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Transcriptomic Response of *Saccharomyces cerevisiae* during Fermentation under Oleic Acid and Ergosterol Depletion

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Abstract: Under anaerobic/hypoxic conditions, *Saccharomyces cerevisiae* relies on external lipid supplements to modulate membrane lipid fraction in response to different stresses. Here, transcriptomic responses of two *S. cerevisiae* wine strains were evaluated during hypoxic fermentation of a synthetic must with/without ergosterol and oleic acid supplementation. In the absence of lipids, the two strains, namely EC1118 and M25, showed different behaviour, with M25 significantly decreasing its fermentation rate from the 72 h after inoculum. At this time point, the whole genome transcriptomic analysis revealed common and strain-specific responses to the lack of lipid supplementation. Common responses included the upregulation of the genes involved in ergosterol biosynthesis, as well as the seripauperin and the heat shock protein multigene families. In addition, the upregulation of the aerobic isoforms of genes involved in mitochondrial electron transport is compatible with the previously observed accumulation of reactive oxygen species in the two strains during growth in absence of lipids. Considering the strain-specific responses, M25 downregulated the transcription of genes involved in glucose transport, methionine biosynthesis and of those encoding mannoproteins required for adaptation to low temperatures and hypoxia. The identification of these pathways, which are presumably involved in yeast resistance to stresses, will assist industrial strain selection.

Keywords: wine fermentation; ergosterol; oxidative stress; *TIR/TIP*; methionine biosynthesis

1. Introduction

During white wine fermentation, *S. cerevisiae* is subjected to (i) high osmotic pressure, (ii) limited nitrogen concentrations, (iii) prolonged hypoxic/anaerobic conditions, (iv) the presence of chemical preservatives (sulphites), (v) increasing ethanol concentrations, and (vi) low temperatures. Thus, white winemaking represents an interesting process to understand yeast response and adaptation to many different stresses occurring during industrial processes. Different mechanisms permitting yeast to grow in hostile and stressful environments have been documented so far. Upon osmotic stress, the High Osmolarity Glycerol (HOG) pathway governs the production of glycerol as a compatible solute [1]. The Target of Rapamycin (TOR) pathway allows yeast cells to adapt to nitrogen limitation conditions by triggering morphological transitions such as pseudohyphal growth and biofilm formation [2,3]. Reactive oxygen species (ROS) generated during wine fermentation trigger the production of proteins needed to protect the cells against oxidative stress [4,5]. Temperature changes activate the production of heat shock proteins (HSPs) through the Heat Shock Response pathway [6,7]. HSPs, involved in folding, stabilization or degradation of protein aggregates, are also controlled by the General Stress Response pathway [5]. This pathway overlaps with the other stress responses.

Modification of the membrane lipid composition is another cellular response elicited by different stress factors which confers so called "cross-protection", i.e., the "increased resistance to multiple stresses conferred by sublethal doses of one stress condition" [8]. Henderson and Block [9] found that bioethanol production and maximum yeast cell density were highly correlated with yeast cell lipid composition Indeed, changes in the relative abundance of saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), and ergosterol allow yeast cells to counteract variations in membrane fluidity and permeability, that can severely impair the activities of transmembrane proteins [10]. *S. cerevisiae* derives cell lipids via the assimilation of external nutritional supplies and/or the activation of lipid biosynthesis. During winemaking, the biosynthesis of unsaturated fatty acids and ergosterol is impaired, as oxygen is required to desaturated SFA and to complete the oxygen-dependent steps of sterol biosynthesis. In these conditions, wine yeasts can assimilate exogenous sterols and UFAs [11,12]. However, it has been observed that some oenological practices, such as excessive must clarification and a short time of contact between grape solids and juice, may significantly reduce the levels of UFA and sterols in must [13]. Thus, in absence of appropriate lipid supplementation, yeast adaptation to the stressful conditions encountered during winemaking, such as high osmotic pressure, prolonged hypoxic/anaerobic conditions, high ethanol concentrations and low temperatures, strictly relies on yeast ability to make optimal use of the dissolved oxygen in the first hours of fermentation [14].

Our previous researches have shown that during fermentation in absence of lipid supplementation, wine strains of *S. cerevisiae* showed different fermentative abilities that were related to their ability to scavenge ROS [4] and activate the transcription of key genes in lipid metabolism [15]. In this context, the aim of this work was to further elucidate the mechanisms involved in *S. cerevisiae* adaptation to fermentation in hypoxic conditions in absence of lipid supplementation. To this end, the genome-wide response of two *S. cerevisiae* strains with different susceptibility to stuck/sluggish fermentation was determined by transcriptomic analysis in two media characterized by the presence and absence of lipid supplementation. This comparative approach allowed the identification of candidate yeast genes commonly regulated in the two strains and specifically associated with resistance to the lack of lipid supplementation.

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions

S. cerevisiae strains used in this study were the industrial wine strain EC1118 (Lalvin, Canada) and the wild-type wine strain M25 belonging to the culture collection of the Department of Agricultural science, University of Sassari (Sassari, Italy). EC1118 was rehydrated according to the manufacturer's instructions. Yeast strains were precultured at 25 °C for 24 h in a rotary shaker (300 rpm) in flasks containing synthetic juice (SJ) prepared as described in [16] and containing glucose 200 g/L, tartaric acid 2 g/L, malic acid 2 g/L, citric acid 0.2 g/L, YNB w/o amino acids and w/o ammonium sulphate 1.7 g/L, ammonium sulphate 2 g/L. pH was adjusted to 3.3 with KOH. Cells from precultures were inoculated to the final concentration of 1×10^6 cell/mL in 500 mL flasks equipped with glass capillary stoppers and containing 350 mL of liquid media. The media used for bench-scale fermentations were SJ and ergosterol/oleic acid supplemented SJ (FSJ: SJ with 15 mg/L ergosterol and 20 mg/L oleic acid). Fermentations were carried out in triplicate in static at 20 °C for 30 days and monitored by measuring weight loss, optical density (A600) and glucose concentration (enzymatic kit Roche Molecular Biochemicals, Laval, QC, Canada).

2.2. RNA Extraction and Microarray Analysis

Yeast cells growing in SJ and FSJ were collected after 72 h from the inoculum, rapidly harvested, washed and stored at −80 °C until RNA extraction. Total RNA was extracted using the hot-phenol method [17]. From total RNA, mRNA was isolated according to [18]. Poly(A)+ RNA purification, amplification, labelling, and cRNA fragmentation were carried out as described previously [19].

