*Article*

# Different Gene Expression Patterns of Hexose Transporter Genes Modulate Fermentation Performance of Four *Saccharomyces cerevisiae* Strains

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**Abstract:** In *Saccharomyces cerevisiae*, the fermentation rate and the ability to complete the sugar transformation process depend on the glucose and fructose transporter set-up. Hexose transport mainly occurs via facilitated diffusion carriers and these are encoded by the *HXT* gene family and *GAL2*. In addition, *FSY1*, coding a fructose/H+ symporter, was identified in some wine strains. This little-known transporter could be relevant in the last part of the fermentation process when fructose is the most abundant sugar. In this work, we investigated the gene expression of the hexose transporters during late fermentation phase, by means of qPCR. Four *S. cerevisiae* strains (P301.9, R31.3, R008, isolated from vineyard, and the commercial EC1118) were considered and the transporter gene expression levels were determined to evaluate how the strain gene expression pattern modulated the late fermentation process. The very low global gene expression and the poor fermentation performance of R008 suggested that the overall expression level is a determinant to obtain the total sugar consumption. Each strain showed a specific gene expression profile that was strongly variable. This led to rethinking the importance of the *HXT3* gene that was previously considered to play a major role in sugar transport. In vineyard strains, other transporter genes, such as *HXT6/7*, *HXT8*, and *FSY1*, showed higher expression levels, and the resulting gene expression patterns properly supported the late fermentation process.

**Keywords:** *HXT*; fructose symporter; late fermentation

## 1. Introduction

In wine alcoholic fermentation, glucose and fructose present in grape must are co-fermented by yeasts to ethanol and carbon dioxide. Grape must usually contains equal or very similar amounts of both sugars [1]. Glucose is known to be the preferred carbon source for *Saccharomyces cerevisiae*. Although fructose is used concomitantly with glucose, the latter is the first sugar to be depleted from the medium during fermentation [2,3]. Consequently, fructose becomes the main sugar present during the late stages of alcoholic fermentation, and wine yeasts have to ferment this non-preferred sugar in the presence of large amounts of ethanol. The stress associated with these conditions, together with nutritional imbalances, may result in sluggish or stuck fermentations [1,4]. Moreover, it has been reported that stuck fermentations are frequently characterized by an unusually high fructose-to-glucose ratio [2]. The ability of wine yeasts to ferment fructose is therefore critically important for the maintenance of a high rate of fermentation at the end of the process and for fermentation of the must to dryness. The reasons for the difference between the glucose fermentation rate and the fructose fermentation rate are unclear, but one of the first steps in hexose metabolism is generally thought to be involved [1].

The first essential step towards the utilization of hexose sugars is their uptake by yeast cells. In yeast, hexose uptake may proceed through facilitated diffusion carriers and energy-dependent active proton-sugar symporters [3,5,6]. Hexose transport in *S. cerevisiae* occurs via facilitated diffusion carriers and these are encoded by several genes, including the *HXT* genes, the *GAL2* gene encoding a galactose transporter, and *SNF3* and *RGT2* encoding two glucose sensors [5,6]. Among the 17 *HXT* genes in *S. cerevisiae*, only seven of them, Hxt1p–Hxt7p, are required for growth on glucose or fructose [3,7]. Although all the hexose transporters in *S. cerevisiae* can also transport fructose, glucose is the preferential sugar for Hxt carriers [3]. The catabolic hexose transporters exhibit different affinities for their substrates; furthermore, the expression of their corresponding genes is controlled by the glucose sensors, according to the availability of carbon sources [8]. Expression of the *HXT*s, all of which exhibit different levels of glucose affinity, is differentially regulated depending on extracellular glucose concentrations [9].

The complete genome sequence of the commercial wine yeast strain EC1118 [10] and the vineyard strain P301 [11] revealed the presence of a new gene (named EC-1O4_6634g in the former study and P301_O3_0021 in the latter study) highly similar to the *S. pastorianus FSY1* gene [10]. This gene was designated *FSY1* as well, and Galeote and colleagues [3] demonstrated that, in *S. cerevisiae*, it encoded a high-affinity fructose/H+ symporter. In the EC1118 genome, *FSY1* is in the C region that resulted from a horizontal gene transfer (HGT) from *Torulaspora microellipsoides*, a distant yeast species identified in the wine environment [12]. This region includes the *FOT* genes that confer a strong competitive advantage during grape must fermentation by increasing the number and diversity of oligopeptides that yeast can utilize as a source of nitrogen, thereby improving biomass formation, fermentation efficiency, and cell viability. Thus, the acquisition of the C region genes is related to better fermentation performance in the nitrogen-limited wine fermentation environment [13].

Moreover, *FSY1* expression is repressed by high concentrations of glucose or fructose and is induced by ethanol as the sole carbon source [3]. This observation leads to suppose that this transporter is active in the last part of the fermentation process, where ethanol concentration is high and fructose residue is more abundant than glucose. Although the presence of *HXT* gene family and *GAL2* gene is well documented in the *Saccharomyces* genus, studies on *FSY1* gene diffusion among *Saccharomyces* strain genomes is lacking, as well as the *FSY1* gene expression level during the fermentation process.

Previously, sugar transporter genes expression studies were mainly focused on a single strain in the fermentation process [14–17]. Therefore, in this work, the gene expression patterns of four *S. cerevisiae* strains (P301.9, R31.3, R008, and EC1118) have been studied at two time points of the late fermentation phase. The aim was to investigate how the strain gene expression pattern modulated the late fermentation process.

## 2. Materials and Methods

### 2.1. Yeast Strains

In the present work, four *Saccharomyces cerevisiae* strains were used: The industrial strain EC1118 (Lallemand Inc.—Montreal, QC, Canada) and three natural strains isolated in a vineyard: P301.9, R008, and R31.3 (University of Padova, Padova, Italy).

### 2.2. Fermentation Trial and Samplings

Fermentations were performed at 25 °C in synthetic grape must MS300 [18] modified for the carbon source: 100 g/L of glucose and 100 g/L of fructose were added instead of 200 g/L of glucose. Three independent biological replicates were carried out for each strain in 1 L bioreactors (Multifors, Infors HT, Basel, CH, Switzerland). These instruments are equipped with sensors to monitor temperature and a flow meter to determine $CO_2$ outflow (red-y mod. GSM-A95A-BN00, Infors HT, Basel, CH, Switzerland) (range 1–20 mL/min). Strict anaerobiosis was not imposed, but fermentation conditions were largely anaerobic due to the design of the bioreactor and the effect of $CO_2$ production. $CO_2$ production was

monitored by the flow meter every 5 min to determine the rate of $CO_2$ production. For each strain, approximately $3 \times 10^6$ cells/mL have been inoculated in 1 L of MS300 must. For each strain, cells were collected for Real Time-PCR assay at two sampling points during the late fermentation phase, when 45 and 60 g/L of $CO_2$ were produced. After sampling, the cells were centrifuged to remove the growth media and immediately frozen at $-80\ °C$. At the same sampling points, 50 mL of fermented media have been collected and frozen for chemical analysis.

