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Identification of Molecular Markers for Early Detection of Sluggish Fermentation Associated with Heat Shock during Alcoholic Fermentation

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Abstract: Problematic fermentations frequently drive economic losses and logistic problems in the winemaking industry. Previous studies have determined thermal conditions leading to problematic fermentations, selecting two contrasting yeast strains for further transcriptomic analysis. *Saccharomyces cerevisiae* SBB11 showed strong thermosensitivity towards heat shock, while *S. cerevisiae* PDM was found to be thermotolerant. The aim of this study was to select genes with significantly upregulated expression to be later used as biomarkers for early detection of sluggish fermentation associated with heat shock. Candidate genes were selected from previously obtained RNA-seq data. Alcoholic fermentations were conducted with 4 *S. cerevisiae* strains SBB11, PDM, M2 and ICV D21. Heat shocks on day 3 of alcoholic fermentation were applied at 36 and 40 °C for 16 h. *S. cerevisiae* cells were collected at different times after heat shock onset for qPCR analysis of candidate gene expression over time. Three genes showed promising results; SSA1, MGA1 and OPI10 significantly increased expression with respect to the control. The selected genes showed increased expression during the first 9 h post heat shock and are proposed for early detection of sluggish fermentations associated with heat shock.

Keywords: *Saccharomyces cerevisiae*; wine; sluggish fermentations; heat shock; qPCR

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1. Introduction

Alcoholic fermentation (AF) is an ancestral biotechnological process mostly carried out by *Saccharomyces* yeasts, mainly *S. cerevisiae*. The core reaction involved in winemaking is the transformation of grape must sugars, glucose and fructose into ethanol and CO_2 [1–3]. However, yeasts must frequently cope with harsh must conditions as AF proceeds, such as high osmolarity, low nutrient contents or rapid nutrient depletion, increasing ethanol content and/or abrupt changes in fermentation temperature. This hostile environment threatens AF completion, potentially leading to sluggish or stuck fermentation [4].

Oenological technology has developed novel tools for AF monitoring and control, avoiding problematic fermentations. However, sluggish or stuck fermentations are still a major issue in the industry, causing logistic and operating difficulties such as inefficient tank occupation. Furthermore, stuck fermentations affect wine quality due to undesired

alterations during the process or after forcing fermentation completion [5]. Hence, detecting and identifying the factors leading to problematic fermentations remains critical. Among the main causes of sluggish or stuck fermentations, the literature cites nitrogen deficiency, the presence of inhibitors or toxic compounds such as grape pesticides and even sudden changes in fermentation temperature [4,6].

In a previous study, our group identified the thermal conditions leading to sluggish fermentations. Briefly, on the third day of AF, heat shocks were carried out for 16 h. Temperatures over 36 °C applied in the early stages of the process led to sluggish fermentations with evidenced delays directly related to increasing temperature. Three *S. cerevisiae* strains (SBB11, PDM and T73) were subjected to heat shock during AF. The heat shock effect on fermentation kinetics differed according to the yeast strain [7]. Post heat shock outcomes depended on yeast thermotolerance and the applied temperature. This study identified two strains with contrasting heat shock behavior. While SBB11 was found to be quite sensitive, PDM was highly thermotolerant [7].

Determining the specific cause leading to sluggish fermentation or needing to restart the process is quite complicated. Indeed, it is often accepted that stuck fermentations are easier to prevent than to treat. Therefore, closely monitoring fermentation temperature is key to preventing altered yeast sugar consumption. In this sense, biomarkers can potentially help avoid or enable early detection of alterations in fermentation performance. In this regard, other studies have proposed the use of biomarkers for desiccation tolerance prediction during selection of industrial wine yeast suitable for producing active dried yeasts (ADYs) [8]. Another study showed the expression levels of yeast nitrogen metabolism genes such as GAP1 and DAL4 and proposed them as biomarkers of nitrogen deficiency during wine fermentation [9]. In this sense, biomarkers for the wine industry could constitute useful tools to detect problematic fermentations.

The aim of this work was to identify early molecular markers able to anticipate problematic fermentations associated with a sudden increase in grape juice temperature. *S. cerevisiae* SBB11 and PDM transcriptomic data previously obtained by RNA-seq [10] allowed for selection of candidate genes with significantly enhanced expression 40 min after heat shock. We evaluated four *S. cerevisiae* strains with different AF performances after heat shock treatment, finding expression of three candidate genes in correlation with fermentation outcome after heat shock. Quantitative real-time PCR (qRT-PCR) was used to estimate the heat shock impact on yeast fermentation performance. Genes SSA1, MGA1 and OPI10 were identified as promising candidates for monitoring of the heat shock effect on yeast fermentation performance.

2. Materials and Methods

2.1. Transcriptomic Analysis and Candidate Gene Selection

Fermentations were conducted with the thermosensitive strain *S. cerevisiae* SBB11 and the thermotolerant strain PDM [7]. Heat shocks were applied at 36 and 40 °C on day 3 of AF. Samples for transcriptomic analysis were collected 40 min after the beginning of heat shock [10] (Figure 1A). After transcriptomic analysis, potential biomarker genes were selected for qRT-PCR evaluation. Briefly, biomarkers exclusively consisted of statistically upregulated genes in sluggish fermentations after heat shock.

