Received: 28 March 2023 | Revised: 2 August 2023 | Accepted: 16 August 2023

DOI: 10.1002/yea.3896

RESEARCH ARTICLE

Yeast WILEY

# Sequential inoculation of flocculent *Torulaspora delbrueckii* with *Saccharomyces cerevisiae* increases color density of Pinot Noir wines

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## Abstract

Pinot noir grapes require careful management in the winery to prevent loss of color density and promote aging stability. Winemaking with flocculent yeast has been shown to increase color density, which is desirable to consumers. This research explored interspecies sequential inoculation and co-flocculation of commercial yeast on Pinot noir wine color. Sedimentation rates of six non-*Saccharomyces* species and two *Saccharomyces cerevisiae* strains were assayed individually and in combination. The most flocculent pairings, *Torulaspora delbrueckii* BIODIVA with *S. cerevisiae* RC212 or VL3, were used to ferment 20 L Pinot noir must. Sequential fermentations produced wines with greater color density at 420 + 520 nm, confirmed by sensory panel. Total and monomeric anthocyanin concentrations were decreased in sequentially fermented wines, despite being the main source of red wine color. BIODIVA adsorbed more anthocyanins than *S. cerevisiae*, indicating a greater number of cell wall mannoproteins in flocculent yeast, that could then result in a later release of anthocyanins and enhance copigment formation in red wines.

KEYWORDS

anthocyanins, fermentation, flocculation, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, wine

## 1 | INTRODUCTION

Phenolic compounds are essential for determining key sensorial attributes in red wines, including color density and hue, mouthfeel aspects such as astringency, and the perception of bitterness (Casassa et al., 2019). Phenolics are extracted during winemaking via techniques that allow for contact between the skins, seeds, and pulp; however, for thin-skinned grape varieties such as *Vitis vinifera* L. cv. Pinot noir, with relatively low concentrations of total anthocyanins, and lack of acylated anthocyanins, it can be more difficult to extract color and tannin from skins compared with other varieties (Carew et al., 2013; Dimitrovska et al., 2011). Lower extraction of phenolic compounds can result in wines with lower color density and decreased color stability during aging (Carew et al., 2013). This reduction in color density with wine age is a concern for winemakers, as it can negatively impact on the assessment of wine quality by consumers, who prefer red wines with greater color density (Parpinello et al., 2009).

The main contributors to the color of young red wines are anthocyanins and their copigments (Boulton, 2001). Pinot noir wines

contain five anthocyanins: cyanidin-, delphinidin-, peonidin-, petunidin, and malvidin-3-monoglucosides, with malvidin-3-glucoside being the most abundant (Dimitrovska et al., 2011). Other red varieties typically possess a sixth anthocyanin, pelargonidin-3-glucoside, which is notably absent in Pinot noir (He et al., 2012). Monomeric anthocyanins are highly reactive, with color stability requiring a complex set of reactions including polymerization and copigmentation. Anthocyanins can polymerize with catechin, epicatechin, kaempferol, proanthocyanidins and quercetin, and form new polymeric pigments such as pyranoanthocyanins (Oliveira et al., 2010). Polymeric pigments are more pH stable than monomeric anthocyanins and generally less susceptible to sulfite bleaching (Somers & Evans, 1974). Copigmentation reactions occur when monomeric anthocyanins form complexes with nonpigmented organic compounds, including amino acids, flavonoids, non-flavonoids, and organic acids, present in the must or wine (Boulton, 2001). Copigmentation has the added benefit of protecting anthocyanins from oxidation, therefore providing a protective role (Boulton, 2001). This phenomenon also means that the levels of anthocyanins in the wine do not necessarily correlate with spectrophotometric measures of wine color density (Boulton, 2001). Since anthocyanins react with tannins to form more stable polymeric pigments, greater extraction of tannins from the grape and contact with oak wood help to stabilize red wine color (González-Centeno et al., 2016). Along with oak, winemakers utilize several other tools to improve extraction and stabilization of phenolic compounds, including prefermentative cold soak and extended maceration, thermovinification, inclusion of whole bunches and stems, microoxygenation (MOX), and choice of yeast and bacterial species and strain (Božič et al., 2020; Casassa et al., 2019; Yang et al., 2021). Although extended fermentation times, higher temperatures and MOX extract greater color from grape skins, there is an increased risk of extracting greater phenolics which contribute to bitterness and astringency (Yang et al., 2021).

The choice of yeast and bacterial species and strain is central to wine color stability, as these microorganisms mediate several color reactions during winemaking. Pyruvic acid and acetaldehyde produced during alcoholic fermentation by yeast can react with monomeric anthocyanins to form the more stable pyranoanthocyanins, vitisin A and B, respectively (Morata et al., 2007). Conversely, lactic acid bacteria (LAB) can degrade pyruvic acid and acetaldehyde during malolactic fermentation (MLF), which along with an increase in pH and adsorption of anthocyanins to bacterial cell walls, can result in significant color bleaching (Wells & Osborne, 2012). Formation of vinylphenolic pyranoanthocyanins depends on the decarboxylation of hydroxycinnamic acids and formation of vinylphenols by yeast, with a high degree of variation in the phenylacrylic acid decarboxylase activity of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts (Božič et al., 2020). Carew et al. (2013) showed that the choice of *S. cerevisiae* strain for alcoholic fermentation directly influences the color density and concentration of monomeric anthocyanins and nonbleachable pigments in Pinot noir wines, with Lalvin RC212 outperforming four other commercial strains across all color measurements. Different yeast species and strains produce diverse levels of metabolites that can increase color density or change the tonality of the wine color. In addition to pyruvic acid and acetaldehyde, yeasts produce polysaccharide-degrading enzymes and cell wall mannoproteins that adsorb grape skin pigments (Caridi, 2013). Yeast behavior also modulates red wine color. Varela et al. (2020) showed that the formation of yeast flocs through cell-to-cell binding by a relatively flocculent *S. cerevisiae* strain, AWRI1759, produced greater color opacity in Shiraz wines, and reduced the need for clarification and filtration. However, AWRI1759 was isolated from Château Cantemerle in Bordeaux, France, and is not commercially available. Since the flocculation ability of commercial *S. cerevisiae* strains is generally poor compared with other yeast species (Rossouw et al., 2015), this study aimed to identify a co-flocculation partner for *S. cerevisiae* to form mixed flocs, a phenomenon observed by Rossouw et al. (2015), selected from a range of commercially available non-*Saccharomyces* spp. used in winemaking. Since sequential and co-inoculation of non-*Saccharomyces* and *S. cerevisiae* has also been shown to improve color density and increase monomeric anthocyanin concentrations (Escribano-Viana et al., 2019; Nardi et al., 2018), formation of flocs may be one of the mechanisms at play and/or this process could be harnessed improve these gains further. We assessed the flocculation capabilities of non-*Saccharomyces* and *S. cerevisiae* cells present at the end of fermentation when fermented independently and sequentially, using sedimentation rate assays. The non-*Saccharomyces* yeast with the highest sedimentation rate, BIODIVA, was trialed alongside two commercial *S. cerevisiae* strains, RC212 and VL3, in small-scale Pinot noir fermentations, followed by color analyses on finished wines to determine whether sequential inoculation of flocculent non-*Saccharomyces* yeast spp. can increase color density in red wines. Lastly, pigmentation adsorption assays were trialed to determine whether the degree of binding between anthocyanins from Pinot noir skins and mannoproteins on the surface of yeast cells could explain differences in color density based on yeast choice.

**Take-away**

- Sequential mixed-species fermentations enhanced Pinot noir color density.
- Presence of flocculent *Torulaspora delbrueckii* lowered wine anthocyanins.
- *T. delbrueckii* BIODIVA adsorbed more anthocyanins than *Saccharomyces cerevisiae* strains.
- Higher color density may arise from co-flocculation between yeast species.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and reagents

Chemicals were of the highest available analytical grade. Ethanol (99.5%) was purchased from ECP Labchem. Bacteriological agar,

casein peptone, D-glucose, glycerol, malvidin chloride, malvidin-3-glucoside, sodium hydroxide and yeast extract were purchased from Merck/Millipore Sigma. Hydrochloric acid was obtained from ThermoFisher Scientific, and potassium metabisulfite (PMS; 97%) was purchased from Acros Organics. All solutions were prepared using deionized water unless Milli-Q water was specified (Millipore).

