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Strain-Specific Responses by *Saccharomyces cerevisiae* to Competition by Non-*Saccharomyces* Yeasts

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**Abstract:** The use of non-*Saccharomyces* yeast species generally involves sequential or co-inoculation of a *Saccharomyces cerevisiae* strain to complete fermentation. While most studies have focused on characterising the impact that *S. cerevisiae* has on the growth and metabolic activity of these non-*Saccharomyces* species, microbial interactions work reciprocally. Antagonism or competition of non-*Saccharomyces* species against *S. cerevisiae* has been shown to impact subsequent fermentation performance. To date, it remains unclear whether these negative interactions are strain specific. Hence, characterisation of strain-specific responses to co-inoculation would enable the identification of specific *S. cerevisiae* strain/non-*Saccharomyces* combinations that minimise the negative impacts of sequential fermentation on fermentation performance. The competitive fitness response of 93 *S. cerevisiae* strains to several non-*Saccharomyces* species was simultaneously investigated using a barcoded library to address this knowledge gap. Strain-specific fitness differences were observed across non-*Saccharomyces* treatments. Results obtained from experiments using selected *S. cerevisiae* strains sequentially inoculated after *Metschnikowia pulcherrima* and *Torulaspora delbrueckii* were consistent with the competitive barcoded library observations. The results presented in this study indicate that strain selection will influence fermentation performance when using non-*Saccharomyces* species, therefore, appropriate strain/yeast combinations are required to optimise fermentation.

**Keywords:** non-*Saccharomyces*; *Saccharomyces cerevisiae*; wine; fermentation; yeast interaction

1. **Introduction**

Inoculation with selected *Saccharomyces cerevisiae* strains is one way to initiate fermentation efficiently in compositionally and microbiologically diverse grape musts [1]. However, inoculated wines are often considered to lack the complexity of their uninoculated counterparts [2]. This lack of complexity may be attributed to a reduction in non-*Saccharomyces* metabolic activity. To address this perceived shortcoming, several non-*Saccharomyces* species have now been isolated and commercialised with the objective of enhancing the aroma and flavour attributes of wine [3]. Species such as *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* are now commercially available as starter cultures and represent practical tools that can be used to reduce alcohol and volatile acidity concentrations in wine and to modulate the concentrations of varietal thiols and esters [4]. Due to the variable capacity of these yeasts to complete sugar consumption [5], strains of *S. cerevisiae* are sequentially inoculated to ensure the completion of alcoholic fermentation. However, inoculation with multiple species can introduce issues associated with microbial compatibility, especially when the purified strains are inoculated at high concentrations [6].

Alongside the characterisation of nutrient requirements, fermentation capabilities and metabolite production of non-*Saccharomyces* species [7–13], studies have investigated the impact of *S. cerevisiae* on growth to maximise non-*Saccharomyces* species’ persistence in grape juice. Several mechanisms of antagonism between *S. cerevisiae* and non-*Saccharomyces* species have been proposed, and these can differ depending on the non-*Saccharomyces*
species, including cell-to-cell contact [14,15], the production of antimicrobial peptides [7,16] and toxic compounds [17].

Conversely, given the difference in inoculation times under a sequential inoculation scenario, initial uptake of nutrients such as amino acids and vitamins by non-Saccharomyces species has been suggested as an impediment for the subsequent growth of S. cerevisiae strains [18]. Non-Saccharomyces species such as T. delbrueckii, M. pulcherrima, Hanseniaspora vineae, H. guilliermondii and Kluyveromyces marxianus have also been shown to significantly decrease the nitrogen availability for S. cerevisiae [19–21]. Aside from nutrient competition, other antagonistic mechanisms of non-Saccharomyces species against S. cerevisiae have been reported, including cell-to-cell contact [20] and the production of killer toxins [22,23].

While the previous investigations have focused on characterising the features relating to competition between specific yeast species, recent research suggests that microbial interactions could be strain-specific [24,25]. Strain selection could therefore represent an important factor in maximising fermentation performance when inoculating with multiple yeast species. The fermentation fitness differences [26] and genetic diversity [27,28] of wine-associated S. cerevisiae strains have been well documented. However, it is unknown if these strain differences affect the ability to compete with other non-Saccharomyces species. This study simultaneously investigated the competitive fitness response of 93 S. cerevisiae wine strains to several non-Saccharomyces species using a genomically barcoded library. Experiments were also performed using single S. cerevisiae strains to further investigate specific strain–species interactions in a sequential inoculation regime.