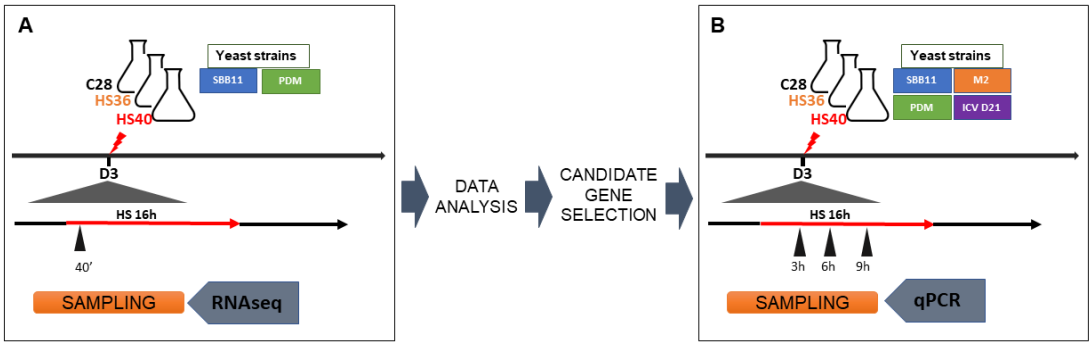


Figure 1. Experimental design. Two *S. cerevisiae* strains previously selected [7] with contrasting behavior upon heat shock were evaluated by transcriptomic response after heat shock [10] (**A**). Candidate gene selection took place after RNA-seq data analysis from samples collected 40 min after heat shock. Candidate gene expression was validated by fermentations with four *S. cerevisiae* strains: SBB11, PDM, M2 and ICV D21. Heat shocks were applied at 36 and 40 °C on day 3 of AF for 16 h. Samples were collected 3, 6 and 9 h after heat shock onset and subjected to qRT-PCR (**B**). Trials considered independent triplicates for each yeast strain.

2.2. Yeast Strains and Inoculum Preparation

In this study, we tested four *S. cerevisiae* strains: SBB11, PDM, Enoferm M2 and Lalvin ICV D21. Strain SBB11 was isolated and selected from spontaneous fermentation of Syrah grapes from Mendoza, Argentina, whereas PDM (Maurivin Co., Mauri, Australia), Enoferm M2 (Lallemand Co., Montreal, QC, Canada) and Lalvin ICV D21 (Lallemand Co.) are commercial strains. Yeast cells were activated, plated and grown on yeast peptone dextrose (YPD) medium. Single colonies were spread into five YPD plates and incubated for 48 h at 28 °C until confluent growth. Yeasts were collected in 100 mL YPD broth and incubated with agitation at 150 rpm for 6 h at 28 °C. For dilution determination and later inoculation in synthetic grape must with an initial cell concentration of 2×10^6 cells/mL, cells were counted in a Neubauer chamber. Synthetic grape must (SM) was formulated with 120 g/L glucose and 120 g/L fructose [11]. Nitrogen content was 140 mg N/L (42 mg N/L as ammonium and 98 mg N/L in amino acid form), and the pH was adjusted to 3.3 with NaOH. Finally, SM was sterilized through 0.2 µm membrane filtration.

2.3. Microvinifications

Fermentations were performed in 500 mL Erlenmeyer flasks containing 300 mL of SM at 28 °C ± 2 °C with a daily manual shaking to simulate pumping over normally performed in industrial winemaking. Flasks were capped with closures enabling carbon dioxide diffusion. Weight loss and must density were monitored daily. Must density was measured using a densitometer (Densito 30 PX, Mettler Toledo Co., Columbus, OH, USA). Fermentation finished when residual sugar concentration dropped below 4 g/L [4]. Wine physicochemical parameters were measured using an FT-IR Alpha Wine Analyzer (Bruker Co., Billerica, MA, USA).

Heat shock impact during AF was evaluated for the 4 yeast strains under 3 treatments in triplicate: heat shock at 36 °C (HS36), heat shock at 40 °C (HS40) and control (C28) at 28 °C. Heat shocks were applied for 16 h on day 3 of AF (must density of 1060 ± 5 mg/L) by placing the flasks in incubators at 36 °C ± 2 °C and 40 °C ± 2 °C. Three hours before heat shock onset, each flask received 200 mg/L of diammonium phosphate (DAP) for nutrition purposes. Figure 1 schematizes the experimental design.

2.4. RNA Extraction and cDNA Synthesis

Samples containing 1×10^7 cells were collected 3, 6 and 9 h after heat shock onset. Samples were centrifuged, and pellets were immediately frozen in liquid nitrogen and stored at −80 °C until use. Samples were subjected to cell wall lysis using sterile glass beads in a Mini-Beadbeater (GlenMills Inc., Clifton, NJ, USA). Briefly, pellets were suspended in

Tri Reagent® from the Molecular Research Center (Genebiotech Co., Gongju-si, Republic of Korea) and subjected to 4 cycles of 30 s in a bead beater. RNA extraction was performed with Tri Reagent® according to the manufacturer's instructions. Finally, RNA pellets were carefully resuspended in 40 µL of sterile ultrapure water. RNA concentration was determined using a Denovix DS-11 FX spectrophotometer, and RNA integrity was verified by electrophoresis in 1% agarose gels. Contaminating DNA was digested by DNase treatment for 1 µg of RNA using RQ1 RNase-free DNase (Promega Co., Madison, WI, USA). cDNA was synthesized from total RNA using reverse transcriptase (RevertAid, Thermofisher Scientific Co., Waltham, MA, USA) and 0.5 µg oligo (dT) in a reaction with a final volume of 20 µL. Following the manufacturer's protocol, cDNA was synthesized at 42 °C for 60 min. Finally, the reaction was stopped at 70 °C for 10 min.