Twelve oligonucleotide yeast genome arrays (YGS98, Affymetrix, Santa Clara, CA, USA) were used as targets for hybridization. Hybridization, washing, staining and scanning of yeast arrays were done as described by the manufacturer (Eukaryotic Arrays GeneChip Expression Analysis and Technical Manual, Affymetrix, Santa Clara, CA, USA). Washing and staining were carried out using the EukGE-WS2v3 fluidics protocol of the Affymetrix MASv5.0 software (Affymetrix, Santa Clara, CA, USA). Scanning of the arrays was done on an Agilent G2500A GeneArray Scanner (Agilent Technologies, Palo Alto, CA, USA). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [20] and are accessible through GEO Series accession number GSE130016 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130016) (access on 18 April 2019).

2.3. Data Analysis

Preliminary analysis of gene expression data was carried out using Affymetrix Microarray Suite v5.0 (MASv5.0). To determine absolute call a statistical algorithm based on the Wilcoxon signed-rank test was used. The detection *p*-value was set at ≤ 0.05 for genes with a present call. For each condition, only the genes with the same change call in the three replicated experiments were included. The effect of the medium (first independent variable with two levels) and of the yeast strain used (second independent variable with two levels) on the transcription level of each gene (dependent variable) was evaluated by fitting a linear model. A gene was considered to show significant differential expression if the *p*-value (adjusted for false discovery rate (FDR) using the Benjamini and Hochberg method [21]) was < 0.05. Significant genes were functionally annotated using the high-throughput enrichment tool DAVID v6.8 [22]. Transcription factor analysis was carried out using YEASTRACT [23].

3. Results

3.1. Fermentation Profiles in Absence of Lipid Supplementation.

The ability of yeast strains to adapt to the lack of lipid supplementation was tested by growing two wine strains of *S. cerevisiae* in synthetic juice lacking lipids (SJ). As a control condition, the same strains were inoculated in SJ enriched with oleic acid and ergosterol (FSJ). While EC1118 was able to complete the fermentation in the two media (Figure 1), M25 completed the fermentation in FSJ but underwent a stuck/sluggish fermentation in SJ (Figure 2). In particular, the fermentative behavior of M25 was significantly lower ($p < 0.05$) in SJ than in FSJ starting from 72 h from the inoculum.

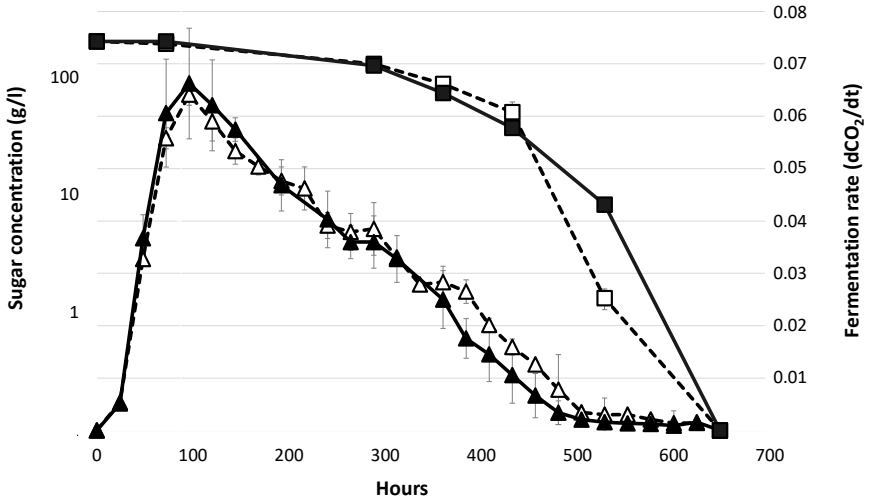

Figure 1. Fermentation kinetics and sugar consumption of EC1118 during growth in FSJ (straight lines) and SJ (dotted lines). Black triangles: fermentation rate in FSJ; white triangles: fermentation rate in SJ; black squares: residual glucose in FSJ; white squares: residual glucose in SJ. Data are means ± standard deviation of three independent biological replicates.

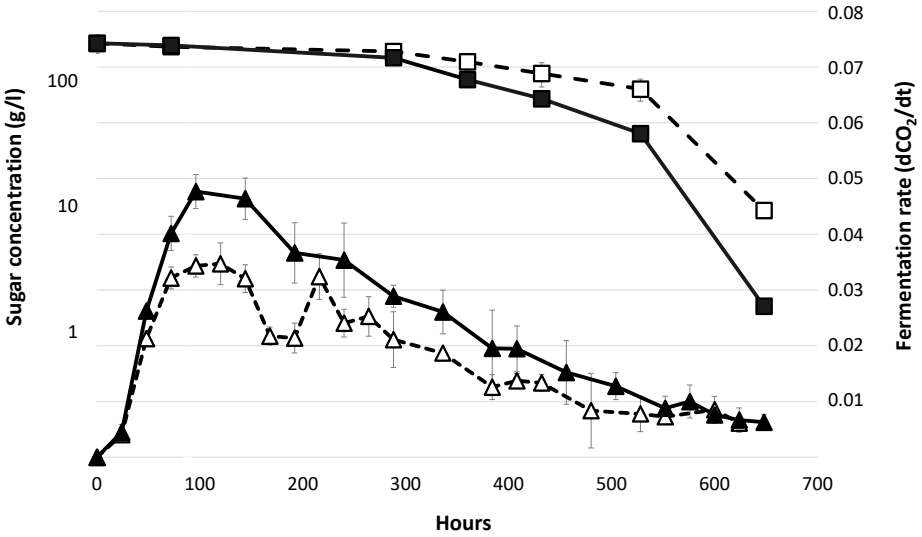

Figure 2. Fermentation kinetics and sugar consumption of M25 during growth in FSJ (straight lines) and SJ (dotted lines). Black triangles: fermentation rate in FSJ; white triangles: fermentation rate in SJ; black squares: residual glucose in FSJ; white squares: residual glucose in SJ. Data are means ± standard deviation of three independent biological replicates.

The reduced fermentative performances were associated with significant lower growth rates and maximum population sizes in M25 growing in SJ than in all the other strain/medium combinations (Table 1).

Table 1. Parameters for the logistic equation fitted on cell viability data of M25 and EC1118 strains growing in absence (SJ) and in presence (FSJ) of oleic acid and ergosterol supplementation.