2. Materials and Methods

2.1. Non-Saccharomyces Strains and Barcoded Library

The strains used in this study are available in The Australian Wine Research Institute (AWRI) culture collection. Five non-Saccharomyces species (Table 1) were used for the competitive fitness experiments and were selected based on their use as commercial starter cultures and their reported natural abundance in grape juice. The S. cerevisiae barcode library is comprised of 93 wine strains (Table S1) that represent several of the phylogenetic clades within this species [26,28]. Each strain contains a unique pair of barcodes inserted in the HO ORF region, allowing the quantification of each strain under mixed culture competitive conditions. Details on the construction of the wine yeast barcoded library (WYBC) and mixed pooled inoculum have been described previously [26].

Table 1. Yeast strains used in this study.

| Species                      | Strain   | Experiments         |
|------------------------------|----------|---------------------|
| Aureobasidium pullulans      | AWRI4229 | WYBC                |
| Hanseniaspora uvarum         | AWRI3580 | WYBC                |
| Lachancea thermotolerans     | AWRI2009 | WYBC                |
| Metschnikovia pulcherrima    | AWRI3050 | WYBC, single strain |
| Torulaspora delbrueckii      | AWRI2845 | WYBC, single strain |
| Saccharomyces cerevisiae     | AWRI2913 | single strain-M. pulcherrina |
| Saccharomyces cerevisiae     | AWRI2863 | single strain-M. pulcherrina |
| Saccharomyces cerevisiae     | AWRI2867 | single strain-M. pulcherrina |
| Saccharomyces cerevisiae     | AWRI1490 | single strain-M. pulcherrina |
| Saccharomyces cerevisiae     | AWRI739  | single strain-T. delbrueckii |
| Saccharomyces cerevisiae     | AWRI1430 | single strain-T. delbrueckii |
| Saccharomyces cerevisiae     | AWRI2851 | single strain-T. delbrueckii |
| Saccharomyces cerevisiae     | AWRI1686 | single strain-T. delbrueckii |

2.2. Barcoded Library Competitive Fitness Experiments

Competitive fitness experiments were performed in a chemically defined medium (CDM) [26]. The overall approach has been described in detail previously [26]. Briefly, each non-Saccharomyces species was inoculated at a concentration of 10⁶ CFU/mL into 100 mL sealed Schott flasks fitted with gas release valves and stirred at 250 rpm for 24 h.
at 18 °C, after which 1 mL of freshly thawed WYBC pool was added into each flask. A control treatment without the presence of a non-\textit{Saccharomyces} species was also included. After 3 days (T1) of growth, samples were taken for DNA extraction, and 1 mL of each flask was used to inoculate another set of treatments and control flasks using a serial batch approach. Sequential inoculations were performed twice for a total of 3 sampling time points (T1–T3). All treatments were performed in triplicate. DNA was also extracted directly from the WYBC pool to use as an intercept (control) in the statistical analyses of the barcode counts' data.

2.3. \textbf{Amplicon Sequencing, Bioinformatic and Data Analyses}

Genomic DNA from each time point was extracted using a Gentra Puregene Yeast/Bact kit (Qiagen, Doncaster, Victoria, Australia) following the manufacturer’s instructions with minor modifications. A total of 3 uL of Zymolyase (10 mg/mL) (MP Biomedicals, NSW, Australia) was used instead of lytic enzyme. Amplicons for Illumina sequencing were generated using primers Illum\_P5\_S50(1-6) and Illum\_P7\_N70(1-12), and PCR conditions as previously described [26]. A total of 300 ng of each PCR reaction was pooled and then cleaned using a NucleoSpin PCR cleanup kit (Machery-Nagel GMBH, Düren, Germany) and sent for sequencing to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) on an Illumina NextSeq500 instrument using 2 × 36 bp run format. Forward and reverse reads were fused using BBMap v.39.90 and converted into fasta files. Unique dual barcodes corresponding to each strain were demultiplexed from fasta files using the Python packages demultiplex and TSSV [29], searching for the unique pair of barcodes in each read allowing no nucleotide mismatches. Demultiplexed reads corresponding to each strain and treatment were counted and imported into R [30] (File S1). Statistical analysis of strain counts was performed using EdgeR [31], as previously described by Schmidt et al. [26], using a pairwise design with the WYBC pool strain counts data as an intercept. Strain fitness was evaluated as the contrast of each time point in each non-\textit{Saccharomyces} treatment against the control treatment (e.g., T3 MP = Timepoint 3 \textit{S. cerevisiae} strain counts (\textit{M. pulcherrima} treatment) − Timepoint 3 \textit{S. cerevisiae} strain counts (WYBC control treatment)) (Tables S2–S5).