## 2.2 | Microbiological handling

Six commercially available non-*Saccharomyces* spp. (BIODIVA, CONCERTO, FROOTZEN, GAÏA, LAKTIA and PRELUDE) and three *Saccharomyces cerevisiae* strains (EC-1118, RC212, VL3) were utilized in this research (Supporting Information: Table S1). The non-*Saccharomyces* spp. were chosen to represent a variety of available enological characteristics (Supporting Information: Table S1). EC-1118 was chosen to produce synthetic wine medium (SWM); RC212 was selected based on its positive impact on Pinot noir wine color (Carew et al., 2013); and VL3 was chosen due to its slight tendency to flocculate (Varela et al., 2020). EC-1118 and FROOTZEN were propagated from cryogenically frozen (−80°C) cultures. All other yeasts were prepared by dissolving active dried pellets in 100 µL sterile Milli-Q water. Yeasts were cultured on yeast-peptone-dextrose (YPD) medium (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ casein peptone, 20 g $L^{-1}$ D-glucose, 20 g $L^{-1}$ bacteriological agar) and grown for 48–72 h at 28°C. To inoculate the microvinifications, single colonies were subcultured into 2 mL YPD medium (without agar) and grown overnight at 28°C, with orbital shaking at 150 revolutions per minute (rpm). A further 10 µL subculture was used to inoculate 50 mL YPD, incubated at 28°C overnight at 150 rpm. For Pinot noir winemaking trials, yeasts were in active dried form and rehydrated according to the manufacturer's instructions.

## 2.3 | Microvinification and sedimentation rate assay

Microvinification of non-*Saccharomyces* spp. and *S. cerevisiae* strains, both individually and in combination via sequential inoculation, were conducted in chemically-defined synthetic grape must (SGM), with constituents reflecting the concentrations of sugars (210 g $L^{-1}$, 1:1 D-glucose:D-fructose), salts, nitrogen and amino acids (300 mg $L^{-1}$ yeast assimilable nitrogen [YAN]), minerals, vitamins, and lipids present in grape must, at pH 3.2 (full composition in Harsch et al., 2010). For each fermentation performed in triplicate, 8 mL SGM was aliquoted into 13-mL Sarstedt polypropylene culture tubes with a 0.5 $mm^2$ lid perforation. Fermentations were inoculated from precultures (washed and resuspended in sterile Milli-Q water) at $5 \times 10^6$ cells $mL^{-1}$, calculated using a Neubauer hemocytometer and Leica light microscope. Sequential fermentations were inoculated with non-*Saccharomyces* spp. ($5 \times 10^6$ cells $mL^{-1}$), followed by *S. cerevisiae* ($5 \times 10^6$ cells $mL^{-1}$) after 24 h. One set of triplicates were uninoculated as negative controls. Fermentations were incubated at 28°C, 100 rpm, and weight loss was monitored daily. Fermentations were monitored until the rates reached ≤0.001 g $L^{-1}$ $h^{-1}$, and then sedimentation rate assays were performed on these end-point samples to assess yeast flocculation ability following the method by Varela et al. (2020). Samples were vortexed and a 1 mL aliquot was centrifuged at 1000*g* for 5 min. The pellet, corresponding to yeast cells, was resuspended in 1 mL SWM (produced from SGM following the protocol of Varela et al. (2020), but using *S. cerevisiae* EC-1118 inoculated at $5 \times 10^6$ cells $mL^{-1}$, fermented at 28°C, 100 rpm shaking, and adjusted to ~12.5% v/v ethanol and pH 3.2) and incubated for 24 h at 28°C, 100 rpm. A 1:4 aliquot of culture and SWM (40 µL:160 µL) was pipetted into a 96-well plate (200 µL total), with three 200 µL SWM samples used as blanks. Optical density (OD) was measured at 600 nm using a SpectraMax iD3 plate reader (precise setting, 10 s medium intensity shaking) to determine the ratio of sample to SWM required to adjust samples to an OD 600 nm of 1. Sample and SWM were pipetted into 1.5 mL Eppendorf tubes and 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 min, then 100 µL was pipetted from just below the liquid surface. A second OD 600 nm reading, following 1 min medium intensity shaking, was taken ($t_{25}$). The sedimentation rate was calculated by dividing the difference between the $t_0$ and $t_{25}$ by the incubation time (25 min).

## 2.4 | Pinot noir fermentation trials

On March 17, 2021, 120 kg of *Vitis vinifera* L. cv. Pinot noir grapes (clone 777), were machine-harvested by Mahi Wines Limited. Grapes transported via truck to the winery in Renwick, Marlborough, New Zealand (NZ), covered and stored overnight at ambient temperature. Approximately 15 kg fruit was transferred to eight 20 L food-grade plastic pails (Mitre 10), which were cleaned prior with a solution of 4.2 g $L^{-1}$ PMS (Esseco) and 4 g $L^{-1}$ anhydrous citric acid (Weifang Ensign), followed by a cold-water rinse. Grapes were hand-macerated using sterile gloves, and 10 ppm free sulfur dioxide ($SO_2$) from a PMS solution dissolved in water was added to each pail. The must was 24.2 °Brix, pH 3.5, titratable acidity (TA) of 7.5 g $L^{-1}$ and 85 mg $L^{-1}$ YAN. Must was inoculated with either *S. cerevisiae* RC212 or VL3 (25 g $hL^{-1}$), or RC212 and VL3 (25 g $hL^{-1}$) in combination with *T. delbrueckii* BIODIVA (25 g $hL^{-1}$). Mixed species fermentations were inoculated sequentially, with *S. cerevisiae* added 24 h after BIODIVA. Each treatment was conducted in duplicate. Pail lids were fitted with airlocks containing 2 mL water, to protect fermentations from spoilage and oxidation. Fermentations were plunged manually twice daily using sterile gloves until they reached 1 °Brix and were then plunged once daily. The exterior and lids were cleaned regularly following plunging using the PMS/citric acid solution. Nutrient additions of 150 ppm diammonium phosphate (DAP) and 300 ppm of yeast autolysate Nutristart Org (Laffort), were made 48 h after the first inoculation (between 12.1 and 15.8 °Brix). After alcoholic

fermentation, triplicate 50 mL samples from each pail were frozen. Wines were hand-pressed using a sterile 24 cm stainless steel colander and 18 cm mesh sieve. Two liters of each wine was transferred into two 1 L Schott bottles. Sterile glass marbles were added to the bottles to prevent ullage. Wines were inoculated for MLF using 10 mg $L^{-1}$ commercial LAB *Oenococcus oeni*, REFLEX MALO 360 (Martin Vialatte), prepared as per the manufacturer's instructions. Bottles were sealed with parafilm, with lids loosely on top. Wines were maintained at ~18°C for the first 2 weeks of MLF, transported by car to Auckland, NZ, and stored at 25°C to further encourage bacterial activity. Once MLF was complete (<0.1 g $L^{-1}$ malic acid), wines were racked and decanted into Schott bottles with an 80-ppm free $SO_2$ addition. Bottles were sealed with parafilm and their lids and settled at 14°C for 6 weeks. After settling, wines received a 5-ppm free $SO_2$ addition, were flushed with nitrogen gas (BOC), sealed, and stored at 14°C until required.

## 2.5 | Basic juice and wine analyses

Juice analysis was conducted at Mahi Wines Limited. An Anton Paar handheld density meter was used to measure °Brix and a Mettler Toledo pH meter and Thermolyne Nuova II stir plate were used to measure pH and TA. YAN was measured using the Megazyme Primary Amino Nitrogen and L-Arginine/Urea/Ammonia assay kits, via microplate procedure and SpectraMax iD3 plate reader. Wine analysis was conducted following the 6-week settling period. Residual sugar and volatile acidity (VA) were measured using the Megazyme D-Fructose/D-Glucose and Acetic Acid assay kits, respectively, performed in microplate format, using a SpectraMax iD3 plate reader. Alcohol content was determined using an Anton Paar Alcolyzer. pH was measured using an SI Analytics Lab 855 pH meter. TA was analyzed using an Eutech 2700 Series pH meter and Dr Schilling burette.

