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

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# 51 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

## 111 1. Introduction

112 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, 113 114 Soltanipour, Ferrari, Pataro, & Donsì, 2021; Chevalier-Lucia & Picart-Palmade, 2019; Gottardi et al., 2021; Mesa et al., 115 2020; Wu et al., 2020). In fact, several papers report the effectiveness of HPH in deactivating pathogenic and spoilage 116 microorganisms in model and real systems (Lanciotti et al., 1994; Guerzoni et al., 2002; Diels et al., 2003; Briñez et al., 117 2006a,b; Taylor et al., 2007; Pathanibul et al., 2009). Spatial pressure and velocity gradients, turbulence, impingement, 118 cavitation and viscous shear have been identified as the primary mechanisms of microbial cell disruption and food 119 constituent modification during HPH treatments. In fact, the mechanical stresses that the product encounters during the 120 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 121 through microbial, bacteriophage and enzyme inactivation (dos Santos Aguilar, Cristianini, & Sato, 2018; Levy, Okun, 122 & Shpigelman, 2021; Liu & Kuo, 2016; Patrignani & Lanciotti, 2016). However, the effectiveness of HPH treatment, 123 124 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 125 126 microorganisms (Patrignani et al., 2016).

127 HPH can also be applied to recover intracellular components through cell disruption or to alter biopolymer (protein and 128 sugar) structures, conferring new physical and physical-chemical properties (Comuzzo & Calligaris, 2019; Patrignani & 129 Lanciotti, 2016). Most of the data presented in the literature deal with the use of HPH in the fruit and vegetable beverage 130 and dairy sectors, while its use in the winemaking process has not been extensively studied (Comuzzo & Calligaris, 2019). 131 In fact, HPH treatments have been mainly reported to improve the microbiological quality of wine by reducing the 132 indigenous microbial cell loads present in must without affecting the nutritional and sensory properties of the final product 133 (Puig, Olmos, Quevedo, Guamis, & Mínguez, 2008). Alternatively, HPH was applied at a high level (200-600 MPa, 1-3 134 passes, or 150 MPa, 10 passes) directly on the microorganisms used as starters to obtain inactive dry yeasts or autolyzed 135 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 136 137 processing parameters could be used to obtain different outcomes. Another interesting application could be represented 138 by high-pressure homogenization when used at sublethal pressures (equal to or lower than 100 MPa) and applied to 139 bacteria or yeasts to improve their metabolic and enzymatic activities, impacting the features of the final products 140 (Lanciotti, Patrignani, Iucci, Saracino, & Guerzoni, 2007; Serrazanetti et al., 2015; Siroli et al., 2020; Tabanelli et al., 141 2014). This aspect could be fundamental for winery innovation and differentiation. For instance, Patrignani et al. (2013) 142 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 143 144 refermentation behaviour, treated cells underwent accelerated autolysis during the ageing period, suggesting that HPH 145 treatment could have stimulated the enzymatic pool involved in the autolytic process. However, in that paper, only 146 phenotypic features were observed without any investigation of the changes involved in the yeast general metabolism. 147 Moreover, Serrazanetti et al. (2015), studying the effect of a 90 MPa HPH treatment on S. bayanus, reported an 148 upregulation of OLE1 and ERG3, which are involved in unsaturated FA biosynthesis, 60 and 100 min after the treatment, 149 suggesting a quick readaptation of gene expression. Although several works on yeast and bacteria have focused on 150 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 151 152 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 153 154 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 155 156 HPH treatment), cultivability and volatile molecule profiles (during 48 h) obtained by incubating HPH-treated cells were 157 compared with those obtained using untreated cells (treated with 0.1 MPa) in synthetic must to obtain data that are 158 exploitable for the oenological field.

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# 2. Materials and methods

#### 163 2.1 Yeast stain applied and culture conditions

164 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 165 (16%), cryotolerance, and fast fermentation kinetics. The strain was grown twice in yeast peptone dextrose (YPD) broth 166 for 24 h at 30 °C before inoculum preparation. After 24 h, the inoculum was centrifuged (9000 rpm, 6 °C, 10 min) to 167 168 remove the medium, and the pellet was resuspended in 600 mL of synthetic-must to reach a concentration of 169 approximately 10<sup>6</sup> CFU/mL before treatment with HPH. The synthetic must was prepared according to the recipe as 170 described in OIV-OENO 370-2012 made by the International Organization of Vine and Wine (OIV, 2012). The synthetic-171 must containing 230 g/L fermentable sugars (115 g/L glucose and 115 g/L fructose) was brought to pH 3.2 and filter 172 sterilized (0.22 µm) (SARTOLAB 180C3) prior to its use.