2.5. Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR)

All primers used in this study were designed using the primer designing tool of the National Center for Biotechnology Information (NCBI, Table 1). The qRT-PCR reaction was performed using SsoAdvanced™ universal SYBR® (Biorad, Hercules, CA, USA). The final volume for the PCR reaction was 10 µL. All PCR reactions were mixed in 96-well optical plates and covered using optical films (Applied Biosystems, Life Technologies Co., Carlsbad, CA, USA). Quantitative real-time PCR assays were carried out in a Step One Plus real-time PCR system (Applied Biosystems Co., San Francisco, CA, USA) using the following amplification conditions: initial enzymatic activation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and elongation). Melting curve analysis verified amplification specificity. Reactions were run in triplicate. Each primer pair included negative controls (without template). Ubiquitin-conjugating enzyme encoding gene UBC6 served as an internal control to normalize expression variability. Gene expression is reported in relation to the internal control gene (UBC6), and data are reported as the ΔCq-target gene/ΔCq-UBC6 ratio. Relative quantification of gene expression was performed according to Pfaffl et al. [12], as modified by Ruijter et al. [13], and considering amplification reaction efficiencies calculated from raw data with LingReg PCR software [14].

Table 1. Primer sequences for qRT-PCR gene amplification.

Gene	Primer Name	Oligonucleotide Sequence (5′-3′end) *
UBC6	UBC6-F	TACTTGGAATCCTGGCTGGT
	UBC6-R	GATCCTGTCGTGGCTTCATC
SSA1	SSA1-F	GAAGTCCGAGATCTTTTCCACTT
	SSA1-R	CCTCTTGGAGCTGGTGGAAT
MGA1	MGA1-F	ATCTCATCCTTCCCCAGACC
	MGA1-R	ATTCAAGATACCGGCGTTGG
OPI10	OPI10-F	CCGCTGATCCGTTTACTGAC
	OPI10-R	TTCCTTGTTCTCGAGGCTCA
YNL194C	YNL194C-F	GATACTAGCAGGTGGCAGGA
	YNL194C-R	TTAAAGCCCGAAGTGGATGC
TIP1	TIP1-F	ATCGCTGCTGCTCTTGCCT
	TIP1-R	AGCGGCAGAGGATGTAGCTT
YBR116C	YBR116C-F	GTGTTGCGTCAAGGGCTGAA
	YBR116C-R	GGCAGCAAGTGACCATCAACC
ECL1	ECL1-F	TGCTCCGAAGATTGTAAGCTG
	ECL1-R	CGGTGGAGTGAGATTATGCG
SSA3	SSA3-F	AGGTAGGCTCTCGAAGGATG
	SSA3-R	GTTCTGCCTCCCTTTCATCG
SPG4	SPG4-F	GGAGACAGTAAAACGCAGGT
	SPG4-R	ACATCGGAACTGTCCTGTGA
HSP12	HSP12-F	CAAGGTCGCTGGTAAGGTTC
	HSP12-R	ACCTTCAGCGTTATCCTTGC

* Primer sequences were obtained using the primer designing tool from NCBI.

2.6. Statistical Analyses

The area under the curve fitted for density vs. time (AUC) allowed for analysis of the overall fermentation performance. This parameter was calculated using the Riemann sum area under the curve (AUC) from inoculation time until day 11. Results are expressed as arbitrary units. Day 11 was chosen for treatment comparison according to the shortest fermentation time. All analyses were carried out with InfoStat version 2020p statistical software (FCA, Universidad Nacional de Córdoba, Argentina). AUC and qRT-PCR data were statistically analyzed using two-way ANOVA, and comparisons were performed with an LSD Fisher test ($p \leq 0.05$).

3. Results

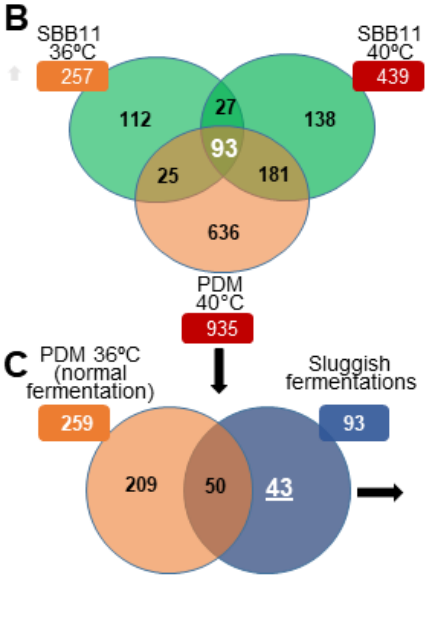
In a previous study, our group found that after a heat shock during the third day of AF, two *S. cerevisiae* strains showed contrasting behavior after sudden temperature increases. *S. cerevisiae* strain SBB11 was found to be the most sensitive to heat shock, with sluggish fermentation at 36 and 40 °C. On the other hand, *S. cerevisiae* PDM was the most thermotolerant strain, with sluggish fermentation only observed after heat shock at 40 °C, with an insensitive response to 36 °C heat shock [7]. Samples from both strains obtained 40 min after heat shock onset were subjected to RNA-seq analysis. Transcriptomic data from SBB11 and PDM [10] allowed for candidate gene selection as early biomarkers of sluggish fermentations after a heat shock.

