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Short-term response of different *Saccharomyces cerevisiae* strains to hyperosmotic stress caused by inoculation in grape must: RT-qPCR study and metabolite analysis

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ABSTRACT

During the winemaking process, glycerol synthesis represents the first adaption response of *Saccharomyces cerevisiae* to osmotic stress after inoculation in grape must. We have implemented an RT-qPCR (Reverse Transcription-quantitative PCR) methodology with a preventive evaluation of candidate reference genes, to study six target genes related to glycerol synthesis (*GPD1*, *GPD2*, *GPP2* and *GPP1*) and flux (*STL1* and *FPS1*), and three *ALD* genes coding for *aldehyde dehydrogenase* involved in redox equilibrium via acetate production. The mRNA level in three strains, characterized by different metabolite production, was monitored in the first 120 min from inoculation into natural grape must. Expression analysis shows a transient response of genes *GPD1*, *GPD2*, *GPP2*, *GPP1* and *STL1* with differences among strains in term of mRNA abundance, while *FPS1* was expressed constitutively. The transient response and different expression intensity among strains, in relation to the intracellular glycerol accumulation pattern, prove the negative feedback control via the HOG (High Osmolarity Glycerol) signalling pathway in *S. cerevisiae* wine strains under winery conditions. Among the *ALD* genes, only *ALD6* was moderately induced in the hyperosmotic environment but not in all strains tested, while *ALD3* and *ALD4* were drastically glucose repressed. The intensity of transcription of *ALD6* and *ALD3* seems to be related to different acetate production found among the strains.

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1. Introduction

In recent years, climate change, and the resulting rise in temperature, has presented formidable challenges to the global agricultural sector. Even the wine industry is facing such a crisis, which poses new problems for winemakers to overcome (Battaglini et al., 2009). Whereas a few years ago the alcohol content of wine was considered one of the significant parameters in judging wine quality, especially in red wines, where reaching a good level of potential ethanol in certain vintages was problematic, today, in some areas of the world, this vision has reversed. In fact, higher temperatures and an increasing must sugar content, causes, in some years, an unacceptable alcohol content from the point of view of quality and health. Even from a technological point of view the new challenges are urgent, and a series of problems that were limited to certain types of wine fermentation, such as that of German Eiswine or Italian Passito, concerning the interaction between the yeast, usually *Saccharomyces cerevisiae*, with a must particularly rich in sugar, have become relevant even in fermentation of common wines. At the end of fermentation, wine yeasts are subject to nutritional and environmental stress related mainly to a lack of nutrients and the accumulation of ethanol, (Bauer and Pretorius, 2000; Alexandre et al., 2001) while at the beginning of fermentation, once inoculated into the must, they face osmotic stress resulting from high sugar concentrations. The response of yeast cells can affect cell viability and influences the progression of the fermentation process (Attfield, 1997; Gibson et al., 2007).

The response of wine yeast to hyperosmotic stress in the early hours of fermentation, resulting from exposure to a high concentration of sugar, has been studied extensively in synthetic media mimicking natural must (Pérez-Torrado et al., 2005; Zuzuarregui and Del Olmo, 2004; Zuzuarregui et al., 2005; Rossignol et al., 2003; Jiménez-Martí et al., 2011a, b). Global transcriptomic and proteomic analysis in this media revealed important changes of

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several genes and proteins including HSP (heat shock protein) (Zuzuarregui et al., 2006) and other genes with unknown functions, such as Y*HR087w* whose encoded protein has been shown to be involved in the stress response (Jiménez-Martí et al., 2011a, b; Gomar-Alba et al., 2012). Other studies showed the upregulation of biosynthetic genes for glycerol and trehalose (Kaeberlein et al., 2002) while Erasmus et al. (2003), using a natural riesling grape must analysed two hours after inoculation, showed the upregulation of glycolysis genes and the genes involved in the pentose phosphate pathway and acetate synthesis, while some genes related to the synthesis of aromatic amino acids, purines and pyrimidines were repressed.

The principal response of the yeast cell to a hyperosmotic environment is the synthesis of metabolites with an osmoprotective effect, in particular glycerol (Nevoigt and Stahl, 1997; Hohmann and Mager, 2007). This molecule, which is produced during the entire fermentation in response to a need to balance the redox equilibrium via NAD+ formation, has a fundamental action as an osmoprotector in the early stages with a high concentration of osmolytes (Hohmann, 2002). The metabolite is produced as the main effect of the HOG (High Osmolarity Glycerol) signalling pathway, a branched MAPK (Mitogen Activated Protein Kinase) signal transduction system (De Nadal et al., 2002; Hohmann, 2009). In this signalling system, the external osmolyte concentration, detected by membrane proteins such as Sln1, rapidly initiates a MAPK signalling cascade culminating in phosphorylation of the Hog1 MAPK. In this way, cytoplasmatic Hog1 is activated and translocates to the nucleus playing direct and indirect roles in a transcriptional response (Hohman, 2009; Brewster and Gustin, 2014). The HOG system induces a series of adaptive changes of the cell at different levels; the study of these changes is complicated by the fact that in grape must, glucose and fructose, acting as osmolytes, are the primary source of carbon for the cell and they exert catabolite repression on the assimilation of slowly fermentable carbon sources (Gancedo, 2008). Among these complex responses, the HOG pathway induces the transcription of biosynthetic genes coding for the enzymes involved in glycerol formation via the Gpd–Gpp pathway: *GPD1*, *GPP1* and *GPP2* but not *GPD2* (Ansell et al., 1997; Rep et al., 2000). As a result, the glycolytic flux is directed mainly toward glycerol formation to the detriment of biomass production (Hohmann and Mager, 2007; Petelenz-Kurdziel et al., 2013). Furthermore, the HOG pathway also contributes to activate various mechanisms that serve to retain intracellular glycerol, as the activation of glycerol carriers coded by gene *STL1* and the closure of the aquaglyceroporin channel Fps1 to prevent the loss of glycerol (see reviews of Hohmann, 2009 and Ahmadpour et al., 2014).

During hyperosmotic shock yeast cells require mechanisms to balance the excess NAD+ generated during the production of glycerol. One mechanism operates through the formation of acetate mediated by NAD+ dependent *aldehyde dehydrogenase* encoded by the genes of the *ALD* family (Miralles and Serrano, 1995; Navarro-Aviño et al., 1999). In particular, NAD+ dependent *ALD3* seems to be responsible for the production of acetate from acetaldehyde during the production of ice wines (Pigeau and Inglis, 2005).

Despite these studies, little information is available on the early molecular responses to an osmotic environment under actual winemaking conditions using wine strains and real grape must. The importance of these studies is justified by the fact that, due to the osmotic adaptation mechanisms mentioned above, more than half of the glycerol and acetate are produced in the early stages of fermentation, and their concentration has an important influence on wine quality (Ribéreau-Gayon et al., 2004).