### 2.3. Chemical Analysis

HPLC analysis was performed to determine the concentrations of residual sugars and ethanol, as described by Lemos Junior [19]. Ten microliters of sample was analyzed by the Waters 1525 HPLC binary pump (Waters, Milford, MA, USA) equipped with a $300 \times 7.8$ mm stainless-steel column packed with Aminex HPX_87H HPLC column (Bio-Rad, Hercules, CA, USA) and a Waters 2414 Refractive Index Detector (Waters, Milford, MA, USA). The analyses were performed isocratically at 0.6 mL/min and 65 °C with a cation-exchange column (300 by 7.8 mm [inner diameter]; Aminex HPX-87H) and a Cation H+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.01 N $H_2SO_4$ as the mobile phase. Ammonia and amino nitrogen were measured by means of specific enzymatic kits (Steroglass, Perugia, Italy) according to the manufacturer's instructions.

### 2.4. RNA Extraction and Reverse Transcription

Total RNA was extracted using the TRIzol® Plus RNA Purification Kit (Ambion, Austin, TX, USA). The concentration, purity, and integrity of RNA samples were determined by spectrophotometric analysis using the SPARK® multimode microplate reader (Tecan Trading AG, Switzerland), considering the absorbance ratio at 260/280 nm and at 230/260 nm. The quality and integrity of RNAs were confirmed by electrophoresis on 1.5% agarose gels under denaturing conditions (2% formaldehyde, *v/v*, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). To obtain DNA-free RNA, the total RNA previously extracted (1 μg) was treated with DNase I (Fermentas, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was synthesized using Revert Aid Reverse Transcriptase (Fermentas, Waltham, MA, USA) according to the manufacturer's instructions using both polyT (16) primers (MWG-biotech; 0.5 μg/μL) and random hexamers (Promega; 0.5 μg/μL). Samples were stored at $-20\ °C$ until Real-Time PCR was run.

The quality control of cDNAs was checked by end-point PCR in a PTC200 thermal cycler (MJ Research Inc., Waltham, MA, USA). Amplification of the gene *APE2* (F—TGCGCATCAATGTAATGTGGAAGCAGAGTA, R—TGAAATCAGGTTCCACGGTTAAATCGTAGTGT) was performed on cDNAs both for checking the reverse-transcription efficiency and for excluding genomic DNA contamination. Amplified samples were run on 1.5% agarose gel containing 1X GelRedTM Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). Run was performed on a horizontal electrophoresis apparatus with TBE 0.5x as the running buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) and the bands were visualized by UV trans-illumination.

### 2.5. Primer Design

PCR primers of the investigated genes for real-time assays are listed in Table 1. They were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 5 May 2017). The yeast database was used to check primer specificity on sequences of other yeast species. Special attention was given to primer length (15–25 bp), annealing temperature (58–62 °C), nucleotides composition, 3′-end stability, and amplicon size (80–200 bp). All primers were synthesized and OPC purified by Metabion International AG (Metabion International AG, Planegg, Germany). After synthesis, the primer specificity was tested by end-point PCR and gel electrophoresis.

**Table 1.** List of the investigated genes and details of primers and amplicons for each gene.

| Gene | Molecular Function (SGD Curated) | Primer Sequence [5′ → 3′] | Amplicon Length | Efficiency % | $R^2$ |
|---|---|---|---|---|---|
| *HXT1* | HeXose Transporter (low-affinity glucose transporter) | F: 5′-GAA GCT GGC AGA ATC GAC GA-3′ <br> R: 5′-TAT GGA TGG TCA GGT GGG CA-3′ | 71 bp | 102.9 | 0.996 |
| *HXT2* | HeXose Transporter (high-affinity glucose transporter) | F: 5′-TGA ACT CCC AGC AAA GCC AA-3′ <br> R: 5′-TCC CAA CCA AAG ACA AAC CCA-3′ | 90 bp | 104.9 | 0.992 |
| *HXT3* | HeXose Transporter (low affinity glucose transporter) | F: 5′-CAC GTT ATT TGG TTG AAG CTG GT-3′ <br> R: 5′-GTT GAA TGA ATG GAT GGT CTG GG-3′ | 93 bp | 101.3 | 0.998 |
| *HXT4* | HeXose Transporter (high-affinity glucose transporter) | F: 5′-TGG TGG TAT GAC ATT CGT TCC-3′ <br> R: 5′-CGC TGA CCT TAT TTG AAA GAG CA-3′ | 101 bp | 104.7 | 0.989 |
| *HXT5* | HeXose Transporter (moderate affinity for glucose) | F: 5′-TCC AAA TCG CCT CCA TTG ACA-3′ <br> R: 5′-AAT ACC ACC AAC GCC CAG TC-3′ | 77 bp | 102.5 | 0.983 |
| *HXT6/7* | HeXose Transporter (high-affinity glucose transporter) | F: 5′-GAC TTT GGA AGA AGT CAA CAC CA-3′ <br> R: 5′-TTC TTC AGC GTC GTA GTT GGC-3′ | 106 bp | 100.0 | 0.998 |
| *HXT8* | Protein of unknown function with similarity to hexose transporters | F: 5′-AAT TCT GTC CAG TGG CGT GT-3′ <br> R: 5′-CGG AAC AAA CGT CAT ACC ACC-3′ | 81 bp | 95.2 | 0.991 |
| *FSY1* | Fructose/H$^+$ symporter (Galeote et al., 2010) | F: 5′-CGA TGT TAA AGG CGG GTG GA-3′ <br> R: 5′-AAC GTG GTG ACT CGG GTA AG-3′ | 98 bp | 95.1 | 0.989 |
| *GAL2* | GALactose metabolism (also able to transport glucose) | F: 5′-GGG TCT GAA GGC TCC CAA AG-3′ <br> R: 5′-ACA AAC AAA GCA AGG AAA CGG T-3′ | 85 bp | 104.7 | 0.981 |

For each different primer pair, the efficiency of RT-PCR (E), slope values, and correlation coefficients ($R^2$) were determined using serial 1:5 dilutions of the template cDNA on the CFX96 cycler—Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Efficiency was considered adequate when ranging from 95% to 105%, and $R^2$ was considered acceptable when greater than 0.98.

### 2.6. Real-Time PCR

Real-Time PCR was carried out on a CFX96 Cycler-Real Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in white-walled PCR plates (96 wells). A ready-to-use master-mix containing a fast proof-reading Polymerase, dNTPs, stabilizers, $MgCl_2$, and Eva Green dye was used according to the manufacturer's instructions ( Bio-Rad Laboratories, Hercules, CA, USA). Reactions were prepared in a total volume of 15μL containing 400 nM each primer (MWG), 1× Sso Fast Eva Green Supermix 2× (Bio-Rad Laboratories, Hercules, CA, USA), and 5 μL cDNA. The cycle conditions were set as follows: Initial template denaturation at 98 °C for 30 s, followed by 40 cycles of denaturation at 98 °C for 2 s, and combined primer annealing/elongation at 60 °C for 10 s. The amount of fluorescence for each sample, given by the incorporation of Eva Green into dsDNA, was measured at the end of each cycle and analyzed via CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Melting curves of PCR amplicons were obtained using temperatures ranging from 65 °C to 95 °C. Data acquisition was performed for every 0.5 °C temperature increase with a 1-s step. For each target gene, each sample was analyzed in triplicate and no-template controls for each primer pair were included in all plates. Gene expression analysis was performed using the CFX-Manager Software v2.0, adopting the $2^{-\Delta\Delta CT}$ method. Four housekeeping genes have been used in Real-Time PCR gene expression analysis: The ALG9 and UBC6 primers designed by Teste [20]; andthe FBA1 and PFK1 primers designed by Cankorur-Cetinkaya [21] and Nadai [22], respectively (Table 2). In this study, the lettering "total expression" indicates the sum of the normalized expression values of the genes, for each strain, relative to one of the two sampling points (45 or 60 g/L of produced $CO_2$).

