persisting into the mid and final fermentation stages (Domizio et al. 2007, Zott et al. 2008, Ciani et al. 2010). *Saccharomyces cerevisiae* has the advantage of flourishing in anaerobic environments, causing high alcohol production, having a tolerance for high ethanol contents, being heat tolerant, and showing an overall dominance over non-*Saccharomyces* yeasts. All of these factors contribute to the large population of *S. cerevisiae* at the end of fermentation (Goddard 2008). Thus, it is likely a combination of factors that cause the succession from non-*Saccharomyces* to *S. cerevisiae*. This characteristic
dominance of *S. cerevisiae* in the final stages of fermentation is common to all fermentations throughout the world (Heard and Fleet 1985, Parapouli et al. 2010).

There are two major sources of microbes in wine fermentations; the first is the vineyard and the second is the winery (Beltran et al. 2002). The winery itself is the source of many microbes found in fermentations and it takes several vintages for a winery to establish yeast diversity (Beltran et al. 2002). The yeasts that dominate in fermentation have been found to originate from the winery (Martini 1993, Beltran et al. 2002, Valero et al. 2005). These fermenting yeasts are both *Saccharomyces* and non-*Saccharomyces* species that are present on the equipment and in the air of the winery (Beltran et al. 2002). Two types of fermentation, spontaneous and inoculated, are used in today’s operational fermentations; they differ in the way the yeasts are introduced. For the last 7000 years, indigenous yeasts colonizing the must spontaneously have been responsible for the fermentation of grapes. In the last 60 years, the use of rehydrated *S. cerevisiae* active dry yeast (ADY) has been implemented for more reliable fermentations (Reed and Chen 1978, Pretorius et al. 1999). Inoculating musts with ADY is advantageous as it allows for more reproducible winemaking, avoidance of ‘stuck’ fermentations, and better control of fermentation speed (Heard and Fleet 1985, Clavijo et al. 2010, Capece et al. 2011).

Spontaneous fermentations can increase species and strain diversity that contributes to unique sensorial attributes (Pretorius et al. 1999, Ugliano and Henschke 2009). However, ‘stuck’ and incomplete fermentations are risks associated with relying on spontaneous fermentations (Heard and Fleet 1985, Francesca et al. 2010). Even with these risks, some winemakers use spontaneous fermentations in order to achieve a high diversity of indigenous yeast strains fermenting the must. The idea is that a diverse population of indigenous yeasts
will produce wines with a more complex aroma and taste (Vilanova et al. 2005, Callejon et al. 2010). In the Okanagan Valley, only commercial strains previously introduced into the winery have been dominant in spontaneous fermentations (Hall et al. 2011, Lange 2012, Faasse unpublished). Thus, the idea that spontaneous fermentations (Vilanova et al. 2005, Callejon et al. 2010) will enhance aroma and taste due to an increase in indigenous strains appears not to be the case in the Okanagan Valley. Nevertheless, the increase in diversity of commercial strains could contribute to unique sensorial attributes of the wine.

Many studies have been conducted on the diversity of yeast species present in spontaneous fermentations comparing white and red wine as well as comparing wines from different regions and different climates (Prakitchiwattana et al. 2004, Cordero-Bueso et al. 2011). The aim of these studies was to determine yeast successional dynamics within vineyards and during fermentation in various regions in order to determine the microbial impact on wine chemistry (Brezna et al. 2010). There are regional variations in the diversity of non-\textit{Saccharomyces} spp. and it likely contributes to regional characteristics of the wine (Brezna et al. 2010, Goddard et al. 2010). Even though non-\textit{Saccharomyces} spp. make limited contributions to the fermentation process, it has been shown that they can contribute to sensorially important metabolites. Thus, they may enhance or augment the impact that \textit{S. cerevisiae} has on sensorial attributes in wine (Ugliano and Henschke 2009, Ciani et al. 2010, Ocón et al. 2010). Thus, the presence of non-\textit{Saccharomyces} species is not necessarily detrimental to the wine, and may enhance positive characters such as fruity aromas in white wines (Parapouli et al. 2010). A recent Australian study detected several compounds that were only present in spontaneous fermentations, or were present in significantly different concentrations compared with inoculated fermentations (Varela et al. 2009). Differences in
spontaneous and inoculated wines have also been detected with regards to alcohol levels, total acid, pH, levels of organic acids and polysaccharides. However, the origins of the specific yeast flora that lead to these differences remain unclear.

Yeasts experience osmotic stress, oxidative stress, heat shock, high ethanol and sulfur dioxide concentrations, and exhaustion of sugar and assimilable nitrogen during vinification (Barrajón et al. 2011). These stresses are taken into account by winemakers during vinification and they can be applied favorably to modify the population dynamics. For example, sulfur dioxide is added to inhibit growth of hyphal fungi and bacteria, which may spoil the wine. This addition of sulfur dioxide also reduces the competition from non-\textit{Saccharomyces} species in the must giving \textit{S. cerevisiae} an opportunity to thrive and complete the fermentation. Temperatures are also controlled during the fermentation to provide the microbial population steady temperatures. Strains of \textit{S. cerevisiae} are specifically selected and used during winemaking to contribute to fermentation-derived attributes. Each strain produces differing amounts of by-products (Romano 2003, Ugliano and Henschke 2009, Styger et al. 2011). There is a lack of information on the sensorial consequences of interactions between \textit{S. cerevisiae} strains. One reason for this is that these studies require intense sampling and analysis in order to provide the replication necessary to achieve a statistically valid result (Blanco et al. 2008, Ugliano and Henschke 2009). It has been found that some fermentations by single culture inoculations lack the aroma and flavour complexity of a more diverse yeast population (Capece et al. 2011). Therefore, a common belief is that diverse populations of yeasts will lead to wines that are more complex. Yeast strains, as co-inocula, exhibit different growth patterns and they interact metabolically in antagonistic, neutral, or stimulatory fashions, which can be beneficial and improve the performance of the
yeasts (Fleet 2003). Apart from aspects of fermentation reliability and processing attributes, ADY strain choice is currently based on several factors: the ability of the yeast to enhance and stabilize colour, production of aromatic volatile esters and higher alcohols, and the production of polyalcohols responsible for structure (Suarez-Lepe and Morata 2012). No information is available from yeast manufacturers about the potential sensory benefits arising from the co-inoculation of multiple *S. cerevisiae* strains.

1.2 The alcoholic fermentation and yeast derived organoleptic compounds

In fermentation, glucose is the preferred energy source for *S. cerevisiae*; however, fructose and other monosaccharides can be converted into intermediates of glycolysis (Kaniak 2004). Through fermentation, glucose is metabolized into pyruvate, which is further metabolized into acetaldehyde and carbon dioxide (CO$_2$) at which point acetaldehyde is reduced to ethanol (Boulton 1996). Fermentation by mainly *S. cerevisiae*, producing ethanol from glucose and fructose, is the primary fermentation of vinification. Typically, grape juice contains 180-220 g/l of sugar prior to fermentation and primary fermentation is considered complete when less than 2 g/l residual sugars remain (Ribereau-Gayon et al. 2006). During primary fermentation, yeasts convert approximately 18 g/l of sugar to 1 % (w/v) ethanol (Ribereau-Gayon et al. 2006). The fermentation pathway is closely related to other biochemical pathways of sugar, lipid, nitrogen, and sulfur metabolism. These pathways produce higher alcohols, polyalcohols, fatty acids, organic acids, and esters that have an impact on the bouquet of the wine (Ribereau-Gayon et al. 2006, Ugiano and Henschke 2009). The production of these volatile and non-volatile compounds is yeast strain specific.
and different strains will contribute different amounts of these by-products as they ferment sugar.

The term bouquet is used to describe the aroma that arises from the chemical reactions of fermentation (Robinson 2006). Thus, the bouquet is formed through an interaction between yeasts and grape compounds. *Saccharomyces cerevisiae* contribute to the bouquet of wine in several ways. The main action is due to enzyme activity, the release of yeast cell components, modification of grape derived non-volatile compounds, and the formation of secondary metabolites (Murat et al. 2001, Starkenmann et al. 2008, Parapouli et al. 2010). Compounds such as esters, acids, and higher alcohols are flavour active and are produced or enhanced by the action of *S. cerevisiae*. The glycolytic pathway is coupled to the reductive formation of alcohol; these pathways produce or are coupled to other metabolic pathways that produce adenosine triphosphate (ATP), precursor molecules, and reducing equivalents for cell growth and maintenance (Ugliano and Henschke 2009). The glycolytic pathway is also associated with pathways that produce both volatile and non-volatile metabolites that impact both the flavour and aroma of the fermentation bouquet (Ugliano and Henschke 2009). Through anabolic and catabolic pathways, *S. cerevisiae* metabolize sugars, amino acids, ammonium, peptides, and sulfate to produce esters, higher alcohols, acids, carbonyls, polysaccharides, and volatile sulfur compounds (Reynolds et al. 2009, Ugliano and Henschke 2009). The formation of these metabolites is dependent on the yeast strains, the nutrients available, and the physicochemical properties of the fermentation (Patel and Shibamato 2002, Swiegers et al. 2005, Ugliano and Henschke 2009). Higher alcohols and esters are yeast derived and greatly contribute to the organoleptic characteristics of wine (Romano et al. 2003, Ugliano and Henschke 2009, Styger et al. 2011).
The formation of higher alcohols is attributed to the Ehrlich pathway, where amino acids are transaminated, decarboxylated and reduced to produce the higher alcohols (Hazelwood et al. 2008). Higher alcohols are also known as fusel alcohols and contribute a desired complexity and increased varietal aromas at levels less than 300 mg/l but at higher concentrations, their contribution is considered undesirable (Ugliano and Henschke 2009, Suarez-Lepe and Morata 2012). These are alcohols that contain two or more carbons and include branched chain alcohols. The production and composition of higher alcohols is dependent on yeast strains, sugar, temperature, pH, and aeration (Ugliano and Henschke 2009). It has been shown that single yeast strains compared to co-inoculations of yeast strains will produce different higher alcohol profiles (Longo et al. 1992, Barrajón et al. 2011). The abundance of compounds in wine contributes to the complex flavour of wines. However, the compounds must reach the aroma threshold and have a high odour activity value (OAV) in order to contribute directly to the aroma of a wine (Grosch 2001).

Esters are produced in one of two ways, either via an esterification reaction mediated by an esterase or via a condensation reaction of higher alcohols with acyl CoA (Lilly et al. 2000). Ethyl esters contribute to fruity aroma characteristics of the fermentation bouquet. There are many esters formed and they create an organoleptic synergy. There is potential for many esters to form in wine because of the different acids and alcohols present (Sumby et al. 2009). It is therefore unreasonable to suggest that one fruity characteristic is the result of the concentration of one ester. Esters are formed through the condensation of higher alcohols with acetyl CoA (Ugliano and Henschke 2009). Strain specific expression of structural genes for esterases is also involved in ester formation (Sumby et al. 2009). Different yeast strains will produce varying types and amounts of esters. The formation of esters is favored by some
strains and by fermentations at lower temperatures. There are four different hypotheses why esters are produced by yeasts (Saerens et al. 2010). The first is to regenerate free acyl CoA without releasing acetic acid. The second is that esters act as unsaturated fatty acid analogues; anaerobic fermentation conditions do not allow unsaturated fatty acids, the major components of cellular membranes, to be made. Therefore, esters may be incorporated into the membranes to maintain the fluidity of the membranes. The third is for detoxification, anaerobic conditions produce saturated fatty acids, and this can cause an arrest in fatty acid synthesis, which can cause a release of medium chained fatty acids (MCFA). These fatty acids are toxic to yeast cells because they increase membrane permeability to protons and can thereby alter the pH of the cell. Esterification converts toxic MCFA to less toxic esters that can diffuse out of the cell. The fourth hypothesis as to why S. cerevisiae produce esters is for the natural dissipation of yeast. Esters are fruity smelling and if yeasts produce esters, it is a lure for Drosophila, which can help disperse yeast back to fruit. Therefore, ester-producing yeasts may prevail.