In this study we applied a validated real-time RT-qPCR technique to study the expression of key genes related to glycerol production and cellular flux in the first two hours of the alcoholic fermentation of grape must. Our work focused on three strains which exhibited different metabolite production. In particular, we studied the time course expression, at the transcriptomic level, of genes involved in the osmotic response by analysing the biosynthetic genes of glycerol *GPD1*, *GPD2*, *GPP1*, *GPP2*, genes *STL1* and *FPS1*, related to glycerol flux, and correlated transcriptomic differences with metabolites production. In addition, three *aldehyde dehydrogenase* genes *ALD3*, *ALD4* and *ALD6* were studied considering the involvement of this enzyme in NADH reconstitution. The objective of this work was to increase knowledge regarding the transcription mechanism which regulates the production of metabolites involved in the osmoadaptation in yeast of oenological interest.

2. Materials and methods

2.1. Yeast strains and media

Eighty *S. cerevisiae* strains, belonging to the culture collection of CRA-Centro di Ricerca per l'Enologia (Asti, Italy) and industrial strain BK1 (Tecnofood, S. Maria della Versa, Italy) were used in this study. For the screening step, the strains were first propagated in YPD (Yeast Peptone Dextrose) (Sigma–Aldrich, Saint Louis, USA) medium for 48 h and then inoculated at 10^6 cells/mL in synthetic medium MNS2 as previously reported (Vaudano et al., 2011). Fermentations were carried out in triplicate using 300 mL Erlenmeyer flasks with 200 mL of must and capped with a Muller valve. The fermentation temperature was set at 20 °C. Ethanol formation was indirectly determined via CO_2 production monitored by weight loss. For grape must fermentations, a 0.22 μm filter sterilized Cortese white grape must was used, the main parameters of the must were: pH 3.30, 235 g/L reducing sugar and 228 mg/L readily assimilable nitrogen. No sulphur dioxide was added during grape crushing and in the must obtained.

In the EF (early fermentation) experiments, the microfermentation in Cortese grape juice was set-up in triplicate. Yeast strains, previously propagated in YPD were drawn during the exponential phase, washed in sterile water and inoculated into grape must at a concentration of 1×10^8 cells/mL. These cell concentrations were necessary to magnify metabolite variation in short-term experiments. The must was maintained at 20 °C and sampled during the first 2 h, at 0, 5, 10, 30, 60, 120 min after inoculation, the pellet was separated from the supernatant by centrifugation at 10,000 *g* for 1 min and the samples were immediately frozen in liquid nitrogen. Several samples were drawn to perform RNA and metabolite analysis.

2.2. RNA extraction and cDNA synthesis

RNA extraction was performed using 2×10^7 cells employing a commercial kit (Omega Biotek Inc., Doraville, USA); DNAse treatment was carried out. Qualitative and quantitative analysis of RNA were performed using a lab-on-chip instrument Experion microcapillary electrophoresis system (Biorad Laboratories Inc., Hercules, USA). cDNA was synthesized from 0.3 μg of total RNA using the two step IScript Select cDNA Synthesis kit (Biorad) as the RT enzyme, with RNAse treatment. The cDNA was conserved at −80 °C.

2.3. Primers and quantitative PCR

All primers were designed using Primer 3 software with the exception of *TAF10*, *TFC1* and *UBC6* which were designed by Teste et al. (2009). Primer specificity was tested *in silico* using BLAST analysis and agarose gel electrophoresis; amplification efficiency

was calculated using the dilution method (Rasmussen, 2001). A description of the genes, primers used, and efficiencies are shown in Tables 1 and 2.

Real-time PCR was performed in 96-well plates on a Biorad ICycler instrument (Biorad) using SYBR Green as the fluorophore. Reactions were carried out in 20 μl volume which contained 2.5 μl cDNA, 0.5 μM forward and reverse primers and 10 μl 2× EVA Green Master mix (Biorad). Each sample was analysed twice and no – template control for each primer was included in all real-time plates. Amplifications were performed under the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 63 °C for 30 s and a final extension at 72 °C for 5 min. At the end of the amplification cycle, a melting analysis was carried out to verify the absence of non-specific amplification. The expression level of a given gene was reported as the quantification cycle (Cq), corresponding to the number of cycles required to reach a predetermined threshold fluorescence. The threshold values were obtained using the automated setting of the instrument software (baseline subtracted curve fit data). The data, expressed as Cq, were imported into a Microsoft Excel data sheet for subsequent analysis.

2.4. Selection of reference genes

We tested the transcriptional variability of nine genes, previously reported in the literature as potential reference genes (RG), including genes that are widely used in normalization studies on *S. cerevisiae*, such as genes encoding actin *(ACT1)* and glyceraldehyde-3-phosphate dehydrogenase, isoenzyme 2 *(TDH2)*, during the first two hours of fermentation in all three *S. cerevisiae* strains (Table 1). The best reference genes were selected using the GeNorm statistical software version 3.3 for the Microsoft Excel method (Vandesompele et al., 2002), considering the M value after stepwise exclusion as reported by the authors.

2.5. Analysis of target genes

The expression analysis of nine target genes was carried out (Table 2). The genes were chosen for their involvement in glycerol metabolism and flux, in particular, the genes coding for biosynthetic enzymes, *GPD2*, *GPP1*, *GPP2*, *GPD1* and the genes coding for transporters *STL1* and a aquaglyceroporin channel *FPS1*. Furthermore, genes involved in acetate production via *aldehyde dehydrogenase*, *ALD3*, *ALD4* and *ALD6* were studied. The relative quantification of mRNA was performed by normalizing the expression value transformed in relative copy numbers (obtained from Cq values and considering the efficiency), to the geometric mean of the RGs. In the calculation of the relative copy number from raw Cq, amplification efficiencies were considered.

2.6. Metabolite analysis

In the screening study, ethanol and residual sugar were determined according to official methods (EUR Lex 31990R2676 EN). Glycerol was determined using an HPLC equipped with a refractometric detector with a Rezex RCM-Monosaccharide column (dimension: 300 × 7.8 mm; particle size 8 μm; Phenomenex, Torrance, USA) using the following conditions: eluent: water; column temperature: 85 °C; flow 0.35 mL/min; injection volume: 20 μL. Acetate was determined using an UV-method enzymatic kit (R-Biopharm AG, Darmstadt, Germany).

In EF experiments, two types of samples were drawn for each time point: intracellular samples obtained by centrifuging the cell pellet without the supernatant and extracellular samples (supernatant). Extracellular samples did not require further processing; cell pellets (intracellular samples) were extracted with 1 mL of sterile water by boiling for 10 min followed by centrifugation at 10,000 *g* for 1 min.

Extracellular acid acetic and ethanol, and extracellular/intracellular glycerol were determined using enzymatic methodologies (R-Biopharm AG) and normalized toward cell concentration expressed as OD.

2.7. Statistics

Analysis of variance (ANOVA) (XLStat, Addinsoft SARL, Paris, France) was performed to evaluate the significance of variation in the gene expression during the two hours of fermentation. The Tuckey test to evaluate group (strain) differences in expression and metabolite synthesis was carried out; the significance level was set at $p \leq 0.05$.

