Co-flocculation of *Saccharomyces cerevisiae* and Non-*Saccharomyces* Yeast Species to Increase Colour Intensity in Pinot noir Wines

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Abstract

Vitis vinifera L. cv. Pinot noir is New Zealand's most important red grape variety and second most planted variety after Sauvignon blanc. Pinot noir is a notoriously difficult grape to work with – in the vineyard it is susceptible to disease and climatic changes, and in the winery, it is a challenge to extract the compounds responsible for colour and then ensure colour stability during aging. The appearance of a wine, of which colour is a crucial component, can shape a consumer's enjoyment and perception of flavour while drinking it. Research has shown that consumers prefer deeper colour in red wines and therefore the development of tools to achieve greater colour intensity in Pinot noir wines could be extremely useful for winemakers and the wine industry.

This research explores the impact of interspecies yeast flocculation and sequential inoculation on Pinot noir colour. Six commercial non-*Saccharomyces* yeast species, LAKTIA (*Lachancea thermotolerans*), CONCERTO (*Lachancea thermotolerans*), GAÏA (*Metschnikowia fructicola*), FROOTZEN (*Pichia kluyveri*), BIODIVA (*Torulaspora delbrueckii*) and PRELUDE (*Torulaspora delbrueckii*), and two commercial *Saccharomyces cerevisiae* strains, VL3 and RC212, were used in microfermentations of synthetic grape must, both individually and in every possible combination of non-*Saccharomyces* and *S. cerevisiae*. Sedimentation rate assays at the end of fermentation were performed to determine how well each individual yeast, and yeast combination, flocculated. The most flocculant individual yeast was BIODIVA and the most flocculant *S. cerevisiae* and non-*Saccharomyces* pairings were VL3 + BIODIVA and RC212 + BIODIVA, suggesting that mixed species flocs may have formed.

These yeast combinations, along with *S. cerevisiae* controls of VL3 and RC212 alone, were used in a 20 L-scale Pinot noir winemaking trial. Both UV/visible spectrophotometric measurement of colour intensity, and sensory evaluation of wine appearance performed by human participants, found that the mixed species fermentations resulted in wines with greater colour intensity compared to the controls. The final VL3 + BIODIVA wines were found to be deeper in colour than the VL3 control wines and while the final RC212 + BIODIVA wines were not found to be different from the RC212 control wines, the control juice used at the beginning of the experiment was significantly deeper in colour intensity than the juice used for the sequential inoculations.

Two methods of measuring anthocyanins in the wines, the Adams-Harbertson assay and high performance liquid chromatography (HPLC), confirmed that the mixed fermentation wines with higher colour intensity, had lower anthocyanin concentrations than the less intense control wines, despite anthocyanins being the principal source of colour in young red wines. This result is likely due to the phenomenon of copigmentation, which can result in wines displaying a deeper colour intensity than would be expected based on their anthocyanin content. The increases in colour intensity may be due to increased flocculation during primary fermentation, between the highly flocculant BIODIVA and the two strains of *S. cerevisiae*. A further trial found that the non-*Saccharomyces* yeast strains used in the sedimentation rate trial adsorbed significantly more pigments from Pinot noir skins than the *S. cerevisiae* strains, with BIODIVA adsorbing the most. Grape pigments are adsorbed onto yeast cell wall mannoproteins and previous research has suggested that BIODIVA and other strains of *T. delbrueckii* have a high concentration of mannoproteins compared to other yeast species. Given that flocculant yeasts have differences in cell wall mannoprotein composition compared to non-flocculant yeasts, the cell wall could be a crucial component behind the mechanism involved in the greater colour intensity of wines inoculated with BIODIVA. Further research is required to confirm this hypothesis and confirm that flocculation is the cause of colour intensity changes or if there is another aspect of BIODIVA metabolism or mixed species interactions resulting in enhanced colour intensity and copigmentation, such as increased production of acetaldehyde or pyruvic acid.

This work expands on recent studies exploring the benefits of sequential inoculation and yeast flocculation and the relationship between yeast behaviour and red wine colour. There are multiple avenues for future research to refine our understanding of mixed-species interactions, including co-flocculation, between yeast species and uncover the mechanisms responsible for the impact of sequential inoculations and flocculation on red wine colour, in particular for Pinot noir, but with further application for other red wine styles.

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Abbreviations

Abbreviation	Definition
°Brix	Degrees Brix
°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
3-AFC	3-Alternative forced choice
4MSP	4-Methyl-4-sulfanylpentan-2-one
A	Absorbance
a.u.	Absorbance units
ACE	Accentuated cut edges
ANOVA	Analysis of variance
AR	Analytical reagent
Ca^{2+}	Calcium ion
cm	Centimetre(s)
CO_2	Carbon dioxide
d	Difference
DAP or $(NH_4)_2HPO4$	Diammonium phosphate
g	Gravitational force equivalent
g	Gram(s)
h	Hour(s)
ha	Hectare(s)
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography

HSD	Honest significant difference
HSO_3-	Hydrogen sulfite (bisulfite ion)
kg	Kilogram(s)
L	Litre(s)
M	Molar concentration
M-3-G	Malvidin-3-glucoside
mg	Milligram(s)
MI	Mannose-insensitive
min	Minute
mins	Minutes
mm	Millimetre(s)
mM	Millimolar
mL	Millilitre(s)
MLF	Malolactic fermentation
MOX	Micro-oxygenation
n	Number
NIR	Near-infrared
nm	Nanometer
ns	Not significant
NZ	New Zealand
OD	Optical density
PMS	Potassium metabisulfite
ppm	Parts per million
RGB	Red green blue
rpm	Revolutions per minute

s	Second(s)
SGM	Synthetic grape must
spp.	Species (plural)
SO_2	Sulfur dioxide
SWM	Synthetic wine medium
TA	Titratable acidity
UV	Ultraviolet
v/v	Volume/volume
VA	Volatile acidity
Vis	Visible
V_{max}	Maximal fermentation rate
YAN	Yeast assimilable nitrogen
YPD	Yeast extract peptone dextrose

Chapter 1. Introduction and Objectives

1.1. Introduction

In general, consumer preference is for deeper colour in red wines; however, in wines made from thin-skinned varieties such as Pinot noir, this can be difficult to achieve due to the nature of the variety. The flocculation of yeast, which sees thousands of cells cluster together and separate from the wine through sedimentation, may have potential to help improve colour intensity in wines such as Pinot noir.

Colour intensity is an important component in the sensory evaluation of red wine. This presents a challenge for winemakers when working with varieties such as Pinot noir, where it is difficult to extract and stabilise the phenolic compounds associated with colour. Preliminary data in the literature has shown that during alcoholic fermentation, inoculation with *Saccharomyces cerevisiae* strains with greater flocculation ability relative to other strains, may have the potential to improve the colour intensity of red wines. Flocculation also has other benefits, such as reduced need for clarification and filtration and greater wine recovery after pressing. However, only a small proportion of wine yeasts flocculate, and the primary yeast used for alcoholic fermentation, *S. cerevisiae*, does not flocculate to the same extent as many non-*Saccharomyces* yeast species. Since co-flocculation between mixed yeast species has been demonstrated, there is significant potential to investigate the use of sequential fermentation with non-*Saccharomyces* species and *S. cerevisiae*, to promote mixed species flocs, thereby increasing wine colour intensity. This research aims to identify the best co-flocculation partner for *S. cerevisiae* from a series of commercially available non-*Saccharomyces* yeast species. The flocculation capabilities of different yeast species and strains can be established through sedimentation rate assays. The best non-*Saccharomyces* candidate will be selected and trialled alongside commercial *S. cerevisiae* strains in a Pinot noir fermentation, with the goal of increased flocculation and therefore increased colour intensity in finished wines.

1.2. Objectives

The objectives of this research are to:

- Identify which commercially available non-*Saccharomyces* yeast species form flocs with *S. cerevisiae* in wine fermentation conditions.

- Establish whether the sequential inoculation and co-flocculation of *S. cerevisiae* and the selected non-*Saccharomyces* yeast during alcoholic fermentation result in deeper colour intensity of Pinot noir wines compared to a control.

Despite its difficult nature, Pinot noir is New Zealand's second most planted grape variety after Sauvignon blanc, and most planted red variety. This research will contribute new knowledge on the co-flocculation abilities of commercially available non-*Saccharomyc*es yeast species with *S. cerevisiae* and how this can achieve greater colour intensity in Pinot noir wines. This interaction between yeast species will be straightforward for New Zealand winemakers to replicate in their own Pinot noir fermentations, resulting in a new tool in the toolbox for winemakers to use to enhance wine colour intensity and stability.

Chapter 2. Literature Review

2.1. Pinot noir wine production in New Zealand

Vitis vinifera L. cv. Pinot noir is a famous grape variety originating from Burgundy, France. Pinot noir is used to produce some of the world's most prestigious, high quality wines from renowned growing regions, including the Grand Cru vineyards of Burgundy, other areas of France and further afield in new world locations such as Oregon, in the United States, and Central Otago, in New Zealand. The finest examples of Pinot noir wines have complex aromatic profiles of ripe red fruit, earthy notes and well-integrated oak characters. On the palate, the wines are balanced with low tannins, high acidity and a silky mouthfeel (Robinson & Harding, 2015). Under optimal conditions Pinot noir vines can produce these aromatically distinctive and high quality wines, but the variety is particularly sensitive to the environment it grows in (Cantu, et al., 2021; Ledderhof et al., 2014).

Pinot noir is New Zealand's second most planted grape variety after Sauvignon blanc, with a vineyard producing area of 5,625 hectares. In the 2020 vintage 34,105 tonnes of fruit was harvested. New Zealand Pinot noir is consumed both domestically and internationally, with 10.3 million litres exported in 2020 (New Zealand Winegrowers, 2021). The cooler, more southerly regions of Marlborough, Central Otago, Wairarapa and North Canterbury are the country's largest producers of Pinot noir. Despite its popularity and importance to the New Zealand wine industry, Pinot noir is a challenging variety for winegrowers. Its thin skins and tight bunches make Pinot noir susceptible to diseases and climatic changes in the vineyard, while poor colour extraction and low pigment stability cause difficulties for winemakers trying to achieve deeper colour intensity (Carew et al., 2013). Government and industry bodies have made considerable investments in research to understand and improve the production of quality Pinot noir in New Zealand. The Ministry of Business, Innovation and Employment and New Zealand Winegrowers have contributed $10.3 million to the Pinot Noir Programme: Quality and Productivity, Diversification, run by the Bragato Research Institute. The programme began in 2017 and will run until September 2022 and the research spans sensory evaluation, chemistry and viticultural and winemaking techniques - all with the objective of producing high quality Pinot noir at higher yields in order to grow returns (Bragato Research Institute, n.d.). Similar goals are shared by key Pinot noir producers around the world, including in Oregon, where research has examined how growers can increase yields while retaining the high quality the

region is known for (Uzes & Skinkis, 2016). The quality of red wines, such as Pinot noir, are judged on several important attributes, including the aromas, mouthfeel, the levels of acidity, tannins and alcohol and most importantly the wine's overall complexity and balance (Parr et al., 2011). The appearance of Pinot noir wine, in particular its colour, is also considered to be an important component of quality. It is the first attribute of a wine to be evaluated and has a strong influence on consumer perception (de Freitas et al., 2017). Colour may also affect the retail price of the wine (Dias Araujo & Kilmartin, 2020) (Figure 2.2).

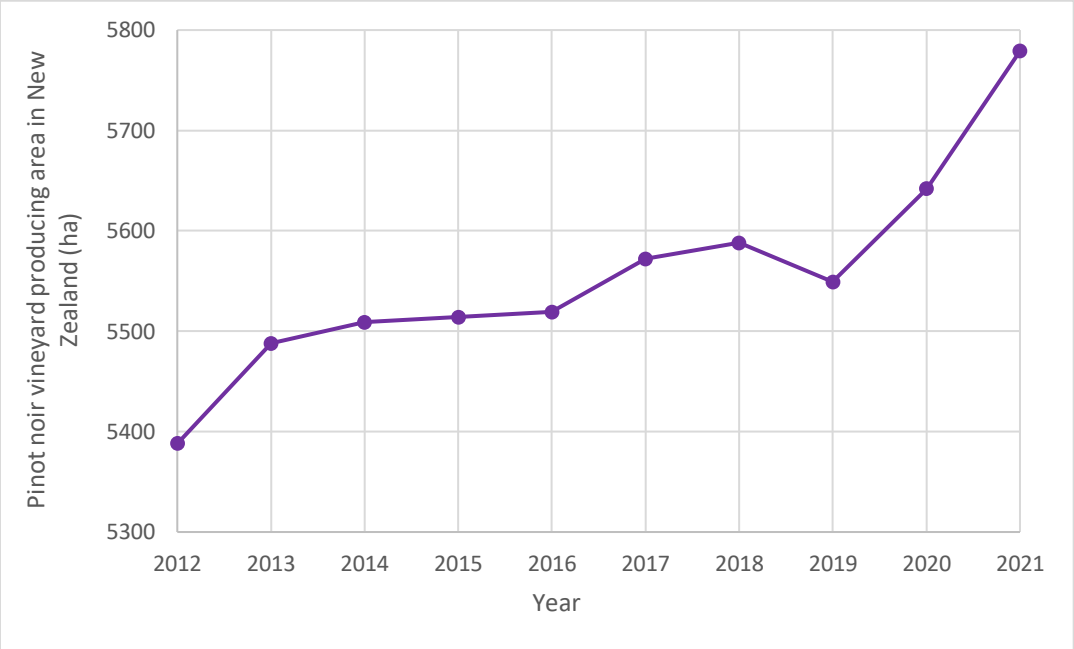


Figure 2.1. Changes in New Zealand Pinot noir vineyard producing area (ha) between 2012-2021 (Adapted from New Zealand Winegrowers, 2021).

2.2. Composition of Pinot noir wines

All wines, including Pinot noir, are comprised primarily of water and ethanol. The main differences between wines produced from different varieties, from different vintages or styles, can be attributed to the comparatively small but significant presence of a variety of compounds that give wines their unique appearances, aromas and palates. Aroma compounds are found in low concentrations but are particularly important for a wine's complexity and flavour profile. Hundreds of volatile aroma compounds have been identified in Pinot noir but only a fraction of these are present in concentrations above their individual odour detection thresholds and contribute to the sensory experience of the wine (Longo, Carew et al., 2020). Other components of Pinot noir include organic acids, sugars, minerals, glycerol and polyphenols, such as tannins,

which play an important role in a wine's mouthfeel, and anthocyanins, which give red wine its colour (Kennedy, 2008).

2.3. Importance of colour in Pinot noir wine

2.3.1 Factors contributing to Pinot noir colour

The principal source of colour in young red wines is free anthocyanins (He et al., 2012). Red wines contain a number of different anthocyanins, usually found in total concentrations of approximately 500 mg/L in young, full-bodied red wines (He et al., 2012). The only anthocyanins found in Pinot noir wines are delphinidin, cyanidin, petunidin, peonidin and malvidin 3-monoglucosides (Gao et al., 1997). Other red wines can also contain a sixth anthocyanin, pelargonidin-3-glucoside (He et al., 2012). Research by Dimitrovska et al., (2011) found that malvidin-3-glucoside was most abundant, making up 67.1% of anthocyanin content in Pinot noir wines. However, free anthocyanins are highly reactive and not particularly stable. Colour stability in red wine is achieved through a complex set of reactions, including polymerisation with catechins and proanthocyanidins, and the formation of new pigments such as pyranoanthocyanins (Oliveira et al., 2010). Pyranoanthocyanins are relatively stable compared to anthocyanins and are not bleached by bisulfite (de Freitas & Mateus, 2011). Certain compounds derived from wine contact with oak can also help stabilise red wine colour (Garcia et al., 2012; Gonzalez-Centeno, 2016). The form of anthocyanins present is heavily influenced by the wine's pH (de Freitas et al., 2017). Anthocyanins are derived directly from the grape skins. However, compared with other red varieties, Pinot noir has a low anthocyanin content, and the form of anthocyanins that are present are less stable (Carew et al., 2013). Because anthocyanins react with tannins to form more stable pigmented polymers, tannin content is also important for red wine colour intensity (Bird, 2010). Achieving an adequate concentration of tannin is also difficult when working with Pinot noir, due to the variety's low ratio of skin tannin in comparison to seed tannin. Seed tannin is much harder to extract and is more likely to be extracted later on in the fermentation (Carew et al., 2013). Therefore, achieving the desired depth of colour in Pinot noir wines has always been a challenge for winemakers, and various techniques have arisen to increase colour intensity.

2.3.2 Consumer and industry colour preferences

The appearance of a beverage will shape a consumer's first impression and then continue to influence their overall enjoyment, or lack of, as they drink it. Philipsen et al. (1995) found that adults are more sensitive to visual changes over flavour changes in beverages. Colour in particular had a significant influence over how the study participants rated a cherry-flavoured beverage, with deeper colour intensity being associated with increased flavour concentration. In formal sensory analysis of wine, colour and clarity are generally the first attributes to be assessed. According to Iland (2000), wines with more colour intensity may have higher concentrations of appealing flavour and aroma compounds and tend to perform well during sensory evaluation. The judgement of a wine's quality is influenced by both intrinsic cues, including the appearance and aroma, and extrinsic cues, such as the price and branding. The opinions of consumers are generally based on their overall enjoyment of a wine, and they will often link the appearance of a wine with the pleasure of drinking it (Charters & Pettigrew, 2007). In comparison, trained wine professionals tend to be less influenced by visual cues and make broader judgements that encompass a wine's complexity and adherence to varietal typicity. Valentin et al. (2016) compared the judgements of French and New Zealand wine professionals to see if colour was a factor when deducing the quality of Pinot noir. Both the French and New Zealand professionals did not consider colour to be a major factor, instead basing their analysis on the perceived balance of each wine. In a recent study involving a panel of New Zealand wine professionals, Parr et al., (2020) found that Pinot noir colour influenced the judges' assessment of the wines at either end of the quality and price spectrum but otherwise, visual influence was not considered to be important. However, the visual appearance of any food or beverage is crucial in influencing consumer opinion. The colour, particularly of red wine, has always been one of the first major characteristics to be evaluated by wine drinkers (de Freitas & Mateus, 2011). Limited research has been conducted on consumer preference for Pinot noir colour, but studies on other grape varieties have found consumers prefer a deeper colour. In research on Italian Novello-style wines, Parpinello et al., (2009) discovered that consumers gave higher quality ratings to wines with more intense colour. In Australia, the market demand for more intensely coloured wines and fruity flavours have led to longer ripening periods for Cabernet Sauvignon (Bindon et al., 2014). There is a general consensus that these findings also apply to other red grape varieties, including Pinot noir. In a New Zealand context, preliminary consumer sensory data from the New Zealand Pinot Noir Programme indicates consumers prefer deeper coloured Pinot noir wines (M. Kinzurik,

personal communication, October 12, 2020). The hue and intensity of Pinot noir colour can also influence its price. Dias Araujo and Kilmartin (2020) found that there was a slight correlation between the retail price of Pinot noir and the total red pigments present in the wine (Figure 2.2), with the most expensive wine exhibiting the deepest colour intensity and the lowest priced wine having the least colour intensity.

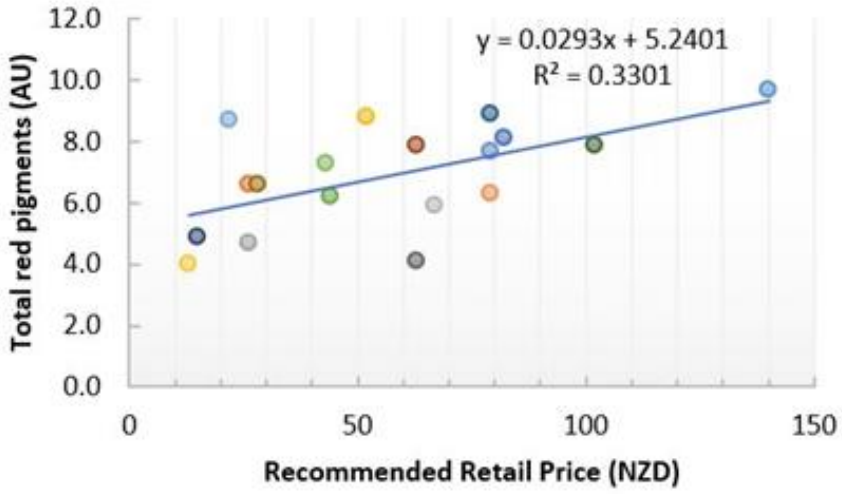


Figure 2.2. Relationship between total red pigments and retail price of NZ Pinot Noir wines (Dias Araujo & Kilmartin, 2020).

2.4. Impact of viticulture and winemaking techniques on Pinot noir wine colour

A wine is the product of its terroir - the sum of terrain, soil, climatic and winemaking influences (Vaudour, 2002). A wine's quality and style are determined by a wide variety of factors in both the vineyard and the cellar. Each of these factors, from the geography of the vineyard site to the fermentation techniques employed by the winemaker, affects the outcome when a glass of the resulting wine is eventually poured. This includes the colour of the wine, which is shaped by terroir much the same as other wine quality attributes. Due to the difficult nature of extraction in Pinot noir winemaking, several techniques have arisen to increase the colour intensity and tannin content of these wines. Aspects of alcoholic fermentation such as temperature, yeast species, and cap management can influence the extraction of anthocyanins (Božič et al., 2020; Chittenden & King, 2020; Gao et al., 1997). Other techniques used before or after fermentation, such as cold soaking, micro-oxygenation (MOX), oak use, extended

maceration and the inclusion of whole bunches during fermentation can also influence Pinot noir colour (Casassa et al., 2019; Casassa et al., 2018). The challenge for viticulturalists and winemakers is first to produce fruit with the highest anthocyanin content, then extract the phenolic compounds responsible for colour and finally, ensure colour stability as the wine ages. The effectiveness of these methods is varied and some are based on tradition rather than science (Casassa et al., 2019).

2.4.1 Clone and rootstock selection

Limited research has been conducted on the influence of clone and rootstock selection on red wine colour, despite these factors having considerable impact on other wine properties, such as the concentrations of aroma compounds or tannins. A comparison of 20 Pinot noir clones by Castagnoli and Carmo Vasconcelos (2006) found differing levels of skin anthocyanin concentration between the clones. Winemakers have preferences for various clones based on the characteristics they confer to finished wine, which can include deeper colour, and will often use a blend of clones to produce the desired result. The Riversun Nursery, the source of vines for many wineries in New Zealand, has a choice of 22 Pinot noir clones, all with different attributes and potential for colour intensity (Riversun, n.d.).

2.4.2 Leaf removal

Research has found that defoliation can positively impact colour in Pinot noir wines (Song et al., 2015; Lee & Skinkis, 2013). Bunches exposed to sunlight and ultraviolet (UV) radiation produced wines with substantially higher colour density, anthocyanins and total pigment content. Lemut et al. (2013) compared the effect of early leaf removal, at pre-flowering and fruitset, with later leaf removal, at veraison, on Pinot noir wines. The researchers found that colour density improved by 33% with pre-flowering leaf removal and 28% with leaf removal at fruitset compared to controls. Leaf removal at veraison increased colour density by 8%. Climate change threatens the effectiveness of this technique, with open canopies risking grape sunburn and potentially hindering the biosynthesis of anthocyanins.

2.4.3 Pre-fermentation cold soak

Cold soaking involves allowing the juice to sit in contact with the skins and seeds prior to alcoholic fermentation, with the goal of extracting phenolic compounds. This period can last from several hours up to 12 days (Aleixandre-Tudo & du Toit, 2018). The temperature is kept

low to prevent the growth of yeast and onset of fermentation. Without the presence of ethanol, colour can be extracted but excess tannin is not. Research by Chittenden and King (2020) found that a five-day cold soak, with no plunging, helped Pinot noir wines develop more stable colour (Figure 2.3).

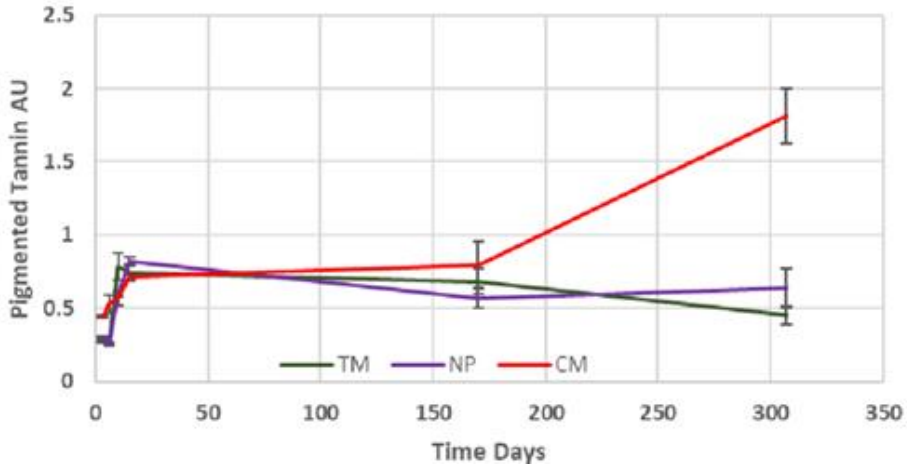


Figure 2.3. Pigmented tannin content (measured in absorbance units) of Pinot noir wines following three winemaking procedures, beginning at day 1. TM: Traditional maceration. NP: Not plunged. CM: Cold maceration and not plunged (Chittenden & King, 2020).

However, the effectiveness of this treatment can change depending on vintage conditions and grape variety. Casassa et al. (2015) found cold soaking improved colour intensity in Barbera D'Asti and Cabernet Sauvignon wines, but decreased colour in Pinot noir. Casassa et al., (2019) compared combinations of four and six winemaking techniques to control wines, across two consecutive vintages. The researchers found that for the cooler 2014 vintage, Pinot noir wines made using a cold soak and partial whole cluster fermentation had the deepest colour intensity. But in 2015 the opposite occurred, with the cold soak treatment appearing to diminish colour saturation.

2.4.4 Alcoholic fermentation

Certain aspects of primary fermentation have a significant impact on the colour intensity and stability of red wines. Given that grape skins are the source of colour in all red wines, cap management is an important consideration for the winemaker. Sparrow et al. (2016) trialled different methods of cap management during alcoholic fermentation of Pinot noir, including conventional daily plunging, a submerged cap and reduced skin particle size, also known as accentuated cut edges (ACE). The ACE technique was found to increase phenolic extraction

and improve the wine's sensory characteristics, including colour. The inclusion of a proportion of whole bunches in Pinot noir fermentations is a popular technique to change the flavour profile of the wine, increasing freshness and vibrancy, but it can also influence colour. Anecdotally, many winemakers believe the presence of stems reduces the colour intensity of the final wine. Limited research on whole bunch influence on Pinot noir colour has been conducted but Sun et al. (2001) trialled several fermentation techniques using Tinta Miúda grapes and discovered that wines made without stems contained higher concentrations of anthocyanins than those made with stems included. The temperature and speed of primary fermentation also play a key role in colour development. The extraction of monomeric anthocyanins during fermentation climbs in line with increases in temperature and then begins to drop when fermentation ends. In contrast, the more stable polymeric anthocyanins start to develop during fermentation and then continue to increase post-fermentation. The use of oak, in the form of barrels, chips or staves, during fermentation and maturation can also influence red wine colour. Oak-derived compounds such as ellagitannins, phenolic acids, and furanic and phenolic aldehydes can help stabilise the colour as a wine matures (Garcia et al., 2012; Gonzalez-Centeno, 2016). Raising the fermentation temperature of Pinot noir from 20 to 30 °C has been found to also stimulate the production of polymeric anthocyanins, even once fermentation has finished (Gao et al., 1997). Innovative new techniques are also being trialled. The correct use of pectolytic enzymes can increase colour intensity, due to the enzymatic breakdown of grape skin cell walls; however, the careful preparation of the enzyme addition is necessary. The presence of ß-glucosidases can instead cause a reduction in colour intensity, as anthocyanins are converted to aglycones (Sacchi et al., 2005). Sparrow et al. (2020) found a supplement made from fresh and fermented Pinot noir skins could be added to wine to improve both the hue and colour stability. However, if grape seeds are included in the supplement, then the formation of non-bleachable pigments is compromised. Most importantly for this research, the winemaker's choice of yeast strain to conduct alcoholic fermentation can have a significant influence on red wine colour. A variety of interactions can take place between yeast, the by-products they release during fermentation and phenolic compounds (Carew et al., 2013). Different impacts on colour are also possible when different yeast species and strains are used sequentially or co-inoculated. This research will consider how yeast behaviour, in particular the ways in which species interact, can be utilised by winemakers to improve Pinot noir colour.

2.4.5 Post-fermentation extended maceration

Winemakers may also choose to keep red wine in contact with the skins once primary fermentation is complete. This period of extended maceration is designed to continue the extraction of phenolic compounds. Generally, this technique leads to an increase in tannin but not anthocyanin content (Sacchi et al., 2005). In a study by Casassa et al., (2019), an extended maceration of 30 days increased the extraction of tannin in Merlot and Cabernet Sauvignon but had a detrimental effect on colour. Limited research has been conducted on varieties with lower phenolic content, such as Pinot noir. However, the negative impact on colour during experiments with other grape varieties suggest this approach should be avoided.