2.4. \textbf{Single Strain Competitive Experiments}

Experiments using single \textit{S. cerevisiae} strains selected according to their competitive fitness performance (high and low fitness performance) were performed in the presence of non-\textit{Saccharomyces} species. A blue fluorescent protein (BFP) gene was introduced in the \textit{HO} ORF of the parental non-barcoded \textit{S. cerevisiae} strains to allow cell counting of \textit{S. cerevisiae} by flow cytometry in the presence of a different yeast species. Transformations were carried out using the lithium acetate-polyethylene glycol method [32]. DNA cassettes for transformation were obtained by PCR from plasmid pCV2\_HO-BFP, a modified version of plasmid pCV2\_BB-BFP [33] containing 312 bp of homology to the \textit{HO} ORF. Positive transformants were screened using geneticin and confirmed by PCR. Competitive experiments were performed in CDM using two non-\textit{Saccharomyces} species (\textit{M. pulcherrima} and \textit{T. delbrueckii}) and eight \textit{S. cerevisiae} strains (Table 1). All treatments were undertaken in triplicate. Non-\textit{Saccharomyces} species were inoculated (10^6 CFU/mL) into 100 mL sealed Schott flasks fitted with gas release valves and stirred at 250 rpm for 24 h after which a \textit{S. cerevisiae} strain was inoculated at a final OD600 of 0.02 (approx. 10^6 CFU/mL). All fermentations were conducted at 18 °C. Samples were taken daily to determine cell concentration using flow cytometry and sugar concentration by enzymatic assay.

2.5. \textbf{Analytical Methods}

Glucose and fructose concentrations were determined enzymatically as previously described by [34] with adaptations described by [35] to allow the measurement in a 96-well microplate format.
Cells were counted by flow cytometry using a Guava® easyCyte 12HT instrument (Merck Millipore, Burlington, MA, USA). Prior to cell counting, cells were diluted in PBS (NaCl 8 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.44 g/L, KH₂PO₄ 0.24 g/L pH 7.4) containing propidium iodide (PI) (1% v/v) to a concentration lower than 5 × 10⁶ cells/mL. Forward and side scatter detectors were used to determine particle size and estimate cell numbers. A minimum of 5000 events with a throughput lower than 500 event/µL was measured in all samples. The viable cell concentration of S. cerevisiae was determined by measuring the number of positive blue fluorescent (405 nm excitation and 448/50 nm detection filter) and PI-negative events per mL.

3. Results and Discussion

3.1. S. cerevisiae Competitive Fitness Response to the Presence of a Non-Saccharomyces Species

The strain-specific effect resulting from preculturing of medium with different non-Saccharomyces species (A. pullulans, H. uvarum, L. thermodolerans, M. pulcherrima and T. delbrueckii) on the growth of S. cerevisiae was investigated. A sequential-batch experiment estimated competitive fitness responses for each S. cerevisiae strain using a pooled-inoculum approach (93 barcoded S. cerevisiae strains). At each time point, the strain-wise barcode counts for samples taken from ferments containing non-Saccharomyces species were determined by sequencing and compared to the strain-wise barcode counts obtained from ferments without non-Saccharomyces species.

Sugar concentrations at the time of passage revealed a differential effect of the non-Saccharomyces species on the general fermentation performance of the mixed cultures (Table 2). Ferments containing A. pullulans and M. pulcherrima displayed the slowest sugar consumption rates (Table 2). Relative to the control condition, all mixed cultures had reduced sugar consumption rates in T2 and T3, suggesting a direct competition/antagonism of these non-Saccharomyces species with S. cerevisiae. While the majority of these non-Saccharomyces species are associated with beneficial organoleptic effects in wine [12,13,36,37], several studies have reported slower fermentation rates when these species were used in co-culture [11,20,37].