3. Results

3.1. Fermentation screening

The variability of metabolite production in the 80 strains, observed at the end of fermentation in a synthetic medium, is

Table 1
Candidate reference genes and relative primers used in RT-qPCR.

Genes	NCBI gene Id	Description	Forward and reverse primer	PCR product size[a]	Efficiency
ACT1	850504	Actin, structural protein involved in cytoskeletal functions	TTT GCC GGT GAC GAC GCT CC CGT CCC AGT TGG TGA CAA TAC CGT	181	2.01
TUB2	850506	Beta-tubulin; involved in microtubules formation	TCT GCG GGC AAC GTG TGG G GGG AGT CGC ATC CTT CGG CC	104	2.04
QCR9	853095	Subunit 9 of the ubiquinol cytochrome-c reductase complex	TGC AGG TGC CTT TGT TTT CCA AAC T TCG TCG TCT CCA TCG CCT GC	123	2.02
PGK1	850370	3-phosphoglycerate kinase, key enzyme in glycolysis and gluconeogenesis	GCC AAG GCC AAG GGT GTC GAA CCA ACC CTT GCC AGC CAG CT	127	1.99
LSC2	853159	Beta subunit of succinyl-CoA ligase, a mitochondrial enzyme of the TCA cycle	TGG TGC CAC CCC TGA GAC CA GCG GCT TCT ACC AGC CCC AG	135	1.97
TDH2	853465	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2	ACG CTG GTG AAG TTT CCC ACG A TGG GTC TCT TTC TTG GAA AGT GGC G	80	2.00
TAF10[b]	851745	Subunit of TFIID and SAGA complexes; involved in RNA polymerase II transcription initiation and in chromatin modification	ATA TTC CAG GAT CAG GTC TTC CGT AGC GTA GTC TTC TCA TTC TGT TGA TGT TGT TG	141	1.96
TFC1[b]	852421	Subunit of RNA polymerase III transcription initiation factor complex	GCT GGC ACT CAT ATC TTA TCG TTT CAC AAT GG GAA CCT GCT GTC AAT ACC GCC TGG AG	223	2.04
UBC6[b]	856837	E2 ubiquitin-conjugating protein UBC6	GAT ACT TGG AAT CCT GGC TGG TCT GTC TC AAA GGG TCT TCT GTT TCA TCA CCT GTA TTT GC	272	1.99

[a] As expected by BLAST on s288c strain.
[b] Primer designed by Teste et al. (2009).

Table 2
Target genes and relative primers used in RT-qPCR.

Genes	NCBI gene Id	Description	Forward and reverse primer	PCR product size[a]	Efficiency
GPD1	851539	Glycerol-3-phosphate dehydrogenase (NAD (+))	GCG AGG GCA AGG ACG TCG AC TGG ATG GCA GCA GAA GCG TTG T	184	1.88
GPD2	854095	Glycerol-3-phosphate dehydrogenase (NAD (+))	TTT CCC AGA ATC CAA AGT CG CTG AGC AGG TGG TGA TCA GA	74	1.96
GPP1	854758	Glycerol-1-phosphatase RHR2	TGC TTT GAA CGC CTT GCC AAA GG ACG GGT ACC AGA GGT GGC GA	55	1.91
GPP2	856791	Glycerol-1-phosphatase HOR2	CAG CAG GTA TTG CCG CCG GA CGG CAT TGT AGC CGC CAA CT	145	2.08
STL1	852149	Glycerol proton symporter of the plasma membrane	ACG CAA GAG GTG CTG CCG TC AGC AAC CCC AAC CGG ACT GT	210	2.00
FPS1	850683	Aquaglyceroporin, plasma membrane channel	AAG TGC GCG GCC TAC TCC CA CTT GCA CTC GGC GGA CCG TT	141	2.00
ALD3	855205	Cytoplasmic aldehyde dehydrogenase; involved in β-alanine synthesis	GCG CAC ATG TTT GCT CGC GA CAA CGC CGG TGT CGC CTG AT	133	2.00
ALD4	854556	Mitochondrial aldehyde dehydrogenase	CGG CTA CGG GTA GAC ACA TT GAT TTA CCA CCC AGC TCC AA	76	1.65
ALD6	856044	Cytosolic aldehyde dehydrogenase	GCC GAC CGT GCT TTC CAC GA TCC CCA CGG GCT AAG GCC AA	161	2.02

[a] As expected by BLAST on s288c strain.

shown in Fig. 1. Considering acetate (Fig. 1a), a variability between 1.45 ± 0.08 (mean ± standard error; n = 3) and 0.72 ± 0.03 g/L was found, with the two strains, ISE19 and ISE121 which were the maximum and minimum of the range respectively. 50% of the strains showed values between 0.87 ± 0.01 and 1.10 ± 0.01 g/L. The acetate detected was considerably higher than the values found in the fermentation using natural grape must, probably due to the different composition of the culture medium compared with the natural grape must, in particular, regarding the content of fatty acids (Delfini et al., 1992). With regards to the production of glycerol (Fig. 1b), the box plot showed that 50% of the strains produced glycerol at a concentration between 4.42 ± 0.20 and 5.23 ± 0.25 g/L; at the extreme limits of production were two strains, ISE19 and ISE90, with values of 6.06 ± 0.16 and 3.77 ± 0.10 g/L respectively. Lower variability was found in ethanol production (Fig. 1c); between the first and third quartiles (50% of the strains), the variation was 0.16% v/v and the most variable strains were ISE90 with 12.19 ± 0.05% (v/v) and ISE36 with 11.57 ± 0.06% (v/v).

Among the 80 strains screened, two strains were selected for subsequent analysis, on the basis of the production of glycerol, using the largest variability and the significance of differences (data not shown) as a criterion. Strain ISE19, which exhibited the highest production of the metabolite, and ISE90, the strain with the lowest production ($p \leq 0.01$), were chosen. Significant differences ($p \leq 0.01$) were found, for these two strains, also in terms of acetate with a final concentration of 1.45 ± 0.08 g/L for ISE19 and 0.92 ± 0.02 g/L for ISE90. The differences in the acetate and glycerol production were also tested in the fermentation using natural must and compared with the commercial BK1 strain. Observing the whole fermentation, monitored using the CO_2 emission converted in ethanol (Fig. 2), ISE19 was less efficient than ISE90 and BK1. This strain had some difficulty in ending the fermentation, which was sluggish and ended after 27 days respect to 22–23 days for ISE90 and BK1 strains. The analysis performed at the end of fermentation showed that, even in this case, ISE19 and ISE90 differ significantly in the production of glycerol and acetate (Table 3). A lower (albeit not statistically significant) production of ethanol was found for ISE19 with respect to the other two strains. The selected commercial strain showed the lowest production of acetate and an intermediate concentration of glycerol among the strains tested.