Regarding the number of genes significantly upregulated according to RNA-seq data, at 36 °C, SBB11 upregulated 257 genes. Similarly, and under the same conditions, the thermotolerant strain PDM upregulated 259 genes (Figure 2A). However, under heat shock at 40 °C, SBB11 upregulated 439 genes, whereas PDM upregulated 935 genes (Figure 2A). Potential biomarker selection only considered genes upregulated under all sluggish fermentation conditions (SBB11 at 36 and 40 °C and PDM at 40 °C). Ninety-three genes were upregulated in sluggish fermentations (Figure 2B). Upregulated genes under heat shock without alteration in AF kinetics (PDM at 36 °C) were excluded from these 93 initially selected genes, leaving 43 genes exclusively upregulated in sluggish fermentations (Figure 2C). Finally, the 10 genes with the highest log2 fold change with respect to control conditions were selected as potential biomarkers for sluggish fermentations associated with heat shock (Figure 2D).

Then, the AF performance and expression of selected genes after heat shock were assessed in four yeast strains following the experimental design previously depicted in Figure 1. Additionally, the parameters AUC and AF duration allowed for statistical comparison of heat shock impacts on the different strains (Table 2). AUC evidenced short-term impacts of heat shock on AF performance, whereas AF duration provides insight into the global impact of heat shock, including yeast resilience to overcome thermal stress. In general, AF performance showed great heterogeneity among the strains after heat shocks (Figure 3). In agreement with our previous observations [7], SBB11 was highly affected by heat shocks both at 36 and 40 °C and was identified as a highly sensitive strain (Figure 3A, Table 2). PDM was not affected by heat shocks at 36 °C, while sluggish fermentation was observed at 40 °C (Figure 3B, Table 2). Similarly to SBB11, M2 was highly affected by heat shock treatments. During the first days after heat shock, differences between 36 and 40 °C resulted in a significantly higher AUC at 40 °C than at 36 °C. However, both heat shock treatments produced a sluggish 30-day fermentation (Figure 3C, Table 2). On the other hand, for ICV D21, 36 and 40 °C AF profiles after heat shock showed statistical differences between them and relative to the control. Interestingly, in this strain, AF profiles of heat shock treatments rapidly reached control values by day 13 (Figure 3D, Table 2).

A

Yeast strain	Treatment	N° of genes	Regulation	N° of genes
SBB11	36vs28	564	UP	257
			DOWN	307
	40vs28	793	UP	439
			DOWN	354
PDM	36vs28	550	UP	259
			DOWN	291
	40vs28	1911	UP	935
			DOWN	976



D

GENES	Log2Fold Change (RNAseq) SBB11 36vs28	SBB11 40vs28	PDM 40vs28
OPI10	1.5	3.3	3.4
MGA1	1.7	2.8	2.3
YNL194C	1.0	2.5	2.6
TIP1	1.0	2.5	1.0
YBR116C	2.0	2.3	3.2
SSA1	1.0	2.1	2.8
ECL1	1.2	2.0	3.4
SSA3	1.5	1.8	3.5
SPG4	1.4	1.2	2.8
HSP12	1.0	1.1	3.0

Figure 2. Candidate gene selection. Table A describes the number of differentially expressed genes in the two *S. cerevisiae* strains (SBB11 and PDM) subjected to heat shock at 36 and 40 °C, showing the number of genes up- and downregulated (**A**). Venn diagrams show overlapping differentially expressed genes in the three treatments with sluggish fermentations (**B**). Venn diagrams show 93 previously selected genes overlapping with upregulated genes in fermentations subjected to heat shock without sluggish fermentation (PDM at 36 °C) (**C**). List of the ten selected genes (**D**).

Table 2. Summary of parameters employed to statistically compare the impact of heat shock treatments on the AUC (arbitrary units) and AF duration (days) in the different strains assessed.

Yeast/Treatment	AUC ± SD (Arbitrary Units)		
	C28	**HS36**	**HS40**
SBB11	11,310.38 ± 3.34 (a)	11,403.32 ± 15.54 (b)	11,472.93 ± 1.7 (c)
PDM	11,334.02 ± 3.71 (a)	11,355.47 ± 33.3 (a)	11,463.07 ± 30.47 (b)
M2	11,167.47 ± 25.76 (a)	11,289.18 ± 28.9 (b)	11,358.97 ± 5.85 (c)
ICV D21	11,183.18 ± 25.36 (a)	11,257.82 ± 18.56 (b)	11,297.42 ± 5.21 (c)
	AF duration ± SD (days)		
SBB11	12.33 ± 0.57 (a)	22 ± 1 (b)	39.66 ± 1.52 (c)
PDM	13.33 ± 0.57 (a)	14.33 ± 0.57 (a)	27 ± 1.73 (b)
M2	9.67 ± 1.15 (a)	30.67 ± 0.58 (b)	30.33 ± 0.58 (b)
ICV D21	9.67 ± 1.15 (a)	13.33 ± 0.58 (b)	13.67 ± 1.15 (b)

Mean and standard deviation values of three replicates. Different letters indicate statistical differences between the treatments (LSD Fisher test $p < 0.05$). Statistical analyses were performed for each strain.

