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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 SO_2-sensitive *L. thermotolerans* strain, Viniflora® Concerto™, in a juice high in SO_2 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*, SO_2

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 this 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 *ser1Δ* 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 (C2987I, 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

TABLE 1. Yeast strains used in this study.

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

TABLE 2. Plasmids and oligonucleotides used in this study.

Plasmid	Description	Source
pWS082	sgRNA cloning vector (Amp^R)	Shaw *et al.* (2019) Addgene plasmid* #90516
pWS173	Cas9 linear co-transformation vector (Kan^R, 2 μ vector)	Addgene plasmid* #90960
pWS-SER1	sgRNA vector targeting *SER1* (Amp^R)	This study

Oligonucleotides	Sequence (5' – 3')	Source
sgSER1 F	gactttGAAGTGTTCTACTTGCAAGG	This study
sgSER1 D	aaacCCTTGCAAGTAGAACACTTCaa	This study
pWS082 seq	GTCATCTGGAGGTCCTGTGTTC	Lang *et al*. (2021)
SER1-HDR F	ATCGAACTGCTAAATATTCCTGACACTCAT GAAGTGTTCTACTTGCAACGTGGTGGCAC	This study
SER1-HDR R	CAGCTGCCAAATTAGTAGCAACGGAAGAA AAACCAGTAGTGCCACCACGTTGCAAGTAGA	This study
SER1 A	CAAAAGAAAAGCCATAATAAGGACA	SGDP**
SER1 D	AGATAGTTCAGTCTCACCCACATTC	SGDP**
SER1 seq	AATGCCTACACCAGTTTTGC	This study

*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.

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 (BIO-33054); 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

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 replica plated 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 10^6 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 according to Gardner *et al.* (2005) with modification. An Agilent 1100 system (Agilent Technologies, USA) was utilised with detection by refractive index detector (G1362A; Agilent Technologies, USA) for glycerol and diode-array detector (G1315A/B; Agilent Technologies, USA) for lactic acid, succinic acid and acetic acid.

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 SO_2/L, 40 mg total SO_2/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 10^7 cells/mL. Monocultures of each strain were also undertaken, using 1 x 10^7 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 10^6 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 acetate esters (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. SO_2 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 SO_2 and 34.4 mg/L total SO_2). 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 10^7 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 lysine 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

S*ER1*(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.

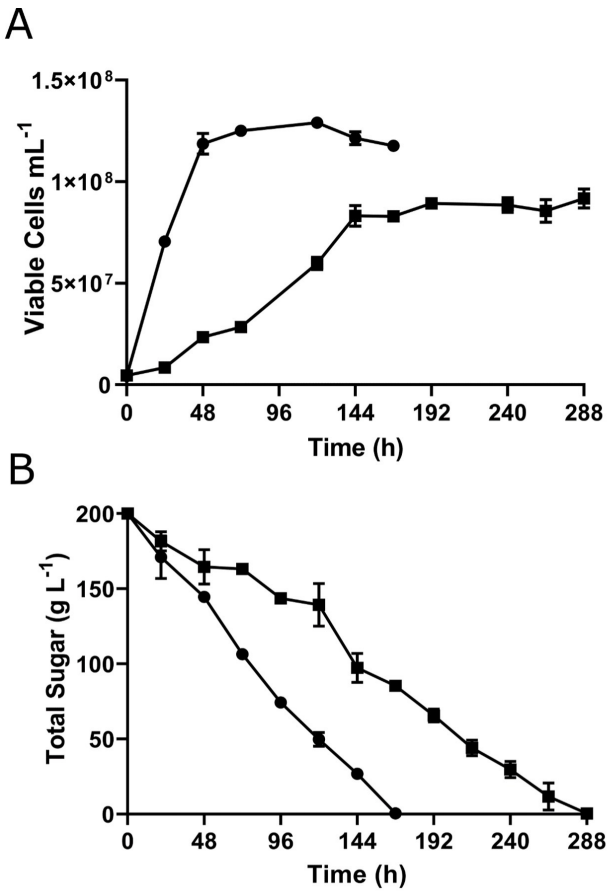

FIGURE 1. Evaluation of fermentation performance and viability of EC1118 and *SER1*(G78R) grown in CDGJM.

