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Molecular adaptation response of *Oenococcus oeni* in non-*Saccharomyces* fermented wines: A comparative multi-omics approach

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ABSTRACT

Oenococcus oeni is the main agent responsible for malolactic fermentation (MLF) in wine. This usually takes place in red wines after alcoholic fermentation (AF) carried out by *Saccharomyces cerevisiae*. In recent years, there is an increasing interest in using non-*Saccharomyces* yeast, usually in combination with *S. cerevisiae*, to improve wine quality. Current studies report a stimulatory effect of non-*Saccharomyces* on MLF, generally related to a decrease in the inhibitor compounds found in wine. In this work, we followed a comparative multi-omics approach, including transcriptomic and proteomic analysis, to study the molecular adaptation of *O. oeni* in wines fermented with *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, two of the most frequently used non-*Saccharomyces*, in sequential inoculation with *S. cerevisiae*. We compared the results to the adaptation of *O. oeni* in *S. cerevisiae* wine to determine the main changes arising from the use of non-*Saccharomyces*. The duration of MLF was shortened when using non-*Saccharomyces*, to half the time with *T. delbrueckii* and to a quarter with *M. pulcherrima*. In this work, we observed for the first time how *O. oeni* responds at molecular level to the changes brought about by non-*Saccharomyces*. We showed a differential adaptation of *O. oeni* in the wines studied. In this regard, the main molecular functions affected were amino acid and carbohydrate transport and metabolism, from which peptide metabolism appeared as a key feature under wine-like conditions. We also showed that the abundance of Hsp20, a well-known stress protein, depended on the duration time. Thus, the use of non-*Saccharomyces* reduced the abundance of Hsp20, which could mean a less stressful wine-like condition for *O. oeni*.

1. Introduction

Oenococcus oeni is the main microbial agent responsible for malolactic fermentation (MLF) in wine (Lonvaud-Funel, 1999). This process occurs naturally, but it can also be brought about through the inoculation of selected strains. MLF usually takes place after alcoholic fermentation (AF) that is driven by oenological yeasts (Ribéreau-Gayon et al., 2006). During AF, the yeasts transform grape must into wine consuming much of the nutrients present in the medium. Moreover, several inhibitor compounds are produced by yeast during this process, such as a high content of ethanol, SO_2 and medium-chain fatty acids, that negatively affect the development of *O. oeni* in wine (Balmaseda et al., 2018; Bech-Terkilsen et al., 2020).

Traditionally *Saccharomyces cerevisiae* has been used as a starter culture for AF (Padilla et al., 2016). However, recent studies on non-conventional wine yeasts other than *S. cerevisiae* have led to their increased use in winemaking. Indeed, different strains of non-*Saccharomyces* are now commercially available, including strains of the *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* species (Capozzi et al., 2015; Roudil et al., 2020). Given that the metabolism of the fermenting yeasts will determine the physicochemical conditions of the wine after AF (Berbegal et al., 2020), directly affecting the development of MLF (Balmaseda et al., 2018; Berbegal et al., 2019). The use of some non-*Saccharomyces* has been linked to the mitigation of certain inhibitory compounds, thereby having a positive effect on MLF (Balmaseda et al., 2021a; Martín-García et al., 2020).

Wine is a very harsh environment, in which *O. oeni* needs to overcome several oenological stress factors (Bech-Terkilsen et al., 2020). The

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genetic characteristics of this lactic acid bacterium (LAB) make it the best adapted to wine-like conditions (Beltramo et al., 2006), with stress response genes such as *clp*, *grpE*, *groES*, *hsp18*, *hdc*, *ftsH*, *cfa* and *trxA* appearing to be essential (Beltramo et al., 2004; Bourdineaud, 2006; Bourdineaud et al., 2003, 2004; Guzzo et al., 2000; Jobin et al., 1997; Margalef-Català et al., 2016; Olguín et al., 2009, 2010; Spano and Massa, 2006). Other genes associated with nitrogen metabolism and translation have also been reported as being essential in order to overcome wine conditions (Margalef-Català et al., 2016).

Researches on induction and repression under wine conditions is essential to better understand the role of these genes. The publication of the first complete genome of the *O. oeni* strain PSU-1 in 2005 (Mills et al., 2005) made it possible to implement omics approaches. Since then a number of authors have studied the *O. oeni* adaptation mechanisms. Silveira et al. (2004) showed that the proteomic profile of *O. oeni* was different in ethanol shock or adaptation, while Cecconi et al. (2009) explored the proteomic profile in the adaptation to wine conditions. In addition, Liu et al. (2017) looked into the transcriptomic changes of acidic shock, and Sternes et al. (2017) investigated the transcriptomic profiles of three *O. oeni* strains in wine. Other works presented combined transcriptomics and proteomics to delve into *O. oeni* stress response. Olguín et al. (2015), for example, studied the ethanol shock after 1 h of incubation, Costantini et al. (2015) explored the adaptation to wine conditions at 24 h, and Margalef-Català et al. (2016) looked at the adaptation to wine conditions during the first 8 h in wine. Finally and more recently, Yang et al. (2020) studied the combined effects of acidic and ethanol stresses. In conclusion, most of the studies about *O. oeni* stress response have focused on abiotic conditions, whereas less is known concerning the effect of biotic factors, such as the use of non-*Saccharomyces* yeast.

The aim of this work was to evaluate the effect of different yeast inoculation strategies on the molecular mechanisms of adaptation of *O. oeni* to wine. With this objective, a transcriptomic and proteomic study of *O. oeni* PSU-1 in wines fermented with *T. delbrueckii* and *M. pulcherrima*, in sequential inoculation with *S. cerevisiae*, and in wine fermented only with *S. cerevisiae*, was carried out.

2. Materials and methods

2.1. Microorganisms and inocula

The yeast strains used were *T. delbrueckii* Biodiva, *M. pulcherrima* Flavia and *S. cerevisiae* Lalvin-QA23, all from Lallemand Inc. (Montréal, Canada). Strain *O. oeni* PSU-1 (ATCC BAA-331) was used for the MLF. To prepare the inocula, yeasts were grown in YPD medium (20 g/L glucose, 20 g/L bacto-peptone, 10 g/L yeast extract) at 28 °C and *O. oeni* was grown in MRSmf (Margalef-Català et al., 2017) at 27 °C with a 10% atmosphere of CO_2.

2.2. Fermentation trials

The must for fermentation was prepared using white grape concentrate (65.4°Bx; Mostos Españoles S.A., Tomelloso, Spain) and sterile Milli-Q purified water to obtain a density of ~1075 g/L (corresponding to ~180 g/L of glucose and fructose). After dilution with water, the must was supplemented with nutrients (0.4 g/L Nutrient Vit Nature™, Lallemand Inc.) and the pH was adjusted to 3.6 with HCl 37% (vol/vol). Finally, the must was sterilized by adding 1% (vol/vol) of dimethyl dicarbonate (Santa Cruz Biotechnology, Inc., Dallas TX, USA) and kept at 4 °C overnight.