# **173** *2.2 HPH treatment*

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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.

# 182 *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.

#### 188 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.

#### 192 2.6 Sample volatile molecule analysis

193 Volatile molecule profiles were determined by solid phase microextraction combined with gas chromatography and mass 194 spectrometry (SPME-GC/MS). A polyacrylate-coated fibre (85 µm) (Supelco Inc., Bellefonte, PA, USA) and a manual 195 SPME holder (Supelco Inc., Bellefonte, PA, USA) were used in this study after preconditioning, according to the 196 manufacturer's instruction manual. Five millilitres of sample was placed in 10 mL glass vials and supplemented with 1 g 197 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 198 199 equilibration and absorption phases were carried out under stirring conditions. The adsorbed volatile molecules were 200 desorbed in a gas chromatograph (GC) injector port in splitless mode at 250 °C for 10 min. GC-MS analyses were 201 performed on an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 202 5975C mass selective detector (Agilent Technologies, Palo Alto, CA, USA) operating in electron impact mode (ionization 203 voltage 70 eV). A Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) was used (Chrompack, 204 Middelburg, The Netherlands). Volatile compounds were separated using helium as the carrier gas (1 mL/min). The 205 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, 206 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 207 208 those contained in the available database (NIST version 2011) and those of pure standards. Quantitative analyses were 209 performed with the internal standard and expressed as equivalent ppm (ppm eq.). For each compound detected, the ppm 210 eq. represents the amount of compound present in the headspace in dynamic equilibrium with the aqueous phase. 211

# 212 2.7 RNA extraction and sequencing

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Milano, Italy). Quality checks, cDNA preparation, 213 214 and library preparation were performed by Galseq (Milan, Italy). Libraries were generated with the Illumina TruSeq 215 Stranded mRNA Library Prep kit and sequenced on an Illumina HiSeq instrument using a 2x150 paired-end strategy. The 216 quality files of the fastq sequencing was initially assessed with the FastQC tool 217 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reference genome for S. cerevisiae 218 (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 219 (https://www.ncbi.nlm.nih.gov/genome/?term=Saccharomyces%20cerevisiae[Organism]&cmd=DetailsSearch). 220 The alignment was performed with the spliced aligner STAR v.2.5.0c (Dobin et al., 2013) using the --quantMode 221 222 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 223 expression analysis was performed using the DESeq2 tool (Love, Huber, & Anders, 2014). The contrast was defined as 224 225 'Case' vs. 'Control', where Case and Control represent the treatment and absence of treatment conditions. Differential 226 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  $|\log_2 Fold change| \ge 1$  and padj < 0.05 were 227 considered significant differentially expressed genes (DEGs) (Table S2). padj is the p value adjusted using the Benjamini-228 Hochberg procedure. All DEGs were annotated according to the Saccharomyces Genome Database (SGD, 229 http://www.yeastgenome.org/), one of the renowned databases specific for S. cerevisiae. Gene Ontology (GO) enrichment 230 231 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<sup>-4</sup> and KEGG 232 233 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/).

236237 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 238 ACT1, BTN2, ATG8, ACC1 and HSP30 were taken from Hu et al. (2019), ADH1 and HXT1 from Nijland et al. (2017), 239 240 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 241 of each extraction were determined by measuring the absorbance at 260 nm and 280 nm using a BioDrop mLITE 242 (BioDrop, Milan, Italy). Reverse transcription into cDNA was performed using the Reverse Transcription System Kit 243 244 (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, 245 Australia). The ACT1 gene was used as a reference, and the fold change was quantified by the  $2^{-\Delta\Delta Ct}$  method (Dong, Hu, 246 247 Fan, & Chen, 2017). All tests were performed at least three times. 248

249 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.

# 256 3. Results

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258 *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.