Heat maps representing normalized data (relative to control values) of AUC and AF duration allowed for comparison of differences among strains. Figure 3E,F show heat maps evidencing AF heterogeneity after heat shock for the different yeast strains. SBB11 and PDM revealed a stronger impact at 40 °C than at 36 °C in both AUC and AF duration (Figure 3E,F). Regarding M2 and ICV D21, AUC heatmap values show a weaker impact of 36 with respect to 40 °C. However, the early effect observed on AUC derived different outcomes, since M2 ended up in a sluggish fermentation, whereas ICV D21 heat shock treatments showed nearly no difference relative to the control (Figure 3E,F).

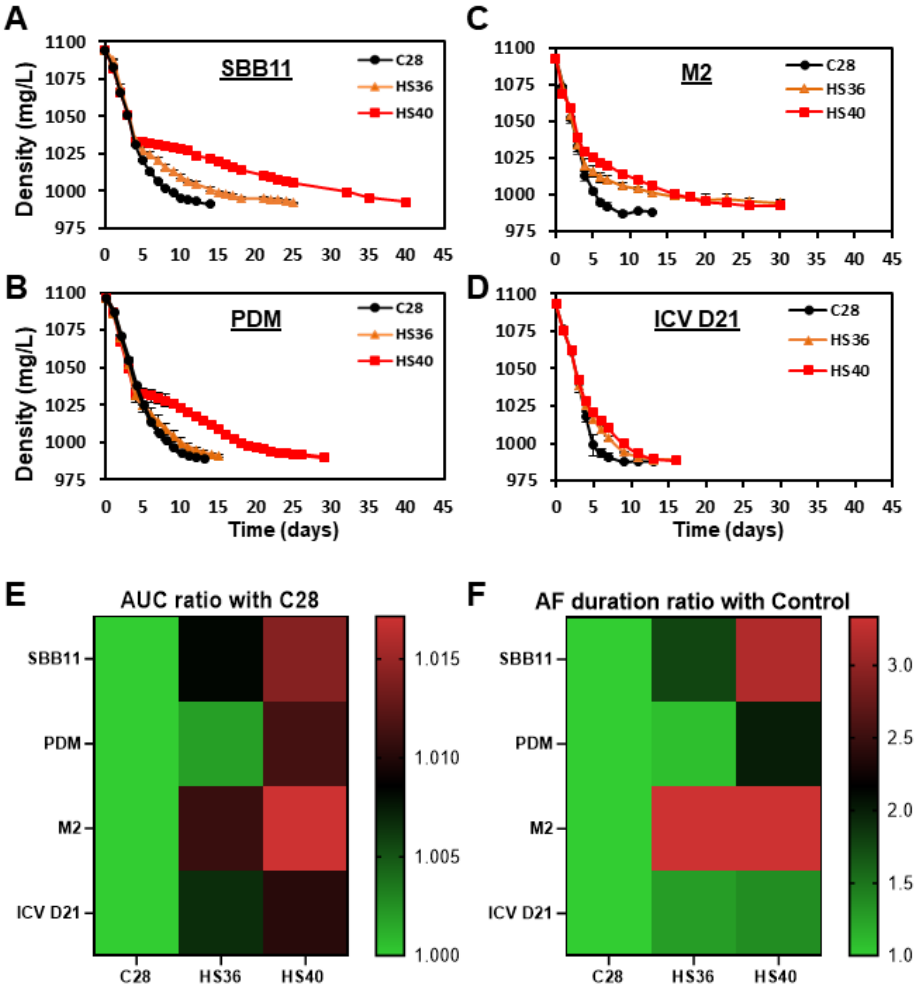


Figure 3. Evaluation of the effect of heat shock at 36 and 40 °C on AF performance for the *S. cerevisiae* strains SBB11 (**A**), PDM (**B**), M2 (**C**) and ICV D21 (**D**). Heat maps represent normalized data (relative to control values, C28) of AUC (**E**) and AF duration values (**F**).

Next, we evaluated candidate gene expression over time for later consideration of putative biomarkers of sluggish fermentations associated with heat shock. Target biomarker genes should increase and show long-lasting expression in sluggish fermentations under winemaking conditions. SBB11 showed inconsistent results in the quantification of gene expression for 6 (TIP1, HSP12, YBR116C, SPG4, YNL194 and ECL1) of 10 candidate genes. Either expression was similar in the control and heat shock conditions or expression differences were transient, disabling marker functionality (data not shown).

Three genes (SSA1, MGA1 and OPI10) showed promising results. SSA1 showed statistical differences relative to the control in SBB11 and ICV D21 samples at almost all time-points at 36 and 40 °C (Figure 4). Since SBB11 was found to be the most heat shock-sensitive strain, expectations were that SSA1 expression was proportional to heat shock temperature. However, expression was higher at 36 than at 40 °C, suggesting that heat

shock response is not proportional to temperature. Expression of SSA1 in PDM showed statistical differences between the control and heat shock at 40 °C (Figure 4), the one condition of sluggish fermentation observed for this strain (Figure 3B). Regarding M2, SSA1 expression was higher than the control at all time points, although statistical differences were only observed 6 h after heat shock onset (Figure 4).