Sample	Medium	Maximum Population Size (10^7 cell/mL)	Growth Rate (h^{-1})	Area under Curve
M25	SJ	1.230 ^{a} ± 0.16	0.094 ^{a} ± 0.05	236.09 ^{a} ± 12.01
	FSJ	1.406 ^{c} ± 0.22	0.156 ^{b} ± 0.11	275.02 ^{b} ± 20.12
EC1118	SJ	1.366 ^{b} ± 0.25	0.351 ^{c} ± 0.29	267.91 ^{b} ± 19.84
	FSJ	1.383 ^{b} ± 0.18	0.298 ^{c} ± 0.25	270.73 ^{b} ± 15.71

$^{a, b, c}$ Superscript letters in the same column indicate significant groups as determined by ANOVA followed by Tukey-HSD test ($p < 0.05$).

3.2. Transcriptomic Response during Fermentation in Absence of Lipid Supplementation

By fitting a linear model to the transcriptional data, it was possible to identify the genes whose expression changed in the two strains as a response to the absence of lipid supplementation. Of the 4176 ORF analyzed, 544 were differentially expressed in SJ (adjusted *p*-value of the medium effect <0.05) (Table S1). Of these, 213 ORFs were commonly regulated regardless of the strain background (strain effect *p*-value > 0.0330 which corresponds to FDR > 0.05). The remaining 331 ORFs were differentially expressed in M25 and EC1118 (strain effect *p*-value < 0.0330)

To gain insight into the common metabolic pathways involved in the response of yeast cells to the lack of lipid supplementation, the 213 ORFs showing the same regulation in the two strains were condensed into Annotation Clusters (AC) using the DAVID web tool (Table 2).

Table 2. Annotation clusters of M25 and EC1118 ORFs showing significant medium and not significant strain effect.

Functional Groups Upregulated in SJ				
Annotation Cluster 1	**Enrichment Score: 9.36**	**Count**	***p*_Value**	**Benjamini**
GOTERM_BP_DIRECT	ergosterol biosynthetic process	13	3.9×10^{-15}	5.4×10^{-13}
UP_KEYWORDS	Sterol biosynthesis	12	1.3×10^{-13}	1.7×10^{-11}
KEGG_PATHWAY	Steroid biosynthesis	8	8.2×10^{-9}	2.5×10^{-7}
UP_KEYWORDS	Lipid biosynthesis	13	7.6×10^{-8}	3.4×10^{-6}
GOTERM_BP_DIRECT	lipid metabolic process	14	7.0×10^{-6}	2.7×10^{-4}
UP_KEYWORDS	Lipid metabolism	14	8.3×10^{-6}	2.8×10^{-4}
Annotation Cluster 2	**Enrichment Score: 6.41**	**Count**	***p*_Value**	**Benjamini**
GOTERM_BP_DIRECT	oxidation-reduction process	24	1.6×10^{-7}	7.0×10^{-6}
UP_KEYWORDS	Oxidoreductase	17	8.5×10^{-5}	1.6×10^{-3}
GOTERM_MF_DIRECT	oxidoreductase activity	17	2.2×10^{-4}	1.3×10^{-2}
Annotation Cluster 3	**Enrichment Score: 3.51**	**Count**	***p*_Value**	**Benjamini**
GOTERM_BP_DIRECT	response to stress	11	1.0×10^{-8}	5.4×10^{-7}
GOTERM_MF_DIRECT	structural constituent of cell wall	9	2.5×10^{-6}	4.6×10^{-4}
GOTERM_BP_DIRECT	fungal-type cell wall organization	10	1.6×10^{-3}	5.2×10^{-2}
GOTERM_CC_DIRECT	fungal-type cell wall	9	1.8×10^{-3}	3.3×10^{-2}
Annotation Cluster 4	**Enrichment Score: 3**	**Count**	***p*_Value**	**Benjamini**
UP_KEYWORDS	Heme	7	7.4×10^{-5}	2.0×10^{-3}
GOTERM_MF_DIRECT	heme binding	6	2.1×10^{-4}	1.9×10^{-2}
Annotation Cluster 5	**Enrichment Score: 2.44**	**Count**	***p*_Value**	**Benjamini**
UP_KEYWORDS	Electron transport	7	4.5×10^{-4}	7.5×10^{-3}
GOTERM_BP_DIRECT	mitochondrial electron transport, ubiquinol to cytochrome c	4	1.8×10^{-3}	5.3×10^{-2}
GOTERM_CC_DIRECT	respiratory chain	4	3.5×10^{-3}	5.3×10^{-2}
Functional Groups Downregulated in SJ				
Annotation Cluster 6	**Enrichment Score: 2.46**	**Count**	***p*_Value**	**Benjamini**
GOTERM_BP_DIRECT	cytoplasmic translation	11	2.5×10^{-4}	6.7×10^{-2}
GOTERM_CC_DIRECT	intracellular ribonucleoprotein complex	14	4.5×10^{-4}	4.2×10^{-2}
UP_KEYWORDS	Ribonucleoprotein	14	5.0×10^{-4}	6.3×10^{-2}
GOTERM_CC_DIRECT	cytosolic large ribosomal subunit	8	9.0×10^{-4}	4.2×10^{-2}
Annotation Cluster 7	**Enrichment Score: 2.21**	**Count**	***p*_Value**	**Benjamini**
UP_KEYWORDS	Purine biosynthesis	4	2.2×10^{-3}	5.6×10^{-2}
GOTERM_BP_DIRECT	purine nucleotide biosynthetic process	4	2.4×10^{-3}	2.8×10^{-1}

Lack of lipid nutrients induced a significant upregulation of the ORFs involved in ergosterol biosynthesis (Figure 3) and in oxidation-reduction processes, particularly those involved in the mitochondrial electron transport such as *COX5A* and *CYC1*.

The stress generated by the absence of lipids induced the upregulation of the ORFs associated with the cell-wall: the seripauperins *PAU3, PAU4, PAU5, PAU10, PAU14, PAU15, PAU20, PAU23* as well as *HSP12*. In addition, other *HSP* genes were upregulated in SJ, particularly *HSP104, HSP12, HSP26, HSP31* and *HSP60*. On the contrary, ORFs involved in cytoplasmatic translation (AC 6) and in purine biosynthesis (AC 7) were downregulated, suggesting reduced protein biosynthesis in SJ.