## 2.6 | Quantitation of wine color parameters

Starting juice, wine samples pre- and post-MLF, and final wines were analyzed with UV/visible spectrophotometry using Eppendorf Uvettes and an Implen NanoPhotometer NP80, following the modified Somers method (Somers & Evans, 1974). Absorbance units (a.u.) at 420 and 520 nm were used to calculate red pigments (A520), brown pigments (A420), total color density (A420 + A520), and hue (A420/A520). Total anthocyanins (malvidin-3-glucoside (M-3-G) equivalents mg $L^{-1}$) were measured in final wines using the Adams–Harbertson assay (Harbertson et al., 2003), adapted for a microplate (Heredia et al., 2006). Color parameters were adjusted to the 1-cm path length of a standard cuvette. The high-performance liquid chromatography (HPLC) method was developed by Giglio et al. (2023), and used to quantitate concentrations of malvidin chloride standards for the Adams–Harbertson anthocyanin assay, and anthocyanin concentrations in four finished wines. An Agilent Technologies PLRP-S polymeric reverse phase column (100 Å, 250 × 4.6 mm, 5 µm) was used on an Agilent Technologies 1200 series HPLC system. The column temperature was held at 40°C and the sample injection volume was 20 µL. Solvents used were: mobile phase A: 1.5% orthophosphoric acid in Milli-Q water (v/v); solvent B: 100% acetonitrile. Gradient conditions were: 0 min (95% solvent A), 16.7 min (90% solvent A), 25 min (88.3% solvent A), 35 min (88.3% solvent A), 55 min (78% solvent A), 56.8 min (50% solvent A), 62 min (50% solvent A), 65 min (95% solvent A), and 75 min (95% solvent A) with an additional 5 min postrun, and flow rate set at 1 mL $min^{-1}$. Cyanidin-, delphinidin-, peonidin-, and petunidin-3-O-glucoside were quantified in 20 µL of filtered wine (pore size 0.45 µm) based on M-3-G standards (mg $L^{-1}$). The HPLC was operated, and data processed using Agilent ChemStation software, version B.04.02.

## 2.7 | Sensory evaluation of wine color

Participants 18 years of age or older, and not colorblind (self-identification), were recruited from staff and students in the Faculty of Science, University of Auckland to determine whether there were visible differences in color parameters between sequential and mono-inoculated (control) Pinot noir wines. For session one, four replicates of each wine were combined, and 30 mL samples, in Arcoroc Viticole 120 mL clear wine glasses, were randomly assigned a three-digit code using random.org. Twenty participants each performed two triangle tests, where they were asked to identify which sample was different, and one 3-alternative forced choice (3-AFC) test where they were asked to identify which sample had the deepest color intensity (two control wines with one sequentially inoculated wine). A Latin square arrangement was used to ensure that the tests contained all possible orders and combinations of the wine samples, which were randomly assigned to six stations. The session was held in a quiet, temperature-controlled room with natural light and a white background. Responses were recorded on paper ballot sheets. Session two was held virtually via an anonymous Google Forms survey. Two 30 mL samples of the VL3 sequential and control wines (each pooled from four replicates) were poured into Arcoroc Viticole 120 mL clear wine glasses and randomly assigned a three-digit code. The four glasses were separated into two stations comprising one of each of the two wines, in random order. Wines were photographed against a white background in neutral lighting using a Nikon DSLR D600 digital camera. Photos were resized using Pixlr photo editing software. Two separate Google Forms surveys, one for each station, were prepared. Forty-one participants were provided with photographs of the two wines they were evaluating, together, individually and from different angles. Participants were asked to rate both wines based on their hue, intensity, and brightness on a 5-point scale anchored from 1 to 5 (red-tinged to purple-tinged, light to deep, and dull to bright, respectively). Participants also rated the extent to which they disagreed or agreed (5-point scale anchored from 1 = *strongly disagree* to 5 = *strongly agree*), with two statements about the color of the

wines: "I like the color of this wine" and "Based on its color, I would expect this wine to be of high quality."

### 2.8 | Yeast pigment adsorption assay

Yeast pigment adsorption was measured by growing yeast on grape skin medium (60 g $L^{-1}$ dried Pinot noir grape skins (defrosted hand-harvested Mt Difficulty grapes from Station Vineyard, Central Otago, NZ), 50 g $L^{-1}$ citric acid monohydrate, 25 g $L^{-1}$ disodium hydrogen phosphate, 20 g $L^{-1}$ D-glucose, 7.5 g $L^{-1}$ casein peptone, 4.5 g $L^{-1}$ yeast extract and 20 g $L^{-1}$ agar). Grape skin medium was prepared as described in Caridi (2013). Grape skins were removed, washed in deionized water, and dried. Skins were incubated at 55°C for 72 h and then ground using a Black and Decker BMC100 blender. The powder was resuspended in water, heated for 5 min at 110°C, and filtered through a 7.5 × 7.5 cm 8-ply woven gauze. The corresponding double amounts of other components, minus the agar, were added and mixed, then heated at 110°C for 5 min. Separately, 40 g $L^{-1}$ agar was dissolved in deionized water and autoclaved. Grape skin and agar solutions were mixed and poured into petri dishes to solidify. Yeast cells were spread-plated and grown anaerobically (to prevent pigment oxidation and mimic fermentation conditions), at 28°C for 10 days, inside an airtight 10 L plastic container purged with nitrogen (BOC gas). Cell biomass was scooped onto sterile loops and photographed. Biomass color was analyzed using the red-green-blue (RGB) color mode in Adobe Photoshop 2020 21.1.0 (Adobe).

### 2.9 | Statistical analyses

Statistical analyses were conducted using Microsoft Excel 16.0 for the Student's *t* test, JASP software 0.14.1 for analysis of variance (ANOVA) and post-hoc Tukey's honest significant difference (HSD) test, and JMP Pro 17 for the Shapiro–Wilk test. RStudio was used to run R version 4.3.0 to calculate fermentation kinetics, including lag time (h), maximal fermentation rate ($V_{max}$) (g $L^{-1}$ $h^{-1}$), and total weight loss (g) using a custom fermentation model with modified Gompertz decay function (Tronchoni et al., 2009). For all statistical analyses performed, the alpha ($\alpha$) used was 0.05.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Mono- and sequentially inoculated non-*Saccharomyces* and *S. cerevisiae* fermentations vary in their fermentation kinetics

Cumulative $CO_2$ loss and fermentation kinetics of six non-*Saccharomyces* and two *S. cerevisiae* commercial yeast strains, individually and when mixed via sequential inoculation, are displayed in Supporting Information: Figure S1 and Table 1. Fermentations in SGM at 28°C took ~2 weeks (329 h) to reach completion and no contamination was detected in the uninoculated control, as seen by the minimal weight loss (Supporting Information: Figure S1). There were no significant differences between lag times across the fermentations, other than inoculated versus uninoculated control (Table 1), likely due to the relatively high amount of variation in weight loss at these early time points. As expected, FROOTZEN (*Pichia kluyveri*) and GAÏA (*Metschnikowia fructicola*) were unable to ferment efficiently or reach completion when present as a single inoculum, demonstrating relatively low $V_{max}$ values and significantly lower total $CO_2$ loss than all other fermentations (Supporting Information: Figure S1 and Table 1). FROOTZEN is designed for sequential inoculation with *S. cerevisiae* to maximize aromatic complexity and strains of *P. kluyveri* typically cannot survive once ethanol concentrations reach 4%–5% (v/v) (Vicente et al., 2021). GAÏA is used as a biocontrol agent in the early stages of winemaking as $SO_2$ alternative, preventing the growth of acetic acid producing spoilage microorganisms (Johnson et al., 2020). FROOTZEN and GAÏA fermentations sequentially inoculated with RC212 or VL3 demonstrated improved fermentation performance compared with the non-*Saccharomyces* yeasts alone, with increased $V_{max}$ values and significantly higher total $CO_2$ loss (Table 1). However, FROOTZEN + RC212, FROOTZEN + VL3, and GAÏA + RC212 exhibited a more sluggish performance throughout when compared with the best-performing yeasts (Supporting Information: Figure S1). All other yeasts, either alone or sequentially inoculated, performed well, with most demonstrating a lag time <24 h (also the time point for sequential inoculation) (Table 1), and reaching their $V_{max}$ between 46 and 60 h (Supporting Information: Figure S1). PRELUDE (*Torulaspora delbrueckii*) was the best-performing non-*Saccharomyces* yeast spp. trialed, with a short lag time, high $V_{max}$ comparable with RC212 and VL3, and high total $CO_2$ loss fermentation (Table 1). Other studies have shown that some *T. delbrueckii* strains can exhibit fermentation rates to rival *S. cerevisiae* in the presence of their preferred nitrogen sources (Su et al., 2020). Monocultures of BIODIVA (*T. delbrueckii*) and CONCERTO (*Lachancea thermotolerans*) displayed poor finishing ability compared with PRELUDE, while LAKTIA (*L. thermotolerans*) was intermediate (Supporting Information: Figure S1 and Table 1). Although BIODIVA and CONCERTO were able to perform alcoholic fermentation, superior alcoholic finishing ability in terms of total $CO_2$ loss was observed when sequentially fermented with RC212 or VL3 (Table 1). This result is likely due to the dominance of *S. cerevisiae*, enabling the fermentations to reach completion without becoming stuck or sluggish (Nissen & Arneborg, 2003).

