Effect of ‘loss of function’ mutation in SER1 in wine yeast: fermentation outcomes in co-inoculation with non-Saccharomyces

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ABSTRACT

In wine fermentation, improved wine complexity and sensorial properties can arise from the use of non-Saccharomyces yeast. Generally less alcohol tolerant, such strains often do not finish fermentation, therefore requiring a second inoculation with the more robust Saccharomyces cerevisiae, usually added on Day 3. This sequential approach affords non-Saccharomyces time to make an impact before being overtaken by S. cerevisiae. However, two inoculations are inconvenient; therefore the identification of a slow growing S. cerevisiae strain that can be used in a single co-inoculation with the non-Saccharomyces yeast is highly attractive.

In this study we investigated the use of the naturally occurring ‘loss of function’ SER1 variant, identified in a Sake yeast, for the purposes of carrying out co-inoculated wine fermentations. The SER1-232(G > C; G78R) change was introduced into the commonly used wine strain, EC1118, via CRISPR/Cas9 editing. In a chemically defined grape juice medium, the SER1(G78R) mutant grew and fermented more slowly and increased acetic acid, succinic acid and glycerol concentrations. Simultaneous inoculation with the slower-growing mutant with a Metschnikowia pulcherrima or Lachancea thermotolerans strain in sterile Sauvignon blanc juice resulted in differences in sensorial compounds, most likely derived from the presence of non-Saccharomyces yeasts. The EC1118 SER1(G78R) mutant completed fermentation with M. pulcherrima, MP2, and in fact improved the viability of MP2 compared to when it was used as a monoculture. The SER1(G78R) mutant also promoted both the growth of the SO₂-sensitive L. thermotolerans strain, Viniflora® Concerto™, in a juice high in SO₂, and its subsequent dominance during fermentation. In co-fermentations with wild-type EC1118, the Concerto™ population was substantially reduced with no significant changes in wine properties. This research adds to our understanding of the use of a novel slow-growing S. cerevisiae yeast in wine fermentations co-inoculated with non-Saccharomyces strains.

KEYWORDS: Saccharomyces cerevisiae, SER1, CRISPR/Cas9, co-inoculation, Metschnikowia pulcherrima, Lachancea thermotolerans, SO₂
INTRODUCTION

A simplistic view of winemaking involves the biochemical transformation of hexose sugars present in grape must to ethanol and carbon dioxide by Saccharomyces cerevisiae and/or other Saccharomycetaceae. S. cerevisiae is generally the dominant fermentative species, as it is able to withstand fermentation-related stresses (especially ethanol), as well as inhibit other species, so that fermentation completes in a timely manner (Albergaria and Arneborg, 2016). As such, it is common practise for grape must to be inoculated with commercial monocultures to ensure fermentation reliability, although wines created in this way can lack sensorial complexity (Padilla et al., 2016). Conversely, wine made by “wild” fermentation, making use of the native microflora present on grapes, can provide a complexity not achieved through monoculture (reviewed in Belda et al., 2017). Whilst these uninoculated or ‘spontaneous/wild’ fermentations are at risk of microbial failure if incorrectly managed, between 3–6 % of wines produced in Australia are made by these means, in particular, within the premium wine sector (The Australian Wine Research Institute, 2019). A more recent and reliable alternative is the use of selected non-Saccharomyces strains co-cultured with S. cerevisiae, which allow increased complexity and mouthfeel (attributes associated with wine quality) and consistent fermentation reliability (Padilla et al., 2016). The use of these techniques is reflected in the increasing commercial availability of non-Saccharomyces starter cultures (including Metschnikowia spp., Lachancea thermotolerans and Torulaspora delbrueckii), either as single strains or mixed with other non-Saccharomyces and/or S. cerevisiae.

Mixed fermentations involving S. cerevisiae and non-Saccharomyces yeast are generally performed using two different inoculation regimes: co-inoculation or sequential inoculation. Co-inoculation involves simultaneous inoculation with more than one yeast, whereas sequential inoculation involves an inoculation with the non-Saccharomyces species, followed by S. cerevisiae some days later to enable fermentation completion (Padilla et al., 2016). Co-inoculation offers benefits, such as acidification in the case of Lachancea thermotolerans (Comitini et al., 2011; Gobbi et al., 2013; Kapsopoulou et al., 2007), decreased acetic acid (Comitini et al., 2011; Zohre and Erten, 2002), increased acetate esters (Varela et al., 2017; Zohre and Erten, 2002) and lower alcohol contents by Metschnikowia pulcherrima (Varela et al., 2017). Whilst both methods can improve wine sensory complexity, sequential inoculation is generally favoured due to the additional control over S. cerevisiae populations through timing and inoculation rate, allowing the expression of non-Saccharomyces characteristics (Vilela, 2020).

Recent advancements in co-inoculation strategies include the use of slow growing S. cerevisiae strains to achieve greater non-Saccharomyces abundance. Albertin et al. (2017) utilised a long-lag-phase S. cerevisiae in co-fermentation with five non-Saccharomyces yeasts: Hanseniaspora uvarum, Candida zemplinina, Metschnikowia spp., Torulaspora delbrueckii and Pichia kluyveri. The long lag phase trait depended on the presence of sulfite, and arose due to reduced activity of the sulfite exporter, SSU1. The resulting wines exhibited increased complexity and fruitiness because of the extended presence of the non-Saccharomyces yeast. Whilst this is one example of a novel inoculation protocol, other yeast mutants that exhibit a long lag phase or slow growth independent of the presence of sulfite would be of merit.

This study targeted the disruption of SER1, encoding 3-phosphoserine aminotransferase, because of its slow-growth phenotype, which was hypothesised as being useful in a co-inoculation regime. The ser1A deletion was first associated with the slow initial growth in a heme-deficient laboratory strain (Reiner et al., 2006). A naturally occurring ‘loss of function’ variant, Ser1p (G78R) in Sake yeast, resulted in reduced growth rate and biomass production (Jung et al., 2018). The introduction of Ser1p (G78R) into wine Saccharomyces genotypes using “self-cloning” techniques has commercial potential, since such strains are classified as non-recombinant in jurisdictions such as Japan and the US (reviewed in Hanlon and Sewalt, 2020). Accordingly, we introduced the Ser1p (G78R) variant into the commonly used wine strain EC1118 by CRISPR/Cas9 gene editing and assessed the fermentation outcomes in monoculture and co-culture with either Lachancea thermotolerans or Metschnikowia pulcherrima, in order to determine its potential for use as a wine starter culture.

MATERIALS AND METHODS

1. Microbial strains and media

All Saccharomyces cerevisiae strains used in this study were derived from the wine strain Lalvin EC1118 (Table 1). Metschnikowia pulcherrima MP2 (Hranilovic et al., 2020) and Lachancea thermotolerans Concerto™ (Chr. Hansen) were used in co-inoculation experiments (Table 1). All yeasts were routinely grown in YEPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 28 °C. When appropriate, media were solidified with agar (20 g/L). Escherichia coli NEB® 5-Alpha (C2987L, New England Biolabs) was used for plasmid transformation and storage. Transformation was carried out according to the manufacturer’s instructions with appropriate antibiotic selection. E. coli were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) and supplemented with kanamycin (50 mg/L) or ampicillin (100 mg/L), depending on plasmid selection. Wizard® Plus SV Minipreps DNA Purification System (Promega) were used to isolate plasmids. Plasmids and oligonucleotides are described in Table 2.