1.3 Common commercial Saccharomyces cerevisiae strains used in the Okanagan Valley, British Columbia

Commercial S. cerevisiae strains originate from indigenous strains of major wine producing areas that have been isolated, analyzed, and are now mass-produced by yeast manufacturers (Lambrechts and Pretorius 2000, Schuller and Casal 2007). Stuck fermentations, which can spoil the final wine product, are more prevalent in spontaneous fermentations than those inoculated with commercial starter strains (Valero et al. 2005, Bisson and Karpel 2010). Spontaneous fermentation is still heavily practiced in ‘Old World’
wine-producing regions contributing to the ‘terroir’. Yeasts, naturally found at the vineyard and winery, are adapted to the environment and promote the development of fermentation-derived compounds complementary to the grapes of the region (Pretorius 2000, Vilanova et al. 2005, Fleet 2008, Barrajón et al. 2009, Tello et al. 2011). However, studies have shown that commercial strains compete with indigenous strains in spontaneous fermentations (Constanti et al. 1997, Beltran et al. 2002, Hall et al. 2011). Commercial strains are sourced based on their fermentation kinetics, nutritional requirements, temperature tolerance, and by-product production. These attributes create competitive strains in a fermentation environment and may reduce strain diversity (Ganga and Martinez 2004, Vilanova et al. 2005, Fleet 2008, Barrajón et al. 2009). Since fermentation-derived by-products are strain specific, using the same commercial strains throughout a region creates continuity in the final wine product but it may reduce a ‘terroir’ character from yeast (Raineria and Pretorius 2000).

In the Okanagan Valley of British Columbia, Canada, the commercial ADY strains Lalvin® Bourgrouge RC212 (RC212) and Lalvin® ICV-D254/Fermol® Premier cru (D254) are commonly used to ferment Pinot Noir and Chardonnay musts, and have been found to dominate fermentations at several wineries (Hall et al. 2011, Lange 2012, Faasse unpublished). Notably, D254 and Fermol Premier cru share the same microsatellite fingerprint and therefore appear to be genetically indistinguishable (Hall et al. 2011). Previously conducted studies have shown that inoculated commercial S. cerevisiae starter strains were not necessarily the dominant or finishing strain (Hall et al. 2011, Lange 2012). In addition, D254 was consistently detected in significant and dominating proportions if the winery had used this strain in previous fermentations at the winery, even in tanks inoculated with RC212. Originally, RC212 was selected by the Burgundy Wine Board (BIVB) to extract
and protect the polyphenols of Pinot Noir. This strain is reported to have limited colour loss, and it consistently produces wine with stable colour (Carew, A. unpublished abstract, Crush 2011 Conference, Adelaide, Australia). It requires large amounts of nutrients to prevent the development of sulfides. In the information supplied by the manufacturer, it is claimed that wines fermented by RC212 have good structure with fruity and spicy characteristics (Lallemand Inc.). The strain D254 was selected from Syrah fermentations in the Rhône Valley in 1998 by the Coopératif du Vin (ICV) Institute. This strain is commonly used in both red and white wines. The information from the manufacturer states that red wines fermented with D254 contribute to high fore-mouth volume, smooth tannins, intense fruits and a slightly spicy finish (Lallemand). In white wines, D254 contributes nutty aromas and a creamy mouthfeel. The strain does not have high nutrient requirements and has a short lag phase in population growth and fermentation kinetics. These two strains have been compared in independent inoculations of cherry musts by Sun et al. (2011) and it was determined that D254 has faster fermentation kinetics and that both strains significantly decreased total acidity, which is important for cherry wine. It produced higher levels of higher alcohols and esters than RC212 (Sun et al. 2011). To my knowledge, these two strains, commonly used and found responsible for co-fermenting Pinot Noir in the Okanagan Valley, have not been studied when they are co-fermenting within in-lab and/or operational settings.

1.3.1 Identifying yeast strains in a fermentation

In an industrial setting, several *S. cerevisiae* strains are typically found co-fermenting wines even where a selected strain has been inoculated. Thus, the inoculated strain does not always dominate the fermentation. Studying mixtures of *S. cerevisiae* strains in fermentation
is difficult. All strains of *S. cerevisiae* are morphologically similar, thus, molecular techniques have been the method of choice when distinguishing different *S. cerevisiae* strains. Molecular techniques developed to distinguish between different strains of *S. cerevisiae* include pulsed field electrophoresis (Grando et al. 1994), mitochondrial DNA restriction (Aigle et al. 1984), and microsatellite finger printing (Legras et al. 2005). Although these methods, particularly the use of microsatellites, can be very accurate, there are drawbacks. For example, sample preparation is time consuming, samples must be plated, incubated, and DNA extracted from colonies prior to the amplification of DNA target regions. Current methods of identifying yeast strains in fermentation are limited to a small sample size due to the time, effort, and resources required to conduct the analysis. Generally, 16-24 colonies are identified per sample. With published sample sizes ranging from 8 – 45 colonies and the time and cost of molecular techniques, the number of colonies that can practically be identified is limited (Howell et al. 2006, Hall et al. 2011). This number is quite minimal given that yeast culture densities reach ~2 x 10^8 cells/ml at the peak of fermentation. Attempts to use stains to distinguish different strains have failed for several reasons; standard fluorescent stains do not remain fluorescent throughout fermentation, distinguishing stain fluorescence from yeast-derived autofluorescence is often problematic, and commercially available stains may alter membrane integrity, which can affect the diffusion of molecules in and out of the cell. In addition, commercially available stains are not effectively inherited by daughter cells in *S. cerevisiae* because budding is asymmetric.

Stains such as FUN-1 and propidium iodide (PI) are commonly used to detect viability in *S. cerevisiae* and act by entering the cell through passive diffusion. Because these stains are limited to viability staining, they are added to a sample after taking the sample
from the fermentation flask and prior to analysis. They cannot be added to a fermentation and
used to track strain populations in vivo. This is because current commercially available stains
and dyes require either cell wall or membrane attachment, which can compromise the
membrane integrity for attachment and entry into the cell. In addition, green fluorescent
proteins cannot be used because they interfere with the regular metabolism of the cell.
Tracking the populations during a metabolic activity is the key to strain-interaction studies;
interfering with the genome, transcription, and with the expression of proteins is not ideal. In
addition, commercially available stains are not effectively inherited by daughter cells in S.
cerevisiae because budding is asymmetric and most parts of the bud are created de novo
(Farkas et al. 1974). Tagging the cell wall is not an option because the polysaccharides of the
cell wall are released into the wine as cell wall reorganization occurs (Llauberes and
Dubourdieu 1987). CellTracker™ Probes for long term tracing of living cells is a probe that
uses an innovative approach to tracing cell populations through several generations
(Molecular Probes, Invitrogen). In order to effectively retain the probe, cells must be
subjected to temperature fluctuations, dimethyl sulfoxide (DMSO), and permeabilization.
Subsequently, the cells are subjected to several stresses during the uptake of the fluorophore.
Once the probe is retained within the cell, the probe is conjugated to glutathione, a ubiquitous
molecule. Additionally, the fluorescence needs to be activated by esterases, and once
activated is only fluorescent up to 72 hours. Ideally, in tracking populations, membrane
integrity should not be compromised at any time because this may interfere with regular cell
growth. In essence, there are three main issues with current cell tracking probes; firstly, the
cells might respond negatively to the probe; secondly, the fluorescent probe may be detected
only during part of the fermentation; and thirdly, it may not be passed on to the budding daughter cell.

1.4 Quantum dots

Quantum dots are semiconducting nanoparticles typically with a cadmium-selenide core and a zinc-sulphide shell coated in an organic polymer such as hydroxyl groups or amide groups. These nanoparticles have impressively narrow emission, are resistant to photo bleaching, and have strong long-lasting fluorescence (Zhou and Gosh 2006). The use of quantum dots as a fluorescence particle has a potential to overcome the short luminescent lifetimes of currently available tracking probes and thereby allowing different strains of cells to be distinguished throughout fermentation, which is generally greater than 100 hours. Because quantum dots have amino or hydroxyl terminals, they are easily conjugated to other organic molecules. A coupling agent such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is commonly used to activate carboxyl groups of organic molecules in preparation for coupling to quantum dots (Zhou and Gosh 2006). With the right ligand, quantum dots can be used as novel cell tracking probes.

1.5 Significance of glutathione

Glutathione (GSH), γ-L-glutamyl-L-cysteinylglycine, is a tripeptide with a low redox potential ($E'_0 = -240$ mV) (Penninckx 2002, Ganguli et al. 2007). It is the most widely found sulfur-containing compound in living organisms and is present at concentrations between 1-10 mM in mammalian cells (Lu 2013). It is important in many biological functions such as antioxidant defense, xenobiotic detoxification, cell cycle regulation, and redox signaling (Lu
Glutathione is synthesized by glutamate cysteine ligase (GCL) and GSH synthetase (GS) (Lu 2013). The only enzyme that can hydrolyze the gamma bond between the glutamate and the cysteine of GSH is γ-glutamyltranspeptidase (GGT), which is expressed extracellularly on certain cell types in *S. cerevisiae* and is located in the vacuole (Meister and Anderson 1983, Mehdi et al. 2001, Bauduoin-Cornu et al. 2012). *Saccharomyces cerevisiae* have been shown to grow on media containing GSH as the sole source of sulfur, indicating that *S. cerevisiae* has transport mechanisms for GSH uptake (Miyake et al. 1998). Bourbouloux et al. (2000) showed that radiolabelled GSH was actively transported across the plasma membrane through Hgt1p transporters of *S. cerevisiae*. Eukaryotes store 80-85% of cellular GSH in the cytosol, 10-15% in the mitochondria, and a small percentage in the endoplasmic reticulum (Penninckx 2002). The thiol moiety provides reductive potential that can defend against oxidative stress (Lu 2013). Glutathione is an effective antioxidant in its reduced form, which is maintained by NADPH-dependent glutathione reductase (Penninckx 2002).

### 1.6 Background of the study

Previous research on *S. cerevisiae* strain diversity at wineries in the Okanagan Valley revealed the prevalence of strains RC212 and D254 co-fermenting Pinot Noir musts (Lange 2012). Because these two yeast strains, RC212 and D254, dominated Pinot Noir fermentations in Okanagan wineries, their strain interaction and contributions of fermentation-derived by-products to wine are of interest. The significance of strain diversity and competition in an industrial setting is evident because each strain provides its unique sensorial attributes. For example, some strains may be more suited to ferment a fruity white
wine than a robust red and *vice versa*. In order to control wine quality, the yeasts best suited to the wine being fermented should dominate the alcoholic fermentation. In addition, commercial strains alone were found to carry out fermentation in both inoculated and spontaneous fermentations, which suggests that fermentation-derived by-products in the Okanagan come only from commercial strains. It is therefore of interest to define the strain competition or interaction between these two prevalent strains as well as to study their fermentation-derived organoleptic by-products.