### 3.2 | Cells extracted from mono-inoculated and sequentially inoculated fermentations with *T. delbrueckii* BIODIVA display significantly higher sedimentation rates

Sedimentation rates of cells harvested from microvinifications at the end of primary fermentation were measured for each mono- and sequential inoculum (Figure 1). Significant differences between the

| Inoculum | Lag time (h) | $V_{max}$ ($gL^{-1}\,h^{-1}$) | Total $CO_2$ loss ($g\,L^{-1}$) |
|---|---|---|---|
| BIODIVA | 16.0 ± 9.43 b | 1.73 ± 0.07 abc | 90.6 ± 2.17 bc |
| BIODIVA + RC212 | 0.00 ± 0.00 b | 1.51 ± 0.13 abcd | 100 ± 3.12 ab |
| BIODIVA + VL3 | 0.00 ± 0.00 b | 1.36 ± 0.12 abcd | 98.3 ± 3.35 ab |
| CONCERTO | 12.7 ± 8.22 b | 1.35 ± 0.35 abcd | 84.3 ± 7.13 c |
| CONCERTO + RC212 | 26.8 ± 18.7 b | 1. ± 0.85 abcd | 98.0 ± 1.11 ab |
| CONCERTO + VL3 | 0.00 ± 0.00 b | 1.32 ± 0.43 abcd | 99.3 ± 2.57 ab |
| FROOTZEN | 5.25 ± 9.09 b | 0.45 ± 0.12 bcd | 38.0 ± 7.90 d |
| FROOTZEN + RC212 | 16.0 ± 4.86 b | 1.15 ± 0.22 abcd | 98.3 ± 3.57 ab |
| FROOTZEN + VL3 | 35.5 ± 3.52 b | 0.98 ± 0.14 abcd | 96.6 ± 4.20 abc |
| GAÏA | 2.40 ± 4.15 b | 0.27 ± 0.10 cd | 19.3 ± 4.96 e |
| GAÏA + RC212 | 39.5 ± 1.33 b | 1.22 ± 0.16 abcd | 100 ± 2.19 ab |
| GAÏA + VL3 | 33.8 ± 14.5 b | 1.45 ± 0.37 abcd | 103 ± 4.58 ab |
| LAKTIA | 0.00 ± 0.00 b | 1.25 ± 0.16 abcd | 95.4 ± 3.90 abc |
| LAKTIA + RC212 | 11.1 ± 3.31 b | 1.52 ± 0.36 abcd | 102 ± 4.15 ab |
| LAKTIA + VL3 | 5.91 ± 2.83 b | 1.47 ± 0.54 abcd | 101 ± 3.92 ab |
| PRELUDE | 0.00 ± 0.00 b | 1.92 ± 0.35 a | 105 ± 3.71 a |
| PRELUDE + RC212 | 0.00 ± 0.00 b | 1.48 ± 0.38 abcd | 101 ± 7.21 ab |
| PRELUDE + VL3 | 0.00 ± 0.00 b | 1.49 ± 0.07 abcd | 106 ± 1.99 a |
| RC212 | 2.74 ± 4.74 b | 1.80 ± 1.17 ab | 103 ± 5.41 ab |
| VL3 | 0.00 ± 0.00 b | 2.19 ± 1.38 a | 102 ± 5.32 ab |
| Uninoculated control | 811 ± 458 a | 0.10 ± 0.07 d | 3.71 ± 0.89 f |

*Note*: Different letters next to the values indicate significant differences for that kinetic parameter (ANOVA followed by Tukey's HSD).

**TABLE 1** Mean and standard deviation of kinetic parameters (lag time [h], $V_{max}$ [$g\,L^{-1}\,h^{-1}$], and total $CO_2$ loss [$g\,L^{-1}$]) from fermentations conducted by six non-*Saccharomyces* spp. (BIODIVA, CONCERTO, FROOTZEN, GAÏA, LAKTIA, and PRELUDE) and two *S. cerevisiae* strains (RC212 and VL3) as monocultures or sequentially inoculated in SGM at 28°C, $n = 3$.

sedimentation rates were evident ($p$ value of <0.001 from ANOVA). Cells from fermentations containing non-*Saccharomyces* yeast *T. delbrueckii* BIODIVA displayed significantly higher sedimentation rates than the other single or sequentially inoculated yeasts, with an average rate of 0.030 $dOD_{600\,nm}\,min^{-1}$ (BIODIVA), 0.028 $dOD_{600\,nm}\,min^{-1}$ (BIODIVA + RC212) and 0.026 $dOD_{600\,nm}\,min^{-1}$ (BIODIVA + VL3) (Figure 1). LAKTIA + RC212 (0.013 $dOD_{600\,nm}\,min^{-1}$) was the only other sample with no significant difference to BIODIVA + RC212, likely due to the greater variation in replicates for the latter sample (Figure 1). There were no significant differences between the other sedimentation rates, except for FROOTZEN, with the lowest mean sedimentation rate (0.0004 $dOD_{600\,nm}\,min^{-1}$). The flocculation capability of cells derived from fermentations inoculated with BIODIVA, either alone or sequentially inoculated with RC212 or VL3, indicates the potential for BIODIVA as a co-flocculation partner for *S. cerevisiae* during fermentation (Figure 1). During routine culture, an early indication of flocculation ability was observed for BIODIVA, as the cells tended to settle to the bottom of culture medium. It is likely that the live cells remaining in the sequential fermentations were predominantly *S. cerevisiae*, particularly since the alcoholic finishing time was improved in BIODIVA fermentations sequentially inoculated with *S. cerevisiae* (Supporting Information: Figure S1 and Table 1). It is also typical for *S. cerevisiae* to outcompete and cause the early death of *T. delbrueckii* in sequential fermentations through cell-to-cell contact mechanisms (Nissen & Arneborg, 2003). However, there are reports of *T. delbrueckii* cells surviving until the end of fermentation alongside *S. cerevisiae* (Wang et al., 2016), which could mean that cells were present in mixed flocs of *T. delbrueckii* and *S. cerevisiae* (Rossouw et al., 2015). Even if the live cells present were predominantly *S. cerevisiae*, dead BIODIVA cells would remain in the lees. Alternatively, the presence of BIODIVA could induce *S. cerevisiae* cells to flocculate, as cell-to-cell contact mechanisms between *S. cerevisiae* and non-*Saccharomyces* yeast spp. significantly modify yeast metabolism (Petitgonnet et al., 2019). Additionally, high levels of mannoproteins released by *T. delbrueckii* strains during early fermentation (Balmaseda et al., 2021) could contribute to sedimentation phenomena, although there is clearly a strain-specific effect, considering that *T. delbrueckii* PRELUDE did not exhibit the same capacity for sedimentation as BIODIVA.