Up- and down-regulated ORFs were further analyzed to identify potential transcription factors involved in the adaptation to the absence of lipid supplementation. Enrichment analysis carried out using the YEASTRACT database showed that Sfp1p is associated with 85.37% and 83.15% of the upregulated and downregulated ORFs, respectively (Table 3). Transcription factors Upc2p, Ecm22p, Hap1p, Mot3p, Rox1p were specifically associated with the upregulated ORFs. In accordance, genes encoding for some of those transcription factors were upregulated in SJ as well: *HAP4* = 0.88 log2

SJ/FSJ, *MOT3* = 1.53 log2 SJ/FSJ, *ROX1* = 1.63 log2 SJ/FSJ. The ORFs showing a repression in absence of lipid supplementation were regulated by the transcription factors Gcn4p and Yap1p.

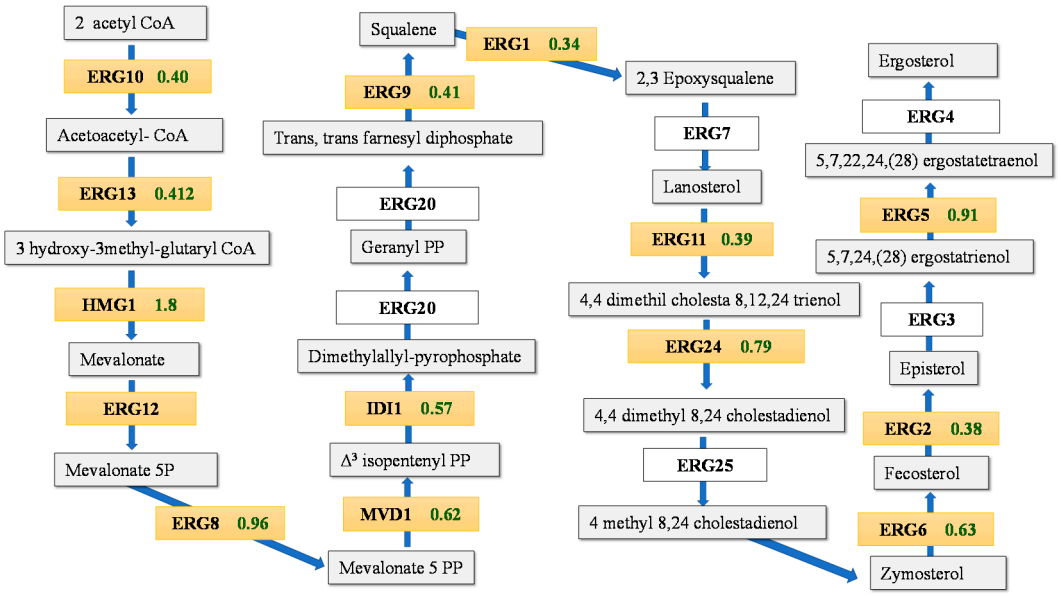

Figure 3. Ergosterol biosynthetic pathway. ORFs showing significant upregulation in SJ with respect to FSJ in EC1118 and M25 are highlighted in yellow. Green numbers indicate mean log2 ratio (SJ/FSJ).

Table 3. Transcription factors associated with upregulated and downregulated ORFs in SJ with respect to FSJ.

Upregulated ORFs			
Transcription Factors	**% in user set**	**% in Yeastract**	***p*-value**
Sfp1p	85.37%	2.42%	4.45×10^{-10}
Hap1p	39.84%	14.08%	1.03×10^{-15}
Rox1p	30.08%	4.10%	6.82×10^{-8}
Mot3p	26.02%	5.43%	5.86×10^{-10}
Ecm22p	23.58%	5.45%	3.75×10^{-9}
Upc2p	21.95%	8.49%	3.80×10^{-13}
Downregulated ORFs			
Transcription Factors	**% in user set**	**% in Yeastract**	***p*-value**
Sfp1p	83.15%	1.71%	1.17×10^{-6}
Yap1p	73.03%	1.88%	6.025×10^{-7}
Gcn4p	70.79%	2.10%	8.73×10^{-9}

3.3. Differences in the Transcriptomic Response of M25 and EC1118 during Growth in Absence of Oleic Acid and Ergosterol Supplementation

Considering that M25 and EC1118 showed a different fermentative behavior in SJ, differences in the transcriptional response of the two strains could provide information about the successful adaptation of EC1118 to the absence of lipid nutrients in the fermentation medium. Thus, the 331 ORFs showing a significant medium and strain effect were clustered according to their functional annotations (Table 4). Expression values of genes differentially expressed in M25 and EC1118 and included in the biological process GO:0009086 "methionine biosynthetic process", in the cellular component GO:0009277 "fungal-type cell wall" and in the cellular component GO:0005887 "integral component of plasma membrane" are reported in Figures 4–6 respectively.

Table 4. Annotation clusters of ORFs differentially expressed in M25 and EC1118 in response to fermentation in absence of oleic acid and ergosterol.

Annotation Cluster 1	**Enrichment Score: 4.43**	**Count**	***p*_Value**	**Benjamini**
GOTERM_BP_DIRECT	cellular amino acid biosynthetic process	23	7.5×10^{-9}	2.6×10^{-6}
GOTERM_BP_DIRECT	methionine biosynthetic process	11	5.4×10^{-7}	1.2×10^{-4}
UP_KEYWORDS	Cysteine biosynthesis	6	1.9×10^{-4}	1.2×10^{-2}
KEGG_PATHWAY	Sulfur metabolism	5	4.7×10^{-3}	6.1×10^{-2}
Annotation Cluster 2	**Enrichment Score: 3.7**	**Count**	***p*_Value**	**Benjamini**
INTERPRO	Stress-induced protein SRP1/TIP1	16	6.4×10^{-11}	3.9×10^{-8}
GOTERM_BP_DIRECT	response to stress	17	7.3×10^{-10}	5.1×10^{-7}
GOTERM_MF_DIRECT	structural constituent of cell wall	17	1.3×10^{-9}	5.4×10^{-7}
GOTERM_CC_DIRECT	fungal-type cell wall	24	2.4×10^{-8}	4.3×10^{-6}
GOTERM_BP_DIRECT	fungal-type cell wall organization	23	6.3×10^{-6}	1.1×10^{-3}
UP_KEYWORDS	Cell wall	13	2.1×10^{-4}	1.1×10^{-2}
GOTERM_CC_DIRECT	cell wall	13	2.3×10^{-4}	2.0×10^{-2}
Annotation Cluster 3	**Enrichment Score: 2.66**	**Count**	***p*_Value**	**Benjamini**
UP_SEQ_FEATURE	transit peptide:Mitochondrion	34	5.8×10^{-4}	1.2×10^{-1}
UP_KEYWORDS	Mitochondrion	62	2.3×10^{-3}	4.2×10^{-2}
Annotation Cluster 7	**Enrichment Score: 2.25**	**Count**	***p*_Value**	**Benjamini**
UP_KEYWORDS	Arginine biosynthesis	5	9.7×10^{-4}	2.4×10^{-2}
Annotation Cluster 8	**Enrichment Score: 1.79**	**Count**	***p*_Value**	**Benjamini**
GOTERM_CC_DIRECT	integral component of plasma membrane	18	2.8×10^{-4}	1.6×10^{-2}