The alcoholic fermentations were carried out in 5 L flasks containing 5 L of must statically at 20 °C. The must was inoculated with the non-*Saccharomyces* strains – *T. delbrueckii* (Td) or *M. pulcherrima* (Mp) – and then inoculated with *S. cerevisiae* after 48 h. Each yeast was inoculated for a population of 2.5×10^6 cells/mL previously grown in YDP liquid medium. There was also a control fermentation with *S. cerevisiae* as a sole starter (Sc). All the fermentations were performed in triplicate. Samples were taken every 48 h to monitor density decrease and yeast population evolution. YPD agar medium was used to calculate the total number of yeasts, and lysine agar medium (Oxoid LTD, England) was used to quantify the non-*Saccharomyces* yeasts (Wang et al., 2015), both after incubation at 28 °C for 48 h. AF was considered to be finished when the sugar (glucose + fructose) content was below 2 g/L.

After AF the wines were centrifuged (8500 ×*g* for 5 min) and then filtered with a 0.22-μm pore size filter (Merck Millipore Steritop™ Sterile Vacuum Bottle-Top Filters, Madrid, Spain). Each AF replicate of 5 L was divided into two 2 L flasks which would correspond to the t_0 MLF and t_f MLF samples. The flasks were inoculated with *O. oeni* for a population of 2×10^7 cells/mL. These fermentations were carried out statically at 20 °C and in triplicate. Samples were taken every 24 h to monitor L-malic acid consumption and bacterial population. To this end, they were plated on MRSmf and incubated at 27 °C in a 10% CO_2 atmosphere for 7 days. MLF was considered to be finished when L-malic acid was below 0.1 g/L.

Sugar content in the final stages of AF and L-malic acid during MLF were determined using the Miura One multianalyser (TDI SL, Gavà, Spain). On completion of AF and MLF, the pH was measured (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain) and primary amino nitrogen (NOPA), NH_4, citric acid and acetic acid quantified using the Y15 multianalyser (Biosystems, Barcelona, Spain). Ethanol concentration was also measured at the end of AF by ebulliometry (Electronic ebulliometer uEBU6576, GabSystem, Moja, Spain) in accordance with the Compendium of International Methods of Analysis of Musts and Wines (OIV, 2009).

2.3. Sampling for omics analysis

For the analysis of *O. oeni*, wines were sampled at the beginning of MLF (1 h after *O. oeni* inoculation, t_0 MLF flask) and at the end of MLF (when [L-malic acid] < 0.1 g/L, t_f MLF flask). Appropriate volumes of wine were centrifuged at 4600 ×*g* for 20 min at 4 °C for each analysis, 1 L for proteomic and 50 mL for transcriptomic analyses. The resulting pellet of 1 L of wine was washed with 10 mM Tris-HCl buffer at pH 8, frozen in liquid nitrogen and kept at −80 °C until protein extraction. For the transcriptomic analyses, the pellet of 50 mL of wine was washed with 10 mM Tris-HCl prepared with diethyl pyrocarbonate-treated water (DEPC), then frozen in liquid nitrogen and kept at −80 °C until RNA extraction.

2.4. RNA and protein extraction

RNA and protein extraction from *O. oeni* were performed following Margalef-Català et al. (2016). For the RNA extraction, the cell pellet was defrosted and washed again with 10 mM Tris-HCl DEPC water. A High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used for the extraction following the manufacturer's instructions, changing the cell lysis for lysozyme dissolved in 10 mM Tris-HCl buffer DEPC at 50 mg/mL for 30 min at 37 °C. Total acid nucleic concentrations were calculated using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Bremen, Germany). The extracted RNA was stored at −80 °C until RNA analysis.

The cell pellets for protein extraction were resuspended in 0.1 M Tris-HCl at pH 7.5, mixed with a protease inhibitor cocktail from Roche. Cells were disrupted using a one-shot disruptor (Constant Systems Ltd.) at 5 °C, applying 2.7 kbar pressure twice. Protein suspension was centrifuged at 4500 ×*g* for 15 min at 4 °C to remove cell debris, the protein concentration was estimated (Bradford, 1976) and the supernatant was frozen in liquid nitrogen until protein analysis.

2.5. Transcriptomic analysis

The transcriptomic analysis was performed by the Centre for Omic

Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat according to optimized protocols. The RNA of each sample was ribo-depleted using a riboPOOL kit (TOOLs Biotech) in accordance with the manufacturer's protocol. Between 60 and 250 ng of ribo-depleted RNA were used to prepare strand-specific barcoded libraries using the Total RNA-seq v2 kit (catalog no. 4475936, ThermoFisher). Each library was quantified by micro-fluid electrolysis in the Agilent TapeStation using the Agilent High Sensitivity Screen Tape kit. The barcoded libraries were amplified with an Ion 540™ Kit-Chef (Ion Torrent) and pooled on 540 chips ready to be sequenced on the GeneStudio S5 System (Ion Torrent). Low-quality reads (phred < 17) were filtered prior to analysis along with those that presented 6-bp length homopolymers. The reads were mapped against the reference genome *Oenococcus oeni* (NC_008528.1) using HISAT2 (v.2.2.0). Aligned reads were annotated and quantified with FeatureCounts (v.2.0.0). Gene expression levels were compared in R (v.3.6.2) using the edgeR package (v.3.268) (Robinson et al., 2010). The samples were normalized by the trimmed mean of M-values (TMM) method, and expression levels were expressed as counts per million (CPM).

2.5.1. *RT-qPCR validation of RNAseq data*

Those genes that exhibited the highest up- or down-regulation in one of the comparisons were selected for real-time qPCR validation of the RNAseq data (Table S1). The expression of these genes was quantified in more than one sample in order to perform multiple comparisons. The OligoPerfect Primer Designer (ThermoFisher) online tool was used for primer design. The RT-qPCR was performed following Olguín et al. (2009). Four constitutive genes (Margalef-Català et al., 2016) were evaluated as internal controls. From these, *gyrA* and *gyrB* – which presented the least variation between samples – were selected. The correlation between the RNAseq data and the values obtained with the RT-qPCR was good with an $R^2 = 0.9$ (Suppl. Fig. 1).