2. Yeast genome editing by CRISPR/Cas9

The SER1-232 (G > C; G78R) mutation - referred to herein as SER1(G78R) - was generated by CRISPR/Cas9 according to Shaw et al. (2019) and https://benchling. com/pub/ellis-crispr-tools. sgRNA sequences and ligation oligonucleotides (sgSER1 F and sgSER1 R; Table 2) for
pWS082 were designed (benchling.com) in such a way that the introduction of the desired SER1(G78R) codon change (GGT > CGT) also disrupted the CRISPR/Cas9 guide sequence, preventing further Cas9 nuclease activity. Guide sequences were confirmed to match the EC1118 genome (taxid:643680) using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). sgRNA sequences were cloned into pWS082 using Golden Gate assembly (Engler et al., 2008) according to Shaw et al. (2019). Ligation reactions were transformed into NEB® 5-Alpha cells (C2987I, New England Biolabs) using standard protocols, plated onto LB with 100 mg/L ampicillin and incubated at 37 °C. Colonies not fluorescing under UV (365 nm) were inoculated into 10 mL LB medium with 100 mg/L ampicillin and incubated at 37 °C (200 rpm). Overnight cultures were used for plasmid purification. To confirm correct integration of sgRNA sequences, plasmids were Sanger sequenced with primer pWS082 seq (Table 2; Australian Genome Research Facility, Australia).

Homology directed repair (HDR) templates were constructed by PCR using overlapping oligonucleotides SER1-HDR F and SER1-HDR R (Table 2). PCR reactions followed a standard 50 µL reaction using Velocity DNA Polymerase (BIO-21098; Bioline) with modifications, i.e., each overlapping oligo (8 µL, 100 µM) was used instead of template DNA. Standard cycling conditions for Velocity DNA Polymerase were used (annealing at 57 °C, 10 sec extension time). Amplified HDR templates were semi-quantified by gel electrophoresis using DNA molecular weight markers (Hyperladder™ 50 bp; Bioline).

A standard lithium acetate transformation introduced CRISPR/Cas9 components into EC1118 to create the SER1(G78R) mutant. Briefly, an overnight culture (1 mL) was inoculated into fresh YEPD (100 mL), grown at 28 °C (140 rpm) to an OD$_{600}$ of 0.4-0.5. Cells were pelleted, twice washed and resuspended in 0.1 M lithium acetate to a final volume of 500 µL. Cell suspension (50 µL) was pelleted and

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**TABLE 1.** Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>EC1118</td>
<td>Lalvin EC1118 (SER1/SER1) wt</td>
<td>Lallemand, France</td>
</tr>
<tr>
<td>SER1(G78R)</td>
<td>EC1118 SER1-232 (G &gt; C; G78R) homozygous allele</td>
<td>This study</td>
</tr>
<tr>
<td>Concerto</td>
<td>L. thermotolerans Viniflora Concerto™</td>
<td>Chr. Hansen, Denmark</td>
</tr>
<tr>
<td>MP2</td>
<td>M. pulcherrima MP2</td>
<td>Hranilovic et al. (2020)</td>
</tr>
</tbody>
</table>

**TABLE 2.** Plasmids and oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>pWS082</td>
<td>sgRNA cloning vector (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Shaw et al. (2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Addgene plasmid* #90516</td>
</tr>
<tr>
<td>pWS173</td>
<td>Cas9 linear co-transformation vector (Kan&lt;sup&gt;+&lt;/sup&gt;, 2 µ vector)</td>
<td>Addgene plasmid* #90960</td>
</tr>
<tr>
<td>pWS-SER1</td>
<td>sgRNA vector targeting SER1 (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study</td>
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<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence (5’ – 3’)</th>
<th>Source</th>
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<tr>
<td>sgSER1 F</td>
<td>gaccttgAAGTGTCTACCTTGCAAGG</td>
<td>This study</td>
</tr>
<tr>
<td>sgSER1 D</td>
<td>aaacCCTTGCAAGTAGAACACTTcCaa</td>
<td>This study</td>
</tr>
<tr>
<td>pWS082 seq</td>
<td>GTCATCTGAGGTTCTGTTTC</td>
<td>Lang et al. (2021)</td>
</tr>
<tr>
<td>SER1-HDR F</td>
<td>ATCGAACTGCTAATATCTTCGACACTCATGAAGTTGTTCTACCTTGCAACGTTGCCGAC</td>
<td>This study</td>
</tr>
<tr>
<td>SER1-HDR R</td>
<td>CAGCTGCAAATATTGCAAAGGAAAACCCATAGTGCCACACCAACGTTGCCGAC</td>
<td>This study</td>
</tr>
<tr>
<td>SER1 A</td>
<td>CAAAAGAAAGCCATAAATAAGGACA</td>
<td>SGDP**</td>
</tr>
<tr>
<td>SER1 D</td>
<td>AGATAGTTCAGTCTCACCACATTC</td>
<td>SGDP**</td>
</tr>
<tr>
<td>SER1 seq</td>
<td>AATGCCTACACCAGTTTGCG</td>
<td>This study</td>
</tr>
</tbody>
</table>

*https://www.addgene.org/.
**SGDP; Saccharomyces Genome Deletion Project [http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html].

Lowercase font represents nucleotides to reconstruct the BsmBI site in the CRISPR/Cas9 sgRNA in the plasmid pWS082. Bold font denotes GGT > CGT codon change for SER1(G78R) construction, whilst the underlined font represents overlapping sequence in construction of double stranded DNA mutation templates.
resuspended with the DNA components (100 ng of BsmBI digested and purified pWS173 plasmid, 200 ng of EcoRV-HF digested and un-purified pWS-SER1 plasmid, ~ 5 µg of HDR template DNA) and sterile ultrapure water to 54 µL. The other transformation components were then added (10 µL salmon sperm DNA (10 mg mL⁻¹), 36 µL lithium acetate (1 M) and 260 µL polyethylene glycol 4000 (50 % w/v)). Reactions were statically incubated at 30 °C for 30 min, followed by 42 °C for 30 min. Collected cells (20,000 x g, 1 min) were resuspended in 1 mL YEPD and incubated at 30 °C for 2.5 h. Cells were then pelleted and resuspended in 200 µL of sterile ultrapure water before plating onto YEPD agar containing 100 mg/L G418 and incubation at 30 °C for 2 days.

G418 resistant colonies were grown in non-selective YEPD at 28 °C with shaking (140 rpm) for 72 h to facilitate plasmid loss. Cultures were streaked onto YEPD agar to select for single colonies, which were then replated on YEPD with and without 100 mg/mL G418 to determine plasmid loss. Genomic DNA was isolated from yeast colonies no longer G418 resistant by the phenol/chloroform glass bead method (Adams et al., 1998). Isolated genomic DNA was subsequently used for amplification of the SER1 gene using primers SER1 A and SER1 D (Table 2) using Velocity DNA Polymerase. Reactions were purified using Wizard® SV Gel and PCR Cleanup System (Promega) and Sanger sequenced (Australian Genome Research Facility, Australia) using primer SER1 seq (Table 2) to confirm correct integration of the HDR template.

3. Evaluation of the SER1(G78R) mutant via fermentation of Chemically Defined Grape Juice Medium

Fermentations were performed in Chemically Defined Grape Juice Medium (CDGJM) containing 200 g/L sugar (100 g/L glucose, 100 g/L fructose) and 450 mg YAN/L (Jiranek et al., 1995). Single colonies of each of EC1118 and SER1(G78R) were each inoculated into 100 mL of 1:1 YEPD and CDGJM as a starter medium, and grown overnight at 28 °C (140 rpm). Initial viable yeast counts were quantified by flow cytometry (Guava® easyCyte™, Luminex) using propidium iodide (PI). Cells were diluted in phosphate buffered saline with 10 µg/mL PI prior to analysis and the counting of 5000 events per sample. PI staining was monitored using the yellow bandpass filter (583/26 nm) in combination with the blue laser (488 nm). Cells were washed and resuspended in CDGJM prior to inoculation at 5 x 10⁴ viable cells/mL. Fermentations were conducted in triplicate at 23 °C with shaking (140 rpm). Regular samples were used to assess viability by flow cytometry (as above) and residual glucose, fructose and total sugar (D-fructose/ D-glucose Assay Kit, Megazyme). Fermentations were considered complete when total residual sugars were less than 2.5 g/L. End-point acetic acid, succinic acid and glycerol were determined by HPLC, as described above. Endpoint ester acetates (propyl acetate, isobutyl acetate, isoamyl acetate, cis-3-hexenyl acetate, 2-phenylethyl acetate and ethyl acetate) were determined by Gas Chromatography-Mass Spectroscopy (GC-MS; Lin et al., 2020).