### 1.6.1 Objectives and hypotheses

The first objective of the study was to follow the interactions between RC212 and D254 in both in-lab and operational fermentations.

- **Hypothesis 1:** The *S. cerevisiae* strain D254 is dominant over RC212 even when it is inoculated with substantially fewer cells than RC212.
- **Hypothesis 2:** RC212 and D254 is prevalent in both the inoculated and spontaneous QGEW 2012 Pinot Noir samples.
- **Hypothesis 3:** The QD-GSH complexes is taken into the cytosol of the yeast strains and passed to daughter cells during budding.
- **Hypothesis 4:** The QD-GSH remains in the yeast cells and fluoresce throughout the fermentation.

In fermentations where RC212 is the inoculant, D254 has been shown to co-ferment Pinot Noir tanks without being added as a starter (Lange 2012, Faasse unpublished). Therefore, it is predicted that D254 will be more competitive than RC212 even when inoculated in a lower concentration than RC212 in the in-lab fermentations. Likewise, under
operational conditions, it is predicted that both RC212 and D254 will be present in both inoculated and spontaneous Pinot Noir fermentations at QGEW’s 2012 vintage.

Based on a study where radiolabeled GSH entered *S. cerevisiae* cells through Hgt1p transporters, it is predicted that QD-GSH complexes will enter the cell and localize within the cytoplasm (Bourbouloux et al. 2000). Because QDs are bright fluorescence particles, it is predicted that they will remain fluorescent throughout fermentation.

The second objective was to compare strain specific fermentation-derived by-products of RC212 and D254 between in-lab and operational fermentations.

- Hypothesis 5: The concentrations of fermentation-derived byproducts in all five inoculation treatments reflect the abundance of RC212 and D254.
- Hypothesis 6: D254 produces greater concentrations of higher alcohols and esters than RC212.
- Hypothesis 7: The RC212 and D254 populations in the QGEW samples reflect the concentrations of fermentation-derived by-products as determined by the in-lab experiments.

I predict that there will be strain specific differences in fermentation by-products between RC212 and D254. Several studies have concluded that different strains of *S. cerevisiae* produce strain specific metabolites (Pretorius et al. 1999, Romano 2003, Styger et al. 2011). Based on these studies, higher alcohols and esters will differ with varying dominance of the two strains (Hernandez et al. 2003, Morata et al. 2003, Romano 2003, Styger et al. 2011). Based on a study by Sun et al. (2011), where RC212 and D254 were compared in cherry wine fermentations, it is predicted that D254 will produce greater concentrations of all higher alcohols and esters than RC212. It is predicted that
dominance of RC212 or D254 in the operational fermentations will reveal similar concentrations of fermentation-derived by-products to the comparable inoculation ratios of the in-lab experiment.
Chapter 2  The interaction of two *Saccharomyces cerevisiae* strains

(Lalvin® RC212 and Lalvin® ICV-D254) and its effect on
fermentation-derived by-products

2.1  Synopsis

Previous studies investigating the population dynamics of yeast in wine fermentations have reported the dominance of two commercial *Saccharomyces cerevisiae* strains, Lalvin® RC212 (RC212) and Lalvin® ICV-D254 (D254). These two strains have been found to co-ferment in multiple tanks at numerous wineries over three years (Hall et al. 2011; Lange 2012). Interactions of *S. cerevisiae* strains can influence the production of fermentation-derived organoleptic compounds, which in turn, may affect the sensory attributes of the wine. Knowledge of the yeast strains fermenting wine must and their effect on the sensory attributes of wine is helpful to winemakers when they make decisions on choosing commercial yeasts. The aim of this study was two-fold: 1) to define the interaction of two strains under different inoculation ratios in controlled in-lab fermentations and in operational fermentations; and 2) to determine the impact that RC212:D254 ratios have on fermentation-derived compounds in both controlled and operational fermentations. The controlled fermentations were conducted in the lab at The University of British Columbia (UBC) Okanagan, whereas the operational fermentations were conducted at Quails’ Gate Estate Winery (QGEW).
2.2 Materials and Methods

2.2.1 Description of *Saccharomyces cerevisiae* strains

The *S. cerevisiae* strains used in this study were Lalvin® RC212 (RC212) and Lalvin® ICV-D254 (D254), which were chosen based on previous studies (Hall et al. 2011; Lange 2012). Lallemand (Montreal, Canada) provided us with both RC212 and D254. The strain D254 and another commercial strain Fermol® Premier Cru were indistinguishable, because they shared the same fingerprint based on microsatellite analysis (Hall et al. 2011). They were consistently found as dominant strains co-fermenting Pinot Noir must in several wineries in the Okanagan Valley over the course of three years in both inoculated and spontaneous fermentations (Table 2.1).
Table 2.1 Relative frequency of occurrence of Lalvin® RC212 (RC212) and Lalvin® ICV-D254 at Quails' Gate Estate Winery (QGEW), Cedar Creek Estate Winery (CCEW) and Tantalus Vineyards (TLS) at Early, Mid, and End stages of fermentation and combined frequency of occurrence for each treatment (n=3).

<table>
<thead>
<tr>
<th>Winery</th>
<th>Year</th>
<th>Fermentation Type</th>
<th>Early</th>
<th>Mid</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RC212</td>
<td>D254</td>
<td>RC212</td>
</tr>
<tr>
<td>QGEW</td>
<td>2010</td>
<td>Inoculated</td>
<td>0.50</td>
<td>0.27</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spontaneous*</td>
<td>0.31</td>
<td>0.38</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>Inoculated</td>
<td>0.71</td>
<td>0.21</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spontaneous</td>
<td>0.04</td>
<td>0.27</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Inoculated</td>
<td>0.85</td>
<td>0.00</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spontaneous</td>
<td>0.13</td>
<td>0.27</td>
<td>0.00</td>
</tr>
<tr>
<td>CCEW</td>
<td>2010</td>
<td>Inoculated L2TD*</td>
<td>0.13</td>
<td>0.13</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inoculated AMH*</td>
<td>0.44</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inoculated RC212*</td>
<td>0.56</td>
<td>0.19</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spontaneous*</td>
<td>0.00</td>
<td>0.81</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Inoculated RC212#</td>
<td>0.71</td>
<td>0.04</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inoculated RB2</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spontaneous</td>
<td>0.00</td>
<td>0.14</td>
<td>0.06</td>
</tr>
<tr>
<td>TLS</td>
<td>2012</td>
<td>Inoculated RC212*</td>
<td>0.63</td>
<td>0.00</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inoculated D254*</td>
<td>0.50</td>
<td>0.04</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spontaneous ^</td>
<td>0.17</td>
<td>0.02</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* n=1  
# n=2  
^ n=4
2.2.2 Overview of experiments

This study consisted of three experiments: 1) an in-lab interaction experiment using microsatellite analysis; 2) an in-lab interaction experiment using quantum dot (QD) analysis; and 3) an operational-level experiment conducted at Quails’ Gate Estate Winery (QGEW). For both the first and third experiments, population dynamics of yeast were monitored throughout the fermentation and fermentation-derived by-products were measured at the end of fermentation. The second experiment was conducted solely to analyze population dynamics of yeast.

2.2.3 Experimental design, fermentation parameters and sampling of in-lab experiments at UBCO.

Fermentations consisted of 100 ml sterile Pinot Noir juice with an °Brix reading of 22. The °Brix is estimated according to the refractive index (RI) indicates the approximate sugar concentration. Fermentation flasks with a sampling port and a gas lock were inoculated with either RC212, D254, or with a mixture of both strains. The treatments (n=3) consisted of different inoculation ratios of RC212: D254 (1:0; 9:1; 3:1; 1:1; and 0:1). Pinot Noir juice was obtained from a Selection Series Wine Kit (WM3245). The juice was prepared by centrifugation for 45 minutes at 5000 rpm. Subsequently, the juice was sequentially filtered through 2.7 μm/1 μm (Whatman filter Thermo Fisher Scientific, Ottawa, Canada), 0.45 μm mixed cellulose ester filter and a 0.22 μm polyvinylidene fluoride (PVDF) filter, respectively (Thermo Fisher Scientific, Ottawa, Canada). The °Brix of filtered juice was determined and adjusted to 22 with sterile Milli-Q water and stored at -20 °C. Sterility of the filtered juice
was confirmed by plating onto yeast extract peptone dextrose (YEPD) media and observing an absence of colonies.

Inoculum was prepared using the following protocol. Each strain was individually rehydrated aerobically in 25 ml of liquid YEPD and shaken overnight (120 rpm) at 28 °C. The cultures were counted using a hemocytometer to determine the cells/ml of the YEPD culture. A 100 ml “starter” inoculum, composed of 50% sterile Pinot Noir grape juice and 50% sterile Milli-Q water, was inoculated at a rate of $1 \times 10^6$ cells/ml for each strain and allowed to grow aerobically shaking at 120 rpm overnight at 28 °C. The starter cultures were counted for cells/ml using a hemocytometer. Replicates from each ratio treatment were inoculated in bulk. The master mixes consisted of 1) 1.2 l of sterile Pinot Noir juice inoculated at a rate of $5 \times 10^6$ cells/ml with RC212 and 2) 700 ml of sterile Pinot Noir juice inoculated at a rate of $5 \times 10^6$ cells/ml with D254. Prior to pouring 100 ml of each bulk inoculum into 250 ml fermentation flasks, the bulk inocula were prepared according to Table 2.2.

<table>
<thead>
<tr>
<th>Inoculation ratio</th>
<th>RC212 Master mix (ml)</th>
<th>D254 Master mix (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>9:1</td>
<td>360</td>
<td>40</td>
</tr>
<tr>
<td>3:1</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>1:1</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>0:1</td>
<td>0</td>
<td>400</td>
</tr>
</tbody>
</table>

Fermentations were conducted in 250 ml fermentation flasks. The flasks were shaken (120 rpm) at 28 °C until the end of the fermentations. To monitor the fermentation progress,
aliquots of 0.5 ml were sampled at the start, early, mid and end stages of fermentation through sampling ports with a 12 cm stainless steel needle and hypodermic syringe. The sampling ports were sanitized with 90% ethanol prior to piercing the septa. Early, mid, and end stages were defined by a °Brix of 17 (~21 hrs.), 13 (~41 hrs.), and < 8 (~ 120 hrs.) respectively. At the end of fermentation, all wines contained < 2 g/l residual sugar as indicated with a D-Glucose/D-Fructose sugar assay kit (Megazyme). At the end of fermentation, the flasks were removed from the incubator and the wine was centrifuged at 3000 rpm for 2 minutes prior to filtration through a PVDF 0.45 um syringe filter (Millipore, Billerica, MA, USA). The samples were transferred to glass vials and stored at -80 °C until the chemical analysis was performed.

During the fermentations, total cell counts were determined with a hemocytometer to monitor population density. To monitor fermentation progress, a handheld refractometer (Thermo Fisher Scientific, Ottawa, Canada) was used to measure the °Brix, which indicates the sugar content of the juice (Petering et al. 1991). The end of fermentation was confirmed with Benedict’s solution (Thermo Fisher Scientific, Ottawa, Canada), a residual sugar indicator, and a D-Fructose and D-Glucose enzyme assay kit (Megazyme, Ireland) using the manufacturer’s protocols. Standard curves were constructed for both the Benedict’s solution and the D-Glucose/D-Fructose enzyme assay kit using known concentrations of D-Glucose and D-Fructose.