2.6. *Proteomic analysis*

The proteomic analysis was performed by the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat according to optimized protocols. For each sample preparation, 30 μg of protein were reduced with 4 mM 1.4-dithiothreitol (DTT) and alkylated with 8 mM iodoacetamide (IAA) before enzymatic digestion using sequencing-grade Trypsin/Lys-C (ThermoFisher Scientific, CA, USA) at an enzyme-to-protein ratio of 1:50. After digestion the TMT 10-plex labelling (ThermoFisher Scientific, CA, USA) was performed in accordance with the manufacturer's instructions. To normalize all the samples in the study we included in each TMT batch a pool of all the samples labelled with the TMT-126 tag. The TMT labelled peptides were then fractionated by Off-gel (Agilent Technologies) following the manufacturer's protocol. Peptides from the 6 fractions obtained were further separated in a C18 reversed phase (RP) nano-column (75 μm I.D.; 15 cm length; 3 μm particle diameter, Nikkyo Technos Co. Ltd., Japan) on an EASY-II nanoLC from ThermoFisher. The chromatographic separation was performed with a 90 min gradient using Milli-Q water and acetonitrile (0.1% formic acid) as a mobile phase at a flow rate of 300 nL/min.

The mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro from ThermoFisher using an enhanced FT-resolution MS spectrum ($R = 30{,}000$ FHMW) followed by a data dependent FT-MS/MS acquisition ($R = 15{,}000$ FHMW, 40% NCE HCD) of the most intense ten parent ions with a charge state rejection of one and a dynamic exclusion of 0.5 min. Protein identification and quantification were performed using Proteome Discoverer software v.1.4.0.288 (ThermoFisher Scientific) with Multidimensional Protein Identification Technology (MudPIT), combining the 6 raw data files (fractions) obtained for each sample. For protein identification, all the MS and MS/MS spectra were analysed using the Mascot search engine (v.2.5). The workflow was set up using the Mascot node combining the *O. oeni* PSU-1 database (1682 entries) and the contaminants database (247 entries). Two missed cleavages were allowed, as were an error of 0.02 Da for an FT-MS/MS fragmentation mass and 10.0 ppm for an FT-MS parent ion mass. TMT-10plex was set as a quantification modification and the oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas the carbamidomethylation of cysteine was set as a static modification. The false discovery rate (FDR) and protein probabilities were calculated by Percolator. For protein quantification, the ratios between each TMT-label and 126-TMT label were used and the quantification results normalized based on the protein median.

2.7. *Statistical analysis*

The transcriptomic CPM values (fold change) were analysed using the quasi-likelihood F-test of the edgeR package (Robinson et al., 2010) and the statistical comparisons were performed following the Benjamini-Hochberg method using a p-value cutoff < 0.05 with an FDR < 0.05.

The statistical analyses for proteomics were performed using Mass Profiler Professional Software (Agilent). Raw data were normalized by Log2 transformation and mean-centred for univariate (Student t-test). $p < 0.05$ was fixed as significant.

A next-generation clustered heat map (NG-CHM) builder (Ryan et al., 2020) with hierarchical clustering using the Euclidean distance metric with the ward agglomeration method was used to analyse the differentially expressed genes (DEG) and proteins (DEP) of some selected clusters of orthologous groups (COG).

3. Results and discussion

3.1. *Fermentation dynamics*

The wines fermented with *S. cerevisiae* as the sole starter had the quickest AF (7 days) compared to the sequential inoculations (Fig. 1). As described in the literature, the duration of AF is usually extended when more than one species is fermenting (Martín-García et al., 2020). However, the viability of *T. delbrueckii* and *M. pulcherrima* decreased rapidly when *S. cerevisiae* was inoculated. No non-*Saccharomyces* was detected by the end of the AF (Fig. 1).

After filtering the wines, *O. oeni* PSU-1 was inoculated. The MLF finished correctly in all the wines (< 0.1 g/L of L-malic acid). Under the studied conditions, it finished in 8 days (Sc wine), 4 days (Td wine) and 2 days (Mp wine) (Fig. 1). It can be seen that the duration of MLF was reduced through the use of non-*Saccharomyces* to a half (Td) or a quarter (Mp) of the time compared to the Sc control. This reduction in MLF duration has recently been addressed in other studies focusing on these yeast–bacteria interactions (Balmaseda et al., 2021a, b; Ferrando et al., 2020; Martín-García et al., 2020). The bacterial viability increased from around 2×10^7 CFU/mL to 10^8 CFU/mL and gradually decreased to the initial concentration over time (Fig. 1). In the case of the non-*Saccharomyces* wines, as the time taken was shorter, the viability at the end of MLF was greater than in the Sc wine.

No relevant differences were observed in the oenological parameters (Suppl. Table 2). Citric acid was consumed during MLF by *O. oeni* in all conditions and especially in the non-*Saccharomyces* fermented wines. A slightly increased concentration of NOPA was quantified in non-*Saccharomyces* wines, as reported in Martín-García et al. (2020).

3.2. *Global analysis of functions affected by the use of different yeast species*

In the RNAseq, 1701 expressed sequence tags (EST) were detected in the *O. oeni* adaptation to wine during MLF (t_0 and t_f samples). Of these, 66 (Sc wine), 69 (Td wine) and 101 (Mp wine) were classified as differentially expressed genes (DEG) according to t-Student test MLF t_f *vs* MLF t_0 (FC > 2, $p < 0.05$) (Fig. 2, Suppl. Table 3). These total numbers exclude the DEG corresponding to tRNAs – 13 for Sc wine, 32 for Td