4. Co-inoculated fermentation in Sauvignon blanc juice

Fermentations were undertaken in filter-sterilised Sauvignon blanc juice (19 °Brix, pH 3.4, 4 g/L-malic acid, 27 mg free SO₂/L, 40 mg total SO₂/L and 250 mg N/L) and inoculated with a 1:1 or 1:9 ratio of S. cerevisiae (EC1118 or SER1(G78R) and non-Saccharomyces (MP2 or Concerto). The total number of cells inoculated was 1 x 10⁶ cells/mL. Monocultures of each strain were also undertaken, using 1 x 10⁶ cells/mL as inoculum. For starter cultures, a single colony of each yeast was inoculated separately into 100 mL of 1:1 YEPD and Sauvignon blanc juice, and grown overnight at 28 °C (140 rpm). Yeast culture density was quantified by flow cytometry and cells washed and resuspended in Sauvignon blanc juice prior to inoculation to 5 x 10⁶ viable cells/mL. Fermentations were conducted in triplicate at 17 °C (140 rpm) and sampled regularly for determination of total cell numbers by plating on WL agar (Oxoid) to differentiate the Saccharomyces (cream-coloured colonies) and non-Saccharomyces (green colonies). Fermentations were considered dry when the total residual sugar was below 2.5 g/L (D-fructose/ D-glucose Assay Kit, Megazyme). End-point acetic acid, succinic acid, lactic acid and glycerol were determined by HPLC, as described above. Endpoint ester acetates (propyl acetate, isobutyl acetate, isoamyl acetate, cis-3-hexenyl acetate, 2-phenylethyl acetate and ethyl acetate) were determined by Gas Chromatography-Mass Spectroscopy (GC-MS; Lin et al., 2020).

5. SO₂ resistance of Concerto in co-inoculated fermentations

To further evaluate the sensitivity of L. thermotolerans Concerto to added sulfite, fermentations were undertaken in Chardonnay juice (22 °Brix, 249 mg YAN/L, 3.3 pH, 16 mg/L free SO₂ and 34.4 mg/L total SO₂). Yeast were grown overnight from a single colony in 100 mL of 1:1 YEPD and Chardonnay juice. Yeast cultures were quantified (Guava® easyCyte™, Luminex), washed and resuspended in Chardonnay juice prior to inoculation at 1 x 10⁶ viable cells/mL at a 1:1 ratio. Fermentations were incubated at 17 °C (140 rpm) for 17 h with regular sampling for determination of the total cell number on YEPD and lysis agar (Fowell, 1965) to differentiate L. thermotolerans.

6. Statistical analysis

Data were analysed using GraphPad Prism 9.0.0 software, with datasets compared with the Unpaired t-Test or One-way Analysis of Variance (ANOVA) with multiple comparisons (Tukey’s test) at the 99 % confidence level. Samples from Sauvignon blanc fermentations were further analysed using a Principal Components Analysis (PCA) using the statistically significant HPLC and GC-MS data at p < 0.01.
RESULTS

1. Introduction of the SER1(G78R) variant into EC1118 via CRISPR/Cas9

SER1(G78R) is a naturally occurring ‘loss of function’ variant identified in a Sake yeast strain by QTL analysis, which is associated with increased chronological age (Jung et al., 2018). The associated glycine to arginine substitution was introduced into the wine yeast EC1118 as a non-synonymous mutation at nucleotide position +232 (G > C i.e., GGG > CGG) by homology directed repair using CRISPR/Cas9 (Shaw et al., 2019). Both the SER1(G78R) naturally occurring ‘loss of function’ variant (Jung et al., 2018) and a SER1 deletion in a ser1Δ heme deficient yeast (Reiner et al., 2006) have been found to reduce growth. Whilst serine biosynthesis via glycolysis is disrupted, the mutants are not auxotrophs, as serine can be produced from glycine via gluconeogenesis (Melcher and Entian, 1992). The reduced growth phenotype may be beneficial in wine co-inoculations, since the persistence of non-Saccharomyces in the later stages of fermentation may result in wine with greater aroma complexity and novelty because of the compounds derived from these yeasts.

The fermentation characteristics of SER1(G78R) were compared to wild type EC1118 in 100 mL fermentations of CDGJM. The mutant exhibited slower initial growth (Figure 1A), similar to that observed in ser1Δ heme-deficient strains (Reiner et al., 2006) and in other strains containing the SER1(G78R) mutation (Jung et al., 2018). EC1118 growth took 48 h to reach stationary phase, whereas SER1(G78R) took 144 h, achieved a reduced viable population (Figure 1A) and took an extra 120 h to complete fermentation (i.e., 288 h vs 168 h) (Figure 1B). Analysis of fermentation relevant metabolites by HPLC showed significantly higher acetic acid (1.90 g/L vs 1.61 g/L), lactic acid (0.07 g/L vs 0.00 g/L), succinic acid (1.32 g/L vs 0.98 g/L), glycerol (4.62 g/L vs 4.30 g/L) and ethanol (102.10 g/L vs 100.20 g/L; Table 3) in the mutant vs the wildtype. The observed increases in succinic acid, acetic acid and glycerol were expected, as they have previously been reported with ser1Δ mutants of the laboratory strain, BY4742, when grown in synthetic must (Chidi et al., 2016). Whilst statistically significant increases in lactic acid and ethanol were also observed, they are unlikely to be sensorially significant. The acetic acid concentrations in both strains were over the sensory threshold (1.19 g/L for white wines; Corison et al., 1979), explaining why they contributed to the wines having a harsh ‘vinegar’ smell.

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FIGURE 1. Evaluation of fermentation performance and viability of EC1118 and SER1(G78R) grown in CDGJM.

Fermentations were conducted in 100 mL of CDGJM containing 200 g/L total sugars and 450 mg/L N (n = 3). Viable cell counts (A) and total sugars (B) were determined by flow cytometry and enzymatic analysis, respectively for EC1118 (●) and SER1(G78R) (■). Error bars denote standard deviation from the mean.
2. Co-inoculated fermentations in Sauvignon blanc juice: fermentation performance and metabolite contribution

Yeasts with slowed initial growth have been shown to be potentially useful in co-inoculation experiments (Albertin et al., 2017). To investigate the effects of SER1(G78R) in co-inoculated winemaking, 100 mL fermentations were undertaken in Sauvignon blanc juice with both S. cerevisiae and non-Saccharomyces monocultures and co-inoculated cultures. Monocultures of EC1118 and SER1(G78R) completed fermentation, whereas MP2 was sluggish with 54.5±5.0 g/L residual sugar when the other fermentations were concluded (Supplementary Table 1). This was likely due to a reduction in viable cells present in the fermentation after 264 h when the ethanol reached 55.7 g/L (5.5% (w/v)) (Figure 2A, Supplementary Table 1). Concerto monocultures largely failed to consume sugars (Figure 2A) with the yeast dying 24 h after inoculation (Supplementary Table 1). These results support the recommendation of the manufacturer that Concerto be used in unsulfured grape juice, as the yeast is sulfite (SO₂)-sensitive at 40 mg/L total SO₂, which is within the range (20–50 mg/L), ordinarily considered non-inhibitory to non-Saccharomyces (Henick-Kling et al., 1998). As with CDGJM, the fermentation duration of SER1(G78R) was significantly extended (+216 h; Table 4) and had a lower population density compared to EC1118 (Figure 2A).