PC: 58,57%
 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).

#### 286 *3.3 Global transcriptional changes*

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287 Gene expression of samples containing HPH-treated and untreated cells was analysed after 1 h of incubation in synthetic 288 must. As shown in Fig. 3, differentially expressed genes (DEGs) of HPH-treated and untreated cells were screened out. 289 A volcano plot (Fig. 3A) plotting log<sub>2</sub> (fold change) against -log<sub>10</sub> (adjusted p value) was generated to represent the 290 expression level difference and the statistically significant degree between the two samples. Each point represents a gene, 291 the blue spots represent significant DEGs, and the red spots represent genes that do not show significant differences. For 292 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 293 294 with padj <0.05 were considered significantly different. According to this consideration, 1220 DEGs were identified, 295 including 562 upregulated genes and 658 downregulated genes. The large number of DEGs suggests that HPH had a 296 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<sub>10</sub> adjusted p value against log<sub>2</sub> 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-

305 CTR\_3: three biological replicates of the control group; HPH\_1-HPH\_3: three biological replicates of the HPH-treated cells. <u>Colour should be used.</u>

#### *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). 

- A 400 В Percentage of DEG: Number of DEGs centage of DEGs Number of DEGs sicol patied e cellulat Golisiappo Cellular component Molecular function С Number of DEGs Percentage DNATE rane trans small mole 15° to the RIAPI metabolic in RIA mall **Biological** process
- 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
   transaction to hundrage
- 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.

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#### 342 *3.6. DEGs involved in cytoplasmic translation*

343 Most of the genes of S. cerevisiae related to cytosolic ribosome machinery were downregulated upon HPH treatment and 344 subsequent 1 h incubation in synthetic must. In fact, at least 100 genes related to ribosomes were DEGs, and their 345 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 346 347 mitochondrial ribosomes (15S rRNA and 21S rRNA) were upregulated upon HPH, with increases of 7.1 and 6.8 Log2 FC, 348 respectively. Moreover, considering the 74 annotated mitoribosomal protein genes (MRPGs), 10 were DEGs, and among 349 them, 8 were upregulated (mainly RSM28, MRPS17 and RSM27), together with genes involved in mitochondrial transport 350 (i.e., TOM5, TOM7, PAM16, MMT1).

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

358 RNA metabolic processes were also upregulated (Table S2 and S6). Sixty-seven genes were involved in transcription by 359 RNA polymerase II. For instance, RPO21, RPC10, RPB4 and RPB9 are genes coding for proteins that are part of the 360 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 361 ribosomal RNA processing (RRP) genes (RRP14, RRP15, RRP17, RRP36, RRP45 and RRP8), and 14 were small 362 363 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 (RRSI), which is required for nuclear 364 365 export of the 60S pre-ribosomal subunit, was upregulated in HPH-treated cells. Other upregulated genes were those 366 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 367 SNU23 and SNU66, both components of U4/U6. U5 small nuclear ribonucleoproteins (snRNP) complex, YHC1, 368 369 component of the U1 snRNP complex required for pre-mRNA splicing; HSH49, U2-snRNP associated splicing factor, 370 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 371 372 snRNPs (Fig. 7).

#### 374 *3.7 DEGs involved in the main metabolic processes and energy metabolism*

375 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 376 377 central carbon metabolism (such as PGK1, PGI1, ENO1, ENO2, TDH3 and TPI1, among the most repressed ones), 378 alcoholic fermentation (i.e., PDC1, PDC5, ADH1, and ADH2), lipid metabolism (in particular those related to fatty acid 379 and sphingolipid biosynthesis, such as FAS1, FAS2, ACC1, FAA1, FAA3, LCB3, ELO2, ELO3, YSR3, LAC1, and ERG10), 380 and biosynthesis of cofactors (ADE12, ADE13 and ADK1 for adenine ribonucleotide biosynthesis, TH14 and TH16 for 381 thiamine biosynthesis) (Tables S2 and S6). Regarding amino acid metabolism, HPH downregulated genes involved in 382 aromatic amino acid metabolism (such as ARO1, ARO2, ARO4, TRP2, TRP3, and TRP5), arginine biosynthesis (ARG2, 383 ARG4, ARG7, CAR1, and CAR2), branched-chain amino acid metabolism (LEU1, LEU4, ILV3 and ILV5), and alanine, 384 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).

