Sublethal HPH treatment is a sustainable tool that induces autolytic-like processes in the early gene expression of Saccharomyces cerevisiae

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Davide Gottardi [a], Lorenzo Siroli [a,b], Giacomo Braschi [a], Samantha Rossi [a], Diana I. Serrazanetti[c], Francesca Patrignani [a,b*], Rosalba Lanciotti [a,b1]

[a] Department of Agricultural and Food Sciences, Campus of Food Science, Piazza Goidanich 60, Cesena (FC), Italy;

[b] Interdepartmental Centre for Agri-Food Industrial Research, Campus of Food Science, Via Quinto Bucci 336, Cesena (FC), Italy

[c] Istituto d'Istruzione Superiore "A. Cecchi", Via Caprile 1, Pesaro (PU), Italy

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[1] Correspondence: rosalba.lanciotti@unibo.it; fancesca.patrignani@unibo.it; Tel.: +39 0547 338132

Abstract

Sublethal HPH treatments have been demonstrated to impact the technological properties and functions of treated microorganisms by inducing specific enzymes/genes or modulating membrane structures and inducing autolysis. In this work, the early effects of a 100 MPa HPH treatment on the winery starter *Saccharomyces cerevisiae* ALEAFERM AROM grown in synthetic must were assessed. While there were no differences in cell cultivability during the first 48 h between treated and untreated cells, a reduction in volatile metabolites released by HPH-treated cells during the first 2 h was observed. This reduction was only temporary since after 48 h, volatile molecules reached similar or even higher concentrations compared with the control. Moreover, the gene expression response of HPH-treated cells was evaluated after 1 h of incubation and compared with that of untreated cells. A massive rearrangement of gene expression was observed with the identification of 1220 differentially expressed genes (DEGs). Most of the genes related to energetic metabolic pathways and ribosome structure were downregulated, while genes involved in ribosome maturation, transcription, DNA repair, response to stimuli and stress were upregulated. These findings suggest that HPH induces or promotes an autolytic-like behaviour that can be exploited in winemaking.

Key words: High-pressure homogenization, *Saccharomyces cerevisiae*, gene expression, volatile molecule profiles, autolysis

1. Introduction

High-pressure homogenization (HPH) has long been considered one of the most promising food processing technologies for the stabilization of liquid food and an alternative to thermal treatment (Bevilacqua et al., 2019; Carpentieri, Soltanipour, Ferrari, Pataro, & Donsì, 2021; Chevalier-Lucia & Picart-Palmade, 2019; Gottardi et al., 2021; Mesa et al., 2020; Wu et al., 2020). In fact, several papers report the effectiveness of HPH in deactivating pathogenic and spoilage microorganisms in model and real systems (Lanciotti et al., 1994; Guerzoni et al., 2002; Diels et al., 2003; Briñez et al., 2006a,b; Taylor et al., 2007; Pathanibul et al., 2009). Spatial pressure and velocity gradients, turbulence, impingement, cavitation and viscous shear have been identified as the primary mechanisms of microbial cell disruption and food constituent modification during HPH treatments. In fact, the mechanical stresses that the product encounters during the HPH process can generate different modifications on food constituents with very interesting industrial applications. For instance, HPH treatments can be exploited to produce stable emulsions, encapsulations and safer products, the latter through microbial, bacteriophage and enzyme inactivation (dos Santos Aguilar, Cristianini, & Sato, 2018; Levy, Okun, & Shpigelman, 2021; Liu & Kuo, 2016; Patrignani & Lanciotti, 2016). However, the effectiveness of HPH treatment, when applied to a food matrix, can depend on several factors, including the level of pressure applied, the number of cycles applied, the type of valve used, the composition of the food matrix, and the cell loads of naturally occurring microorganisms (Patrignani et al., 2016).

HPH can also be applied to recover intracellular components through cell disruption or to alter biopolymer (protein and sugar) structures, conferring new physical and physical-chemical properties (Comuzzo & Calligaris, 2019; Patrignani & Lanciotti, 2016). Most of the data presented in the literature deal with the use of HPH in the fruit and vegetable beverage and dairy sectors, while its use in the winemaking process has not been extensively studied (Comuzzo & Calligaris, 2019). In fact, HPH treatments have been mainly reported to improve the microbiological quality of wine by reducing the indigenous microbial cell loads present in must without affecting the nutritional and sensory properties of the final product (Puig, Olmos, Quevedo, Guamis, & Mínguez, 2008). Alternatively, HPH was applied at a high level (200–600 MPa, 1–3 passes, or 150 MPa, 10 passes) directly on the microorganisms used as starters to obtain inactive dry yeasts or autolyzed cells to be used in the wineries as nutrients for starter cultures or colloidal supplements during wine ageing (Dimopoulos, Limnaios, Aerakis, Andreou, & Taoukis, 2021; Voce, Calligaris, & Comuzzo, 2021). In this regard, modulation of HPH processing parameters could be used to obtain different outcomes. Another interesting application could be represented by high-pressure homogenization when used at sublethal pressures (equal to or lower than 100 MPa) and applied to bacteria or yeasts to improve their metabolic and enzymatic activities, impacting the features of the final products (Lanciotti, Patrignani, Iucci, Saracino, & Guerzoni, 2007; Serrazanetti et al., 2015; Siroli et al., 2020; Tabanelli et al., 2014). This aspect could be fundamental for winery innovation and differentiation. For instance, Patrignani et al. (2013) applied HPH treatment (90 MPa) on different strains of *S. cerevisiae* and *S. bayanus* to be used as tirage solutions for sparkling wine refermentation. While the treatment poorly affected yeast viability immediately after HPH treatment and refermentation behaviour, treated cells underwent accelerated autolysis during the ageing period, suggesting that HPH treatment could have stimulated the enzymatic pool involved in the autolytic process. However, in that paper, only phenotypic features were observed without any investigation of the changes involved in the yeast general metabolism. Moreover, Serrazanetti et al. (2015), studying the effect of a 90 MPa HPH treatment on *S. bayanus*, reported an upregulation of *OLE1* and *ERG3*, which are involved in unsaturated FA biosynthesis, 60 and 100 min after the treatment, suggesting a quick readaptation of gene expression. Although several works on yeast and bacteria have focused on functional, cell membrane or few gene modifications upon HPH treatments, no data have been reported regarding the whole genome response in relation to the application of a sublethal HPH treatment. Since the comprehension of genetic changes is fundamental to explain the phenotypic behaviour of a specific strain, also from the perspective of its application in industry, the present work wanted to study the early effects of sublethal HPH treatment (100 MPa) on the transcriptome of a commercial strain of *S. cerevisiae* ALEAFERM AROM used in winemaking to better understand the shift towards an autolytic feature that can then be exploited for oenological purposes. In particular, the gene expression (within 1 h of HPH treatment), cultivability and volatile molecule profiles (during 48 h) obtained by incubating HPH-treated cells were compared with those obtained using untreated cells (treated with 0.1 MPa) in synthetic must to obtain data that are exploitable for the oenological field.

2. Materials and methods

2.1 Yeast stain applied and culture conditions

This experiment was performed with the commercial product ALEAFERM AROM (Alea Evolution s.r.l.), a *Saccharomyces cerevisiae* strain specific for white wines endowed with technological properties such as alcohol tolerance (16%), cryotolerance, and fast fermentation kinetics. The strain was grown twice in yeast peptone dextrose (YPD) broth for 24 h at 30 °C before inoculum preparation. After 24 h, the inoculum was centrifuged (9000 rpm, 6 °C, 10 min) to remove the medium, and the pellet was resuspended in 600 mL of synthetic-must to reach a concentration of approximately 10^6 CFU/mL before treatment with HPH. The synthetic must was prepared according to the recipe as described in OIV-OENO 370-2012 made by the International Organization of Vine and Wine (OIV, 2012). The synthetic-must containing 230 g/L fermentable sugars (115 g/L glucose and 115 g/L fructose) was brought to pH 3.2 and filter sterilized (0.22 μm) (SARTOLAB 180C3) prior to its use.

2.2 HPH treatment

Three hundred out of 600 mL of the synthetic-must containing *S. cerevisiae* were treated with HPH. Homogenizing treatment was performed using a PANDA continuous high-pressure homogenizer (Niro Soavi, Parma, Italy). The inlet temperature of the cell suspension was 25 °C with an increasing rate of 2.0 °C/10 MPa. Cells were treated at 100 MPa for a few milliseconds and cooled by using a thermal exchanger (Niro Soavi, Parma, Italy), resulting in an outlet temperature of 15 °C. Upon treatment, 300 mL was split into three independent Erlenmeyer flasks (n=3). The remaining 300 mL of the synthetic-must containing *S. cerevisiae* was treated at 0.1 MPa and divided into three Erlenmeyer flasks and used as a control.

2.3 Fermentation and sample collection

All Erlenmeyer flasks (containing HPH-treated samples and controls) were incubated at 18 °C. Samples were collected after 1, 2, and 48 h for plate count and volatile molecule profile analyses and after 1 h for gene expression. Immediately after collection, samples for gene expression were mixed with 10 mL RNAprotect (Qiagen, Milano, Italia) and stored at -20 °C prior to RNA extraction.

2.5 Microbiological analyses

Samples collected at 1, 2, and 48 h upon HPH treatment were immediately serially diluted in sterile saline solution (NaCl, 0.9%). The dilutions were plated onto YPD agar and incubated at 30 °C for 48 h before counting the colonies.