All co-inoculated fermentations completed fermentation. EC1118 quickly dominated the fermentations when co-inoculated with MP2, successfully outcompeting Metschnikowia regardless of the inoculation regime (Figure 2B) and with the fermentations having the same duration as the EC1118 monoculture (Table 4, Figure 2A). The population dynamics of EC1118 and Concerto mimicked that of MP2, with EC1118 rapidly dominating the fermentation resulting in no detectable Concerto cells by WL plating after 72 h (Figure 2C).

Interestingly, the slower-growing SER1(G78R) mutant permitted greater populations of non-Saccharomyces in co-culture than did EC1118. This was evident in SER1(G78R) fermentations co-inoculated with MP2 (Figure 2D). Thus, while EC1118 dominated the culture early on and no colonies of MP2 were noted under either inoculation regime (Figure 2B), this was not the case for the mutant (Figure 2D). The maximum cell number reflected the amount inoculated for the given inoculation ratio; MP2 CFUs declined after 168 h, eventually dropping to zero at the end of fermentation. The largely viable SER1(G78R) population was responsible for the MP2 co-inoculated fermentations finishing after 408 h (Figure 2D), in contrast to the MP2 monoculture. The duration of the mixed fermentation was identical to SER1(G78R) as a monoculture (Figure 2A). In both cases, the Metschnikowia population declined with increasing ethanol content as fermentation progressed (Figure 2A and 2D).

The growth behaviour of the Concerto Lachancea in monoculture and co-culture with SER1(G78R) was surprisingly different, with the presence of the slow-growing mutant enabling the growth of the SO₂-sensitive Lachancea in SO₂ conditions that were lethal in the Concerto monoculture (Figure 2A). This led to Concerto dominating the co-inoculated fermentation in numbers greater than observed with EC1118 (Figures 2C and 2E), and an earlier completion of fermentation than the SER1(G78R) monoculture (i.e., 240 h vs 408 h; Table 4). These results allude to a synergistic interaction between the two yeasts when in co-culture in sulfured Sauvignon blanc juice, the basis of which is unclear. Whilst the fermentation was extended in the co-inoculations with the SER1(G78R) mutant compared to EC1118, the presence of Concerto, and to a lesser extent MP2, was anticipated to influence the chemical composition of the resultant wines.

HPLC analysis of the completed wines targeted organic acids, glycerol and ethanol, as well as lactic acid, which is reportedly produced by L. thermotolerans in a strain-dependent manner (Hranilovic et al., 2020; Vaquero et al., 2020). Interestingly, lactic acid did not differ between EC1118 or SER1(G78R) and Concerto in the monocultures and co-inoculated fermentations (Table 4). Furthermore, no differences were seen in any of the other quantified metabolites in the wines produced by EC1118 in monoculture and co-inoculated fermentations (Table 4). This can likely be attributed to the dominance of EC1118 in these fermentations (Figure 2A).

The comparison of monocultures of EC1118 and its SER1(G78R) mutant showed the impact of the ‘loss of function’ mutation in glycolysis (Supplementary Figure 1). 3-phosphoglycerate feeds directly into serine biosynthesis with theSER1 encoded phosphoserine transaminase catalysing a bi-directional reaction between 3-phospho.pyruvate and 3-phospho-L-serine. Disruption ofSER1 is postulated to result in a feed-back by the accumulated precursors with the glycolytic flux favouring acetic acid production. This is corroborated by the large increase in acetic acid in wines produced by the SER1(G78R) mutant compared to EC1118 (1.56 g/L vs 0.09 g/L; Table 4). Such concentrations can

### Table 3: Fermentation duration (h) and metabolite content (g/L) of ‘wines’ produced by EC1118 and SER1(G78R) when fermenting in CDGJM.

<table>
<thead>
<tr>
<th></th>
<th>Duration</th>
<th>Acetic Acid</th>
<th>Lactic Acid</th>
<th>Malic Acid</th>
<th>Succinic Acid</th>
<th>Glycerol</th>
<th>Ethanol</th>
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<tr>
<td>EC1118</td>
<td>168 ± 0.00</td>
<td>1.61 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>3.25 ± 0.03</td>
<td>0.98 ± 0.01</td>
<td>4.30 ± 0.03</td>
<td>100.20 ± 0.49</td>
</tr>
<tr>
<td>SER1(G78R)</td>
<td>288 ± 0.00*</td>
<td>1.90 ± 0.03*</td>
<td>0.07 ± 0.00*</td>
<td>3.20 ± 0.02</td>
<td>1.32 ± 0.04*</td>
<td>4.62 ± 0.03*</td>
<td>102.10 ± 0.25*</td>
</tr>
</tbody>
</table>

*Denotes significant difference (Unpaired t-test; p < 0.01) between the strains for the given parameter.
be considered a fault, as they exceed the odour threshold (1.19 g/L for white wines; Corison et al., 1979) and contributed to an unpleasant 'vinegar-like' aroma as seen in CDGJM. In contrast, the marginal change in succinic acid (3.85 g/L (EC1118) vs 3.55 g/L (SER1(G78R)); Table 4) indicated that flux through the tricarboxylic acid cycle was not greatly affected by the mutation. The 29.5 % increase in glycerol by the mutant (8.59 g/L vs 6.63 g/L; Table 4), also points to a modulation of glycolysis between 3-phosphoglycerate and glyceraldehyde-3-phosphate due to the SER1 disruption (Supplementary Figure 1). This increase would likely alter the perception of sweetness, but not viscosity (Noble and Bursick, 1984), as detectable changes are noted at 5.2 g/L and 25 g/L, respectively. The strain’s classification in Australia as genetically modified prevented sensory analysis due to containment regulations.

The slow growth of SER1(G78R) enabled non-Saccharomyces to persist in co-inoculated fermentations, thereby producing significant differences between the monoculture and most co-fermentations in the resulting wines. Acetic acid and glycerol yields were affected by the non-Saccharomyces co-inoculated with SER1(G78R) and the inoculation regime (Table 4). Co-fermentation with Metschnikowia (MP2) resulted in 11 % more glycerol when inoculated at 9 times the Saccharomyces rate. This was associated with a 73 % decrease in acetic acid (0.41 vs 1.56 g/L) and a 2 % decrease in ethanol (-2.58 g/L) compared to the SER1(G78R) monoculture (Table 4). These findings mirror earlier observations (Hranilovic et al., 2020), where MP2 also increased glycerol and decreased ethanol and acetic acid in monocultures and sequentially inoculated fermentations. Sadoudi et al. (2017) also demonstrated decreased acetic acid (40 %) and increased glycerol (12 %) in a sequential culture of S. cerevisiae/M. pulcherrima (inoculated 1:10) compared to the pure S. cerevisiae culture.

In the co-fermentations with Concerto, L. thermotolerans dominated and glycerol levels fell to between those of the SER1(G78R) monoculture and the EC1118; with the
TABLE 4. Fermentation duration (h) and metabolite content (g/L) of monoculture and co-inoculated fermentations in Sauvignon blanc juice.