**Table 2.** List of the reference genes and details of primers and amplicons for each gene.

| Gene | Reference | Primer Sequence [5′ → 3′] | Amplicon Length | Efficiency % | $R^2$ |
|------|-----------|---------------------------|-----------------|--------------|-------|
| *ALG9* | [20] | F: 5′-CAC GGA TAG TGG CTT TGG TGA ACA ATT AC-3′<br>R: 5′-TAT GAT TAT CTG GCA GCA GGA AAG AAC TTG GG-3′ | 156 bp | 95.7 | 0.996 |
| *UBC6* | [20] | F: 5′-GAT ACT TGG AAT CCT GGC TGG TCT GTC TC-3′<br>R: 5′-AAA GGG TCT TCT GTT TCA TCA CCT GTA TTT GC-3′ | 272 bp | 99.0 | 0.985 |
| *FBA1* | [21] | F: 5′-GGT TTG TAC GCT GGT GAC ATC GC-3′<br>R: 5′-CCG GAA CCA CCG TGG AAG ACC A-3′ | 125 bp | 102.4 | 0.998 |
| *PFK1* | [22] | F: 5′-GAG GTT GAT GCT TCT GGG TTC CGT-3′<br>R: 5′-TGT GGC GGT TTC GTT GGT GTC G-3′ | 138 bp | 97.7 | 0.998 |

### 2.7. Statistical Analysis

The statistical data analysis was performed using XLSTAT software, vers. 2016.02 (Addinsoft, Paris, France). Data were subjected to Student's *t*-test or simple analysis of variance (one-way ANOVA) followed by the Tukey's *post-hoc* test. The analysis was carried out comparing the averages of three independent replicates, and differences were considered statistically significant for *p*-value lower than 0.05.

## 3. Results

### 3.1. Yeasts Fermentation Process

Fermentations in synthetic must were run using three *S. cerevisiae* strains, P301.9, R31.3, and R008, isolated from a vineyard and the commercial starter EC1118. The aim was to check strain behavior during the alcoholic fermentation focusing on the last part of the

fermentation, starting form half of the total expected $CO_2$ (45 g/L) to the end of the process (late fermentation phase).

The fermentation profiles of the four strains were determined monitoring the fermentation rate ($dCO_2/dt$) (Figure 1).
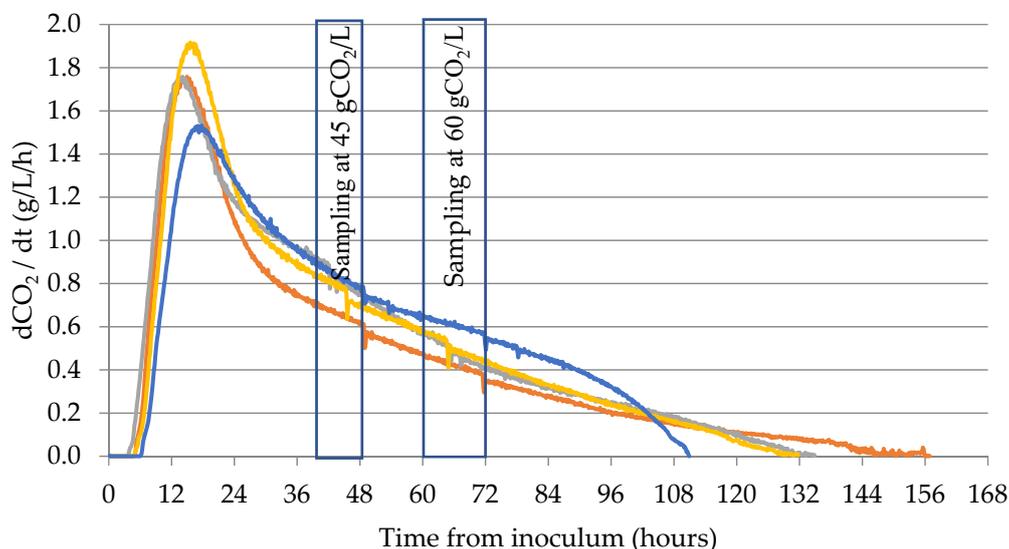


**Figure 1.** $CO_2$ production kinetics of strains R008 (—), R31.3 (—), P301.9 (—), and EC1118 (—). Data of $dCO_2/dt$ are the average of three biological replicates.

During fermentation process, the fermentation rate peaked (Vmax) just before the entry into the yeast stationary growth phase and declined thereafter until the end of the fermentation [23]. Strain P301.9 showed the highest Vmax value (1.93 $gCO_2/L/h$), reached after 15.58 h from inoculum, while the lowest was obtained for EC1118 (1.53 $gCO_2/L/h$) reached in 16.75 h from inoculum. Intermediate values were registered for strains R008 and R31.3 (Table 3).

**Table 3.** Parameters of the fermentation kinetics.

| Strain | $CO_2$ Production Peak (g/L/hours) | Peak Time (hours) | Sampling Time 45 g/L (hours) | Sampling Time 60 g/L (hours) | Fermentation Time (hours) |
|--------|-----------------------------------|-------------------|------------------------------|------------------------------|---------------------------|
| EC1118 | 1.53 ± 0.05 [A] | 16.75 ± 0.85 [B] | 50.3 ± 2.7 [A] | 73.9 ± 3.7 [B] | 110.2 ± 1.7 [A] |
| R008 | 1.76 ± 0.00 [B] | 14.91 ± 0.33 [A] | 46.3 ± 4.9 [A] | 67.5 ± 3.1 [AB] | 156.9 ± 1.0 [C] |
| R31.3 | 1.76 ± 0.05 [B] | 14.08 ± 1.08 [A] | 41.8 ± 1.2 [A] | 64.6 ± 1.2 [A] | 134.9 ± 4.2 [B] |
| P301.9 | 1.93 ± 0.05 [C] | 15.58 ± 0.84 [AB] | 43.2 ± 4.1 [A] | 63.5 ± 1.5 [A] | 131.7 ± 4.2 [B] |

Data are expressed as the average of three biological replicates ± standard deviation. Uppercase letters indicate significant differences obtained from Tuckey post-hoc test after ANOVA analysis of variance between the strains (*alpha* = 0.05).

During the late fermentation phase, strains R008, R31.3, and P301.9 showed a marked decrease of the fermentation rate that is more evident in the last part of the fermentation. This trend was responsible for the increase of the fermentation time, with respect to the commercial strain EC1118. Therefore, EC1118 was the first strain to complete the fermentation, followed by P301.9, R31.3, and R008.