2.6 Sample volatile molecule analysis

Volatile molecule profiles were determined by solid phase microextraction combined with gas chromatography and mass spectrometry (SPME–GC/MS). A polyacrylate-coated fibre (85 µm) (Supelco Inc., Bellefonte, PA, USA) and a manual SPME holder (Supelco Inc., Bellefonte, PA, USA) were used in this study after preconditioning, according to the manufacturer's instruction manual. Five millilitres of sample was placed in 10 mL glass vials and supplemented with 1 g of NaCl and 10 µL of 4-methyl-2-pentanol (Sigma, Milan, Italy) (final concentration 4 mg/L) as an internal standard. The samples were then equilibrated for 10 min at 45 °C. The SPME fibre was exposed to each sample for 30 min. Both the equilibration and absorption phases were carried out under stirring conditions. The adsorbed volatile molecules were desorbed in a gas chromatograph (GC) injector port in splitless mode at 250 °C for 10 min. GC–MS analyses were performed on an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5975C mass selective detector (Agilent Technologies, Palo Alto, CA, USA) operating in electron impact mode (ionization voltage 70 eV). A Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) was used (Chrompack, Middelburg, The Netherlands). Volatile compounds were separated using helium as the carrier gas (1 mL/min). The temperature program was 50 °C for 2 min, then programmed at 1.5 °C/min to 65 °C and finally at 4.5 °C/min to 220 °C, which was maintained for 20 min. The injector, interface and ion source temperatures were 250, 250 and 230 °C, respectively. The identification of the molecules detected was confirmed by comparing mass spectra of compounds with those contained in the available database (NIST version 2011) and those of pure standards. Quantitative analyses were performed with the internal standard and expressed as equivalent ppm (ppm eq.). For each compound detected, the ppm eq. represents the amount of compound present in the headspace in dynamic equilibrium with the aqueous phase.

2.7 RNA extraction and sequencing

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Milano, Italy). Quality checks, cDNA preparation, and library preparation were performed by Galseq (Milan, Italy). Libraries were generated with the Illumina TruSeq Stranded mRNA Library Prep kit and sequenced on an Illumina HiSeq instrument using a 2x150 paired-end strategy. The quality of the fastq sequencing files was initially assessed with the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reference genome for *S. cerevisiae* (GCF_000146045.2_R64_genomic.fna.gz) and the associated GFF annotation file (GCF_000146045.2_R64_genomic.gff.gz) were downloaded from the genome database of the NCBI repository (https://www.ncbi.nlm.nih.gov/genome/?term=Saccharomyces%20cerevisiae[Organism]&cmd=DetailsSearch).
The alignment was performed with the spliced aligner STAR v.2.5.0c (Dobin et al., 2013) using the --quantMode TranscriptomeSAM and GeneCounts parameters. Bam indexing was performed using samtools (H. Li et al., 2009). Bam/Bai pairs were manually inspected using the Integrative Genomics Viewer (Robinson et al., 2011). Differential gene expression analysis was performed using the DESeq2 tool (Love, Huber, & Anders, 2014). The contrast was defined as 'Case' vs. 'Control', where Case and Control represent the *treatment* and *absence of treatment* conditions. Differential analysis was performed on raw counts and visualization on rlog-transformed counts. The sample counts used for the analyses are reported in the supplementary table (**Table S1**). Only genes with |log2Fold change | ≥ 1 and padj < 0.05 were considered significant differentially expressed genes (DEGs) (Table S2). padj is the p value adjusted using the Benjamini–Hochberg procedure. All DEGs were annotated according to the Saccharomyces Genome Database (SGD, http://www.yeastgenome.org/), one of the renowned databases specific for *S. cerevisiae*. Gene Ontology (GO) enrichment analysis, Slim Term Mapper and KEGG pathways were performed using SGD, YeastMine (https://yeastmine.yeastgenome.org/yeastmine/begin.do), filtering enriched GOs at p value < $1x10^{-4}$ and KEGG mapper (https://www.genome.jp/kegg/mapper/). In addition, the functions of the DEGs and the related biological

pathways were also confirmed one by one through functional annotation of the same websites and related reports. The interaction networks of DEGs were obtained using the STRING v11.5 database (http://string-db.org/).

2.8 Validation of RNA-seq data

A total of eight DEGs were selected, and related primers for these eight genes were applied. In particular, primers for ACT1, BTN2, ATG8, ACC1 and HSP30 were taken from Hu et al. (2019), ADH1 and HXT1 from Nijland et al. (2017), MET17 from Wang et al. (2018) and ARO10 from Lee, Sung, Kim, & Hahn (2013). Primer sequences are reported in Table S3. RNA was extracted using MasterPureTM Complete DNA and RNA Purification (Lucigen). The yield and purity of each extraction were determined by measuring the absorbance at 260 nm and 280 nm using a BioDrop mLITE (BioDrop, Milan, Italy). Reverse transcription into cDNA was performed using the Reverse Transcription System Kit (Promega, Wisconsin, WI, United States) following the manufacturer's instructions. Reverse transcription quantitative PCR (RT–qPCRqPCR) was performed using a Rotor gene 6000 thermal cycler (Corbett Life Science, Mortlake, NSW, Australia). The ACT1 gene was used as a reference, and the fold change was quantified by the $2^{-\Delta\Delta Ct}$ method (Dong, Hu, Fan, & Chen, 2017). All tests were performed at least three times.

2.9 Statistical analysis

All the results were averages of at least three measurements taken from three experimental replicates. One-way ANOVA and Tukey's HSD test were carried out on the values found for the different parameters analysed, as well as on the absolute areas of the volatile compounds detected by SPME–GC–MS. Significant differences were considered at $p < 0.05$. ANOVA and principal component analysis (PCA) were carried out by the software Statistica for Windows (StatSoft, Tulsa, OK, USA), Version 8.0.

3. Results

3.1-Cell viability and volatile molecule profiles

Compared with control cells, the cultivability and viability of *S. cerevisiae* ALEAFERM AROM were not affected by sublethal HPH treatment upon 1, 2 and 48 h incubation in a synthetic must rich in fermentable sugars (230 g/L). In fact, cell counts were the same as those counted in control samples treated only with 0.1 MPa (**Fig. 1**).

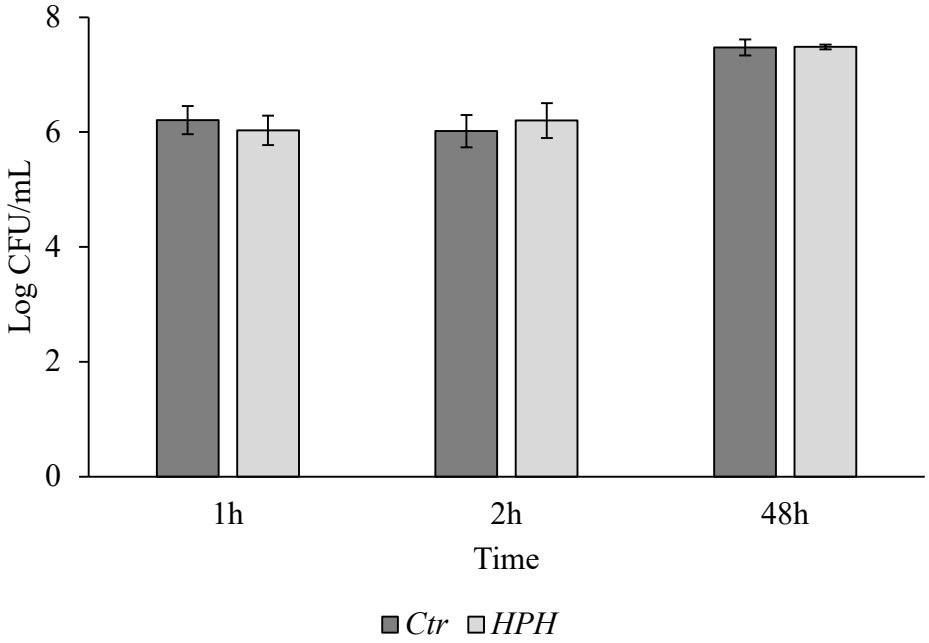

Figure 1. Counts of yeasts incubated in synthetic-must after 1, 2 and 48 hours upon HPH treatment. Colony forming units (CFU) were obtained by plate counts and reported as concentration (Log CFU/mL). Cells were treated or not (Ctr) with HPH (100 MPa x 1 passage) before incubation. The trial was performed in triplicate (n=3).

At the same sampling times considered for cell counts, volatile molecule compounds were measured in treated and untreated samples. Approximately 41 volatile compounds were identified using SPME/GC–MS, mainly belonging to ketones, acids, alcohols, and aldehydes (**Table 1**). After 1 h of incubation in synthetic must, treated and untreated cells generated similar profiles but a higher concentration of ethanol and acetic acid in the control. After 2 h, HPH-treated cells produced fewer ketones (especially methyl isobutyl ketone, 4-methyl-2-hexanone and 2,6-dimethyl-4-heptanone),

alcohols (such as ethanol, isoamyl alcohol, 1-hexanol, 1-octanol and furfuryl alcohol), acids (mainly acetic acid) and esters (es. ethyl acetate), while higher amounts of phenyl-ethanol ($p < 0.05$) were found. Higher production of this molecule was maintained up to 48 h after incubation, when a significantly higher release of benzaldehyde, ethanol, isoamyl alcohol, acetic acid, and octanoic acid ethyl ester was also observed in HPH-treated cells ($p < 0.05$).

To better highlight the effects of HPH on the volatilome of treated cells, SPME/GC–MS data were analysed by PCA (**Fig. 2**). The projection of the samples is reported in Fig. 2a, where PC1 and PC2 can explain approximately 59 and 31% of the total variance among the samples, respectively. Three main clusters were observed. The first one included all the samples collected after 1 h of incubation and the sample with HPH-treated cells after 2 h of incubation. The second cluster contained the untreated cells incubated for 2 h, while the third cluster was represented by samples collected after 48 h.