2.4.6 Sulfur dioxide

The addition of sulfur dioxide (SO_2), commonly used in the wine industry as an antioxidant and to prevent unwanted microbial growth, can impact the colour of red wine in a variety of complex ways. According to He et al. (2012), the amount of free sulfur dioxide is the most important factor contributing to the colour of a young red wine. At red wine pH, generally between 3.3 to 3.6, sulfur dioxide is mainly present as the bisulfite ion (HSO_3-). The bisulfite ion binds with monomeric anthocyanins to produce a colourless compound, anthocyanin-4-bisulfite (Jurd, 1964). This reaction creates a bleaching effect on red wine colour but it is reversible. Not all anthocyanins are susceptible to bisulfite bleaching, such as polymeric pigments which are generally resistant. The extent of the colour loss is dependent on the wine's pH, with less bleaching occurring at lower pH due to a decreased concentration of the bisulfite ion (Jurd, 1964). Sulfur dioxide can also play a positive role in the development of red wine colour. Bakker et al. (1998) found the extraction of anthocyanins from Tinta Roriz grapes increased with larger additions of sulfur dioxide prior to primary fermentation. In the long term, the presence of sulfur dioxide helps preserve the desired hue of red wines as it prevents browning while the wine matures.

2.4.7 Malolactic fermentation

Malolactic fermentation (MLF) can result in colour loss in red wine, due to a reduction in polymeric pigments. These pigments can be formed via reactions with acetaldehyde and pyruvic acid, both of which are degraded by the MLF bacteria *Oenococcus oeni* (Osborne & Burns, 2015). In a study of four Spanish red varieties, all wines saw significant decreases in colour intensity following MLF (Martínez-Pinilla et al., 2011). For Pinot noir, Carew et al.

(2013) found that the *S. cerevisiae* Lalvin RC212 yeast strain produced wines with high anthocyanin concentrations; however, this benefit was then lost as the effect was reversed following MLF.

2.4.8 Micro-oxygenation

Micro-oxygenation (MOX) is the periodic addition of small quantities of oxygen to wine and is generally carried out as the wine matures after primary fermentation. A high ratio of anthocyanin to flavan-3-ol content contributes to MOX-induced colour changes. These compounds decrease during MOX treatments and the number of polymeric pigments increases, which leads to greater colour intensity. Durner et al., (2015) found the colour intensity of Pinot noir wines could be enhanced by a MOX dosage of 20 mg/L/month; however, this positive effect was vintage dependent. In recent work by Yang et al. (2021), 30-day MOX treatments led to deeper colour intensity in Pinot noir wines, despite also causing a decrease in anthocyanins. The MOX wines saw a loss of malvidin-3-glucoside and total anthocyanins over the 30-day period, indicating that the treatments may not be suitable for light-coloured wines with low initial anthocyanin content. The MOX treatments increased the concentration of acetaldehyde in the wine, which can react with anthocyanins to form pyranoanthocyanins, which contribute to colour intensity.

2.5. Impact of yeast species, strain and behaviour during alcoholic fermentation on red wine colour intensity

The fermentative powers of yeast are integral to the winemaking process. Yeast convert sugar into ethanol and carbon dioxide (CO_2), turning grapes into wine. During this process, many complex reactions are occurring, and the species, strain and behaviour of the yeast population can alter the composition and quality of the final wine (Darriet et al., 1995; Howell et al., 2004; Pretorius et al., 1999; Sweigers et al., 2005). At the beginning of primary fermentation, a diversity of yeast species is present on the fruit. As fermentation progresses, the population of *S. cerevisiae*, despite being very small initially, increases and takes over while non-*Saccharomyces* species die out (Goddard, 2008). Fermentation is almost always completed by *S. cerevisiae* due the unique characteristics that allow it to survive in wine conditions. Winemakers have the choice of allowing fermentation to occur spontaneously or adding yeast themselves. Using a commercial yeast means a winemaker has more control over the fermentation process and can specifically select a yeast that will enhance or diminish certain

characteristics in the final wine (Borneman, et al., 2016). This includes red wine colour, as the behaviour and by-products of the yeast performing primary fermentation can have a considerable impact on colour intensity.

2.5.1 Yeast behaviour

The behaviour of and interaction between yeast cells varies greatly depending on the species and strain. The diversity of yeast activity means that the particular yeast chosen by a winemaker to initiate a red wine fermentation can have a significant impact on the colour intensity of the wine. Yeast can affect the colour of red wine in several complex ways. Yeast cells react directly with phenolic compounds and can also react with their own by-products to influence the properties of the wine. For example, the production of stable pyranoanthocyanins, such as vitisins, is important for colour retention as a wine matures. Pyranoanthocyanins are formed when anthocyanins react with metabolites produced by yeast during fermentation, such as pyruvic acid and acetaldehyde (Marquez et al., 2013). These pigments are more resistant to the bleaching effect of sulfur dioxide than anthocyanins, further improving colour longevity. Vinylphenolic pyranoanthocyanins are another type of pyranoanthocyanins, and their formation is dependent on the decarboxylation of hydroxycinnamic acids and formation of vinylphenols. Božič et al. (2020) screened two commercial *S. cerevisiae* and 93 non-commercial non-*Saccharomyces* wine yeasts for their level of hydroxycinnamate decarboxylase activity and therefore potential to form vinylphenolic pyranoanthocyanins. The results ranged from 0% to 91.1%, indicating large variation across yeast species and strains. Colour can also be affected by the flocculation tendencies of yeast, which will be investigated in this research.

2.5.2 Impacts of different yeast species and strain on wine colour

The nature of all the above reactions is primarily dictated by the species and strain of yeast, which has been highlighted in research on the effect of yeast choice on red wine colour. In Pinot noir, Carew et al. (2013) found that the *S. cerevisiae* strain Lalvin RC212 significantly outperformed the four other yeast strains tested. The wine fermented by RC212 had significantly higher concentrations of total pigments, free anthocyanins and nonbleachable pigments. The wine's high colour density was maintained during six months of bottle aging. Van Rensburg et al., (2007) genetically modified the commercial wine yeast *S. cerevisiae* strain Anchor VIN13 to enhance the activity of enzymes that degrade polysaccharides. They found

that the glucanase- and xylanase-producing VIN13 strain increased colour intensity in Pinot noir wine, as well as increasing phenolic compounds and decreasing wine turbidity. The choice of yeast can also influence red wine colour, as yeast strains can adsorb pigments from the grape skins during fermentation. The pigments adsorb on the mannoproteins found on yeast cell walls, increasing colour extraction from the fruit. Caridi (2012) refined an existing screening method to investigate the pigment adsorption ability of 20 yeast strains. The yeasts were grown on grape skin medium plates and the colour of the resulting yeast biomass was analysed using Adobe Photoshop, revealing a diversity of pigment adsorption levels and implications for wine colour. Yeast also interact with each other in different ways. The use of mixed blends of yeast or staggered inoculation with different yeasts is a common technique in winemaking to increase complexity. There are no consistent outcomes when different wine yeasts are used sequentially or in tandem, as interactions between non-*Saccharomyces* and *S. cerevisiae* are dependent on the strain of each species (Bordet et al., 2020). Research has found some yeast combinations can have an effect on red wine colour. Nardi et al., (2018) trialled the co-inoculation of the non-*Saccharomyces* species *Torulaspora delbrueckii* with *S. cerevisiae* in Barbera wines, which resulted in deeper colour intensity compared to *S. cerevisiae* alone. The characteristics of individual yeast species and strains have a significant impact on wine properties, including colour intensity. This variability can occur due to a range of factors, from the by-products secreted during fermentation, yeast enzyme activity, colour adsorption ability to interactions with other yeast.

2.6. Yeast flocculation

2.6.1 Process of yeast flocculation

Flocculation is a biochemical process that also requires physical interaction. Flocculation is where yeast cells cluster together through cell-to-cell binding and form aggregates called flocs. These flocs can contain thousands of yeast cells and will separate and rapidly settle to the bottom of the vessel through sedimentation (Soares, 2010). Flocculation occurs when protruding glycoproteins on the surface of yeast cells, called flocculins, bind to the α-mannan carbohydrates on the cell walls of other yeast cells (Rossouw et al., 2015). Calcium is necessary for this process to occur, as Ca^{2+} binds to flocculins and ensures their structure is correct. Mannose residues are always present in the cell walls; therefore, it is the presence or absence of flocculins that determines a cell's flocculation capability (Verstrepen et al., 2003). Flocculation can be reversed with the use of a chelating agent which removes the essential

calcium ions, or the addition of mannose which reduces potential flocculin binding sites by displacing the mannose residues on cell walls (Vidgren & Londesborough, 2011).

2.6.2 Role of yeast flocculation

Flocculation is believed to be a protective mechanism in response to environmental stress (Rose, 1984). The flocs formed also provide excellent conditions for cell mating, which increases the probability of long-term survival of a yeast population (Goossens et al., 2015). Only a small fraction of wine yeasts can flocculate. The ability of *S. cerevisiae* strains to flocculate is controlled by a family of *FLO* genes, of which nine have been identified. These *FLO* genes encode flocculin proteins. Transcription of the *FLO* genes is affected by stress factors including the nutritional condition of the cells (Verstrepen et al., 2003). Three important flocculation phenotypes have been identified in *S. cerevisiae*, Flo1 type, New-Flo type and mannose-insensitive (MI) type, which are classified based on their sensitivity or insensitivity to different sugars (Govender et al., 2011). Flocculation in yeast strains with the Flo1 phenotype are inhibited only by mannose, indicating that the flocculins on these cells will only bind with mannose. Flocculins of New-Flo phenotype strains can bind with a wider range of sugars, including mannose, sucrose, maltose, maltotriose and glucose. Attempts to define the precise mechanisms behind flocculation have been further complicated by the discovery of the third phenotype, the MI type, which is unaffected by mannose and does not require calcium for flocculation. Flocculation in these strains is induced by ethanol (Dengis et al., 1995). Research has shown that the longer the protein produced by the *FLO* gene, the stronger the flocculation potential of the strain. Of the nine key *FLO* genes, *FLO1* contains the most repeats and is the longest and therefore the most likely to lead to increased flocculation (Vidgren & Londesborough, 2011). It is possible to use genetic engineering to control the timing and extent of yeast flocculation; however, genetic modification is strictly regulated and generally disapproved of by the wider public, meaning that industrial applications are more limited and depend on the regulatory environment of the country in which it will be used (Verstrepen et al., 2003).

2.6.3 Factors affecting yeast flocculation

There are three groups of factors affecting yeast flocculation rates; the genetics of the yeast strain used, and two different types of external factors, those that influence the activation and activity of *FLO* genes, and those that influence the likelihood of cells physically interacting

with each other (Verstrepen et al., 2003). Environmental factors include pH, ethanol content, sugar content, temperature, and oxygen availability. The pH of the medium can alter the charge of the cell's surface, while reductive winemaking conditions inhibit flocculation (Jin & Speers, 2000). The effect of temperature is strain-dependent, with yeasts with the NewFlo phenotype requiring a narrower temperature range in order to flocculate, rather than the Flo1 phenotype which can withstand lower temperatures. At moderate levels, ethanol can enhance flocculation, whereas large quantities of fermentable sugars can occupy the flocculins and therefore prevent binding. During fermentation, the concentration of yeast cells must be high enough to ensure plenty of contact between cells so flocs can form. Similarly, the hydrodynamic conditions of the fermentation need to encourage movement of and therefore contact between cells but must not be so aggressive as to break apart the flocs that form. The properties of the yeast flocs formed can also influence flocculation rates, with their shape, density and size affecting how quickly sedimentation occurs (Vidgren & Londesborough, 2011).

2.6.4 Co-flocculation

Generally, *S. cerevisiae* does not flocculate to the same degree as certain non-*Saccharomyces* species. Rossouw et al., (2015) demonstrated for the first time that both flocculant and non-flocculant *S. cerevisiae* strains could form mixed species flocs with certain non-*Saccharomyces* yeast species, including *Cryptococcus flavescens*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum* (Figure 2.4). This process, known as co-flocculation, is a key concept for this research. The researchers found that the flocculation rates of mixed-species cultures were more than 10% higher than if the *S. cerevisiae* and other species had flocculated on their own. Co-flocculation had previously only been observed between yeast and bacteria (Peng et al., 2001).

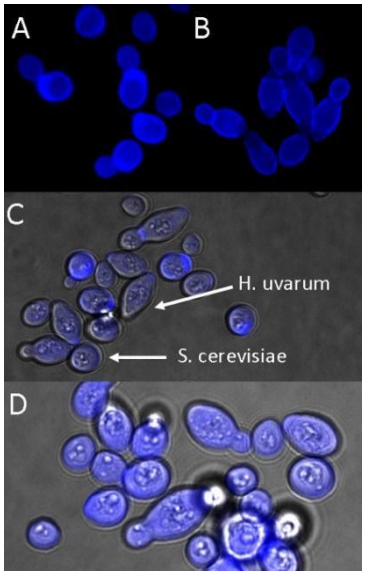


Figure 2.4. Fluorescent microscopy images showing pure cultures of A) *S. cerevisiae* and B) *Hanseniaspora uvarum* and C) and D) the flocculation of *S. cerevisiae* (circular shaped cells) and *H. uvarum* (elongated cells) in mixed species flocs (Rossouw et al., 2015).

2.7. Benefits of yeast flocculation in winemaking

In winemaking, the timing of flocculation is important, as the clustering of yeast cells too early can lead to stuck or sluggish ferments with unacceptable levels of residual sugar (Vidgren & Londesborough, 2011) or the production of harsh off-flavours (Verstrepen et al., 2003). If flocculation occurs successfully near the end of primary fermentation, the benefits are threefold, with the potential to increase colour intensity, retain greater volumes of wine following racking and assist the processes of clarification and filtration.

2.7.1 Colour intensity

Most significantly for wines made from thin-skinned grape varieties, such as Pinot noir, effective yeast flocculation can result in greater colour intensity. Varela et al. (2020) tested 95 strains of *S. cerevisiae* using a new high throughput sedimentation rate assay to determine flocculation abilities. The strains tested were either commercially available or from the Australian Wine Research Institute Wine Microorganism Culture Collection. Based on the results of the assay, two highly flocculant strains were selected for a winemaking trial. One was a commercial strain, AWRI1688 (ZYMAFLORE VL3), and one strain was from the Australian Wine Research Institute collection, AWRI1759 (isolated from Château Cantemerle, Bordeaux). AWRI1759 significantly increased the opacity of Shiraz wines in comparison to

the other selected yeast strains and the control. While these results indicate the potential impact of flocculation on red wine colour, AWRI1759 is not commercially available for winemakers to use.

2.7.2 Wine recovery after racking

Flocculation is an efficient, cost-effective and environmentally friendly technique to remove the majority of yeast cells from wine (Vidgren & Londesborough, 2011). When flocculation happens at the end of primary fermentation, compact sedimentation of the lees occurs. This means that a greater volume of wine can be recovered after racking (Govender et al., 2011). Varela et al. (2020) found that the flocculation of yeast AWRI1688 resulted in a compact, low-volume lees which then improved the wine yield by 1% compared to another trial strain. The researchers note that while this may seem like a small increase, in an industry context, where wine can be produced in tanks with capacities in the thousands of litres, this small increase could equate to a large volume of additional wine. The ability to produce more wine from the same quantity of fruit is always in the best commercial interests of winemakers and catalogue profiles of winemaking yeasts often include their flocculation abilities as a selling point.

2.7.3 Clarification and filtration

The bulk of commercial wine undergoes the process of clarification to ensure microbial stability and meet consumer expectations of clear wines, free of sediment or haze. Various methods are available to winemakers, including the addition of fining agents, cold stabilisation, centrifugation, flotation, racking and filtration. While clarification is considered an essential part of wine production, depending on the methods employed, the process can alter the appearance and flavour of the wine, stripping colour and aroma compounds (Govender et al., 2011). It can also be costly and time-consuming. Flocculation and therefore increased sedimentation can help simplify the process and reduce the need for clarification and filtration (Rossouw et al., 2015).

2.8. Flocculation analysis methods

The flocculation ability of yeast is usually determined by measuring its sedimentation rate (Vidgren & Londesborough, 2011). The proportion of the yeast cells that drop out of suspension over a set amount of time is calculated by cell counts, optical density measurements or other means.

2.8.1 Flocculation analysis in brewing

The wine industry has many parallels with the brewing industry, where flocculation of *S. cerevisiae* is also important. For brewers, the Helm's test is the industry standard for measuring flocculation. The sedimentation rate of yeast cells is observed in a calcium sulfate solution to determine the type and extent of any flocculation. The test aims to replicate brewing conditions to provide more accurate and industry-relevant results (Vidgren & Londesborough, 2011). The American Society of Brewing Chemists (ASBC) has developed an alternative technique to measure flocculation, known as the absorbance method. A calcium sulfate solution is also used but the flocculation ability of the yeast is determined by absorbance, providing a more objective result than the Helm's test (ASBC, 1996).

2.8.2 Flocculation analysis in winemaking

In the wine industry, the principles of the Helm's test were applied by Varela et al., (2020) where a high-throughput sedimentation rate assay and a competitive sedimentation rate assay were used to evaluate the flocculation of wine yeasts in wine conditions. The results of the two assays reflected each other, with the same strains identified as having strong or weak flocculation abilities. The high-throughput sedimentation rate assay can be replicated in industrial laboratories and involves measuring a sample's change in optical density at 600 nm over a 20-minute settling period. The method allows for the environmental factors that affect flocculation to be controlled, while having the potential to be modified to allow flocculation to be measured in different conditions, including at various pH levels, sugar concentrations, temperatures or with single or mixed cultures.

2.9. Colour analysis methods

Interest in red wine colour blossomed in the 1930s. Colour intensity in particular was considered one of the most important factors in determining price, alongside alcohol content (Heredia & Guzmán-Chozas, 1993). Since then, various methods of analysing red wine colour have been developed.

2.9.1 UV/visible spectrophotometry

Many analyses of red wine colour incorporate some form of UV/visible spectrophotometry. A spectrophotometric colour assay was first developed by Somers and Evans (1974, 1977) and the methods later adapted by Iland (2000). The broad set of measurements is still commonly

used in the wine industry today and involves recording the absorbances of wines at 280, 420 and 520 nm to determine the concentrations of red and brown pigments, total colour intensity, total phenols and hue of the wine. Additions of acetaldehyde, hydrochloric acid and potassium metabisulfite can also be made to the wine for further colour analyses, including the concentration of SO_2-resistant pigments, red pigments in the absence of bleaching effects, total red pigments, monomeric anthocyanins and chemical age of the wine. The full range of measurements and calculations is outlined in Table 2.1. In particular, red pigments and brown pigments, where higher values represent higher concentrations. The sum of the red and brown pigments then gives the total colour intensity. For hue, a lower value indicates a higher ratio of red pigments to brown pigments, while a higher value represents increased brown pigments compared to red pigments. The methods for colour intensity and hue can also be used for grape juice as well as finished wine (Burin et al., 2010), to detect any initial differences between the colour of the juice used in fermentations.

Table 2.1: Spectrophotometric measures and calculations to determine red wine colour according to methods adapted from Iland et al. (2000)

Wine property	**Absorbance**	**Comment**
Red pigments	A_{520}	Absorbance at 520 nm for the undiluted wine
Brown pigments	A_{420}	Absorbance at 420 nm for the undiluted wine
Total phenols	A_{280}	After dilution in 1 M HCl
SO_2-resistant pigments	A_{520} (SO_2)	Absorbance at 520 nm after excess SO_2 added
Red pigments in the absence of bleaching effects	A_{520} (CH_3CHO)	Absorbance at 520 nm after excess acetaldehyde added
Total red pigments	A_{520} (HCl)	Absorbance at 520 nm after dilution in 1 M HCl

Monomeric anthocyanins	A_{520} (HCl) - A_{520} (SO_2)	Total red pigments minus SO_2-resistant pigments
Total colour intensity	$A_{520} + A_{420}$	Sum of brown and red pigments
Hue	A_{420} / A_{520}	Ratio of brown to red pigments
Chemical age	A_{520} (HCl) / A_{280}	Ratio of total pigments to total phenols

2.9.2 Adams-Harbertson assay

The Adams-Harbertson assay uses spectrophotometry to measure phenolic compounds in wine, including tannins, iron-reactive phenols and most importantly for colour, anthocyanins and pigmented polymers. Harbertson et al. (2003) adapted the method for the wine industry from Hagerman and Butler (1978) who originally developed the process to measure tannin in grains. A 96-well microplate reader version of the assay has since been refined by Heredia et al. (2006), reducing costs and allowing a larger number of samples to be analysed at once. The Adams-Harbertson assay provides an accurate and efficient measure of phenolic compounds, including those that influence wine colour, without the expensive materials and equipment required to perform HPLC. Iron-reactive phenolics, tannins, anthocyanins and long and short polymeric pigments can all be measured using the assay. Converting the method from a traditional spectrophotometer to a microplate reader further lowers the cost and increases the speed of the analysis, which is crucial for decision-making in a winery during the busy harvest period (Heredia et al., 2006).

2.9.3 High performance liquid chromatography (HPLC)

HPLC is a precise tool for identifying and measuring individual compounds through separation. HPLC can detect multiple compounds in a single analysis, is easily automated and only a small sample size is required (Burin et al., 2011). HPLC can identify the anthocyanins commonly found in red wines, such as malvidin-3-glucoside, as well as other compounds that can influence colour hue and intensity like proanthocyanidins and acetaldehyde (Elias et al., 2008; Vrhovsek et al., 2001). While HPLC is extremely accurate, it faces the disadvantages of being time-consuming and requiring high setup and running costs. Further sample preparation is also necessary if using HPLC to analyse juice, as sugars and other substances that could interfere

with the analysis need to be removed (Yamamoto et al., 2015). It is generally used in research or large laboratories rather than for day-to-day analysis in a busy winery. Researchers continue to refine HPLC techniques and Laitila et al. (2019) have developed a new method using HPLC in combination with mass spectrometry to provide detailed information in a single analysis about the anthocyanins present in a wine sample.

2.9.4 Near-infrared (NIR) spectroscopy

While techniques such as HPLC can provide accurate results and commonly used in research, there is considerable interest in other methods with the potential to meet industry demand for fast and inexpensive analysis that can be performed in-house. The NIR region is part of the electromagnetic spectrum between 750 and 2500 nm and can be used to measure different compounds (Fernández-Novales, 2011). NIR spectroscopy provides a real-time fingerprint of the chemical and physical properties of a sample. This includes colour parameters and research by Cozzolino et al. (2004) found it was possible to use visible (Vis) and NIR spectroscopy to predict the concentrations of malvidin-3-glucoside and pigmented polymers during primary fermentation. While there is a high initial cost of purchasing an NIR spectrophotometer, NIR spectroscopy provides rapid results and requires minimal sample processing before analysis (Fernández-Novales, 2011). NIR spectroscopy has its limitations, as it only provides qualitative data; however, it is a practical tool for frequently and quickly monitoring changes during red wine fermentation (Cozzolino et al., 2006).

2.9.5 Sensory evaluation

When analysing red wine colour, it is beneficial to determine if any chemical differences are also visible to humans, as during regular consumption it is the human perception of colour intensity that affects judgements about the quality of a wine. Wine appearance can be evaluated using both difference and descriptive tests, to compare colour between wines or gather detailed information about the colour attributes of a wine. Sensory evaluation is frequently used in Pinot noir quality research and previous studies have involved both consumers and wine professionals analysing Pinot noir (Durner et al., 2010; Parr et al., 2020). Mohekar et al. (2017) used triangle tests to determine if a panel of untrained wine consumers could differentiate between control Pinot noir samples and samples spiked with trans-2-decenal, the aroma compound found when grapes are contaminated by the brown marmorated stink bug. This was followed by paired preference tests, to find the point at which consumers would reject the wine.

Tomasino and Bolman (2021) used triangle tests to investigate how two aroma compounds, β-ionone and β-damascenone, affected consumer perception of Pinot noir samples. Further descriptive tests were performed on samples where differences were identified. Using sensory evaluation as a tool to analyse only the appearance of a wine is useful in that the participants are not required to undergo the sometimes-extensive training needed to evaluate the organoleptic qualities of a wine. When analysis of the aromatic profile of a wine is required, the panel must first be trained to accurately identify and then quantify the intensity of certain aromas, for example ‘passionfruit’ or ‘green capsicum’. This process generally requires expert participants and can include multiple training sessions, which means significant time commitments from those involved (Benkwitz et al., 2012). Researchers must also take steps to exclude results from panel members who provide inconsistent responses (Brien et al., 1987). Similar training is required for evaluation of a wine’s palate, but simple visual assessment is much more straightforward. In recent studies Božič et al. (2021) asked panellists to rank Pinot noir wines for three colour descriptors (brown colour, purple colour and colour intensity) and Longo, Pearson et al. (2020) provided four colour descriptors (dense, red, brown and purple) for panellists to judge wines on during research about Australian Pinot noir. Sensory evaluation is a source of both quantitative and qualitative data that can be directly applied to winemaking decisions, so the final wine appeals to the target consumer.

Chapter 3. Materials and Methods

3.1. Laboratory reagents

All solutions were prepared using deionised water, except for the glutathione solution used in synthetic grape must (see Section 3.2.2), in which MilliQ water was used. The source of laboratory reagents used in this research is presented in Table 3.1.

Table 3.1: List of laboratory reagents

Chemical	**Source**
Growth and fermentation media (see Section 3.2)	Duchefa Biochemie (Haarlem, Netherlands), Merck (Kenilworth, New Jersey, United States), Mt Difficulty Wines (Bannockburn, New Zealand), Sigma-Aldrich (St. Louis, Missouri, United States)
Model wine components (see Section 3.2.5)	Sigma Aldrich (St. Louis, Missouri, United States), Thermo Fisher Scientific (Waltham, Massachusetts, United States)
Buffer components (see Section 3.2.6)	Merck (Kenilworth, New Jersey, United States), Sigma Aldrich (St. Louis, Missouri, United States), Thermo Fisher Scientific (Waltham, Massachusetts, United States)
Analytical reagent (AR) grade ethanol 99.5%	ECP Labchem (Auckland, New Zealand)
Malvidin chloride	Sigma-Aldrich (St. Louis, Missouri, United States)
Cleaning chemicals (Tristel, Decon 90)	Tristel (Cambridge, United Kingdom), Decon Laboratories (Hove, United Kingdom)

Hydrochloric acid	Thermo Fisher Scientific (Waltham, Massachusetts, United States)
Sodium hydroxide	Sigma-Aldrich (St. Louis, Missouri, United States)
Glycerol	Sigma-Aldrich (St. Louis, Missouri, United States)

3.2. Media

3.2.1 Yeast extract peptone dextrose (YPD)

YPD is a rich complete medium used for the standard propagation of yeast. Liquid YPD was prepared using a mix of 20 g/L peptone, 10 g/L yeast extract and 20 g/L D-glucose dissolved in deionised water. For solid YPD plates, 20g/L of agar was added. All YPD media were sterilised in an autoclave at 121 °C for 20 mins.

3.2.2 Grape skin medium

Grape skin medium is a form of YPD infused with dried grape skins used to assay yeast pigment adsorption. Frozen *Vitis vinifera* L. cv. Pinot noir grapes from Mt Difficulty's Station Vineyard in Central Otago, New Zealand (NZ), hand-harvested on March 27, 2018, were defrosted and the skins were manually removed. The skins were washed in deionised water and gently dried on paper towels. The skins were incubated for three days at 55 °C until dry and then ground into a powder using a Black and Decker BMC100 blender. The powder was suspended in deionised water and heated for 5 mins at 110 °C to extract pigments from the grape skins. The solution was filtered through a 7.5 cm × 7.5cm 8 ply woven gauze and measured. The corresponding double amounts of the other components, minus the agar, were added and mixed (Table 3.2). The solution was heated at 110 °C for 5 mins. Separately, 40 g/L agar was dissolved in deionised water and sterilised by autoclaving. The grape skin and agar solutions were mixed together and poured into petri dishes to solidify.

Table 3.2: Final composition of grape skin medium plates

Component	**Concentration (g/L)**

Dried Pinot noir grape skins	60
Citric acid monohydrate	50
Disodium hydrogen phosphate	25
D-Glucose	20
Casein peptone	7.5
Yeast extract	4.5
Agar	20

3.2.3 Synthetic grape must (SGM)

SGM is a chemically defined medium that mimics natural grape juice for use in trial fermentations. SGM was prepared according to an adapted version of the protocol developed by Henschke and Jiranek (1993) and Harsch et al. (2010). The constituents of the SGM (Table 3.3) reflect the ratio of sugars, amino acids, minerals, vitamins, salts, lipids and glutathione present in Marlborough Sauvignon blanc juice. Heat-sensitive SGM components were filter-sterilised with a 0.45 µm pore sized filter, otherwise the solutions were sterilised by autoclaving at 121 °C for 20 mins.