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.

TABLE 3. Fermentation duration (h) and metabolite content (g/L) of 'wines' produced by EC1118 and SER1(G78R) when fermenting in CDGJM.

	Duration	Acetic Acid	Lactic Acid	Malic Acid	Succinic Acid	Glycerol	Ethanol
EC1118	168 ± 0.00	1.61 ± 0.03	0.00 ± 0.00	3.25 ± 0.03	0.98 ± 0.01	4.30 ± 0.03	100.20 ± 0.49
SER1(G78R)	288 ± 0.00*	1.90 ± 0.03*	0.07 ± 0.00*	3.20 ± 0.02	1.32 ± 0.04*	4.62 ± 0.03*	102.10 ± 0.25*

*Denotes significant difference (Unpaired t-test; $p < 0.01$) between the strains for the given parameter.

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 (SO_2)-sensitive at 40 mg/L total SO_2, 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 SO_2-sensitive *Lachancea* in SO_2 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 the *SER1* encoded phosphoserine transaminase catalysing a bi-directional reaction between 3-phosphooxypyruvate and 3-phospho-L-serine. Disruption of *SER1* 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

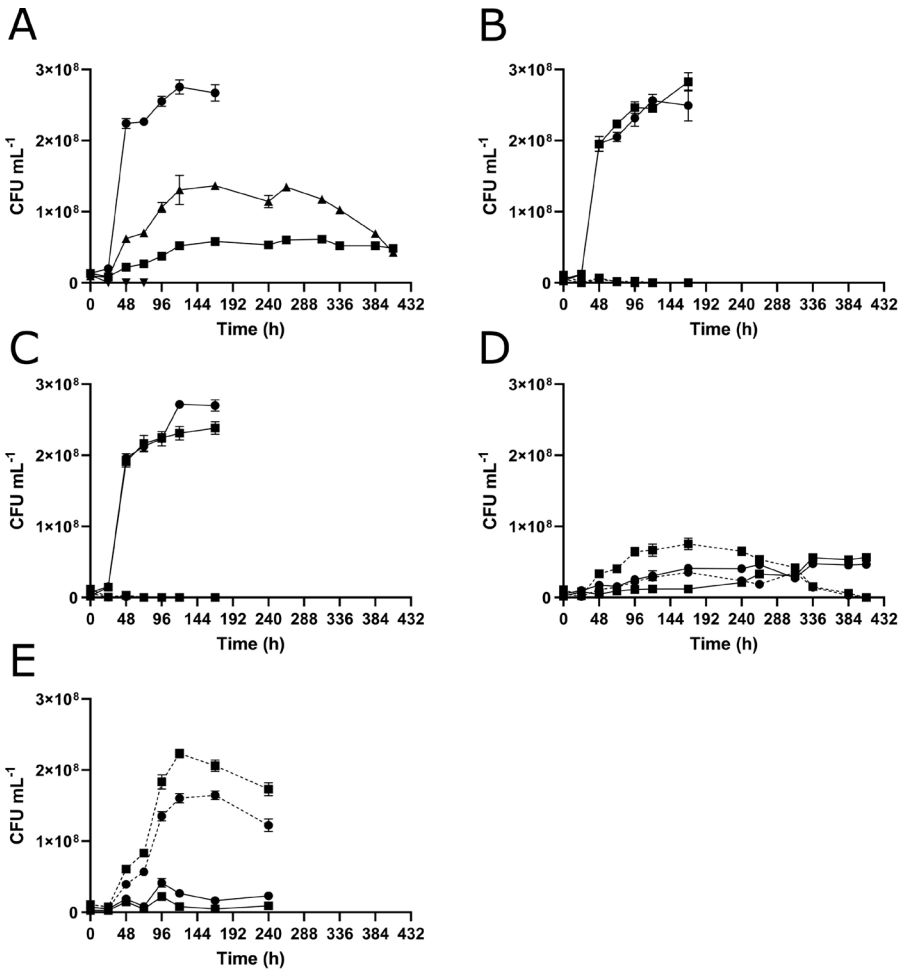

FIGURE 2. Viable yeast counts from Sauvignon blanc juice fermentations.