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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. <u>Colour should be used.</u>

#### 394 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<sup>+</sup> and P-type ATPase (*PMA1, PMA2, ENA1*),
mitochondrial ATP synthase (*ATP1, ATP2*), vacuolar H<sup>+</sup>-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*).

# 401 *3.9 DEGs involved in stress and chemical response*

402 Sixty-three genes related to chemical, osmotic and oxidative stress were upregulated in response to HPH (Table S2 and 403 S5), including chaperone- or chaperone-related genes (*CDC37, CUR1, SIS1, HSP4, HSP82, SSA4*), components of the 404 MAPK pathway (*RLM1, RPI1, STE12, MID2, MTL1, PAF1, CLB2* and *BAR1*, Fig. 8); osmotic stress genes (*MOT3,* 

- 405 *ROX1*); and genes involved in DNA repair (*PPH3, RAD59, POL32, RAD10, PSY3, DPB1, WSS1, IRC4, AHC1, MPH1,*
- 406 SHU2, RAD34, and YKU70). On the other hand, 49 stress-related genes were downregulated, including genes coding for
- 407 peroxidase and oxidoreductase (such as *GRX2, TSA1, PRX1* and *CTT1*) (Table S2 and S6).
- 408



 409 (1) 122316 (c) Kamebias Laboratories
 410 Figure 8. Visualization of KEGG MAPK signalling pathway genes related to *Saccharomyces cerevisiae*. The red genes are those upregulated 1 h upon HPH treatment. <u>Colour should be used.</u>

413 *3.10 Interactions of the identified DEGs* 

The results were integrated and predicted in the STRING database (<u>http://stringdb.org/</u>) (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

- 420 processes, such as the polymerase II transcription process (*TFB5, TOA1, RPB4, TAF7*) and histone acetylation (*AHC1,*
- 421 *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). <u>Colour should be used.</u>

428 The downregulated genes clustered in two main groups, one with closer and more extensive interactions related to 429 ribosome components and another with wider and restricted interactions containing genes involved in principal cell 430 metabolism (confidence score > 0.9) (**Fig. 10**). The genes present in these two clusters were mainly involved in glycolysis 431 and the TCA cycle (including *PFK2*, *TKL2*, *PGI1*, *ENO1*, *ENO2*, *TDH2*) and cytosolic ribosome biogenesis and 432 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). <u>Colour should be</u>
<u>used.</u>

# 3. Discussion

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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.

447 Additionally, in our work, no significant modifications in cell viability were observed on *S. cerevisiae* ALEAFERM 448 AROM treated at 100 MPa and subsequently incubated in synthetic must for 48 h, demonstrating the high strain tolerance 449 to pressure stress. Although species and strain dependent, this feature has already been well documented in the literature 450 (Comuzzo & Calligaris, 2019; Dimopoulos, Tsantes, & Taoukis, 2020; Iwahashi, Nwaka, & Obuchi, 2000; Patrignani et 451 al., 2013). According to Serrazanetti et al. (2015), immediately after sublethal treatment (80 MPa), only 30% of *S. bayanus* 452 L951 cells collapsed or lost their turgor due to cell wall and membrane damage. However, during the following 2-3 h of 453 treatment, the number of collapsed cells decreased, while the percentage of budding cells doubled. Although these 454 structural changes were transient, the quick stress induced by sublethal levels of HPH seemed sufficient to promote cell 455 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 456 different yeasts prior to their use in the preparation of tirage solutions for sparkling wines. The effects on yeast cell 457 458 viability, fermentation, and death kinetics during secondary fermentation and ageing (where autolytic phenomena take 459 place) were determined. The results showed that the cell counts of S. bayanus and S. cerevisiae strains were not affected 460 by an HPH of 90 MPa, and all strains were able to multiply and start fermentative activity during the preparation of the 461 triage solution. The SEM microphotographs obtained on the wines aged for 40 days indicated an acceleration of the 462 autolytic phenomena for all the strains treated with HPH treatment, although no viability changes were observed during 463 the cell pretreatment. These results may have great potential for sparkling wine producers who seek to accelerate ageing 464 on lees without detrimental effects on product quality. In fact, in the oenological field, yeast autolysis is an enzymatic 465 self-degradation process of cellular constituents (Martínez et al., 2018) that leads to changes in wine volatile profiles and 466 sensory characteristics.