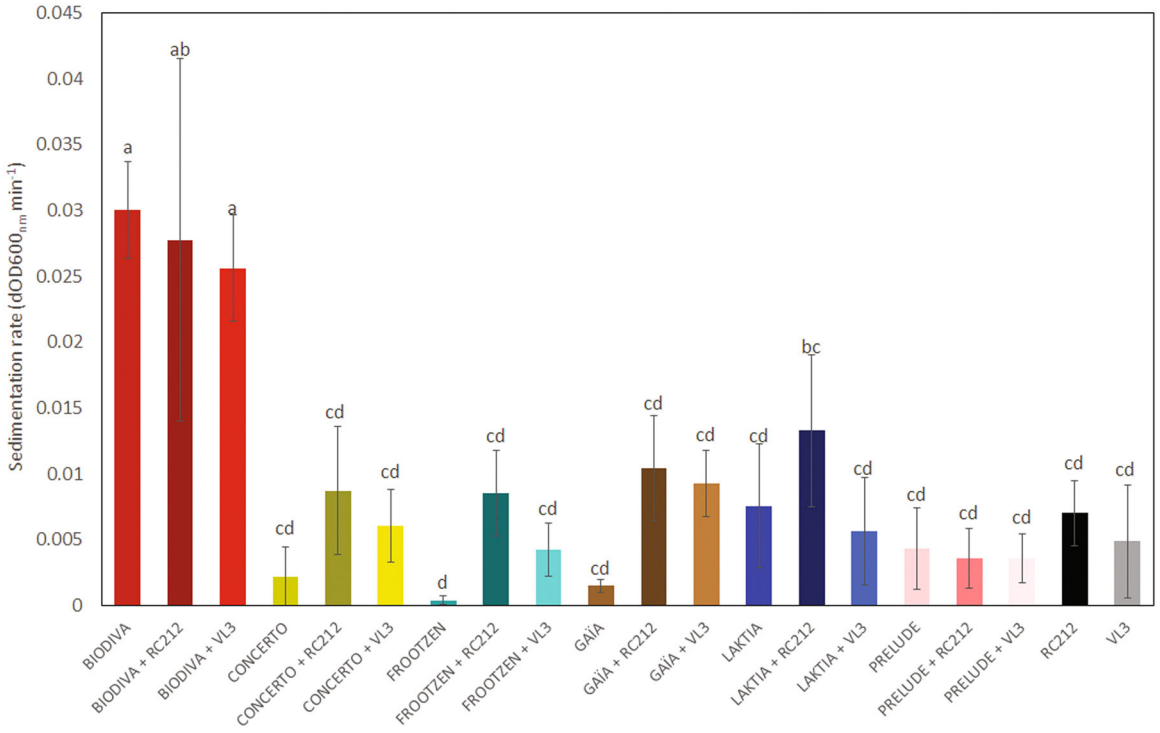


**FIGURE 1** Sedimentation rate ($dOD_{600\,nm}\ min^{-1}$) of mono- and sequentially inoculated yeasts following fermentation in SGM at 28°C, $n = 3$. Error bars represent 95% confidence intervals. Different letters above each bar indicate significant differences (ANOVA followed by Tukey's HSD).

### 3.3 | Sequential inoculation of BIODIVA with RC212 and VL3 results in comparable fermentation performance to *S. cerevisiae* controls during Pinot noir fermentation

The most flocculent non-*Saccharomyces* and *S. cerevisiae* yeasts after sequential fermentation, *T. delbrueckii* BIODIVA with *S. cerevisiae* RC212 or VL3, were selected for Pinot noir winemaking trials to determine whether the use of these yeast in sequential fermentations could increase wine color density. Eight 20-L Pinot noir fermentations, inoculated in duplicate with RC212, VL3, BIODIVA + RC212 (24 h sequential) and BIODIVA + VL3 (24 h sequential) were monitored for changes in °Brix and temperature (Supporting Information: Figure S2). Each fermentation performed consistently, with alcoholic fermentation complete after 10 days (240 h) (final °Brix levels between −0.5 and −1.1) (Supporting Information: Figure S2). The peak of fermentation for all treatments occurred between 39.5 and 71 h, reflected by an increase in must temperature (Supporting Information: Figure S2). A later temperature increase (169.5–189 h) was due to fermentations being moved into the sun to ensure successful completion to dryness. The eight fermentations were split into two 1 L Schott bottles and inoculated with *O. oeni* REFLEX MALO 360 for MLF. These 16 wines were analyzed for basic parameters after the completion of MLF (2 months) and a subsequent 6-week settling period (Supporting Information: Table S2). All wines were confirmed as dry with <1 g $L^{-1}$ residual sugar and with low VA (<1 g $L^{-1}$ acetic acid). *T. delbrueckii* is known for its low acetic acid production (Renault et al., 2009). All wines had <0.05 g/L of malic acid remaining, exception for one VL3 fermentation at 0.11 g $L^{-1}$ (Supporting Information: Table S2). This result shows that BIODIVA does not inhibit MLF. Work by Balmaseda et al. (2021) has suggested that BIODIVA may even help to optimize MLF due to its high compatibility with *O. oeni*. The alcohol content was standard for Pinot noir, ranging from 13.05% v/v for the BIODIVA + RC212 wines to 13.49% v/v for the VL3 wines (Supporting Information: Table S2). Sequentially fermented wines had lower alcohol than mono-inoculated RC212 and VL3 wines, with a significant result for BIODIVA + VL3. This result is consistent with other reports noting the ability of sequential and co-fermentations with non-*Saccharomyces* spp. to reduce alcohol concentration in finished wines, thanks to these species being less efficient at converting sugars to ethanol (Maturano et al., 2019). TA and pH levels varied slightly, with the TA ranging from 5.04 to 5.16 g $L^{-1}$, but with no significant differences (Supporting Information: Table S2). The pH ranged from 3.53 for BIODIVA + RC212 and RC212 to pH 3.64 for BIODIVA + VL3 (significantly higher compared with the other wines) (Supporting Information: Table S2), all within the typical range for Pinot noir (Carew et al., 2013; Casassa et al., 2019). The fermentation performance and lack of unwanted characters indicate that BIODIVA can be successfully used as a fermentation partner with *S. cerevisiae* for Pinot noir.

### 3.4 | Spectrophotometric analyses of wine color indicates that sequential fermentations with *T. delbrueckii* BIODIVA and *S. cerevisiae* increase the color density of finished Pinot noir wines

Color density and hue of 16 Pinot noir wines (four inoculation treatments with four replicates from two pails divided into two (1A, 1B, 2A and 2B), at each production stage (juice, wines pre-MLF, wines post-MLF, and wines after $SO_2$ addition and 6 weeks settling), were measured using UV/visible spectrophotometry to determine the evolution of color parameters over time (Table 2). Significant differences were found between treatments at the juice, pre-MLF and wine stage for red pigments, brown pigments, and color density. No significant differences were evident for hue at any winemaking stage, nor were there any differences for any color parameters at the post-MLF stage. The RC212 juice displayed significantly higher values for red pigments, brown pigments, and total color density compared with other juice samples (color density of 8.26 ± 0.86 a.u. compared with 5.37 ± 0.84 a.u. for BIODIVA + RC212) (Table 2). After the completion of primary fermentation, at the pre-MLF stage, RC212 wines were no longer significantly different for red pigments, brown pigments, or color density, compared with BIODIVA + RC212 and BIODIVA + VL3, suggesting that the sequential inoculation, and use of BIODIVA, was able to increase the color density during fermentation. VL3 wines had significantly lower color parameters than BIODIVA + VL3. Post-MLF, the wines were indistinguishable based on spectrophotometry; however, at the final wine stage, differences once again became apparent. Final wines produced using BIODIVA + VL3 were significantly higher in red pigments, brown pigments, and color density compared with all other wines, including VL3 (color density of 6.62 ± 0.35 a.u. vs. 4.95 ± 0.91 a.u.) (Table 2). Given that the RC212 juice had much higher color parameters than the BIODIVA + RC212 juice, the observation that there were no significant differences between these treatments at the later stages of winemaking indicate the increase in color pigments and density through sequential inoculation with BIODIVA.