2.2.4 In-lab quantum dot conjugation and inoculation

A red QD emitting at 631 nm was fed to RC212, and a blue QD emitting at 495 nm was fed to D254. An additional treatment of strains in a 1:1 ratio without any QDs was run
simultaneously as a negative control for autofluorescence. To allow for yeast to take up quantum dots, QDs were first conjugated to glutathione (GSH), a naturally occurring antioxidant in wine, which is also a yeast nutrient. Quantum dots (Crystalplex, Pittsburgh, USA) were bound to GSH with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in 10 mM borate buffer at pH 6.8. The conjugations, one for each type of QD, were performed in 2 ml glass vials with 3.17 nmol of QD in 1 ml of 10 mM borate buffer at 6.8 pH, EDC was added in excess at 400 mg to 104.52 nmol of GSH (1 QD: 33 GSH). The vials were vortexed for 20 minutes and then dialyzed against 1 l of sterile water for 30 minutes. The QDs were transferred to scintillation vials and stored at 4 °C after the addition of 1 ml of 10 mM borate buffer at pH 6.8. Figure 2.1 shows how the QD-GSH complex is proposed to look after conjugation.
Figure 2.1 Likely Quantum Dot-Glutathione complex post conjugation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) shown with 8 glutathione molecules to each Quantum Dot (QD). There are 33 sites for possible GSH conjugation. Note either carboxyl group of reduced glutathione may be activated by EDC and therefore either carboxyl group may conjugate to the QDs.

For treatments with QDs, 3 ml 1.05 mM of red QD-GSH was added to the RC212 starter fermentation and 3 ml 1.05 mM of blue QD-GSH was added to the D254 starter fermentation and was allowed to incubate overnight shaking at 120 rpm at 28 °C prior to co-inoculation. Ratio treatments were prepared identically to what is described in section 2.2.3.
2.2.5 Strain quantification using Quantum Dot (QD) analysis

Samples were taken at Early (20 hours), Mid (45 hours), and End of fermentation (120 hours). They were loaded onto cover slides to visualize the populations with an Olympus FluoView™ FV1000 confocal microscope (Markham, Ontario, Canada). Spectral scans and bright light images were taken for each cover slide. Images were processed with Normal processing type and Background correction on. Thresholds for the three channels were adjusted to reduce overlap from auto-fluorescence. Sobel 5 x average filter was applied to each image and the fluorescent scan superimposed on the bright light image to confirm fluorescence was from yeast. The images were imported to FijiImageJ, converted to binary images, and automatically counted (Schindelin et al. 2012).

2.2.6 Experimental design, fermentation parameters, and sampling of the operational experiment at QGEW.

During the 2012 harvest, Pinot Noir grapes were picked, crushed, and treated with 40 ppm of SO$_2$ in the winery and placed into six separate 5300 L fermentation tanks. Grapes from blocks that were planted in 2005 were placed in fermentation tanks T1, T2, and T3 (inoculated tanks, n = 3). Grapes from blocks planted in 1990 were placed in fermentation tanks T4, T5, and T6 (spontaneous tanks, n = 3). The three tanks T1, T2, and T3 were inoculated with the commercial S. cerevisiae ADY strain RC212 at approximately 20 g/hl. The ADY was rehydrated in 40 °C water, stirred, and an equal amount of Pinot Noir must was gradually added over 30 minutes to reduce osmotic stress. Once rehydrated, the inoculant was added during pump-over.

Samples were taken from all six tanks at four stages; cold-soak, early, mid and end of
fermentation. Cold-soak was sampled after SO₂ addition at 8-15 °C and 22-24° Brix. Early, mid and end fermentation samples were taken at 20-16°, 15-10° and <5° Brix, respectively. Samples were collected using a sanitized stainless steel collection apparatus at a depth of approximately 0.7 m. The collection apparatus was sanitized with 90% ethanol in between tanks. Samples were placed in sterilized, sealed plastic or glass bottles and transported to the laboratory at UBC Okanagan for further processing. Samples for fermentation-derived by-product analysis were collected only at the end of fermentation.

2.2.7 Strain quantification using microsatellite analysis

The first in-lab experiment, which used microsatellite analysis, was sampled at the Start, Early, Mid and End of fermentation. The samples were plated in duplicate on YEPD agar and incubated at 28 °C for 48 hours. The operational samples from QGEW were collected at cold soak, early, mid, and end stages of fermentation and then plated onto YEPD as describe for the first in-lab experiment. Twenty colonies from each in-lab sample (total = 1200 colonies) were randomly chosen for extraction and amplification. For each of the operational samples, sixteen colonies (total = 384) were randomly chosen for extraction and amplification.

DNA was extracted using a SIGMA Extract-N-Amp™ DNA Extraction and Dilution kit, (Sigma-Aldrich, Oakville, Canada) using the manufacturer’s protocol with an Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler (Foster City, USA). Once the extraction was complete, the plate was vortexed and centrifuged for 30 seconds at 3000 rpm. The plate was either stored at -20 °C or amplified at this point.
Amplification of the 1200 in-lab isolates was performed with primer sets amplifying the loci C11 and SCYOR267c (Legras et al. 2005). These two loci were chosen because RC212 is heterozygous and D254 is homozygous at both of these loci (Hall et al. 2011). Additionally, the two loci are separated by 78 base pairs, which allowed for simultaneous analysis and resulted in two fragments for RC212 and one fragment for D254. The primers used to define these loci, C11 and SCYOR267c, were modified to include the M-13 tack-on sequence TCC CAG TCA CGA CGT at the 5’ end of the forward primer (Integrated DNA Technologies Inc., Coralville, USA). The M-13 tack-on sequences were tagged with the fluorogenic compounds PET and VIC prior to amplification (Applied Biosystems, Foster City, USA). The final volume for these single loci microsatellite Polymerase Chain Reactions (PCR) was 12.5 μl. It included the following reagents: 2.5 μl of 5X colorless GoTaq® reaction buffer (Promega®, USA), 1.00 μl of dNTP mix (10 mM), 0.20 μl of the appropriate 5’-labeled M-13 tack-on sequence (10 μM), 0.5 μl of the forward primer (1 μM), 0.5 μl of the reverse primer (10 μM), 0.20 μl of each dye (VIC or PET), 0.40 μl of 1% BSA (10 mg/ml), 1.25 μl of MgCl2 (25 mM), 0.10 μl of GoTaq® DNA Polymerase (Promega®, USA), 1.00 μl of genomic yeast DNA, and 5.55 μl of sterilized Milli-Q water to make up the volume to 12.5 μl. A quarter of the colonies were amplified with the primers (tagged with the dye PET) for the loci C11 (Legras et al. 2005). Another quarter were amplified with the primers for the loci C11 (tagged with the dye VIC). Another quarter were amplified with the primers (tagged with the dye Pet) for the loci SCYOR267c (Legras 2005). The final quarter of the colonies were amplified with the primers (tagged with the dye VIC) for the loci SCYOR267c. After amplification, one colony from each sample and each amplification type were multiloaded. An Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler (Foster
City, USA) with the following program: 94 °C for 3 min (1 cycle), 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 min (35 cycles) and 72 °C for 10 min (1 cycle) was used to amplify the samples.

Amplification of the 384 operational isolates was performed with a multiplex of 6 different primer sets that define a microsatellite fingerprint. The six microsatellite loci chosen were: C4, C8, C3, SCYOR267c, YPL009c (Legras et al. 2005) and YML091C (Perez et al. 2001). The forward primer of these primer sets was labeled by Applied Biosystems (Foster City, USA) to include the fluorogenic compounds NED (C4 and C8), PET (SCYOR267c), VIC (YPL009c) or 6-FAM (C3 and YML091C). PCR reactions were run with the following recipe: 1.60 μl sterile DNA grade H₂O, 3 μl GoTaq 5X buffer, 0.75 μl dNTPs (10 mM each), 1 μl BSA (10 mg/ml), 1.4 μl MgCl₂ (25 mM), 0.75 μl C4 forward and reverse primers each (10 mM), 0.4 μl C8 forward and reverse primers each (10 mM), 0.15 μl C3 forward and reverse primers each (10 mM), 0.6 μl YML091C forward and reverse primers each (10 mM), 0.4 μl YPL009c forward and reverse primers each (10 mM), 0.2 μl SCYOR267c forward and reverse primers each (10 mM), 0.25 μl GoTaq DNA polymerase (5 U/ μl) and 2 μl of genomic yeast DNA for a total reaction volume of 15 μl. Amplification was performed on an Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler (Foster City, USA) with the following program: 94 °C for 3 min (1 cycle), 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 min (35 cycles) and 72 °C for 10 min (1 cycle).

All amplifications were confirmed by visualization of successful fragments on a 1% (w/v) agarose gel under UV light in a Gel Logic 400 Imaging System (Mandel, Rochester, NY). Samples were submitted for fragment analysis by the Fragment Analysis and DNA Sequencing Service (FADSS) at The University of British Columbia Okanagan, which uses
an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, USA). The microsatellite data were compared to a *S. cerevisiae* microsatellite database, constructed at UBC Okanagan of commercial strains, to confirm the presence of either RC212 or D254.

### 2.2.8 GC-MS and GC-FID analysis of in-lab and operational samples.

Analysis was carried out for a total of 11 compounds, 4 of these (ethyl butyrate, isoamyl acetate, hexanol, and 2-phenylethylalcohol) were quantified with gas chromatography-mass spectrometry (GC-MS) at UBC Okanagan. The other 7 compounds, ethyl acetate, acetaldehyde, methanol, 1-propanol, isobutanol, active amyl alcohol (2-methyl-1-butanol), and isoamyl alcohol (3-methyl-1-butanol) were quantified by ETS laboratories (St. Helena, California) using gas chromatography flame ionization detector (GC-FID). The procedure used for the quantification was an American Association for Laboratory Accreditation (A2LA) method, which meets the ISO/IEC 17025:2005 standard operating procedure # G002 for GC-FID.