467 Although no differences in viability were observed, HPH-treated yeast showed a shift in volatile molecule production. In 468 fact, within the first 2 h of incubation in synthetic must, HPH-treated cells produced fewer ketones, alcohols, acids and 469 esters, while they generated higher amounts of 2-phenylethanol (p < 0.05). 2-Phenylethanol, other than representing an 470 important wine aroma with a characteristic rose-like scent able to impart positive sensorial features to the final wine, was 471 proposed as a possible quorum sensing molecule for S. cerevisiae produced during the stationary phase. Its presence 472 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 473 474 benzaldehyde (able to confer almond notes), ethanol, isoamyl alcohol (alcoholic, winey, fruity notes), acetic acid, and 475 ethyl octanoate (fruity, fat aroma) (p < 0.05) compared with the control. Additionally, Comuzzo et al. (2015) showed an 476 increase in alcohols, particularly 2-phenylethanol, and esters, mainly ethyl octanoate, in S. bayanus powder after HPH 477 treatment, confirming the changes in the volatile molecule patterns. Moreover, according to the authors, the presence of 478 higher amounts of alcohols and ethyl esters also represented evidence about the ability of HPH to induce autolysis in wine 479 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. 480 481 The use of autolyzed cells obtained with HPH can have significant relapses on winemaking protocols (Voce et al., 2021). 482 In fact, in the field of winemaking, where commercial starters are usually applied, positive modifications of the sensorial 483 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 484 485 from a phenotypic point of view (Comuzzo et al., 2015; Patrignani et al., 2013; Voce et al., 2021). Therefore, in this work, 486 we focused on the gene expression profile of HPH-treated cells after incubation for 1 h in synthetic must confirm the 487 induction of the autolytic process. As expected, compared to control samples, HPH induced an entire reprogramming of 488 the cell translational apparatus, as shown in Figure 3, with 1220 differentially expressed genes between HPH-treated 489 samples and their controls. (X. Li et al., 2020; Xu, Wang, & Li, 2014a, 2014b) studied the autolysis of brewer's yeasts 490 through transcriptomic, proteomic and microarray analyses. They observed a high proportion of downregulated genes 491 with respect to upregulated ones. In particular, X. Li et al. (2020) observed 1187 or 971 downregulated and 115 or 39 492 upregulated genes, depending on the process applied to induce autolysis (i.e., enzymatic process or heat treatment, 493 respectively). Similar behaviour was also reported during autolysis of *Kluyveromyces marxianus* subjected to vacuum 494 negative pressure and high temperature (X. Li et al., 2022). In our work, HPH induced the upregulation of 562 genes and 495 the downregulation of 658. The higher ratio of downregulated/upregulated genes that was observed may depend on the 496 HPH treatment, performed with one pass at sublethal pressure, that does not induce a complete autolytic process. In fact, 497 to obtain complete autolysis of all the cells, higher pressures and more passes are required (Voce et al., 2021). Sublethal 498 HPH treatment downregulated genes involved in cytoplasmic translation and metabolic pathways, while it upregulated 499 those contributing to transcription, ribosome biogenesis and stress response. To the best of our knowledge, there are no 500 published data that describe the gene expression of S. cerevisiae upon sublethal HPH treatment in must. However, 501 according to X. Li et al. (2020), genes involved in energy production/utilization and protein metabolism were 502 downregulated during autolysis of baker's yeast upon enzymatic processing or heat treatment, while genes related to cell 503 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 504 505 translation (X. Li et al., 2022). Xu et al. (2014a), instead, studied spontaneous autolysis in lager brewer's yeast with a 506 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 507 508 translation.