Table 2. Sugar concentration (g/L) at each time point (3 days after S. cerevisiae inoculation). All data points correspond to the mean of three replicates ± sd.

| Treatment                        | T1       | T2       | T3       |
|----------------------------------|----------|----------|----------|
| Aureobasidium pullulans          | 159 ± 5  | 175 ± 25 | 145 ± 20 |
| Hanseniaspora uvarum             | 73 ± 3   | 90 ± 4   | 81 ± 2   |
| Metschnikowia pulcherrima        | 126 ± 2  | 141 ± 8  | 128 ± 5  |
| Torulaspora delbrueckii          | 92 ± 8   | 121 ± 4  | 93 ± 8   |
| Lachancea thermodulerans         | 84 ± 4   | 97 ± 5   | 71 ± 4   |
| WYBC control                     | 101 ± 3  | 83 ± 1   | 69 ± 7   |

S. cerevisiae strain-specific fitness responses to different non-Saccharomyces treatments varied depending on the non-Saccharomyces species present (Figure 1). Clustering of treatments resolved three groups which included T. delbrueckii and H. uvarum in one group, M. pulcherrima and L. thermodolerans in the second and the third containing A. pullulans as the only member (Figure 1). These observations indicate that S. cerevisiae strain fitness variability is species-dependent.
Figure 1. Fitness (log$_2$ FC relative to control conditions) of *Saccharomyces cerevisiae* strains in the presence of a non-*Saccharomyces* species. Data are clustered across fitness and treatments. Strains with an asterisk (*) showed a log$_2$ FC $\geq 1$ across all treatments. AP: *Aureobasidium pullulans*, HU: *Hanseniaspora uvarum*, TD: *Torulaspora delbrueckii*, MP: *Metschnikowia pulcherrima* and LT: *Lachancea thermotolerans*.

Inspection of the fitness response of specific *S. cerevisiae* strains revealed 12 strains with a log$_2$ fold change (FC) $\geq 1$ (Figure 1, highlighted in red) and 30 strains with a log$_2$ FC $\leq -1$ across all treatments. The largest increase in relative abundance was observed in strain AWRI1709 (log$_2$ FC of 11.8) in the presence of *A. pullulans* (Figure 1) (Table S4).
The increased relative abundance of AWRI1709 suggests that this specific strain might have the ability to overcome the nutrient competition previously associated with *A. pullulans* [37]. Mapping onto pre-existing wine yeast phylogenies of the 12 strains that showed a log₂ FC ≥ 1 revealed that none of these strains corresponded to the previously reported *Prize de Mousse* (PdM) clade [28], which is represented by 17 strains in the WYBC. Furthermore, from these 17 strains, 10 showed a log₂ FC ≤ −1, and 4 showed a negative log₂ FC in all treatments. Strains belonging to the PdM clade are considered highly domesticated to the grape juice/wine environment; however, the data suggests that these strains might not be the fittest in a highly competitive scenario.

All treatments showed a higher number of strains with reduced representation in the community relative to the control condition (MP: 45 strains down, 23 strains up | TD: 44 down, 29 up | HU: 41 down, 30 up | LT: 50 down, 22 up | AP: 37 down, 28 up, Figure 1). Although it is not possible to determine if the strains showing a relative increase in abundance are indeed growing at faster rates when a non-*Saccharomyces* species is present, the sugar consumption data suggest the contrary, where strains with a positive log₂ FC are likely less affected by the antagonism/competition of these non-*Saccharomyces* species.

3.2. Single Inoculum Competition Experiments

To confirm the barcoded competitive growth assay results, eight *S. cerevisiae* strains that showed either a strongly positive or negative fitness response to the presence of two non-*Saccharomyces* species (Table 1, Figure 1) were individually assessed in dual-species fermentation experiments.