Table 3.3: SGM components

Carbon source, salts	**Concentration (g/L)**	**Trace minerals**	**Concentration (µg/L)**
D-Glucose	105	$MnCl_2.4H_2O$	198.2
D-Fructose	105	$ZnSO_4.7H_2O$	287.5
Potassium tartrate	5	$CuSO_4.5H_2O$	70.1
Malic Acid	3	H_3BO_3	25.3
Citric Acid	0.2	$CoCl_2.6H_2O$	5.7
K_2HPO_4	1.14	$NaMoO_4.2H_2O$	23.8
$MgSO_4.7H_2O$	1.23	KIO_3	24.2
$CaCl_2.2H_2O$	0.44	$FeSO_4.7H_2O$	10.8

Vitamins	**Concentration (mg/L)**	**Amino acids**	**Concentration (mg/L)**
Myo-inositol	100	L-Alanine	100
Pyridoxine hydrochloride	2	L-Arginine-HCl	400
Nicotinic acid	2	L-Aspartic acid	50
Ca-pantothenate	1	L-Asparagine	10
Thiamine hydrochloride	0.5	L-Cysteine	5
p-Aminobenzoic acid	1.0	L-Glutamic acid	100
Riboflavin	1.2	L-Glutamine	125
Biotin	0.125	L-Glycine	5
Folic Acid	0.2	L-Histidine	20
		L-Isoleucine	25
		L-Leucine	25
		L-Lysine-HCl	5
		L-Methionine	10
		L-Phenylalanine	40
		L-Proline	300
		L-Serine	60
		L-Threonine	75
		L-Tryptophan	10
		L-Tyrosine	10
		L-Valine	30
Lipids, glutathione	**Concentration (mg/L)**	**Nitrogen supplement***	**Concentration (mg/L)**
Ergosterol	15	$(NH_4)_2HPO4$	352
Tween 80	0.5 (mL/L)	**Total Yeast Assimilable Nitrogen (YAN) = 300 ppm*	
Glutathione	50		

3.2.4 Synthetic wine medium (SWM)

A 200 mL portion of SGM was fermented to produce SWM in a 250 mL flask with an airlock. SGM was inoculated with *Saccharomyces cerevisiae* wine yeast Lalvin EC-1118, with an

inoculum of 5×10^6 cells/mL. The fermentation was incubated at 28 °C, with 100 revolutions per minute (rpm) shaking, and monitored by weight loss once per day. After 13 days the rate of fermentation had slowed to 0.004 g/L of CO_2 released/ hr (dCO_2/dt) and primary fermentation was considered complete. The SWM was then cold stabilised at 4° C in a cold room for four days. SWM was centrifuged at 3000 *g* for 5 mins to remove the yeast lees. The pH was adjusted from 3.246 to 3.203 with hydrochloric acid and the alcohol was adjusted from 13.78% to 12.68% with deionised water. The SWM was then filter-sterilised with a 0.45 µm pore sized filter and stored at room temperature in a 250 mL Schott bottle.

3.2.5 Model wine solution

A model wine solution was prepared for the dilution of wine samples during colour analysis (see Section 3.6). The components, 2 g of potassium bitartrate and 60 mL of ethanol, were added to a 500 mL Schott bottle, which was filled up to 500 mL with deionised water. The pH was adjusted down to 3.5 using hydrochloric acid.

3.2.6 Assay buffers

Two buffer solutions (see Table 3.4) were prepared based on the methods of Heredia et al. (2006), for use in an assay to determine anthocyanin concentration in red wines (see Section 3.6.2). Both buffers were made in 500 mL Schott bottles.

Table 3.4: Buffer components

Solution name	Component	Concentration and preparation
Buffer A	Glacial acetic acid Sodium chloride 10% Sodium hydroxide Deionised water	200 mM 170 mM Adjust to pH 4.9 Fill to 500 mL
Buffer D	Maleic acid Sodium chloride 10% Sodium hydroxide Deionised water	200 mM 170 mM Adjust to pH 1.8 Fill to 500 mL

3.2.7 Long term storage medium

For long term storage, yeast cultures were streaked out and grown on solid YPD plates. Single colonies were then added to 200 µL liquid YPD in 96 well plates and kept at 28°C, with 150 rpm shaking, for two days. Forty-four µL of an 80% glycerol solution was added to each culture (final concentration of 15% glycerol), which were then stored at -80 °C.

3.2.8 Grape juice

During grape harvest, approximately 120 kg of *V. vinifera* L. cv. Pinot noir grapes, clone 777, was provided by Mahi Wines. The grapes were machine-picked on March 17, 2021, from the Ward Farm vineyard, located near Ward in Marlborough, NZ. The fruit was transported to the Mahi winery in Renwick, Marlborough, by truck. Juice analysis was conducted in the winery's laboratory (Table 3.5). An Anton Paar handheld density meter was used to measure °Brix and a Mettler Toledo benchtop pH meter and Thermolyne Nuova II stir plate were used to measure pH. The titratable acidity (TA) of the juice was also measured with the same pH meter and stir plate, using the TA method of titration to an end point of pH 8.2. A 0.1 M sodium hydroxide solution was used for the titration. The fruit in the picking bins was covered and stored overnight at ambient temperature in the winery. It was then used fresh in winemaking trials the following day. The level of yeast assimilable nitrogen (YAN) was later measured from juice samples taken during harvest and frozen, using the Megazyme Primary Amino Nitrogen and L-Arginine/Urea/Ammonia assay kits. The microplate procedures for the two assays were performed according to the manufacturer's instructions, using a SpectraMax iD3 plate reader.

Table 3.5: Pinot noir harvest juice analysis

Parameter	**Value**
°Brix	24.2
pH	3.5
TA	7.5 g/L
YAN	85 mg/L

3.3. Yeast

All wine yeasts used in this research were commercially available in New Zealand (Table 3.6). The non-*Saccharomyces* species chosen were all suited to red winemaking. The two *Saccharomyces cerevisiae* yeasts were selected based on the recommendation of RC212 for Pinot noir winemaking (Carew et al., 2013), and relatively high flocculation capabilities of VL3 (Varela et al., 2020). EC-1118 was used to produce SWM, as it is an efficient yeast for fermentation with good finishing ability, but was not part of the wider research.

Table 3.6: List of yeast species and strain

Strain	**Species**	**Source**	**Commercial purpose**
Lalvin RC212	*Saccharomyces cerevisiae*	Lallemand	Extract and protect polyphenols in Pinot noir
ZYMAFLORE VL3	*Saccharomyces cerevisiae*	Laffort	Produce elegant white wines with thiol-type varietal aromas
LEVEL2 LAKTIA	*Lachancea thermotolerans*	Lallemand	Raise acidity by producing high levels of lactic acid during fermentation
VINIFLORA CONCERTO	*Lachancea thermotolerans*	CHR Hansen	Mimic wild fermentation and enhance complexity
IOC GAÏA	*Metschnikowia fructicola*	Lallemand	Bioprotection and suppression of the growth of microbes responsible for acetic acid production
VINIFLORA FROOTZEN	*Pichia kluyveri*	CHR Hansen	Mimic wild fermentation and enhance complexity by boosting production of thiol aromas
LEVEL2 BIODIVA	*Torulaspora delbrueckii*	Lallemand	Increase complexity by enhancing varietal characteristics, increasing ester production and improving mouthfeel

VINIFLORA PRELUDE	*Torulaspora delbrueckii*	CHR Hansen	Increase wine body and improve palate and mouthfeel, while generating low volatile acidity
Lalvin EC-1118	*Saccharomyces cerevisiae*	Lallemand	Reliable fermentation kinetics and low sensory impact

3.3.1 Routine culture

All yeasts were in active dried form except for EC-1118 and FROOTZEN. VL3 was used in active dried form in the winemaking trials but was prepared from frozen stocks (-80 °C) for the microvinification experiments. For microvinification, active dried yeast was prepared by suspending pellets in deionised water in 1.5 mL Eppendorf tubes. Tubes were vortexed and two sterile toothpicks were used to streak each yeast onto a solid YPD plate. EC-1118, FROOTZEN and VL3, which were stored at -80 °C, were thawed and individually streaked onto solid YPD plates. Yeasts were propagated at 28 °C for two to three days, depending on when visible single colonies had grown on the plates. A single colony from each plate was suspended in 2 mL liquid YPD in a 13 mL culture tube. The 2 mL cultures were grown at 28 °C with 150 rpm shaking for three days. The tubes were vortexed and 10 µL of the culture was pipetted into flasks containing 50 mL of liquid YPD. The 50 mL cultures were left overnight at 28°C with 150 rpm shaking. During the Pinot noir winemaking trials, all yeasts used (BIODIVA, RC212, and VL3) were in active dried form and were rehydrated according to the manufacturer's instructions.

3.4. Winemaking

3.4.1 Microvinification

A total of 63 microfermentations were conducted, comprising 21 different fermentation treatments in triplicate. Sarstedt polypropylene 13 mL-culture tubes containing 8 mL of SGM were inoculated with each yeast individually and/or each *Saccharomyces cerevisiae* yeast in combination with each non-*Saccharomyces* yeast (see Section 4.2). Three culture tubes were left uninoculated as negative controls. A 0.5 mm^2 pinhole was made in each culture tube lid to allow for the release of CO_2. Prior to inoculation, cell concentrations of the 50 mL yeast pre-cultures were determined using a Neubauer haemocytometer. The cultures were transferred to

a 50 mL falcon tube and centrifuged for 5 mins at 3000 *g*. The supernatant was removed and 40 mL of deionised water was added to the remaining yeast pellet and vortexed. A 1/50 dilution was used, with 10 µL of the solution and 490 µL of deionised water pipetted into an Eppendorf tube. The tube was vortexed, 10 µL was pipetted onto the haemocytometer and a cover slip placed on top. The number of cells on five of the 25 squares was counted under 400 × magnification using a Leica light microscope. The mean number of cells was used to calculate the volume of each yeast culture to be used to inoculate the SGM at an inoculum of 5×10^6 cells/mL.

The mixed species fermentations (numbers 25-60, see Table 4.1) were sequentially inoculated, first with the non-*Saccharomyces* species and then the *S. cerevisiae* 24 h later. Tubes were weighed before and after the second inoculation so that the weight difference was accounted for in weight loss calculations. Culture tubes were kept in an incubator at 28 °C, with 100 rpm shaking, and weighed once per day to monitor fermentation progress. Fermentations were considered complete after they had lost approximately 10% of their weight and reached a weight loss rate of ~0 dCO_2/dt. Products used during microvinification and winemaking are presented in Table 3.8.

3.4.2 Primary fermentation

The day after the Pinot noir grapes were harvested (see Section 3.2.8), approximately 15 kg of fruit was transferred to each of eight 20 L food grade plastic pails, which had been cleaned with a 4.2 g/L potassium metabisulfite (PMS) and 4 g/L citric acid solution and rinsed with cold water. Triplicate 50 mL juice samples were collected from each bucket and frozen at -20 °C prior to inoculation. An addition of 10 ppm sulfur dioxide, in the form of PMS dissolved in water, was made to each bucket. Lids fitted with airlocks were used to protect the fermentations from oxidation and spoilage. Each trial fermentation was conducted in duplicate. The yeasts trialled included co-inoculations of *S. cerevisiae*, RC212 or VL3, and *Torulaspora delbrueckii*, BIODIVA, alongside controls of *S. cerevisiae* alone (Table 3.7). The co-fermentations were sequentially inoculated, with the juice inoculated first with BIODIVA, then the *S. cerevisiae* yeast, either RC212 or VL3, after 24 hours. All yeasts were rehydrated according to the manufacturer's instructions. The temperature and °Brix of each fermentation was monitored once or twice daily using an Anton Paar handheld density meter until they all reached lower than 0 °Brix. The fermentations were hand plunged using gloves twice per day until they reached 1 °Brix and were then plunged once daily. The exterior and lids of the buckets were

regularly cleaned following plunging with a sulfur citric solution to prevent spoilage. Nutrient additions of 300 ppm Nutristart Org (Laffort), a complete nutrient made from yeast autolysate, and 150 ppm diammonium phosphate (DAP) were made two days after the first inoculations, when the fermentations were between 12.1 and 15.8 °Brix. When primary fermentation was complete, triplicate 50 mL wine samples from each bucket were collected and frozen. The wines were hand-pressed using a 24 cm stainless steel colander and an 18 cm mesh sieve. A sample from each bucket was transferred into two 1 L Schott bottles and the remaining wine was returned to Mahi Wines. Clean glass marbles were added to the bottles to increase the volume of the wine and prevent ullage.

Table 3.7: Yeast combinations used in the Pinot noir fermentation trial

Bucket	**Yeast name**	**Yeast species**
1	VL3 + BIODIVA	*Saccharomyces cerevisiae, Torulaspora delbrueckii*
2	VL3 + BIODIVA	*Saccharomyces cerevisiae, Torulaspora delbrueckii*
3	RC212 + BIODIVA	*Saccharomyces cerevisiae, Torulaspora delbrueckii*
4	RC212 + BIODIVA	*Saccharomyces cerevisiae, Torulaspora delbrueckii*
5	VL3	*Saccharomyces cerevisiae*
6	VL3	*Saccharomyces cerevisiae*
7	RC212	*Saccharomyces cerevisiae*
8	RC212	*Saccharomyces cerevisiae*

3.4.3 Malolactic fermentation

Once primary fermentation was complete, the bottles were inoculated for malolactic fermentation using the lactic acid bacteria *Oenococcus oeni*, strain REFLEX MALO 360. REFLEX MALO 360 was prepared according to the manufacturer's instructions and added at a rate of 10 mg/L. The temperature of the bottles was maintained at an average of 18 °C. While undergoing malolactic fermentation, the bottles were transported from Marlborough to Auckland by car. In Auckland, the bottles were stored at 25 °C to encourage bacterial activity.

Throughout malolactic fermentation, Schott bottle lids were placed loosely on the bottles. Malic acid levels were tested using a Megazyme L-malic acid assay kit. Once malolactic fermentation was complete (< 0.1 g/L malic acid present), the bottles were racked off the heavy lees into 500 mL and 250 mL Schott bottles. An addition of 80 mg/L SO_2, in the form of a PMS solution, was made to the bottles, which were then sealed with parafilm and stored at 14 °C.

3.4.4 Settling

The wine was left to settle for six weeks. The wine in the 500 mL Schott bottles was then racked off the lees and divided into three smaller Schott bottles, one 250 mL bottle and two 100 mL bottles. Each new bottle received a 5 mg/L SO_2 addition, in the form of a PMS solution. The bottles were flushed with nitrogen gas and sealed with parafilm to prevent oxidation, then returned to the incubator to be stored at 14 °C.

3.4.5 Wine analysis

Analysis of the 16 final wines was conducted following the six-week settling period. Measures of residual sugar and volatile acidity (VA) were obtained using the Megazyme D-Fructose/D-Glucose and Acetic Acid assay kits, performed as per the manufacturer's instructions. Both assays were carried out in the microplate format, using 96 well plates and a SpectraMax iD3 plate reader. Alcohol content was determined using an Anton Paar Alcolyzer Wine M and pH was measured using a SI Analytics Lab 855 pH meter. The TA was analysed via the titration method to an end point of pH 8.2 using a 0.1 M sodium hydroxide solution, Eutech 2700 Series pH meter and Dr Schilling burette.

Table 3.8: Products used in winemaking

Product	**Contents**	**Origin**
Potassium Metabisulfite NZ	Potassium metabisulfite	Esseco
Potassium Metabisulfite, 97%, extra pure	Potassium metabisulfite	Acros Organics
Yeast (see Table 3.6)	-	-

Nutristart Org	Organic nutrient from yeast (*Saccharomyces cerevisiae*) autolysate	Laffort
Diammonium Phosphate Food Grade	Diammonium phosphate	Redox
REFLEX MALO 360	*Oenococcus oeni* bacteria	Martin Vialatte
L-Malic Acid Assay Kit	Buffer plus L-glutamate and sodium azide, NAD+ plus PVP, Glutamate-oxaloacetate transaminase suspension, L-Malate dehydrogenase suspension	Megazyme
Acetic Acid Assay Kit	Buffer plus sodium azide, NADH, ATP, PEP and PVP, CoA, D-Lactate dehydrogenase, phosphotransacetylase and pyruvate kinase suspension, Acetate kinase suspension, Acetic acid standard solution	Megazyme
D-Fructose/D-Glucose Assay Kit	Buffer plus sodium azide, $NADP^+$, ATP and PVP, Hexokinase plus glucose-6-phosphate dehydrogenase suspension, Phosphoglucose isomerase suspension, D-Glucose plus D-fructose standard solution	Megazyme
Primary Amino Nitrogen Assay Kit	*N*-acetyl-L-cysteine tablets, Ortho-phthaldialdehyde in ethanol, Isoleucine standard solution	Megazyme
L-Arginine/Urea/Ammonia Assay Kit	Buffer plus sodium azide, NADPH, Glutamate dehydrogenase	Megazyme

	suspension, Urease solution, Arginase suspension, Ammonia standard solution, L-Arginine powder standard	
Citric Acid Anhydrous 30-100 MESH	Citric acid	Weifang Ensign
Nitrogen, compressed	Nitrogen gas	BOC

3.5. Sedimentation rate assays

High-throughput sedimentation rate assays were used to measure the flocculation of yeast cells at the end of the microfermentations following the method from Varela et al. (2020).

3.5.1 Sample preparation

The 8 mL fermentations were vortexed and a 1 mL sample was taken and centrifuged at 1000 *g* for 5 min. The supernatant was removed and the yeast pellet was resuspended in 1 mL SWM (Section 3.2.4) and vortexed. The samples were incubated for 24 hours at 28 °C and 100 rpm.

3.5.2 Optical density measurements

Samples were vortexed and a 1:4 aliquot of 40 µL of the culture and 160 µL of SWM was pipetted into a 96 well plate. Three 200 µL samples of SWM were used as blanks. The optical density (OD) of the samples was measured at 600 nm using a SpectraMax iD3 plate reader. All OD readings were conducted on the precise setting, with 10 s of medium intensity shaking. Based on these values, the ratio of sample to SWM required to adjust all samples to an OD 600 nm of 1 was calculated. The appropriate volumes of the samples and SWM were pipetted into 1.5 mL Eppendorf tubes, which were vortexed. A 100 µL subsample was pipetted into a new 96 well plate and OD 600 nm was measured after 1 min medium intensity shaking to determine an initial reading (t_0). The original subsamples were incubated for 25 mins before 100 µL was pipetted from just below the liquid surface into a 96 well plate. A second OD 600nm reading, following 1 min medium intensity shaking, was taken (t_{25}). The sedimentation rate was calculated by dividing the difference between the two OD 600 nm readings (t_0 and t_{25}) by the incubation time (25 mins).

3.6. Colour analysis

Several methods were used to determine the colour intensity of the Pinot noir wines. The initial juice samples, pre-malolactic fermentation wine samples, post-malolactic wine samples and final wines were all analysed using UV/visible spectrophotometry. The final wines also underwent two additional colour analysis methods, the Adams-Harbertson red wine phenolics assay to determine anthocyanin content and sensory evaluation via human participants.

3.6.1 UV/visible spectrophotometry

A version of the colour assay developed by Somers and Evans (1974, 1977) was used to analyse all the samples collected at four different stages during the winemaking process. The methods were adapted from Iland (2000). A 1.5 mL sample of each juice or wine was pipetted into a 1.5 mL Eppendorf tube. The samples were centrifuged at 14680 *g* for 8 mins. A 1 mL subsample was pipetted into an Eppendorf Uvette disposable cuvette, which was placed into an Implen NanoPhotometer NP80 with cuvette capability. The UVette's 2 mm path length option was used to measure the samples against a deionised water blank at two wavelengths, 420 nm (A_{420}) and 520 nm (A_{520}). The absorbance values were automatically adjusted to a 1 cm path length by the NanoPhotometer and the readings were used to calculate the red pigments (A_{520}), brown pigments (A_{420}), total colour intensity ($A_{520} + A_{420}$) and hue (A_{420} / A_{520}) of the samples.

3.6.2 Adams-Harbertson anthocyanin assay

The anthocyanin concentration of the finished wines was analysed using methods from the Adams-Harbertson red wine phenolics assay (Harbertson et al., 2003) and volumes adapted by Heredia et al. (2006) for a microplate reader. The assay determines the anthocyanin concentration of a wine sample, equivalent to malvidin-3-glucoside concentration in mg/L. A series of pure malvidin-3-glucoside standards were prepared to construct a standard calibration curve to calculate the concentrations in the wine samples. For the first standard, 1 mg malvidin chloride was dissolved in 2 mL AR grade ethanol. A series of half dilutions were then carried out until eight 1 mL standards had been produced, ranging in concentration from 443 mg/L to 3.5 mg/L. The concentration of three of the standards (1. 443 mg/L, 4. 55.4 mg/L and 8. 3.5 mg/L) was validated using high performance liquid chromatography (HPLC) (see section 3.6.3). All samples and standards were assayed in triplicate. Two readings were required for the assay, measurement A and measurement D. To prepare the wine samples, 1.5 mL was pipetted into a 1.5 mL Eppendorf tube and centrifuged for 5 mins at 3000 *g*. For measurement

A, 500 µL of each sample and standard was pipetted into a 1.5 mL Eppendorf tube and then 1 mL of buffer A (see Table 3.4) was added. The tubes were mixed by inversion and a 300 µL aliquot was pipetted into a well on a 96 well plate. The microplate was incubated at room temperature for 10 mins and then the absorbance at 520 nm was determined using a SpectraMax iD3 plate reader. For measurement D, 50 µL of each sample and standard was pipetted into a microplate well and then diluted with a 50 µL addition of model wine solution (see Section 3.2.5). Two hundred µL of buffer D (see Table 3.4) was added to each well and mixed by pipetting. The microplate was incubated at room temperature for 10 mins and then the absorbance at 520 nm was determined using a SpectraMax iD3 plate reader. The difference between the two absorbance values (measurement D – measurement A) was calculated. A calibration curve was created with the results of the assay for the standards. The calibration curve was then used to convert the final values for the wine samples into mg/L of malvidin-3-glucoside.

3.6.3 High performance liquid chromatography (HPLC)

An Agilent Technologies 1200 series HPLC system was used for further analysis. The system was comprised of a G1311A quaternary pump, G2260A autosampler, G1316A column compartment and G1315D diode array detector. The system was calibrated before use for a reversed-phase Phenomenex Kinetex C18 column (2.6 µm, 100 mm x 4.6 mm, 100 Å pore size), which was used to determine anthocyanin concentrations. For each analysis, a 100 µL sample was pipetted into a Thermo Scientific 0.1 mL glass micro-insert for an Interlab 1.5 mL amber vial, which was then sealed with an Agilent screw cap ready for injection. The injection volume was 20 µL, the column was held at 25 °C and the flow rate set at 1 mL/min. Three solvents were used, Milli-Q water, a 5% (v/v) acetic acid solution and 100% acetonitrile. The gradient conditions used were 0 min (40% solvent A, 55% solvent B, 5% solvent C), 3.2 min (20% solvent A, 75% solvent B, 5% solvent C), 6.4 min (95% solvent B, 5% solvent C), 9.6 min (95% solvent B, 5% solvent C), 16 min (90% solvent B, 10% solvent C), 22.4 min (85% solvent B, 15% solvent C), 25.6 min (80% solvent B, 20% solvent C), 30.4 min (60% solvent B, 40% solvent C), 33.6 min (55% solvent B, 45% solvent C), 36.8 min (40% solvent A, 55% solvent B, 5% solvent C), and 40 min (40% solvent A, 55% solvent B, 5% solvent C), followed by a 3 min post-run. The HPLC system was operated and the data processed using Agilent ChemStation software, version B.04.02. The HPLC methods were used to determine the malvidin-3-glucoside concentration of four of the final wines (2A.V+B, 3B.R+B, 6B.V,

8B.R), which included one example of each yeast combination. Samples were centrifuged at 5 mins at 3000 *g* prior to the analysis. HPLC was also used to confirm the concentration of malvidin chloride standards used in the Harbertson-Adams anthocyanin assay (section 3.6.2). For the three standards analysed (numbers 1. 443 mg/L, 4. 55.4 mg/L and 8. 3.5 mg/L), a 500 µL sample was added to 1 mL of Buffer A (see Table 3.4) to replicate the assay and aliquots of these solutions were analysed.

3.6.4 Sensory evaluation

Two sensory evaluation sessions were held so that human participants could assess the appearance of the final Pinot noir wines. In the first session, 20 participants performed differentiation tests to determine if there was a visible difference in colour between the wines inoculated sequentially with BIODIVA and VL3 and the control wines inoculated with VL3 only, as well as a difference between the wines inoculated sequentially with BIODIVA and RC212 and the control wines inoculated with RC212 only. Four 150 mL Schott bottles of each wine were combined and 30 mL samples were measured and poured into Arcoroc Viticole 120 mL clear wine tasting glasses. Each wine sample was randomly assigned a three-digit code using the random number generator from random.org. Two types of differentiation tests were carried out at six stations. Each station involved two triangle tests and one 3-alternative forced choice (3-AFC) test. The triangle tests featured two samples of the same wine and one of a different wine and participants were asked to identify the different sample. In the 3-AFC tests, two control wines were presented alongside one mixed inoculation wine. Participants were asked to identify which of the samples was deepest in colour intensity (see Appendix 8.1). A Latin square arrangement was used to ensure all possible orders and combinations of the wine samples were used for the two tests. The different possibilities were each randomly assigned to a station. The session was held in a quiet, temperature-controlled room with natural light. Each set of three wines was placed on an A4 piece of white paper as a background so that participants could more accurately evaluate the colour of the samples. All responses were recorded on paper ballot sheets. The second sensory evaluation session was held virtually via a Google Forms survey. Following the results of the first session, only the VL3 mixed and control wines were compared. A 15 mL sample of each of the four VL3 mixed wines (1A, 1B, 2A, 2B) was combined in a beaker, as was a 15 mL sample of each of the four VL3 control wines (5A, 5B, 6A, 6B). A 30 mL sample of each combined wine was poured into two Arcoroc Viticole 120 mL clear wine tasting glasses and randomly assigned a three-digit code using the

random number generator from random.org. The four wine glasses were separated into two stations comprising one of each of a VL3 mixed wine and VL3 control wine, in different orders. The wines were photographed against a plain white paper background in neutral lighting using a Nikon DSLR D600 digital camera. The photos were resized using Pixlr photo editing software to ensure uniformity. Two separate Google Forms surveys (see Appendix 8.2), reflecting each of the stations, were prepared. In each survey, participants were provided with photographs of the two wines they were evaluating together, as well as of the wines individually and from different angles. In the survey the participants were asked to rank each of the two wines on three colour attributes - hue, brightness and intensity - on a five-point scale. They were also asked to rank the extent to which they agreed or disagreed on a five-point scale with two statements about the colour of the wines. The statements were 'I like the colour of this wine' and 'Based on its colour, I would expect this wine to be of high quality'. A total of 41 responses across the two stations were recorded. The participants recruited for both sensory sessions were all over the age of 18 and not colour blind (self-identification). The sensory evaluation sessions were approved by the University of Auckland Human Participants Ethics Committee on July 20, 2021, reference number UAHPEC22881.

3.7. Pigment adsorption

3.7.1 Yeast growth

The eight yeasts used in the microvinification and sedimentation rate trials (see Table 3.6) were grown on grape skin medium plates to observe their pigment adsorption capability. The yeasts were routinely cultured on YPD plates for two days and then 10 µL of biomass of each yeast was collected using a sterile loop. The biomass was suspended in 200 µL sterile water which was then mixed and pipetted onto a grape skin medium plate (see Section 3.2.2) and distributed evenly on the surface using a sterile L-shaped glass spreader. Two grape skin medium plates were inoculated with the non-*Saccharomyces* yeasts and one plate was inoculated with the *S. cerevisiae* yeasts. A YPD plate was also inoculated with each yeast as a control. The plates were placed inside 4 L plastic food grade pails, which were flushed with nitrogen for 1 minute and then sealed with the plastic lid and parafilm to prevent oxidation of the skin pigments and induce a semi-fermentative response of the yeast. The plates were incubated at 28 °C and left to grow for 10 days.

3.7.2 Colour analysis

After 10 days, the biomass on each plate was gently mixed using a sterile loop. Three 10 µL samples from each plate were collected in 10 µL sterile loops and photographed using a Samsung Galaxy S8 smartphone from a height of 10 cm. The camera settings were ISO 200, focal length 4.2 mm, shutter speed 1/50 s and aperture F/1.7. The photos were analysed using the colour sampler tool in Adobe Photoshop version 23.0.2, which assigns a value representing the colour intensity of red, green and blue (RGB) in a defined area. The RGB values for three 5 × 5 pixel areas in each loop were recorded.

3.8. Data analysis

Two types of software were used for data analysis. In Microsoft Excel 16.0, the Student's t-test was performed to analyse the data sets that involved comparisons of two groups. This included the results of the sensory evaluation sessions, which compared the mixed fermentations of VL3 and BIODIVA to the control VL3-only fermentations and the mixed fermentations of RC212 and BIODIVA to the control RC212-only fermentations. Using the software JASP 0.14.1, analysis of variance (ANOVA) and post-hoc Tukey's honest significant difference (HSD) tests were performed for four sets of data, the results of the sedimentation rate assay, the anthocyanin assay, HPLC analysis and the pigment adsorption experiment. For the results of the spectrophotometric analysis of colour, JASP 0.14.1 was used to perform ANOVA. As the samples were compared in small groups for each of four colour parameters at four stages of the winemaking process, post-hoc Dunn's tests were carried out following the ANOVA, as Dunn's test is suited to small sample sizes. For all statistical analyses performed, the alpha (α) used was 0.05, meaning the probability of making a type I error (where the null hypothesis is true but is rejected), was 5%.