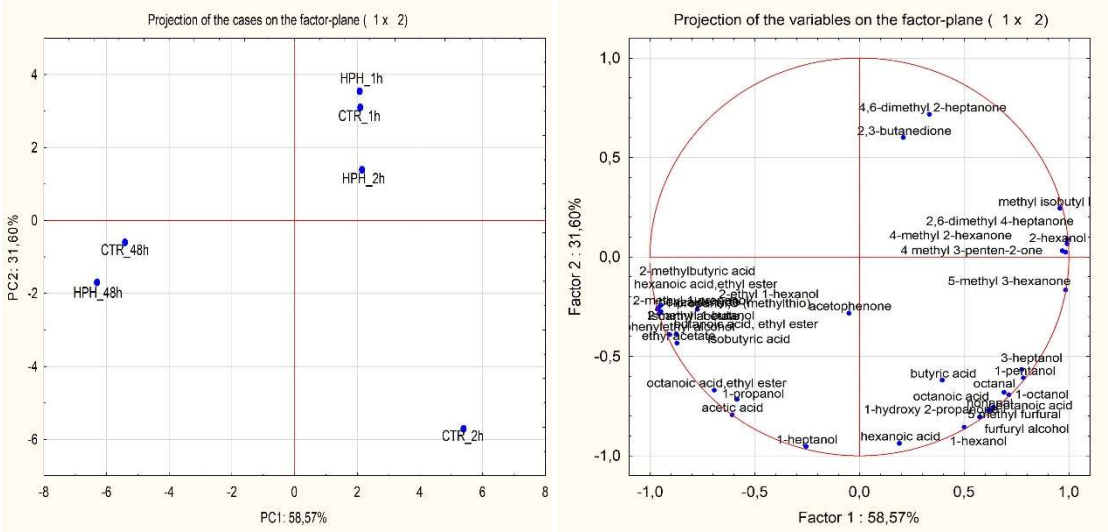

Figure 2. Projection on the factor plane (1x2) of HPH-treated (HPH) and untreated (CTR) cells of *S. cerevisiae* incubated in synthetic must for 1, 2 and 48 h upon treatment (a) and the respective variables (b).

3.3 Global transcriptional changes

Gene expression of samples containing HPH-treated and untreated cells was analysed after 1 h of incubation in synthetic must. As shown in **Fig. 3**, differentially expressed genes (DEGs) of HPH-treated and untreated cells were screened out. A volcano plot (**Fig. 3A**) plotting $\log_2$ (fold change) against $-\log_{10}$ (adjusted p value) was generated to represent the expression level difference and the statistically significant degree between the two samples. Each point represents a gene, the blue spots represent significant DEGs, and the red spots represent genes that do not show significant differences. For a global perspective on gene expression upon HPH, a heatmap representing the transcription levels of all DEGs was also generated, showing a complete rearrangement of gene expression between treated and untreated cells (**Fig. 3B**). DEGs with padj <0.05 were considered significantly different. According to this consideration, 1220 DEGs were identified, including 562 upregulated genes and 658 downregulated genes. The large number of DEGs suggests that HPH had a global effect on *S. cerevisiae*.

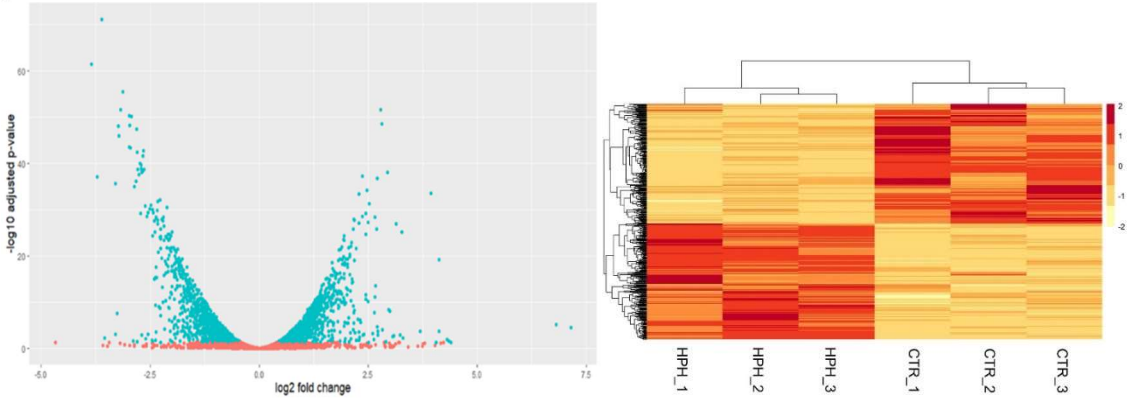

Figure 3. Screening and expression profiling of DEGs in HPH-treated and untreated cells of *S. cerevisiae* incubated for 1 h in synthetic must. A) Volcano plot for all differentially expressed genes (DEGs), which plotted the $-\log_{10}$ adjusted p value against $\log_2$ fold change (FC). Each point represents a gene, the green dot represents a significant DEG, and the red dot represents a gene that did not show a significant difference. B) Heatmap for DEGs using hierarchical clustering analysis. The signal ratios are shown in a red–yellow colour scale, where red represents upregulation and light yellow represents downregulation. Each column represents the expression value of the RNASeq performed on HPH-treated (HPH) and untreated (CTR) cells upon 1 h incubation in synthetic must, while each row represents a DEG. CTR_1-

CTR_3: three biological replicates of the control group; HPH_1-HPH_3: three biological replicates of the HPH-treated cells. **Colour should be used.**

3.4 GO classification analyses

To gain insight into the functional categories, DEGs were categorized into functional GO categories (Table S4) using the Yeast Gene Ontology (GO)-Slim bioprocess mapper (Dwight et al., 2002). **Figure 4** shows the distributions of the GO terms in the biological process, molecular function, and cellular component categories. The most dominant subcategories were involved in cytoplasm, nucleus, and mitochondrion (27.97, 24.28, 19.20%) for cellular component (**Fig. 4a**); in structural constituent of ribosome, transmembrane transporter activity, and oxidoreductase activity (9.62, 6.38, 6.30%) for molecular function (**Fig. 4b**); in cytoplasmic translation, rRNA processing, and transcription by RNA polymerase II (8.60, 8.12, 6.64%) for biological process (**Fig. 4c**). Looking more specifically at the functions, downregulated genes were mainly involved in cytoplasmic translation, cellular amino acid metabolic process, and nucleobase-containing small molecule metabolic process, while upregulated genes were mainly involved in transcription by RNA polymerase II, rRNA processing, and response to chemicals (**Table S5-S6**). Related to KEGG pathways, HPH induced a downregulation of 214 genes involved in all metabolic pathways (carbohydrates, lipids, and amino acids), 129 in biosynthesis of secondary metabolites and 86 genes of ribosome, while it induced an upregulation of 26 genes involved in metabolic pathways (mainly sulfur metabolism, acylglycerol degradation, arginine biosynthesis and aromatic amino acid metabolism), 19 genes of spliceosome and 12 in MAPK signalling pathway (**Table S2**).

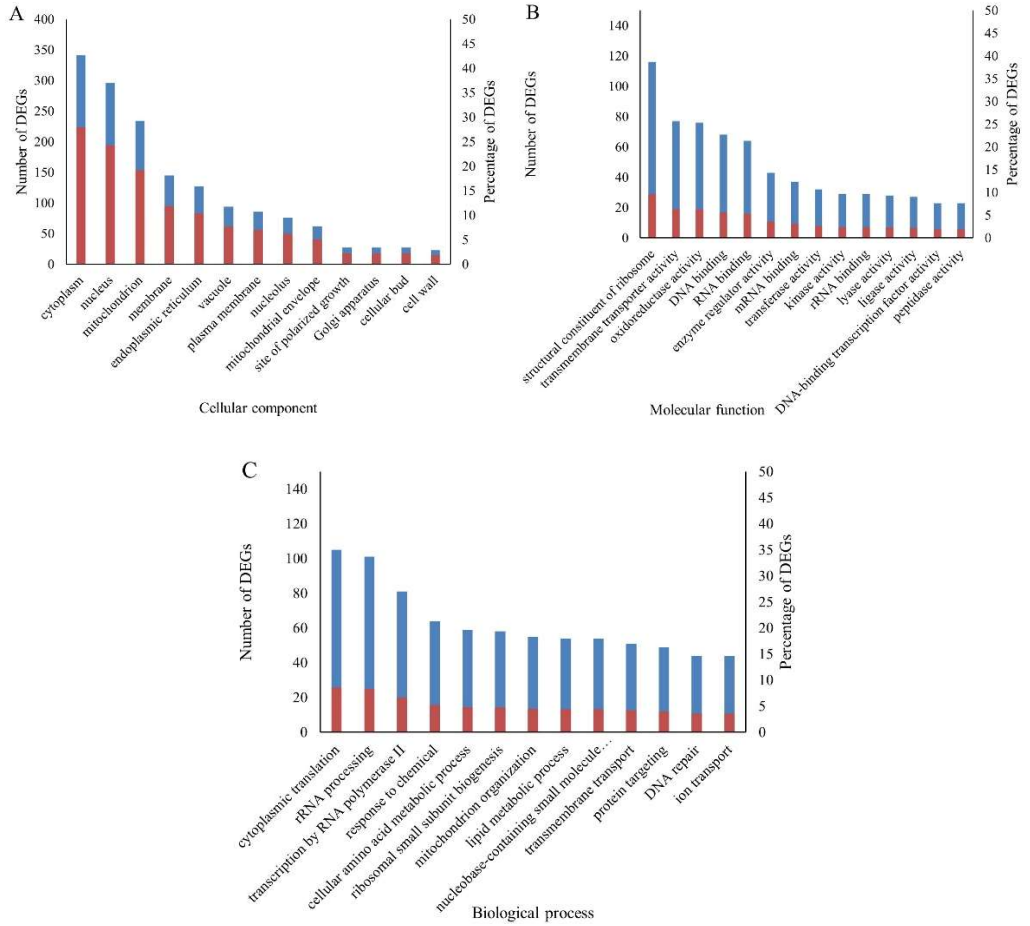

Figure 4. Classifications of the DEGs belonging to cells treated or untreated with HPH and subsequently grown for 1 h in synthetic must. The DEGs were classified into 3 functional categories using the Yeast GO-Slim bioprocess mapper: cellular component (a), molecular function (b) and biological process (c). **Colour should be used.**

3.5 RNA-seq expression validation by quantitative real-time PCR

To quantitatively determine the reliability of the transcriptome results, we detected the expression of eight candidate DEGs using reverse transcription quantitative PCR (RT–qPCR). These candidates included four upregulated (MET17, ARO10, BTN2, and ATG8) and four downregulated genes (ADH1, HXT1, ACC1, and HSP30). A high consistency was displayed between the RNA-seq and RT–qPCR data (**Fig. 5**), proving the validity of RNA-seq data for genes with distinct transcript abundance.