Viable yeast counts from WL agar plating of either monocultures (A) of EC1118 (●), *SER1*(G78R) (■), MP2 (▲) or Concerto (▼) or co-inoculated cultures of (B) EC1118 with MP2, (C) EC1118 with Concerto, (D) *SER1*(G78R) with MP2 or (E) *SER1*(G78R) with Concerto. Solid lines in co-inoculated fermentations denote *S. cerevisiae* populations, whereas dashed lines denote non-*Saccharomyces* populations. Square (■) markers in co-inoculated fermentations correspond to a 1:9 inoculation ratio, whereas circles (●) correspond to a 1:1 inoculation ratio.

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.

Strain(s)	Duration	Acetic Acid	Lactic Acid	Malic Acid	Succinic Acid	Glycerol	Ethanol
EC1118 (monoculture)	168 ± 0.00c	0.21 ± 0.07de	0.69 ± 0.02a	3.97 ± 0.01a	3.85 ± 0.01b	6.63 ± 0.03e	86.29 ± 0.11a
EC1118 + MP2 (1:1)	168 ± 0.00c	0.24 ± 0.02de	0.70 ± 0.15a	3.96 ± 0.02a	3.79 ± 0.03b	6.65 ± 0.06e	85.95 ± 0.45a
EC1118 + MP2 (1:9)	168 ± 0.00c	0.12 ± 0.11e	0.77 ± 0.04a	4.05 ± 0.02a	3.75 ± 0.04b	6.69 ± 0.01e	86.13 ± 0.34a
EC1118 + Concerto (1:1)	168 ± 0.00c	0.26 ± 0.02de	0.68 ± 0.05a	3.91 ± 0.02a	3.81 ± 0.03b	6.63 ± 0.00e	86.22 ± 0.05a
EC1118 + Concerto (1:9)	168 ± 0.00c	0.22 ± 0.03de	0.72 ± 0.02a	3.92 ± 0.03a	3.81 ± 0.03b	6.61 ± 0.01e	86.37 ± 0.15a
SER1(G78R) (monoculture)	408 ± 0.00a	1.56 ± 0.11a	0.55 ± 0.09a	4.12 ± 0.16a	3.55 ± 0.02c	8.59 ± 0.04c	85.87 ± 0.14a
SER1(G78R) + MP2 (1:1)	408 ± 0.00a	1.28 ± 0.03b	0.74 ± 0.06a	4.06 ± 0.06a	3.48 ± 0.02c	8.83 ± 0.03b	85.66 ± 0.06a
SER1(G78R) + MP2 (1:9)	408 ± 0.00a	0.41 ± 0.10cd	0.72 ± 0.12a	3.83 ± 0.11a	3.15 ± 0.08d	9.60 ± 0.07a	83.29 ± 0.38b
SER1(G78R) + Concerto (1:1)	240 ± 0.00b	0.62 ± 0.02c	0.58 ± 0.16a	3.31 ± 0.19b	3.79 ± 0.02b	7.36 ± 0.12d	86.22 ± 1.25a
SER1(G78R) + Concerto (1:9)	240 ± 0.00b	0.39 ± 0.01d	0.58 ± 0.01a	3.08 ± 0.04b	4.05 ± 0.01a	7.22 ± 0.05d	86.50 ± 0.60a

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), whist 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 - lead 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,

TABLE 5. Acetate ester content (µg/L) of monoculture and co-inoculated fermentations in Sauvignon blanc juice.