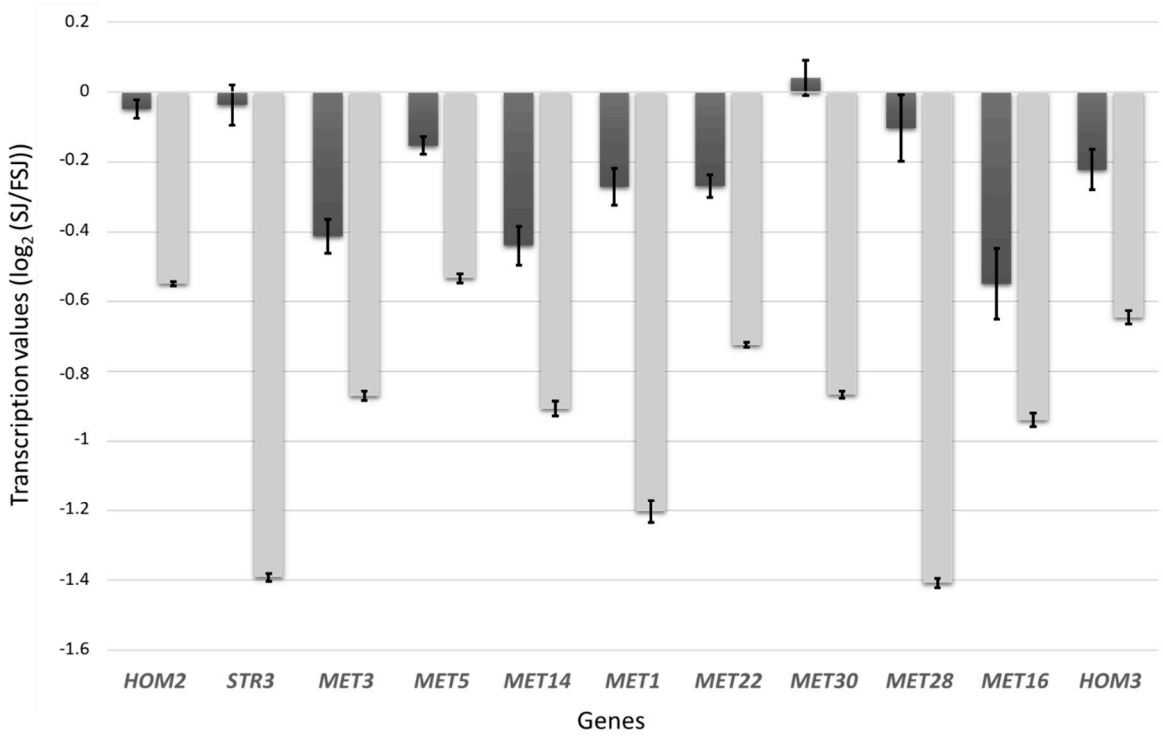

Figure 4. Transcription values of genes differentially expressed in EC1118 (black bars) and M25 (gray bars) in the functional category GO:0009086 "methionine biosynthetic process". Data are mean log2 ratio (SJ/FSJ) ± standard deviation of three independent biological replicates.

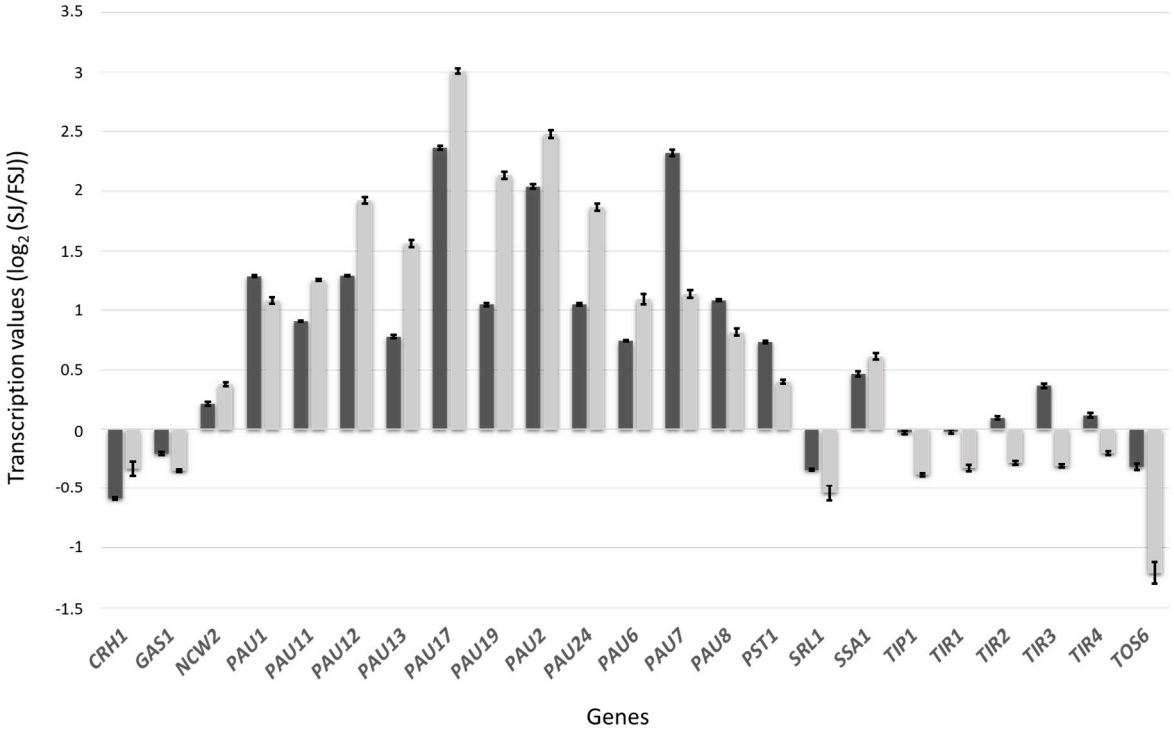

Figure 5. Transcription values of genes differentially expressed in EC1118 (black bars) and M25 (gray bars) in the functional category GO:0009277 "fungal-type cell wall". Data are mean log2 ratio (SJ/FSJ) ± standard deviation of three independent biological replicates.