Chapter 4. Results

4.1. Introduction

The results of three stages of experiments are presented in this chapter. First, microfermentations and sedimentation rate assays were performed to assess the flocculation abilities of six non-*Saccharomyces* and two *Saccharomyces cerevisiae* commercial yeast strains, both individually and when used in sequential inoculations. The best performing yeast combinations were then selected for use in a Pinot noir winemaking trial performed in an industry setting. The trial involved eight small scale fermentations to determine whether the sequential inoculation of a highly flocculant combination of non-*Saccharomyces* and *S. cerevisiae* yeast strains would produce wines with deeper colour intensity than *S. cerevisiae* controls. The trial included two mixed species fermentations, VL3 + BIODIVA and RC212 + BIODIVA, with BIODIVA (species *Torulaspora delbrueckii*) selected as the non-*Saccharomyces* co-flocculating partner, to be compared with control fermentations of *S. cerevisiae* commercial wine strains VL3 and RC212. The mixed and control fermentations were each carried out in duplicate due to grape availability. Following fermentation, a series of analyses were performed on samples collected throughout the winemaking process and the final wines produced. The colour of the wines was analysed using spectrophotometry, the Adams-Harbertson anthocyanin assay, high performance liquid chromatography (HPLC) and sensory evaluation. Results of standard industry wine analyses are also presented. Finally, an additional experiment was performed to determine the pigment adsorption ability of each yeast trialled in the sedimentation rate assays.

4.2. Sedimentation rate assays

The purpose of the sedimentation rate assays was to identify the most flocculant non-*Saccharomyces* yeast candidates, both as an individual and when sequentially inoculated with each of two commercial strains of *S. cerevisiae*, following fermentation in wine-like conditions. A total of 63 synthetic grape must (SGM) fermentations were performed, comprising triplicate fermentations of each yeast alone and in pairwise non-*Saccharomyces* and *S. cerevisiae* combinations (Table 4.1). At the end of primary fermentation, sedimentation rate assays were performed on the yeast cells to assess the flocculation capability of each single yeast species and each pairwise combination.

Table 4.1: Yeast combinations trialled in microfermentations prior to sedimentation rate assays

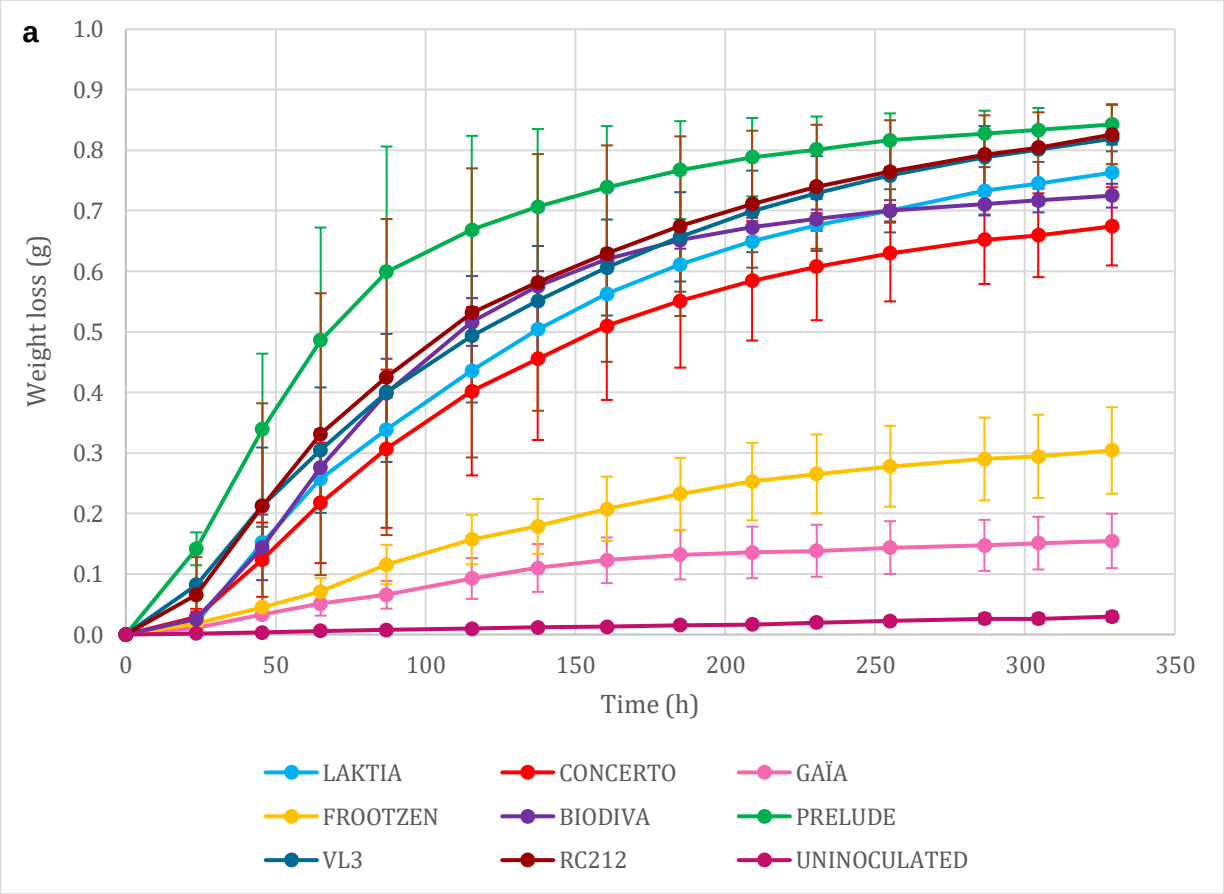
Fermentation number	**Strain name**	**Species**
1-3	LAKTIA	*Lachancea thermotolerans*
4-6	CONCERTO	*Lachancea thermotolerans*
7-9	GAÏA	*Metschnikowia fructicola*
10-12	FROOTZEN	*Pichia kluyveri*
13-15	BIODIVA	*Torulaspora delbrueckii*
16-18	PRELUDE	*Torulaspora delbrueckii*
19-21	VL3	*Saccharomyces cerevisiae*
22-24	RC212	*Saccharomyces cerevisiae*
25-27	LAKTIA + VL3	*Lachancea thermotolerans; Saccharomyces cerevisiae*
28-30	CONCERTO + VL3	*Lachancea thermotolerans; Saccharomyces cerevisiae*
31-33	GAÏA + VL3	*Metschnikowia fructicola; Saccharomyces cerevisiae*
34-36	FROOTZEN + VL3	*Pichia kluyveri; Saccharomyces cerevisiae*
37-39	BIODIVA + VL3	*Torulaspora delbrueckii; Saccharomyces cerevisiae*
40-42	PRELUDE + VL3	*Torulaspora delbrueckii; Saccharomyces cerevisiae*

43-45	LAKTIA + RC212	*Lachancea thermotolerans; Saccharomyces cerevisiae*
46-48	CONCERTO + RC212	*Lachancea thermotolerans; Saccharomyces cerevisiae*
49-51	GAÏA + RC212	*Metschnikowia fructicola; Saccharomyces cerevisiae*
52-54	FROOTZEN + RC212	*Pichia kluyveri; Saccharomyces cerevisiae*
55-57	BIODIVA + RC212	*Torulaspora delbrueckii; Saccharomyces cerevisiae*
58-60	PRELUDE + RC212	*Torulaspora delbrueckii; Saccharomyces cerevisiae*
61-63	Uninoculated control	N/A

4.2.1 Microvinification

Microfermentations carried out in SGM were monitored via their cumulative weight loss, as a proxy for the CO_2 released during primary fermentation (Bely et al., 1990). Microfermentations of the individual yeasts took two weeks to complete primary fermentation at 28 °C, with the exception of FROOTZEN and GAÏA, which were sluggish from the beginning and unable to finish (Figure 4.1a). All fermentations, except for FROOTZEN and GAÏA, adhered to standard fermentation kinetics, where the production of CO_2 begins more slowly with a short fermentative lag, followed by an exponential increase in weight loss and then a plateau. For all the fermentations except for FROOTZEN and GAÏA, the lag phase was less than 24 h, with a noticeable increase in CO_2 production after the second weight measurement was taken (Figure 4.1a). All of the yeasts fermented independently, except for GAÏA, reached their maximal fermentation rate (V_{max}) between day 2 (46 hours) and day 4 (87 hours) (Figure 4.1b). FROOTZEN reached its V_{max} within the first 4 days of fermentation but only reached 0.002 g/L/h, with a consistently low rate of fermentation throughout monitoring. The rate of fermentation for GAÏA followed a similar trajectory, reaching a small peak (0.001 g/L/h) on day 5 (116 hours). The best-performing individual yeast was PRELUDE, a strain of *T.*

delbrueckii, based on its cumulative weight loss of 0.843 g and V_{max} of 0.009 g/L/h. PRELUDE was followed by the two *S. cerevisiae* yeasts, RC212 and VL3, with V_{max} values of 0.007 and 0.006 g/L/h, respectively (Figure 4.1b).



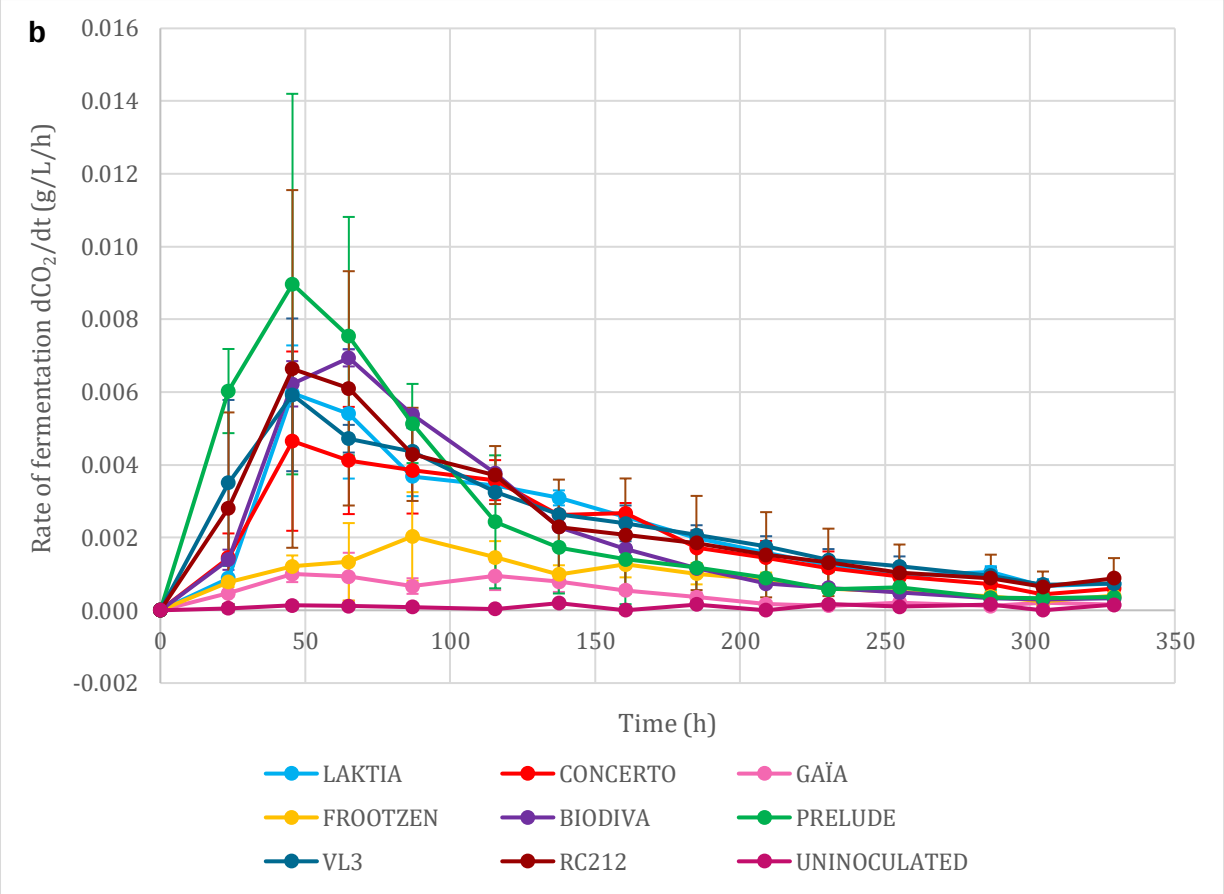
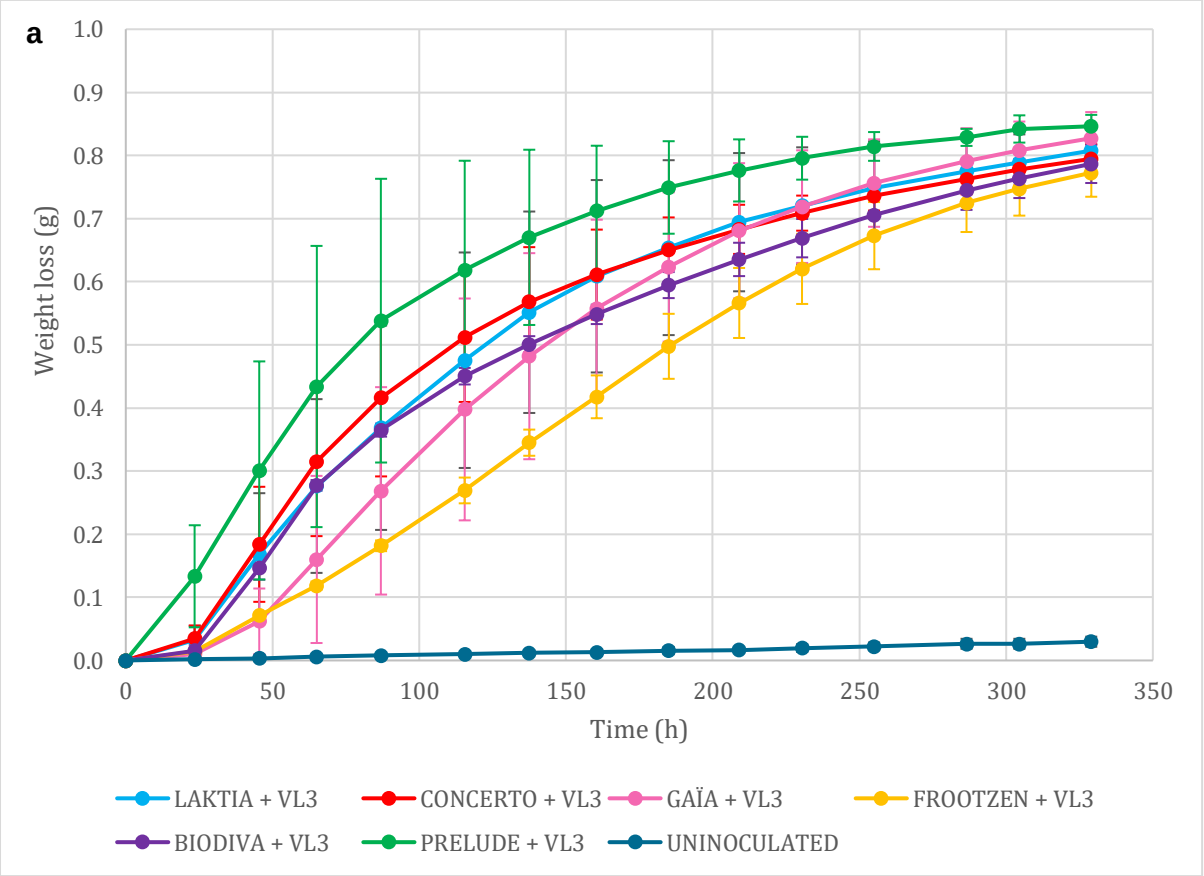


Figure 4.1. Fermentation progress of six non-Saccharomyces spp. (LAKTIA, CONCERTO, GAIA, FROOTZEN, BIODIVA and PRELUDE) and two commercial Saccharomyces cerevisiae yeast strains (VL3 and RC212) in SGM at 28 °C. An uninoculated control is included. a) Cumulative weight loss (g) over time (h). b) Rate of fermentation dCO_2/dt (g/L/h) over time (h). Data points shown are the means of triplicate fermentations. Error bars represent the 95% confidence intervals.

In addition to the individual yeast fermentations, each of the six non-*Saccharomyces* yeast spp. were sequentially inoculated with two different strains of *S. cerevisiae*, RC212 and VL3. These fermentations were carried out since *S. cerevisiae* is the yeast species that is almost exclusively responsible for completing alcoholic fermentation of wine in industry (Goddard, 2008; Maicas, 2020); however, the majority of *S. cerevisiae* strains have low flocculation capabilities on their own but are capable of flocculating with other species (Rossouw et al., 2015). The purpose of the mixed fermentations was to see which non-*Saccharomyces* yeast spp. would pair best with commercial *S. cerevisiae* strains to encourage flocculation, while providing desirable fermentation kinetics and fermentation completion. Pairwise fermentations with *S. cerevisiae* VL3, saw all of the mixed fermentations reach completion within 336 hours (two weeks)

(Figure 4.2a). When the two yeasts that failed to complete fermentation alone (FROOTZEN and GAÏA) (Figure 4.1a and b) were sequentially inoculated with VL3, these fermentations were able to reach completion; however, FROOTZEN + VL3 was sluggish, and the rate of fermentation was consistently lower relative to the other mixed fermentations throughout the 14-day period (Figure 4.2a and b). Of the mixed VL3 fermentations, the highest total weight loss was shown by PRELUDE + VL3 (0.846 g) (Figure 4.2a) and the highest V_{max} values were displayed by CONCERTO + VL3 (0.008 g/L/h) and PRELUDE + VL3 (0.008 g/L/h) (Figure 4.2b).



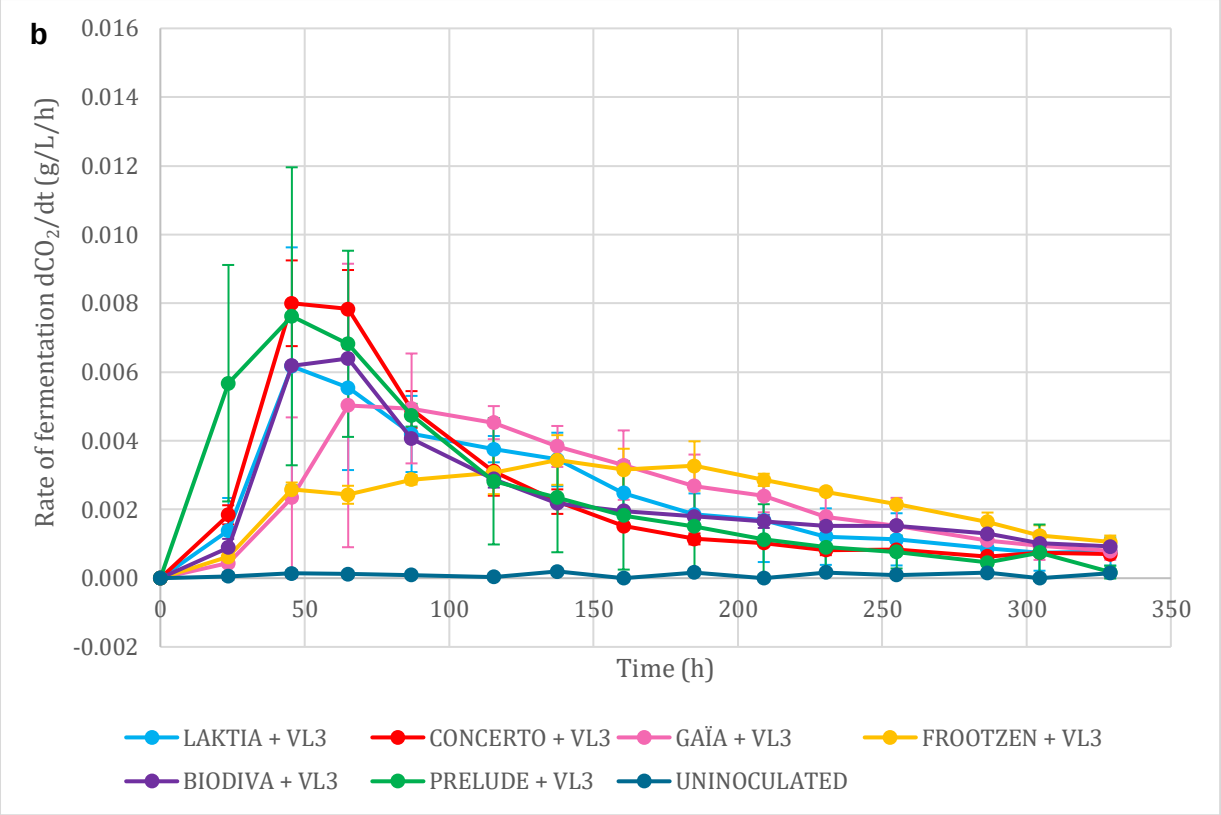


Figure 4.2. Fermentation progress of six non-Saccharomyces spp. (LAKTIA, CONCERTO, GAIA, FROOTZEN, BIODIVA and PRELUDE) sequentially inoculated with *S. cerevisiae* strain VL3 in SGM at 28 °C. An uninoculated control is included. a) Cumulative weight loss (g) over time (h). b) Rate of fermentation dCO_2/dt (g/L/h) over time (h). Data points shown are the means of triplicate fermentations. Error bars represent the 95% confidence intervals.

Each commercial non-*Saccharomyces* yeast was also sequentially inoculated with *S. cerevisiae* strain RC212. Similar to the FROOTZEN + VL3 and GAÏA + VL3 fermentations, FROOTZEN + RC212 and GAÏA + RC212 displayed slightly sluggish fermentation progress (Figure 4.3a) with rates of fermentation that peaked later and at lower values (0.005 and 0.004 g/L/h, respectively) than the other fermentations (Figure 4.3b). Both of the fermentations were still able to reach completion, reaching a rate close to zero g/L/h by 329 hours (Figure 4.3b). Each non-*Saccharomyces* spp. and RC212 yeast combination finished primary alcoholic fermentation, with final cumulative weight losses ranging between 0.784 g (CONCERTO + RC212) and 0.816 g (LAKTIA + RC212). The fastest rate of fermentation (0.008 g/L/h) was shown by PRELUDE + RC212 and the highest cumulative weight loss (0.816 g) was shown by LAKTIA + RC212, although there is significant overlap between error bars.

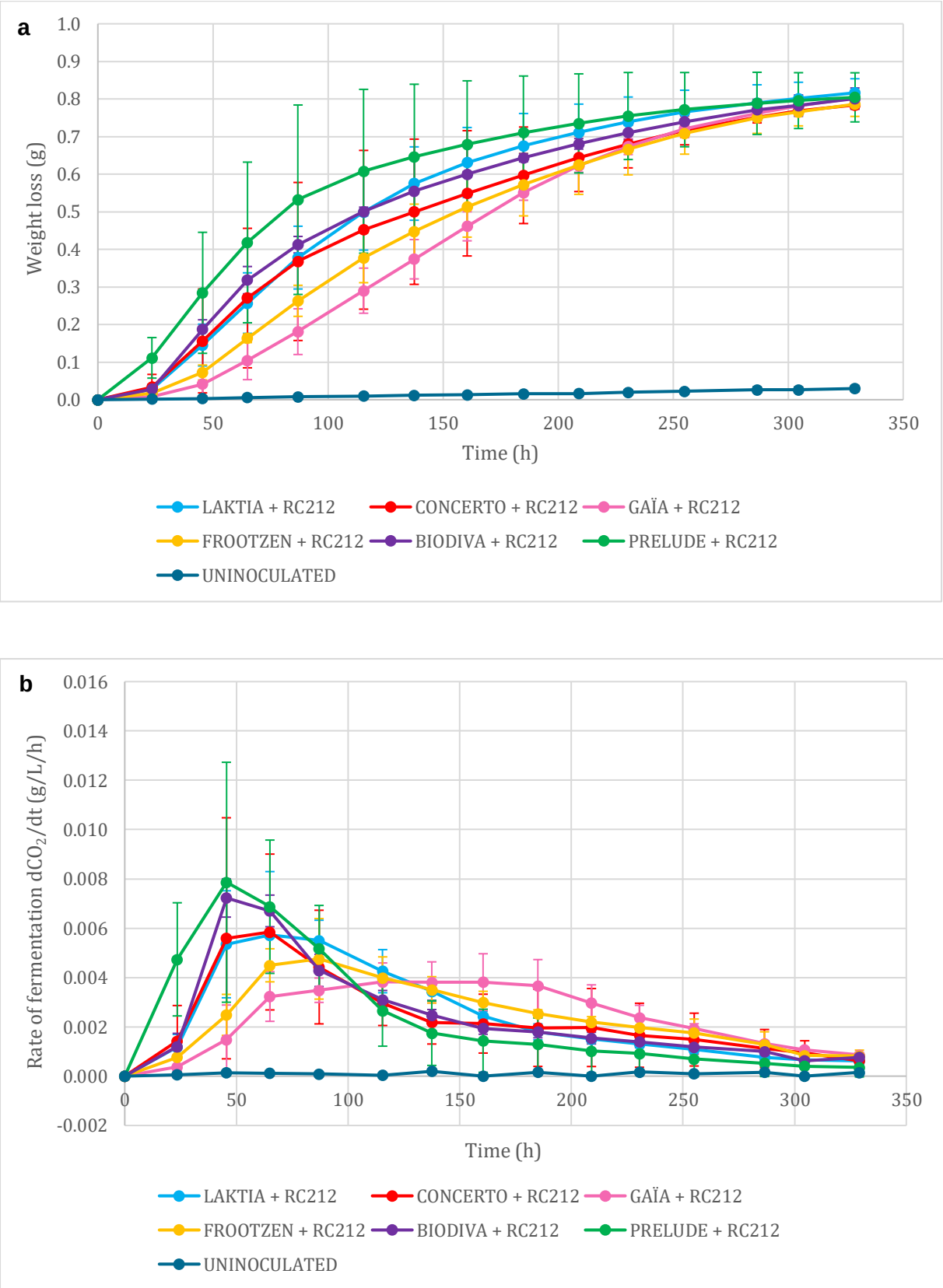


Figure 4.3. Fermentation progress of six non-Saccharomyces spp. (LAKTIA, CONCERTO, GAIA, FROOTZEN, BIODIVA and PRELUDE) sequentially inoculated

with *S. cerevisiae* strain RC212 in SGM at 28 °C. An uninoculated control is included. a) Cumulative weight loss (g) over time (h). b) Rate of fermentation dCO_2/dt (g/L/h) over time (h). Data points shown are the means of triplicate fermentations. Error bars represent the 95% confidence intervals.

Overall, the non-*Saccharomyces* yeast spp. LAKTIA, CONCERTO, BIODIVA and PRELUDE displayed similar fermentation kinetics regardless of whether they were fermented independently or in a pairwise combination with RC212 or VL3. In contrast, fermentations inoculated with FROOTZEN and GAÏA both failed to finish fermentation without the sequential inoculation of a *S. cerevisiae* yeast strain. PRELUDE, alone and when mixed, was consistently among the fastest to produce the most CO_2 and reach the highest rates of fermentation. Of all the yeast combinations trialled, the best performing fermentations appeared to be PRELUDE alone, which reached the highest fermentation rate of 0.009 g/L/h and PRELUDE + VL3 which lost the largest amount of total weight, 0.846 g. Another observation to note was that there was greater variation across the triplicate fermentations for these two yeast combinations, in comparison to the other fermentations, as indicated by the large error bars.

4.2.2 Sedimentation rate assays

The sedimentation rate of a yeast is an indication of how well it flocculates, with a higher sedimentation rate signifying a higher rate of flocculation (Varela et al., 2020). The sedimentation rates of the 63 microfermentations (Section 4.2.1) were determined after primary fermentation was complete. Figure 4.4 shows the results from the sedimentation rate assay. An ANOVA performed on the results found significant differences between the yeasts (fermented independently and sequentially), with a p value of < 0.001. The highest sedimentation rates were demonstrated by the fermentations that included the non-*Saccharomyces* yeast BIODIVA (*T. delbrueckii*). The highest rate recorded was 0.030 dOD_{600nm}/min shown by the BIODIVA cells when fermented alone. This was followed by the sedimentation rates of BIODIVA + RC212 (0.028 dOD_{600nm}/min) and BIODIVA + VL3 (0.026 dOD_{600nm}/min). Following the ANOVA, a Tukey's honest significant difference (HSD) post-hoc test showed significant differences ($p = < 0.05$) between the three BIODIVA fermentations and all other yeast combinations except for LAKTIA + RC212 (0.013 dOD_{600nm}/min) when compared to BIODIVA + VL3. All the other fermentations were comparable and not significantly different from each other, with a total of 15 out of 20 yeast combinations having a mean sedimentation

rate of between 0.001 and 0.010 dOD_{600nm}/min. The exception was FROOTZEN alone, which had the lowest mean sedimentation rate of 0.0004 dOD_{600nm}/min and was significantly different from the three BIODIVA fermentations and LAKTIA + RC212. Therefore, BIODIVA, a strain of *T. delbrueckii*, clearly outperformed the other non-*Saccharomyces* yeast spp. in terms of flocculation capability when fermented alone, and as the best co-flocculation partner for *S. cerevisiae* strains VL3 and RC212. During routine culture it was observed that the BIODIVA yeast cells settled to the bottom of the YPD liquid and were more difficult to resuspend than the other yeasts, an indication of BIODIVA's tendency to flocculate.