Ethyl butyrate, isoamyl acetate, hexanol, and 2-phenylethylalcohol were quantified with GC-MS. For GC-MS, conducted at UBC Okanagan, 4-methyl-2-pentanol was used as the internal standard (IS) for analysis at a concentration of 1.615 mg/l in each sample and in each concentration of pure standards for construction of a standard curve (Callejon et al. 2010, Barrajón et al. 2011). Duplicates of seven different concentrations of the pure compounds in solvent were used to construct a standard curve. The compound 2-phenylethylalcohol is found at much higher concentrations than the other compounds. Therefore, it was added at about a 10-fold higher concentration to each standard concentration. Standards contained the concentrations detailed in Table 2.3.
Table 2.3 Concentrations of each compound in the standards used for GC-MS in mg/l.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ethyl Butyrate</td>
<td>17.50</td>
</tr>
<tr>
<td>Isoamyl Acetate</td>
<td>17.52</td>
</tr>
<tr>
<td>Hexanol</td>
<td>16.28</td>
</tr>
<tr>
<td>2-Phenylethanol</td>
<td>204.00</td>
</tr>
<tr>
<td>4-methyl-2-pentanol</td>
<td>1.62</td>
</tr>
</tbody>
</table>

A Varian/Agilent CP-3800 GC equipped with a VF-5MS 30 m x 0.25 mm FactorFour capillary column and with a CP-8400 auto sampler was used for a splitless analysis. The injector was ramped from 40 °C to 100 °C at 10 °C/min. The oven was ramped from 40 °C to 240 °C at 10 °C/min. A solvent delay of 2.5 minutes was used. Samples were extracted with liquid-liquid extraction using a 1:1 ratio of pentane and diethyl ether as the solvent. 5 ml of sample, 5 μl of 1615 mg/l 4-methyl-2-pentanol, and 5 ml of solvent were shaken vigorously in large test tubes. The solution settled for 1 hour and the extract was transferred from the top layer to GC-MS vials. The retention times, the mass to charge ratios used for analysis, the boiling point, and concentrations previously reported in wine, aroma threshold, aroma descriptor, molecular mass, CAS-RN, and the structure are listed in Table 2.4.
Table 2.4 Table of analyzed aroma compounds with reported concentration in wine, aroma threshold, descriptors, structure, molar mass, CAS registry numbers, boiling points, retention times, and mass to charge ratios used in analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in wine (mg/l)</th>
<th>Aroma threshold (mg/l)</th>
<th>Aroma descriptor</th>
<th>Structure</th>
<th>MW (g/mol)</th>
<th>CAS-RN</th>
<th>BP (°C)</th>
<th>Retention time (min)</th>
<th>M/Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetaldehyde</td>
<td>10-75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>sherry, nutty, bruised apple&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>44.05</td>
<td>75-07-0</td>
<td>20.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Higher alcohols</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexanol</td>
<td>0.3-12.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Green, grass&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>102.17</td>
<td>111-27-3</td>
<td>155-159</td>
<td>15.529</td>
<td>55.8, 84.8</td>
</tr>
<tr>
<td>isobutanol</td>
<td>9.0-174&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Fusel, spritous&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>74.12</td>
<td>78-83-1</td>
<td>108</td>
<td>6.405</td>
<td>56.9, 72.9</td>
</tr>
<tr>
<td>methanol</td>
<td>30-35</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt; = 350 mg/kg</td>
<td>No organoleptic character</td>
<td></td>
<td>32.04</td>
<td>67-56-1</td>
<td>64.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-methyl-1-butanol</td>
<td>6-110&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>Alcohol, nail polish</td>
<td></td>
<td>88.148</td>
<td>137-32-6</td>
<td>127.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methyl-1-butanol</td>
<td>6.0-490&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Harsh, nail polish&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>88.148</td>
<td>123-51-3</td>
<td>131.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-phenylethanol</td>
<td>4.0-197&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Floral, rose&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>122.16</td>
<td>60-12-18</td>
<td>219-221</td>
<td>22.876</td>
<td>121.8</td>
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<tr>
<td>propanol</td>
<td>9.0-68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>500&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Pungent, harsh&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>60.10</td>
<td>71-23-8</td>
<td>97</td>
<td></td>
<td></td>
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</table>

(continued on next page)
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in wine (mg/l)</th>
<th>Aroma threshold (mg/l)</th>
<th>Aroma descriptor</th>
<th>Structure</th>
<th>MW (g/mol)</th>
<th>CAS-RN</th>
<th>BP (°C)</th>
<th>Retention time (min)</th>
<th>M/Z</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Esters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>5.0-63.5(^a)</td>
<td>12.27(^b)</td>
<td>VA, nail polish(^a)</td>
<td></td>
<td>88.11</td>
<td>141-78-6</td>
<td>77.1</td>
<td>2.789</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fruity (&gt;100mg/ml)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Solvent, balsamic(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethyl butyrate</td>
<td>0.01-1.8(^a)</td>
<td>0.02(^b)</td>
<td>Floral, fruity(^ab)</td>
<td></td>
<td>116.16</td>
<td>105-54-4</td>
<td>120-121</td>
<td>4.845</td>
<td>70.8, 116.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strawberry, sweet(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>0.03-5.52(^b)</td>
<td>0.16(^b)</td>
<td>Banana, fruity(^b)</td>
<td></td>
<td>130.19</td>
<td>123-92-2</td>
<td>142</td>
<td>7.711</td>
<td>70.8, 130.7</td>
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<tr>
<td><strong>Internal standard</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-methyl-2-pentanol</td>
<td>IS</td>
<td>IS</td>
<td>Not present in wine</td>
<td></td>
<td>102.17</td>
<td>108-11-2</td>
<td>131.6</td>
<td>10.165</td>
<td>68.9, 84.8</td>
</tr>
</tbody>
</table>

\(^a\)Swiegers et al. 2005
\(^b\)Sumby et al. 2009
\(^c\)Comi et al. 2001
\(^*\) in 10% Ethanol
\(^**\) in wine
2.2.9  Data analysis

All statistical analyses were conducted using JMP® (Version 9.0.1, SAS Institute Inc., Cary, NC, USA). Frequency of occurrence of the strains RC212 at QGEW, Cedar Creek Estate Winery (CCEW), and at Tantalus Vineyards (TLS) were obtained from sample sizes of 16 isolates per tank per stage, except for in 2010 where only 8 isolates were analyzed at QGEW. For the all of the experiments, a global test of means, a one-way Analysis of variance (ANOVA) at an $\alpha = 0.05$ level was used. ANOVA was used to determine whether the in-lab inoculation treatments were significantly different from each other, whether there was a significant change in strain population within in-lab inoculation treatments between sampling time points, and also to determine whether the in-lab and the operational samples had significantly different amounts of each compound analyzed. The null and alternate hypotheses for the one-way ANOVA’s were: $H_0: \mu_i = \mu_j$, and $H_1: \mu_i \neq \mu_j$ for any $i$ and $j$ where $i \neq j$. When significance was indicated, a multiple comparison Tukey’s Honest Significant Difference (HSD) test was used also at an $\alpha = 0.05$ level. The null and alternate hypotheses for the Tukey’s HSD were $H_0: \mu_i = \mu_j$, and $H_1: \mu_i \neq \mu_j$ for at least one pair of $i$ and $j$ where $i \neq j$. A correlation analysis was conducted on the microsatellite analysis and the quantum dot analysis; a correlation quantifies how related two variables are. Regression in bivariate fit model was used to see the regression of the percentage of RC212 to the resulting concentrations of fermentation-derived by-products of the in-lab experiments. The null and alternate hypotheses for the regressions were $H_0: \beta_1 = 0$, and $H_1: \beta_1 \neq 0$ for the regression model $y = \beta_0 + \beta_1x + \varepsilon$. The concentrations of all fermentation-derived compounds analyzed
were used to evaluate the grouping of treatments by hierarchical cluster analysis using Ward’s method with Euclidean distance.

Principal component analysis (PCA) was used to derive a small number of principal components (linear uncorrelated combinations) between the in-lab treatments and the operational samples using the resulting fermentation-derived compounds. PCA were performed on the correlation matrix of the concentrations of fermentation-derived by-products of all replicates for both in-lab and operational samples. Linear discriminant canonical analysis of covariances using fermentation-derived compound data from of both the in-lab experiment and the operational samples was also analyzed. Both PCA and canonical analyses were conducted using JMP® (Version 9.0.1, SAS Institute Inc., Cary, NC, USA).

2.3 Results

2.3.1 Strain population dynamics throughout fermentation of in-lab fermentations

Based on microsatellite analyses, all treatments remained in the inoculated ratio throughout the fermentation ($p < 0.0001$), whereas all ratio treatments of RC212: D254 (1:0, 9:1, 3:1, 1:1, and 0:1) were significantly different from each other as indicated by a one-way ANOVA; $n = 3, p < 0.0001$ (Table 2.5).
Table 2.5 Percent Lalvin® RC212 identified in the in-lab experiment by microsatellite analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Start</th>
<th>Early</th>
<th>Mid</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC212</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>D254</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1:1</td>
<td>45.7 ± 12.4a</td>
<td>43.0 ± 13.2a</td>
<td>34.7 ± 12.3a</td>
<td>50.0 ± 24.4a</td>
</tr>
<tr>
<td>3:1</td>
<td>66.7 ± 12.4b</td>
<td>60.0 ± 13.2b</td>
<td>45.0 ± 13.2b</td>
<td>61.0 ± 25.9b</td>
</tr>
<tr>
<td>9:1</td>
<td>77.0 ± 6.2c</td>
<td>88.0 ± 12.0c</td>
<td>73.3 ± 8.5c</td>
<td>76.0 ± 18.2c</td>
</tr>
</tbody>
</table>

Values with the same letters are not significantly different according to the Tukey test (95%).

n=3, 20 colonies sampled per flask per time point

The quantum dot analysis fully supported the results of the microsatellite analysis (Table 2.6).

Table 2.6 Percent Lalvin® RC212 identified in the in-lab experiment by quantum dot analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Early</th>
<th>Mid</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 1</td>
<td>59.4 ± 6.7a</td>
<td>58.3 ± 11.4a</td>
<td>50.7 ± 7.7a</td>
</tr>
<tr>
<td>3 to 1</td>
<td>71.8 ± 5.5b</td>
<td>74.8 ± 2.5b</td>
<td>65.5 ± 8.3b</td>
</tr>
<tr>
<td>9 to 1</td>
<td>82.9 ± 4.8c</td>
<td>81.9 ± 1.2c</td>
<td>81.8 ± 1.4c</td>
</tr>
</tbody>
</table>

Values with the same letters are not significantly different according to the Tukey test (95%).

n=3, average of 320 yeast cells sampled per flask per time point

This resulted in a significant correlation; r = 0.85 between the two types of analyses (Figure 2.2).
Figure 2.2 Correlation between frequency of RC212 data collected by microsatellite (MS) analysis and quantum dot (QD) analysis, correlation coefficient 0.85.

The kinetics of the QD-GSH fermentation and the unlabeled fermentations show no significant difference (Figure 2.3).
Figure 2.3 Fermentation kinetics of a QD-GSH tagged fermentation (Sugar ◆, Cells/ml ■) and an untagged fermentation (Sugar ◆, Cells/ml ■). There does not appear to be any significant change in fermentation kinetics in the tagged fermentation.

In addition, pure culture treatments (1:0 and 0:1) and co-inoculation treatments (1:1, 3:1, and 9:1) were not significantly different with respect to fermentation kinetics when provided with the same fermentation conditions. Additionally, confocal imaging showed localization of QD-GSH in both the cytoplasm and the vacuoles of mother and daughter cells, indicating that the QDs were successfully inherited by daughter cells (Figure 2.4).
Figure 2.4 QD-GSH localized in the cytoplasm (a, b, c) and in the vacuoles (d, e, f) transferred from mother cell to daughter cell. Superimposed fluorescence image and bright field image (a, d). Fluorescence image (b, e). Bright field image (c, f).

2.3.2 Strain population dynamics throughout fermentation at QGEW

In 2012, the inoculant RC212 dominated the inoculated fermentations with minimal influence from D254, whereas D254 dominated spontaneous tanks with minimal influence from RC212. This was in contrast to the 2010 vintage where RC212 and D254 were co-dominant in both inoculated and spontaneous fermentations. At QGEW during the 2012
vintage, an increase of D254 from 26 % in the early stage to 67 % in the end stages of spontaneous fermentation occurred. Most vintages from the different wineries demonstrated a dominance of D254 (≥ 14 % frequency of occurrence) in the spontaneous fermentations at either early, mid, or end stages (Table 2.1).