3.2. *Metabolite production during early fermentation (EF)*

Metabolite analyses over the first two hours of fermentation with regard to the production of ethanol, acetate, intra- and extracellular glycerol are shown in Fig. 3. Ethanol (Fig. 3a) increased in all strains after 5–10 min from inoculation into the must, growing constantly during all the experiments and at 120 min reached values between 3.4 ± 0.1 and 8.0 ± 0.2 mmole/OD. At this time point, ISE19 and ISE90 strains showed superior ethanol content, with respect to commercial strain BK1, and reached values which were 115–135% higher. Acetate production (Fig. 3b) showed

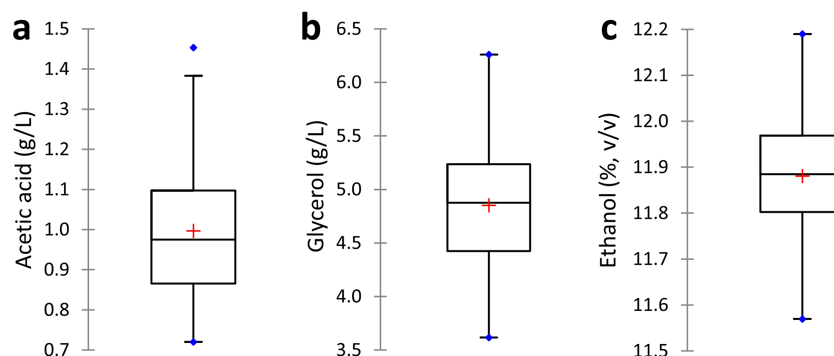


Fig. 1. Metabolite variability in 80 strains determined at the end of fermentation in MNS2 synthetic must. a) acetate, b) glycerol, c) ethanol. Legend: diamond: max and min value; box: interquartile range (IQR) (25th, 75th), band in box: median; + in box: mean; whiskers: lowest and highest data within ±1.5 IQR.

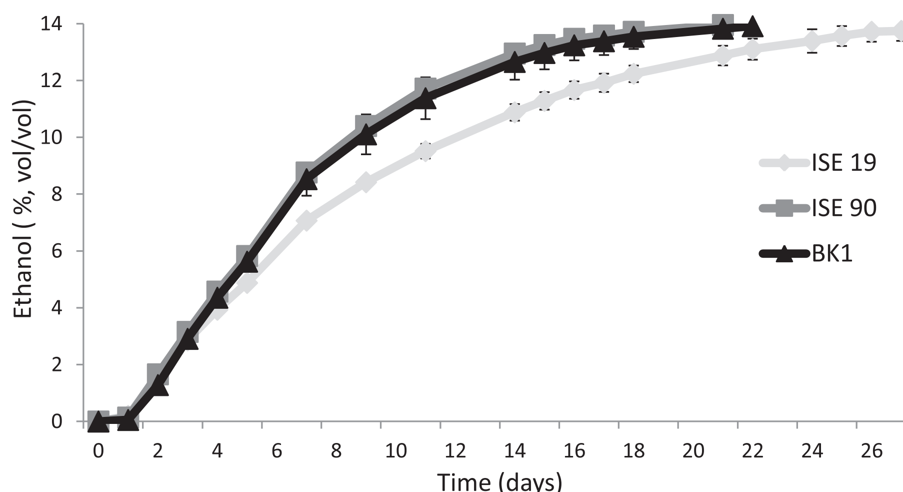


Fig. 2. Fermentation performance of strains ISE19, ISE90 and BK1 in Cortese grape must, monitored by weight loss as described in Materials and methods section. Error bars represent standard error of the mean (n = 3).

increments starting from 30 min after inoculation; at 120 min strain BK1 was different with a lower production, by about 50%, compared with the other strains, ISE19 and ISE90, which showed a similar metabolite production. Analysis of the extracellular glycerol (Fig. 3c) showed a trend that is substantially similar to that of acetate with a rapid increase from 30 min. In this case, BK1 and ISE19 were different in comparison to strain ISE90 with a lower rate of accumulation, presenting a 25–30% lower concentration at 120 min. The accumulation of intracellular glycerol (Fig. 3d) showed relevant differences with respect to the metabolites previously described. The inoculation of strains ISE19 and ISE90 into the must induced the accumulation of glycerol in the cell with a greater increase between 10 and 30 min. After 30 min, the accumulation was interrupted and the concentration remained essentially unchanged until the end of the experiment. Once again, strain BK1 differed in its accumulation of intracellular glycerol: starting from inoculation, this strain accumulated glycerol at a lower rate with respect to the other two strains, but continuously up to 120 min and did not exhibit any stopping of the accumulation. At 120 min, the concentrations of the three strains didn't show any significant differences. Interestingly, cell growth measured as OD shows an increase starting from 30 min in strain ISE19 and 60 min in ISE90, while growth is not observed in the case of strain BK1 (Fig. 3e).

3.3. Choice of reference genes

With the aim to select the best RGs set for the normalization of target genes expression in EF experiment, a preventive evaluation of nine candidate RGs were performed using the statistical method GeNorm algorithm (Vandesompele et al., 2002). Genes were classified according to the M value, which represents the arithmetic average of pairwise variation, V, intended as the standard deviation of the ratio between the expression of a particular gene and all other candidate genes. After a stepwise exclusion, the software excludes the least stable gene (with the higher M value) and recalculates the M value, identifying the two steadily expressed ones.

GeNorm analysis performed for single strains showed that, in ISE90 and ISE19, the two most stable genes during the experiments were *UBC6* and *TAF10*, whereas for the commercial BK1 strain, genes *UBC6* and *PGK1* were the most stable (data not shown). Analysis of the entire dataset composed of all the samples of the three strains (Fig. 4) showed a general increment of variability, i.e., a higher M value, with respect to the analysis of single strain samples. Stepwise analysis allowed the identification of *TAF10* and *UBC6* as more stable genes followed by *TFC1*, the third in order of stability, on the basis of the M values. *TFC1* was included in the reference set following the GeNorm author instructions, which recommends the use of three reference genes during normalization. This reference gene system was used for normalization of target genes employing the geometric mean of their expression value in relative copy number. The use of this reference system, calculated over the three strains together, allowed evaluation of the differences in mRNA level among different strains, in addition to calculating the changes of transcription during the experiment for each strain individually.

Table 3
Analysis of wines obtained with fermentation of Cortese must.