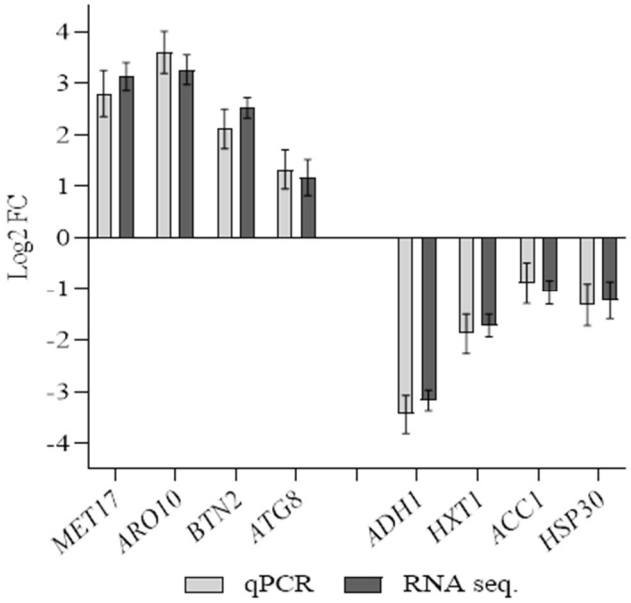

Figure 5. Validation of RNA sequencing data through qPCR. For qPCR data, the fold change indicates the ratio of the mRNA level of genes in the acetic acid group against the control group. ACT1 served as a reference in qPCR.

3.6. DEGs involved in cytoplasmic translation

Most of the genes of *S. cerevisiae* related to cytosolic ribosome machinery were downregulated upon HPH treatment and subsequent 1 h incubation in synthetic must. In fact, at least 100 genes related to ribosomes were DEGs, and their expression was significantly reduced (mainly *RPS3*, *RPS12*, *RPL3*, *RPS7A, RPL2B* and *RPP1B*) (**Fig. 6**; Tables S2 and S5). On the other hand, looking at the mitoribosome, the two rRNAs that compose the small and large subunits of the mitochondrial ribosomes (*15S rRNA* and *21S rRNA*) were upregulated upon HPH, with increases of 7.1 and 6.8 Log2 FC, respectively. Moreover, considering the 74 annotated mitoribosomal protein genes (MRPGs), 10 were DEGs, and among them, 8 were upregulated (mainly *RSM28, MRPS17* and *RSM27*), together with genes involved in mitochondrial transport (i.e., *TOM5, TOM7, PAM16, MMT1*).

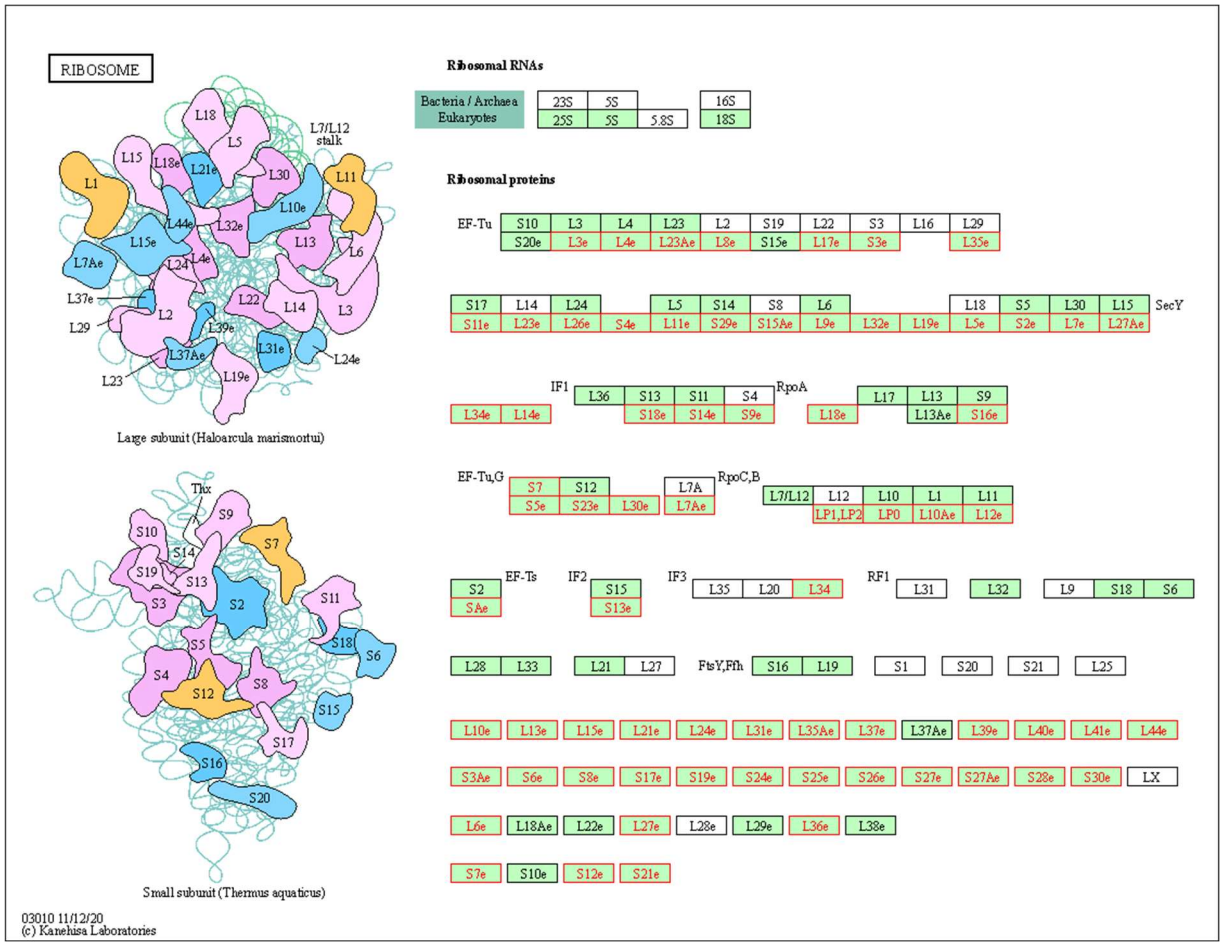

Figure 6. Visualization of KEGG ribosome pathway genes related to *Saccharomyces cerevisiae*. The red genes are those downregulated 1 h upon HPH treatment. **Colour should be used.**

RNA metabolic processes were also upregulated (Table S2 and S6). Sixty-seven genes were involved in transcription by RNA polymerase II. For instance, *RPO21*, *RPC10*, *RPB4* and *RPB9* are genes coding for proteins that are part of the RNA polymerase II subunits, while *TAF7*, *TAF8*, *TAF11*, and *TAF13* are basal transcription factors belonging to the TATA binding protein-associated factor (TAF) genes. Among the 61 genes involved in rRNA processing, 6 were ribosomal RNA processing (RRP) genes (*RRP14, RRP15, RRP17, RRP36, RRP45* and *RRP8*), and 14 were small nucleolar RNAs (snoRNAs, mainly *snR55, snR39B, snR83, snR51, snR17a*, and *snR78*), both involved in ribosome biogenesis and rRNA maturation. Even the regulator of ribosome synthesis gene (*RRS1*), which is required for nuclear export of the 60S pre-ribosomal subunit, was upregulated in HPH-treated cells. Other upregulated genes were those related to mRNA maturation. Other genes involved in mRNA export to the cytoplasm (*HRB1*), or mRNA decapping (*EDC1*) were also upregulated. Eventually, 19 genes involved in the spliceosome machinery were upregulated, such as *SNU23* and *SNU66*, both components of U4/U6. U5 small nuclear ribonucleoproteins (snRNP) complex, *YHC1*, component of the U1 snRNP complex required for pre-mRNA splicing; *HSH49*, U2-snRNP associated splicing factor, *SLU7*, an RNA splicing factor, and *PRP21*, *PRP24* and *PRP3* required for spliceosome assembly, *SPP2* required for the final stages of spliceosome maturation and activation, and *SME1*, *SMD3*, part of the spliceosomal U1, U2, U4, and U5 snRNPs (Fig. 7).

3.7 DEGs involved in the main metabolic processes and energy metabolism

From a metabolic point of view, HPH-treated cells incubated for 1 h in synthetic must showed a downregulation of almost all the cell metabolic pathways (**Fig. 7**, Table S2). Specifically, downregulation included most of the genes involved in central carbon metabolism (such as *PGK1, PGI1, ENO1, ENO2, TDH3* and *TPI1,* among the most repressed ones), alcoholic fermentation (i.e., *PDC1, PDC5, ADH1,* and *ADH2*), lipid metabolism (in particular those related to fatty acid and sphingolipid biosynthesis, such as *FAS1, FAS2, ACC1, FAA1, FAA3, LCB3, ELO2, ELO3, YSR3, LAC1*, and *ERG10*), and biosynthesis of cofactors (*ADE12, ADE13* and *ADK1* for adenine ribonucleotide biosynthesis, *THI4* and *THI6* for thiamine biosynthesis) (Tables S2 and S6). Regarding amino acid metabolism, HPH downregulated genes involved in aromatic amino acid metabolism (such as *ARO1, ARO2, ARO4, TRP2, TRP3,* and *TRP5*), arginine biosynthesis (*ARG2, ARG4, ARG7, CAR1,* and *CAR2*), branched-chain amino acid metabolism (*LEU1, LEU4, ILV3* and *ILV5*), and alanine, aspartate and glutamate metabolism (*GDH1, GDH3, GLT1, AAT2,* and *ASP1*). Upregulation was only related to some

genes involved in phenylalanine, tyrosine, and tryptophan biosynthesis (*ARO7, ARO9*, and *ARO10*), arginine biosynthesis (*ARG3* and *ARG80*), sulfur metabolism and assimilatory sulfate reduction (*MET3, MET16, and MET14*) (Tables S2 and S5).