The increase in color density observed for the sequentially inoculated wines with BIODIVA and *S. cerevisiae* RC212 and VL3 could be a result of many different phenomena. As shown by Varela et al. (2020), the flocculation ability of BIODIVA may have been one factor driving the change in color density. Addition of exogenous flocculants have been shown to clarify haze in beer samples, resulting in enhanced color (Gassara et al., 2015). Paszkot and Kawa-Rygielska (2022) also showed that darker-colored beers are characterized by lower levels of attenuation by yeast species that flocculate during the early stages of fermentation, compared with those producing lighter beers. In addition to the physiological process of flocculation, there is also the possibility that other cell-to-cell interactions or cell signaling occurring during the mixed-species fermentation may have altered

**TABLE 2** Average values and standard deviations for red pigments, brown pigments, total color density, and hue of mono- and sequentially inoculated Pinot noir wines at four stages of the winemaking process ($n = 6$ for juice and premalolactic [MLF] stages, $n = 4$ for post-MLF and wine stages).

| Treatment | Stage | Red pigments ($A_{520}$) | Brown pigments ($A_{420}$) | Color density ($A_{420} + A_{520}$) | Hue ($A_{420}/A_{520}$) |
|---|---|---|---|---|---|
| BIODIVA + RC212 | Juice | 3.33 ± 0.63 b | 2.04 ± 0.21 b | 5.37 ± 0.84 b | 0.62 ± 0.06 a |
| BIODIVA + VL3 | Juice | 3.90 ± 0.58 b | 2.43 ± 0.23 b | 6.33 ± 0.81 b | 0.63 ± 0.04 a |
| RC212 | Juice | 5.30 ± 0.65 a | 2.97 ± 0.21 a | 8.26 ± 0.86 a | 0.56 ± 0.03 a |
| VL3 | Juice | 3.86 ± 0.86 b | 2.38 ± 0.36 b | 6.24 ± 1.22 b | 0.63 ± 0.05 a |
| BIODIVA + RC212 | Pre-MLF | 3.66 ± 0.23 ab | 1.96 ± 0.09 ab | 5.62 ± 0.32 ab | 0.53 ± 0.01 a |
| BIODIVA + VL3 | Pre-MLF | 3.77 ± 0.18 a | 2.06 ± 0.08 a | 5.82 ± 0.24 a | 0.55 ± 0.02 a |
| RC212 | Pre-MLF | 3.65 ± 0.14 ab | 2.03 ± 0.06 a | 5.67 ± 0.21 ab | 0.56 ± 0.01 a |
| VL3 | Pre-MLF | 3.41 ± 0.08 b | 1.89 ± 0.08 b | 5.30 ± 0.13 b | 0.55 ± 0.02 a |
| BIODIVA + RC212 | Post-MLF | 2.55 ± 0.09 a | 2.14 ± 0.12 a | 4.70 ± 0.20 a | 0.84 ± 0.02 a |
| BIODIVA + VL3 | Post-MLF | 2.75 ± 0.08 a | 2.31 ± 0.12 a | 5.07 ± 0.18 a | 0.84 ± 0.03 a |
| RC212 | Post-MLF | 2.55 ± 0.08 a | 2.17 ± 0.08 a | 4.71 ± 0.14 a | 0.85 ± 0.03 a |
| VL3 | Post-MLF | 2.56 ± 0.14 a | 2.15 ± 0.08 a | 4.71 ± 0.22 a | 0.84 ± 0.03 a |
| BIODIVA + RC212 | Wine | 2.56 ± 0.36 b | 2.17 ± 0.23 b | 4.73 ± 0.57 b | 0.85 ± 0.06 a |
| BIODIVA + VL3 | Wine | 3.85 ± 0.17 a | 2.77 ± 0.18 a | 6.62 ± 0.35 a | 0.72 ± 0.02 a |
| RC212 | Wine | 2.65 ± 0.36 b | 2.24 ± 0.13 b | 4.89 ± 0.46 b | 0.86 ± 0.09 a |
| VL3 | Wine | 2.71 ± 0.65 b | 2.24 ± 0.26 b | 4.95 ± 0.91 b | 0.84 ± 0.10 a |

*Note*: Different letters next to the values indicate significant differences for that color parameter specifically at that stage (ANOVA followed by Tukey's HSD).

**TABLE 3** Average total anthocyanin concentrations (equivalent to malvidin-3-glucoside (M-3-G) mg $L^{-1}$) from mono- and sequentially inoculated Pinot noir wines postmalolactic fermentation and settling, measured using the Adams–Harbertson assay ($n = 12$; four wines measured in triplicate).

| Wine | Total anthocyanins (M-3-G equivalents mg $L^{-1}$) |
|---|---|
| BIODIVA + RC212 | 255.74 ± 18.43 a |
| BIODIVA + VL3 | 206.25 ± 31.24 b |
| RC212 | 257.29 ± 8.66 a |
| VL3 | 252.34 ± 31.45 a |

*Note*: Sample values with the same letter are not significantly different (ANOVA and Tukey's HSD, $p < 0.05$).

yeast metabolism, to directly or indirectly influence wine composition (Petitgonnet et al., 2019). Two previous studies investigating changes in physiochemical properties of wines sequentially fermented with *T. delbrueckii* (not BIODIVA) and *S. cerevisiae* found that the inclusion of the non-*Saccharomyces* yeast increased monomeric anthocyanin levels but did not result in significant differences in color density (Chen et al., 2018; Escribano-Viana et al., 2019). However, Nardi et al. (2018) performed sequential fermentation with *T. delbrueckii* BIODIVA and *S. cerevisiae* RBS 133 in a Barbera fermentation and observed higher color density, suggesting that the increase in color density may be a characteristic of BIODIVA specifically. Nardi et al. (2018) hypothesized that the increase in color density was due to increased pyruvic acid production of *T. delbrueckii*, as shown by Belda et al. (2015), as pyruvic acid reacts with anthocyanins to form vitisin A, a stable pigment that is not subject to the bleaching effects of $SO_2$ (Morata et al., 2007).

For all treatments except BIODIVA + RC212, color density was highest at the juice stage. Loss of color density throughout the stages of fermentation is widely reported and steps such as $SO_2$ additions have a known color bleaching effect (Harbertson et al., 2003). MLF also results in a loss of wine color as LAB deplete both pyruvic acid and acetaldehyde, which otherwise stabilize color pigments (Wells & Osborne, 2012). The slight increase in color density in the final wines after 6 weeks of settling was likely due to the release of compounds from the lees that contribute to wine color stability. Lees aging also helps maintain the monomeric anthocyanin content (Palomero et al., 2007). The decrease in color density between the initial juice and final wines reiterates how important it is for winemakers to extract and retain as much color density as possible throughout fermentation.

## 3.5 | Sequentially inoculated wines with higher color densities have lower concentrations of total and monomeric anthocyanins

The Adams–Harbertson phenolics assay was used to determine the total anthocyanin concentration (equivalent to M-3-G concentration (mg $L^{-1}$)) of the final wines (four wines per treatment) after the 6-week settling period, to determine whether the increase in color density in the BIODIVA + VL3 sequentially fermented wine was due to higher anthocyanins. Statistically significant differences (ANOVA with $p < 0.001$) were evident across the four treatments (Table 3). The BIODIVA + VL3 wine had significantly lower total anthocyanins (206.25 ± 31.24 mg $L^{-1}$) than the BIODIVA + RC212, RC212 and VL3 wines (mean of these samples at 255.12 ± 19.51 mg $L^{-1}$). No other significant differences were evident. Since there was no difference in color density between BIODIVA + RC212 and RC212, it was expected that the total anthocyanin concentrations would be similar between these treatments. However, although there was a significant difference between BIODIVA + VL3 and VL3 wines, the direction of the result was surprising, as the BIODIVA + VL3 wines had higher color density than the VL3 wines when analyzed using UV/visible spectrophotometry. To investigate this result further, HPLC was used measure total and monomeric anthocyanins (cyanidin-3-glucoside, delphinidin-3-glucoside, malvidin-3-glucoside, peonidin-3-glucoside, and petunidin-3-glucoside) for the four samples with the highest and lowest color densities from each pairwise comparison: BIODIVA + RC212 1B (highest) versus RC212 2B (lowest), and BIODIVA + VL3 2A (highest) versus VL3 1B (lowest) (Supporting Information: Table S3). Malvidin-3-glucoside was found at the highest concentration in all four wines, ranging from 170.06 ± 0.104 mg $L^{-1}$ (RC212 2B) to 125.33 ± 0.18 mg $L^{-1}$ (BIODIVA + VL3 2A), confirming reports in the literature for Pinot noir (Dimitrovska et al., 2011). Cyanidin-3-glucoside was not detected in any of the four wines. Zhang et al. (2019) reports that cyanidin-3-glucoside is typically found in very low concentrations in several *Vitis vinifera* cultivars, including Pinot noir. The RC212 and VL3 wines had the highest concentrations total and monomeric anthocyanins, while BIODIVA + VL3 2A had the lowest (Supporting Information: Table S3), consistent with the results from the Adams–Harbertson assay (Table 3). However, unlike in the Adams–Harbertson assay, which used an average of the total anthocyanin levels from four biological replicates of BIODIVA + RC212, the results from HPLC using the BIODIVA + RC212 1B sample only (highest color density) showed intermediate levels of total and monomeric anthocyanins (Supporting Information: Table S3). These results corroborate previous reports demonstrating that anthocyanin concentrations do not necessarily reflect the color density of the wines (Escribano-Viana et al., 2019; Somers & Evans, 1974). One of the main reasons for the lack of correlation between anthocyanins and color density could be due to pyranoanthocyanin formation, suggested to be responsible for the lack of correlation for Pinot noir wines in Yang et al. (2021). Given that *T. delbrueckii* can produce higher levels of pyruvic acid, it is possible that vitisin A concentrations were higher in the sequentially fermented wines. Non-*Saccharomyces* spp. can also produce higher levels of acetaldehyde, resulting in elevated vitisin B (Medina et al., 2016), although the *T. delbrueckii* yeast trialed in this study did not produce elevated acetaldehyde. Alternatively, copigmentation, where anthocyanins can react with themselves to form dimers, or with various colorless compounds called copigments (Boulton, 2001), can also result in a