2.3.3 Fermentation-derived by-products specific to each strain in in-lab samples

The compounds analyzed had previously been observed as fermentation-derived by-products specific to *S. cerevisiae* strains (Swiegers et al. 2005, King et al. 2008, Sun et al. 2011). There was a positive linear relationship between the abundance of RC212 and the quantity of 4 compounds, including acetaldehyde \( p = 0.0023 \), propanol \( p < 0.0001 \), isobutanol \( p < 0.0001 \), and 3-methyl-1-butanol (isoamyl alcohol) \( p = 0.0268 \), as well as the total concentration of higher alcohols \( p < 0.0001 \) (Table 2.7).

| Table 2.7 Fermentation-derived compounds and their linearity with respect to an increase in Percent Lalvin® RC212 and a decrease in Percent Lalvin® D254. |
|---------------------------------|------------------|-------------|
| Compound                        | Linearity        | \( p \) value |
| acetaldehyde\(^*\)              | Positive         | 0.0023      |
| **Higher alcohols**             |                  |             |
| hexanol                         | ND               |             |
| isobutanol\(^*\)                | Positive         | <0.0001     |
| methanol                        | ND               |             |
| 2-methyl-1-butanol\(^*\)        | Negative         | 0.007       |
| 3-methyl-1-butanol\(^*\)        | Positive         | 0.0268      |
| 2-phenylethanol                 | Negative         | 0.6643      |
| propanol\(^*\)                  | Positive         | <0.0001     |
| Total higher alcohols           | Positive         | <0.0001     |
| **Esters**                      |                  |             |
| ethyl acetate\(^*\)             | Negative         | 0.0237      |
| ethyl butyrate                   | Negative         | 0.715       |
| isoamyl acetate                 | Negative         | 0.0953      |

\(^*\) significant correlation to strain proportion \( \alpha = 0.05 \)
On the other hand, there was a negative linear relationship between the abundance of RC212 and the quantity of ethyl acetate $p = 0.0237$ and 2-methyl-1-butanol $p = 0.007$.

Because of the successive inoculation ratios of RC212: D254, 1:0, 9:1, 3:1, 1:1, 0:1, an increase in RC212 was synonymous with a decrease in D254. The compounds; ethyl butyrate, isoamyl acetate, or 2-phenylethylalcohol, were not significantly affected by the abundances of these two strains. Compounds with a significant regression to strain proportion are considered to be influenced by either RC212 or D254. The concentrations of the analyzed compounds are summarized for the five successive inoculation ratio treatments as well as the two operational, inoculated and spontaneous, QGEW treatments in Table 2.8.
Table 2.8 Summary of fermentation-derived compounds for each in-lab fermentation treatment as well as for Quails’ Gate Estate winery samples. Concentrations in mg/l (n=3).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RC212</th>
<th>RC212: D254</th>
<th>Quails’ Gate Estate Winery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9:1</td>
<td>3:1</td>
<td>1:1</td>
</tr>
<tr>
<td>acetaldehyde(^1)</td>
<td>22.0 ± 4.6(^{ab})</td>
<td>17.0 ± 2.7(^{bc})</td>
<td>17.7 ± 2.5(^{bc})</td>
</tr>
<tr>
<td>hexanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>isobutanol(^1)</td>
<td>270.7 ± 8.3(^a)</td>
<td>218.7 ± 3.8(^b)</td>
<td>173.0 ± 14.2(^c)</td>
</tr>
<tr>
<td>methanol(^1)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-methylbutanol(^1)</td>
<td>47.0 ± 3.5(^{cd})</td>
<td>46.7 ± 1.2(^{cd})</td>
<td>45.3 ± 4.5(^d)</td>
</tr>
<tr>
<td>3-methylbutanol(^1)</td>
<td>280.7 ± 10.7(^a)</td>
<td>272.0 ± 7.8(^{ab})</td>
<td>254.7 ± 18.2(^{ab})</td>
</tr>
<tr>
<td>2-phenylethanol(^1)</td>
<td>14.5 ± 5.1(^a)</td>
<td>14.5 ± 5.1(^{a})</td>
<td>27.3 ± 6.1(^{cd})</td>
</tr>
<tr>
<td>propanol(^1)</td>
<td>47.3 ± 3.2(^{ab})</td>
<td>42.3 ± 1.5(^{ab})</td>
<td>41.0 ± 2.0(^{bc})</td>
</tr>
<tr>
<td>Σ higher alcohols(^1)</td>
<td>669.0 ± 23.4(^a)</td>
<td>594.1 ± 14.6(^b)</td>
<td>541.3 ± 32.9(^{bc})</td>
</tr>
<tr>
<td>Esters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethyl acetate(^2)</td>
<td>17.0 ± 3.6(^c)</td>
<td>22.0 ± 2.7(^{bc})</td>
<td>22.3 ± 5.1(^{bc})</td>
</tr>
<tr>
<td>ethyl butyrate(^1)</td>
<td>0.5 ± 0.0(^{bc})</td>
<td>0.5 ± 0.0(^{bc})</td>
<td>0.5 ± 0.0(^c)</td>
</tr>
<tr>
<td>isovalyl acetate(^2)</td>
<td>1.4 ± 0.0(^{bc})</td>
<td>1.5 ± 0.0(^{abc})</td>
<td>1.6 ± 0.0(^{c})</td>
</tr>
</tbody>
</table>

Values within a row with the same letters are not significantly different according to the Tukey test (95%).

ND: not detected.

Significance level of one way ANOVA across the row \(^1\) \(p < 0.0001; \(^2\) 0.01 > \(p > 0.0001; \(^3\) 0.05 > \(p > 0.01.


* (Perez-Coello et al. 1999, Callejon. et al. 2010)

*(Vilanova and Martinez 2007)
Acetaldehyde increased as the abundance of RC212 increased (Table 2.7). The maximum concentration of acetaldehyde produced by RC212 was seen in the pure inoculum treatment with 22 mg/l (Table 2.8). This was not significantly different from the concentrations seen in the 9:1 and the 3:1 treatments, respectively. However, it was significantly different from the concentrations reported for the 1:1 ratio and the pure inoculum treatment of D254, $p < 0.0001$.

For the higher alcohols, neither hexanol nor methanol was detected. Isobutanol concentration was also related to RC212 abundance (Table 2.7). Isobutanol concentrations for all in-lab treatments were all significantly different from each other, $p < 0.0001$ (Table 2.8). The pure inoculum of RC212 produced 270.7 mg/l, the 9:1 ratio produced 218.7 mg/l, the 3:1 ratio treatment produced 173 mg/l, the 1:1 ratio treatment produced 138.3 mg/l, and the pure D254 inoculum treatment produced 89 mg/l.

The concentration of 2-methyl-1-butanol, also known as active amyl alcohol, increased with the abundance of D254 (Table 2.7). The pure D254 inoculum treatment had 53.7 mg/l, which was similar to all other in-lab concentrations except for the 3:1 inoculation treatment, which had only 45.3 mg/l active amyl alcohol (Table 2.8). The pure inoculum of RC212 contained 47 mg/l of active amyl alcohol.

Reported concentrations of 3-methyl-1-butanol, commonly known as isoamyl alcohol, increased with the abundance of RC212 (Table 2.7). Isoamyl alcohol was found at 280.7 mg/l in the in-lab pure RC212 inoculum treatment and at 249.7 mg/l in the pure D254 inoculum treatment (Table 2.8). These were significantly different from each other, $p < 0.001$, but not significantly different to any of the ratio inoculations.
The concentration of 2-phenylethanol did not relate to the concentration of RC212 or D254 (Table 2.7). The 9:1 inoculation treatment contained the lowest concentrations of 2-phenylethanol with 14.5 mg/l and was significantly different to the higher 27.3 mg/l detected in the 3:1 inoculation ratio treatment, \( p = 0.05 \) (Table 2.8). The pure RC212 inoculum treatment, 1:1 inoculation ratio treatment, and pure D254 inoculum treatment were not significantly different from each other.

Propanol showed a positive relationship to RC212 (Table 2.7), the maximum concentration was from the pure inoculum RC212 treatment at 47.3 mg/l (Table 2.8). Successive concentrations of 42.3 mg/l, 41 mg/l, 39 mg/l and 34 mg/l were reported for 9:1, 3:1, 1:1 and 0:1 treatments respectively (Table 2.8). With respect to propanol, the pure inoculum RC212 treatment showed similarity to the 9:1 and the 3:1 treatment, the pure inoculum D254 treatment showed similarity to the 3:1 and the 1:1 treatment.

As seen in Table 2.7, there was a significant increase in the total higher alcohols produced as the population of RC212 increased, \( p = 0.05 \). In the in-lab fermentations, the RC212 pure inoculum produced a concentration of 669 mg/l of the analyzed higher alcohols, which were significantly different to all other treatments (Table 2.8). The 9:1 inoculation ratio treatment was similar to the 3:1 inoculation ratio treatment. The 3:1 inoculation ratio treatment was similar to the 1:1 concentration and to the pure D254 inoculum treatment.

With respect to the esters, ethyl acetate concentration increased as the abundance of D254 increased (Table 2.7). The 1:1 inoculation ratio treatment contained the highest concentration of ethyl acetate followed by the pure inoculum D254 treatment, the 3:1 ratio inoculation treatment, 9:1 ratio inoculation treatment, the pure RC212 inoculation treatment
respectively (Table 2.8). These concentrations were not significantly different from each other $p = 0.01$.

Ethyl butyrate did not show a significant correlation to strain proportion in the controlled fermentations (Table 2.7). Ethyl butyrate concentrations were significantly different for the 3:1 inoculation ratio treatment and the pure D254 inoculum treatment. However, all treatments contained approximately 0.5 mg/l of ethyl butyrate.

Isoamyl acetate showed a trend to increase as D254 population increased, although this was not significant (Table 2.7). The 3:1 inoculation ratio treatment had a concentration of 1.6 mg/l, which was the highest concentration of isoamyl acetate recorded. The other in-lab concentrations were not significantly different from each other (Table 2.8).

### 2.3.4 Fermentation-derived by-products in operational QGEW samples

The 2012 inoculated QGEW samples were dominated by RC212, and the spontaneous QGEW samples were dominated by D254 (Table 2.1). The acetaldehyde concentrations in the samples were not significantly different from each other (Table 2.8). For the alcohols; isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and the total higher alcohol concentrations were significantly greater in the inoculated samples, $p < 0.0001$ (Table 2.8). Hexanol, methanol, and 2-phenylethanol concentrations were greater, but not significantly, in the spontaneous samples. For the esters, the QGEW samples were not significantly different from each other for ethyl acetate, ethyl butyrate, or isoamyl acetate (Table 2.8).
2.3.5 Clustering of treatments based on chemical composition

A hierarchical analysis of chemical analysis results from the in-lab experiments using Ward’s method showed that the 1:0 ratio treatment of RC212 clusters together with the ratio treatment of 9:1 (RC212: D254). Both mixed ratios of 3:1 and 1:1 (RC212: D254) cluster together with the single inoculum treatment of D254. This suggests that D254 has a greater effect on fermentation-derived compounds than RC212 even when D254 has a significantly lower population. The samples from QGEW, which were inoculated with RC212 and had 75% frequency of occurrence of RC212 at the end of fermentation, formed a cluster and so did the QGEW spontaneous samples, which had 56% D254 at the end of fermentation (Table 2.1). A biplot of a PCA, showed all treatments were separate, but showed minor overlap (Figure 2.5).
Figure 2.5 PCA biplot, 73.5% of the variance in the model is explained by principal component 1 (53.1%) and principal component 2 (20.4%).

The ellipses were added post-computation by hand as a visual aid. The first principal component represents 53.3% of the variance and the second principal component represents 20.4%. In a hierarchical cluster analysis seen in the dendrogram in Figure 2.6, the single inoculum treatment of RC212 clusters together with the ratio treatment of 9:1 (RC212:D254).
Figure 2.6 Hierarchical cluster analysis using the Ward method based on fermentation-derived compounds.