Strain(s)*	Propyl Acetate	Isobutyl Acetate	Isoamyl Acetate	cis-3-Hexenyl Acetate	2-Phenylethyl Acetate	Ethyl Acetate
EC1118	2.66 ± 0.21d	1.58 ± 0.12cd	35.88 ± 7.17b	56.39 ± 2.05a	17.51 ± 1.36b	2184 ± 177.0c
EC1118 + MP2 (1:1)	2.65 ± 0.22d	1.51 ± 0.11d	35.38 ± 10.60b	60.95 ± 3.77a	19.59 ± 0.95b	2238 ± 230.1c
EC1118 + MP2 (1:9)	2.97 ± 0.52d	1.65 ± 0.23cd	36.23 ± 15.98b	53.86 ± 11.66a	20.53 ± 4.06b	2996 ± 419.2bc
EC1118 + Concerto (1:1)	2.76 ± 0.12d	1.59 ± 0.04cd	26.9 ± 8.41b	56.76 ± 3.09a	19.74 ± 1.24b	2432 ± 142.1bc
EC1118 + Concerto (1:9)	2.88 ± 0.71d	1.71 ± 0.55cd	36.22 ± 8.96b	53.39 ± 0.99a	18.74 ± 0.91b	2228 ± 334.1c
SER1(G78R)	8.64 ± 1.17c	2.37 ± 0.32bcd	21.84 ± 2.86b	63.18 ± 9.45a	18.64 ± 1.58b	2363 ± 189.2bc
SER1(G78R) + MP2 (1:1)	11.29 ± 1.34b	3.15 ± 0.40b	21.98 ± 5.76b	54.36 ± 11.30a	22.03 ± 2.10b	3598 ± 519.6b
SER1(G78R) + MP2 (1:9)	14.85 ± 0.46a	14.06 ± 0.32a	75.86 ± 22.13a	60.97 ± 5.62a	46.33 ± 2.38a	8801 ± 526.9a
SER1(G78R) + Concerto (1:1)	4.42 ± 0.18d	2.75 ± 0.16bc	15.21 ± 7.37b	8.96 ± 0.81b	2.31 ± 0.21c	3342 ± 135.6bc
SER1(G78R) + Concerto (1:9)	3.87 ± 0.50d	3.28 ± 0.59b	15.93 ± 5.66b	5.54 ± 1.03b	1.90 ± 0.19c	3275 ± 631.1bc

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 SO_2 sensitivity

The influence of co-culture with *Saccharomyces* on the SO_2 sensitivity of Concerto was investigated by carrying out a repeat of the fermentations in another white variety, Chardonnay, containing 34.4 mg/L total SO_2. 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 10^7 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 SO_2 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 SO_2 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.