Despite the different fermentation trends, EC1118, P301.9, and R31.3 completed the sugar transformation, while R008 left a fructose residue of 7.62 g/L (Table 4). The ethanol concentration at the end of the fermentation reflected the sugar consumption. No significant differences in ethanol production were found among strains EC1118, P301.9, and R31.3. Strain R008 did not reach the same ethanol level, due to the sugar residues (Table 4).

**Table 4.** Ethanol and sugar residues at the end of the fermentations.

|  | Ethanol (% vol.) | Glucose (g/L) | Fructose (g/L) |
|---|---|---|---|
| EC1118 | 11.49 ± 0.04 [B] | nd | nd |
| P301.9 | 11.45 ± 0.04 [B] | nd | nd |
| R31.3 | 11.38 ± 0.26 [B] | nd | nd |
| R008 | 10.64 ± 0.07 [A] | nd | 7.62 ± 0.04 |

Data are expressed as the average of three biological replicates ± standard deviation. Uppercase letters indicate significant differences obtained from Tuckey post-hoc test after ANOVA analysis of variance between the strains (*alpha* = 0.05). nd: not detected.

During the late fermentation phase, two samplings were performed at 45 g/L (half of the $CO_2$ produced) and 60 g/L of $CO_2$ produced. Due to differences in strain fermentation rates, these values were reached at different times.

The mean total sugar residue of the four strains was 79.69 ± 4.17 g/L at 45 g/L of $CO_2$ produced and 40.80 ± 5.96 g/L at 60 g/L of $CO_2$ produced (Supplementary Table S1). ANOVA analysis of variance found no significant differences among the strains' sugar residue at both sampling points (*alpha* = 0.05). This result indicates that the sampling time have been correctly chosen in order to harvest cells in the same physiological state.

At 45 g/L of $CO_2$ produced, strain EC1118 showed the lowest ratio between fructose and the total sugar residue (60.30%), corresponding to the most balanced intake of fructose and glucose with respect to the other strains, which have shown significantly higher ratios. The same pattern has been observed at 60 g/L of produced $CO_2$. For all strains, this ratio increased from 45 to 60 g/L of produced $CO_2$, during the late fermentation phase, confirming the glucophilic aptitude of *S. cerevisiae* (Table 5). Strain R31.3 showed the highest increase of the ratio between fructose and the total sugar residue from 45 to 60 g/L of produced $CO_2$ (12.27%), followed by P301.9 (10.11%), R008 (8.92%), and EC1118 (6.57%).

**Table 5.** Fructose/total sugars ration and amino nitrogen residue at 45 and 60 g/l of produced $CO_2$.

|  | Fructose/Total Sugars (%) | | | $NH_2$ (mg/L) | | |
|---|---|---|---|---|---|---|
|  | 45 g/L | 60 g/L | *p* | 45 g/L | 60 g/L |  |
| EC1118 | 60.30 ± 0.43 [A] | 66.87 ± 0.70 [A] | <0.0001 *** | 61.17 ± 2.35 | 59.87 ± 3.10 | Ns |
| R008 | 66.05 ± 0.29 [B] | 74.97 ± 1.65 [B] | 0.012 * | 55.75 ± 1.95 | 63.10 ± 1.70 | * |
| R31.3 | 67.01 ± 1.08 [B] | 79.27 ± 1.84 [B] | 0.016 * | 51.93 ± 7.35 | 56.57 ± 6.33 | * |
| P301.9 | 66.56 ± 0.74 [B] | 76.67 ± 1.72 [B] | 0.002 ** | 38.03 ± 4.29 | 39.20 ± 3.21 | Ns |

Data are expressed as the average of three biological replicates ± standard deviation. Uppercase letters indicate significant differences obtained from Tukey post-hoc test after ANOVA analysis of variance between the strains (*alpha* = 0.05). (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$, Ns (not significative) between the percentage of fructose on total sugar residue at 45 and 60 g/L of produced $CO_2$ by Student's *t* test.

At the two sampling points, assimilable nitrogen (ammonium and amino acids) residues were measured. At the beginning of the fermentation process, the assimilable nitrogen present in the synthetic must (300 mg/L) was at a high level and provided the suitable nitrogen amount required by the yeast strains to complete the fermentation when 200 g/L of sugar are present in the must [24]. Generally, ammonium nitrogen is completely consumed during the first part of the fermentation (up to Vmax) that corresponds to the last part of the exponential growth [23]. The amino-nitrogen residues are reported in Table 5 for each sampling point.

The amino-nitrogen residues reflected strain fermentation rates during the exponential growth phase. The three strains that showed high Vmax values left lower amino-nitrogen residues than EC1118. Among the three strains, different nitrogen consumptions were observed. R31.3 and P301.9, which showed a similar fermentation trend, revealed different nitrogen consumptions. Between the two sampling points, no further nitrogen consumption was apparently registered. This could be due to the nitrogen release by cell lysis that occurred during the stationary phase [14].

### 3.2. Expression of Hexose Transporters Genes during Late Fermentation

During the late fermentation phase, two cell samplings were performed, at 45 and 60 g/L of $CO_2$ produced, to analyze the gene expression of hexose transporters that are known to be active during the fermentation process, namely *HXT 1-8*, *GAL2*, and *FSY1*. All the investigated genes were present in the strain genome, but *FSY1* was not found in strain R008 [11].