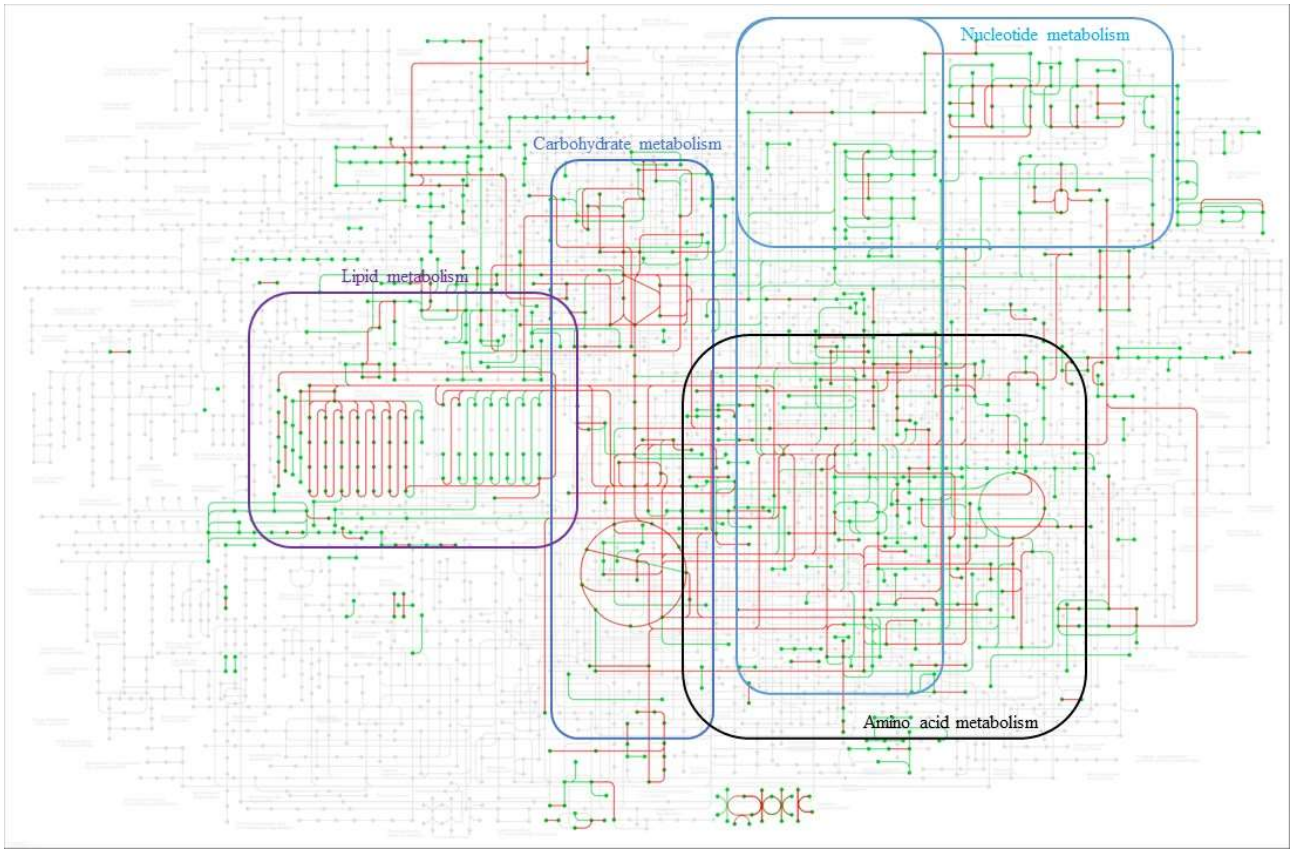

Figure 7. Visualization of KEGG Metabolic pathways present in *Saccharomyces cerevisiae* (green). The red nodes and lines represent all the pathways affected by the downregulated genes 1 h upon HPH treatment. **Colour should be used.**

3.8 Transmembrane transport

Even the uptake and transport of various compounds and metals were inhibited by HPH. In total, 90 genes related to transmembrane transport were downregulated, such as plasma membrane H^+ and P-type ATPase (*PMA1, PMA2, ENA1*), mitochondrial ATP synthase (*ATP1, ATP2*), vacuolar H^+-ATPase (*VMA2, VMA3, VMA5*), amino acid transport (*AGP1, AGP2, GAP1, PUT4, SAM3, HNM1, TAT2*, and *YPQ2*), sugar transport (*HXT1, HXT17, CIN10, GLK1, HXK1*), and zinc and iron transport (*FTR1, SIT1, ZRT1, ZRT2, ZRC1*).

3.9 DEGs involved in stress and chemical response

Sixty-three genes related to chemical, osmotic and oxidative stress were upregulated in response to HPH (Table S2 and S5), including chaperone- or chaperone-related genes (*CDC37, CUR1, SIS1, HSP4, HSP82, SSA4*), components of the MAPK pathway (*RLM1, RPI1, STE12, MID2, MTL1, PAF1, CLB2* and *BAR1,* **Fig. 8**); osmotic stress genes (*MOT3, ROX1*); and genes involved in DNA repair (*PPH3, RAD59, POL32, RAD10, PSY3, DPB1, WSS1, IRC4, AHC1, MPH1, SHU2, RAD34*, and *YKU70*). On the other hand, 49 stress-related genes were downregulated, including genes coding for peroxidase and oxidoreductase (such as *GRX2, TSA1, PRX1* and *CTT1*) (Table S2 and S6).

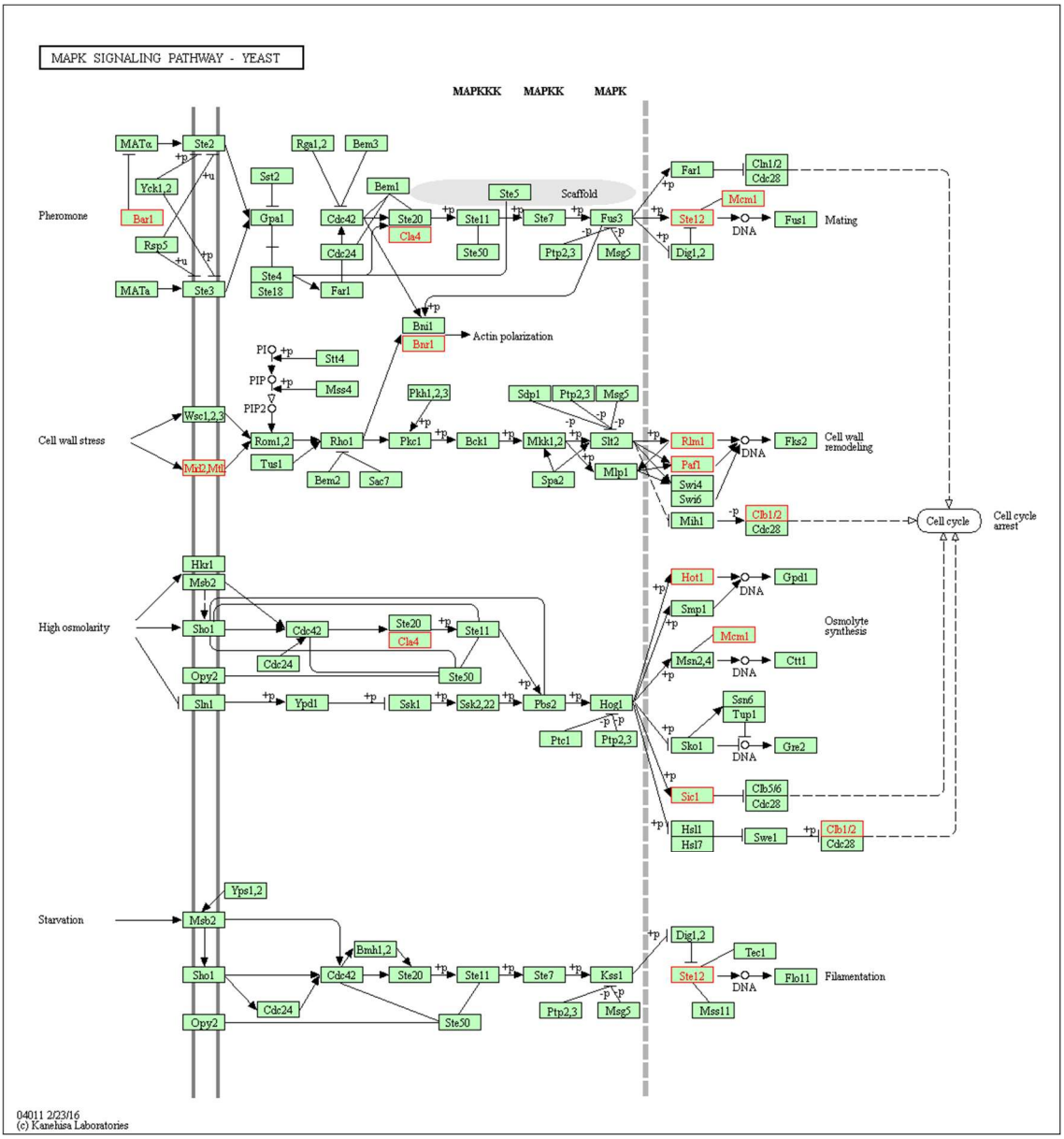

Figure 8. Visualization of KEGG MAPK signalling pathway genes related to *Saccharomyces cerevisiae*. The red genes are those upregulated 1 h upon HPH treatment. **Colour should be used.**

3.10 Interactions of the identified DEGs

The results were integrated and predicted in the STRING database (http://stringdb.org/) (Szklarczyk et al., 2015). **Figure 9** shows the different interaction networks of upregulated and downregulated DEGs after 1 h of HPH treatment and subsequent incubation in synthetic must. Among the upregulated DEGs, two main clusters with close and extensive interactions (confidence score > 0.9) were formed, containing genes involved in ribosome biogenesis (such as *RRP8, RRP15, RRP17, RRP36, and RRP45*) and spliceosome formation (including *SME1, HSH49, SMD2, SMB2, and PRP3*). Moreover, another cluster with less extensive interactions made of genes involved in the regulation of primary metabolic processes, such as the polymerase II transcription process (*TFB5, TOA1, RPB4, TAF7*) and histone acetylation (*AHC1, EAF6, SAS3, YNG1, EAF7*), was observed.