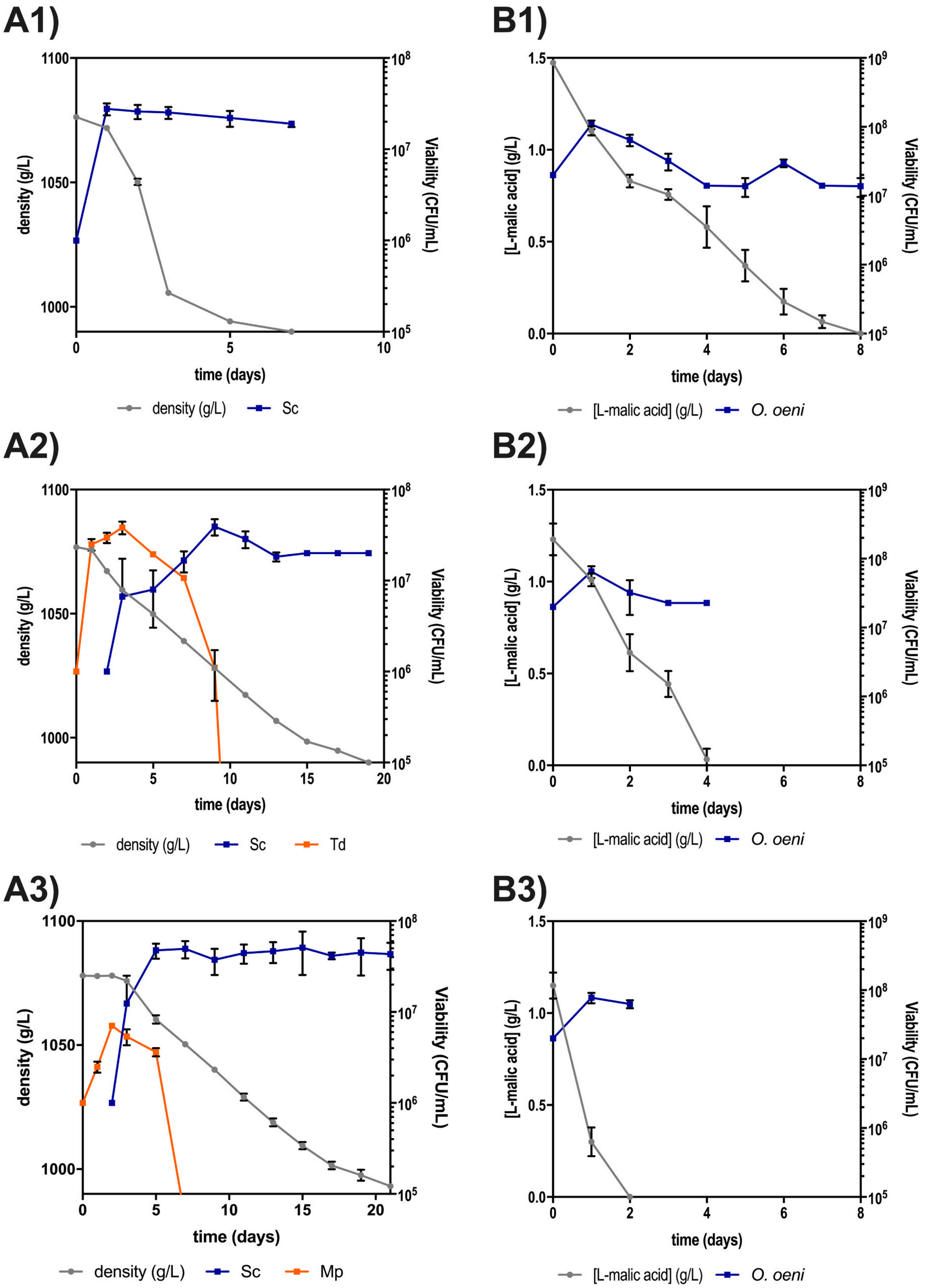
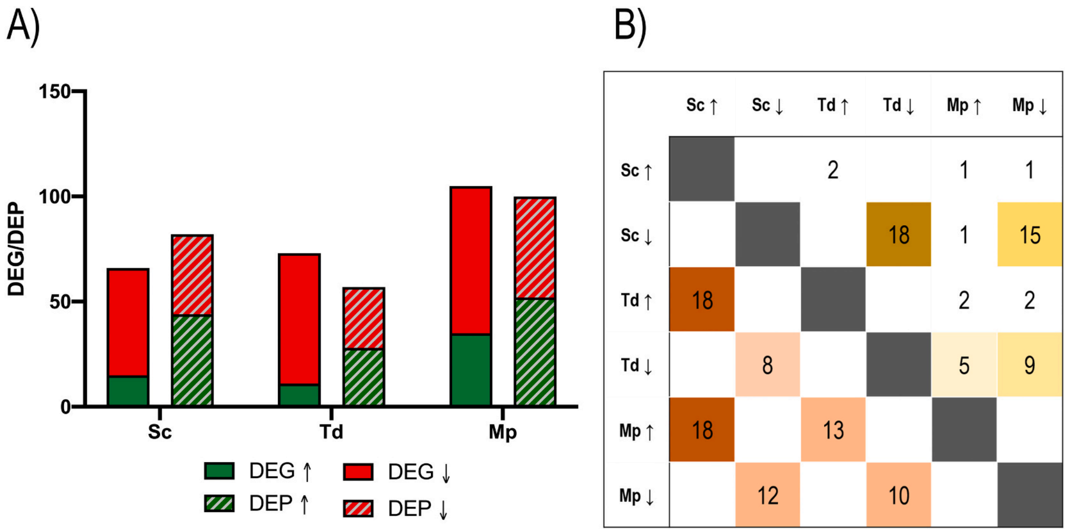


Fig. 1. Alcoholic (AF) and malolactic (MLF) fermentation dynamics, where density and L-malic acid decrease, respectively, are shown together with the viability of the fermenting agents. A1) AF of *S. cerevisiae*. A2) *T. delbrueckii* sequential AF. A3) *M. pulcherrima* sequential AF. B1) MLF in *S. cerevisiae* wine. B2) MLF in *T. delbrueckii* sequential AF. B3) MLF in *M. pulcherrima* sequential AF. Sc, Td and Mp refer to *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima*, respectively. Values shown are the mean of triplicates ± SD.



	Sc ↑	Sc ↓	Td ↑	Td ↓	Mp ↑	Mp ↓
Sc ↑			2		1	1
Sc ↓				18	1	15
Td ↑	18				2	2
Td ↓		8			5	9
Mp ↑	18		13			
Mp ↓		12		10		

Fig. 2. Total differentially expressed genes (DEG) and proteins (DEP) of *O. oeni* PSU-1 during its adaptation in the studied wines. A) Distribution of DEPs and DEGs of *O. oeni* in each wine with increased (↑) or decreased (↓) regulation or abundance, respectively. B) Table of coincident DEG (upper-right corner) and DEP (lower-left corner) with increased (↑) or decreased (↓) regulation/abundance of *O. oeni* in each condition. Cell shadow intensity corresponds to increasing number of DEGs/DEPs for each comparison. Sc, Td and Mp refer to wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation and *M. pulcherrima* sequential inoculation, respectively. Locus tags corresponding to tRNAs were excluded from this representation.

wine and 27 for Mp wine – which will be discussed later.

The number of DEG detected here (Fig. 2, Suppl. Table 3) was quite modest compared to other studies of *O. oeni* wine adaptation mechanisms (Margalef-Català et al., 2016). This might be addressable to the fact the study compared the molecular changes of the bacterium in different wines, where the only difference was the fermenting yeast. Nevertheless, the *O. oeni* functions were most differently expressed when fermenting in Mp wine. Regarding these, the most shared ESTs involved the down-regulated DEGs (Fig. 2). Sc wine presented 18 downregulated DEGs shared with Td wine and 15 with Mp wine. Only nine were common between Td and Mp wines. In contrast, less homogeneity was observed in the up-regulated DEGs for the tested comparisons.