509 Cytoplasmic translation is a complex process in which ribosomes synthesize proteins after the process of DNA-to-RNA 510 transcription. The production of yeast ribosomes requires the dedication of a considerable part of RNA polymerase II 511 activity to RP gene transcription (Pérez-Ortín, Medina, & Jordán-Pla, 2011). Even 90% of yeast spliceosome activity is 512 dedicated to the production of mature RP mRNAs, and 34% of total ribosomes work in the translation of RP transcripts. 513 Furthermore, small nucleolar RNAs (snoRNAs) are fundamental for the posttranscriptional modifications of rRNAs. 514 Therefore, changes in the expression of one of these three components may affect ribosome biogenesis but also the entire

515 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 516 517 of 86 genes coding for ribosome components (mainly RPs). Due to this dramatic reduction in RPG expression, yeast cells 518 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 519 520 polymerase II (RPO21, RPB4, RPB9, RPC10) or those involved in RNA polymerase II transcription initiation (es. TAF4). 521 RNA polymerase II is the enzyme responsible for mRNA and snRNA transcription (Hsin & Manley, 2012). Transcription 522 was also promoted by the downregulation of ASCI, which may even favour frameshifting. Although it induced a 523 downregulation of RPGs, HPH-treated cells also showed an upregulation of 8 MRPGs, RPs produced outside the 524 mitochondria but that are meant for that organelle. This entire behaviour was already observed in yeast cells that 525 underwent cell death upon acetic acid and ethanol stress (R. Li et al., 2017; Monticolo, Palomba, & Chiusano, 2021). In 526 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 527 machinery may also be suggested by the upregulation of snoRNAs. These molecules are involved in different biological 528 processes, including rRNA modifications. For instance, snR17A is involved in cleaving and editing primary rRNA 529 530 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, 531 532 changes in snoRNA expression levels suggest different patterns of rRNA structure organization during exposure to HPH. 533 Under stress conditions, yeast cells should form so-called "stress ribosomes" (Ghulam, Catala, & Abou Elela, 2020). 534 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 535 536 hypothesized due to all modifications reported. Posttranslational modifications of mRNA were also affected by HPH. For 537 instance, EDC1, which is involved in mRNA decapping, was upregulated. This may lead to increased mRNA decay. 538 Eventually, HPH induced an upregulation of at least 23 genes involved in the spliceosome. The spliceosome is the cellular 539 machinery that regulates both quantitative and qualitative gene expression through mRNA splicing (Chanarat & Svasti, 540 2020). However, it can also repress protein-coding gene expression by promoting nuclear turnover of spliced RNA 541 products (Volanakis et al., 2013). Reprogramming of the overall translation apparatus, together with rRNA modifications 542 and mRNA decapping, are relevant events that have been reported in yeast during programmed cell death induced by 543 acetic acid (Monticolo et al., 2021). If translational machinery and transcription processes were altered from one side, 544 then all metabolic pathways were downregulated. For instance, the expression of genes involved in glycolysis (30 DEGs) 545 and the TCA cycle (17 DEGs) was reduced. Although low gene expression is not directly correlated with protein 546 abundance and/or activity, this aspect was supported by the lower ethanol concentration measured in samples containing 547 HPH-treated cells during the first 2 h of incubation compared with control samples. This was supported by the fact that 548 the key genes of alcoholic fermentation (PDC1, PDC5, ADH1, and ADH2) were especially downregulated. Even electron 549 transport and membrane-associated energy processes were downregulated upon HPH treatment. In fact, our results 550 showed that membrane H<sup>+</sup> and P-type ATPase (*PMA1, PMA2, ENA1*), mitochondrial ATP synthase (*ATP1* and *ATP2*), 551 and vacuolar H<sup>+</sup>-ATPase (VMA1, VMA2 and VMA3) were all downregulated, as previously reported during autolysis (Xu 552 et al., 2014a, 2014b). Lipid metabolism was also downregulated, with 50 DEGs involved in sphingolipid metabolism, 553 fatty acid degradation and biosynthesis. This is slightly in contrast with what was reported by (Serrazanetti et al., 2015), 554 who observed higher expression of OLE1, ERG3, and ERG11 after HPH. However, in that case, another yeast species 555 was used, and the cells were grown in Sabouraud medium and not synthetic must, as in our case. The stressful condition 556 related to the synthetic must (high sugar content) may have played a role in slower membrane readaptation. Even amino 557 acid metabolism and related transporters were downregulated, in line with what was reported by Xu et al. (2014a, 2014b). 558 Specific upregulation of ARO9 and ARO10 (1.8 and 3.2 Log<sub>2</sub> FC) was observed as described by the same authors. These 559 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 560 turned into 2-phenylethanol through alcohol dehydrogenase (ADH). The main genes encoding alcohol dehydrogenases 561 562 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 563 564 necessary to increase the concentration of 2-pheilethanol by 6.5-fold (Shen, Nishimura, Matsuda, Ishii, & Kondo, 2016). 565 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 566 downregulated may suggest that the upregulation of ARO10 is maintained for a longer time or may even impact the 567 568 production of isoamyl alcohol from leucine in the late phase of incubation (Yuan, Chen, Mishra, & Ching, 2017). The 569 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 570 571 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 572 573 signalling, yeast detoxification, and lifespan extension. According to Xu et al. (2014b), during autolysis, yeast cells 574 activate responses to DNA damage, pH, and starvation, while they reduce genes that react to chemical stimulus, oxidative 575 stress, salt stress, and osmotic stress. In our case, sublethal HPH activated the expression of genes with chaperone or 576 chaperone-like functions, MAPK pathway-related and DNA repair. Among the chaperone genes, CDC37, CDC31, SSA4, 577 CUR1, and SIS1 are all proteins involved in the sorting, recovery, or removal of misfolded proteins. Moreover, HSP42