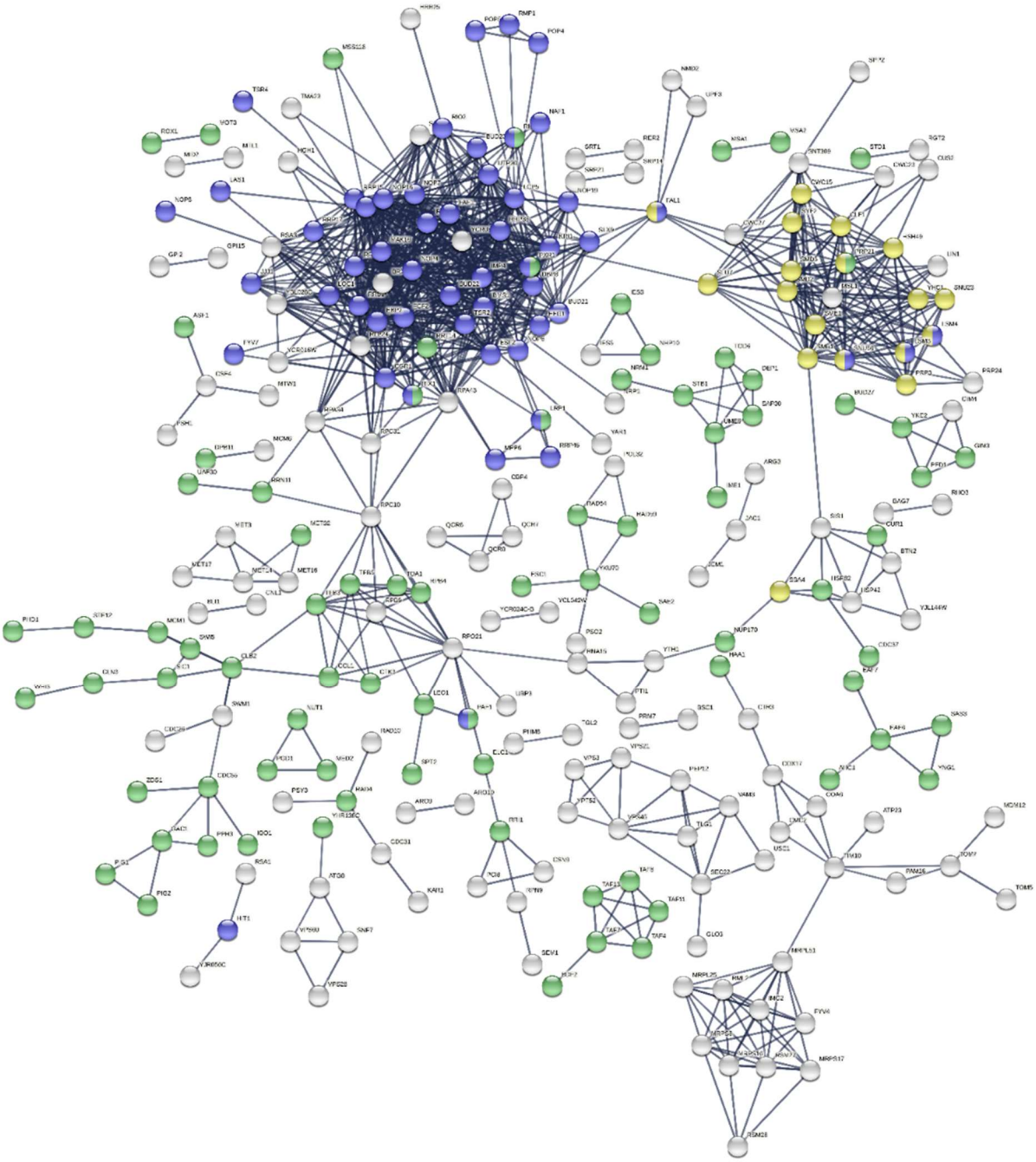

Figure 9. Interaction network of upregulated DEGs with a confidence score > 0.9. Interactions are indicated by edges, with thicker edges having stronger associations. The edges indicate both functional and physical protein associations. Colours represent genes in specific GO biological processes (green: regulation of primary metabolic process; blue: rRNA processing; yellow: spliceosome). **Colour should be used.**

The downregulated genes clustered in two main groups, one with closer and more extensive interactions related to ribosome components and another with wider and restricted interactions containing genes involved in principal cell metabolism (confidence score > 0.9) (**Fig. 10**). The genes present in these two clusters were mainly involved in glycolysis and the TCA cycle (including *PFK2, TKL2, PGI1, ENO1, ENO2, TDH2*) and cytosolic ribosome biogenesis and translation (including RPL and RPS genes).

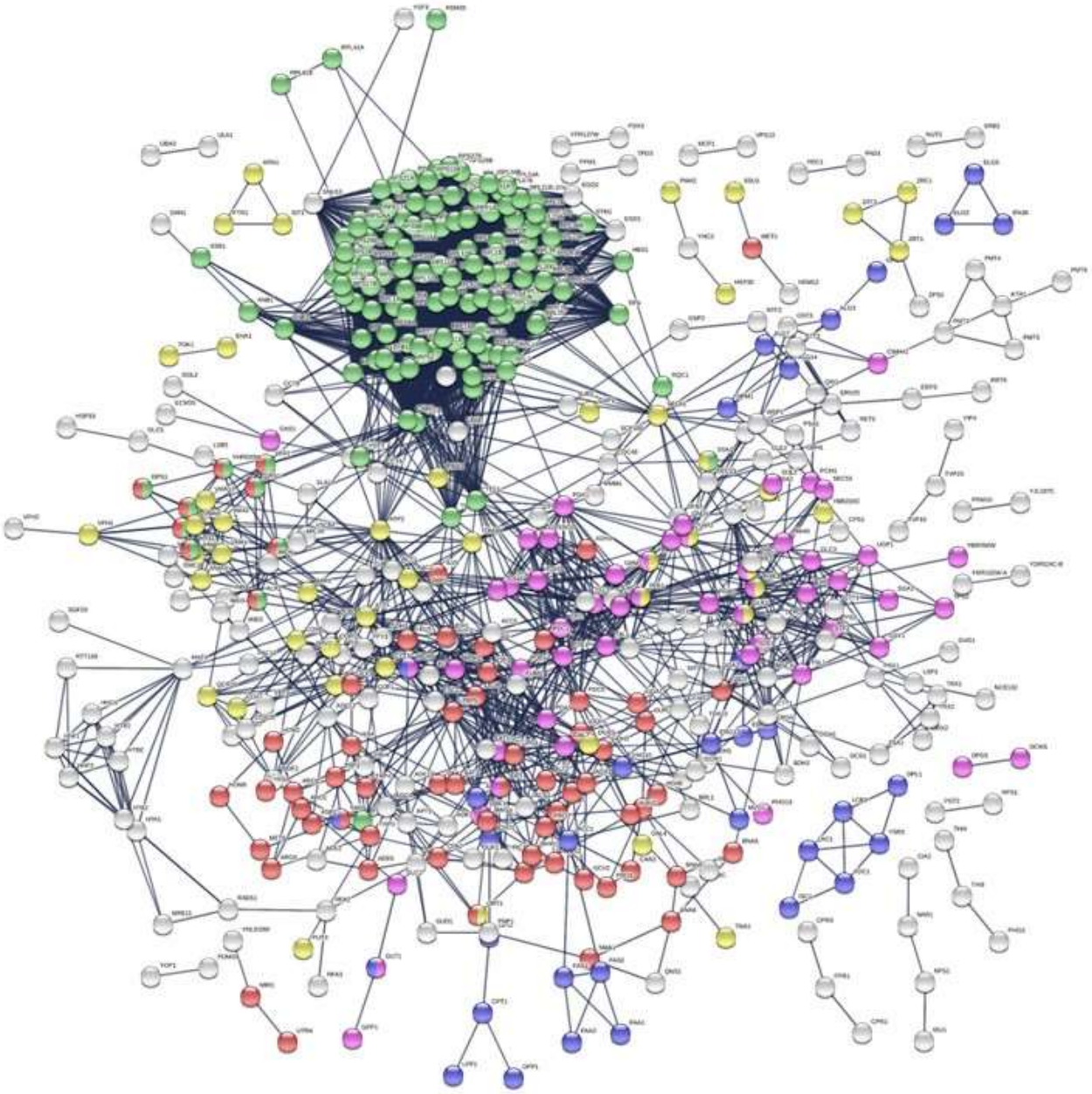

Figure 10. Interaction network of downregulated DEGs with a confidence score > 0.9. Interactions are indicated by edges, with thicker edges having stronger associations. The edges indicate both functional and physical protein associations. Colours represent genes in specific GO biological processes (green: translation; yellow: transmembrane transport; red: amino acid metabolic process; pink: carbohydrate metabolic process; blue: lipid metabolic process). **Colour should be used.**

3. Discussion

The use of HPH to modulate microbial functions, including microbial metabolism, has been described by several authors. In particular, sublethal treatments, 100 MPa or lower, should not affect the cultivability of *S. cerevisiae* or other microorganisms, as reported by (Braschi et al., 2021; Comuzzo et al., 2015; Patrignani et al., 2013; Patrignani, Vannini, Kamdem, Lanciotti, & Guerzoni, 2009; Siroli et al., 2020). Indeed, Lanciotti et al. (2007) reported that the application of HPH at sublethal levels (50-100 MPa) was useful to modify the metabolism of the pretreated cells of lactic acid bacteria without any changes in cell viability.

Additionally, in our work, no significant modifications in cell viability were observed on *S. cerevisiae* ALEAFERM AROM treated at 100 MPa and subsequently incubated in synthetic must for 48 h, demonstrating the high strain tolerance to pressure stress. Although species and strain dependent, this feature has already been well documented in the literature (Comuzzo & Calligaris, 2019; Dimopoulos, Tsantes, & Taoukis, 2020; Iwahashi, Nwaka, & Obuchi, 2000; Patrignani et al., 2013). According to Serrazanetti et al. (2015), immediately after sublethal treatment (80 MPa), only 30% of *S. bayanus* L951 cells collapsed or lost their turgor due to cell wall and membrane damage. However, during the following 2-3 h of treatment, the number of collapsed cells decreased, while the percentage of budding cells doubled. Although these

structural changes were transient, the quick stress induced by sublethal levels of HPH seemed sufficient to promote cell autolysis during wine ageing (Comuzzo et al., 2015; Patrignani et al., 2013). However, the autolysis of yeast takes place very slowly, and the process lasts from a few months to several years. Patrignani et al. (2013) applied HPH at 90 MPa to different yeasts prior to their use in the preparation of tirage solutions for sparkling wines. The effects on yeast cell viability, fermentation, and death kinetics during secondary fermentation and ageing (where autolytic phenomena take place) were determined. The results showed that the cell counts of *S. bayanus and S. cerevisiae* strains were not affected by an HPH of 90 MPa, and all strains were able to multiply and start fermentative activity during the preparation of the triage solution. The SEM microphotographs obtained on the wines aged for 40 days indicated an acceleration of the autolytic phenomena for all the strains treated with HPH treatment, although no viability changes were observed during the cell pretreatment. These results may have great potential for sparkling wine producers who seek to accelerate ageing on lees without detrimental effects on product quality. In fact, in the oenological field, yeast autolysis is an enzymatic self-degradation process of cellular constituents (Martínez et al., 2018) that leads to changes in wine volatile profiles and sensory characteristics.