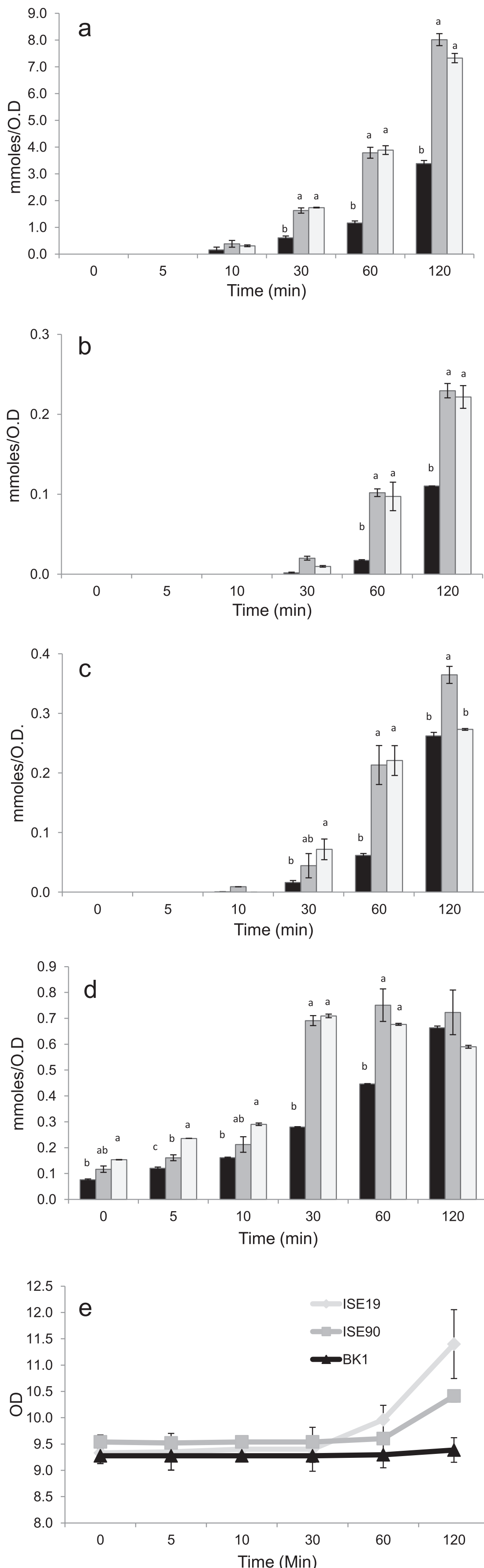
Strain	Glycerol (g/L)	Acetate (g/L)	Ethanol (%, v/v)
ISE19	6.42 ± 0.09^a	0.74 ± 0.02^a	13.75 ± 0.20^a
ISE90	5.83 ± 0.05^b	0.63 ± 0.02^b	14.03 ± 0.14^a
BK1	6.17 ± 0.05^a	0.52 ± 0.01^c	13.92 ± 0.18^a

Data are the average of three replicate ± standard error of the mean.
Lower case letters indicate statistically significant differences among strains.

3.4. Expression of glycerol synthesis genes: GPD1 GPD2, GPP2, GPP1

The normalized mRNA levels of the four genes involved in the synthesis of glycerol are shown in Fig. 5. Time points EP and 0 showed the mRNA level at the exponential phase in YPD and at time of the inoculum, before the effect of hyperosmosis. Comparing the two genes encoding for the first enzymatic step, *glycerol-phosphate-dehydrogenase GPD1* and *GPD2*, the second gene is less expressed with an average initial mRNA level in the strains about one order of magnitude lower than *GPD1.* The gene *GPD1*, despite being the most transcribed during the initial phase, showed a weak induction in the strains tested in the experiment; among strains, a significant change ($p \leq 0.05$) was detected only in strain BK1 which showed, with respect to time zero, a five-fold increase in the level of mRNA at 10 min, later reducing the transcription. Between 10 and 60 min significant differences in strains ISE19, ISE90 and BK1 were found.

GPD2 does not seem to be significantly induced with the exception of strain BK1 ($p \leq 0.05$), where it showed a maximum



five-fold increase from 30 to 60 min with respect to time zero. In this strain, relative mRNA level was significantly higher than the other two strains at 30 and 60 min.

The two genes *GPP2* and *GPP1* coding for the second enzymatic step *glycerol-3-phosphatase* which leads to the formation of glycerol, showed similar mRNA levels at time zero (Fig. 5). Regarding gene *GPP2*, after the first 5 min, it showed a potent but transient induction, in all strains tested, ($p \leq 0.01$), reaching its maximum after 30 min followed by an immediate repression. The intensity of this induction varied significantly depending on the strain; in BK1 *GPP2* is more induced with a maximum around 190-fold higher with respect to the initial value after 30 min of exposure to high sugar concentrations, while in strains ISE90 and ISE19 had a mRNA peak at 30 min of approximately 90- and 25-fold compared with time zero, respectively.

The *GPP1* gene (Fig. 5) was strongly induced ($p \leq 0.01$), even if to a lesser extent than *GPP2*, and with maximum mRNA level varying, also in this case, depending on the strain. In strain BK1, *GPP1* had a maximum induction at 30 min of about 90-fold compared with time zero. The other two strains showed a significantly less induction which was delayed by 30 min with respect to BK1. As in the isogene *GPP2*, the response of *GPP1* to the hyperosmotic environment was transient, showing a drastic reduction of mRNA level after 30–60 min.

3.5. Expression of glycerol flux genes: STL1 and FPS1

The two genes coding for aquaglyceroporin *FPS1* and glycerol transporter *STL1* showed different transcriptional behaviour during the experiment, although starting from similar initial values (Fig. 6). *FPS1* is expressed constitutively showing no significant changes during the experiment; *STL1* on the contrary showed significant variation ($p \leq 0.01$) with a strong induction starting from 10 min after inoculation into the must. Also, for this gene, the transcription response was transient being drastically repressed after 30 min. The two strains, BK1 and ISE90, showed a maximum mRNA level at 30 min of about 80 and 40-fold, respectively, in comparison to time zero; while ISE19 had a peak of mRNA at 10 min with an 80-fold intensity with respect to 0 min. Also for this gene, at the peak of transcription, strain BK1 exhibited a greater mRNA level with respect to the other two strains.

3.6. Expression of aldehyde-dehydrogenase genes ALD3, ALD4, ALD6

The genes coding for *aldehyde-dehydrogenase* (Fig. 7) show different mRNA levels at the beginning of the experiments. In particular, considering the moment of inoculation, *ALD4* was transcribed by three orders of magnitude less than *ALD6* and four orders less than *ALD3*. Furthermore, *ALD4* also tends to reduce its mRNA level ($p \leq 0.01$) becoming undetectable at 120 min in BK1 and was in very low concentrations in the other two strains. *ALD3* was transcribed to a greater extent during the experiment respect the other two isogenes, but showed a big difference among the two strains, ISE19 and ISE90, compared with the commercial strain BK1. Despite this, evolution during the experiment was very similar among strains after 10 min, with a drastic repression ($p \leq 0.01$) of

Fig. 3. Ethanol (a), acetate (b), extracellular glycerol (c), intracellular glycerol (d), production by *Saccharomyces cerevisiae* strains BK1 (black bars), ISE90 (grey bars), ISE19 (light grey bars) after inoculation into grape must; (e) cell concentration calculated as optical density (OD). When reported, lower case letters indicate the time point at which statistically significant differences were found among strains. In a, b, c, d, concentrations are expressed in mmoles normalized toward OD. Error bars represent standard error of the mean ($n = 3$).