MGA1 in SBB11 performed as a good biomarker at 36 °C, whereas at 40 °C, expression differences were only observed 3 h after heat shock onset (Figure 4). In ICV D21, MGA1 was found to be a suitable biomarker, with statistically difference between heat shock and the control but evidencing a decreased expression over time. In the case of PDM, MGA1 expression increased at all time points at 40 °C, whereas, as expected, no differences were observed at 36 °C (Figure 4). Regarding M2, statistical differences were observed at 40 °C 6 h after heat shock, whereas at 36 °C, the observed higher expression was not statistically significant (Figure 4).

Finally, regarding OPI10, SBB11 and ICV D21 at 36 and 40 °C, showed statistically higher expression than the control. Non-significant increased expression was observed at 9 h and 40 °C in SBB11 samples or 6 h at 36 °C in ICV D21 (Figure 4). As expected for PDM, OPI10 expression was statistically higher than the control at all time points at 40 °C, whereas expression at 36 °C was nearly the same as the control (Figure 4). OPI10 expression for M2 at 40 °C was statistically higher than the control, whereas at 36 °C, expression was statistically different 6 h after heat shock onset (Figure 4).

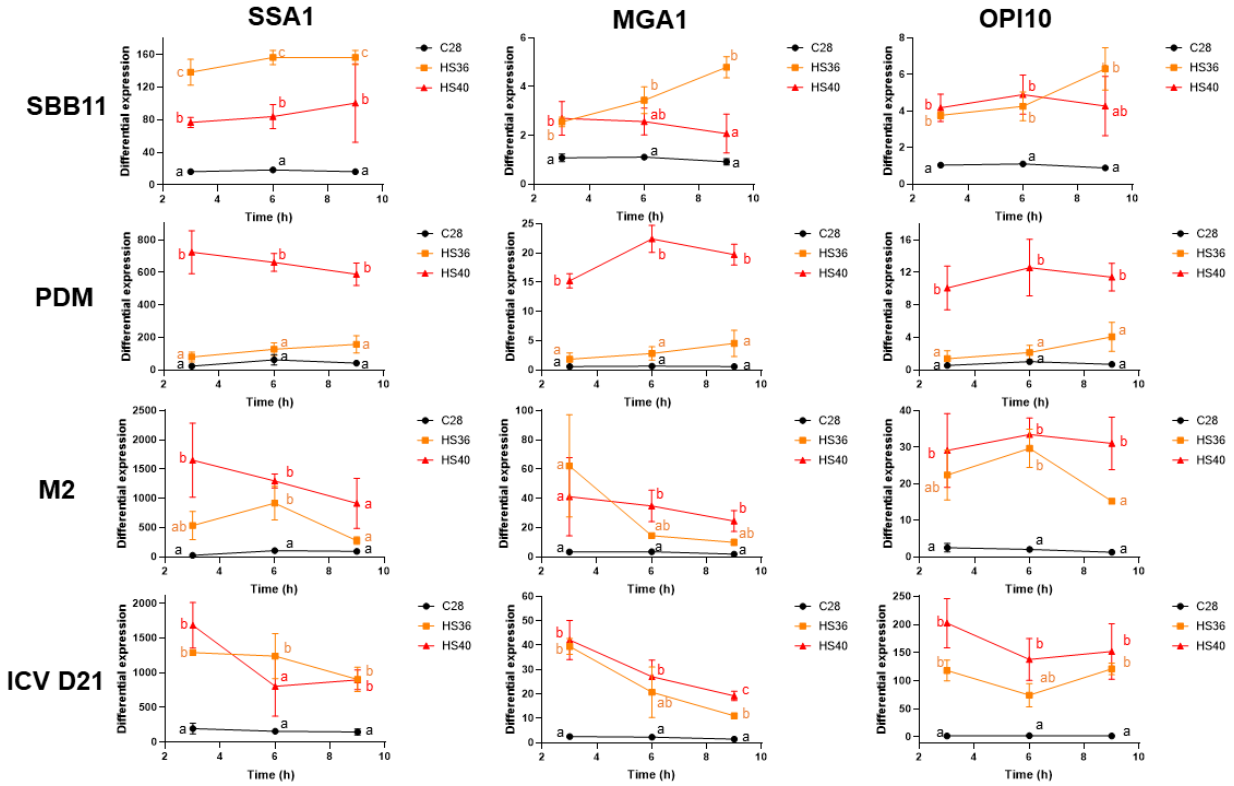


Figure 4. qRT-PCR quantification of differential gene expression with respect to housekeeping gene UBC6 for genes SSA1, MGA1 and OPI10. Gene expression for strains SBB11, PDM, M2 and ICV D21 is shown for the control conditions C28 and heat shocks at 36 and 40 °C. Graphs represent mean values and standard errors for the fold change in gene expression relative to housekeeping gene expression (UBC6), as calculated according to the ΔCq-target gene/ΔCq-UBC ratio [12]. Different letters indicate statistical differences among treatments (C28, HS36 and HS40) for each strain at every time point evaluated.