Comparing strains gene expression (Figure 2), each strain evidenced a specific pattern both at 45 and at 60 g/L of $CO_2$ produced.
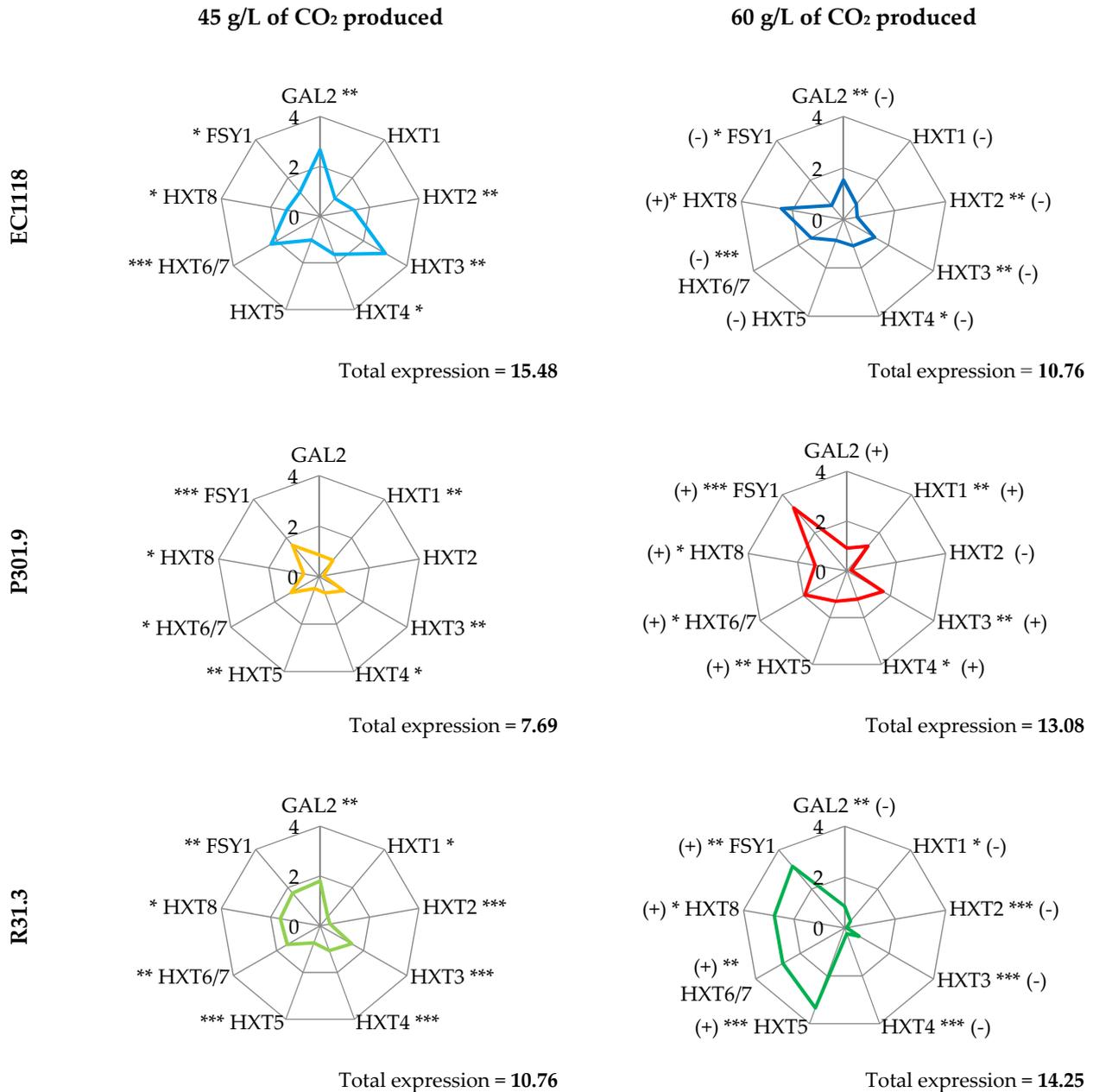
**45 g/L of $CO_2$ produced**    **60 g/L of $CO_2$ produced**
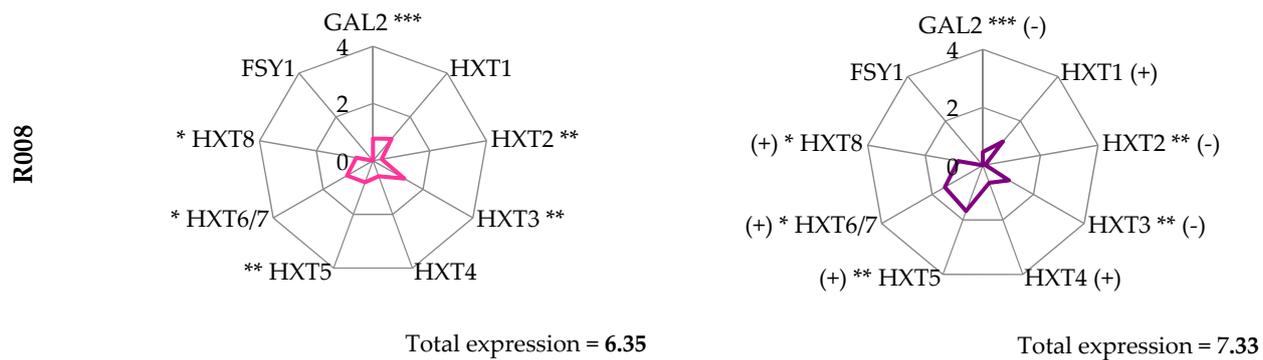


**Figure 2.** *Cont.*

**Figure 2.** Gene expression pattern of hexose transporters. Letters indicate significant differences in the gene expression values obtained from ANOVA analysis of variance followed by the Tukey post-hoc test between the strains, at 45 or 60 g/L of $CO_2$ (*alpha* = 0.05). (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$ between gene expression values at 45 and 60 g/L of produced $CO_2$ by Student's *t* test for each yeast strain.

The global expression rate was calculated by summing the expression rate of each single gene analyzed. At 45 g/L of $CO_2$ produced, EC1118 showed the highest expression rate (15.48) and strain R008 showed the lowest (6.35). EC1118 was the only strain that decreased the global expression rate at 60 g/L of $CO_2$ produced (10.76), reaching a global expression rate value lower than P301.9 and R31.3 (13.08 and 14.25, respectively). R008, which left a sugar residue at the end of the fermentation, showed the lowest level of the global expression rate at both sampling times. Regarding the good fermenting yeasts (EC1118, P301.9, and R31.3), in the two vineyard strains, the expression of the tested genes increased at 60 g/L of $CO_2$ produced. On the contrary, in EC1118, most of the tested genes showed a decrease of expression.

When the expression of each gene was compared among the four strains (Table 6), different expression levels were always detected suggesting a strain-specific expression pattern at the two sampling points and trend.

*HXT3* was the most expressed gene at 45 g/L (in EC1118 relative expression 3.004). This gene encodes a low-affinity transporter, and its expression requires the presence of glucose, but it is only weakly dependent on sugar concentration [25]. It has been demonstrated that, among the *HXT* family, *HXT3* has the highest capacity to support fermentation. In fact, Hxt3p is the only carrier that is expressed throughout the fermentation, consistent with the fact that it plays a key role in the process [26]. The robust expression and high stability of Hxt3p during the stationary phase is consistent with its capacity to maintain a high fermentation rate during starvation when expressed alone [7].

Transport-kinetic data support the idea that Hxt3p is the primary low-affinity transporter during stationary phase. All these previous findings were related to strain V5 isolated from Champagne [26], such as EC1118. In the other vineyard strains, the expression level is nearly half, and at 65 g/L, it decreased in EC1118, R008, and R31.3 but increased in P301.9. In the two vineyard strains that completed fermentation (P301.9 and R008), *HXT3* was not the most expressed strain both at 45 and 60 g/L of produced $CO_2$. This finding caused us to rethink the role of the Hxt3 transporter during fermentation. The main contribution of *HXT3* to maintain a high fermentation ratio during the late fermentation phase, as suggested by the literature, could be limited to specific yeast genotypes.

The second most expressed gene at 45 g/L of produced $CO_2$ was *GAL2.* Substrate-inducible and glucose repressible galactose transporter Gal2p, which is more than 60% identical to the Hxt transporters, mediates the transport of galactose by the mechanism of facilitated diffusion. It has been demonstrated that the Gal2p also mediates the transport of glucose in the *HXT1-7* null mutant [27]. This finding suggested that *GAL2* can play a role when glucose concentration is low such as in the late fermentation phase, although the expression level is reduced at 60 g/L of $CO_2$ produced with the exception of P301.9.