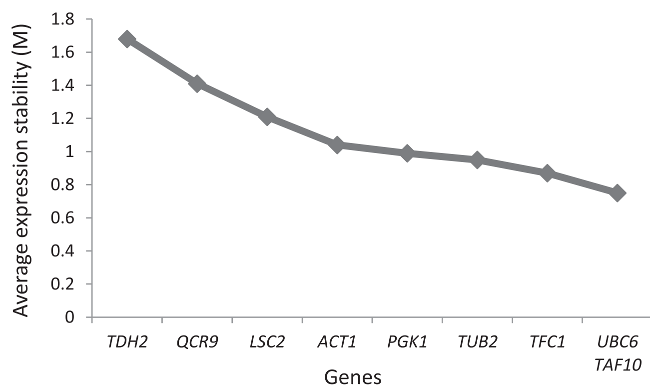


Fig. 4. Average expression stability values (M) of the remaining control genes during stepwise exclusion of the least stable gene, performed by GeNorm software on the candidate reference genes in all fermentations.

transcription. *ALD6*, although presenting similar values in all strains at time zero, showed different trends for ISE19 and ISE90 with respect to the commercial strain BK1. The former two strains showed an *ALD6* induction ($p \leq 0.05$) at 5 min after inoculation with a maximum between 10 and 30 min, their mRNA levels then decreased drastically. On the contrary, strain BK1 showed *ALD6* mRNA that, after 5 min, reduced its abundance ($p \leq 0.01$) to almost undetectable levels at 120 min.

4. Discussion

The introduction of *S. cerevisiae* into a medium rich in sugar, such as grape must, causes at the molecular level, a series of adaptive responses that influence the yeast implantation in grape must and the progression of fermentation, influencing in particular the duration of the lag phase. While the immediate response to osmotic stress by *S. cerevisiae* has been widely studied in laboratory conditions using synthetic media, there is little information on what happens in real conditions using natural grape juice. This is important considering that we found significant differences in the expression of genes when studied during fermentation in natural and synthetic must (unpublished results), and it is difficult to compare the observations for yeast fermented in synthetic media to the behaviour in natural grape must under winery conditions (Viana et al., 2014). Our work investigated the genes related to the osmoprotective metabolite glycerol under real fermentation conditions using natural grape must, from the period of inoculation up to 120 min of fermentation.

Starting from a preliminary screening of the collection we have verified the metabolic variability of eighty genetically characterized strains of *S. cerevisiae*. We identified two strains, ISE19 and ISE90, exhibited significative metabolic differences in acetate and glycerol production; these metabolic characteristics have been confirmed in natural must and compared with a commercial strain BK1. In particular, among the strains analyzed, ISE19 proved to be less efficient during fermentation with a sluggish end stage and produced significantly higher levels of acetate and glycerol. This is probably caused by a higher rate of diversion of the glycolytic flow towards glycerol and acetate, which is reflected in a lower ethanol production that has been observed in this strain. The commercial strain BK1 proved to be efficient in fermentation and produced a lower concentration of acetate.

Based on these data we hypothesized that the intensity of the response of the yeast strain during the early stages of fermentation, through the mechanisms of adaptation to hyperosmosis generated by high concentrations of sugars, may concur with the

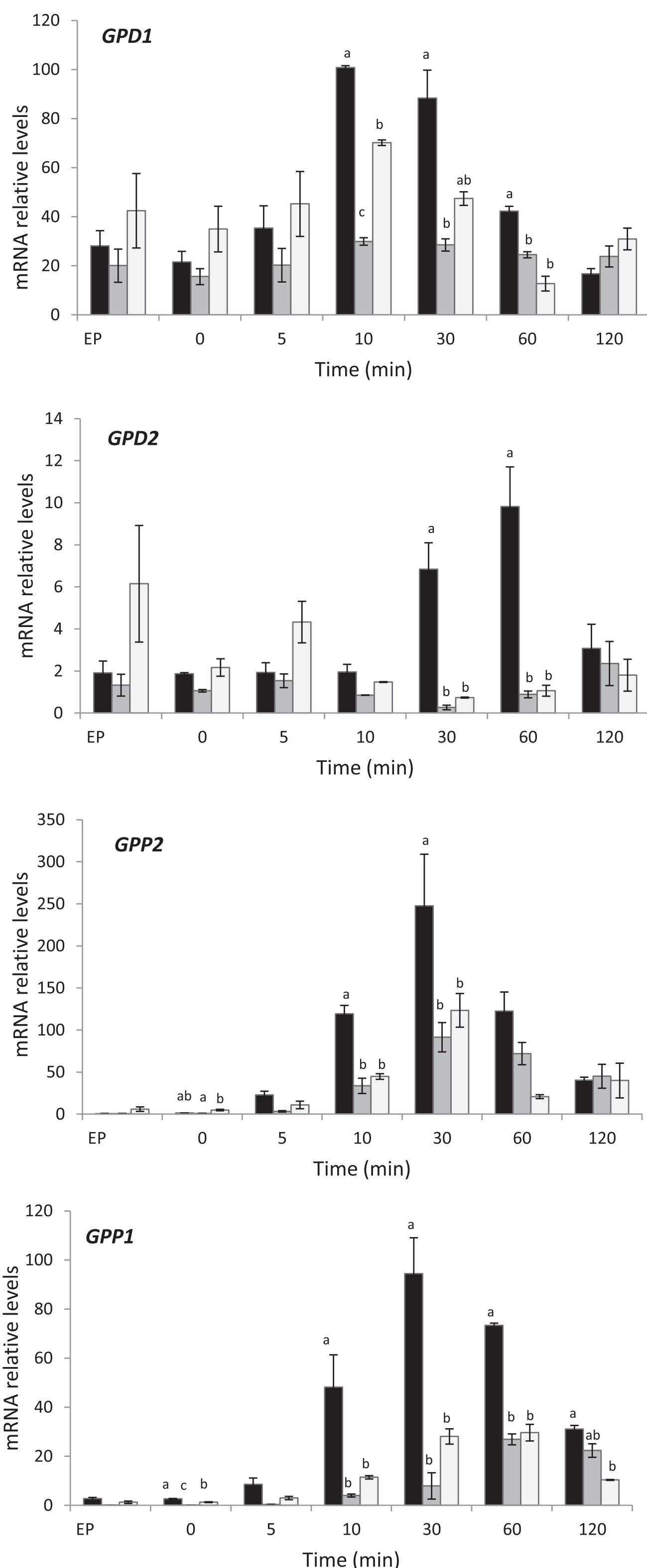


Fig. 5. Expression of glycerol synthesis genes *GPD1*, *GPD2*, *GPP1* and *GPP2* in *Saccharomyces cerevisiae* strains BK1 (black bars), ISE90 (grey bars), ISE19 (light grey bars) after inoculation into grape must up to 120 min mRNA levels are normalized using a geometric mean of the reference genes *TFC1*, *TAF10* and *UBC6* expression values transformed in relative copy numbers. Point EP represents the expression value at the exponential phase in YPD pre-propagation. When reported, lower case letters indicate the time point at which statistically significant differences were found among strains. Differences in expression relative to time are reported in the text. Error bars represent standard error of the mean ($n = 3$).