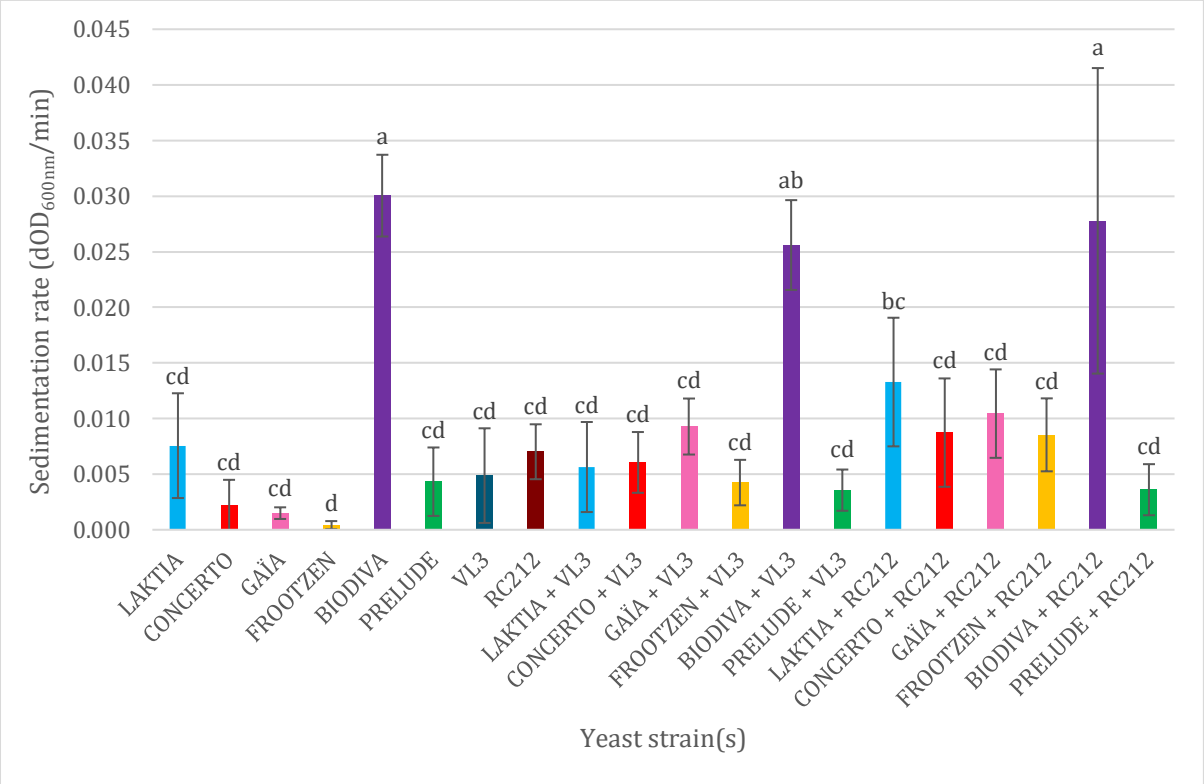


Figure 4.4. Sedimentation rate (dOD600nm/min) of individual and mixed yeasts following fermentation in SGM at 28 °C. Bars are coloured based on the presence of each non-*Saccharomyces* spp. Samples shown are the means of triplicate fermentations and the error bars represent 95% confidence intervals. Different letters above the data indicate significant differences (ANOVA followed by Tukey's HSD).

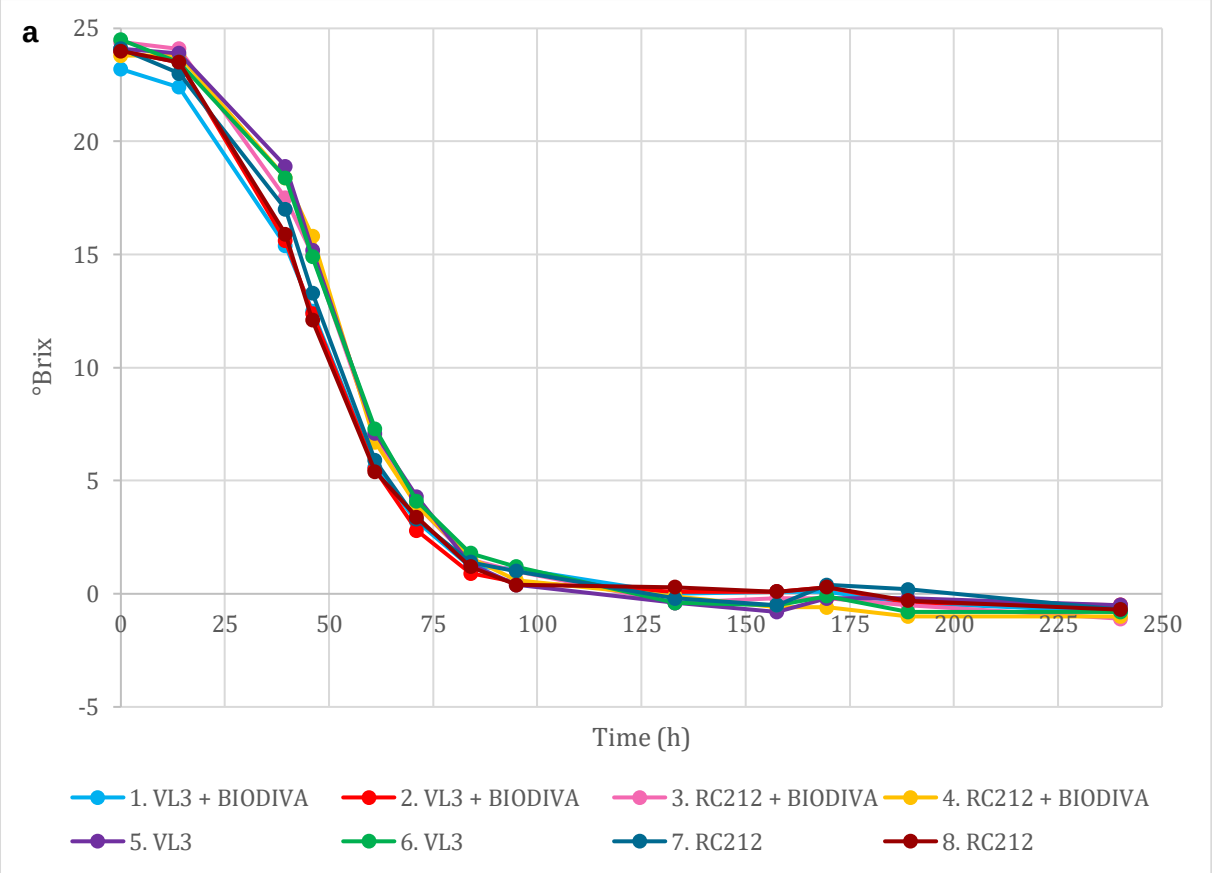
4.3. Pinot noir winemaking

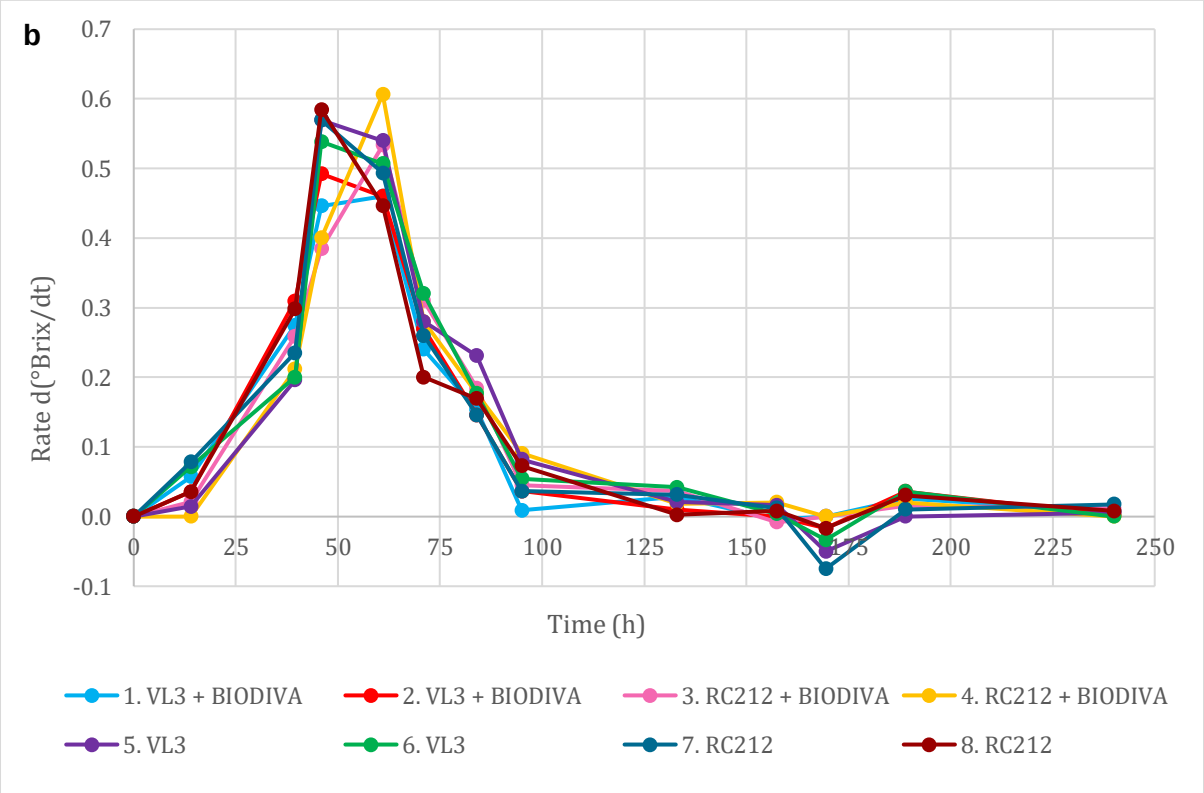
The results of the sedimentation rate assays were used to select the yeast combinations to be trialled in 20 L Pinot noir fermentations, carried out in a commercial winery under industry

conditions. The non-*Saccharomyces* yeast with the highest flocculation capability, BIODIVA, was used to inoculate Pinot noir, followed by sequential inoculation of *S. cerevisiae* after 24 h. Two concurrent trials with sequential inoculation of BIODIVA were performed; one with commercial *S. cerevisiae* yeast strain RC212, chosen based on its popularity in the wine industry for fermenting Pinot noir (Carew et al., 2013), or commercial *S. cerevisiae* yeast strain VL3, chosen as a control based on its slight ability to flocculate as previously shown in work by Varela et al. (2020). The purpose of carrying out the Pinot noir winemaking was to compare the colour parameters of these mixed inoculation wines with control *S. cerevisiae* wines, inoculated with RC212 and VL3 alone. The results would indicate whether the flocculation of non-*Saccharomyces* and *S. cerevisiae* yeast cells during fermentation had an impact on colour intensity, in comparison to the control *S. cerevisiae* fermentations. Two replicate fermentations of each yeast combination were performed.

4.3.1 Fermentation data

The eight 20 L Pinot noir fermentations were monitored with once or twice daily testing of °Brix and temperature (°C). The eight fermentation buckets began with a starting °Brix value between 24.5 and 23.2 (Figure 4.5). Primary alcoholic fermentation ended after 240 hours (10 days) with °Brix levels between -0.5 and -1.1 (Figure 4.5a). The rate of fermentation was consistent across all of the eight fermentations with maximal fermentation rates (V_{max}) between 0.460 and 0.607 d°Brix/dt reached on either day 2 or day 3 of fermentation (between 46 and 61 hours) (Figure 4.5b). The length of the lag phase of each fermentation was slightly different, with some, such as 7. RC212, having lag phases of less than 14 hours and others, such as 4. RC212 + BIODIVA, having a longer lag phase of more than 14 hours. By day 2 of fermentation (40 hours), all fermentations had moved out of the lag phase and exponential sugar consumption was observed for the next 3 days. The rate of fermentation began to slow after 84 hours (on day 4 of fermentation).


a
°Brix
Time (h)
1. VL3 + BIODIVA
2. VL3 + BIODIVA
3. RC212 + BIODIVA
4. RC212 + BIODIVA
5. VL3
6. VL3
7. RC212
8. RC212


b
Rate d(°Brix/dt)
Time (h)
1. VL3 + BIODIVA
2. VL3 + BIODIVA
3. RC212 + BIODIVA
4. RC212 + BIODIVA
5. VL3
6. VL3
7. RC212
8. RC212

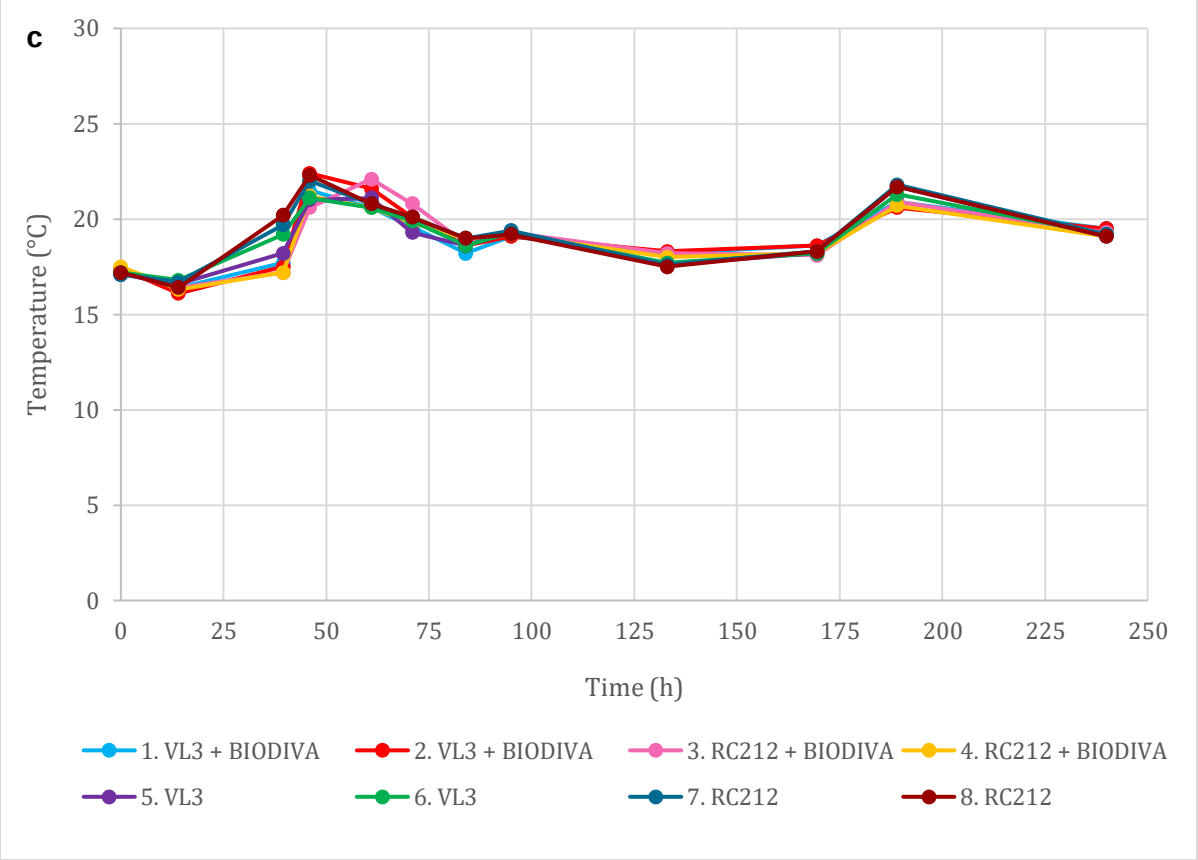


Figure 4.5. Fermentation of eight 20 L Pinot noir fermentations, performed by *T. delbrueckii* BIODIVA sequentially inoculated with *S. cerevisiae* RC212 or VL3, compared to *S. cerevisiae* RC212 and VL3 control fermentations. a) Sugar consumption (°Brix) over time (h). b) Rate of fermentation (d°Brix/dt) over time (h). c) Temperature (° C) over time.

The peak of fermentation between 46 and 61 hours (days 2 to 3) was also reflected in the changing temperature of the fermentations over time (Figure 4.5) (note that temperature control was not available in the winery). All fermentations, except 3. RC212 + BIODIVA (3.R+B) and 5. VL3 (5.V), reached their highest temperatures between 21.1 °C and 22.4 °C, at 46 hours (day 2). In contrast, fermentation 3.R+B and fermentation 5.V reached their highest temperatures at 61 hours (day 3), of 22.1 °C and 21.1 °C respectively. The highest temperature recorded was 22.4 °C by fermentation 2.V+B. These peaks in temperature on days 2 and 3 were a sharp increase from the starting temperatures, which were all between 17.1 and 17.5 °C on the day of the first inoculations. The standard temperature range for a red wine fermentation is between 20 °C and 35 °C (Cooke & Berg, 1983). The later temperature increase, at 189 hours (day 8), was due to the fermentations being moved outdoors during the day, to be exposed to

warmth from the sun. As the rate of fermentation slowed, this approach was used to stimulate the yeast and ensure each bucket completed the final stage of fermentation and did not result in wines with high levels of residual sugar. Overall, the best performing fermentations were the two RC212 + BIODIVA replicates, which reached the highest V_{max} of 0.607 d°Brix/dt (4.R+B) and the highest sugar consumption, with a total °Brix loss of 25.5 (3.R+B).

4.3.2 Wine analysis

Additional analyses of two of the fermentations were conducted by The Coterie, a contract winemaking facility in Marlborough, NZ (Table 4.2). One of the RC212 control wines (8.R) was analysed on March 27, representing day 8 of the 10 days of fermentation, and one of the VL3 + BIODIVA wines (2.V+B) was analysed on April 1, two days after fermentation was finished. These analyses confirmed that the wines had completed primary fermentation, with levels of residual sugar (1.8 g/L and 0.4 g/L, respectively) well within the 0 to 4.9 g/L range required for a wine to be considered dry. All wines displayed very similar values for pH, titratable acidity (TA), alcohol concentration, volatile acidity and concentration of malic acid.

Table 4.2: Results of two Pinot noir fermentation analyses performed by The Coterie

Wine parameter	**Fermentation 8.R (analysis on day 8 of 10 days of fermentation)**	**Fermentation 2.V+B (analysis two days after completion of fermentation)**
pH	3.59	3.56
Titratable acidity (g/L)	6.8	6.6
Residual sugar (g/L)	1.8	0.4
Alcohol (% v/v)	13.51	13.46
Volatile acidity (g/L)	0.275	0.380
Malic acid (g/L)	1.7	1.8

Following pressing, two 1 L wine samples were collected from each fermentation for transport via vehicle from Renwick to Auckland (772 km). The bottles were labelled according to their original fermentation number (for example, wine 1A.V+B and wine 1B.V+B were taken from

fermentation 1.V+B). The resulting 16 wines were inoculated with commercial *O. oeni* strain REFLEX MALO 360 for malolactic fermentation (MLF) separately. The final wines were analysed after MLF (two months) and a subsequent six-week settling period were complete. The 16 wines showed consistent results across the six standard wine parameters measured (Table 4.3). All wines were dry with less than 1 g/L of residual sugar. The wines also had low levels of volatile acidity (acetic acid), all with less than 1 g/L of each. All of the wines had less than 0.05 g/L of malic acid remaining, except for wine 5B.V which had a slightly higher concentration of 0.11 g/L. Slightly more differences between the wines were shown in the analyses of alcohol content and acidity (pH and TA). Alcohol content ranged from 12.83% (v/v) in wine 2B.V+B up to 13.59% (v/v) in wine 6B.V. Only one other wine (2A.V+B) was within the 12% (v/v) range, with 12.89% (v/v). The other 14 wines all had an alcohol content above 13% (v/v). TA levels also varied slightly, with results ranging from 4.91 g/L (2B.V+B) to 5.27 g/L (4B.R+B). The lowest pH of 3.50 was shown by wine 4A.R+B and two wines shared the highest pH of 3.66, 2A.V+B and 2B.V+B. Overall, there was minimal variation between the wines. The wine 2B.V+B differed the most from the others, with the lowest alcohol content, lowest TA and equal highest pH.

Table 4.3: Results of final wine analyses

Wine	**pH**	**Titratable acidity (g/L)**	**Alcohol (% v/v)**	**Residual sugar (g/L)**	**Volatile acidity (g/L)**	**Malic acid (g/L)**
1A.V+B	3.64	5.05	13.13	0.04	0.38	< 0.05
1B.V+B	3.61	5.12	13.36	0.06	0.35	< 0.05
2A.V+B	3.66	5.12	12.89	0.06	0.32	< 0.05
2B.V+B	3.66	4.91	12.83	0.04	0.27	< 0.05
3A.R+B	3.54	5.19	13.20	0.06	0.38	< 0.05
3B.R+B	3.57	5.00	13.21	0.04	0.28	< 0.05
4A.R+B	3.50	5.19	13.43	0.14	0.32	< 0.05

4B.R+B	3.51	5.27	13.33	0.07	0.29	< 0.05
5A.V	3.56	5.05	13.52	0.06	0.24	< 0.05
5B.V	3.54	5.05	13.39	0.11	0.24	0.11
6A.V	3.52	5.05	13.47	0.06	0.25	< 0.05
6B.V	3.53	5.00	13.59	0.06	0.30	< 0.05
7A.R	3.53	5.12	13.38	0.08	0.28	< 0.05
7B.R	3.53	5.19	13.50	0.12	0.34	< 0.05
8A.R	3.53	5.12	13.43	0.06	0.28	< 0.05
8B.R	3.54	5.19	13.56	0.07	0.23	< 0.05

The mean results of the standard wine analyses for all VL3 + BIODIVA wines, compared with the mean results for all VL3 control wines, are presented in Table 4.4. The overall results for most of the wine parameters are reasonably consistent between mixed and control wines. The most significant difference was shown in the pH, with an average of 3.64 found in the VL3 + BIODIVA wines compared to 3.53 of the VL3 control wines. There were also significant differences in the alcohol content and the concentration of volatile acidity between the mixed and control wines.

Table 4.4: Means of standard analyses for final wines (n = 4) where fermentation was performed by BIODIVA sequentially inoculated with *S. cerevisiae* VL3, compared with VL3 control wines. Significant differences, calculated using the Student's t test, are indicated by asterisks, with more asterisks representing a higher level of significance - ns (not significant), * (*p* value < 0.05), ** (*p* value < 0.01) and * (*p* value < 0.001).**

Wine	pH[1]	Titratable acidity (g/L)	Alcohol (% v/v)	Residual sugar (g/L)	Volatile acidity (g/L)	Malic acid (g/L)
VL3 + BIODIVA	3.64	5.05	13.05	0.05	0.33	< 0.05
VL3	3.54	5.04	13.49	0.07	0.26	< 0.05
Significance	***	ns	*	ns	*	ns

[1]The mean was calculated by transforming pH into H+ concentration.

The means of the standard analyses of the RC212 + BIODIVA wines and the RC212 controls were more consistent than the VL3 wines (see Table 4.5). The means of the results for pH, TA and residual sugar were exactly the same between mixed and control wines, while the VA content differed slightly. The only significant difference was between the alcohol levels, with a 13.29% v/v average for the RC212 + BIODIVA wines and a 13.47% average for the RC212 control wines.

Table 4.5: Means of standard analyses for final wines (n = 4) where fermentation was performed by BIODIVA sequentially inoculated with *S. cerevisiae* RC212, compared with RC212 control wines. Significant differences, calculated using the Student's t test, are indicated by asterisks, with more asterisks representing a higher level of significance - ns (not significant), * (*p* value < 0.05), ** (*p* value < 0.01) and * (*p* value < 0.001).**

Wine	pH[1]	Titratable acidity (g/L)	Alcohol (% v/v)	Residual sugar (g/L)	Volatile acidity (g/L)	Malic acid (g/L)
RC212 + BIODIVA	3.53	5.16	13.29	0.08	0.32	< 0.05
RC212	3.53	5.16	13.47	0.08	0.28	< 0.05
Significance	ns	ns	*	ns	ns	ns

[1]The mean was calculated by transforming pH into H+ concentration.

4.4. Wine colour analysis

The main purpose of this research was to evaluate the impact of yeast flocculation through sequential inoculation on colour intensity of Pinot noir wines; therefore, following the trial fermentations, several colour analyses were performed on the finished wines. The main form of colour analysis used was UV/visible spectrophotometry, which was performed on all samples at each production stage, including the initial juice samples, the wines prior to MLF, the wines after MLF, and the final wines following a SO_2 addition and settling period of six weeks. Measuring the final wines, as well as the samples taken at earlier stages, was carried out in order to provide insight into how the wine colour parameters evolved over time, and how different steps in the winemaking process may affect the colour intensity of the wines. The colour of the final wines was also evaluated further using alternative analysis methods, including an anthocyanin assay, HPLC and sensory evaluation, to provide further information about the nature of the differences in colour.

4.4.1 UV/visible spectrophotometry

UV/visible spectrophotometry was used to determine the concentration of red pigments (absorbance at 520 nm) and brown pigments (absorbance at 420 nm), plus the total colour intensity (420 + 520 nm) and hue (420 / 520 nm) of each sample. The results for each of these categories for the two VL3 + BIODIVA fermentations and two VL3 control fermentations, at four different stages during the winemaking process (juice, pre-malolactic fermentation, post-malolactic fermentation and finished wine), are presented in Table 4.6. The juice used in the VL3 mixed and control fermentations displayed no significant differences across all four colour parameters. Results for the total colour intensity ranged from 6.15 ± 0.43 absorbance units (a.u.) for fermentation 1.V+B to 6.51 ± 1.18 a.u. for fermentation 2.V+B. Following primary fermentation, ANOVA and post-hoc Dunn's tests showed that there were significant differences (p = < 0.05) between the pre-malolactic fermentation (MLF) wines for all four colour attributes. The two VL3 + BIODIVA wines were significantly different from the VL3 control wines for both red pigments and total colour intensity. In both of these categories, the VL3 + BIODIVA wines showed higher values than the VL3 controls. The highest concentration of red pigments (3.83 ± 0.26 a.u.) and the highest colour intensity (5.85 ± 0.36 a.u.) were both shown by fermentation 1.V+B, while the lowest values (3.38 ± 0.11 a.u. for red pigments; 5.24 ± 0.16 a.u. for colour intensity) were both from fermentation 5.V. Fermentation 2.V+B was also significantly different from the VL3 control wines for the two other colour attributes, brown pigments and hue, but fermentation 1.V+B was not.

Following MLF, further changes in the wines' colour profiles were apparent. Fermentation 2.V+B continued to be significantly different, with higher values in each category, from fermentation 6.V for red pigments, brown pigments and total colour intensity. Fermentation 1.V+B and fermentation 5.V both showed no significant differences from any of the other wines. The highest colour intensity was displayed by fermentation 2.V+B with 5.21 ± 0.08 a.u. followed by fermentation 1.V+B (4.92 ± 0.08 a.u.). There were no significant differences in hue for any of the post-MLF wines. At the final stage in the winemaking process, fermentation 2.V+B had the highest results for red pigments (3.96 ± 0.19 a.u.), brown pigments (2.91 ± 0.11 a.u.) and total colour intensity (6.88 ± 0.30 a.u.). This wine was significantly different from fermentation 6.V in all three categories and from fermentation 5.V for red pigments and total colour intensity. Overall, the highest colour intensity (6.88 ± 0.30 a.u.) was seen in the final wines, in fermentation 2.V+B.

Table 4.6: Mean concentration and standard deviation of red pigments and brown pigments, total colour intensity and hue of Pinot noir wines, where fermentation was performed by *T. delbrueckii* BIODIVA sequentially inoculated with *S. cerevisiae* VL3, compared to *S. cerevisiae* VL3 control fermentations, at four stages of the winemaking process (n = 3 for juice and pre-MLF stages, n = 2 for post-MLF and wine stages). Different letters next to the data indicate significant differences for that colour parameter at that stage (ANOVA followed by Dunn's post hoc test).