4. Discussion

The aim of this study was to find genes for early detection of sluggish fermentation associated with sudden temperature increase through qPCR. Based on previous transcriptomic data, a group of potential biomarker genes was selected and analyzed over time. Different expressions of these genes could ease early detection of sluggish fermentation, preventing AF delays. One frequent practice for sluggish fermentation reactivation is reinoculation with fresh yeast cultures. In this sense, biomarkers could allow for early corrective actions when conditions are less harsh than at the end of AF (low nutrient and high ethanol contents), increasing chances of successful AF reactivation [6]. The experimental design simulated winemaking conditions under which a temperature increase could remain several hours before being detected and corrected. Thus, biomarkers should maintain high differential expression for at least 9 h after heat shock onset.

The present study showed contrasting behaviors in AF performance of four different *S. cerevisiae* strains after heat shock. Few studies have evaluated sudden temperature increases during the first days of tumultuous AF as a triggering factor of sluggish fermentations. We identified the most thermosensitive strain (SBB11) and the most thermotolerant strain (PDM), along with strains showing similar early responses upon heat shock but different later outcomes (M2 and ICV D21).

S. cerevisiae strains are phenotypically diverse. Thermotolerance and ethanol tolerance have long been research targets [15–18]. During red wine fermentation, temperature can reach values as high as 40 °C, constituting a major concern in the winemaking industry [19]. As previously mentioned, control systems currently employed in wineries attempt to prevent sudden temperature increases during AF. However, fermentation in large tanks with high thermic inertia (where must temperature is not easily decreased after a heat shock) is associated with higher probabilities of heat impact on fermenting yeasts [7,20,21]. Consequently, a better understanding of *S. cerevisiae* phenotypic diversity in terms of thermotolerance becomes strategic [22].

Although the heat shock effect has been previously studied in *S. cerevisiae*, few studies have considered heat shock effects during AF [23,24]. Strassburg et al. [25] assessed sudden temperature increases and gradual adaptation to high temperatures, suggesting differences in transcriptomic and metabolic responses in both cases. Beyond the different responses to heat shock, the ultimate consequence of heat damage is cell death [25]. We previously evidenced that cell death was the main cause of AF delay after heat shock, suggesting that the inability of yeasts to overcome heat stress culminates in cell death [7]. Since restarting sluggish fermentations mainly involves reinoculation with fresh yeast cultures, information on cell viability is highly valuable. Moreover, biomarker expression, together with viability data, constitutes valuable information for early diagnosis and rescue of potentially sluggish fermentations.

Among 10 preliminarily selected genes, 3 fulfilled biomarker requirements. These genes (SSA1, MGA1 and OPI10) had increased expression in heat shock-affected AF. Regarding gene function, SSA1 plays a key role in misfolded protein degradation and belongs to the cytoplasmic Hsp70s of the SSA family [26–28]. SSA1 selectively binds to unfolded proteins and participates in the degradation of protein aggregates in the ubiquitin-proteosome system and the lysosomal pathways through chaperone-mediated autophagy [29,30]. Considering the accumulation of misfolded protein aggregates as one of the first heat shock consequences, chaperone proteins such as SSA1 are relevant in early heat shock response (ESR) [31]. Increased SSA1 expression has also been reported after high-pressure carbon dioxide (HPCD) treatment in *S. cerevisiae* [32]. On the other hand, the transcription factor HSF1 (heat shock factor 1) is the principal regulator of the heat shock response (HSR) in eukaryotes. In unstressed cells, Hsf1 is sequestrated by Ssa1 interaction. Upon heat shock, Hsf1 and Ssa1 dissociate within 5 min of heat stress, coincident with HSR induction. This interaction has been suggested as key for regulation of Hsf1 transcriptional activity during optimal non-stress conditions, supporting the relevant role of SSA1 in heat shock response regulation [28,33,34]. In our study, SSA1 showed contrasting expression in comparison to

the other two genes analyzed, reaching the highest values in our assays (Figure 4) and becoming a highly sensitive biomarker for early heat shock detection. In addition, changes in expression magnitude during heat shock depend on environmental severity [31]. However, SSA1 expression in SBB11 is higher at 36 than at 40 °C (Figure 4). In particular, SBB11 was the most heat shock-sensitive strain, not only considering AF performance (Figure 3) but also since our previous study showed 92% reduced cell viability at 40 °C [7]. Therefore, after a 40 °C heat shock, cells might lose viability as other cell-death mechanisms takes place.

MGA1 encodes a protein similar to heat shock transcription factor (HSF1) that is also involved in pseudohyphal growth (https://www.yeastgenome.org/locus/S000003481 (accessed on 12 December 2022). Additionally, Mga1p, Ste12p and Gat3p, are transcription factors that regulate filamentation and activate pseudohyphal/invasive growth genes downregulated by melatonin [35]. Another study suggested that MGA1 participates in the osmotic stress response [36]. Finally, a recent study reported the downregulation of MGA1 during freezing–thawing tolerance in *S. cerevisiae* [37]. In the present study, increased MGA1 expression was observed at most time points evaluated after heat shock. Similarly to SSA1 expression in SBB11, higher MGA1 expression occurred at 36 than at 40 °C.