These DEGs were classified into clusters of orthologous groups (COGs) in order to better understand the main biological processes affected by the use of non-*Saccharomyces* (Fig. 3). Globally, most of the DEGs corresponded to poorly characterized COGs, these being the uncharacterized proteins whose gene expression varied the most. Apart from these, the COGs that clustered most DEGs were those related to transcription. *O. oeni* behaved similarly in all three wines in up- or down-regulation, with a slight difference in down-regulation in the Mp wine. A similar response for all wines was also observed as regards carbohydrate transport and metabolism. COGs related to cellular process and signaling did not present many DEGs, especially in the case of the *T. delbrueckii* wines. Interestingly, DEGs related to amino acid transport and metabolism were downregulated in non-*Saccharomyces* wines, whereas in the Sc wine they were up-regulated. As regards nucleotide transport and metabolism, a dramatic up-regulation was observed in the Td wines compared to the slight down-regulation in the Sc wine.

After proteomic analysis, 741 proteins were identified. Of these, 658 were considered for analysis because they were present in over 70% of the samples in at least one of the conditions. An unpaired Student's *t*-test MLF t_f *vs* MLF t_0 ($FC > 1.5$, $p < 0.05$) was performed after $\log_2$ normalization, resulting in 82 differentially expressed proteins (DEPs) in the Sc wine, 57 in the Td wine and 100 in the Mp wine (Fig. 2, Suppl. Table 4). Overall, the number of DEPs that presented an increased abundance was similar to that of DEPs with decreased abundance.

The protein study through proteomics presented a greater number of shared DEPs than DEGs in *O. oeni* adaptation for the three tested wines (Fig. 2). Like we observed with DEGs, the highest number of shared DEPs was found when comparing the Td and Mp wines with the Sc wine. 18 DEPs with increased abundance in Td and Mp wines were common to Sc and 13 of them were shared between Td and Mp wines. Similar behaviour was observed with DEPs with decreased abundance (Fig. 2).

Analogously to DEGs, DEPs were classified into COGs (Fig. 3). It should be noted that a small number of DEPs corresponded to poorly characterized COGs. In this regard, the most variable COGs were related to metabolism, mostly involving amino acid and carbohydrate transport. A homogeneous response was observed in amino acid transport and metabolism COGs with the exception of the least abundant DEPs in Mp wine. Indeed, changes in these COGs are associated with adaptation in wine-related conditions (Margalef-Català et al., 2016). Also, *O. oeni* proteome was enriched by lipid transport and metabolism proteins with an increased abundance.

3.3. Main metabolisms modified by the use of different fermenting yeasts

3.3.1. Carbohydrate transport and metabolism

The carbohydrate metabolism of *O. oeni* in Td wines showed fewest changes in the proteome during MLF. The two DEPs with the highest increase in abundance in *O. oeni* from the Mp and Sc wines – but not the Td wine – were 2-hydroxyisocaproate dehydrogenase (OEOE_RS05695) and phosphoketolase (OEOE_RS05700) (Suppl. Table 4). These two proteins gathered in the same cluster due to high values of differential expression compared to the other DEPs (Cluster I, Fig. 4A). In the Sc wines, *O. oeni* increased the abundance of these two proteins more than 10-fold at the end of MLF, these being by far the most abundant DEPs of all the proteins detected. The enzyme 2-hydroxyisocaproate dehydrogenase (HicD) is associated with the production of 2-hydroxyisocaproic acid (HICA) from leucine. This compound has an antifungal activity (Axel et al., 2016) and its production by *Lactobacillus* and *Leuconostoc* species has been described in various fermented foods (Axel et al., 2016; Park et al., 2017). There are no previous reports about HicD or HICA in *O. oeni*. The role of this compound in microbial interaction may be worth considering in future MLF research.

As for phosphoketolase (PK), this is a key enzyme in the heterolactic fermentation of sugars, known as the phosphoketolase pathway. This protein catalyzes the cleavage of D-xylulose 5-phospate and inorganic phosphate to form acetyl phosphate and D-glyceraldehyde 3-phosphate.