**Table 6.** Normalized expression of hexose transporter genes.

| Gene | Strain | 45 g/L of $CO_2$ Produced | | 60 g/L of $CO_2$ Produced | |
|---|---|---|---|---|---|
| *GAL2* | EC1118 | 2.660 ± 0.655 | C | 1.533 ± 0.434 | B |
| | R008 | 0.775 ± 0.184 | A | 0.465 ± 0.082 | A |
| | R31.3 | 1.801 ± 0.519 | B | 0.851 ± 0.521 | A |
| | P301.9 | 0.862 ± 0.236 | A | 0.914 ± 0.131 | AB |
| *HXT1* | EC1118 | 0.925 ± 0.239 | B | 0.771 ± 0.130 | B |
| | R008 | 0.996 ± 0.125 | B | 1.072 ± 0.116 | BC |
| | R31.3 | 0.475 ± 0.045 | A | 0.356 ± 0.075 | A |
| | P301.9 | 0.849 ± 0.111 | B | 1.300 ± 0.254 | C |
| *HXT2* | EC1118 | 1.364 ± 0.565 | B | 0.536 ± 0.385 | B |
| | R008 | 0.304 ± 0.198 | A | 0.089 ± 0.017 | A |
| | R31.3 | 0.399 ± 0.044 | A | 0.084 ± 0.045 | A |
| | P301.9 | 0.181 ± 0.052 | A | 0.167 ± 0.035 | A |
| *HXT3* | EC1118 | 3.004 ± 0.977 | B | 1.385 ± 0.134 | BC |
| | R008 | 1.257 ± 0.106 | A | 1.031 ± 0.123 | AB |
| | R31.3 | 1.454 ± 0.395 | A | 0.648 ± 0.101 | A |
| | P301.9 | 1.117 ± 0.183 | A | 1.662 ± 0.399 | C |
| *HXT4* | EC1118 | 1.642 ± 0.455 | C | 1.090 ± 0.224 | C |
| | R008 | 0.578 ± 0.234 | A | 0.640 ± 0.126 | B |
| | R31.3 | 1.065 ± 0.230 | B | 0.240 ± 0.128 | A |
| | P301.9 | 0.686 ± 0.194 | AB | 1.209 ± 0.474 | C |
| *HXT5* | EC1118 | 1.028 ± 0.466 | B | 0.859 ± 0.257 | A |
| | R008 | 0.806 ± 0.281 | AB | 1.673 ± 0.754 | A |
| | R31.3 | 0.731 ± 0.158 | AB | 3.341 ± 1.546 | B |
| | P301.9 | 0.505 ± 0.116 | B | 1.301 ± 0.338 | A |
| *HXT6/7* | EC1118 | 2.252 ± 0.181 | C | 1.439 ± 0.050 | A |
| | R008 | 1.045 ± 0.129 | A | 1.502 ± 0.499 | A |
| | R31.3 | 1.511 ± 0.447 | B | 2.782 ± 0.938 | B |
| | P301.9 | 1.267 ± 0.277 | AB | 1.931 ± 0.613 | AB |
| *HXT8* | EC1118 | 1.354 ± 0.496 | B | 2.430 ± 0.870 | B |
| | R008 | 0.593 ± 0.097 | A | 0.862 ± 0.314 | A |
| | R31.3 | 1.613 ± 0.390 | B | 2.782 ± 1.106 | B |
| | P301.9 | 0.606 ± 0.213 | A | 1.289 ± 0.692 | A |
| *P301_O30021* | EC1118 | 1.254 ± 0.496 | A | 0.712 ± 0.256 | A |
| | R31.3 | 1.709 ± 0.308 | A | 3.164 ± 1.018 | B |
| | P301.9 | 1.617 ± 0.203 | A | 3.301 ± 0.727 | B |

Uppercase letters indicate significant differences in the gene expression values obtained from ANOVA analysis of variance followed by the Tukey post-hoc test between the strains, at 45 or 60 g/L of $CO_2$ (*alpha* = 0.05).

The *HXT6* and *HXT7* genes encode the most closely related (they differ only in two amino acid residues), high-affinity transporters. Due to this high sequence similarity, the same primer couple was used for qPCR quantification. They are highly expressed at very low glucose concentrations, non-fermentable carbon sources (ethanol, glycerol), maltose and/or galactose, and repressed by moderate and high glucose concentrations [25]. Previous findings evidenced that the two genes displayed a similar expression profile during alcoholic fermentation. In strain V5, they displayed a strong burst of expression following the entry into the stationary phase and the respective proteins remained abundant through the late fermentation phase [26]. The EC1118 expression value at 45 g/L and 60 g/L of $CO_2$ produced confirmed the previous finding. The vineyard strains evidence an opposite trend. At 60 g/L, an increase in the expression level was observed for all the strains. These findings suggest that *HXT6* and *HXT7* have a major role in maintaining the high fermentation rate during the late fermentation phase.

The expression pattern and function of the hexose transporter encoded by *HXT8* is still poorly defined [28]. It has 70% similarity with *HXT6* [29]. It appears to be unable to

support growth on glucose of the *HXT1-7* null strain [30]. However, when overexpressed in an *HXT1–17 GAL2* null strain, it was able to restore growth on glucose, fructose, and mannose, confirming that it is a functional hexose transporter [31]. The expression of Hxt8p is induced by low and repressed by high levels of glucose [32]. This finding was confirmed by the gene expression values displayed by the four strains. All yeast showed an increase in the expression level from 45 to 60 g/L of $CO_2$ produced, particularly in EC1118 and R31.3 that registered the highest expression values.

Hxt5p has a moderate affinity for glucose and a low affinity for fructose and for mannose [33]. It is apparently regulated by the growth rate of the cells rather than by the external glucose concentration [34]. In addition, it is upregulated upon nitrogen and carbon starvation [35]. Therefore, it could be of interest during the late fermentation phase. The expression level in EC1118 is stable at 45 and 60 g/L, and in vineyard strains, a marked increase was evidenced, particularly in the case of R31.3 where the expression level rose almost 5-fold.

The *HXT2* and *HXT4* genes, as well as *HXT5*, have been classified as genes encoding transporters with moderate affinity for glucose (Km values around 10 mM) [27,36] that are maximally expressed at a low concentration of glucose and repressed in the presence of high glucose concentrations [25,37]. Despite moderate affinity, the *HXT2* expression pattern showed a decreasing expression level from 45 to 60 g/L, and specially for vineyard strains was very low. These results were consistent with previous findings that evidenced a strong activation of the *HXT2* gene only during the first hours of fermentation, returning to very low levels during the growth phase. This transient induction of *HXT2* is somewhat contradictory to the known regulation of this gene, as it has been shown to be repressed by high glucose concentrations [38]. *HXT2* appeared to be able to bypass glucose repression during the lag phase but returned to a repressed state as growth resumed. Conversely, they did not observe an induction of *HXT2* at the end of fermentation when the hexose concentration became low and sub-repressive [26].

On the contrary, *HXT4* expression showed a strain-mediated trend: Decreasing in EC1118 and markedly so in R31.3, constant in R008, and increasing in P301.9.