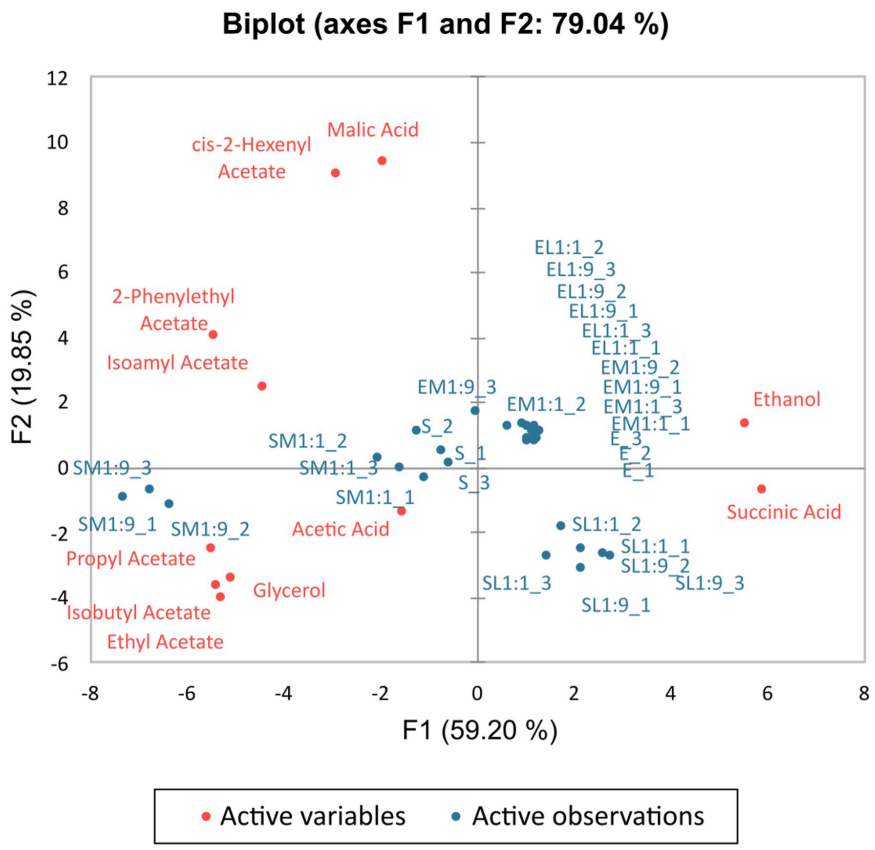

FIGURE 3. Principal component analysis of acetate esters, organic acids and glycerol for individual fermentations in Sauvignon blanc wine fermentations.

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.

DISCUSSION

In winemaking, mixed fermentation by *S. cerevisiae* and non-*Saccharomyces* yeast is a common method for introducting 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. cerevisiase* 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 SO_2-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

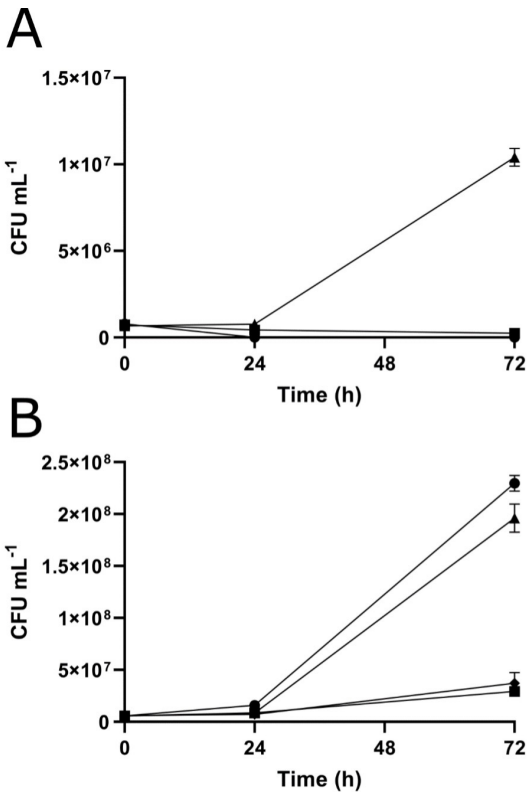

FIGURE 4. Viable cell densities of Concerto or S. cerevisiae after 72 h in Chardonnay juice.

Fermentations (100 mL) were conducted in Chardonnay juice containing 34.4 mg/L total SO_2. 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.

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

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 sulfite, 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 (Gobbi *et al.*, 2013; Hranilovic *et al.*, 2018; Kapsopoulou *et al.*, 2005; Whitener *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. thermotolernas* 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; Gobbi *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 NAD^+ 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, and 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 SO_2 sensitivity associated with Concerto in sulfured Sauvignon blanc and Chardonnay juice. The results for the *SER1*(G78R) co-fermentations are

therefore promising, as they allow the use of SO_2 sensitive non-*Saccharomyces* yeasts - which might offer other interesting oenological properties - in what would otherwise be lethal concentrations of SO_2. Similar findings were made for the EC1118 co-inoculated cultures when plating on selective lysine media (Figure 4A), even though the Concerto concentrations were reduced. As it is well known that acetaldehyde accumulation generally occurs during the yeast growth phase (Amerine and Ough, 1964; Weeks, 1969), the binding of SO_2 by acetaldehyde is a probable cause of this observation. The fact that this phenomenon was observed with both wild-type and mutant *S. cerevisiae* strains implies that other slow-growing yeast (e.g., Albertin *et al.*, 2017) possess this ability. Further research would be of benefit in order to both understand this phenotype and develop novel strains for use in co-inoculation and SO_2 sequestration.

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 SO_2 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 wine-making practice of using SO_2 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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