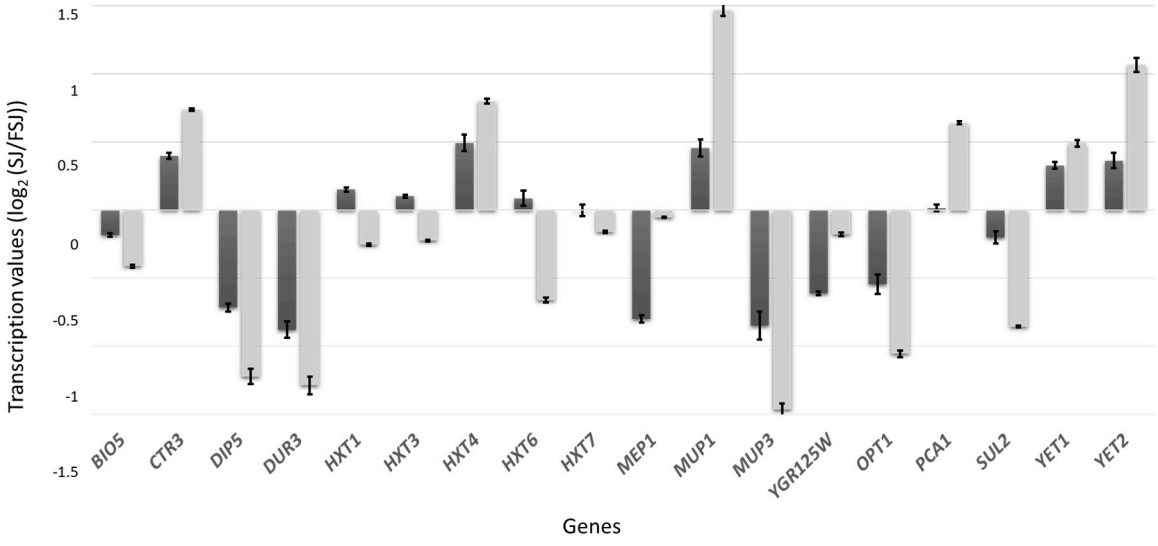

Figure 6. Transcription values of genes differentially expressed in EC1118 (black bars) and M25 (gray bars) in the functional category GO:0005887 "integral component of plasma membrane". Data are mean log2 ratio (SJ/FSJ) ± standard deviation of three independent biological replicates.

4. Discussion

During winemaking, yeasts modulate membrane fluidity in response to stressful conditions such as high ethanol content, low temperatures, hyperosmosis, etc. This modification, which requires changes in the UFA/SFA ratio as well as in the ergosterol content, is severely affected by prolonged hypoxic conditions [10]. In this context, the aim of this work was to elucidate the transcriptomic response of yeast during fermentation in absence of lipid supplementation. To do this, we firstly

identified the conditions and the phase of growth in which to cultivate and collect cells of two *S. cerevisiae* strains, namely EC1118 and M25.

In particular, we used two synthetic musts characterized by the absence (SJ) and the presence (FSJ) of ergosterol and oleic acid. We previously found that the amounts of acetic acid produced by EC1118 and M25 in SJ increased with respect to FSJ [24]. In more detail, acetic acid increased from 1.03 g/L to 1.37 g/L and from 0.91 g/L to 1.13 g/L in EC1118 and M25, respectively. Considering that higher acetic acid concentrations are indicative of an impaired lipid biosynthesis [25], SJ appeared the adequate medium to evaluate the yeast response to the specific stress induced by lack of lipids. In the chosen conditions the two strains showed a different susceptibility to undergo stuck/sluggish fermentation, in accordance with previous observations [4,15,26]. In particular, EC1118 showed identical fermentation profiles in the two media, while M25 was unable to complete the fermentation in SJ. It has been observed that high nitrogen content and low lipid supplementation, such as in SJ, exerted a negative effect on yeast cell viability [27]. Notably, ammonium was the most effective trigger of cell death at the end of fermentation [27]. Thus, the fermentative behavior of M25 and EC1118 in SJ could be related to differences in cell viability of the two strains in the late stationary phase. However, we found that M25 had higher cell viability than EC1118 after 480 h from the inoculum in SJ ($2.7 \pm 0.5 \times 10^7$ UFC/mL in M25 *vs* $7.3 \pm 0.7 \times 10^6$ UFC/mL in EC1118). Thus, cell viability at the end of the stationary phase did not correctly indicate the outcome of the fermentation. Bisson [25] suggested that the number of cells in the exponential phase is an important parameter in determining the fermentation outcome. Accordingly, we found that the fermentation rate of M25 was related to lower cell populations in SJ as compared to FSJ during the first days of fermentation. Thus, in consideration of the observation that the reduction of the fermentation rate was statistically significant from the 72 h after the inoculum, cells of EC1118 and M25 were sampled in SJ and FSJ at this specific time point for the genome-wide transcriptome analysis.

The implementation of a full factorial design (2×2) allowed us to identify common as well as strain-specific responses to the lack of lipid supplementation. The most significant common response to the growth in SJ was the strong induction of the transcription of genes associated with ergosterol biosynthesis. This behavior was expected considering that *ERG* genes are transcriptionally activated in response to ergosterol depletion [28]. During hypoxic growth, low ergosterol levels cause an increase in membrane fluidity thus activating the HOG pathway. This pathway induces the activation and accumulation of the transcription factor Upc2p (*UPC2* = 1.026 log2 SJ/FSJ; this work) [29]. Upc2p binds the sterol regulatory element (SRE) in the promoter of many ERG genes activating their transcription [30]. In addition, Upc2p controls the expression of two sterol transporters, encoded by *AUS1* (0.90 log2 SJ/FSJ) and *PDR11* (0.74 log2 SJ/FSJ), allowing yeast cells to take up sterols form the medium when biosynthesis is compromised [31,32]. Consistent with the observation that ergosterol levels play a role in oxygen sensing and signaling [33], Upc2p also regulates the expression of hypoxic genes particularly the *PAU* genes and those involved in mannoprotein and heme synthesis [34,35]. Accordingly, we measured a significant induction of 19 *PAU* genes in the medium lacking lipid supplementation. The *PAU* genes are the largest gene family in *S. cerevisiae* comprising 24 members that encode proteins involved in the remodeling of cell wall [36]. *PAU* genes play an important role during alcoholic fermentation as they are highly upregulated during fermentation in response to wine-making stress and hypoxia [35,37–39]. Nineteen copies of *PAU* genes are located in subtelomeric regions, and five copies are found in internal regions. It has been suggested that the subtelomeric *PAU* genes are more weakly inducible than the internal ones [39]. Our results, however, showed that 18 out of the 19 *PAU* genes upregulated in SJ were located in the subtelomeric regions, confirming what already observed for *PAU20* by Luo and van Vuuren [35]. Other hypoxic genes significantly induced in M25 and EC1118 in response to the lack of lipid were involved in heme synthesis, particularly *HEM1* (0.12 log2 SJ/FSJ), *HEM2* (0.73 log2 SJ/FSJ), *HEM12* (0.14 log2 SJ/FSJ), *HEM14* (0.11 log2 SJ/FSJ), *HEM15* (0.15 log2 SJ/FSJ) and *HEM25* (0.51 log2 SJ/FSJ).