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Duration</th>
<th>Acetic Acid</th>
<th>Lactic Acid</th>
<th>Malic Acid</th>
<th>Succinic Acid</th>
<th>Glycerol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC1118 (monoculture)</td>
<td>168 ± 0.00c</td>
<td>0.21 ± 0.07de</td>
<td>0.69 ± 0.02a</td>
<td>3.97 ± 0.01a</td>
<td>3.85 ± 0.01b</td>
<td>6.63 ± 0.03e</td>
<td>86.29 ± 0.11a</td>
</tr>
<tr>
<td>EC1118 + MP2 (1:1)</td>
<td>168 ± 0.00c</td>
<td>0.24 ± 0.02de</td>
<td>0.70 ± 0.15a</td>
<td>3.96 ± 0.02a</td>
<td>3.79 ± 0.03b</td>
<td>6.65 ± 0.06e</td>
<td>85.95 ± 0.45a</td>
</tr>
<tr>
<td>EC1118 + MP2 (1:9)</td>
<td>168 ± 0.00c</td>
<td>0.12 ± 0.11e</td>
<td>0.77 ± 0.04a</td>
<td>4.05 ± 0.02a</td>
<td>3.75 ± 0.04b</td>
<td>6.69 ± 0.01e</td>
<td>86.13 ± 0.34a</td>
</tr>
<tr>
<td>EC1118 + Concerto (1:1)</td>
<td>168 ± 0.00c</td>
<td>0.26 ± 0.02de</td>
<td>0.68 ± 0.05a</td>
<td>3.91 ± 0.02a</td>
<td>3.81 ± 0.03b</td>
<td>6.63 ± 0.00e</td>
<td>86.22 ± 0.05a</td>
</tr>
<tr>
<td>EC1118 + Concerto (1:9)</td>
<td>168 ± 0.00c</td>
<td>0.22 ± 0.03de</td>
<td>0.72 ± 0.02a</td>
<td>3.92 ± 0.03a</td>
<td>3.81 ± 0.03b</td>
<td>6.61 ± 0.01e</td>
<td>86.37 ± 0.15a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Duration</th>
<th>Acetic Acid</th>
<th>Lactic Acid</th>
<th>Malic Acid</th>
<th>Succinic Acid</th>
<th>Glycerol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>SER1(G78R) (monoculture)</td>
<td>408 ± 0.00a</td>
<td>1.56 ± 0.11a</td>
<td>0.55 ± 0.09a</td>
<td>4.12 ± 0.16a</td>
<td>3.55 ± 0.02c</td>
<td>8.59 ± 0.04c</td>
<td>85.87 ± 0.14a</td>
</tr>
<tr>
<td>SER1(G78R) + MP2 (1:1)</td>
<td>408 ± 0.00a</td>
<td>1.28 ± 0.03b</td>
<td>0.74 ± 0.06a</td>
<td>4.06 ± 0.06a</td>
<td>3.48 ± 0.02c</td>
<td>8.83 ± 0.03b</td>
<td>85.66 ± 0.06a</td>
</tr>
<tr>
<td>SER1(G78R) + MP2 (1:9)</td>
<td>408 ± 0.00a</td>
<td>0.41 ± 0.10cd</td>
<td>0.72 ± 0.12a</td>
<td>3.83 ± 0.11a</td>
<td>3.15 ± 0.08d</td>
<td>9.60 ± 0.07a</td>
<td>83.29 ± 0.38b</td>
</tr>
<tr>
<td>SER1(G78R) + Concerto (1:1)</td>
<td>240 ± 0.00b</td>
<td>0.62 ± 0.02c</td>
<td>0.58 ± 0.16a</td>
<td>3.31 ± 0.19b</td>
<td>3.79 ± 0.02b</td>
<td>7.36 ± 0.12d</td>
<td>86.22 ± 1.25a</td>
</tr>
<tr>
<td>SER1(G78R) + Concerto (1:9)</td>
<td>240 ± 0.00b</td>
<td>0.39 ± 0.01d</td>
<td>0.58 ± 0.01a</td>
<td>3.08 ± 0.04b</td>
<td>4.05 ± 0.01a</td>
<td>7.22 ± 0.05d</td>
<td>86.50 ± 0.60a</td>
</tr>
</tbody>
</table>

Letters denote significant difference between values in columns (Tukey’s HSD test; p < 0.01).
*Monocultures of Concerto and MP2 did not finish fermentation, having 166.20 ± 5.26 g/L and 54.46 ± 5.01 g/L residual sugar respectively. Refer to Supplementary Table 1.

lowest concentrations measured in the high (1:9) inoculum ratio (Table 4). These findings allude to Concerto being a high producer of glycerol, a phenotype commonly observed in L. thermotolerans yeast (Benito et al., 2016b; Comitini et al., 2011; Gobbi et al., 2013; Kapsopoulou et al., 2007).

Succinic acid differed between the two non-Saccharomyces and different inoculation ratios (Saccharomyces:non-Saccharomyces). Co-inoculation of SER1(G78R) with MP2, gave significantly lower succinic acid at the (1:9) ratio compared to the SER1(G78R) monoculture (3.15 g/L vs 3.55 g/L; Table 4). The small reduction in succinic acid at the 1:1 ratio with MP2 was not significant (3.48 g/L vs 3.55 g/L). This differs from Hranilovic et al. (2020), who reported MP2 to produce either more or comparable amounts of succinic acid to that of EC1118 grown in CDGJM or grape juice, respectively. Co-inoculation of SER1(G78R) with Concerto gave increased succinic acid. When inoculated equally, succinic acid (3.79 g/L) was similar to the EC1118 fermentations (3.81 g/L), whilst the higher inoculum of Concerto (1:9) resulted in a 6.3 % increase (4.05 g/L). These concentrations are considerably higher than in the commercial white wines surveyed by Coulter et al. (2004) (0.1–1.6 g/L; ave. 0.6 g/L).

Whilst increased succinic acid in wine can be identifiable as a salty sour taste (Coulter et al., 2004), sensory analysis was not undertaken here due to regulatory restrictions.

Malic acid degradation by wine yeast is a useful attribute providing there is an alternative to the malolactic fermentation undertaken by lactic acid bacteria to modulate acidity and aroma (Du Plessis et al., 2017). While EC1118 has minimal impact on malic acid (Redzepovic et al., 2003), degradation by non-Saccharomyces was checked for. As expected, concentrations in EC1118-dominated fermentations were unchanged in the Sauvignon blanc juice (Table 4). SER1(G78R) in monoculture vs 1:1 inoculation with MP2 changed malic acid little. However, significant reductions were seen, albeit only for co-inoculation of SER1(G78R) with Concerto at 1:1 or 1:9, these being 20 % and 25 %, respectively (Table 4). This range agrees with reports of L-malic acid degradation being strain-dependent (reviewed in Benito, 2018).