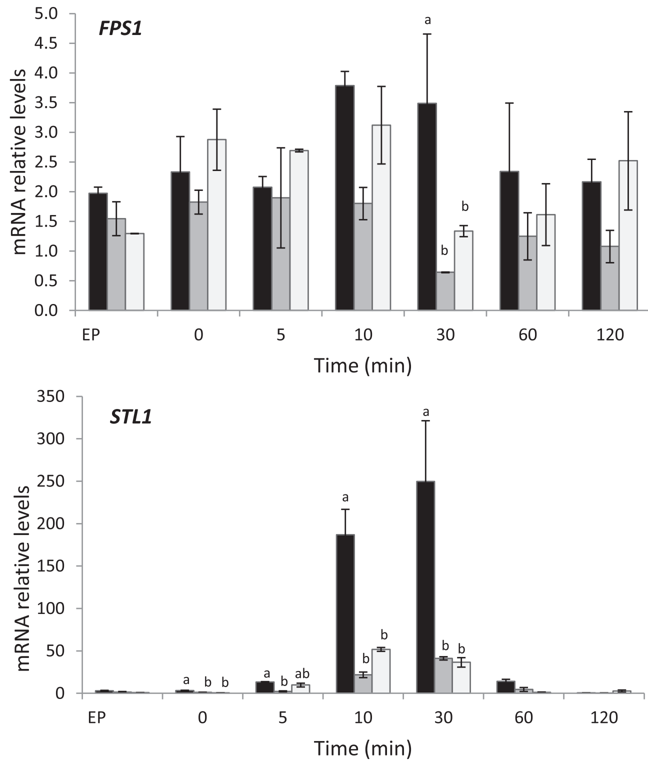


Fig. 6. Expression of glycerol flux genes *STL1* and *FPS1* in *Saccharomyces cerevisiae* strains BK1 (black bars), ISE90 (grey bars), ISE19 (light grey bars) after inoculation into grape must. mRNA levels are normalized using a geometric mean of the reference genes *TFC1*, *TAF10* and *UBC6* expression values transformed in relative copy numbers. Point EP represents the expression value at the exponential phase in YPD pre-propagation. When reported, lower case letters indicate the time point at which statistically significant differences were found among strains. Differences in expression relative to time are reported in the text. Error bars represent standard error of the mean (n = 3).

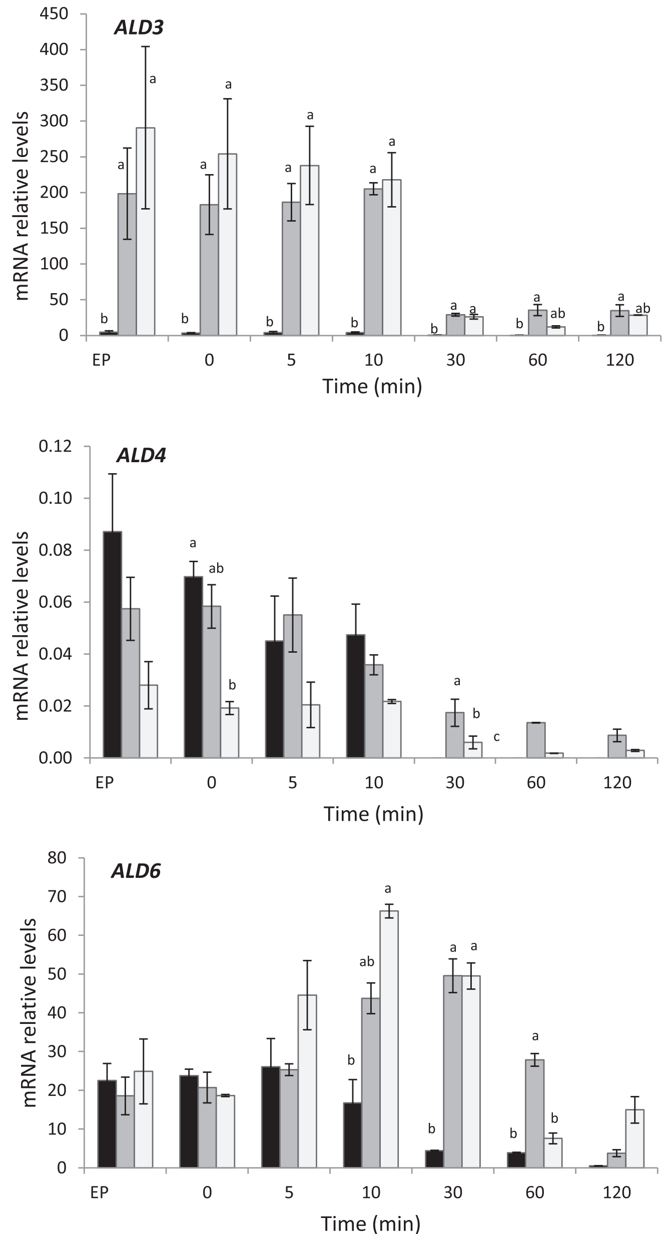


Fig. 7. Expression of *aldehyde dehydrogenase* genes *ALD3*, *ALD4* and *ALD6* in *Saccharomyces cerevisiae* strains BK1 (black bars), ISE90 (grey bars), ISE19 (light grey bars) after inoculation into grape must. mRNA levels are normalized with geometric mean of the reference genes *TFC1*, *TAF10* and *UBC6* expression values transformed in relative copy numbers. Point EP represents the expression value at the exponential phase in YPD pre-propagation. When reported, lower case letters indicate the time point at which statistically significant differences were found among strains. Differences in expression relative to time are reported in the text. Error bars represent standard error of the mean (n = 3).

determination of differences in the production of metabolites at the end of fermentation and thus, wine quality.

From a metabolic point of view, observing the production of intra- and extracellular glycerol, acetate and ethanol in the first two hours of fermentation, no significant differences between the two strains, ISE19 and ISE90, were detected and they produced a similar concentration of these products, with the exception of extracellular glycerol. Interesting data were found for the accumulation of intracellular glycerol in these two strains. The reaction to the hyperosmotic environment was evident between 10 and 30 min after inoculation; the cell in this short period accumulated glycerol and then reached a "plateau" of concentration, as previously observed (Hohmann and Mager, 2007; Petelenz-Kurdziel et al., 2013). The entry into this plateau phase coincides with the beginning of the rise in extracellular glycerol which starts to increase from 30 min. These data indicate a rapid reaction of the cell which in the first minutes of fermentation faces a hyperosmotic change resulting in the production and accumulation of glycerol, reaching an optimal intracellular concentration; once the plateau phase has been reached, the cell begins to diffuse glycerol into the extracellular environment (Hohmann and Mager, 2007). Different behaviour was observed for strain BK1; this strain exhibits a delay in the production of all the metabolites, with intracellular glycerol not reaching the plateau level after two hours of monitoring. It is interesting to see a correlation between the accumulation of intracellular glycerol with cell growth data: only in two strains, i.e., ISE90 and ISE19, a cellular growth is observed and they started to grow only when the intracellular glycerol reached the plateau level. This data can be related to observations which indicate that when the cell is involved in an osmotic response, the glycolytic flux towards glycerol is at the expense of biomass and therefore the cell does not replicate (Petelenz-Kurdziel et al., 2013).