*HXT1*, as well as *HXT3*, is a low-affinity carrier and it is strongly expressed at the beginning of fermentation, while its expression level decreased rapidly during the growth phase, consistent with the *HXT1* gene being induced by high sugar concentrations [26]. R31.3 seemed to confirm this previous finding as it showed a very low expression level decreasing from 45 to 60 g/L produced $CO_2$. At the two sampling points, similar expression levels were found in EC1118 and R008. Surprisingly, P301.9 showed a notably increased value at 60 g/L of $CO_2$ suggesting a role of *HXT1* in supporting the fermentation ratio during the late fermentation phase. The *FSY1* gene was firstly isolated in *Saccharomyces pastorianus* and its protein was the first fructose permease identified in a yeast species [39]. The EC1118 complete genome sequence revealed the presence of a gene sequenced responsible for a protein highly similar to that encoded by the *S. pastorianus'* specific fructose symporter *FSY1* gene [10]. The functional analysis of the EC1118 *FSY1* gene demonstrated that it encodes for a high-affinity fructose/H+ symporter [3]. Since hexoses are transported by facilitated diffusion via hexose carriers (Hxt), which prefer glucose to fructose, the utilization of fructose by wine yeast is critically important in the late phase of the fermentation where fructose is the most present sugar. Previous findings demonstrated that, in EC1118, *FSY1* was repressed by high concentrations of glucose or fructose and was highly expressed on ethanol as the sole carbon source [3]. Interestingly, the *FSY1* expression pattern was very different between EC1118 and the other strains that possessed this gene (R31.3 and P301.9). Indeed, they showed a marked increase of the expression level at 60 g/L of $CO_2$ produced. This finding suggested that due to the expression level, this gene could have a different impact on strain fermentation rate during late fermentation.

The expression percentage of each transporter-coding gene is reported in Figure 3.

| | EC1118 | | P301.9 | | R31.3 | | R008 | |
|---|---|---|---|---|---|---|---|---|
| | *45 g/L* | *60 g/L* | *45 g/L* | *60 g/L* | *45 g/L* | *60 g/L* | *45 g/L* | *60 g/L* |
| GAL2 | 17.2 | 14.3 | 11.2 | 7.0 | 16.7 | 6.0 | 12.2 | 6.3 |
| HXT1 | 6.0 | 7.2 | 11.0 | 9.9 | 4.4 | 2.5 | 15.7 | 14.6 |
| HXT2 | 8.8 | 5.0 | 2.4 | 1.3 | 3.7 | 0.6 | 4.8 | 1.2 |
| HXT3 | 19.4 | 12.9 | 14.5 | 12.7 | 13.5 | 4.5 | 19.8 | 14.1 |
| HXT4 | 10.6 | 10.1 | 8.9 | 9.2 | 9.9 | 1.7 | 9.1 | 8.7 |
| HXT5 | 6.6 | 8.0 | 6.6 | 9.9 | 6.8 | 23.5 | 12.7 | 22.8 |
| HXT6/7 | 14.5 | 13.4 | 16.5 | 14.8 | 14.0 | 19.5 | 16.4 | 20.5 |
| HXT8 | 8.7 | 22.6 | 7.9 | 9.9 | 15.0 | 19.5 | 9.3 | 11.8 |
| FSY1 | 8.1 | 6.6 | 21.0 | 25.2 | 15.9 | 22.2 | | |
| Total (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Variation (%) | −30,5 | | +70.0 | | +32.4 | | +15.4 | |

**Figure 3.** Different utilization of the tested hexose transporter genes among yeast strains. Data per each gene are expressed as percentage calculated on the total expression, for each strain and time point (45–60 g/L of produced $CO_2$). The difference between the total expression values from 45 to 60 g/L of produced $CO_2$ is reported as percentage of variation.

In EC1118, the decrease in the expression level from 45 to 60 g/L of produced $CO_2$ is 30,5%, while in the other strains the increase is from +15.4 in R008 to +70% in P301.9. At 45 g/L in EC1118, the expression of *HXT3*, *GAL2*, and *HXT6/7* accounted for more than 50% of the overall gene expression and at 60 g/L, *HXT8* was the most expressed gene. At 45 g/L in P301.9, *FSY1* is the most expressed gene followed by *HXT6/7* and *HXT3*. At 60 g/L, all these genes showed an increase in the expression level that is more evident in *FSY1*. In R31.3 at 45 g/L, *GAL2* is the most expressed gene and *HXT6/7*, *HXT8*, and *FSY1* showed similar expression percentages. At 60 g/L, they accounted for more than 50% of the overall gene expression the showed the highest increase in total expression (+76%), followed by R31.3 and R008. At 45 g/L of produced $CO_2$, strain EC1118 showed the highest total expression followed by R31.3, P301.9, and R008, while at 60 g/L of produced $CO_2$, strain R31.3 showed the highest total expression followed by P301.9, EC1118, and R008 (Figure 3).

At 45 g/L of produced $CO_2$, the most expressed genes in EC1118 were *HXT3*, *GAL2*, and *HXT6/7*. The expression pattern of *HXT3* and *HXT6/7* confirmed previous findings that assessed *HXT3* as the most highly expressed *HXT* gene during alcoholic fermentation and *HXT6/7*, expressed throughout fermentation [40]. *HXT3* and *HXT8* (together with *HXT1*) were the most expressed gene in R008, although at lower levels than that of EC1118. Interestingly, in P301.9, the most expressed gene was *FSY1* followed by *HXT6/7* and *HXT3*; in R31.3, *FSY1* showed an expression level similar to the most expressed genes, *HXT8* and *GAL2*. At 60 g/L of produced $CO_2$ in EC1118, a strong increase of *HXT8* was registered, while in P301.9, *FSY1* reached the 25% of the total gene expression. In R31.3, the *GAL2* gene expression strongly dropped down, while *FSY1*, *HXT8*, *HXT6/7*, and *HXT5* increased. Although the general low gene expression level in R008 caused an increase in the expression of *HXT5* and *HXT6/7*, *HXT8* was found (Figure 3).

## 4. Discussion

The hexose transporters' activity during late fermentation phase is crucial to ensure the complete transformation of the sugar into ethanol. In fact, a high fructose/glucose ratio may cause sluggish and stuck fermentations with high levels of fructose residue, which is a major problem in the wine industry [3,41].

The transport of glucose and fructose in the yeast *Saccharomyces cerevisiae* plays a crucial role in controlling the rate of wine fermentation, and the yeast fermentation performance is strongly influenced by the hexose carrier set-up [7,26,42]. *Saccharomyces cerevisiae* is the organism provided with the highest number of hexose transporter genes. In its genome, twenty genes encode hexose transporter-like proteins (*HXT1* to *HXT17*, *GAL2*, *SNF3*, and *RGT2*) [43].