Acetate esters result from esterification of ethanol or higher alcohols (from Ehrlich degradation of amino acids; reviewed in Belda et al., 2017) with acetyl-CoA and are important contributors to wine aroma. MP2 is reported to increase acetate ester production (Hranilovic et al., 2020). The end-point ester analysis in this study revealed several differences. Monocultures of EC1118 and SER1(G78R) differed significantly in propyl acetate (pear-like), but values (Table 5) remained well below the sensory threshold of 4.7 mg/L for white wines (Miller, 2019). In terms of the comparison between EC1118 mono- and co-inoculated fermentations, no differences were found; this is probably because the latter were dominated by EC1118, which is “defined as a neutral strain” (Marcon et al., 2018). In the case of SER1(G78R) mono- vs co-cultures, many differences were evident. Co-culture with MP2 - particularly at the 1:9 ratio - led to significant increases in propyl, isobutyl, isoamyl, 2-phenylethyl and ethyl acetates, ranging in magnitude from 1.7- to 5.9-fold (Table 5). In this case, the largest quantitative increase (in ethyl acetate) reached 8.8 mg/L, thereby exceeding the sensory threshold (7.5 mg/L; Guth, 1997) at which fruity aromas are perceived, but remaining well below 100 mg/L, at which a ‘nail polish remover’ fault is evident (Sumby et al., 2010). Isobutyl acetate (up 5.9 x to 14 µg/L) has a sensory threshold of 1.6 µg/L, at which a fruity, apple- and banana-like scent is imparted (Haggerty, 2016). Isoamyl acetate (up 3.5x to 76 µg/L) exceeds the 30 µg/L sensory threshold of this ‘banana-like’ acetate ester (Guth, 2004). Sensory analysis was reviewed in Belda and different inoculation ratios (Saccharomyces:non-Saccharomyces). Co-inoculation of SER1(G78R) with MP2, gave significantly lower succinic acid at the (1:9) ratio compared to the SER1(G78R) monoculture (3.15 g/L vs 3.55 g/L; Table 4). The small reduction in succinic acid at the 1:1 ratio with MP2 was not significant (3.48 g/L vs 3.55 g/L). This differs from Hranilovic et al. (2020), who reported MP2 to produce either more or comparable amounts of succinic acid to that of EC1118 grown in CDGJM or grape juice, respectively. Co-inoculation of SER1(G78R) with Concerto gave increased succinic acid. When inoculated equally, succinic acid (3.79 g/L) was similar to the EC1118 fermentations (3.81 g/L), whilst the higher inoculum of Concerto (1:9) resulted in a 6.3 % increase (4.05 g/L). These concentrations are considerably higher than in the commercial white wines surveyed by Coulter et al. (2004) (0.1–1.6 g/L; ave. 0.6 g/L). Whilst increased succinic acid in wine can be identifiable as a salty sour taste (Coulter et al., 2004), sensory analysis was not undertaken here due to regulatory restrictions.
TABLE 5. Acetate ester content (µg/L) of monoculture and co-inoculated fermentations in Sauvignon blanc juice.

<table>
<thead>
<tr>
<th>Strain(s)*</th>
<th>Propyl Acetate</th>
<th>Isobutyl Acetate</th>
<th>Isoamyl Acetate</th>
<th>cis-3-Hexenyl Acetate</th>
<th>2-Phenylethyl Acetate</th>
<th>Ethyl Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC1118</td>
<td>2.66 ± 0.21d</td>
<td>1.58 ± 0.12cd</td>
<td>35.88 ± 7.17b</td>
<td>56.39 ± 2.05a</td>
<td>17.51 ± 1.36b</td>
<td>2184 ± 177.0c</td>
</tr>
<tr>
<td>EC1118 + MP2 (1:1)</td>
<td>2.65 ± 0.22d</td>
<td>1.51 ± 0.11d</td>
<td>35.38 ± 10.60b</td>
<td>69.95 ± 3.77a</td>
<td>19.59 ± 0.95b</td>
<td>2238 ± 230.1c</td>
</tr>
<tr>
<td>EC1118 + MP2 (1:9)</td>
<td>2.97 ± 0.52d</td>
<td>1.65 ± 0.23cd</td>
<td>36.23 ± 15.98b</td>
<td>53.86 ± 11.66a</td>
<td>20.53 ± 4.06b</td>
<td>2996 ± 419.2bc</td>
</tr>
<tr>
<td>EC1118 + Concerto (1:1)</td>
<td>2.76 ± 0.12d</td>
<td>1.59 ± 0.04cd</td>
<td>26.9 ± 8.41b</td>
<td>56.75 ± 3.09a</td>
<td>19.74 ± 1.24b</td>
<td>2432 ± 142.1bc</td>
</tr>
<tr>
<td>EC1118 + Concerto (1:9)</td>
<td>2.88 ± 0.71d</td>
<td>1.71 ± 0.55cd</td>
<td>36.22 ± 8.96b</td>
<td>53.39 ± 0.99a</td>
<td>18.74 ± 0.91b</td>
<td>2282 ± 334.1c</td>
</tr>
<tr>
<td>SER1(G78R)</td>
<td>8.64 ± 1.17c</td>
<td>2.37 ± 0.32bcd</td>
<td>21.84 ± 2.86b</td>
<td>66.38 ± 4.35a</td>
<td>18.64 ± 1.58b</td>
<td>2363 ± 189.2bc</td>
</tr>
<tr>
<td>SER1(G78R) + MP2 (1:1)</td>
<td>11.29 ± 1.34b</td>
<td>3.15 ± 0.40b</td>
<td>21.98 ± 5.76b</td>
<td>54.36 ± 11.30a</td>
<td>22.03 ± 2.10b</td>
<td>3598 ± 519.6b</td>
</tr>
<tr>
<td>SER1(G78R) + MP2 (1:9)</td>
<td>14.85 ± 0.46a</td>
<td>14.06 ± 0.32a</td>
<td>75.86 ± 22.13a</td>
<td>60.97 ± 5.62a</td>
<td>46.33 ± 2.38a</td>
<td>8801 ± 526.9a</td>
</tr>
<tr>
<td>SER1(G78R) + Concerto (1:1)</td>
<td>4.42 ± 0.18d</td>
<td>2.75 ± 0.16bc</td>
<td>15.21 ± 7.37b</td>
<td>8.96 ± 0.81b</td>
<td>2.31 ± 0.21c</td>
<td>3342 ± 135.6bc</td>
</tr>
<tr>
<td>SER1(G78R) + Concerto (1:9)</td>
<td>3.87 ± 0.50d</td>
<td>3.28 ± 0.59bc</td>
<td>15.93 ± 5.66b</td>
<td>5.54 ± 1.03b</td>
<td>1.90 ± 0.19c</td>
<td>3275 ± 631.1bc</td>
</tr>
</tbody>
</table>

Letters denote significant difference between values in the same column (Tukey’s HSD test (p < 0.01)).

*Monocultures of Concerto and MP2 did not finish fermentation; data available in Supplementary Table 1.

1997). 2-Phenylethyl acetate concentrations were below the sensory threshold of 250 µg/L (Guth, 1997). Overall, these results reflect the enhancement of acetate esters, as previously described for fermentations utilising M. pulcherrima (Hranilovic et al., 2020).

The significant differences between SER1(G78R) monocultures compared with co-cultures with Concerto comprised reductions in propyl, cis-3-hexenyl and 2-phenylethyl acetates (Table 5). Propyl acetate was reduced to below its sensory threshold, presumably modulating its pear-like aroma. cis-3-hexenyl acetate (fruity, green tea aroma) does not have a reported sensory threshold in wine; therefore the influence of the ~10-fold reduction in this compound is unclear.

The Principal Component Analysis (PCA) of the HPLC and GC-MS data sought to define the relationship between fermentation regime and wine metabolites (Figure 3). Four distinct groups were identified within the samples. The first (top right) comprised solely the fermentations containing both monoculture and co-inoculated EC1118, the clustering likely due to a lack of significant differences between the sensory compounds analysed (Table 4 and 5). This is not surprising given the predominance of EC1118 in these fermentations (Figure 2A–2C). For the SER1(G78R) fermentations co-inoculated with MP2, there were two clusters: one (far left) comprised the 1:9 co-inoculation ratio of SER1(G78R) and MP2, which featured high acetate esters (propyl, isobutyl, isoamyl, 2-phenylethyl and ethyl acetates) and glycerol. The other group (centre left) comprised SER1(G78R)/MP2 1:1 co-inoculations and SER1(G78R) monoculture fermentations; this group is largely influenced by the production of acetic acid - a phenotype associated with SER1(G78R) fermentations.