Although no differences in viability were observed, HPH-treated yeast showed a shift in volatile molecule production. In fact, within the first 2 h of incubation in synthetic must, HPH-treated cells produced fewer ketones, alcohols, acids and esters, while they generated higher amounts of 2-phenylethanol ($p < 0.05$). 2-Phenylethanol, other than representing an important wine aroma with a characteristic rose-like scent able to impart positive sensorial features to the final wine, was proposed as a possible quorum sensing molecule for *S. cerevisiae* produced during the stationary phase. Its presence promotes cell adhesion, cell elongation, bipolar growth, and pseudohyphae formation (Jagtap, Bedekar, & Rao, 2020). After 48 h of incubation, the higher production of 2-phenylethanol was maintained together with a significant increase in benzaldehyde (able to confer almond notes), ethanol, isoamyl alcohol (alcoholic, winey, fruity notes), acetic acid, and ethyl octanoate (fruity, fat aroma) ($p < 0.05$) compared with the control. Additionally, Comuzzo et al. (2015) showed an increase in alcohols, particularly 2-phenylethanol, and esters, mainly ethyl octanoate, in *S. bayanus* powder after HPH treatment, confirming the changes in the volatile molecule patterns. Moreover, according to the authors, the presence of higher amounts of alcohols and ethyl esters also represented evidence about the ability of HPH to induce autolysis in wine yeast. From this perspective, from a technological point of view, the application of sublethal HPH stress to the initial yeast starter could represent a tool to promote autolytic processes that can then impart specific features to the final product. The use of autolyzed cells obtained with HPH can have significant relapses on winemaking protocols (Voce et al., 2021). In fact, in the field of winemaking, where commercial starters are usually applied, positive modifications of the sensorial properties of the final wine could be considered a strategy to expand product diversity. The fact that HPH can induce autolytic processes in yeasts and how this can impact wine production has been already described in several publications from a phenotypic point of view (Comuzzo et al., 2015; Patrignani et al., 2013; Voce et al., 2021). Therefore, in this work, we focused on the gene expression profile of HPH-treated cells after incubation for 1 h in synthetic must confirm the induction of the autolytic process. As expected, compared to control samples, HPH induced an entire reprogramming of the cell translational apparatus, as shown in Figure 3, with 1220 differentially expressed genes between HPH-treated samples and their controls. (X. Li et al., 2020; Xu, Wang, & Li, 2014a, 2014b) studied the autolysis of brewer's yeasts through transcriptomic, proteomic and microarray analyses. They observed a high proportion of downregulated genes with respect to upregulated ones. In particular, X. Li et al. (2020) observed 1187 or 971 downregulated and 115 or 39 upregulated genes, depending on the process applied to induce autolysis (i.e., enzymatic process or heat treatment, respectively). Similar behaviour was also reported during autolysis of *Kluyveromyces marxianus* subjected to vacuum negative pressure and high temperature (X. Li et al., 2022). In our work, HPH induced the upregulation of 562 genes and the downregulation of 658. The higher ratio of downregulated/upregulated genes that was observed may depend on the HPH treatment, performed with one pass at sublethal pressure, that does not induce a complete autolytic process. In fact, to obtain complete autolysis of all the cells, higher pressures and more passes are required (Voce et al., 2021). Sublethal HPH treatment downregulated genes involved in cytoplasmic translation and metabolic pathways, while it upregulated those contributing to transcription, ribosome biogenesis and stress response. To the best of our knowledge, there are no published data that describe the gene expression of *S. cerevisiae* upon sublethal HPH treatment in must. However, according to X. Li et al. (2020), genes involved in energy production/utilization and protein metabolism were downregulated during autolysis of baker's yeast upon enzymatic processing or heat treatment, while genes related to cell wall organization and biogenesis, starvation and DNA damage response were upregulated. In another work, autolysis of *K. marxianus* revealed a downregulation of genes related to purine and pyrimidine metabolism, DNA transcription and translation (X. Li et al., 2022). Xu et al. (2014a), instead, studied spontaneous autolysis in lager brewer's yeast with a microarray and observed a downregulation of carbohydrate and energy metabolism, cellular amino acid metabolic processes, cell response to various stresses (such as oxidative stress, salt stress, and osmotic stress), transcription and translation.

Cytoplasmic translation is a complex process in which ribosomes synthesize proteins after the process of DNA-to-RNA transcription. The production of yeast ribosomes requires the dedication of a considerable part of RNA polymerase II activity to RP gene transcription (Pérez-Ortín, Medina, & Jordán-Pla, 2011). Even 90% of yeast spliceosome activity is dedicated to the production of mature RP mRNAs, and 34% of total ribosomes work in the translation of RP transcripts. Furthermore, small nucleolar RNAs (snoRNAs) are fundamental for the posttranscriptional modifications of rRNAs. Therefore, changes in the expression of one of these three components may affect ribosome biogenesis but also the entire energy balance and vital state of the cells or the way back around. Genes involved in ribosome biogenesis were among

those affected the most by HPH treatment. *S. cerevisiae* ALEAFERM AROM subjected to HPH showed a downregulation of 86 genes coding for ribosome components (mainly RPs). Due to this dramatic reduction in RPG expression, yeast cells enhanced the gene transcription process by upregulating factors involved in gene repression/promotion and assembly of the transcriptional machinery. In fact, among the upregulated genes, there are those coding for components of RNA polymerase II (*RPO21, RPB4, RPB9*, *RPC10*) or those involved in RNA polymerase II transcription initiation *(es. TAF4)*. RNA polymerase II is the enzyme responsible for mRNA and snRNA transcription (Hsin & Manley, 2012). Transcription was also promoted by the downregulation of *ASC1*, which may even favour frameshifting. Although it induced a downregulation of RPGs, HPH-treated cells also showed an upregulation of 8 MRPGs, RPs produced outside the mitochondria but that are meant for that organelle. This entire behaviour was already observed in yeast cells that underwent cell death upon acetic acid and ethanol stress (R. Li et al., 2017; Monticolo, Palomba, & Chiusano, 2021). In particular, it was hypothesized that yeast cells might use mitochondrial ribosomes or change the ribosome components to synthesize additional proteins to resist external stress (R. Li et al., 2017). An overall readaptation of the ribosome machinery may also be suggested by the upregulation of snoRNAs. These molecules are involved in different biological processes, including rRNA modifications. For instance, *snR17A* is involved in cleaving and editing primary rRNA transcripts, while *snR39B*, *snR54*, *snR57* and *snR78* guide methylation or pseudouridylation of the rRNAs large and small subunit. These posttranscriptional modifications increase the diversity of rRNA composition and activity. Therefore, changes in snoRNA expression levels suggest different patterns of rRNA structure organization during exposure to HPH. Under stress conditions, yeast cells should form so-called "stress ribosomes" (Ghulam, Catala, & Abou Elela, 2020). These "stress machineries" are obtained by the differential usage of paralogues. In our work, it was not possible to observe a direct change in the relative proportion between orthologues, but the formation of a "stress ribosome" may be hypothesized due to all modifications reported. Posttranslational modifications of mRNA were also affected by HPH. For instance, *EDC1*, which is involved in mRNA decapping, was upregulated. This may lead to increased mRNA decay. Eventually, HPH induced an upregulation of at least 23 genes involved in the spliceosome. The spliceosome is the cellular machinery that regulates both quantitative and qualitative gene expression through mRNA splicing (Chanarat & Svasti, 2020). However, it can also repress protein-coding gene expression by promoting nuclear turnover of spliced RNA products (Volanakis et al., 2013). Reprogramming of the overall translation apparatus, together with rRNA modifications and mRNA decapping, are relevant events that have been reported in yeast during programmed cell death induced by acetic acid (Monticolo et al., 2021). If translational machinery and transcription processes were altered from one side, then all metabolic pathways were downregulated. For instance, the expression of genes involved in glycolysis (30 DEGs) and the TCA cycle (17 DEGs) was reduced. Although low gene expression is not directly correlated with protein abundance and/or activity, this aspect was supported by the lower ethanol concentration measured in samples containing HPH-treated cells during the first 2 h of incubation compared with control samples. This was supported by the fact that the key genes of alcoholic fermentation (*PDC1, PDC5, ADH1*, and *ADH2*) were especially downregulated. Even electron transport and membrane-associated energy processes were downregulated upon HPH treatment. In fact, our results showed that membrane H^+ and P-type ATPase (*PMA1, PMA2, ENA1*), mitochondrial ATP synthase (*ATP1* and *ATP2*), and vacuolar H^+-ATPase (*VMA1*, *VMA2* and *VMA3*) were all downregulated, as previously reported during autolysis (Xu et al., 2014a, 2014b). Lipid metabolism was also downregulated, with 50 DEGs involved in sphingolipid metabolism, fatty acid degradation and biosynthesis. This is slightly in contrast with what was reported by (Serrazanetti et al., 2015), who observed higher expression of *OLE1, ERG3*, and *ERG11* after HPH. However, in that case, another yeast species was used, and the cells were grown in Sabouraud medium and not synthetic must, as in our case. The stressful condition related to the synthetic must (high sugar content) may have played a role in slower membrane readaptation. Even amino acid metabolism and related transporters were downregulated, in line with what was reported by Xu et al. (2014a, 2014b). Specific upregulation of *ARO9* and *ARO10* (1.8 and 3.2 Log_2 FC) was observed as described by the same authors. These two genes encode an aminotransferase and a decarboxylase, respectively, that play an important role in the Ehrlich pathway. For instance, through these enzymes, phenylalanine is converted into phenylacetaldehyde, which can then be turned into 2-phenylethanol through alcohol dehydrogenase (ADH). The main genes encoding alcohol dehydrogenases in *S. cerevisiae* are 5 (*ADH1, ADH2, ADH3, ADH4*, and *ADH5*). The final reaction can be catalysed by any of these alcohol dehydrogenases (Dai, Xia, Yang, & Chen, 2021). However, only the coexpression of *ADH* and *ARO10* seems necessary to increase the concentration of 2-pheilethanol by 6.5-fold (Shen, Nishimura, Matsuda, Ishii, & Kondo, 2016). In our work, three out of five *ADH* genes were downregulated. However, an increase in 2-phenylethanol was observed after 2 and 48 h of incubation in samples containing HPH-treated cells. The fact that *BAT2* and *ADH7* were not downregulated may suggest that the upregulation of *ARO10* is maintained for a longer time or may even impact the production of isoamyl alcohol from leucine in the late phase of incubation (Yuan, Chen, Mishra, & Ching, 2017). The production of these volatile molecules, as mentioned already before, represents an important aspect in winemaking since they confer rose-like and winey/fruity notes, respectively. Other upregulated genes of the sulfur assimilation pathway, in particular, *MET3*, *MET14*, and *MET16,* are involved in the first three steps of sulfate reduction to sulfite. According to Hine et al. (2015), the production of hydrogen sulfide is a biosynthetic intermediate that possesses other functions for signalling, yeast detoxification, and lifespan extension. According to Xu et al. (2014b), during autolysis, yeast cells activate responses to DNA damage, pH, and starvation, while they reduce genes that react to chemical stimulus, oxidative stress, salt stress, and osmotic stress. In our case, sublethal HPH activated the expression of genes with chaperone or chaperone-like functions, MAPK pathway-related and DNA repair. Among the chaperone genes, *CDC37*, *CDC31*, *SSA4*, *CUR1*, and *SIS1* are all proteins involved in the sorting, recovery, or removal of misfolded proteins. Moreover, HSP42