Fermentation	Stage	Red pigments (A_{520})	Brown pigments (A_{420})	Colour intensity ($A_{520} + A_{420}$)	Hue (A_{420} / A_{520})
1.V+B	Juice	4.01 ± 0.86 a	2.50 ± 0.31 a	6.51 ± 1.18 a	0.63 ± 0.06 a
2.V+B	Juice	3.78 ± 0.26 a	2.37 ± 0.16 a	6.15 ± 0.43 a	0.63 ± 0.01 a
5.V	Juice	3.87 ± 0.14 a	2.43 ± 0.01 a	6.30 ± 0.15 a	0.63 ± 0.02 a
6.V	Juice	3.84 ± 1.36 a	2.34 ± 0.56 a	6.18 ± 1.92 a	0.62 ± 0.07 a
1.V+B	Pre-MLF	3.83 ± 0.26 a	2.03 ± 0.10 ab	5.85 ± 0.36 a	0.53 ± 0.01 b
2.V+B	Pre-MLF	3.71 ± 0.08 a	2.09 ± 0.05 a	5.79 ± 0.12 a	0.56 ± 0.00 a
5.V	Pre-MLF	3.38 ± 0.11 b	1.86 ± 0.05 b	5.24 ± 0.16 b	0.55 ± 0.00 ab
6.V	Pre-MLF	3.45 ± 0.03 b	1.92 ± 0.11 b	5.37 ± 0.08 b	0.56 ± 0.03 ab
1.V+B	Post-MLF	2.71 ± 0.09 ab	2.21 ± 0.01 ab	4.92 ± 0.08 ab	0.82 ± 0.03 a
2.V+B	Post-MLF	2.80 ± 0.05 a	2.42 ± 0.03 a	5.21 ± 0.08 a	0.86 ± 0.01 a

5.V	Post-MLF	2.66 ± 0.12 ab	2.21 ± 0.03 ab	4.88 ± 0.15 ab	0.83 ± 0.03 a
6.V	Post-MLF	2.45 ± 0.01 b	2.08 ± 0.06 b	4.54 ± 0.05 b	0.85 ± 0.03 a
1.V+B	Wine	3.73 ± 0.03 ab	2.63 ± 0.08 ab	6.37 ± 0.12 ab	0.70 ± 0.02 b
2.V+B	Wine	3.96 ± 0.19 a	2.91 ± 0.11 a	6.88 ± 0.30 a	0.74 ± 0.01 ab
5.V	Wine	2.95 ± 0.98 b	2.36 ± 0.38 ab	5.31 ± 1.36 b	0.82 ± 0.14 ab
6.V	Wine	2.47 ± 0.29 b	2.21 ± 0.06 b	4.59 ± 0.35 b	0.86 ± 0.08 a

The four colour parameters for the RC212 + BIODIVA fermentations and RC212 control fermentations were also compared with samples taken at four stages of the winemaking process (juice, pre-malolactic fermentation, post-malolactic fermentation and finished wine) (Table 4.7). Unlike in the VL3 fermentations, the juice used for the RC212 + BIODIVA and RC212 control fermentations showed significant differences at the first stage, before any winemaking activities had started. The juice of both RC212 + BIODIVA fermentations (3.R+B and 4.R+B) was significantly different from the juice of the RC212 control fermentations (7.R and 8.R) for red pigments, brown pigments and total colour intensity. For each of these parameters, the RC212 control fermentations displayed higher values, indicating higher concentrations of red pigments, brown pigments and deeper colour intensity. The highest juice colour intensity of 8.55 ± 0.79 a.u. was seen in fermentation 7.R, while the juice with the lowest colour intensity of 4.74 ± 0.50 a.u. was present in fermentation 4.R+B. Differences in the hue of the juice were less pronounced, with only fermentation 4.R+B being significantly different from fermentations 4.R+B and 7.R. After primary fermentation was complete, at the pre-MLF stage, the wines no longer displayed any differences in red pigment content or total colour intensity. There was a difference in brown pigments between fermentation 4.R+B, with a result of 1.95 ± 0.04 a.u., and the higher concentration of 2.05 ± 0.06 a.u. shown by fermentation 8.R. The two RC212 + BIODIVA wines (3.R+B and 4.R+B) had significantly different hues from the two RC212 wines (7.R and 8.R).

Following MLF, differences became more apparent. Fermentation 3.R+B showed the highest results for red pigments (2.62 ± 0.09 a.u.), brown pigments (2.24 ± 0.03 a.u.) and total colour intensity (4.86 ± 0.12 a.u.) and was significantly different from the other RC212 + BIODIVA wine (4.R+B). It was also different from one of the RC212 control wines (8.R), but only for red pigments. There were no differences in hue at this stage. The final wines showed only one significant difference across all four colour attributes. The two RC212 + BIODIVA wines were significantly different from each other for brown pigments, with fermentation 3.R+B having a higher value (2.35 ± 0.17 a.u.) compared to fermentation 4.R+B (2.00 ± 0.02 a.u.). Of the finished wines, the highest colour intensity of 5.12 ± 0.61 a.u. was displayed by fermentation 3.R+B. Overall, the colour intensity was highest at the juice stage, with values of 8.55 ± 0.79 a.u. and 7.97 ± 0.99 shown by the two RC212 controls (7.R and 8.R, respectively).

Table 4.7: Mean concentration and standard deviation of red pigments and brown pigments, total colour intensity and hue of Pinot noir wines, where fermentation was performed by *T. delbrueckii* BIODIVA sequentially inoculated with *S. cerevisiae* RC212, compared to *S. cerevisiae* RC212 control fermentations, at four stages of the winemaking process (n = 3 for juice and pre-MLF stages, n = 2 for post-MLF and wine stages). Different letters next to the data indicate significant differences for that colour parameter at that stage (ANOVA followed by Dunn's post hoc test).

Fermentation	Stage	Red pigments (A_{520})	Brown pigments (A_{420})	Colour intensity ($A_{520} + A_{420}$)	Hue (A_{420} / A_{520})
3.R+B	Juice	3.82 ± 0.4 bc	2.18 ± 0.16 bc	6.00 ± 0.56 bc	0.57 ± 0.02 b
4.R+B	Juice	2.84 ± 0.32 c	1.90 ± 0.17 c	4.74 ± 0.50 c	0.67 ± 0.02 a
7.R	Juice	5.52 ± 0.6 a	3.04 ± 0.19 a	8.55 ± 0.79 a	0.55 ± 0.03 b
8.R	Juice	5.08 ± 0.74 ab	2.90 ± 0.26 ab	7.97 ± 0.99 ab	0.57 ± 0.03 ab
3.R+B	Pre-MLF	3.69 ± 0.35 a	1.97 ± 0.14 ab	5.65 ± 0.49 a	0.53 ± 0.01 b
4.R+B	Pre-MLF	3.64 ± 0.06 a	1.95 ± 0.04 b	5.59 ± 0.09 a	0.54 ± 0.01 b
7.R	Pre-MLF	3.59 ± 0.16 a	2.00 ± 0.07 ab	5.59 ± 0.23 a	0.56 ± 0.00 a
8.R	Pre-MLF	3.70 ± 0.13 a	2.05 ± 0.06 a	5.76 ± 0.19 a	0.55 ± 0.01 a
3.R+B	Post-MLF	2.62 ± 0.09 a	2.24 ± 0.03 a	4.86 ± 0.12 a	0.86 ± 0.02 a
4.R+B	Post-MLF	2.49 ± 0.02 b	2.04 ± 0.03 b	4.54 ± 0.05 b	0.82 ± 0.00 a

7.R	Post-MLF	2.60 ± 0.09 ab	2.23 ± 0.03 a	4.83 ± 0.06 a	0.86 ± 0.04 a
8.R	Post-MLF	2.49 ± 0.02 b	2.10 ± 0.02 ab	4.59 ± 0.03 ab	0.84 ± 0.00 a
3.R+B	Wine	2.77 ± 0.44 a	2.35 ± 0.17 a	5.12 ± 0.61 a	0.85 ± 0.07 a
4.R+B	Wine	2.36 ± 0.15 a	2.00 ± 0.02 b	4.35 ± 0.13 a	0.85 ± 0.06 a
7.R	Wine	2.64 ± 0.55 a	2.27 ± 0.07 ab	4.91 ± 0.61 a	0.88 ± 0.16 a
8.R	Wine	2.66 ± 0.31 a	2.21 ± 0.20 ab	4.87 ± 0.51 a	0.84 ± 0.02 a

4.4.2 Adams-Harbertson anthocyanin assay

A red wine phenolics assay was used to determine the anthocyanin concentration (equivalent to malvidin-3-glucoside concentration in mg/L) of the final wines. ANOVA was used to compare the four wines fermented with sequential inoculation of BIODIVA then VL3 and the VL3 control wines. There were significant differences between the wines with a *p* value of < 0.001. The results of a Tukey's HSD post-hoc test found that one of the VL3 + BIODIVA wines (2.V+B) was significantly different from the other three wines, with a lower anthocyanin concentration of 180.38 mg/L compared to a mean of 245.60 mg/L for the other three wines (Table 4.8). This result was surprising as the VL3 + BIODIVA wines were found to have deeper colour intensity than the VL3 control wines when analysed using UV/visible spectrophotometry (see Section 4.4.1). The other VL3 + BIODIVA wine (1.V+B) and the two VL3 control wines were not significantly different from each other. The wine with the highest anthocyanin concentration of 255.38 mg/L was one of the VL3 control wines, 6.V.

Table 4.8: Anthocyanin concentration (equivalent to malvidin-3-glucoside (M-3-G) mg/L) of wines (n = 2) where fermentation was performed by BIODIVA sequentially inoculated with VL3 compared to VL3 control fermentations. Different letters next to the results indicate significant differences (ANOVA followed by Tukey's HSD).

Fermentation	Yeast	Anthocyanins (M-3-G equivalents) mg/L
1	VL3 + BIODIVA	232.13 ± 10.78 a
2	VL3 + BIODIVA	180.38 ± 20.59 b
5	VL3	249.30 ± 45.54 a
6	VL3	255.38 ± 8.94 a

ANOVA performed on the RC212 + BIODIVA mixed wines and RC212 control wines also found statistically significant differences with a *p* value of 0.020. A Tukey's HSD post-hoc test showed a significant difference between the two RC212 + BIODIVA fermentations, with 3.R+B having the lowest anthocyanin concentration of 245.45 mg/L while 4.R+B had the

highest concentration of 266.03 mg/L (Table 4.9). There were no significant differences when comparing the mixed fermentations to the control fermentations.

Table 4.9: Anthocyanin concentration (equivalent to malvidin-3-glucoside (M-3-G) mg/L) of wines (n = 2) where fermentation was performed by BIODIVA sequentially inoculated with RC212 compared to RC212 control fermentations. Different letters next to the results indicate significant differences (ANOVA followed by Tukey's HSD).

Fermentation	Yeast	Anthocyanins (M-3-G equivalents) mg/L
3	RC212 + BIODIVA	245.45 ± 21.98 b
4	RC212 + BIODIVA	266.03 ± 3.17 a
7	RC212	251.07 ± 2.21 ab
8	RC212	263.52 ± 8.18 ab

4.4.3 HPLC

Following the UV/visible spectrophotometric analyses of the wines, the individual VL3 + BIODIVA and VL3 wines with the highest (2A.V+B) and lowest (6B.V) colour intensities were selected for further analysis using HPLC. Five types of anthocyanins (malvidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-glucoside) and the total anthocyanin content, were measured and the results are presented in Table 4.10. The anthocyanin malvidin-3-glucoside was found at the highest concentration in both wines, at 125.33 ± 0.18 mg/L (2A.V+B) and 168.09 ± 0.14 mg/L (6B.V). The anthocyanin cyanidin-3-glucoside was not detected in either wine. The wines were significantly different from each other, with the control wine (6B.V) having consistently higher concentrations across every category. The total anthocyanin content was 149.24 ± 0.21 mg/L for wine 2A.V+B and 202.67 ± 0.12 mg/L for the control wine 6B.V. The HPLC results reflect the findings of the anthocyanin assay, with wines produced using mixed species having lower anthocyanin concentrations than the control wines (see Section 4.4.2).

Table 4.10: Concentration (equivalent to malvidin-3-glucoside mg/L) of five types of anthocyanins and the total anthocyanins detected in a wine where fermentation was performed by BIODIVA sequentially inoculated with VL3 compared to a VL3 control fermentation (n = 2). Different letters next to the results indicate significant differences for that type of anthocyanin (ANOVA followed by Tukey's HSD).

Wine	Malvidin-3-glucoside (mg/L)	Peonidin-3-glucoside (mg/L)	Petunidin-3-glucoside (mg/L)	Cyanidin-3-glucoside (mg/L)	Delphinidin-3-glucoside (mg/L)	Total anthocyanins (mg/L)
2A.V+B	125.33 ± 0.18 b	6.14 ± 0.04 b	12.28 ± 0 b	Not detected	5.50 ± 0 b	149.24 ± 0.21 b
6B.V	168.09 ± 0.14 a	8.62 ± 0 a	17.89 ± 0.02 a	Not detected	8.07 ± 0.03 a	202.67 ± 0.12 a

The same HPLC analysis was performed on two RC212 wines (3B.R+B and 8B.R) and returned similar results (Table 4.11). The wines were significantly different from each other for the individual anthocyanins detected and the total anthocyanin content. Wine 8B.R had higher concentrations of the individual anthocyanins and a higher total anthocyanin content of 202.96 ± 0.07 mg/L, whereas wine 3B.R+B contained 161.33 ± 0.66 mg/L. Cyanidin-3-glucoside was not found in either wine. Similar to the anthocyanin assay results, the HPLC results are surprising, as it would be logical that a high concentration of total anthocyanins would contribute deeper colour intensity to the wine. Both forms of anthocyanin analysis found the mixed species wines to have lower anthocyanin concentrations; however, during the spectrophotometric analysis it was the mixed species wines that displayed the highest colour intensity (see Section 4.4.1).

Table 4.11: Concentration (equivalent to malvidin-3-glucoside mg/L) of five types of anthocyanins and the total anthocyanins detected in a wine where fermentation was performed by BIODIVA sequentially inoculated with RC212 compared to a RC212 control fermentation (n = 2). Different letters next to the results indicate significant differences for that type of anthocyanin (ANOVA followed by Tukey's HSD).

Wine	Malvidin-3-glucoside (mg/L)	Peonidin-3-glucoside (mg/L)	Petunidin-3-glucoside (mg/L)	Cyanidin-3-glucoside (mg/L)	Delphinidin-3-glucoside (mg/L)	Total anthocyanins (mg/L)
3B.R+B	132.88 ± 0.52 b	6.94 ± 0.08 b	14.52 ± 0.04 b	Not detected	6.98 ± 0.03 b	161.33 ± 0.66 b
8B.R	170.06 ± 0.04 a	8.41 ± 0.02 a	17.20 ± 0.04 a	Not detected	7.28 ± 0.02 a	202.96 ± 0.07 a

4.4.4 Sensory evaluation

During sensory analysis, the mixed fermentation VL3 + BIODIVA wines were compared to the solo VL3 wines, as were the mixed RC212 + BIODIVA wines with the RC212 control. This was carried out to determine whether the colour parameters measured for each set of wines was perceptible by the human eye. The first sensory evaluation session involved 20 participants, 11 females and 9 males. Each participant visited one station and completed two triangle tests and one 3-AFC test. In total, each of the mixed versus control wines was compared 20 times in triangle tests and 10 times in 3-AFC tests. For the VL3 comparisons, both types of sensory tests resulted in significant differences between the control and mixed inoculation wines (Table 4.12). There were 11 out of 20 correct answers for the triangle tests and 7 out of 10 correct answers for the 3-AFC tests. For the RC212 comparisons, there were 8 out of 20 correct answers for the triangle tests and 3 out of 10 correct answers for the 3-AFC tests, resulting in no significant differences between the wines (Table 4.12). The results of the sensory differentiation tests reflect the outcome of the spectrophotometric analysis, with no difference in colour intensity between the RC212 wines and a significant difference in colour intensity found between the VL3 wines (see Section 4.3.1).

Table 4.12: Significance of results obtained from two types of sensory test comparing the colour intensity of mixed fermentation wines (VL3 + BIODIVA and RC212 + BIODIVA) and control wines (VL3 and RC212). *P* values were calculated using the Student's t test.

Wine comparison	Type of sensory test	*P* value	Significance
VL3 + BIODIVA and VL3 control	Triangle	0.0376	*
VL3 + BIODIVA and VL3 control	3-AFC	0.0196	*
RC212 + BIODIVA and RC212 control	Triangle	0.3385	ns
RC212 + BIODIVA and RC212 control	3-AFC	0.7012	ns

As the first sensory evaluation session found differences between the VL3 + BIODIVA and VL3 wines, a second round of sensory analysis was performed to provide further information about how the wines differed and whether these differences changed the way that the participants perceived wine quality and preference. A total of 41 participants completed a survey (see Appendix 8.2) comparing a combined VL3 + BIODIVA wine sample to a combined VL3 wine sample. Each sample was given a score between one and five for three colour attributes (hue, intensity and brightness) and for how much the participants agreed or disagreed with two statements about the wine ("I like the colour of this wine" and "Based on its colour, I would expect this wine to be of high quality"). The average scores for the VL3 + BIODIVA wine and VL3 wine across the five questions are presented in Figure 4.6. Significant differences were found between the two wine samples for all three colour attributes. The largest difference was shown for the hue descriptor, with the VL3 + BIODIVA wine receiving an average score of 3.98 and the VL3 wine receiving a score of 2.34, meaning the mixed fermentation wine was more purple-tinged while the control wine was more red-tinged. The VL3 + BIODIVA wine was ranked as deeper in colour intensity than the VL3 wine, with scores of 4.39 versus 3.05. A significant difference was also found for the brightness attribute, with the VL3 + BIODIVA wine ranked as duller, with a score of 2.59 and the VL3 wine rated as brighter with a score of 3.10. There was no significant difference between respondents' average answers for the statement "I like the colour of this wine", with VL3 + BIODIVA receiving a score of 3.85 and VL3 receiving a similar score of 3.68. The participants did however give a significantly different overall score for the other statement included in the survey, "Based on

its colour, I would expect this wine to be of high quality". The VL3 + BIODIVA wine received a higher score of 3.76 compared to the VL3 wine's 3.22, meaning the respondents thought the mixed fermentation wine was likely to be of higher quality than the control.

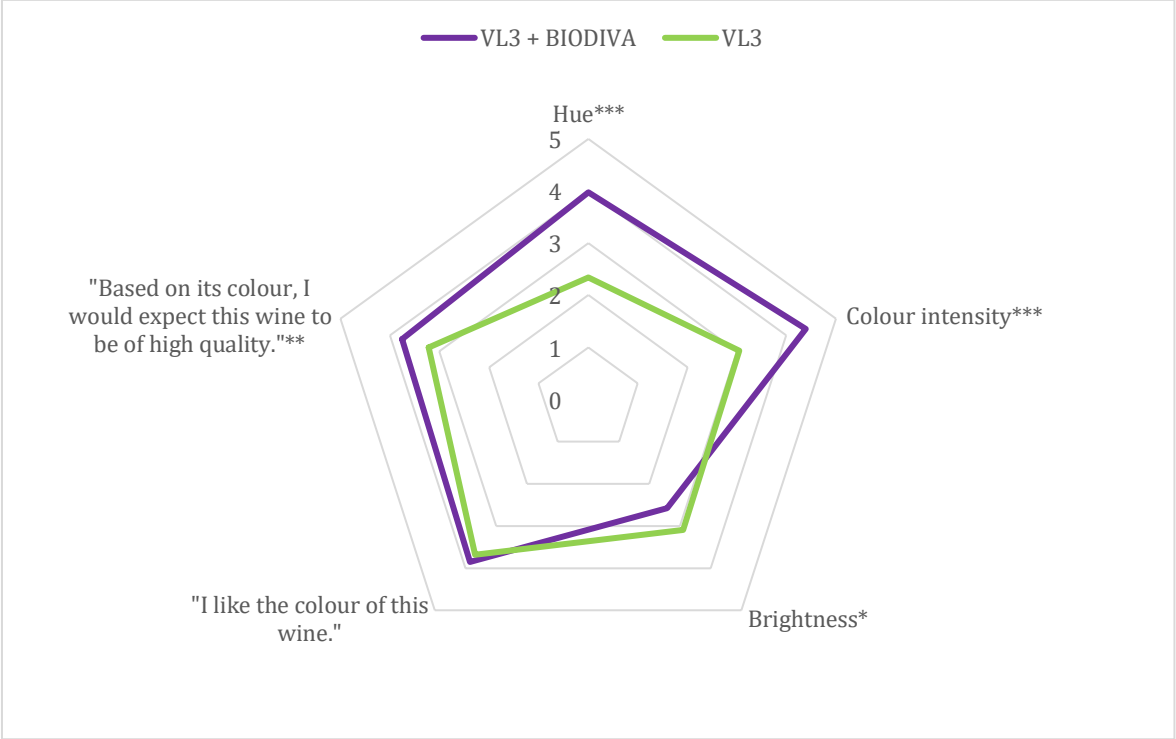
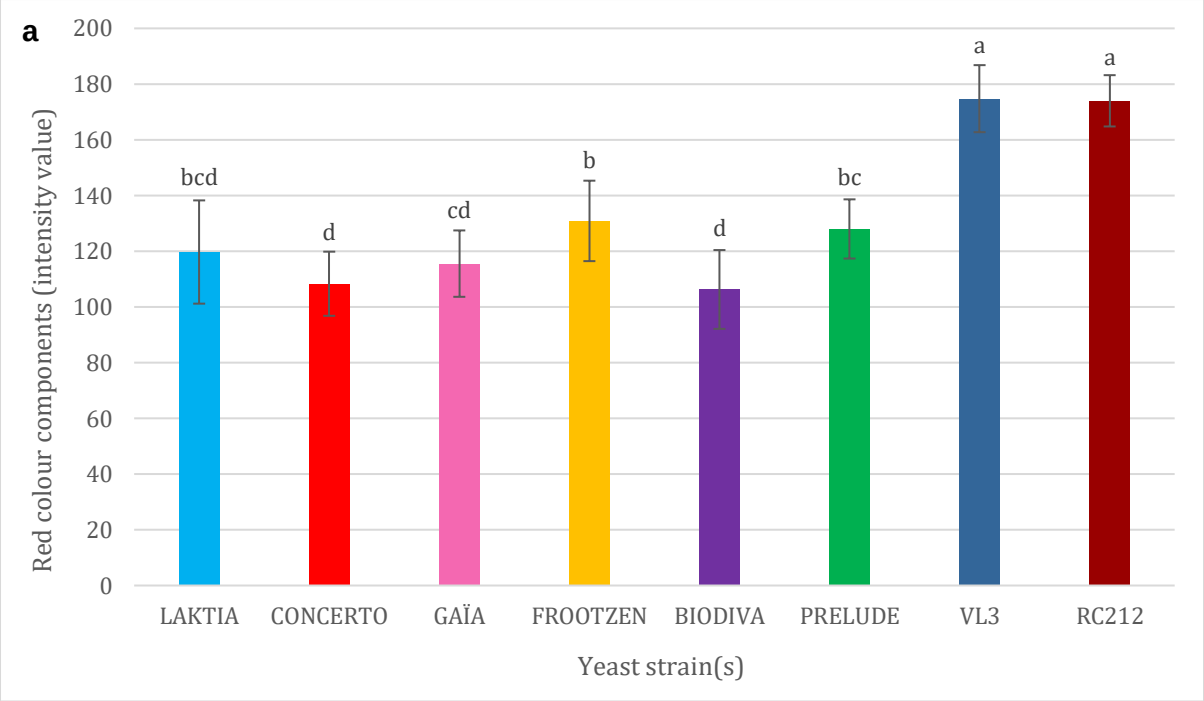


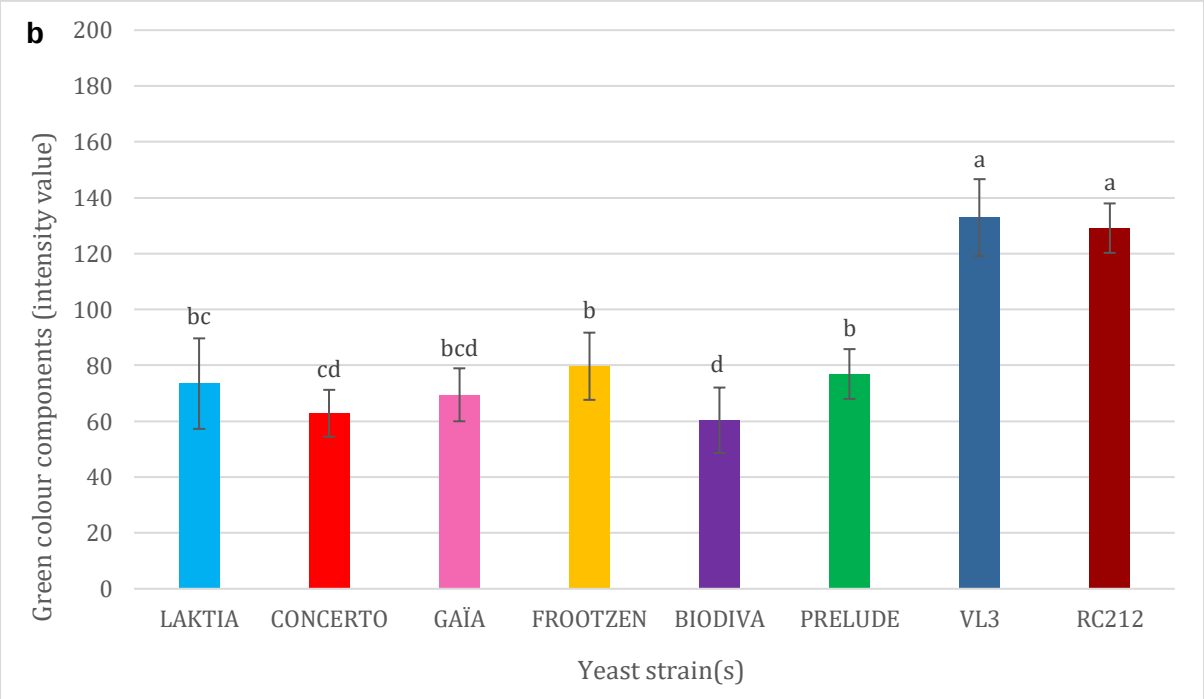
Figure 4.6. Spider plot showing the results of sensory analysis comparing the appearance of wines where fermentation was performed by BIODIVA sequentially inoculated with VL3 (purple line), compared with VL3 control wines (green line). Hue is rated from red-tinged (1) to purple-tinged (5), intensity is rated from light (1) to deep (5) and brightness is rated from dull (1) to bright (5). The two statements are rated from strongly disagree (1) to strongly agree (5). The scores for each wine are the means of responses from 41 survey participants. Significant differences, calculated using the Student's t test, are indicated by asterisks, with more asterisks representing a higher level of significance - * (*p* value < 0.05), ** (*p* value < 0.01) and * (*p* value < 0.001).**

4.5. Yeast pigment adsorption

A separate, complementary trial was conducted to assess the pigment adsorption abilities of the eight yeasts (six non-*Saccharomyces* and two *S. cerevisiae*) used in this research. The yeasts were individually grown in fermentation conditions on grape skin medium plates for 10 days. The biomass colour at the end of the 10 days reflected the binding of grape skin pigments to

the yeast cells. Biomass samples of each yeast were photographed and analysed in Adobe Photoshop's red-green-blue (RGB) colour mode. The resulting intensity values for each of the red, green and blue colour components are presented in Figure 4.7. The results were consistent across the three colour components. The two *S. cerevisiae* yeasts, VL3 and RC212, displayed the highest mean intensity values, and therefore the lowest pigment adsorption, in all three colour categories. Both yeasts were significantly different from all six non-*Saccharomyces* yeasts for red, green and blue components. VL3 was slightly higher than RC212 each time, with the highest values for red components (175 ± 12), green components (133 ± 14) and blue components (98 ± 13) but the differences were not found to be significant during statistical analysis. The lowest intensity values, and therefore highest pigment adsorption, was demonstrated across all three colour components by the yeast BIODIVA. For red colour components, BIODIVA was significantly different from four of the other yeasts (FROOTZEN, PRELUDE, RC212 and VL3) with the lowest mean value of 106 ± 14 (Figure 4.7a). For green colour components, BIODIVA was significantly different from five of the other yeasts (FROOTZEN, LAKTIA, PRELUDE, RC212 and VL3) with the lowest mean value of 60 ± 12 (Figure 4.7b). For blue colour components, BIODIVA was significantly different from three of the other yeasts (FROOTZEN, RC212 and VL3) with the lowest mean value of 47 ± 10 (Figure 4.7c). Overall, the interaction between the yeasts and grape skin pigments differed significantly across yeast species and strains, with BIODIVA (*T. delbrueckii*) consistently adsorbing the most colour and VL3 (*S. cerevisiae*) adsorbing the least colour. The contrast between the level of pigment adsorption displayed by these two yeast species is visible in photographs taken of the yeast biomass samples (Figure 4.8).


a
Red colour components (intensity value)
200
180
160
140
120
100
80
60
40
20
0
bcd
d
cd
b
d
bc
a
a
LAKTIA
CONCERTO
GAÏA
FROOTZEN
BIODIVA
PRELUDE
VL3
RC212
Yeast strain(s)


b
Green colour components (intensity value)
200
180
160
140
120
100
80
60
40
20
0
bc
cd
bcd
b
d
b
a
a
LAKTIA
CONCERTO
GAÏA
FROOTZEN
BIODIVA
PRELUDE
VL3
RC212
Yeast strain(s)

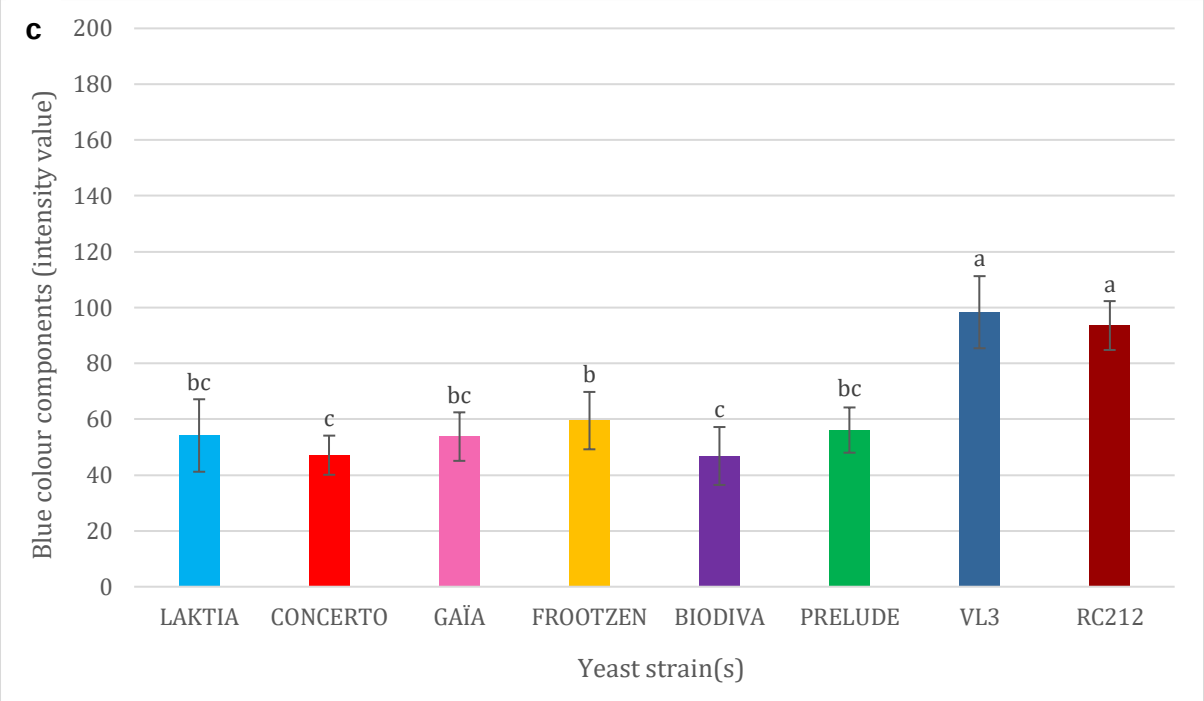


Figure 4.7. Intensity values (assigned by Adobe Photoshop RGB colour mode) of colour components in pigments adsorbed by six non-*Saccharomyces* spp. (LAKTIA, CONCERTO, GAIA, FROOTZEN, BIODIVA and PRELUDE) and two commercial *S. cerevisiae* yeast strains (VL3 and RC212) during growth on grape skin medium plates. Samples shown are the means of 9 colour samples (for the two *S. cerevisiae* strains) or 18 colour samples (for the non-*Saccharomyces* spp.). The error bars represent 95% confidence intervals. Different letters above the data indicate significant differences (ANOVA followed by Tukey's HSD). a) Red colour components. b) Green colour components. c) Blue colour components.