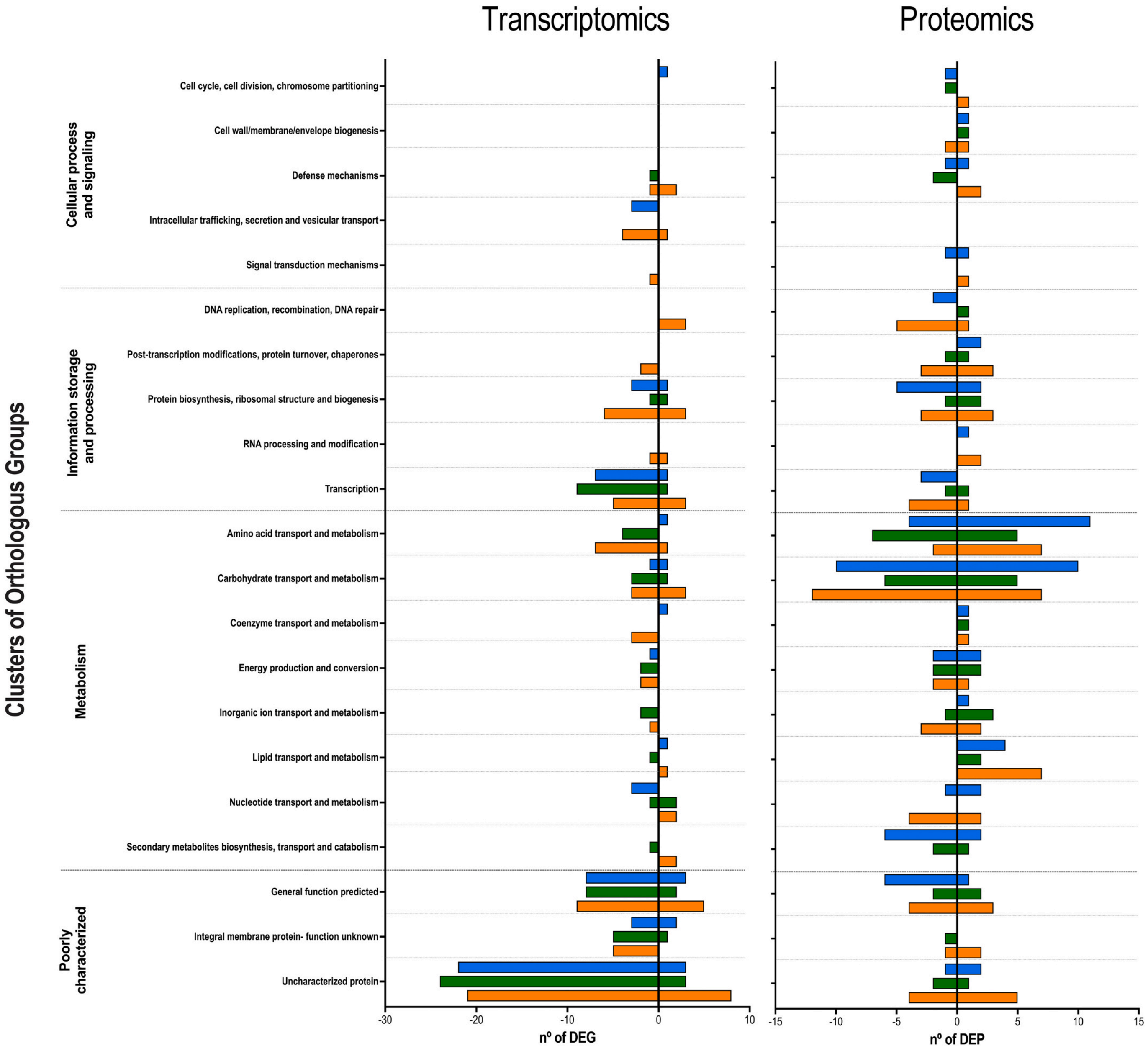


Fig. 3. Distribution of differentially expressed genes (DEG) and proteins (DEP) in *O. oeni* fermenting in the three different wines organized in clusters of orthologous groups (COGs). Positive bars represent the number of increased regulation/abundance of DEGs/DEPs. Negative bars represent the number of decreased regulation/abundance of DEGs/DEPs. Absence of bars means no DEGs/DEPs for that COG. Blue, green and orange refer to wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation and *M. pulcherrima* sequential inoculation, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although residual sugars were barely consumed at the end of MLF in our study (Suppl. Table 2), some authors have described how most of the ATP formed from the phosphoketolase pathway in the presence of ethanol occurs once L-malic acid has been consumed (Contreras et al., 2018), as occurs with other alternative energy sources such as citric acid (Bartowsky and Henschke, 2004) and mannoproteins (Balmaseda et al., 2021c). The increased abundance of PK at the end of MLF could be indicative of the metabolic changes undergone by *O. oeni* in response to L-malic acid exhaustion. The increase in PK synthesis would prepare the cell for the consumption of other energy sources, in this case, sugars. However, this was only detected in *O. oeni* from the Mp and Sc wines and not from the Td wines, where the activation of PK may have occurred later.

Cluster II (Fig. 4A) mainly included proteins from the central pyruvate metabolism (*i.e.*, pyruvate oxidase and enolase) that increased their abundance during MLF in the different wines. It should be noted that all the DEPs in Td wines in this cluster are coincident in Sc wines, meaning that *O. oeni* had more similar behaviour regarding these proteins in the Td and Sc wines. However, other DEPs were detected exclusively in *O. oeni* from the Sc and Mp wines. The increased abundance of these proteins at the end of MLF may be associated with the metabolic switch in response to L-malic acid exhaustion, as hypothesized for PK.

The proteins that decreased their abundance were grouped in cluster III (Fig. 4A). The function of those included in this cluster was varied, including some sugar transporters and glycosyl transferases associated with exopolysaccharide synthesis. The down-regulation of gene encoding for many of these proteins has previously been described (Margalef-