and HSP82 are chaperones induced in response to DNA stress. The fact that HPH may have caused DNA stress conditions is also supported by the observation that genes related to DNA repair (*PPH3, RAD59, POL32, RAD10, PSY3, DPB1, WSS1, IRC4, AHC1, MPH1, SHU2, RAD34*, and *YKU70*) were upregulated, reflecting possible severe DNA damage. (X. Li et al., 2020). Eventually, downregulated genes were more related to oxidoreductive processes (such as *GRX2*, *TSA1*, *CTT1*) and involved in the osmotic stress response (*ENA1* and *MET22*). Eventually, 12 genes in the MAPK pathway were upregulated. They act as sensors or modulators of cell wall integrity; therefore, their downregulation is associated with autolytic processes (X. Li et al., 2020). The fact that sublethal treatment did not affect cell viability may explain why we did not observe their complete downregulation. In fact, despite a few specific processes or pathways that may suggest that the cells are trying to cope with the external stress imposed, all the other results show that the production and utilization of energy are significantly inhibited. This is in line with what has been reported by several authors (X. Li et al., 2020; Xu et al., 2014b), describing autolysis as a process in which cells gradually lose their activity, resulting in accelerated cell death.

4. Conclusions

The results of this work showed for the first time that HPH treatment may induce a perturbation of the whole gene expression profile similar to what was observed in brewer's yeast autolysis or cell death upon chemical stress. The fact that the viability of the yeast upon HPH did not significantly change may be because a nonlethal treatment was imposed. However, the application of such a level of pression was able to induce gene reprogramming, which was able to modify yeast metabolism and lead to the production of alcohols that have a positive effect on the volatilome and sensorial features of the product at 48 h. Moreover, HPH pretreatment induced an overall status of autolysis. This feature could be interesting, especially for white wines from Burgundy, Champagne, Cava or Franciacorta, to accelerate the process and reduce the cost of production. Although these are preliminary results and they regard only the very early response to HPH, further studies are required to better explain how gene expression and metabolites will evolve and change during alcoholic fermentation and to implement specific protocols for winemaking.

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Appendix A. Supplementary material

Supplementary Table 1 (Table S1)
Supplementary Table 2 (Table S2)
Supplementary Table 3 (Table S3)
Supplementary Table 4 (Table S4)
Supplementary Table 5 (Table S5)
Supplementary Table 6 (Table S6)

Author contributions

Davide Gottardi: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing - original draft, Writing - review & editing, Visualization; **Lorenzo Siroli**: Data curation, Formal analysis, Writing - review & editing; **Giacomo Braschi**: Data curation, Formal analysis, Visualization, Software, Writing - review & editing; **Samantha Rossi**: Software, Visualization, Writing - review & editing; **Diana I. Serrazanetti**: Conceptualization, Investigation, Methodology; **Francesca Patrignani**: Conceptualization, Project administration, Writing - review & editing; **Rosalba Lanciotti**: Conceptualization, Project administration, Writing - review & editing

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Tables

Table 1. Aroma compounds (expressed as equivalent ppm) detected by GC–MS-SPME in synthetic must before and after incubation with HPH-treated (100 MPa x 1 passage) and untreated (Ctr) S. cerevisiae. Samples were collected after 1, 2 and 24 h of incubation. The variability coefficient ranged between 5% and 7%. * indicates statistically significant differences within the same time point ($p < 0.05$). The results are the mean of 3 biological repetitions (n=3).

	Synthetic-must	1 h Ctr	1 h HPH	2 h Ctr	2 h HPH	24 h Ctr	24 h HPH
Aldehydes							
hexanal	1.20	0.00	0.00	0.00	0.00	0.00	0.00

heptanal	0.65	0.00	0.00	0.00	0.00	0.00	0.00
octanal	0.34	0.21	0.07	0.86*	0.08	0.00	0.00
nonanal	0.29	0.34	0.38	1.47*	0.38	0.22	0.35
benzaldehyde	0.30	0.00	0.00	0.00	0.00	0.12	0.63*
5 methyl-furfural	0.00	0.00	0.00	3.45*	0.15	0.00	0.00
total	*0.93*	*0.55*	*0.45*	*5.78**	*0.61*	*0.34*	*0.98**
Ketones							
diacetyl	0.00	0.14	0.67	0.00	0.87	0.15	0.16
methyl isobutyl ketone	1.39	4.41	4.49	4.38*	3.43	0.60	0.54
5-methyl 3-hexanone	0.00	0.67	0.71	1.15	0.67	0.08	0.07
4-methyl 2-hexanone	0.65	1.67	1.92	2.24*	1.57	0.41	0.45
4,6-dimethyl 2-heptanone	0.00	0.22	0.15	0.00	0.00	0.00	0.00
4 methyl 3-penten-2-one	3.03	5.77	5.63	7.02	6.84	2.29	2.69
2,6-dimethyl 4-heptanone	0.30	14.78	14.38	17.76*	12.70	0.12	0.29
1-hydroxy 2-propanone	0.00	0.00	0.00	2.10*	0.13	0.00	0.00
acetophenone	0.00	0.00	0.00	0.09	0.25	0.08	0.12
total	*5.38*	*27.66*	*27.95*	*34.73**	*26.45*	*3.73*	*4.32*
Alcohols							
ethanol	0.55	15.08*	11.20	18.43*	15.16	76.10	90.82*
1-propanol	0.00	0.28	0.21	0.41	0.17	0.45	0.47
isobutanol	1.40	0.00	0.00	0.00	0.00	4.22	4.05
isoamyl alcohol	0.00	1.65	1.44	2.50*	1.50	28.75	31.36*
2-hexanol	1.44	1.77	1.65	2.04	1.71	0.55	0.56
3-heptanol	0.44	0.45	0.35	0.74	0.35	0.24	0.29
1-pentanol	0.20	0.22	0.23	0.64	0.25	0.08	0.14
1-hexanol	0.34	0.83	0.73	5.57*	1.11	1.07	1.22
1-Heptanol	0.06	0.68	0.65	1.72*	0.90	1.23	1.62
2-Ethylhexanol	0.40	0.00	0.00	0.00	0.00	0.24	0.28
1-octanol	0.00	0.31	0.25	1.95*	0.80	0.12	0.13
furfuryl alcohol	0.00	0.00	0.00	3.38*	0.13	0.00	0.00
methionol	0.00	0.00	0.00	0.00	0.00	0.78	0.89
2-phenylethanol	0.00	0.19	0.32	2.18	3.96*	7.74	9.62*
total	*4.84*	*21.46**	*17.04*	*39.57**	*26.04*	*121.56*	*141.44**
Acids							
Acetic acid	0.00	1.12*	0.30	7.40*	2.38	8.53	9.50*
propanoic acid, 2-methyl	0.00	0.00	0.00	0.11	0.15	0.50	0.67
butanoic acid	0.00	0.00	0.00	0.31	0.31	0.08	0.09
Butanoic acid, 2-methyl-	0.00	0.00	0.00	0.00	0.00	0.69	1.00
hexanoic acid	0.00	0.00	0.00	1.74*	0.81	0.71	0.69
heptanoic acid	0.00	0.00	0.00	1.58*	0.51	0.00	0.00
octanoic acid	0.00	0.00	0.00	1.91*	0.51	0.00	0.00
total	*0.00*	*1.12**	*0.30*	*13.05**	*4.67*	*10.52*	*11.94**
Esters							
ethyl acetate	0.37	0.79	0.88	2.05*	0.58	5.88	5.15
butanoic acid, ethyl ester	0.00	0.00	0.00	0.00	0.00	0.25	0.21

1-butanol,3-methyl-acetate	0.00	0.00	0.00	0.00	0.00	0.43	0.64
hexanoic acid, ethyl ester	0.00	0.00	0.00	0.00	0.00	0.75	0.74
octanoic acid, ethyl ester	0.00	0.10	0.15	1.07*	0.06	1.25	2.07*
total	*0.37*	*0.89*	*1.03*	*3.12**	*0.64*	*8.56*	*8.81*