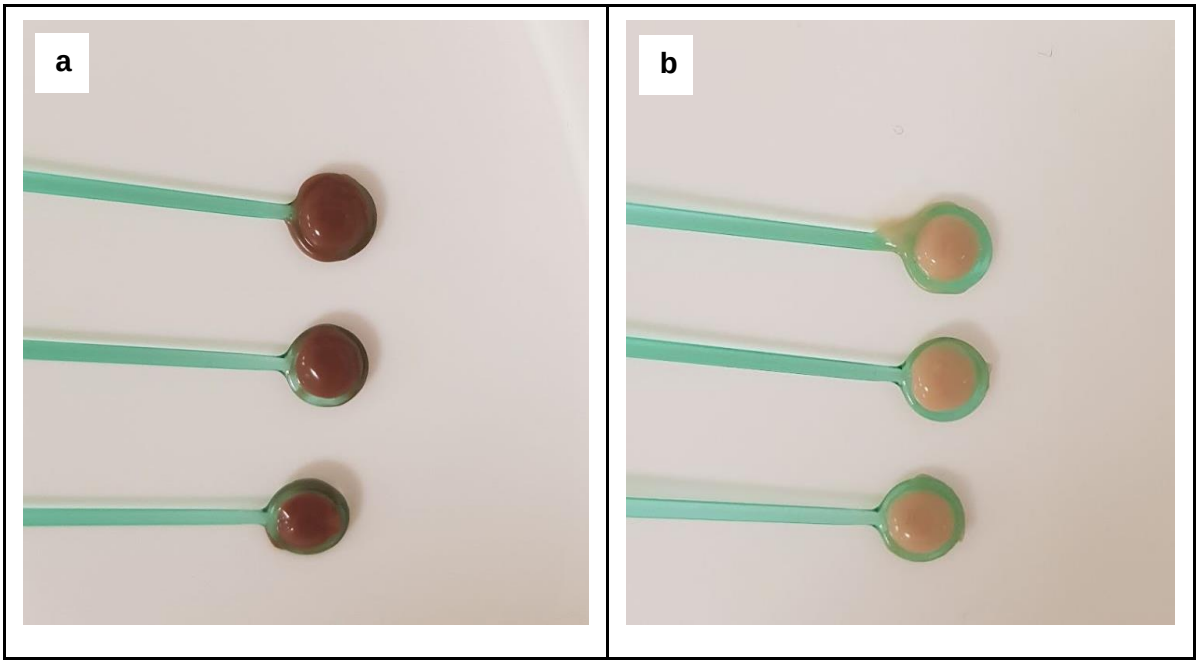


Figure 4.8. Photographs of yeast biomass samples taken after 10 days of growth on grape skin medium plates. a) BIODIVA (*T. delbrueckii*). b) VL3 (*S. cerevisiae*).

Chapter 5. Discussion

5.1. Summary of results

Eight commercial yeast strains, six non-*Saccharomyces* and two *S. cerevisiae*, were trialled in this project. Firstly, the yeasts were used in individual and mixed non-*Saccharomyces* and *S. cerevisiae* microfermentations of synthetic grape must, where a range of fermentation abilities was observed. The fermentations were followed by sedimentation rate assays, where the flocculation ability of each individual yeast strain and each non-*Saccharomyces* and *S. cerevisiae* combination was determined. BIODIVA, a strain of *T. delbrueckii*, displayed significantly greater flocculation ability than the other yeasts trialled, both individually and in combination with *S. cerevisiae* strains VL3 and RC212. To determine whether the increased flocculation capabilities would impact on wine colour, a Pinot noir winemaking trial was then performed, using a sequential inoculation of BIODIVA and VL3 to perform primary fermentation compared to a VL3 control, and a sequential inoculation of BIODIVA and RC212 compared to a RC212 control. Standard wine analyses and a series of colour analyses were performed on the resulting Pinot noir wines. UV/visible spectrophotometry found that the mixed fermentation wines, using BIODIVA and either VL3 or RC212, generally displayed greater colour intensity compared to the *S. cerevisiae* controls. Further colour analysis techniques, including the Adams-Harbertson anthocyanin assay and HPLC, found that the wines with the highest colour intensities also typically had the lowest anthocyanin concentrations. Separately, a pigment adsorption trial found that the six non-*Saccharomyces* yeast strains used in this research adsorbed significantly more pigments from grape skins than the two *S. cerevisiae* strains.

5.2. *S. cerevisiae* and non-*Saccharomyces* yeast strains display diverse fermentation abilities

During the microvinification trials, both the FROOTZEN and GAÏA yeasts failed to complete alcoholic fermentation of SGM when inoculated independently. The fermentations were sluggish, with a long lag phase and low V_{max} compared to the other yeasts used in the trial. Of the eight yeasts trialled, the most effective individual fermenter was PRELUDE (*T. delbrueckii*), followed by RC212 and VL3 (both strains of *S. cerevisiae*).

5.2.1 FROOTZEN (*P. kluyveri*) displays poor fermentation kinetics

FROOTZEN is a strain of *P. kluyveri* designed for use in mixed species fermentations to enhance aromatic complexity by mimicking a wild fermentation, while allowing the winemaker more control. FROOTZEN should be used as the first strain in a sequential inoculation, followed by a strain of *S. cerevisiae* after two days (Anfang et al., 2010; Chr. Hansen, n.d.a). It was unsurprising that FROOTZEN fermented poorly when used to initiate fermentation on its own, given that *P. kluyveri* cannot survive once ethanol levels reach 4-5% (v/v) (Vicente et al., 2021). A sequential inoculation with *S. cerevisiae* is required to continue alcoholic fermentation beyond this point (Vicente et al., 2021). *S. cerevisiae* is uniquely suited to wine fermentation conditions and dominates the mid and later stages of alcoholic fermentation by outcompeting other species (Albergaria & Arneborg, 2016; Goddard, 2008). This is demonstrated by the different fermentation kinetics seen when FROOTZEN was sequentially inoculated with the two *S. cerevisiae* strains used in this research, VL3 and RC212, compared to when FROOTZEN was used alone. The fermentations were still sluggish to begin with, reflecting FROOTZEN's poor fermenting abilities, but they were both able to reach completion following the addition of *S. cerevisiae*. While *P. kluyveri* is unsuitable as a solo fermenter, it plays a specific, beneficial role when used appropriately in industry conditions. Previous research by Anfang et al. (2010) found the co-fermentation of *P. kluyveri* and VL3 in Sauvignon blanc resulted in an increase in varietal thiols, which contribute fruity characteristics to the wine.

5.2.2 GAÏA (*M. fructicola*) performs fermentation poorly

Similar to FROOTZEN, GAÏA is also designed for use in sequential inoculations. A strain of *M. fructicola*, GAÏA has minimal fermenting ability and is not intended to contribute to the primary fermentation process at all (Lallemand, n.d.a; Prior et al., 2019; Su et al., 2020). Instead, it is meant to be used as a method of biocontrol, as an alternative to SO_2 at the start of the winemaking process, particularly during pre-fermentation maceration or must transport. GAÏA works by preventing the growth of spoilage microorganisms that produce acetic acid, but it has weak fermentation abilities with low resistance to ethanol. Johnson et al. (2020) found that GAÏA reduced the growth of spoilage yeast *Hanseniaspora uvarum* during pre-fermentation cold soaking of Pinot noir fruit. Previous research by Boscaino et al. (2019) has also shown using *M. fructicola* in mixed fermentations with *S. cerevisiae* can increase the concentration of esters and terpenes, which enhance the aromatic complexity of wines;

however, the primary use of the species is as a biocontrol agent, making GAÏA an impractical choice for solo fermentation. These characteristics were reflected in the results of the microvinification trials, where the GAÏA fermentation of SGM was sluggish and failed to reach completion. When sequentially inoculated with VL3 or RC212 (both *S. cerevisiae*), the GAÏA fermentations adhered more closely to standard fermentation kinetics and were able to finish.

5.2.3 PRELUDE (*T. delbrueckii*) demonstrates strong fermentation abilities

The best performing fermenter, PRELUDE, is a strain of *T. delbrueckii*, which is believed to be the most frequently used non-*Saccharomyces* yeast species in winemaking (Benito, 2018; Zhang et al., 2018). In fermentation conditions with its preferred nitrogen sources, which are remarkably similar to those of *S. cerevisiae*, *T. delbrueckii* exhibits a cell growth and fermentation rate to rival *S. cerevisiae* (Su et al., 2020). *T. delbrueckii* is considered to be a relatively powerful fermenter compared to most other non-*Saccharomyces* species. It has a moderate resistance to ethanol, usually up to 9% v/v, and is capable of surviving in high sugar musts, allowing it to operate as a solo fermenter in wine conditions (Benito, 2018). PRELUDE was the first strain of *T. delbrueckii* to be made commercially available. It has many benefits for winemakers, including low levels of volatile acidity, promotion of malolactic fermentation (MLF), improved flavour complexity due to the high concentrations of esters and increased production of mannoproteins, which can alter the mouthfeel of the final wine (Chr. Hansen, n.d.b; Hranilovic et al., 2018). PRELUDE's strong fermentation abilities were clearly demonstrated in the microvinification trials, where it reached the highest V_{max} and had the largest cumulative weight loss. PRELUDE was equally effective as a fermenter when sequentially inoculated with the two strains of *S. cerevisiae*, VL3 and RC212, each outperforming almost all the other mixed fermentations. PRELUDE's fermentative abilities, combined with its potential to improve wine quality, prove why it is so popular with winemakers looking to diversify their usage of commercial yeast starters.

5.2.4 VL3 and RC212 (both *S. cerevisiae*) are effective fermenters

The two *S. cerevisiae* strains, VL3 and RC212, were almost on par with PRELUDE's fermentation abilities. Effective fermentation is to be expected from strains of *S. cerevisiae*, as this species is commonly referred to as 'the wine yeast' due to its unique characteristics and dominance during alcoholic fermentation (Jolly et al., 2014). In addition, VL3 and RC212 are both commercial wine strains, which are part of a phylogenetically distinct group, known as

the 'Wine' cluster of *S. cerevisiae*, that have been selected for their winemaking capabilities (Borneman et al., 2016; Liti et al., 2009; Schacherer et al., 2009). *S. cerevisiae* can survive the harsh environmental conditions found during alcoholic fermentation, such as the low pH, high ethanol level, limited oxygen and lack of certain nutrients. Despite being present in low numbers on the fruit initially, the *S. cerevisiae* population will quickly grow and overtake other species during fermentation (Albergaria & Arneborg, 2016). The development of commercial active dried yeast allows winemakers to kickstart fermentation with a selected strain of *S. cerevisiae*, rather than waiting for it to begin spontaneously, which reduces production times and removes the pre-fermentation opportunity for unwanted microbes to grow (Benito et al., 2019). While interest is growing in the use of non-*Saccharomyces* yeasts to increase wine quality by adding complexity on the nose and palate and improving mouthfeel, *S. cerevisiae* still plays a necessary role in alcoholic fermentation. The majority of non-*Saccharomyces* yeasts are poor fermenters and do not tolerate climbing ethanol levels or the presence of sulfur dioxide (Jolly et al., 2014). Thus *S. cerevisiae* will always be crucial to the winemaking process to ensure a quick and complete fermentation (Jolly et al., 2014). The microvinification trials clearly demonstrate how non-*Saccharomyces* yeast species that fail, or are slow to complete fermentation on their own, benefit from the addition of *S. cerevisiae*. The fermentation performances of FROOTZEN, GAÏA, LAKTIA and CONCERTO were all improved by the sequential inoculation of either VL3 or RC212.

5.3. BIODIVA (*T. delbrueckii*) has a high flocculation capability, both alone and in mixed species fermentations

BIODIVA is a strain of *T. delbrueckii*, developed to increase aromatic complexity, intensify varietal characters, improve mouthfeel, while producing low levels of volatile acidity (Hranilovic et al., 2018; Lallemand, n.d.b). BIODIVA is recommended for use with fermentations of Chardonnay, Sémillon, Pinot noir and Shiraz. Previous research has found that BIODIVA is a reasonable fermenter, as was also shown by its performance in this project (Figure 4.1), and can be used in sequential inoculations for high sugar musts to produce late harvest wines (Azzolini et al., 2012). BIODIVA can also produce other metabolites of benefit to wine quality, such as glycerol, which can improve a wine's body and texture (González-Royo et al., 2015). In the sedimentation rate assays, BIODIVA had a significantly higher sedimentation rate, and therefore was more flocculant, than all other yeasts tested. The sequential inoculation of VL3 or RC212 did not change the outcome, with the mixed species

BIODIVA fermentations again displaying a significantly different and much higher rate of flocculation than the other combinations. Flocculation is a complex phenomenon and is hard to control (Verstrepen et al., 2003). It has not yet been confirmed as to why yeast cells flocculate, but it is believed to be in response to environmental stress and may also provide optimal conditions for cell mating (Goossens et al., 2015; Rose, 1984). The likelihood of flocculation occurring is due to three factors – yeast genetics and two categories of environmental conditions, those that influence the activity of *FLO* genes and those that influence the changes of the yeast cells making physical contact (Verstrepen et al., 2003). Given that the external factors, such as temperature and pH, were controlled and accounted for during the experiment, the high flocculation shown by BIODIVA is most likely due to both its genetics, and the ways in which the strain interacts with its environment. The flocculation ability of a particular yeast is due to the presence or absence of *FLO* genes, which encode flocculin proteins (Verstrepen et al., 2003). Flocculins protrude from the cell wall and bind to the α-mannan carbohydrates on the cell walls of other yeast cells, creating flocs (Rossouw et al., 2015). The genome of BIODIVA has not been sequenced but based on the results of the sedimentation rate assays, it should contain *FLO* genes that are transcribed into active proteins. It is also likely that any *FLO* genes present in BIODIVA produce relatively long proteins, as the longer the protein the stronger the strain's flocculation potential (Vidgren & Londesborough, 2011). BIODIVA flocculins may also be of the New-Flo phenotype, which means that they can bind with a wider range of sugars, rather than just mannose, or the MI phenotype, which is not affected by mannose and does not need the presence of calcium for flocculation. This could be confirmed by using next generation sequencing to obtain either the whole genome sequence or the transcriptome of BIODIVA to identify the sequences or expression of genes responsible for flocculation. As the sedimentation rate assay measured flocculation at the end of fermentation, it may be possible that ethanol played a role, inducing flocculation as the ethanol level increased. However, BIODIVA was visibly flocculating and more difficult to handle during routine culture in YPD, where no ethanol is produced, indicating that BIODIVA is capable of forming flocs without the influence of ethanol. Given that the flocculation rate remained high when BIODIVA was sequentially inoculated with VL3 or RC212, the trial shows that BIODIVA is either capable of forming mixed species flocs with *S. cerevisiae*, and/or that its presence triggers the flocculation of *S. cerevisiae* strains VL3 and RC212. Since the sedimentation rate was measured at the end of fermentation, the measure of flocculation was specific for the cells present at fermentation completion. The *S. cerevisiae* and BIODIVA mixed fermentations performed slightly better than BIODIVA alone, as shown by

the cumulative weight loss curves (Figures 4.1, 4.2, 4.3), justifying the assumption that the *S. cerevisiae* strains likely took over the fermentations once added and would predominate at the end of fermentation. Considering that the sedimentation rate remained high, co-flocculation may have taken place with BIODIVA cells, or the presence of BIODIVA cells induced the flocculation of *S. cerevisiae*. A previous study has shown that *T. delbrueckii* is capable of surviving until the end of fermentation alongside *S. cerevisiae*, although neither of the two strains tested were BIODIVA (Wang et al., 2016). In this project, the outcome of the sedimentation rate assays was similar for both of the two *S. cerevisiae* strains (VL3 and RC212), which showed low sedimentation rates as individuals and high sedimentation rates in mixed fermentations, suggesting co-flocculation may be occurring between both combinations of *S. cerevisiae* and BIODIVA. Future research could confirm if this was the case and explore the interactions between BIODIVA and *S. cerevisiae* in more depth. This could be carried out by labelling the yeast cells as in Varela et al. (2020), or plating and genotyping them, to assess the ratio of BIODIVA to *S. cerevisiae* cells present at the end of primary fermentation. Microscopy could also be used to visualise co-flocculation, as in Touhami et al. (2003), where atomic force microscopy was used to observe flocculation of yeast cells in both the exponential phase and the stationary phase of growth, or in Rossouw et al. (2015), where the researchers used fluorescent microscopic imaging of yeast cells to visually confirm that two different species had formed mixed species flocs (see Figure 2.4). The other strain of *T. delbrueckii* included in the trial, PRELUDE, was much less flocculant than BIODIVA and was not significantly different from most of the other yeasts that were assayed. A strong flocculation ability appears to be unique to the strain of BIODIVA, rather than a characteristic of *T. delbrueckii* as a species. Alternatively, BIODIVA may have similar flocculation abilities to other *T. delbrueckii* strains, but the particular conditions of this experiment, such as the specific temperature, pH, or osmolarity, triggered a high rate of flocculation. If the transcriptome of BIODIVA was sequenced, it could be compared to the available transcriptomic sequence data of another *T. delbrueckii* strain, COFT1 (Tondini et al., 2019), to find differences in expression between flocculation-related genes. A future study could also trial more strains of *T. delbrueckii* using the sedimentation rate assay to see if others also exhibit the same high flocculation capabilities as BIODIVA. Regardless, the observation of increased sedimentation rate for mixed *T. delbrueckii* and *S. cerevisiae* fermentations contributes to the idea that physical cell-cell contact may have adapted as a survival mechanism for select yeast during fermentation, providing mutual benefits for multiple species present in a wild fermentation (Goossens et al., 2015; Rose, 1984).

5.4. Mixed species sequential *T. delbrueckii* and *S. cerevisiae* Pinot noir fermentations perform as well as *S. cerevisiae* fermentations

5.4.1 Fermentation

During the Pinot noir winemaking trials, the four *T. delbrueckii* and *S. cerevisiae* mixed fermentations (duplicates of VL3 + BIODIVA and RC212 + BIODIVA) and four *S. cerevisiae* control fermentations (duplicates of VL3 and RC212) performed equally as well in terms of fermentation efficiency, with the fermentation kinetics and temperature showing consistent performance throughout the process. The three yeast strains used (BIODIVA, VL3 and RC212) are all commercially available products, in the form of active dried yeast, and as such have been through development and testing to ensure that when used as intended, with high quality grapes, they are very unlikely to result in stuck or sluggish fermentations. As a non-*Saccharomyces* yeast, *T. delbrueckii* is generally a less powerful fermenter than *S. cerevisiae* (Benito, 2018), but its inclusion as part of the mixed species sequential inoculations in the Pinot noir trials did not appear to hinder the progress or prevent the completion of primary fermentation. This result is to be expected, as *T. delbrueckii* is frequently used in winemaking (Zhang et al., 2018), which would not be the case if it negatively affected fermentation performance. This result is noteworthy in that Pinot noir winemakers can have confidence in using sequential fermentations of non-*Saccharomyces* spp. with *S. cerevisiae*, as an alternative to the more risky spontaneous fermentations.

5.4.2 Wine analysis

The results for the standard wine parameters were consistent across all wines and within the ranges expected for Pinot noir. These analyses further reinforced that commercial mixed species *T. delbrueckii* and *S. cerevisiae* fermentations can perform just as well as *S. cerevisiae* alone, producing wines with very similar levels of titratable acidity, residual sugar and malic acid. MLF was completed with no issues and there were no differences in final malic acid concentrations between the mixed and control wines, except for one wine (5B.V) which had a slightly higher concentration but was still within the acceptable range. Previous research by Balmaseda et al. (2021) found that the presence of BIODIVA may even help optimise MLF, as BIODIVA is more compatible with *O. oeni*, the bacteria inoculated for MLF, than the other yeast strains tested. The only wine parameter where significant differences were found in both the VL3 and RC212 mixed and control wines was the alcohol content. In both cases the control

S. cerevisiae wines had higher alcohol levels than the mixed BIODIVA and *S. cerevisiae* wines. This result is consistent with extensive previous research on the ability of sequential inoculations of non-*Saccharomyces* and *S. cerevisiae* yeast strains to reduce alcohol content of wines compared to *S. cerevisiae* alone (Maturano et al., 2018; Puškaš et al., 2020). This phenomenon is due to non-*Saccharomyces* species generally being less efficient at converting sugar to ethanol (Contreras et al., 2014). The production of lower alcohol wines is a growing area of interest, as consumer preferences evolve and winemakers develop new techniques to control sugar and alcohol levels in the face of climate change. From the results of this project, it appears that BIODIVA is one of the non-*Saccharomyces* strains with the ability to produce wines with lower ethanol concentrations. In the comparison of the VL3 mixed and control wines, there was also a significant difference in the concentration of volatile acidity (primarily acetic acid), which at high levels results in unwanted sensory characteristics. The BIODIVA and VL3 mixed fermentations produced wines with a higher mean acetic acid concentration compared to the control VL3 fermentations. Previous research has shown that the use of non-*Saccharomyces* species during primary fermentation can result in increased production of acetic acid (Röcker et al., 2016; Shekhawat et al., 2017). Unlike other non-*Saccharomyces* species, *T. delbrueckii* is known for its low production of acetic acid (Renault et al., 2009). Shekhawat et al. (2017) found that *T. delbrueckii* and *S. cerevisiae* mixed fermentations could produce high levels of acetic acid but only when the dissolved oxygen level of the wine was also high. While the VL3 wines showed differences in acetic acid levels when *T. delbrueckii* was used in sequential inoculation, there were no differences between the RC212 mixed and control wines. Overall, it is unclear if the strain of *T.delbrueckii* used in this research, BIODIVA, has a positive or negative influence on the production of acetic acid in Pinot noir wines. The pairing of *T. delbrueckii* and *S. cerevisiae* in red wine fermentations continues to be of interest to winemakers and scientists alike (Benito, 2018; Benito et al., 2019; Chen et al., 2018; Escribano-Viana et al., 2019, Vejarano et al., 2021), and these results support the case for future research into the benefits of co-inoculation with these two yeasts, particularly if the strain of *T. delbrueckii* involved is BIODIVA.

5.5. The sequential inoculation of *T. delbrueckii* and *S. cerevisiae* increases Pinot noir wine colour intensity

Two strains of *S. cerevisiae* (VL3 and RC212) were trialled in sequential inoculations with a strain of *T. delbrueckii* (BIODIVA) for the fermentation of Pinot noir. Both yeast combinations

appeared to increase the colour intensity of Pinot noir wines compared to *S. cerevisiae* controls. VL3 with BIODIVA produced final wines with deeper colour intensity compared to controls, while RC212 with BIODIVA resulted in wines with the same level of colour intensity; however, the initial juice used for the sequential inoculations was significantly lower in colour intensity than the juice used for the controls. Following these analyses, further experiments were performed to explore the mechanism behind the differences in colour intensity (see Section 5.7 and Section 5.9).

5.5.1 The sequential inoculation of BIODIVA (*T. delbrueckii*) and VL3 (*S. cerevisiae*)

The results of both UV/visible spectrophotometry and sensory evaluation confirmed that the sequential inoculation of BIODIVA (*T. delbrueckii*) and VL3 (*S. cerevisiae*) resulted in Pinot noir wines with deeper colour intensity than VL3 control wines. Significant differences were found in the colour intensities of the mixed and control wines when using UV/visible spectrophotometry and when the wines were visually examined by panellists. BIODIVA was used in the winemaking trial due to its high flocculation rate in combination with VL3, as previous research has shown that increased flocculation can result in increased colour intensity (Varela et al., 2020). Deeper colour intensity was the outcome for the VL3 + BIODIVA wines in this research. Given that all external factors that affect flocculation were controlled for during the design of this experiment, and that the combination of the two *S. cerevisiae* strains with BIODIVA was shown to result in high end-of-ferment sedimentation rates when sequentially inoculated, there is a strong indication that the higher colour intensity was due to increased flocculation. Albeit, there is the possibility that flocculation was not the sole reason for higher colour intensity, as there may be another characteristic of the BIODIVA yeast, or the interaction between *S. cerevisiae* and BIODIVA, that can impact colour intensity. Further experiments will be required to confirm whether this is the case. Sedimentation rate assays were not performed following the Pinot noir fermentations, which could be measured in future to confirm that co-flocculation was occurring in the red wine matrix, alongside microscopy to visualise the interactions between the yeast cells during winemaking. Techniques used by Mencher et al. (2021) could be replicated to observe yeast cells and confirm if flocculation is causing the increase in colour intensity. The researchers used semi-permeable membranes and hollow fibre filters to prevent cells from making physical contact while still growing in the same medium. This process could be performed with BIODIVA and VL3 to prevent flocculation during fermentation and if a colour intensity increase is still observed, it can be

concluded that flocculation was not the cause. BIODIVA is a strain of *T. delbrueckii*, which has been tested in previous research looking to improve the colour of red wines. Escribano-Viana et al. (2019) and Chen et al. (2018) both used strains of *T. delbrueckii*, not including BIODIVA, and sequentially inoculated them with *S. cerevisiae*, to make Tempranillo and Merlot wines, respectively. Escribano-Viana et al. (2019) used two strains of each species from the Institute of Grapevine and Wine Sciences in Logroño, Spain, while Chen et al. (2018) used a *T. delbrueckii* strain from the Institute of Industrial Fermentation collection in Madrid, Spain and the *S. cerevisiae* strain 7VA from the Polytechnic University of Madrid. Both studies found that the mixed fermentations increased the concentration of anthocyanins compared to control wines; however, there were minimal differences in colour intensity, indicating that anthocyanin content does not always correlate with overall colour intensity, which is also an observation in this study. Nardi et al. (2018) carried out a similar study with Barbera wines but used BIODIVA as the strain of *T. delbrueckii* and analysed colour intensity. The researchers found that the combination of BIODIVA and a commercial strain of *S. cerevisiae*, RBS 133, increased colour intensity compared to *S. cerevisiae* controls, hypothesised to be due to *T. delbrueckii*'s high production of pyruvic acid (Nardi et al., 2018). Pyruvic acid reacts with anthocyanins to form vitisin A, a stable pigment that is not subject to the bleaching effects of SO_2. Research by Belda et al. (2015) found that in fermentations involving *T. delbrueckii*, both as a solo fermenter and in sequential inoculation with *S. cerevisiae*, higher production of pyruvic acid was observed; however, the strain of *T. delbrueckii* used in the study was Viniferm NS TD, not BIODIVA. Future research could explore whether the production of high levels of pyruvic acid is unique to Viniferm NS TD, or is also a characteristic of other strains of *T. delbrueckii*, such as BIODIVA. The role of flocculation is unclear, but the results of this research suggest that it could be the source of increased colour intensity in Pinot noir wines. It is important to note that strains of *T. delbrueckii* differ and not all are as highly flocculant as BIODIVA, as evidenced by the performance of PRELUDE, also *T. delbrueckii*, in the sedimentation rate assays. There are no previous studies examining the impact of VL3 on red wine colour, as VL3 is intended for use in white wine fermentations, particularly Sauvignon blanc. This is due to its ability to generate high levels of varietal thiols, including 4-methyl-4-sulfanylpentan-2-one (4MSP), thanks to a full-length copy of the β-lyase encoding *IRC7* gene (Laffort, n.d.; Roncoroni et al., 2011; Winter et al., 2011). VL3 does however demonstrate relatively strong flocculation capabilities for an *S. cerevisiae* strain (Varela et al., 2020), which may have influenced the level of co-flocculation in the Pinot noir fermentations and therefore the colour of the final wines in this experiment. Further research is required to confirm whether flocculation, or

another behaviour or metabolic pathway, is responsible for deeper colour intensity in Pinot noir wines. As mentioned above, this could include closer examination, through microscopy and further sedimentation rate assays, of the mechanism of flocculation and the cell wall interactions that occur when BIODIVA and other strains of *T. delbrueckii* are used in sequential inoculations with *S. cerevisiae*. It is also important to winemakers and consumers to consider the sensory impact of these yeast combinations. Only the appearance of the VL3 + BIODIVA and VL3 wines was analysed during this project but future work could determine if any changes were noticeable in chemical aroma profile of the wines, as well as sensorially on the nose or palate in terms of aroma and mouthfeel attributes.