greater color intensity than expected based on the anthocyanin concentration. Copigmentation can be responsible for up to 30%–50% of the color in a young red wine (Boulton, 2001). Polymeric material (PM), such as mannoproteins, which are typically produced in high concentrations during early fermentation and throughout the growth of some strains of *T. delbrueckii*, have the potential to adsorb monomeric anthocyanins and improve color density and stability of red wines (Balmaseda et al., 2021). *T. delbrueckii* strains also release mannoproteins during lees aging (Balmaseda et al., 2021). These PMs, which also include polysaccharides, could harbor a pool of scavenged pigments to be released later on in the fermentation. Flocculent yeast, like BIODIVA, are also known to differ in the composition of their cell wall mannoproteins (Saulnier et al., 1991), which links together the production of PMs with flocculation capacity, and the potential to produce wines with greater color densities.

## 3.6 | Sensory analysis confirms that sequentially inoculated fermentations have higher color density and are considered to be of higher quality by panelists

Sensory analysis was used to compare the appearance of sequentially fermented wines, BIODIVA + RC212 versus RC212, and BIODIVA + VL3 versus VL3, to determine whether the color parameters measured were perceptible to humans. For the RC212 comparisons, there were 8/20 and 3/10 correct answers for the triangle and 3-AFC tests, respectively, resulting in no significant differences between the wines ($p = 0.339$ and $p = 0.701$). For the VL3 comparisons, the triangle and 3-AFC tests resulted in significant differences between the control and mixed inoculation wines, with 11/20 and 7/10 correct answers for the triangle and 3-AFC tests, and participants selecting the BIODIVA + VL3 wine as having the higher color intensity ($p = 0.038$ and $p = 0.020$, respectively). The outcomes of these sensory differentiation tests reflect the spectrophotometric analysis, with no difference in color intensity between the RC212 wines, but a significant difference between the VL3 wines. A second sensory analysis was performed to provide further information about how the BIODIVA + VL3 and VL3 wines differed and whether these differences changed the way that the participants perceived wine quality and preference. A total of 41 participants rated the wines from 1 to 5 based on three color attributes (hue, intensity, and brightness) and their degree of agreement regarding two statements about the wine ("I like the color of this wine" and "Based on its color, I would expect this wine to be of high quality") (Figure 2). Significant differences were found between the two wines for all three color attributes. The largest difference was shown for hue, with BIODIVA + VL3 receiving a higher mean score than VL3 (3.98 vs. 2.34), meaning the sequentially fermented wine was more purple-tinged while the control wine was more red-tinged. Copigmentation can result in a shift in the wavelength where the maximum absorbance is found, which can increase blue or purple tones (Boulton, 2001). BIODIVA + VL3 was rated as deeper in color intensity than VL3 (4.39 vs. 3.05), in agreement with spectrophotometric analyses and the previous sensory result. A significant difference was also found for brightness, with BIODIVA + VL3 rated as duller than VL3 (2.59 vs. 3.10) (Figure 2). There was no significant difference between respondents' average answers for the statement "I like the color of this wine," between BIODIVA + VL3 and VL3. However, participants gave a significantly different overall score for the statement "Based on its color, I would expect this wine to be of high quality." BIODIVA + VL3 received a higher score of 3.76 versus 3.22 for VL3. Therefore, respondents considered the sequentially fermented wine to be of higher quality, possibly due to its deeper color and/or greater purple tones, and in spite of it being rated as duller. Other studies have shown that wine experts rate duller Pinot noir wines as being lower in quality (Valentin et al., 2016), but the increased color density may have offset this, and/or the acceptance of dullness may be different for consumers versus experts. Previous studies examining consumer preferences for red wine color indicate that consumers prefer deeper color densities (Parpinello et al., 2009), but as far as we are aware, this is the first study to report consumer preference for deeper-colored Pinot noir wines.

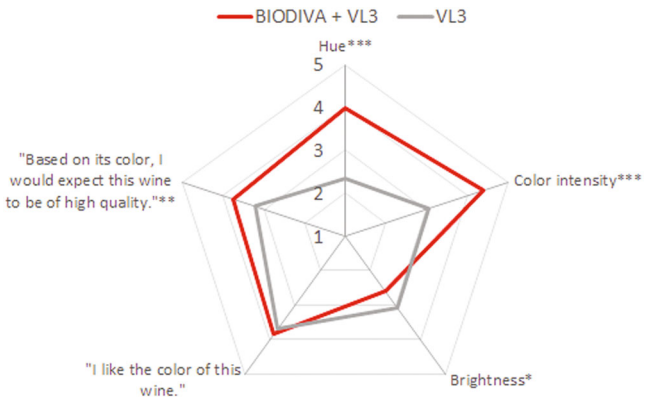


**FIGURE 2** Spider plot displaying the results of sensory analysis comparing the appearance of Pinot noir wines fermented using BIODIVA sequentially inoculated with VL3 (red line), compared with wines fermented using the VL3 mono-inoculum (gray line). Hue is rated from red-tinged (1) to purple-tinged (5), intensity from light (1) to deep (5) and brightness 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. The data was checked for normality using the Shapiro–Wilk test, before the calculation of significant differences using the Student's t test, as indicated by asterisks = *($p$ value < 0.05), **($p$ value < 0.01) and ***($p$ value < 0.001).

## 3.7 | *T. delbrueckii* BIODIVA adsorbs more grape skin pigments than *S. cerevisiae* RC212 and VL3 and the other non-*Saccharomyces* yeasts evaluated

The pigment adsorption abilities of the eight yeasts used in this research (six non-*Saccharomyces* and two *S. cerevisiae*) was conducted to determine whether pigment adsorption to cell wall mannoproteins

could provide further explanation for the higher color density, but lower anthocyanin levels, in Pinot noir wines sequentially fermented with BIODIVA and *S. cerevisiae*. Intensity values for red (R), green (G), and blue (B) color components from the cell biomass of each yeast are presented in Figure 3. Results were consistent across the three color components: *S. cerevisiae* RC212 and VL3 displayed the highest mean intensity values, and therefore the lowest pigment adsorption, in all three color categories with no significant differences between them. Both yeasts were significantly different from all six non-*Saccharomyces* yeasts for R, G and B components, indicating that pigment adsorption differs significantly between yeast species and that non-*Saccharomyces* species (*L. thermotolerans*, *M. fructicola*, *P. kluyveri* and *T. delbrueckii*) generally adsorb more pigments than *S. cerevisiae*, indicating higher levels of cell wall mannoproteins (Caridi, 2013).