The study of gene expression was preceded by examining potential reference genes, as prior validation of the reference system is an essential prerequisite for reliable expression data as reported in MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines (Bustin et al., 2009). After analysis using the GeNorm software (Vandesompele et al., 2002) *TFC1*, *TAF10*, *UBC6* were found to be the most stable genes when

analysing the entire data set of samples of the three strains, confirming the finding observed in *S. cerevisiae* during long-term growth with glucose (Teste et al., 2009).

The expression of genes related to the biosynthesis of glycerol and its transport highlights the role of the MAP-HOG signalling system in the hyperosmotic stress response under real wine fermentation conditions for the three strains in this study. The results obtained in our study confirm the importance of the *GPD1* gene in this stress response, its expression level during the experiment being around 10-fold higher than the paralog *GPD2* gene, which is not under HOG control. Despite this, the *GPD1* gene is not strongly induced during the experiment with the exception of strain BK1; these observations indicate that the gene is also influenced by other factors in addition to the HOG signalling system or that HOG control occurs only partially (Remize et al., 2003). Another hypothesis is that the cell implements, for biosynthesis involving *glycerol-3-phosphate dehydrogenase*, other types of regulation, e.g., at the post-transcriptional, translational or post-translational level (Bouwman et al., 2011). On the contrary, the two isogenes responsible for coding *glycerol-3-phosphatase*, *GPP1* and *GPP2* and glycerol transporter *STL1* are strongly activated, and even here BK1 shows greater expression than the other strains. Genes controlled by the HOG system, *GPD1*, *HOR2*, *RHR2* and *STL1*, undergo upregulation between 10 and 60 min followed by a downregulation phase. This particular "transient" expression behaviour was previously observed using synthetic must (Rep et al., 1999; Pérez-Torrado et al., 2005).

These observations combined with the generally higher expression of glycerol biosynthetic genes and transporter *STL1* gene in strain BK1, along with the glycerol metabolic data, may indicate a negative feedback regulation mechanism in the signalling system. In this view, the cells repress the transcription of "glycerol related genes" involved in the synthesis and uptake of the metabolite, when they reach an optimum intracellular level, which is visible from a metabolic point of view when they achieve the "plateau" phase. Strain BK1 cells were shown to be less efficient in reaching this optimum and try to enhance glycerol production and accumulation, inducing higher gene transcription. This mechanism has proven to work for the HOG signalling cascade and even has an impact on the expression of HOG controlled genes (Hohmann, 2009).

Hyperosmotic stimulation has no effect at the transcriptional level on the *FPS1* gene, coding for the aquaglyceroporin channel, which showed no significant variation during the experiments. The action of the HOG1 regulation system on FPS1 is based on other mechanisms, activating or not the channel closure, which regulates the glycerol flux (Lee et al., 2013). In particular, when yeast reaches an optimal level of internal glycerol, the channel can be opened and glycerol freely flows into the extracellular environment. (Geijer et al., 2012; Ahmadpour et al., 2014). This phenomena was visible, in our experiments, for strains ISE19 and ISE90 where extracellular glycerol started to grow when the intracellular glycerol reached the plateau level.

In our study, the *ALD6* gene was the only one among the three genes coding for *acetaldehyde-dehydrogenase* to show weak induction in two of the three strains studied, although after 30 min of hyperosmotic exposure a decrease in its concentration was observed. *ALD6* has been indicated as mainly responsible for the production of acetate during fermentation as noted by Saint-Prix et al. (2004) and Erasmus et al. (2003) and it is not repressed by glucose (Dickinson, 1996). Our data seem to confirm this observation also at the beginning of fermentation and in this case the higher expression of *ALD6* in ISE19 and ISE90 may to be correlated with the higher production of acetate in these two strains found in the first 120 min. Regarding *ALD3*, encoding for *aldehyde dehydrogenase* NAD+ dependent, in strains ISE19 and ISE90 we observed a high concentration of initial mRNA but with a drastic repression after 10 min. The glucose repression by inoculation into a sugar-rich medium (Navarro-Aviño et al., 1999) was activated with a certain delay and seems to be consistent with the general observation made in our study that some time (minutes) is necessary to cause transduction of the environment change signals to transcriptional effectors. In addition, for this gene there is a great difference in the mRNA level in strain BK1, being much lower with respect to the other two strains, ISE19 and ISE90, although the repression trend is similar. Although the principal role of the *ALD3* gene is in β-alanine production, the differences in mRNA level may explain the differences found in acetate production, considering the non-specific action of *aldehyde dehydrogenase* encoded by this gene (White et al., 2003). Among the three *ALD* genes studied, the gene coding for the K+ mitochondrial *aldehyde dehydrogenase ALD4* appears to have a minor role in acetate production during these early fermentative stages considering the low abundance of its transcription. Our data confirm, at the transcriptomic level, the repression operated by glucose (Llorente and Nunez de Castro, 1977).

Observing the metabolic and transcriptomic data here reported it is clear that yeasts, under winemaking conditions, are able to respond and adapt themselves, after a few minutes, to the hyperosmotic environment by implementing mechanisms that lead to the accumulation of intracellular glycerol until this reaches an optimal level for osmoprotection. Once adapted, yeast cells can turn on their metabolism for biomass production, determining the exit from the lag phase.

Significant results, coming from the present investigation, underline differences among strains both at transcriptional and metabolite level. In particular, as compared to the other two strains tested (ISE 90 and ISE19), commercial strain BK1 showed differences in the mRNA levels of genes involved in glycerol metabolism and acetate synthesis via *aldehyde dehydrogenase*. These transcriptomic differences can probably account for the different production of metabolites detected for this strain in the first 120 min.

For what concerns ISE19 and ISE90 (which were selected on the basis of a different glycerol production at the end of fermentation), experimental data highlight that these differences are not ascribable to a different response to hyperosmotic environment during the first two hours of fermentation, since their profiles both at transcriptomic and metabolite level are very similar. Conversely, in strain BK1 the low acetate concentration, detected both at the beginning and at the end of fermentation, is likely related to a different expression level during the first 120 min of mRNAs encoding ALD. Further studies are necessary to validate this finding on a larger number of yeast strains.

From a technological perspective, these observations can be helpful in the selection of wine yeasts, considering that the latency period represents the moment of greatest vulnerability from a competitive point of view, as wild non-*Saccharomyces* and *Saccharomyces* yeast, already adapted and able to potentially start and dominate the fermentation, are present in the must. Furthermore, understanding the mechanisms of adaptation can be helpful in the implementation of premultiplication and inoculation protocols which could shorten the latency phase and favour the beginning of exponential multiplication, resulting in a competitive advantage for the inoculated strain.

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