A canonical plot has a strong representation of grouping as indicated by a low Wilk’s Lambda value (6.517E-10), and a p-value of <0.0001 (Figure 2.7).
Figure 2.7 Canonical plot with all variables included. Wilk's Lambda value of 6.517E-10 and $p < 0.0001$ concludes the corresponding function explain the group membership well. The model explains 99.1% of the variance in the model.

The first canonical variable (CV) explains 99.1% of the variance in the model shared between the linear combination of variables with an Eigenvalue of 4652.59. The groups calculated by the canonical function match the treatments and each treatment represents a distinguishable group. The RC212 inoculated samples from QGEW, which were dominated
by RC212 had a relatively short distance to the in-lab pure inoculum RC212 treatment samples. Similarly, the spontaneous samples from QGEW were dominated by D254 (Table 2.1), and had a relatively short distance to the in-lab pure inoculum D254 treatment based on the second canonical variable.

2.4 Discussion

2.4.1 Strain interaction between Lalvin® RC212 and Lalvin® ICV-D254

The first hypothesis, that D254 is more dominant over RC212 even when it is inoculated with substantially fewer cells than RC212, was not supported in this study. Both microsatellite and quantum dot analyses demonstrate that ratios of 1:1, 3:1, and 9:1 of RC212:D254 from the in-lab experiments do not significantly change during the fermentations. Thus, under controlled conditions, neither of these strains was more competitive than the other. At wineries under operational conditions, we observed a different pattern; D254 usually enters tanks inoculated with RC212 and co-ferments with or dominates over the inoculant, which was consistent with the initial prediction (Lange et al. 2012). In addition, pure culture treatments (1:0 and 0:1) and co-inoculation treatments (1:1, 3:1, and 9:1) were not significantly different with respect to fermentation kinetics when provided with the same fermentation conditions. Since D254 seems competitive in an operational setting, this indicates that D254 could be better suited to industrial tank environments than under the controlled fermentation conditions studied. Varying temperatures, nitrogen levels, sulfur levels, and other parameters could contribute to the success of D254 in the wineries (Goddard et al. 2010).
The second hypothesis, that RC212 and D254 are prevalent strains in both the inoculated and spontaneous Pinot Noir fermentations at QGEW was not supported during the 2012 vintage. In 2012, the inoculant RC212 dominated the inoculated fermentations with minimal influence from D254, whereas D254 dominated spontaneous tanks with minimal influence from RC212. This was in contrast to the 2010 vintage where RC212 and D254 were co-dominant in both inoculated and spontaneous fermentation (Lange 2012). The increase of D254 in the spontaneous fermentations from 27 % at the early stage to 67 % at the end suggests that D254 was very successful at entering and co-fermenting Pinot Noir spontaneously at QGEW (Hall et al. 2011, Lange 2012, Faasse unpublished). These findings are consistent with Hall et al. (2011) who found D254 in frequencies greater than 25 % at the end of spontaneous Pinot Noir fermentations. Although the 2012 results did not support our hypothesis, which was based on the 2010 results, it allowed us to compare in-lab data to operational data where D254 and RC212 were present in almost pure cultures.

The third hypothesis, that the QD-GSH complexes are taken into the cytosol of the yeast strains and passed to daughter cells during budding was partially supported in this study. Confocal imaging showed that QD-GSH complexes were localized in both the cytoplasm and the vacuoles. In addition, the QDs were successfully passed to daughter cells during budding (Figure 2.4). Cytoplasm localization supports the initial prediction that QD-GSH enters through the plasma membrane. There are two mechanisms by which the QD-GSH could enter the vacuole from the cytoplasm. It may be transferred from the cytoplasm directly into the vacuole (Mehdi and Penninckx 1997) or the QD-GSH could be engulfed by phagocytosis by the cell resulting in vacuolar localization (Bourbouloux et al. 2000, Zuzuki et al. 2002).
The fourth hypothesis, QD-GSH remain in the yeast cells and fluoresce throughout the fermentation was supported in this study. It was also found that the two methods used to quantify different strains fermenting the must correlated well with each other. Nevertheless, the quantum dot method had many advantages, including the ability to quantify a larger number of cells in less time, at lower cost, and has the potential to produce more accurate and consistent results than microsatellite analysis. The kinetics of the QD-GSH fermentation and the unlabeled fermentations show no significant difference (Figure 2.3), which implies that the QD-GSH treatment does not interfere with the yeast’s ability to ferment or grow. We are unaware of any studies that have looked at the kinetics of QD-GSH.

2.4.2 Fermentation-derived by-products associated with a specific strains

The fifth hypothesis, that the concentrations of fermentation-derived by-products in all five inoculation treatments reflect the abundance of RC212 and D254 was partially supported. Regressions of fermentation-derived by-products and abundance of RC212 from the in-lab treatments revealed a significant relationship for 7 of the 12 fermentation-derived by-products analyzed (Table 2.7).

The sixth hypothesis, that D254 produces greater concentrations of higher alcohols and esters than RC212 was not supported in this study. Most of the compounds showing strain specificity from this study were higher alcohols. However, in this study, RC212 produced significantly greater concentration of total higher alcohols. The main higher alcohols; isobutanol, isoamyl alcohol, and active amyl alcohol, are reported to contribute desirable characteristics at less than 300 mg/l but at concentrations greater than 400 mg/l they contribute solvent like aromas (Rapp and Versini 1996, Swiegers et al. 2005, Ribereau-
Gayon et al. 2006). Interestingly, higher alcohol production from amino acids is proportional to the nitrogen content up to a limit of 400 mg/l where the higher alcohol concentrations plateau, except for propanol, which continues to increase as nitrogen increases (Boulton et al. 1996).

The seventh and final hypothesis, that the RC212 and D254 populations in the QGEW samples reflect the concentrations of fermentation-derived by-products as determined by the in-lab experiments, was partially supported. Only a few of the fermentation-derived by-products related to RC212 or D254 showed the same trend in the QGEW samples. For example, in support of it, a positive correlation was found with the concentration of isobutanol with an increase in RC212 abundance in the in-lab experiment and also in the QGEW samples. In contrast to these findings, Sun et al. (2011) found that D254 produced higher concentrations of isobutanol than RC212. Isobutanol is formed by the Ehrlich pathway via decarboxylation and reduction of an α-keto acid in amino acid metabolism. Isobutanol is a derivative of valine and is described as fusel and spirituous at concentrations above 40 mg/l (Swiegers et al. 2005, Hazelwood et al. 2008). All of the in-lab treatments recorded concentrations of isobutanol exceeding the aroma threshold. These findings suggest that there may be a detectable strain contribution of isobutanol in QGEW fermentations. In contrast to the final hypothesis, RC212 was a higher acetaldehyde producer than D254 in-lab, but at the operational level this relationship was not detected. Many of the compounds that were found to have strain specificity in this study can be considered to be detrimental to wines if detected above certain levels. Acetaldehyde, which has a descriptor of sherry, nutty and bruised apple, had the highest concentration in the in-lab fermentations at 22 mg/l.
in the pure RC212 inoculum. This did not exceed the aroma threshold of 100 mg/l (Swiegers et al. 2005). Therefore, under in-lab conditions, RC212 did not produce high enough levels of acetaldehyde to contribute to a negative impact on wine quality. Acetaldehyde is produced by \textit{S. cerevisiae} at concentrations between 0.5-286 mg/l, but it is detected in wines at concentrations between 10-75 mg/l (Boulton et al. 1996, Swiegers et al. 2005). It has been found that yeast strains that produce more acetaldehyde result in greater production of the anthocyanins vitisin A and B, which are important in colour stabilization in red wine (Morata et al. 2003).

Active amyl alcohol, 2-methyl-1-butanol, is formed through the Ehrlich pathway and is a derivative of leucine (Hazelwood et al. 2008). It has a descriptor of alcohol and nail polish (like isoamyl alcohol). In the in-lab study active amyl alcohol increased in concentration as the population of D254 increased. However, QGEW samples had higher concentrations of active amyl alcohol in the inoculated than in the spontaneous samples, which does not support the last hypothesis. Thus, factors other than the dominance of D254 are responsible for the results that were found under operational conditions.

Isoamyl alcohol is produced by the Ehrlich pathway and is a derivative of isoleucine (Hazelwood et al. 2008). Isoamyl alcohol is described as harsh with notes of nail polish at concentrations greater than 30 mg/l (Swiegers et al. 2005). The in-lab pure RC212 treatment had a significantly greater concentration of isoamylalcohol than D254. However, it was still well within the range detected in wines of 6 - 490 mg/l (Swiegers et al. 2005). The inoculated samples from QGEW showed a significantly greater concentration than the spontaneous samples, supporting the seventh hypothesis. In contrast, in cherry wine, RC212 produces a significantly lower amount of isoamyl alcohol than D254 (Sun et al. 2011).
Propanol was found in significantly lower concentrations in the QGEW samples than in the in-lab samples. The QGEW samples contained about half the concentration of propanol compared to the in-lab samples and showed no similarity to the in-lab results; therefore, contradicting the seventh hypothesis. The greatest concentration of propanol recorded was in the pure RC212 inoculum treatment with a concentration of 46.33 mg/l, well below the aroma threshold. Sun et al. (2011) conducted a study with cherry wine where RC212 and D254 were compared in pure inoculum treatments. Their findings contradicted ours as they found that D254 produced a significantly higher concentration of propanol. Propanol is described as pungent and harsh at concentrations greater than 500 mg/l and it is a derivative of threonine (Boulton et al. 1996, Swiegers et al. 2005). The production of propanol is related to the original nitrogen content and yeast growth, and this may be the reason for the different findings between our study and Sun et al. (2011)(Giudici et al. 1993, Rapp and Versini 1996).

The total higher alcohols in the QGEW samples was higher in the inoculated than in the spontaneous fermentation, which supports the seventh hypothesis. These results along with the finding that the concentrations were found above the aroma threshold, suggests that RC212 or D254 could influence the sensory attributes of the wine (Callejon et al. 2010, King et al. 2011).

The esters analyzed were not significantly different in the in-lab samples, so the results cannot support the seventh hypothesis. Nonetheless, all concentrations of ethyl acetate in this study were in the desirable range, which is greater than the aroma threshold at 12 mg/l and lower than 100 mg/l (Sumby et al. 2009). Ethyl acetate has a desirable fruity aroma at concentrations less than 100 mg/l but an aroma described as balsamic and solvent at
concentrations higher than 100 mg/l (Sumby et al. 2009). It is formed from ethanol and acetyl-CoA (Fujii et al. 1994).

The concentrations of ethyl butyrate in the QGEW samples were not significantly different from each other. No treatment differences were observed for the concentration of ethyl butyrate even though previous studies have detected strain differences in ethyl butyrate production (Lilly et al. 2006, Saerens 2006, Saberi et al. 2012). Ethyl butyrate does not have different production by RC212 and D254 under these conditions. Ethyl butyrate is formed mostly through esterification of fatty acids (Suomalainen 1981).

Similarly, this study did not find any strain specificity or significant difference between the QGEW samples in the concentration of isoamyl acetate, which is described as having a banana aroma at concentrations greater than 0.16 mg/l (Swiegers et al. 2005). Isoamyl acetate is formed by an acetyltransferase with isoamyl alcohol and acetyl-CoA (Lilly et al. 2000). In cherry wine, D254 produced a significantly greater amount of isoamyl acetate than RC212 (Sun et al. 2011).