The lowest intensity values, and therefore highest pigment adsorption, was demonstrated across all three color components by BIODIVA. For R, BIODIVA was significantly different from FROOTZEN, PRELUDE, RC212, and VL3, with the lowest mean value of 106 ± 14.2 (Figure 3). For G, BIODIVA was significantly different from FROOTZEN, LAKTIA, PRELUDE, RC212, and VL3, with the lowest mean value of 60.3 ± 11.7 (Figure 3). For B, BIODIVA was significantly different from FROOTZEN, RC212, and VL3, with the lowest mean value of 46.8 ± 10.3 (Figure 3). However, it must be noted that BIODIVA did not significantly differ from CONCERTO or GAÏA in any of the three color components. Overall, the interaction between the yeasts and grape skin pigments differed significantly across yeast species and strains, with BIODIVA consistently adsorbing the most color and *S. cerevisiae* adsorbing the least. The contrast between the level of pigment adsorption displayed by these two yeast species is clearly visible in photographs taken of the yeast biomass samples (Supporting Information: Figure S3). The higher color adsorption of *T. delbrueckii* BIODIVA can provide some insight into the increased color density of the Pinot noir wines that were sequentially inoculated. Since grape skin pigments are adsorbed onto yeast cell wall mannoproteins, *T. delbrueckii* BIODIVA had higher concentrations of cell wall mannoproteins than the other yeasts tested. This result is also in agreement with findings from Balmaseda et al. (2021), who studied mannoprotein content in several non-*Saccharomyces* spp. and *T. delbrueckii* strains. It is known that cell wall mannoproteins are higher in flocculent yeast, also a characteristic of BIODIVA. The implication of mannoproteins in both wine color stability and flocculation provides further clues toward the mechanism behind the increase color of wines produced with the sequential inoculation of BIODIVA. Interestingly, a low pigment adsorption ability is generally thought to be positive for red wine color, 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. For example, *S. cerevisiae* RC212 has been shown to increase Pinot noir color density (Carew et al., 2013) due to its limited adsorption of polyphenols according to Lallemand Inc. In this trial, we verified the low adsorption rate of RC212. However, there may be cases where it is beneficial for yeast cells to extract and adsorb a lot of pigments from the grape skins that can then be later released into the wine (Morata et al., 2007). Over time, yeast cells lose viability and undergo autolysis as metabolic activity decreases and ethanol concentration increases. Through autolysis, adsorbed pigments can also be released back into the wine (Balmaseda et al., 2021). Therefore, it could be beneficial to have *S. cerevisiae* strains with low adsorption, dominating the latter part of fermentation, but high adsorption of

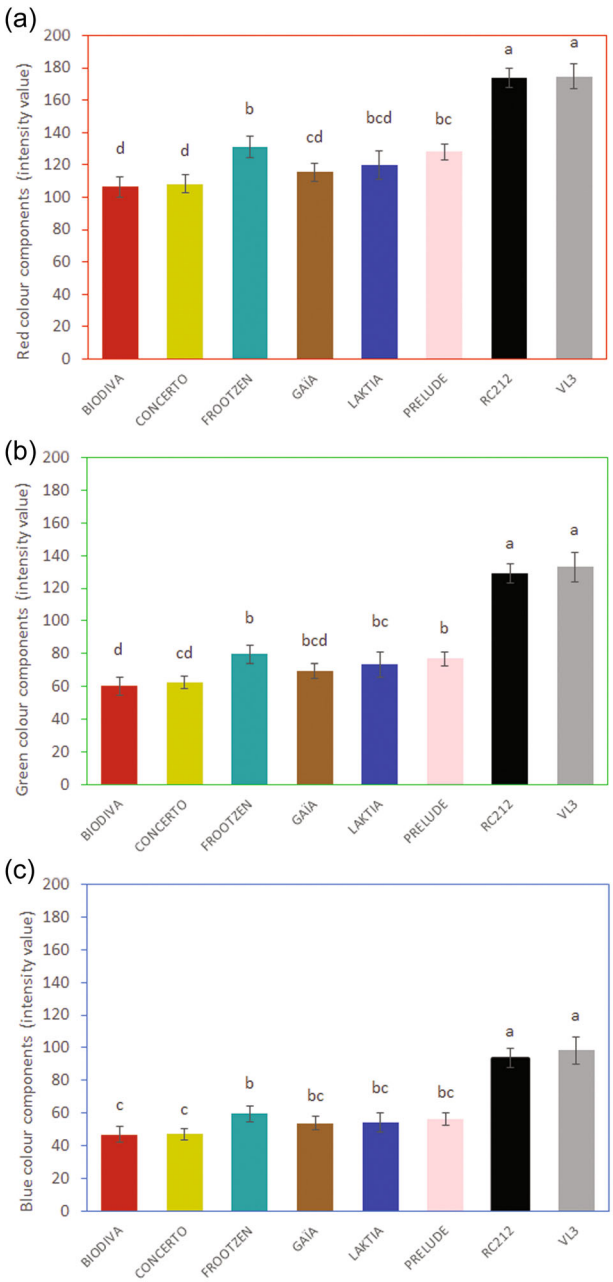


**FIGURE 3** Mean intensity values (assigned by Adobe Photoshop RGB color mode) of red (a), green (b), and blue (c) color components in pigments adsorbed by six non-*Saccharomyces* spp. (BIODIVA, CONCERTO, FROOTZEN, GAÏA, LAKTIA, and PRELUDE) ($n$ = 18) and two commercial *S. cerevisiae* yeast strains (RC212 and VL3) ($n$ = 9) during growth on Pinot noir grape skin medium. Error bars represent 95% confidence intervals. Different letters above each bar indicate significant differences (ANOVA followed by Tukey's HSD).

non-*Saccharomyces* spp., which autolyze during early fermentation. Importantly, the pigment adsorption by yeast cells is only one of several ways that yeasts interact with grape skin pigments, as there are many other mechanisms that can influence anthocyanin concentrations and color density (Caridi, 2013). Further research into the cell wall and grape skin pigment interactions occurring when BIODIVA is used in red wine fermentations would be useful to determine the mechanism behind the increased wine color density. Several unique properties of BIODIVA could play a role, including high pigment adsorption, flocculation behavior, and the production of mannoproteins, pyruvic acid and/or acetaldehyde. Future work could also investigate the long-term color impacts of sequential fermentation with BIODIVA and measure the formation of stable polymeric pigments in aged wines.

To conclude, we analyzed the sedimentation rates of six non-*Saccharomyces* spp. and two *S. cerevisiae* strains to identify potential co-flocculation partners to use in sequentially inoculated Pinot noir fermentations to increase color density in finished wines. Fermentations sequentially inoculated with the most flocculent cells at the end of fermentation, *T. delbrueckii* BIODIVA and *S. cerevisiae*, RC212 or VL3, produced wines with increased color density and lower monomeric and total anthocyanins. Pinot noir winemakers can utilize BIODIVA for sequential fermentations with *S. cerevisiae* to increase color density without the risk of overextracting phenolics causing bitterness and astringency.

## AUTHOR CONTRIBUTIONS

**Katasha S. McCullough**: Methodology; validation; investigation; data curation; formal analysis; writing—original draft; writing—review and editing. **Yi Yang**: Methodology; investigation; writing—review and editing. **Melodie A. Lindsay**: Methodology; investigation; writing—review and editing. **Neill Culley**: Methodology; investigation; resources; writing—review and editing. **Rebecca C. Deed**: Conceptualization; supervision; funding acquisition; project administration; resources; writing—original draft; writing—review and editing.

## ACKNOWLEDGMENTS

The authors would like to thank Mahi Wines Ltd, with special thanks to Brian Bicknell and Phoebe Cathcart, for their generous support of this project and donation of Pinot noir grapes. We would also like to thank Mt Difficulty Wines for the Pinot noir grapes used in the pigment adsorption assay. Thanks to Paul Kilmartin for providing the HPLC standards, Cristian Hernandez for technical assistance, and to the participants who offered their time for the sensory panel. Katasha S. McCullough was supported by a Bragato Trust Scholarship and a University of Auckland Research Masters Scholarship.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** McCullough, K. S., Yang, Y., Lindsay, M. A., Culley, N., & Deed, R. C. (2023). Sequential inoculation of flocculent *Torulaspora delbrueckii* with *Saccharomyces cerevisiae* increases color density of Pinot Noir wines. *Yeast*, *40*, 493–505. https://doi.org/10.1002/yea.3896