5.5.2 The sequential inoculation of BIODIVA (*T. delbrueckii*) and RC212 (*S. cerevisiae*)

Unlike the VL3 mixed and control wines, no significant differences were found between the RC212 mixed and control wines. Both the spectrophotometric analysis and sensory evaluation confirmed that the RC212 + BIODIVA and RC212 wines had very similar levels of colour intensity. However, it is important to acknowledge the differences between fermentations in the initial Pinot noir juice. While the VL3 mixed and control wines began the trial with juice of the same level of colour intensity, the two RC212 control fermentations started with juice that was significantly deeper in colour intensity than the two RC212 + BIODIVA fermentations. Therefore, the final results for the colour intensity reflect a closing of the gap between the mixed and control wines. The significant differences between the RC212 + BIODIVA and RC212 wines were lost during the fermentation process, suggesting the yeast strain(s) used had an influence on colour intensity. Much like in the comparison of the VL3 wines, the mixed fermentation of RC212 + BIODIVA appears to increase colour intensity compared to the RC212 control. This may be due to increased flocculation, or it could be a result of other changes in yeast behaviour because of their inclusion in a mixed-species fermentation. Involvement in a mixed culture alters yeast metabolic activity and changes the way cells communicate and behave, which then influences wine composition (Zilelidou & Nisiotou, 2021). Interactions between *T. delbrueckii* and *S. cerevisiae* during fermentation, such as cell signalling, may affect colour intensity of the wine. Alternatively, the change in colour intensity may be a result of characteristics of BIODIVA as an individual yeast strain, such as high levels of pigment adsorption or increased production of pyruvic acid as shown by other strains of *T. delbrueckii* (Belda et al., 2015). RC212 alone has previously been shown to increase colour intensity in Pinot noir wines compared to other yeast treatments (Carew et al.,

2013), which the researchers suggested could be due to higher levels of pigment adsorption by the yeast or through RC212 producing more acetaldehyde, which contributes to stable red wine colour through acetaldehyde-mediated dimer formation via an ethyl bridge. In this research, given that RC212 was used in both fermentations, its contribution to colour intensity should be equivalent for both wines. Therefore, the changes in colour intensity documented during the winemaking process are specifically due to the inclusion of BIODIVA in sequential fermentation with RC212. Overall, while the final RC212 + BIODIVA and RC212 wines displayed no differences, when considering the whole process from fruit to glass, the RC212 trial reflects the outcome of the VL3 trial and confirms that the sequential inoculation of BIODIVA and *S. cerevisiae* contributes greater colour intensity to Pinot noir wines, which we hypothesise to be the result of increased flocculation during primary fermentation, corroborating initial observations made by Varela et al. (2020).

5.6. Red wine colour intensity is lost during the winemaking process

Differences in colour intensity were not only seen between the mixed and control wines but also between the same individual wines at different stages of the winemaking process. This was the case for both the VL3 trial and RC212 trial. The highest colour intensity was seen at the juice stage and it then decreased over time. Previous research has shown certain steps of the winemaking process have a negative impact on red wine colour intensity. The addition of SO_2, used as a preservative and antimicrobial agent, has a bleaching effect on young red wines (He et al., 2012) and the process of malolactic fermentation also strips colour from red wine, as both pyruvic acid and acetaldehyde are degraded by the bacteria involved, in this case *O. oeni*, (Martínez-Pinilla et al., 2011; Osborne & Burns, 2015). The lowest overall colour intensities were seen in the post-MLF wines, followed by the final wines. MLF is known to reduce colour intensity of red wines (Abrahamse & Bartowsky, 2012; Martínez-Pinilla et al., 2011). The slight increase in colour intensity seen in the final wines after six weeks of settling is likely due to the presence of lees in the wine during this time. The dead yeast cells release compounds that contribute to wine colour stability and lees aging also helps maintain the monomeric anthocyanin content (Moreno-Arribas et al., 2008; Palomero et al., 2007). The overall decrease in colour intensity between the initial juice and final wines in this project shows how important it is for winemakers to use specific techniques to extract and retain as much colour intensity as possible before, during and after fermentation. One of these

techniques is to select a particular yeast species or strain known to have a positive impact on colour intensity.

5.7. *S. cerevisiae* control fermentations produce wines with higher anthocyanin concentrations than fermentations sequentially inoculated with *T. delbrueckii* and *S. cerevisiae*

Following the measurement of the colour intensity of the final wines, further analysis was carried out to help explain the results and explore the interactions occurring between yeast cells and other components of the wine, such as anthocyanins, the main source of colour in red wine (He et al., 2012). Two different methods of analysis, HPLC and the Adams-Harbertson assay, confirmed that the VL3 control wines had significantly higher concentrations of anthocyanins than the VL3 + BIODIVA wines. This was true of the total concentration as well as for each of the individual anthocyanins measured by HPLC. For the RC212 wines, there were significant differences between the individual wines but not an overall difference between the RC212 + BIODIVA and RC212 wines. Free anthocyanins are the main source of colour in young red wines (He et al., 2012); however, in this research the anthocyanin concentrations did not reflect the colour intensity of the wines. The VL3 + BIODIVA wines had significantly less anthocyanins than the VL3 control wines, despite having a significantly higher colour intensity. These results support previous and emerging research about the relationship between anthocyanins and the colour intensity of red wines. Somers, who pioneered the early spectrophotometric methods used to analyse wine colour, assessed 32 red wines and found no correlation between anthocyanin content and total colour intensity (Somers & Evans, 1974). Similar results were reported by Chen et al. (2018) and Escribano-Viana et al. (2019), who used *T. delbrueckii* and *S. cerevisiae* in sequential inoculations, then measured both the anthocyanin concentration and colour intensity of the wines and found no clear relationship. Further research has since explored the reasons for these findings and increased understanding of the phenomenon of copigmentation in red wines. Copigmentation is a complex process where coloured anthocyanins interact with other phenols such as flavonols, flavanols and hydroxycinnamic acids, which are colourless and known as copigments (Cavalcanti et al., 2010; Zhang et al., 2016). This interaction causes a red wine to exhibit greater colour intensity than would be expected based on its anthocyanin content and can account for 30 to 50% of the colour in a young red wine (Boulton, 2001). Previous research has also produced examples of Pinot noir wines with low anthocyanin concentrations despite high colour intensity, which is

believed to be due to the formation of pyranoanthocyanins (Yang et al., 2021). The occurrence of copigmentation in the VL3 + BIODIVA wines could explain why they displayed greater colour intensity despite the relatively low anthocyanin concentration. Copigmentation can also result in a shift in the wavelength where the maximum absorbance is found, which can give a red wine more of a blue or purple tone (Boulton, 2001). This was observed in the second sensory evaluation session, where the VL3 + BIODIVA wine was found to be significantly different in hue, with more purple tones, compared to the VL3 control wine which was more red-tinged. Copigmentation is complex and is affected by several factors, such as the pH, the nature of the cofactor and pigment and the ratio of cofactor to pigment (Boulton, 2001). The yeast strain(s) performing fermentation can also affect copigmentation and ongoing colour stability in different ways, for example by the level of hydroxycinnamate decarboxylase (HCDC) activity or through reactions between anthocyanins and metabolites released by the yeast, such as acetaldehyde (Morata et al., 2015). Medina et al. (2016) found that the co-fermentation of Tannat juice by some non-*Saccharomyces* and *S. cerevisiae* yeast species increased the production of acetaldehyde compared to a *S. cerevisiae* control. Acetaldehyde reacts with anthocyanins to form the stable pigments vitisin A and vitisin B, which can contribute to long term colour intensity, and this research by Medina et al. (2016) revealed for the first time that non-*Saccharomyces* yeast species were capable of producing vitisin B. The researchers indicated that further studies would be necessary to explain why the interaction between species resulted in increased acetaldehyde production and interspecies flocculation could be one hypothesis to explore. In the study, the mixed fermentation of a commercial strain of *T. delbrueckii* and *S. cerevisiae* did not result in increased acetaldehyde concentration, so further experimentation would be required to determine if mixed BIODIVA and *S. cerevisiae* fermentations would result in more acetaldehyde and therefore more stable pigments in Pinot noir wines. More widely there is the potential for future research to confirm if the use of BIODIVA or other strains of *T. delbrueckii* result in increased copigmentation in Pinot noir wines and explore how and why this occurs. One approach would be to measure wine polymeric material. During fermentation, yeast produce wine polymeric material, such as polysaccharides and mannoproteins, and yeast flocculation is controlled by cell wall mannoproteins, flocculins. Previous research has shown that wine polymeric material improves the colour stability of red wines (Escot et al., 2001; Gonçalves et al., 2018). As flocculant yeast strains differ in molecular weight and composition of cell wall mannoproteins (Saulnier et al., 1991), there may be key differences in BIODIVA's composition that are the cause of the increased colour intensity, lower anthocyanins and likely copigmentation, in parallel with the

increased flocculation observed in this project. Future research analysing wine polymeric material could explore this hypothesis further.

5.8. Consumers perceive purple-tinged Pinot noir wines with deep colour intensity to be of high quality

The second sensory evaluation session delved deeper into the visual differences between the VL3 + BIODIVA wines and VL3 control wines. The mixed fermentation wine samples were found to be deeper in colour intensity, more purple-tinged and less bright according to the participants surveyed. The results also found that participants were significantly more likely to agree with the statement "Based on its colour, I would expect this wine to be of high quality" for the VL3 + BIODIVA wine than for the VL3 wine. From this we can conclude that the participants, a group of 41 regular red wine consumers, associate deeper colour and purple tones with higher quality Pinot noir. Previous studies have found that consumers prefer greater colour intensity in red wines (Bindon et al., 2014; Parpinello et al., 2009), which was in agreement with the results from this sensory analysis. Based on the published literature, this is believed to be the first study showing this consumer opinion specifically for Pinot noir. A key difference between this experiment and previous research was that the consumer panel ranked the VL3 + BIODIVA wine, which was perceived to be of higher quality, as duller than the VL3 wine. This result does not reflect previous research where consumers rated bright Pinot noir wines as higher quality than dull Pinot noir wines (Valentin et al., 2016). The findings of the sensory evaluation cannot be applied to wine professionals, as research indicates that experts assess the appearance of a Pinot noir wine differently from consumers. They are more likely to disregard a Pinot noir's appearance and judge it on the overall balance and complexity (Parr et al., 2020; Valentin et al. 2016). Interestingly, while the participants rated the VL3 + BIODIVA wine as more likely to be of high quality than the VL3 wine, the wines received equal scores for agreement with the statement "I like the colour of this wine". Despite equating deeper and more purple colour with higher quality, the participants liked both the deeper, purple colour and the lighter, red colour. This is not surprising given that people who chose to complete the survey, would likely be interested in wine, and may have more knowledge on wine styles coming in many varying shades and intensities.

5.9. Non-*Saccharomyces* yeast species generally adsorb more pigments from grape skins than *S. cerevisiae*

The pigment adsorption levels of eight yeast strains, representing five species, were analysed as part of this research in order to shed further light on the role of cell wall mannoproteins and the adsorption of grape anthocyanins. The findings confirm that pigment adsorption differs significantly between yeast strains and species, with the non-*Saccharomyces* species (*L. thermotolerans, M. fructicola, P. kluyveri* and *T. delbrueckii*) generally adsorbing more pigments from Pinot noir grape skins than *S. cerevisiae*. BIODIVA, the strain of *T. delbrueckii* used in the Pinot noir winemaking trials due to its high flocculation capability, adsorbed the most pigments while the two commercial *S. cerevisiae* strains, VL3 and RC212, adsorbed the least. The influence of yeast pigment adsorption on red wine colour is complex. The grape skin pigments are adsorbed onto yeast cell wall mannoproteins. A previous study by Balmaseda et al. (2021) found that two commercial strains of *T. delbrueckii* (BIODIVA followed by Viniferm NS TD) resulted in the highest concentrations of mannoproteins compared to other yeast strains, which may be the reason for the high pigment adsorption BIODIVA displayed during this research. The implication of mannoproteins in both wine colour stability and flocculation provides further clues towards the mechanism behind the increase colour of wines produced with the sequential inoculation of BIODIVA. Generally, a low pigment adsorption ability is thought to be positive for red wine colour, as the pigments remain in the wine rather than binding to the yeast cells and are not removed when the wine is racked off the lees. RC212 has previously been shown to increase Pinot noir colour intensity (Carew et al., 2013) and its manufacturers claim its limited adsorption of polyphenols is the reason for this (Lallemand, n.d.c). RC212's low adsorption rate was verified in this trial, as it had a significantly lower pigment adsorption ability than BIODIVA and the other non-*Saccharomyces* yeasts, much like the other *S. cerevisiae* strain, VL3. However, the binding that occurs during pigment adsorption is weak and the process can be easily reversed. This means it can sometimes be beneficial if yeast cells extract and adsorb a lot of pigments from the grape skins that can then be later released into the wine (Morata et al., 2015). Over time, yeast cells lose viability and undergo autolysis as metabolic activity decreases and ethanol concentration increases. When this happens, the yeasts release their intracellular content back into the medium, including adsorbed pigments (Balmaseda et al., 2021). Therefore, depending on the conditions and the specific behaviour of the yeast strain involved, a high pigment adsorption may have a positive or negative effect on red wine colour intensity. One such yeast behaviour is flocculation, which

is also controlled by cell wall mannoproteins and can have an impact on colour intensity. Importantly, the adsorption of pigments by yeast cells is only one of several ways that yeasts interact with grape skin pigments (Caridi, 2013), so we cannot conclude for certain that the results of the pigment adsorption trial directly relate to the results of the Pinot noir colour intensity analysis. It is unclear whether the high pigment adsorption by BIODIVA yeast cells during fermentation contributed positively or negatively to the Pinot noir colour intensity seen during this research. If positive, the high pigment adsorption shown by BIODIVA could be another potential cause for the increase in colour intensity shown by the mixed fermentation wines, either instead of or in combination with flocculation. Alternatively, BIODIVA's high pigment adsorption might reduce colour intensity, but the potentially increased flocculation outweighs this effect, still resulting in an overall boost to colour intensity. Ultimately there is only a partial correlation between red wine colour and grape pigment adsorption and there are many other yeast characteristics that can influence anthocyanin content and colour intensity (Caridi, 2013). Further research into the cell wall and grape skin pigment interactions occurring when BIODIVA is used in Pinot noir fermentations would be useful to determine if the strain's high pigment adsorption contributes positively or negatively to wine colour over time.

5.10. Limitations of the research

Most of the limitations within this work were due to time constraints. The study would have benefited from further analyses following the Pinot noir winemaking trial, such as microscopic imaging and PCR genotyping of the yeast cells present, along with the ratios of each species, at the end of each fermentation. End of fermentation samples of plated yeast isolates were taken prior to the sedimentation rate assays, but time constraints prevented further analysis of these samples. As these isolates have placed in long term storage, there is potential for genotyping to be carried out in future. It would also have been interesting to have performed a second set of sedimentation rate assays following the Pinot noir fermentations, to see if the sedimentation rates of the yeast species used in the winemaking trial reflected the results originally obtained following the microfermentations using the same species. Quantification of other wine components known to affect colour intensity, such as acetaldehyde, hydroxycinnamic acid, pyruvic acid, and WPM including polysaccharides and mannoproteins, would have helped to determine why colour intensity changes were observed in the wines. These types of measures would be interesting to include in follow up studies.

While efforts were made to control for all external factors during the winemaking trial, a winery is not a sterile environment. It is uncertain what impact other yeast strains and microorganisms present in the winery may have had on the fermentations. However, because the experiment was performed in industry conditions, the results are more likely to be useful to winemakers, who would be replicating the research in their own wineries.

Chapter 6. Conclusion

6.1. Summary of research

Flocculation of yeast cells during primary fermentation is an emerging topic of interest in wine science research. When flocculation occurs near the end of fermentation there are three key benefits for winemakers; increased colour intensity in red wines, more efficient filtration and a greater volume of wine can be retained following racking. This project explored how the co-flocculation of non-*Saccharomyces* and *S. cerevisiae* yeast strains influences the colour intensity of Pinot noir, a lightly coloured red variety known to challenge winemakers who want to extract more colour from the fruit. Traditionally, non-*Saccharomyces* yeast species have been viewed as unwanted contaminants in the winemaking process but are now becoming increasingly popular in the industry as a method of replicating wild fermentations and increasing the complexity of a wine's flavour profile. Non-*Saccharomyces* yeasts are also generally more flocculant than *S. cerevisiae* but are capable of forming mixed species flocs with *S. cerevisiae*. In this research, microfermentations in SGM, followed by sedimentation rate assays at the end of fermentation, were performed to assess the flocculation abilities of six non-*Saccharomyces* (LAKTIA (*Lachancea thermotolerans*), CONCERTO (*Lachancea thermotolerans*), GAÏA (*Metschnikowia fructicola*), FROOTZEN (*Pichia kluyveri*), BIODIVA (*Torulaspora delbrueckii*), PRELUDE (*Torulaspora delbrueckii*)) and two *S. cerevisiae* (VL3, RC212) yeast strains, both as individuals and in mixed species fermentations. The most flocculant combinations (BIODIVA and VL3; BIODIVA and RC212) were used in sequential inoculations in a Pinot noir winemaking trial and compared to VL3 and RC212 control wines. Chemical and sensory analysis of the final wines found that the use of the sequential inoculations resulted in an increase in wine colour intensity, but lower anthocyanin concentrations based on both the Adams-Harbertson assay and HPLC, compared to the controls. This result adds to the findings in the literature demonstrating that there is not a correlation between wine colour intensity and anthocyanin concentrations. This outcome was likely due to the phenomenon of copigmentation, where anthocyanins react with colourless phenols to form copigments and cause a red wine to display greater colour intensity than would be expected based on its anthocyanin content. Further research is required to determine if the changes in colour intensity and the observed copigmentation are due to mixed species flocculation, a different aspect of BIODIVA metabolism, such as increased production of

acetaldehyde, pyruvic acid, or mannoproteins), or other interactions between BIODIVA *S. cerevisiae*, such as cell-cell signalling. It is also possible that more than one mechanism is responsible. The eight yeasts were also screened for their pigment adsorption ability, which revealed that the non-*Saccharomyces* strains generally adsorbed more pigments from grape skins than the *S. cerevisiae* strains. The implication of cell wall mannoproteins in the increased pigment adsorption, as well as their roles in flocculation and copigmentation, provide an important avenue for future research to determine the exact mechanism/s for the increased colour intensity of the mixed and sequential fermentations.

6.2. Significance for winemaking

The appearance of a wine is the first sensory component to be evaluated by wine consumers and professionals alike (de Freitas et al., 2017; de Freitas & Mateus, 2011). The colour intensity of Pinot noir, known to be light due to the nature of the variety and challenges of colour extraction, is an important consideration for winemakers (Carew et al., 2013). Those wishing to increase sales must appeal to consumers, who prefer deeper colour intensity in red wines (Bindon et al., 2014; Parpinello et al., 2009). Achieving high colour intensity early in the winemaking process is important, as later steps such as SO_2 additions and MLF have bleaching effects (He et al., 2012; Martínez-Pinilla et al., 2011; Osborne & Burns, 2015). Various techniques exist across the vineyard and the winery to increase colour intensity and recent research has found that the flocculation of yeast cells during primary fermentation can contribute greater colour intensity to red wines (Varela et al., 2020). In this project, six non-*Saccharomyces* and two *S. cerevisiae* yeast strains were screened to identify the most flocculant combination for use in a Pinot noir winemaking trial. The use of BIODIVA (*T. delbrueckii*) in sequential inoculation with both *S. cerevisiae* strains VL3 and RC212 appeared to increase wine colour intensity compared to *S. cerevisiae* controls. While further research is required to establish the exact cause of the colour intensity changes seen in this study, we can conclude that the inclusion of BIODIVA in sequential inoculation with strains of *S. cerevisiae* can have a significant impact on Pinot noir colour intensity. BIODIVA and the other yeast strains trialled in this project are all commercially available in New Zealand, should winemakers wish to conduct their own trials using the same yeast combinations. Winemakers may also wish to use the findings of this research to explore how to maximise the other benefits offered by yeast flocculation, increased wine recovery from the lees and improved clarification and filtration. When used successfully, flocculation can benefit all forms of wine production in some way,

not just in Pinot noir or red wines. Many wineries already utilise commercial yeast products, meaning there would be no significant extra costs if they were to purchase alternative strains for use in flocculation and colour trials. A straightforward technique to improve Pinot noir colour intensity is of great value to the New Zealand wine industry. Pinot noir is our most planted red variety and considerable investment has been made into research examining how we can improve the quality of New Zealand Pinot noir wines and therefore increase sales at home and around the world. The production of high quality wines provides economic benefits for both individual wineries and the New Zealand wine industry as a whole.

6.3. Future research

The potential for yeast flocculation to increase colour intensity of red wines appears to be a recent discovery. Flocculation is not covered in a comprehensive review by Tofalo et al. (2021) on how microorganisms affect red wine colour. The focus is instead on how yeast cells interact with anthocyanins, either directly through cell wall adsorption and via enzymes such as β-glycosidase, or indirectly through metabolites produced during fermentation. Recent research by Varela et al. (2020) discovered that using a highly flocculant strain of *S. cerevisiae* increases the colour intensity of Shiraz wines and the results of this project show that using a flocculant non-*Saccharomyces* species in a sequential inoculation with *S. cerevisiae* increases the colour intensity of Pinot noir wines. Future research can explore this phenomenon further. Two key topics of potential future work have emerged following this project, firstly the mechanism of flocculation and its relationship to red wine colour and secondly the characteristics and behaviour of the yeast *T. delbrueckii*, particularly the BIODIVA strain. It would be valuable to use microscopy to examine the cell-to-cell interactions during interspecies flocculation more closely and also to replicate the methods of Mencher et al. (2021) to trial mixed fermentations where cell-to-cell contact is prevented and see if an increase in colour intensity is still observed. This would determine whether flocculation is impacting colour intensity or not. Future work could also involve experiments comparing the properties of various strains of *T. delbrueckii* and how they each impact red wine colour. One interesting candidate for such a study is PRELUDE, the other strain of *T. delbrueckii* trialled in this research alongside BIODIVA. BIODIVA and PRELUDE displayed equivalent pigment adsorption ability but BIODIVA was significantly more flocculant. Both of these *T. delbrueckii* yeasts could be compared to each other in sequential inoculations with *S. cerevisiae* of Pinot noir. Analysing the colour of the wines produced would help determine if pigment adsorption or flocculation has more of an

influence on wine colour intensity, or whether they are complementary phenomena depending on yeast cell wall composition and mannoproteins. Studying the sensory impact of flocculant yeast strains, such as BIODIVA, on the nose and palate of Pinot noir wines would make an interesting follow up to the sensory evaluation of wine appearance performed as part of this project, particularly since the fermentation performance of the mixed sequential fermentation was equivalent to *S. cerevisiae* alone. Previous research has found that the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* has a positive influence on wine aroma when used to ferment Verdejo white wines, where the production of varietal thiols was increased (Belda et al., 2017), and also Cabernet Sauvignon red wines, where the production of esters was increased (Zhang et al., 2021). Neither of the studies trialled the same combinations of yeast strains used in this project, so future research could determine if these results could be replicated in Pinot noir fermentations, using BIODIVA as the strain of *T. delbrueckii* and VL3 or RC212 as the strain of *S. cerevisiae*. Overall, limited research has been conducted on the effect of yeast flocculation on Pinot noir colour but work so far indicates that this mechanism could be of great benefit to the wine industry and is a worthy subject for future studies.

Chapter 7. References

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Chapter 8. Appendices

8.1. Sample ballot form used during sensory evaluation session 1

DIFFERENTIATION TESTS
(Triangle Tests and 3-Alternate Forced Choice Test)

Station 1

Instructions:

You will be evaluating wines presented to you in 3 sets. Each set contains 3 glasses of Pinot noir wine. You are required to view the samples only (do not smell or taste) and evaluate their appearance.

Set 1

For set 1, answer the question: Which of the three samples is different from the other two?

Circle the code of the different sample:

937 698 733

Set 2

For set 2, answer the question: Which of the three samples is different from the other two?

Circle the code of the different sample:

970 147 535

Set 3

For set 3, answer the question: Which of the three samples is deeper in colour intensity than the other two?

Circle the code of the sample with the deepest colour intensity:

390 215 137

8.2. Sample Google Forms survey used during sensory evaluation session 2

Wine Colour Survey

Project: Impact of yeast behaviour on colour intensity in Pinot noir wines
Principal Investigator: Dr Rebecca Deed (rebecca.deed@auckland.ac.nz)
Student Researcher: Katasha McCullough (kmcc100@aucklanduni.ac.nz)

Please read the Participant Information Sheet before completing the survey:
https://bit.ly/3CPfrkU

If you choose to provide your email address it will not be linked to the data received in the survey. By completing and submitting the survey you consent to participating in this research.

Approved by the University of Auckland Human Participants Ethics Committee on 20/07/2021 for three years. Reference Number UAHPEC22881.

*Required

1. Please confirm you meet the participant criteria. *

Tick all that apply.

- [ ] I am over the age of 18.
- [ ] I am not colour blind.

2. Please check the boxes to proceed. *

Tick all that apply.

- [ ] I have read the Participant Information Sheet and I understand the nature of the research.
- [ ] I have had the opportunity to ask questions and they have been answered to my satisfaction.
- [ ] I agree to take part in this research.
- [ ] I understand that I am free to withdraw my participation at any time up until I submit this form.

3. If you would like to receive a summary of the findings, please enter your email address.

Section 1:
Wine 394

You are individually evaluating two Pinot noir wines, wine 394 and wine 536.

Four photos, two of the two wines together and two of wine 394 alone, are provided below for your reference.

In section 1, you will be asked to rank wine 394 on three colour attributes - hue, brightness and intensity - on a five point scale.

You will also be asked about the extent to which you agree or disagree with two statements about the colour of wine 394.

1. Both wines

2. Both wines

3. Wine 394

4. Wine 394

4. Select the number on the scale that most represents the HUE of wine 394. *

Mark only one oval.

	1	2	3	4	5	
Red-tinged	◯	◯	◯	◯	◯	Purple-tinged

5. Select the number on the scale that most represents the COLOUR INTENSITY of wine 394. *

Mark only one oval.

	1	2	3	4	5	
Light	◯	◯	◯	◯	◯	Deep

6. Select the number on the scale that most represents the BRIGHTNESS of wine 394. *

Mark only one oval.

	1	2	3	4	5	
Dull	◯	◯	◯	◯	◯	Bright

7. Statement: "I like the colour of this wine." Select the number on the scale that most represents how much you agree with this statement for wine 394. *

Mark only one oval.

	1	2	3	4	5	
Strongly disagree	◯	◯	◯	◯	◯	Strongly agree

8. Statement: "Based on its colour, I would expect this wine to be of high quality." Select the number on the scale that most represents how much you agree with this statement for wine 394. *

Mark only one oval.

	1	2	3	4	5	
Strongly disagree	◯	◯	◯	◯	◯	Strongly agree

Section 2: Wine 536

You are individually evaluating two Pinot noir wines, wine 394 and wine 536.

Four photos, two of the two wines together and two of wine 536 alone, are provided below for your reference.

In section 2, you will be asked to rank wine 536 on three colour attributes - hue, brightness and intensity - on a five point scale.

You will also be asked about the extent to which you agree or disagree with two statements about the colour of wine 536.

1. Both wines

2. Both wines

3. Wine 536

4. Wine 536

9. Select the number on the scale that most represents the HUE of wine 536. *

Mark only one oval.

	1	2	3	4	5	
Red-tinged	◯	◯	◯	◯	◯	Purple-tinged

10. Select the number on the scale that most represents the COLOUR INTENSITY of wine 536. *

Mark only one oval.

	1	2	3	4	5	
Light	◯	◯	◯	◯	◯	Deep

11. Select the number on the scale that most represents the BRIGHTNESS of wine 536. *

Mark only one oval.

	1	2	3	4	5	
Dull	◯	◯	◯	◯	◯	Bright

12. Statement: "I like the colour of this wine." Select the number on the scale that most represents how much you agree with this statement for wine 536. *

Mark only one oval.

	1	2	3	4	5	
Strongly disagree	◯	◯	◯	◯	◯	Strongly agree

13. Statement: "Based on its colour, I would expect this wine to be of high quality." Select the number on the scale that most represents how much you agree with this statement for wine 536. *

Mark only one oval.

	1	2	3	4	5	
Strongly disagree	◯	◯	◯	◯	◯	Strongly agree