The molecular function of these hexose transporters is redundant, as none of these transporters are essential for growth on glucose. The cooperation among the involved transporters determines the effectiveness of the sugar-uptake systems. In *S. cerevisiae*, two uptake systems have been described: The first, a constitutive low-affinity system involving *HXT2*, *HXT6*, and *HXT7*, and the second, a glucose-repressed high-affinity system responsible for the high-affinity transporters, *HXT1*, *HXT3*, or *HXT4* [27]. In contrast, the expression of *HXT5* [33] is influenced by the growth rate of cells and not by the extracellular glucose concentration. Strains lacking *HXT1* through *HXT7* genes are unable to grow on glucose, fructose, or mannose and have no glycolytic flux [8,27]. Despite extensive research, the functions of Hxt8-Hxt17 have remained poorly defined. *HXT8*, induced by low levels of glucose, appears to function as a glucose transporter since it can partially complement the glucose growth defect of the *hxt* null mutant [30]. Only recently, Treu and colleagues [44] found that the *HXT8* gene was expressed during the first stage of synthetic grape must fermentation, although at a lower level than *HXT1–7* genes. *HXT12* was found to be a pseudogene and only recently Jordan and co-workers [45] described Hxt13p, Hxt15p, Hxt16p, and Hxt17p as a novel type of polyol transporters, not involved in glucose and fructose transport. Another gene with similarity to the *HXT* family is present in the yeast genome: *GAL2*. It encodes a galactose transporter that is also a high-affinity glucose transporter [36]. The *FSY1* gene, encoding for a fructose/H+ symporter, previously identified in *S. pastorianus* [39], has been discovered and characterized in the EC1118 *Saccharomyces cerevisiae* strain [3,5]. In 2014, Treu and colleagues [11] found the fructose transporter in a vineyard strain (P301) with the same origin as the strains tested in this study. Following P301 transcriptome analysis under fermentation conditions reported that *FSY1* gene was expressed at lower levels with respect to other hexoses transporter genes [44]. Notwithstanding, *FSY1* gene is potentially very interesting as in the late fermentation phase, fructose is the main sugar residue due to yeast glucophilic behavior.

With the aim to evaluate the role of the different hexose transporter gene expressions in the strain ability to complete sugar fermentation, four *S. cerevisiae* strains were considered: he industrial wine EC1118 and the vineyard P301.9, R31.3, and R008. Previous results found that the strain R008 genome does not contain the *FSY1* gene [11].

Kinetics of carbon dioxide production differed among the tested strains, evidencing optimal (EC1118), intermediate (P301.9 and R31.3), and poor (R008) fermentation trends. During early fermentation (up to the fermentation rate peak, Vmax) that was consistent with the yeast exponential growth phase [23], vineyard strains showed better fermentation performance than EC1118, particularly P301.9. During late fermentation (after the fermentation rate peak to the end of the process), an opposite trend was observed. EC1118 kept the fermentation rate at a higher level than the other strains and completed the fermentation earlier. Strain R008 showed the lowest fermentation rate during the late fermentation phase, leaving a fructose residue and producing about 1% less ethanol. The fermentation trends were consistent with the fructose/total sugar ratio and amino acid consumption found at 45 g/L. EC1118 that showed the lowest fermentation level during early fermentation consumed the lowest amount of amino acid and fructose. P301.9 showed the highest Vmax value and consumed the highest number of amino acids, although the fructose/total

sugar ratio showed no significant difference with the other vineyard strains. No significant differences were found between amino acid residues at 45 and 60 g/L in EC1118 and P301.9, the best fermenting strains. On the opposite, R31.3 and R008 showed a significant increase in amino nitrogen residues. This could be due to higher cell mortality level at 60 g/L that could also be responsible for the longer fermentation time.

All the *HXT* genes involved in glucose/fructose transport (*HXT1-8*) and *GAL2* and *FSY1* genes were tested by means of Real-Time PCR, and their relative gene expression has been determined. The results evidenced that all the genes tested were expressed during late fermentation regardless of their attitude to be inhibited by high or low glucose regulation pathways.

High-affinity transporter gene *HXT6-7*, intermediate affinity *HXT2* and *HXT4*, *HXT8*, and *GAL2* are induced by a low glucose concentration, therefore an increase in their expression is expected. On the contrary, low-affinity transporter genes *HXT1* and *HXT3*, induced by high glucose, were supposed to decrease their expression. EC1118 showed a decrease expression for all the genes, except for *HXT8*. In P301.9, all gene expressions increased, except for *HXT2* that remained unchanged. R31.3 showed a similar trend to EC1118, except for *HXT6/7* and *HXT8* that increased their expression level. Despite the very low total expression level, R008 showed an intermediate trend as *HXT6/7* and *HXT8* increased as in P301.9 and *HXT2*, *HXT3*, and *GAL2* decreased as in EC1118. These results suggested that the "low-glucose" signal that controls high- and intermediate-affinity transporters gene expression turned up at higher sugars concentration in EC1118 cells than the others. On the contrary, the "high-glucose" signal that controls low-affinity transporters gene expression turned up at higher sugar concentrations in P301.9 cells than the others.

*HXT5* gene expression was maximally induced upon glucose and nitrogen depletion [34]. In this work, the *HXT5* gene expression increased in R31.3, P301.9, and R008 from 45 to 60 g/L of produced $CO_2$, while EC1118 remained low and constant. These findings are consistent with strain nitrogen consumption values as the vineyard strains consumed more nitrogen that EC1118 during early fermentation.

Previous works demonstrated that in EC1118, *FSY1* was repressed by high concentrations of glucose or fructose and was highly expressed on ethanol as the sole carbon source [3]. Surprisingly, in this study, *FSY1* was only poorly expressed at 45 and at 60 g/L in EC1118, evidencing a decrease in the expression from 45 to 60 g/L. On the contrary, in the two other strains containing this gene, the expression level was very high, and a marked increase was evident from 45 to 60 g/L. Therefore, the *FSY1* gene seems to play an important role in the sugar uptake of these yeast strains. EC1118 possessed the lowest "fructose/total residual sugars" ratio values (F/T ratio) at both sampling times. These data suggested that EC1118 is more capable of handling the F/T ratio than the other strains during early fermentation, leaving less fructose during late fermentation. In this condition, P301.9 and R31.3 took advantage of the high-affinity fructose/H+ symporter FSY1 to complete sugar consumption. Therefore, the high level of expression assured the fructose consumption during the late fermentation phase. This is not the case for R008, as they do not possess the *FSY1* gene. The lack of this gene, together with a low gene expression of all the genes of the HXT transport system, could be the cause of the poor fermentation performance during late fermentation.

## 5. Conclusions

In the fermentation trial, all the hexose transporter genes were expressed at both 45 and 60 g/L $CO_2$. This indicated that the corresponding sugar concentration range supported both the high and low glucose-dependent transporters.

The very low total gene expression of the transporters and the poor fermentation performance of R008 suggested that the overall expression level is a determinant to maintain a high fermentation rate during the late fermentation phase.

Each yeast showed a specific gene expression profile that was strongly variable among the strains. This led to rethinking the importance of the *HXT3* that was previously consid-

ered to play a major role in sugar transport during the overall fermentation process. The main contribution of *HXT3* to maintain a high fermentation ratio during late fermentation phase [7,40] could be limited to specific yeast genotypes. In different genetic contexts, other genes such as *HXT6/7*, *HXT8*, and *FSY1* were the most expressed and therefore responsible for sugar transport in the late fermentation phase.

Finally, two trends emerged from the data collected: EC1118, that reduced the gene expression of "low/high glucose"-induced genes, and P301.9, showing a gene expression increase. These findings suggest a strain-specific response of the "high/low glucose"-dependent genes that control most of the sugar transport in the yeast cell.

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