Final wine volatile composition was dependent on the inoculation regime, with the higher inoculation ratio (i.e., greater MP2 inoculum; Figure 2D) resulting in largely different fermentations compared to lower inoculation densities, which resulted in fermentations more similar to the SER1(G78R) monocultures. The final group (bottom right) comprised all fermentations involving SER1(G78R)/Concerto co-inoculations, the Concerto monoculture being non-viable. The resultant wines reflect the dominance of the Concerto population in both co-fermentations, and thereby the characteristics of this non-Saccharomyces (Figure 2E). These fermentations were typically characterised by a lower production of some acetate esters, in particular cis-3-hexenyl acetate and 2-phenylethyl acetate.

3. S. cerevisiae/Concerto co-inoculated fermentations in Chardonnay juice confirm alleviation of SO₂ sensitivity

The influence of co-culture with Saccharomyces on the SO₂ sensitivity of Concerto was investigated by carrying out a repeat of the fermentations in another white variety, Chardonnay, containing 34.4 mg/L total SO₂. Plating on lysine media revealed the persistence of L. thermotolerans in co-inoculation (EC1118 or SER1(G78R), compared to a loss of L. thermotolerans viability in the monoculture after 24 h (Figure 4A). By the 72 h time-point in the Sauvignon blanc fermentation, Concerto had reached 5.7 x 10⁷ CFU/mL when co-inoculated with SER1(G78R), whereas no L. thermotolerans colonies were seen when co-inoculated with EC1118. These results imply that the SO₂ resistance effect is due to the presence of S. cerevisiae biomass. To test this, an equivalent experiment was undertaken using heat-inactivated EC1118 or SER1(G78R) cells. In this instance, however, all fermentations lost viability after 24 h (data not shown), indicating that the SO₂ resistance phenotype experienced by Concerto when co-inoculated with S. cerevisiae strains involves biologically functional yeast and not just biomass. Further work is required to elucidate the precise mechanism involved.
DISCUSSION

In winemaking, mixed fermentation by *S. cerevisiae* and non-*Saccharomyces* yeast is a common method for introducing complexity. Typically, the yeasts are added sequentially, starting with non-*Saccharomyces* to alter the aroma profile (and reduce ethanol), followed a few days later by the addition of the ethanol-tolerant *Saccharomyces* to finish the fermentation. As *Saccharomyces* implants quickly and outcompetes the non-*Saccharomyces*, focus has been on maximising the non-*Saccharomyces* population and persistence during fermentation, in particular when yeasts are inoculated together rather than sequentially. One approach is to extend the *Saccharomyces* lag phase prior to exponential growth. Albertin *et al.* (2017) were the first to modulate the lag phase using sulfite through the breeding of *S. cerevisiae* strains with short and long lag phases associated with the sulfite pump (*SSU1*) translocations. The authors reported improved wine fruitiness and complexity in co-inoculated fermentations with non-*Saccharomyces* when using the long-lag phase strains or low inoculation rates of the short-lag phase strains.

‘Loss of function’ mutations that result in protracted growth independent of sulfite provide an alternative paradigm. Deletion of the SER1 (3-phosphoserine aminotransferase) required for serine and glycine biosynthesis from glycolysis (Supplementary Figure 1) was first demonstrated to negatively impact the initial growth phase of a heme-deficient *S. cerevisiae* strain (Reiner *et al.*, 2006). A naturally occurring ‘loss of function’ variant, SER1(G78R), was later identified in Sake yeast in a QTL study of chronological aging (Jung *et al.*, 2018); the SER1(G78R) mutant exhibited slowed growth and reduced biomass in synthetic media (yeast nitrogen base, YNB) containing either glucose (0.5, 2 or 10 %) or galactose (2 %) as the carbon source. In the present study transfer of the SER1(G78R) mutation to a wine genotype, namely EC1118, also resulted in a slow-growth phenotype and reduced biomass (total cell number) when grown in Chemically Defined Grape Juice Medium (Figure 1). The use of unsulfured CDGJM confirmed the growth phenotype of SER1(G78R) as SO₂-independent in contrast to the ‘SSU1 translocation’ strains described by Albertin *et al.* (2017). The SER1(G78R) mutant was evaluated against EC1118 in co-inoculated fermentations with *L. thermotolerans* strain Concerto or *M. pulcherrima* strain MP2 in Sauvignon blanc juice. The results (Figure 2) supported

![Figure 3](https://via.placeholder.com/150)

**Figure 3.** Principal component analysis of acetate esters, organic acids and glycerol for individual fermentations in Sauvignon blanc wine fermentations.

Red represents loadings of compounds analysed and blue represents the PC scores of individual fermentations. Samples suffixed with E = EC1118 monoculture, S = SER1(G78R) monoculture, EM = EC1118/MP2 co-inoculation, EL = EC1118/Concerto co-inoculation, SM = SER1(G78R)/MP2 co-inoculation and SL – SER1(G78R)/Concerto co-inoculation. 1:1 and 1:9 denote the inoculation ratio used.
the hypothesis that the use of a slower-growing SER1(G78R) mutant results in a larger and more persistent population of non-Saccharomyces yeast; for example, SER1(G78R) was able to support a large population of MP2 at both inoculation ratios before a decline in MP2 occurred in the later stages, and with SER1(G78R) finishing the fermentation. This pattern was similar to the sequential fermentation reported by Contreras et al. (2014), where the M. pulcherrima CFUs declined and a subsequent inoculation with an S. cerevisiae strain after 50 % sugar consumption allowed the completion of the fermentation. The decline most likely reflects the low ethanol tolerance of M. pulcherrima (3–5 %), with only a few tolerating up to 9 % (Barbosa et al., 2018).

The influence of the wild-type and SER1(G78R) mutant on the expression of non-Saccharomyces attributes related to wine composition was investigated as part of the Sauvignon blanc juice fermentations. L. thermotolerans was chosen because of its ability to produce lactic acid (Benito et al., 2015; Gibson et al., 2018; Jolly et al., 2014; Kapsopoulou et al., 2007; Morata et al., 2018), a trait whose exploitation has proven beneficial for the bioacidification of low acid Merlot wine to improve sensory properties (Hranilovic et al., 2021). M. pulcherrima was chosen for its ability to produce high concentrations of some acetate esters and lower alcohol content during wine fermentations (Hranilovic et al., 2020; Varela et al., 2016).

The relative abundance of EC1118 in the co-inoculated fermentations was reflected in the lack of significant variation in the metabolite profiles of the resultant wines (Tables 4 and 5). This scenario is typical of co-inoculated fermentations and is one reason why winemakers rely on a sequential inoculation regime (Vilela, 2020) to ensure expression of non-Saccharomyces attributes (Dutraive et al., 2019; Englezos et al., 2018; Hranilovic et al., 2020; Lin et al., 2020; Lu et al., 2017). On the other hand, SER1(G78R) co-inoculated fermentations resulted in largely greater numbers of non-Saccharomyces yeasts, which is reflected in the concentrations of key metabolites (Table 4) and volatile compounds analysed (Table 5).