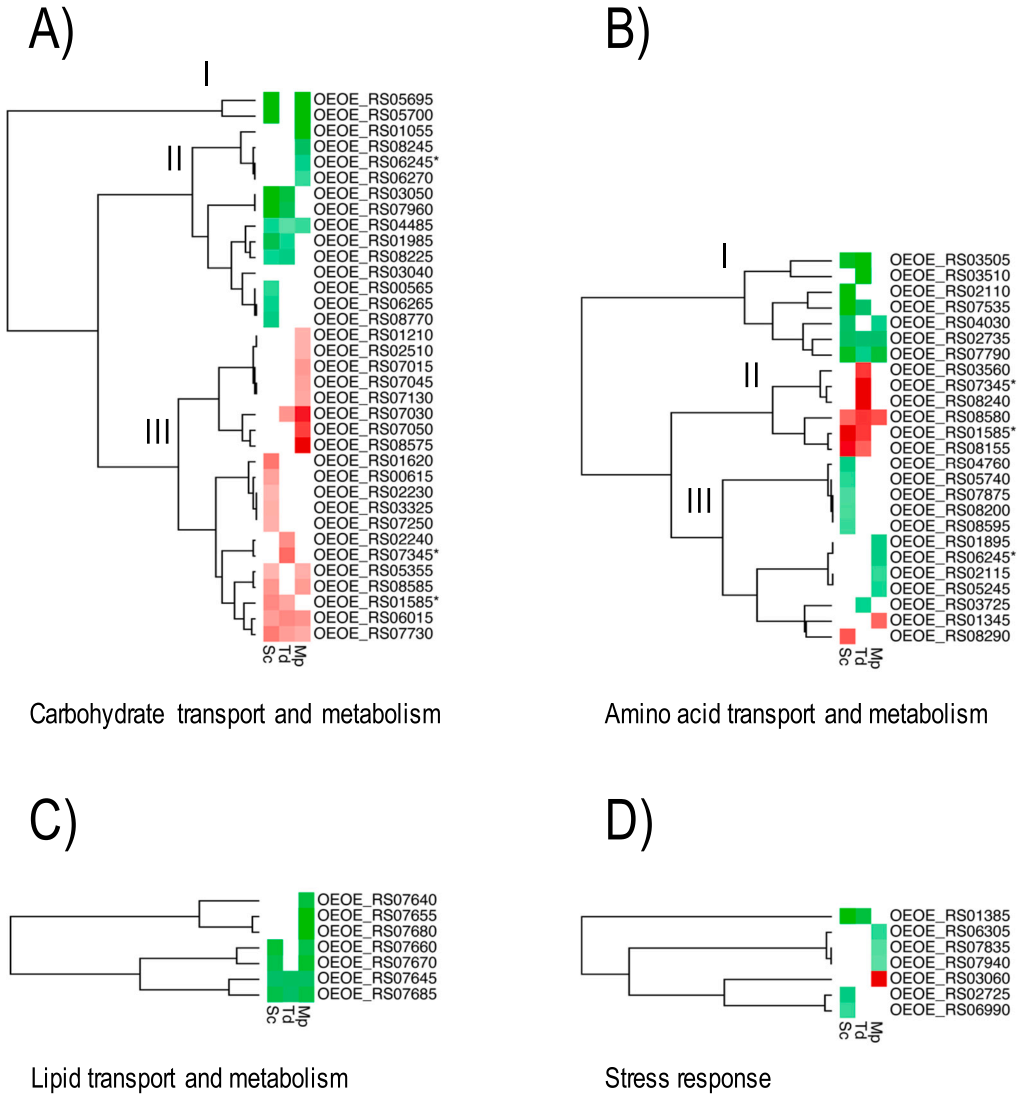


Fig. 4. Heat map and clusterization of some differentially expressed proteins (DEP) in *O. oeni* PSU-1 fermenting in the three different wines. A) DEP of carbohydrate transport and metabolism COG (cluster of orthologous groups). B) DEP of amino acid transport and metabolism COG. C) DEP of lipid transport and metabolism COG. D) DEP of some stress response-related locus tags. Sc, Td and Mp refer to wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation and *M. pulcherrima* sequential inoculation, respectively. Total length of the dendrogram represents 100% similarity. Green (increased) and red (decreased) intensity represents increasing fold change for each DEP. No colour means no DEP. Colour intensity is relative to each heat map. *Locus tag classified in more than one COG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Català et al., 2016) in response to wine conditions (OEOE_RS06015, OEOE_RS01620, OEOE_RS02240, OEOE_RS07345, OEOE_RS07030, OEOE_RS07030, OEOE_RS07015, OEOE_RS02510, OEOE_RS07050, OEOE_RS07045 and OEOE_RS01210; Suppl. Table 4). Interestingly, most of the DEPs included in cluster III showed a different behaviour depending on the yeast strain/s inoculated into the must. Only two DEPs

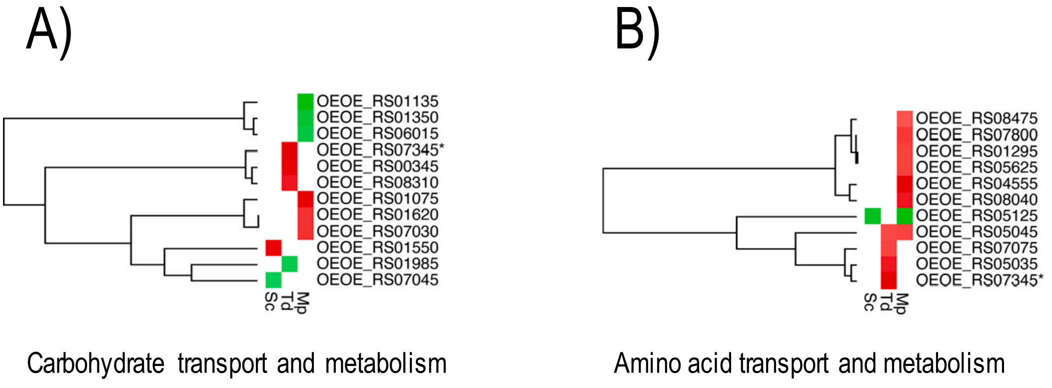


Fig. 5. Heat map and clusterization of some differentially expressed genes (DEG) in *O. oeni* PSU-1 fermenting in the three different wines. A) DEG of carbohydrate transport and metabolism COG (cluster of orthologous groups). B) DEG of amino acid transport and metabolism COG. Sc, Td and Mp refer to wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation and *M. pulcherrima* sequential inoculation, respectively. Total length of the dendrogram represents 100% similarity. Green (increased) and red (decreased) intensity represents increasing fold change for each DEG. No colour means no DEG. Colour intensity is relative to each heat map. *Locus tag classified in more than one COG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were coincident in all conditions: acetoin reductase (OEOE_RS07730) and one aldehyde-alcohol dehydrogenase (OEOE_RS06015). The inhibition of these two proteins in response to wine conditions has been already described by Margalef-Català et al. (2016).

The transcriptomic analysis detected fewer changes than the proteomic analysis. The detected DEGs associated with carbohydrate metabolism showed some coincidences with the DEPs from this functional category. The changes in gene expression showed mostly down-regulated genes (Supp. Table 3, Fig. 5A), some of them related to sugar transport and exopolysaccharide synthesis, as seen in the proteomic analysis. Specific behaviour depending on the yeast strain inoculated is also evident in the DEG profiles, in which all the genes are differentially expressed exclusively in one of the three conditions.

3.3.2. Amino acid transport and metabolism

The changes observed in the proteome of *O. oeni* relating to amino acid transport and metabolism showed that 11 of the 17 DEPs with increased abundance were associated with peptidase activity and peptide transport (Fig. 4B, Suppl. Table 4). Cluster I (Fig. 4B) included mostly proteins relating to peptide transport (OEOE_RS02110, OEOE_RS03505, OEOE_RS03510 and OEOE_RS04030; Suppl. Table 4). It also included a dipeptidase (OEOE_RS02735) that has previously been reported as being differentially increased in response to wine conditions (Margalef-Català et al., 2016). Remarkably, this peptidase and one ABC-type peptide transport protein (OEOE_RS07790) increased their abundance in all three wines. These two proteins showed a common behaviour in all the conditions.

However, most of the *O. oeni* DEPs related to amino acid metabolism behaved differently depending on the yeast strain/s used in AF. In fact, cluster III includes two peptidases differentially expressed only in the *O. oeni* from Sc wines (OEOE_RS08595, OEOE_RS08200; Suppl. Table 4). In addition, glutathione reductase was one of the proteins with increased abundance only in Sc wine. It has previously been related to stress response and adaptation to wine conditions (Cecconi et al., 2009; Margalef-Català et al., 2016; Silveira et al., 2004).

Cluster III also grouped some DEPs only detected in *O. oeni* from the Mp wines and one from the Td wine. These differences in the *O. oeni* proteomic profile indicate that the nitrogen composition in wines after AF, which depends on yeast strain metabolism, may have greatly influenced *O. oeni* peptide utilization. According to Ritt et al. (2008), the level of biosynthesis of *O. oeni* peptidases depends on the peptides present in the medium. The same authors report that the peptides from yeast decreased the levels of peptidase activity and down-regulated peptidase gene transcription. In this study, *O. oeni* from Sc wine showed the highest number of DEPs with increased abundance related to peptidase function. This could be related to the lower availability of peptides at the end of MLF in Sc wine compared to the wines inoculated with non-*Saccharomyces*. The double-inoculated yeast population in Td and Mp wines with non-*Saccharomyces* and *S. cerevisiae* might account for the higher concentration of peptides at the end of MLF. However, the relationship between peptide composition and *O. oeni* peptidase activity requires further investigation. The knowledge about the use of peptides by *O. oeni* and the role of these compounds in its survival in wine would contribute to the proposal of new strategies to improve MLF in the cellar. Peptide composition may be key in the formulation of MLF activators.