In agreement with the WYBC library experiments, sugar consumption kinetics (Figure 2A,C) were delayed in all treatments. Although slower sugar consumption rates were explicitly observed in all treatments containing *M. pulcherrima* (Figure 2A), ferments inoculated with *S. cerevisiae* strains AWRI2867 and AWRI1490 contained high residual sugar concentrations after 40 days of fermentation (10.7 ± 1.7 and 7.3 ± 4.5 g/L). Despite the observed delay in sugar consumption compared to the control condition, all treatments containing *T. delbrueckii* finished fermentation after 14 days (Figure 2C). In both non-*Saccharomyces* treatments, *S. cerevisiae* strains that showed a positive and negative log₂ FC in competitive experiments correlated with faster and slower sugar consumption rates, respectively (Figure 2A,C). The correlation between the two experimental outcomes confirms that there is *S. cerevisiae* strain-dependent variation in response to a non-*Saccharomyces* species. These results suggest that the previously reported detrimental effects on fermentation performance caused by non-*Saccharomyces* species [9,11,20] could be ameliorated by inoculation with specific *S. cerevisiae* strains. Furthermore, in the case of *M. pulcherrima*, the differences observed between strains describe the possibility of a stuck fermentation if specific *S. cerevisiae* strains are selected for sequential inoculation (Figure 2A).
Figure 2. Fermentation kinetics and relative growth in CDM of *Saccharomyces cerevisiae* strains in the presence of *Metschnikowia pulcherrima* (A,B) and *Torulaspora delbrueckii* (C,D). + and - WBYC strains refer to strains that showed a positive or negative log2 FC in the WYBC library competitive fitness experiments for the specific non-*Saccharomyces* treatment (Figure 1). The arrows in panels (A,C) indicate the time point when all *S. cerevisiae* controls finished fermentation. The relative growth in panels (B,D) are expressed as a percentage of cells in the specified timepoint relative to control conditions without the presence of a non-*Saccharomyces* species. All data points correspond to the mean of three replicates ± sd.

Determination of *S. cerevisiae* cell concentration by flow cytometry during the initial stages of fermentation revealed detrimental effects on cell growth that differed between strains and non-*Saccharomyces* treatments (Figure 2B,D). In the presence of *M. pulcherrima* (Figure 2B), *S. cerevisiae* strains differed in the cell concentration they were able to achieve relative to the control condition. These differences in cell concentration correlated with results obtained in the competitive fitness experiments (Figure 1). In treatments including *T. delbrueckii*, differences between positive and negative WYBC were only apparent in day 5 and an overall strong inhibition of cell growth was observed (Figure 2D). It can be speculated that larger differences in cell growth between positive and negative WYBC occurred later in fermentation, reflecting the differences observed in sugar consumption rates (Figure 2C). Other studies have reported similar effects on the growth of *S. cerevisiae* when using *T. delbrueckii* in a sequential inoculation regime; however, the mechanisms of inhibition have not yet been determined [20,38,39]. Despite this, consumption of more than 50% of the available sugar was already observed by day 5, indicating that *T. delbrueckii* was a significant contributor to sugar utilisation in this treatment. The overall cell counts confirm that *S. cerevisiae* strains are affected differently by the antagonism/competition caused by these non-*Saccharomyces* species. Comparison between treatments also highlights different mechanisms of antagonism/competition between *M. pulcherrima* and *T. delbrueckii* that should be further investigated.
4. Conclusions

The competitive fitness response to the presence of five non-\textit{Saccharomyces} species was investigated in 93 \textit{S. cerevisiae} wine strains using a genomically barcoded library of wine yeast. Analysis of unique barcode counts revealed considerable differences in the response of \textit{S. cerevisiae} strains to the different non-\textit{Saccharomyces} treatments. Dual species experiments that evaluated individual \textit{S. cerevisiae} strain responses confirmed the presence of strain-specific fitness variation in response to a non-\textit{Saccharomyces} species with consequences to fermentation timeframes. The information presented in this study will serve as an initial tool for the screen of yeast species interaction and selection of compatible strains that enhance fermentation performance.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7030165/s1, Table S1: Strain names of the barcoded library, Table S2: Log2 FC of barcoded strains at timepoint 1, Table S3: Log2 FC of barcoded strains at timepoint 2, Table S4: Log2 FC of barcoded strains at timepoint 3, Table S5: Metadata of raw barcode count files, File S1: Raw barcode count files.

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