High acetic acid and volatile acidity are often tackled through mixed fermentation involving L. thermotolerans (Benito et al., 2015; Benito, 2018; Comitini et al., 2011) or M. pulcherrima (Hranilovic et al., 2020; Varela et al., 2016). Similar findings were obtained for the fermentations co-inoculated with SER1(G78R), in which the high amounts of acetic acid produced by SER1(G78R) (Supplementary Figure 1) decreased as a result of the uptake

FIGURE 4. Viable cell densities of Concerto or S. cerevisiae after 72 h in Chardonnay juice. Fermentations (100 ml) were conducted in Chardonnay juice containing 34.4 mg/L total SO2. Concerto was present as a (A) monoculture (○) or co-inoculated 1:1 with EC1118 (△) or SER1(G78R) (□) with a cell density determined as CFU/mL by lysine plating. S. cerevisiae population counts (B) were determined by YEPD plating, for EC1118 (○) and SER1(G78R) (□) monocultures, or Concerto cultures co-inoculated 1:1 with EC1118 (△) or SER1(G78R) (◊). In mixed culture fermentations, values from lysine plating were subtracted from total values determined by YEPD plating.
and metabolism of hexose by low acetic acid-producing non-Saccharomyces yeasts (Table 4). As such, concentrations of acetic acid were close to the legal limits ( > 1.5 g/L in Australia; Coultier et al., 2004) in fermentations using SER1(G78R) in monoculture and with MP2 (1:1), but were significantly reduced due to the high inoculation rates of non-Saccharomyces (9:1). Further work is required to evaluate different strain combinations and inoculation regimes in conditions more related to winemaking; e.g., non-sterile conditions and on larger scales to determine the degree of implantation and effect on wine composition (Lin et al., 2022). The influence of parameters such as sulfate, pH and temperature on microbial interaction and succession will also need to be examined.

The other organic acids of interest in terms of wine acidity were lactic acid and malic acid. Malic acid in wine is generally known for its harsh acidic quality and is decarboxylated to lactic acid by lactic acid bacteria, such as Oenococcus oeni or Lactobacillus species, to improve sensorial properties and improve wine stability (Bartowsky, 2014). Some yeasts, such as Schizosaccharomyces pombe, are known to completely degrade malic acid as they possess malate permease and malic acid decarboxylase (Benito et al., 2016a). Malic acid degradation is also associated with L. thermotolerans strains (Gobby et al., 2013; Hranilovic et al., 2018; Kapsopoulou et al., 2005; Whiten et al., 2017), as observed here, although only L. thermotolerans-dominated fermentations reduced malic acid by up to ~25 %. Interestingly, this was not reflected in an increase in lactic acid, a phenotype typically associated with L. thermotolerans yeast; however, Concerto is considered a poor producer of lactic acid, as in some cases it will not produce significantly more of it than some S. cerevisiae strains (Vaquero et al., 2020).

Glycerol and ethanol modulation using non-Saccharomyces yeast has been extensively researched with a view to producing ‘reduced alcohol’ wines. Both species used in this study are able to increase glycerol (Contreras et al., 2014; Gobby et al., 2013; Hranilovic et al., 2020; Kapsopoulou et al., 2007) and decrease ethanol content (Contreras et al., 2014; Hranilovic et al., 2018; Hranilovic et al., 2020; Hranilovic et al., 2021; Varela et al., 2016). Glycerol production was improved when MP2 was co-cultured with SER1(G78R). Meanwhile, the influence of Concerto was more complicated, producing less glycerol than the SER1(G78R) monoculture, although it was still significantly higher than in all EC1118 fermentations (Table 4). SER1(G78R) can still be regarded as a superior glycerol producer compared to EC1118, but yield was dependent upon its abundance in co-fermentations (Figure 2E). Increased glycerol production in S. cerevisiae yeast occurs with a concurrent increase in acetic acid (Eglinton et al., 2002; Remize et al., 1999; van Wyk et al., 2020), which is needed to balance the NAD+/NADH ratio, which tends towards NADH by glycerol biosynthesis. An increase in acetic acid and glycerol was observed in the SER1(G78R) monoculture, but not in the non-Saccharomyces co-inoculated cultures, indicating the addition of Concerto and MP2 as a way of addressing redox balance. Previously, Sadoudi et al. (2017) demonstrated an increase in glycerol and decrease in acetic acid in M. pulcherrima/S. cerevisiae sequential fermentations, with the presence of M. pulcherrima altering the expression of the genes involved in acetic acid biosynthesis in S. cerevisiae. This could explain the reduction of acetic acid in the co-inoculated fermentations in this study. Another plausible explanation is that MP2-derived acetic acid is reduced through the production of acetate esters, a phenomenon observed in studies involving M. pulcherrima (Binati et al., 2020; Contreras et al., 2014; Hranilovic et al., 2020; Sadoudi et al., 2012; Varela et al., 2016). Van Wyk et al. (2020) demonstrated that the overexpression of alcohol acetyltransferase (ATF1) in S. cerevisiae strains that accumulate both glycerol and acetic acid due to NAD-dependent glycerol-3-phosphate dehydrogenase (GPD1) overexpression resulted in lower acetic acid and higher acetate ester concentrations. Whilst outside the scope of this paper, it would be interesting to assess ATF1 and GPD1 transcript levels in M. pulcherrima strains to determine if this is also the case.

Many authors have noted specific increases in acetate esters in mixed fermentations involving M. pulcherrima. In this study, five esters (ethyl acetate, isoamyl acetate, 2-phenylethyl acetate, isobutyl acetate and cis-3-hexenyl acetate) were assessed based on prior reports of their increase with M. pulcherrima inclusion in fermentation (Contreras et al., 2014; Hranilovic et al., 2020; Sadoudi et al., 2012; Varela et al., 2016). Propyl acetate was also analysed. In most instances, there were statistically significant increases in acetate esters with the higher MP2 inoculations correlating to higher acetate esters (Table 5).

Hranilovic et al. (2018) also demonstrated an increase in acetate esters by Concerto when sequentially inoculated with S. cerevisiae in Shiraz fermentations. Specifically, significant increases were observed in ethyl acetate, ethyl phenylacetate, isoamyl acetate and isobutyl acetate. In this study, whilst higher mean concentrations of propyl acetate and isobutyl acetate were observed, ethyl acetate and isobutyl acetate significantly increased at the higher inoculation ratio, isoamyl acetate, cis-3-hexenyl acetate and 2-phenylethyl acetate were reduced (Table 5). Differences between studies may be related to wine type (red vs white) and variety (Shiraz vs Sauvignon blanc). Hranilovic et al. (2018) further reported on vintage-dependent variability in acetate esters, in particular isoamyl acetate: one juice co-inoculated with Concerto had significantly higher concentrations compared to S. cerevisiae, but the inverse was found in a subsequent vintage. Such trends highlight the importance of juice composition in winemaking outcome.

Arguably, the most interesting observation of the present study was the alleviation of SO₂ sensitivity associated with Concerto in sulfured Sauvignon blanc and Chardonnay juice. The results for the SER1(G78R) co-fermentations are
CONCLUSION

The transfer of a novel and naturally-occurring SER1 variant (SER1(G78R)) to the wine yeast EC1118 via CRISPR/Cas9 results in a slower-growth phenotype. This mutation may also have the added advantage over previously reported slow-growing yeasts of SO2, not needing to elicit slow growth, thereby giving it broader applicability. This phenotype benefited novel mixed cultures in which the mutant was co-inoculated with non-Saccharomyces as it resulted in a greater expression of positive sensory effects imparted by the non-Saccharomyces. Furthermore, this strain can be used with non-Saccharomyces strains that have low sulfite tolerance, thereby permitting the standard practice of using SO2 to prevent oxidation and microbial spoilage. Further evaluation is required of this strain in combination with, for example, other non-Saccharomyces and inoculation ratios to determine whether it would be suitable for use as a ‘mixed’ starter culture comprising multiple yeast species. The construction of this strain is an example of ‘self-cloning’ and, as such, is likely to be permitted for use in countries such as Japan and USA where regulations are less stringent than in Europe and Australia (Hanlon and Sewalt, 2020). To date, Australia’s ‘clean green image’ in winemaking and regulation of GMOs prevents the use of genetically modified yeast and bacteria, apart from variants isolated from the vineyard and point mutations generated by mutagenesis.

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