578 and HSP82 are chaperones induced in response to DNA stress. The fact that HPH may have caused DNA stress conditions 579 is also supported by the observation that genes related to DNA repair (PPH3, RAD59, POL32, RAD10, PSY3, DPB1, 580 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, 581 582 *CTT1*) and involved in the osmotic stress response (*ENA1* and *MET22*). Eventually, 12 genes in the MAPK pathway were 583 upregulated. They act as sensors or modulators of cell wall integrity; therefore, their downregulation is associated with 584 autolytic processes (X. Li et al., 2020). The fact that sublethal treatment did not affect cell viability may explain why we 585 did not observe their complete downregulation. In fact, despite a few specific processes or pathways that may suggest 586 that the cells are trying to cope with the external stress imposed, all the other results show that the production and 587 utilization of energy are significantly inhibited. This is in line with what has been reported by several authors (X. Li et 588 al., 2020; Xu et al., 2014b), describing autolysis as a process in which cells gradually lose their activity, resulting in 589 accelerated cell death. 590

## 4. Conclusions

593 The results of this work showed for the first time that HPH treatment may induce a perturbation of the whole gene 594 expression profile similar to what was observed in brewer's yeast autolysis or cell death upon chemical stress. The fact 595 that the viability of the yeast upon HPH did not significantly change may be because a nonlethal treatment was imposed. 596 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 597 598 of the product at 48 h. Moreover, HPH pretreatment induced an overall status of autolysis. This feature could be 599 interesting, especially for white wines from Burgundy, Champagne, Cava or Franciacorta, to accelerate the process and 600 reduce the cost of production. Although these are preliminary results and they regard only the very early response to HPH, 601 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. 602

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# 608 Appendix A. Supplementary material

- 609 Supplementary Table 1 (Table S1)
- 610 Supplementary Table 2 (Table S2)
- 611 Supplementary Table 3 (Table S3)
- 612 Supplementary Table 4 (Table S4)
- 613 Supplementary Table 5 (Table S5)
- 614 Supplementary Table 6 (Table S6)
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# 617 Author contributions

Davide Gottardi: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing - original
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Investigation, Methodology; Francesca Patrignani: Conceptualization, Project administration, Writing - review &
editing; Rosalba Lanciotti: Conceptualization, Project administration, Writing - review &

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**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
<u>Aldehydes</u> hexanal	1.20	0.00	0.00	0.00	0.00	0.00	0.00
nexanar	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*
<u>Ketones</u>							
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
<u>Alcohols</u>							
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					• • •		o <b>z</b> ot
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
nexanoic 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*	4.0/	10.52	11.94*
Estors							
ethyl acetate	0.37	0.70	0.88	2.05*	0.58	5 99	5.15
butanoic acid ethyl ester	0.37	0.79	0.00	0.00	0.58	0.25	0.21
cauntore acra, curyr 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