The in-lab controlled fermentations allowed for strain specific fermentation-derived by-products to be analyzed. Strain specific concentrations of acetaldehyde, ethyl acetate, propanol, active amyl alcohol, isoamyl alcohol, isobutanol, and total higher alcohols were detected in the in-lab experiments. Isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and ethyl acetate were strain specific and were detected above aroma threshold in the in-lab experiments. This indicates that strain proportion in the fermentation can have a direct effect on the production of these compounds. Isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol are all derivatives of amino acids. The concentrations seen here were likely a reflection on amino acid content of the initial must. The trends seen in this study, between
fermentation-derived by-product concentrations and yeast strain presence, were in contrast to the study of Sun et al. (2011) who analyzed cherry wine. This is likely due to initial amino acid content of the musts, specifically valine, leucine, and isoleucine. Cherry juice has much lower concentrations of these amino acids than grape juice (Van Gorsel et al. 1992). It was evident in comparisons with the literature that initial must contents will play a large role in the fermentation-derived by-products produced (Guitart et al. 1999, Hernandez-Orte et al. 2002, Bell and Henschke 2005, Vilanova et al. 2007, Carrau et al. 2008, Garde-Cerdan and Ancin-Azpilicueta 2006). At an operational level, several factors affect the production of fermentation-derived by-products; temperature, amino acid content of the must, grape quality, vine age, geographical region, climate, and vintage (Goddard et al. 2008, Styger et al. 2011).

In this study, the concentration of methanol in the in-lab fermentation samples was reported as less than 5 mg/l; which was likely the detection limit of the method. However, methanol concentrations in the QGEW samples reached 62.3 and 76 mg/l for the inoculated and spontaneous samples, respectively. Methanol is a well known toxin as it releases formaldehyde and formic acid when it is metabolized in the liver, however the food and drug administration (FDA) advises that it is safe for adults to ingest 500 mg/day of methanol, and the international organization of vine and wine (OIV) has a limit of 300 mg/l of methanol. Methanol is produced a result of a few reactions; the first is through the hydrolysis of pectin, which is increased when commercial pectic enzymes are added to the tanks (Boulton et al. 1996). Pectin concentrations depend on the extent of grape maceration (Boulton et al. 1996). The second is the breakdown of dimethyldicarbonate (DMDC), a fungicide, where 200 mg/l DMDC results in 96 mg/l methanol (Boulton et al. 1996). Methanol can also be produced at
concentrations up to 80 mg/l as yeast cell walls break down during fermentation (Boulton et al. 1996).

In this study, hexanol was not detected. Hexanol was run as a pure standard and was detectable at 0.1 mg/l, which is below the common concentration range of 0.3-12 mg/l detected in wine (Swiegers et al. 2005). It is possible that the precursors necessary for the production of hexanol were not present in the juice, or filtered out of the juice prior to inoculation, that was used for the controlled fermentation. Hexanol is reductively formed by S. cerevisiae from hexanal, which is a derivative of linoleic acid, which is formed during processing of the must (Ribereau-Gayon et al. 2000). In the QGEW samples, there was no significant difference in hexanol concentration detected in the spontaneous compared to the inoculated samples.

2.4.3 Clustering and variance between treatments based on chemical composition

Further multivariate analysis revealed the effect that strain composition and other factors present at the operational scale had on the fermentation-derived by-products partially supporting the seventh hypothesis. The PCA plot, Figure 2.5, shows that in principal component 1 there was a greater variance between the controlled fermentations and the QGEW fermentations than there was within strain treatments in controlled fermentations. This finding indicates that other variables such as must composition or vinification techniques contribute to a greater difference in wine composition than the strain populations. Intriguingly, principal component 2 supports the idea that the amount of variance between the QGEW inoculated and spontaneous fermentations was similar to the amount of variance between the pure inocula of RC212 and D254. This finding suggests that there was indeed a
strain effect on the QGEW samples. However, other parameters such as vine age and overall grape quality may also affect this variance.

The dendrogram, Figure 2.6, suggests that the presence of D254 at such a low ratio does not significantly affect the final wine product. The next cluster includes both mixed ratios of 3:1 and 1:1 (RC212: D254) together with the single inoculum treatment of D254. This suggests that under these controlled conditions, if there was a 25% presence of D254 the final wine product would more closely resemble a pure D254 fermentation than a pure RC212 fermentations. This may be consistent with the findings of Sun et al. (2011), who found that D254 produces greater amounts of higher alcohols and esters than RC212 in cherry wine. The inoculated QGEW samples cluster separately from the spontaneous QGEW samples indicating that these two techniques result in significantly different wines.

The groups calculated by the canonical function match the treatments, and each treatment represents a separate group as seen in the canonical plot, Figure 2.7. The groupings of these treatments were nicely separated; the controlled successive ratio treatments align between the controlled pure inoculation treatments of RC212 and D254. Interestingly, the 1:1 inoculation ratio treatment results in a grouping closer to the single inoculation D254 group than to the RC212 group. This may indicate that D254 could contribute more of the analyzed fermentation-derived characteristics than RC212 when fermented under controlled conditions at an even inoculation ratio, even though the ratio was maintained throughout fermentation. The CVA separated the QGEW samples; the spontaneous sample was more aligned with the single inoculation treatments of D254 along CV2, which could be due to the strong presence of D254 in those tanks. Notably, along CV1, which described 90.1 % of the variance, the in-lab experiments were well separated from both QGEW samples. This suggests that most of
the variance between the samples and the QGEW samples was not driven by the strain composition. However, there was a similar amount of separation on CV2, which described 8.0% of the variance, between the spontaneous and inoculated QGEW samples and the pure RC212 and pure D254 samples. This suggests that the variance in operational samples could be attributed to strain presence.

There was a more defined treatment separation in the canonical plot than in the PCA plot. This was likely due to the fact that the canonical plot takes into account correlations between the variables, and can cross-correlate to obtain the greatest group separation. In the PCA plot, none of the variables were correlated and in this case, the separation was not as evident between all of the treatments. Since linked metabolic pathways produce fermentation by-products, it seems reasonable that the production of by-products would correlate depending on the metabolic pathways that were up-regulated. The hierarchical analysis, PCA, and CVA all suggest that at a 1:1 inoculation ratio D254 had a greater impact than RC212. This agrees with the findings of Sun et al. (2011) where D254 produced greater concentrations of fermentation-derived by-products overall than did RC212.
Chapter 3  Conclusion

3.1 Conclusion summary

This study was conducted to gain a better understanding of how yeast populations influence fermentation-derived characteristics at three wineries of the Okanagan, British Columbia. Previous studies revealed the presence of two commercial yeast strains Lalvin® RC212 (RC212) and Lalvin® ICV-D254 (D254) at several wineries in the Okanagan (Hall et al. 2011, Lange 2012, Faasse unpublished). This study had two main objectives; the first was to track strains RC212 and D254 in controlled (in-lab) and operational fermentations at Quails’ Gate Estate Winery (QGEW) and the second was to determine the strain specific metabolic contributions of RC212 and D254. The first objective was addressed and populations were tracked throughout fermentation with both microsatellite analysis and quantum dot (QD) analysis. The initial hypothesis proposed that D254 would be more vigorous and outcompete RC212, because D254 had been found to be very successful in tanks inoculated with RC212 in several wineries in three different years. The findings did not support this hypothesis. The results indicated that the strains RC212 and D254 were equally competitive, grow with the same success, and thereby maintain inoculation ratios under the controlled fermentation conditions. In-lab population data from five inoculation ratios suggested that RC212 and D254 did not compete with one another. However, under operational growth conditions these strains may interact differently. For example, in the QGEW fermentations, strain populations were dynamic indicating that several niche factors can contribute to strain dominance as suggested by Goddard et al. (2010) and Faasse (unpublished). The second objective, to determine strain specific fermentation by-products of RC212 and D254, was accomplished by analyzing the by-products with GC-MS and GC-
FID. The initial hypothesis suggested that all five of the inoculation ratios of the in-lab fermentations would show different metabolic profiles. Indeed, there were several compounds that showed a correlation to either RC212 or D254 populations. Interestingly, when compared to the wine samples from QGEW, only concentrations of isoamyl alcohol, isobutanol and total higher alcohols were consistent with the in-lab findings based on populations of RC212 and D254. Additionally, some of the concentrations in the winery samples exceeded the pure strain inoculations indicating that other factors affect the concentrations of fermentation-derived compounds. Several of the higher alcohols did not follow the same patterns as previously reported in the literature; likely due to the initial amino acid content of the must (Hazelwood et al. 2008).

3.2 Novelty of the research

This study was novel for several reasons. It was the first study to achieve statistically significant in-lab results that could be used to apply strain specific trends to operational samples. Studies on strain competition and fermentation-derived by-products have previously compared co-inoculations, and even successive inoculation ratios; however, they have not been compared with fermentations at an operational scale (Howell et al. 2005, King et al. 2008, Saberi et al. 2012, Sadoudi et al. 2012). The results of this study suggest factors other than those associated with yeast presence were affecting the production of the analyzed fermentation-derived by-products in the winery. Both the population tracking method and the results obtained on strain interactions during alcoholic fermentation were novel. This was the development of a new yeast strain tracking method using quantum dots. The results indicate that the QDs were successfully inherited by daughter cells and remained fluorescent.
throughout fermentation. The well-correlated data between microsatellite analysis and quantum dot analysis indicate that using quantum dots to track strain populations may be a useful method.

3.3 Suggestions for further research

The findings that RC212 and D254 were not competing in in-lab conditions but appear competitive under operational fermentations, opens up an array of new research questions. For example, what factors at a winery contribute to strain dominance during fermentations (Goddard et al. 2010). Further study is also necessary to determine the parameters affecting fermentation-derived by-product production by yeasts at the winery. In addition, further information on the regulation of the metabolic pathways involved in the production of dominant fermentation-derived by-products would provide insight to how winemakers can monitor their tanks to enhance the complexity of their wine.

The results of this study encourage the comparison of other persistent strains of *S. cerevisiae* found at local wineries to be made. It would be useful to continue conducting successive ratios of different yeast strains to determine the strain-derived characteristics. The successive strain ratio treatments and analytical measurements of compounds would benefit from a sensorial panel as well. In the future, it would be beneficial to conduct the controlled fermentations in the same juice as the winery fermentations. Using the same must for in-lab and operational fermentations would decrease the variance from initial must content. The comparison of the yeast strains will provide more information to the local winemakers on what their yeast strains contribute to the wine, and how the strain interactions in the tanks progress.
More research on QD-GSH uptake mechanism and uptake kinetics would benefit the development of the strain tracking method. A better understanding of the mechanism would allow for further development and improved applications. Currently, seven different QD colours are available and seven yeast strains could potentially be tracked throughout fermentations simultaneously. Several strains are present in winery fermentations; strain tracking with QD-GSH could either be analyzed with a confocal microscope or with a fluorescence activated cell sorter (FACS). Additionally, it would be useful to further investigate which parameters affect these persistent yeast strains in order to modify winemaking techniques to favour the success of desirable strains. Further information on the regulation of the metabolic pathways involved in the production of dominant fermentation-derived by-products would provide insight to how winemakers can monitor their tanks to enhance the complexity of their wines. Cells sorted with a FACS could be identified, cDNA could be extracted, and expression of metabolically important enzymes could be analyzed. Understanding how winery practices influence the \textit{S. cerevisiae} populations would bridge the gap between knowing the strain effects and controlling the strain effects in the wine.
References


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