Hemes are prosthetic groups of cytochromes and some oxygen-binding proteins. Considering that oxygen is required for its synthesis, heme plays a central role in oxygen-mediated gene expression in yeast [40]. Several transcription factors are involved in this mechanism: Hap1p, Hap2/3/4/5p, Rox1p and Mot3p. Interestingly, *HAP4*, *ROX1* and *MOT3* were significantly induced in SJ. *HAP1*, which is constitutively transcriptionally active, was found to be not differentially expressed in SJ and FSJ media. Hap1p (heme activated protein) activates the transcription of many aerobic genes involved in the aerobic respiratory chain (such as *CYC1* = 1.56 log2 SJ/FSJ, *COX4* = 0.48 log2 SJ/FSJ, *COX5a* = 0.63 log2 SJ/FSJ, *COX8*= 0.66 log2 SJ/FSJ, *CYT1*= 1.02 log2 SJ/FSJ), in sterol synthesis (*HMG1* = 1.80 log2 SJ/FSJ, *ERG1, ERG5, ERG11,ERG2,ERG29, ERG6, ERG26*) as well as the transcription factor *ROX1*. In the cell nucleus, Rox1p in combination with Mot3p forms a complex with Ssn6p/Tup1p, which results in the repression of hypoxic genes [41]. Rox1p and Mot3p may also function independently of each other [42]. In particular, Mot3p inhibits Ecm22p, but not Upc2p.

In our experimental conditions, the absence of lipid nutrients induced the expression of both hypoxic (through Upc2p) and aerobic (through the activator Hap1p and the repressors Rox1p and Mot3p) genes. Even though this could be seen as a contradictory behavior, it should be noted that the transition from aerobiosis to anaerobiosis is a gradual process in which microbial cells pass several stages of adaptation. In the absence of oxygen, the concentrations of heme and sterols decrease gradually, via their dilution in the process of cell division. Also, aerobic and hypoxic genes in most cases are regulated autonomously from each other to allow a fine-tune adaptation to various oxygen concentrations. In particular, the activation of the transcription of genes involved in sterol synthesis mediated by Upc2p in response to the decrease in sterol concentration is independent of Hap1p, Rox1p, and the level or presence of heme [33,34]. Also *PAU* gene induction is Rox1p independent [39], and data from previous studies have suggested that the hypoxic induction of *PAU* genes is Hap1p independent as well [43].

Analysis of functional categories enrichment showed that a lack of lipid nutrients induced a stress response in yeast cells. Besides the upregulation of the already cited *PAU* genes, many genes encoding for Heat Shock Proteins (*HSPs*) were also induced in SJ. *HSPs* are required for the folding and maintenance of newly translated proteins, the refolding of misfolded proteins, and the disassembly of protein aggregates [6]. Considering that ethanol accumulation leads to the denaturation of cellular proteins, the induction of *HSPs* is a well-known stress response mechanism during alcoholic fermentation [44]. Specific activities of *HSPs* have been reported. While Hsp70p unselectively recognize unfolded or misfolded proteins, the Hsp90s (*HSP82* and *HSC82*) activity is specifically targeted to the stabilization of kinases and transcription factors such as Swe1p, Gcn2p, and Hap1p [6]. Hsp104p and Hsp12p have been shown to directly influence yeast tolerance to ethanol through disaggregation of denaturated proteins and protection of membrane integrity, respectively [45]. Thus considering, the significantly higher induction of *HSPs* genes, particularly *HSP12*, was expected as cells growing in SJ were unable to counteract ethanol stress by increasing the ergosterol content in cellular membranes [46].

Oxidative damage to cell structures is another well-known stress experienced by yeast growing in hypoxic conditions, particularly in absence of lipid supplementation. Specifically, our previous results showed that Reactive Oxygen Species (ROS) increased in M25 and EC1118 during fermentation in SJ medium starting from the third day after the inoculum [4]. Under hypoxic conditions, yeast cells modify the mitochondrial respiratory chain to limit ROS production. A key enzyme in this process is the cytochrome c oxidase (*COX*) which regulates the electron flow through the respiratory chain. *S. cerevisiae* contains two oxygen-regulated subunits 5 of *COX*: *COX5a* and *COX5b*. Similarly, the cytochrome c exists in two isoforms encoded by *CYC1* and *CYC7* [47]. *COX5a* and *CYC1* are expressed in normoxia whereas *COX5b* and *CYC7* are expressed under hypoxia [48]. Interestingly, we found that in absence of lipid supplementation, the two strains induced the transcription of the normoxic subunits *COX5a* and *CYC1*, suggesting an inadequate adaptation to the hypoxic conditions of growth which could be the cause of the observed ROS accumulation [4]. In addition, significant levels of ROS are also produced in hypoxia through NAD(P)H-dependent pathways [49]. Notwithstanding ROS

accumulation, genes differentially regulated in the two media by the transcription factor Yap1p were mainly involved in the cytoplasmatic translation (synthesis of ribosomes, purines, amino-acid transport, etc.) and not in the oxidative stress response. Particularly, the glutaredoxin system (*GSH1, GLR1*), superoxide dismutase (*SOD1, SOD2*), catalase activity (*CAT1*, *CTT1*), glutathione peroxidase (*GPX2*), and thiol-specific peroxidases (*TSA1, AHP1*) were not differentially expressed in SJ as compared to FSJ. In accordance, neither M25 nor EC1118 showed appreciable catalase (CAT) activities in SJ [4]. The repression of these ROS scavenging systems in SJ is even more surprisingly given that the addition of oleic acid and ergosterol in FSJ decreased intracellular ROS levels and oxidative damage in M25 and EC1118 [24].