Although few studies have addressed OPI10 function in *S. cerevisiae*, this gene has a possible role in phospholipid biosynthesis [38], and an increase in protein abundance has been reported in response to DNA replication stress [39]. OPI10 showed interesting results for all evaluated strains, with increased expression correlating with AF affected by heat shocks.

Despite the described role these three genes play in heat stress response and the co-expression we demonstrated in the present work, no evidence of mutual physical interaction in *S. cerevisiae* has been reported until now. However, the STRING database (a predictive tool that relates proteins and checks putative interactions), in combination with species homology, predicted the participation of all three genes in a heat shock response [40]. As a result, STRING output shows pathway SCE-3371453 from the Reactome database (https://reactome.org/content/detail/R-SCE-3371453 (accessed on 10 March 2023), describing the regulation of HSF1-mediated heat shock response [41]. Based on the homology between HSF1 and MGA1 and considering that SSA1 belongs to the HSP70 family, an interaction between MGA1 and SSA1 could be inferred. Although this constitutes weak evidence of a putative real interaction, it may lead to further experimental work on physical or genetic regulation depending on heat shock conditions. Nonetheless, as mentioned, SSA1, MGA1 and OPI10 are also involved in the general stress response; therefore, increasing expressions may be also observed in response to other stressors during AF, such as those naturally occurring under control conditions also considered in this study (AF in the absence of heat shock). Therefore, defining basal gene expression under control conditions becomes essential. Studies addressing heat shock response mechanisms in *S. cerevisiae* strains with different thermotolerance are currently in progress. Such studies will certainly shed light to better understand gene expression after heat shock and further identify new biomarker genes.

The differential expression of each gene assessed in this study showed wide ranges for the four strains. This could be attributed to differences in gene roles and promotors, as well as distinctive strain characteristics. In this study, control conditions allowed for determination of statistical differences between treatments and their respective controls. However, in a winery, no control fermentations are available; hence, defining a gene expression threshold for alert triggering is challenging. Defining a "safe zone" and a "danger zone" according to gene expression could be possible, as shown in Figure 5. Thresholds between zones are different for each strain; therefore, wineries could establish basal biomarker values for frequently employed strains.

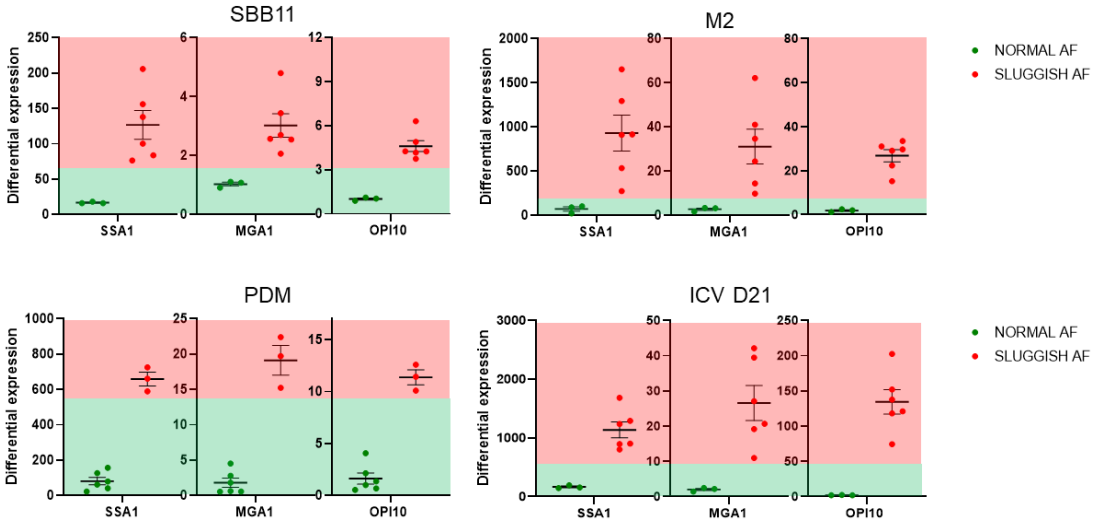


Figure 5. Safe (light green) and danger (pink) zones defined by differential expression of biomarker genes (SSA1, MGA1 and OPI10) for the four strains (SBB11, PDM, M2 and ICV D21). Red dots represent gene expression in sluggish fermentations until 9 h after heat shock onset. Green dots represent gene expression in normal fermentations until 9 h after heat shock onset and control treatments.

This study is a first approach towards defining early detection tools of sluggish fermentations associated with heat shock. Overall, the results show that under most conditions, biomarker measurements allow for prediction of sluggish fermentations. Hence, combined use of the three biomarkers proposed herein is highly recommended. Although molecular methods such as qPCR are not widely used in practical applications, such techniques could contribute to preventing sluggish fermentations. In this regard, molecular methods are becoming increasingly accessible and attractive to the industry. Indeed, qPCR commercial kits are currently available for *Dekkera bruxellensis* detection in wines [42]. Moreover, considering yeast diversity regarding heat shock response, further studies addressing the evaluation of these biomarkers in a larger number of *S. cerevisiae* strains would certainly provide a more precise understanding of their potential in oenological practice. Additionally, combination with other determinations such as yeast viability by flow cytometry could enable a more comprehensive prediction.

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