The implementation of a full experimental design allowed us to identify not only the common response to yeast growth in SJ, but also to detect the genes that were differentially expressed in the two strains. Considering that EC1118 and M25 displayed a different fermentative behavior, these genes could reveal the mechanism involved in yeast adaptation to the fermentation in absence of lipid supplementation. Of particular interest are the "fungal-type cell wall" and "integral component of plasma membrane" terms in the Gene Ontology (GO) knowledgebase that comprise 24 and 18 ORFs, respectively, differentially expressed in the two strains. This suggests an important role of these subcellular components in the adaptation to the growth in absence of lipid supplementation.

The in-depth analysis showed that the "fungal-type cell wall" GO term contains 11 *PAU* genes, 5 *TIP/TIR* genes as well as *CRH1, GAS1, NCW2, PST1, SRL1*, *SSA1* and *TOS6*. Regarding *PAU* genes, only *PAU1* and to a larger extent *PAU7*, were induced more in EC1118 than in M25, while the others showed an opposite behavior. Interestingly, *PAU7* was the only *PAU* gene, among the 19 identified in this study, with an internal chromosomic position. Proteins encoded by *CHR1* (chitin transglycosylase) and *GAS1* (Beta-1,3-glucanosyltransferase) are involved in cell wall assembly. *NCW2*, *PST1* and *SRL1* encode cell wall proteins associated with cell wall stresses. Of interest is the strong repression of *TOS6* in M25. *TOS6* encodes a glycosylphosphatidylinositol-dependent cell wall protein whose expression is decreased in response to ergosterol perturbation, suggesting a higher impact of ergosterol deprivation in M25. The importance of mannoproteins, particularly the *TIR* genes in yeast adaptation to stressful conditions has been well documented. Induction of *TIR* genes has been related to hypoxia, high pressure and low temperature conditions [36,50]. It was also observed that a wine strain which was improved in its ability to grow at low temperatures through evolutionary engineering overexpressed *TIR1*, *TIR4* and *TIR3* [51]. In addition, the overexpression of *TIP1* improved yeast fermentation activity at low temperatures [52]. It has been postulated that the induction of *TIP/TIR* genes allow yeast cells to adapt to the reduced membrane fluidity caused by the aforementioned stress factors [36]. In accordance with the importance of sterol and unsaturated fatty acids in regulating membrane fluidity, the induction of *TIR* genes by Upc2p and Ecm22p occurs in response to sterol depletion [34]. In this context, the downregulation of *TIP1, TIR1, TIR2, TIR3* and *TIR4* in M25 growing in SJ could severely affect its ability to adapt to the absence of lipids. On the contrary, EC1118 showed not significant changes in *TIP1* and *TIR1* and an induction of *TIR2, TIR3* and *TIR4* in SJ.

The ORFs represented in the "integral component of plasma membrane" GO term were associated with the transport of sugars (*HXT1, HT3, HXT4, HXT5, HXT7*), nitrogen sources (*MEP1,OPT1, DIP5*, *MUP3*, *DUR3*), cadmium (*PCA1*), sulphate (*SUL2*) and copper (*CTR3*) inside the cell. Of interest are the hexose transporters *HXT1*, *HXT3* and *HXT6* that showed an opposite behavior in M25 (repressed) and EC1118 (upregulated) during yeast growth in SJ. These genes encode for low affinity glucose transporters induced at high sugar concentrations [53] and thus essential in the fermentation of SJ and FSJ (200 g/L glucose). It could be postulated that the repression of these transporters in M25 is reducing the amount of intra-cellular glucose that is used by this strain, thus causing the subsequent stuck/sluggish fermentation.

Finally, the annotation cluster with the higher statistical significance was related to the "methionine biosynthetic process" GO term. All of the ORFs included in this term showed a higher repression in M25 than in EC1118, suggesting a reduced methionine synthesis in the former strain. Methionine acts

as a ROS scavenger and protects cells from oxidative stress [54]. Indeed, sulfur in methionine is a direct target of ROS, as it can be oxidized to sulfoxide. In addition, it has been shown that protein expressed at sites of ROS production, such as in the mitochondrial respiratory chain are enriched in methionine [55]. Thus, in M25 growing in SJ, the repression of genes involved in methionine biosynthesis together with the already discussed lack of induction of oxidative stress responsive genes, could explain the accumulation of ROS and the inability to complete the fermentation in medium lacking lipid nutrition. It has been observed that the addition of 1 g/L of acetaldehyde to *S. cerevisiae* cells growing in YEPD induced the expression of genes involved in sulfate uptake and homocysteine synthesis (*SUL2, MET2, MET3, MET14, MET16, MET10, MET25*) [56]. Thus, it could be hypothesized that the observed differences in the expression of genes of the sulfur metabolism in EC1118 and M25, could be due to differences in the levels of acetaldehyde produced by the two strains in SJ. In this respect, previous works have shown that during the fermentation of wine-based media, M25 and EC1118 produced 65.00 mg/L and 23.40 mg/L of acetaldehyde, respectively [57,58]. Notwithstanding the relevant differences in the conditions reported by [56] and those in this work, the aforementioned hypothesis deserves subsequent evaluation.

5. Conclusions

A genome-wide analysis of two wine yeast strains with different fermentative behaviors revealed common and strain-specific responses. Common responses observed in the absence of lipids regarded the induction of the expression of genes involved in ergosterol biosynthesis, as well as genes, such as those in seripauperin and heat shock protein multigene families, related to the adaptation to wine-making stresses. The activation of some aerobic genes, particularly those involved in the mitochondrial respiratory chain, could explain previous results showing ROS accumulation in these strains when growing in absence of lipid supplementation. However, genes directly involved in the oxidative stress response were not differentially expressed in the two tested conditions. In this context, the higher inhibition of the methionine biosynthetic process in M25, which underwent sluggish fermentation, suggested an important role of the sulfur-mediated ROS scavenging system in SJ. In addition, M25 was characterized by the downregulation of the mannoprotein encoding genes *TIP1, TIR1, TIR2, TIR3* and *TIR4*, previously associated with yeast adaptation to stress conditions such as low temperatures, high ethanol, and hypoxia. In conclusion, the results obtained in this work provide a valuable dataset to further investigate the mechanisms involved in yeast adaptation during growth in the absence of lipid supplementation and to assist in industrial strain selection.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/5/3/57/s1, Table S1: ORFs differentially expressed in EC1118 and M25 during fermentation in absence of oleic acid and ergosterol.

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