Altogether the results highlight the relevance of wine nitrogen composition and the ability of *O. oeni* to adapt to its environment. Liu et al. (2010) have reported the essential role of peptidases for bacterial growth and survival as they are encoded in all LAB genomes. It is no coincidence that more than 20 peptidases are described in the *O. oeni* PSU-1 genome.

Cluster II (Fig. 4B) included the proteins that decreased their abundance. Interestingly, 5 of the 6 DEPs in this cluster were identified as threonine dehydrogenase-like proteins. The down-regulation of three of these (OEOE_RS08580, OEOE_RS08240 and OEOE_RS03560; Suppl. Table 4) has previously been reported in response to wine conditions (Margalef-Català et al., 2016). The precise function of these proteins is not clear enough for their possible role to be discussed. Indeed, some of these enzymes were identified generically as alcohol dehydrogenases in earlier versions of the PSU-1 genome annotation.

The transcriptional analyses of the amino acid metabolism showed mostly down-regulated genes in *O. oeni* from Td and Mp wines (Fig. 5B). The predominant function among these proteins was related to amino acid and peptide transport, but different DEGs were detected depending on the wine. The only up-regulated gene was a carboxypeptidase of *O. oeni* from Sc wine (Suppl. Table 3). Again, the results seem to point to a different regulation of nitrogen utilization in *O. oeni* that would be conditioned by the nitrogen composition of the wine after AF, a consequence of the yeast strain metabolism.

3.3.3. Lipid transport and metabolism

As far as lipid metabolism is concerned, all the DEPs showed an increase in abundance (Fig. 4C). The *O. oeni* from the Mp wine showed the highest number of proteins (7 in total) responding to wine conditions in this metabolic category. All the DEPs were identified with functions relating to fatty acid (FA) biosynthesis (*i.e.*, acyl and carboxyl transfer and metabolism). The DEPs detected in the *O. oeni* from the Sc and Td wines were coincident with those detected in the Mp wine. This would mean that the activation of FA biosynthesis is a common mechanism in *O. oeni,* although the response in terms of the number of DEPs was stronger in Mp wines than in Sc and Td wines. No coincidences were found in this case with the transcriptomic response. This lack of correspondence is normal, since transcriptional regulation might be different from post-transcriptional mechanisms that influence protein translation.

The changes in FA composition to adapt to wine stress factors such as ethanol and low pH have been described in *O. oeni* (Grandvalet et al., 2008). The different DEP profiles observed in *O. oeni* may be related to differences in wine composition after AF due to the metabolism of specific yeast strains. The observed increase in the abundance of proteins associated with FA biosynthesis and the maintenance of other proteins related to this function (no decreases in protein concentration were detected) stands out as the key role of the changes in the membrane to adapt to stress conditions. According to this, the preadaptation of *O. oeni* starters, inducing changes in the cell membrane, may be a useful tool to improve MLF performance, as suggested by other authors (Chu-Ky et al., 2005).

3.3.4. Stress-related mechanisms

Some DEPs associated with the described *O. oeni* stress response mechanism were able to be identified (Fig. 4D; Suppl. Table 4). The *O. oeni* from the Mp and Sc wines showed the increase in abundance of three stress-related proteins, although there was no coincidence between them. The *O. oeni* from the Sc wine showed an increased concentration of one ATPase (OEOE_RS03185), the ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones (OEOE_RS02725), dTDP-glucose 4,6-dehydratase (OEOE_RS06990) and the heat shock protein Hsp20 (OEOE_RS01385). The increase in Hsp20 was also detected in the Td wine. In the case of the Mp wine, *O. oeni* showed an increased abundance of D-alanine-D-alanine ligase (OEOE_RS03220), DnaJ chaperone (OEOE_RS06305), the universal stress protein UspA (OEOE_RS07940) and thioredoxin (OEOE_RS07835). All these have previously been associated with the stress response to wine conditions (Beltramo et al., 2006; Margalef-Català et al., 2016; Olguín et al., 2010; Yang et al., 2020).

The differences observed in *O. oeni* in the three wines were detected in a small number of DEPs, meaning that the general stress response must have been quite similar, leaving aside the yeast strain/s responsible for AF. Notwithstanding, the stress protein Hsp20 proved to be indicative of adaptation to more difficult wine conditions. This heat shock protein showed a greater increase in those wines that had a longer MLF. Hsp20 increased 4-fold in the Sc wine and 3-fold in the Td wine, while no changes were detected in the Mp wine. This follows the correlation between a greater increase in protein abundance and a slower MLF. Also known as Lo18 or Hsp18, Hsp20 has long been described as one of the main stress-responding proteins in *O. oeni* with chaperone and membrane protection functions (Coucheney et al., 2005b; Guzzo et al., 1997). Indeed, it has also been proposed as a stress response marker of interest to predict MLF performances (Coucheney et al., 2005a; Olguín et al., 2010).

3.3.5. *Differential expression of tRNAs*

Some DEGs relating to tRNAs were detected in the samples (Fig. 6). Little information about the impact of the regulation of tRNAs on MLF performance is available in the literature. Overall, tRNAs are associated with greater protein biosynthesis and thus higher metabolic activity (Raina and Ibba, 2014) that may be related to fast fermentations. In this regard, *O. oeni* strains with higher metabolic activity exhibit an up-regulation in tRNAs (Sternes et al., 2017). No information is available as regards the adaptation of a specific strain.

The main changes in tRNA expression were observed in the Td and Mp wines, whereas few changes were detected in the Sc wine. *O. oeni* presented a general down-regulation of tRNAs at the end of MLF in Td wine. On the contrary, *O. oeni* showed a general up-regulation of tRNAs in Mp wine. There was not a clear correlation between tRNA transcriptional regulation and MLF performance. Further research is needed to understand the possible impact of the changes in tRNA expression on *O. oeni* metabolism.

3.3.6. *Other modified metabolisms*

In addition to the already highlighted metabolisms, a number of other functional categories were affected during MLF. The gene expression of many transcriptional regulators was down-regulated (Suppl. Table 3). The inhibition of some ribosomal proteins was also detected in both the proteomic and the transcriptomic analyses (Suppl. Tables 3 and 4). Again, the response was highly dependent on the yeast strain/s responsible for AF.

As for the L-malic acid consumption, the malolactic enzyme (OEOE_RS07545) showed a similar increase in abundance (2–3-fold) in the three wines studied (Suppl. Table 4). Therefore, it was not possible to correlate the increase in this enzyme with the different duration of MLF.

4. Conclusion

The specific DEGs and DEPs detected in *O. oeni* PSU-1 depending on the yeast strain/s used in the AF show that wine composition, which results from the metabolic traits of each yeast, greatly influences the

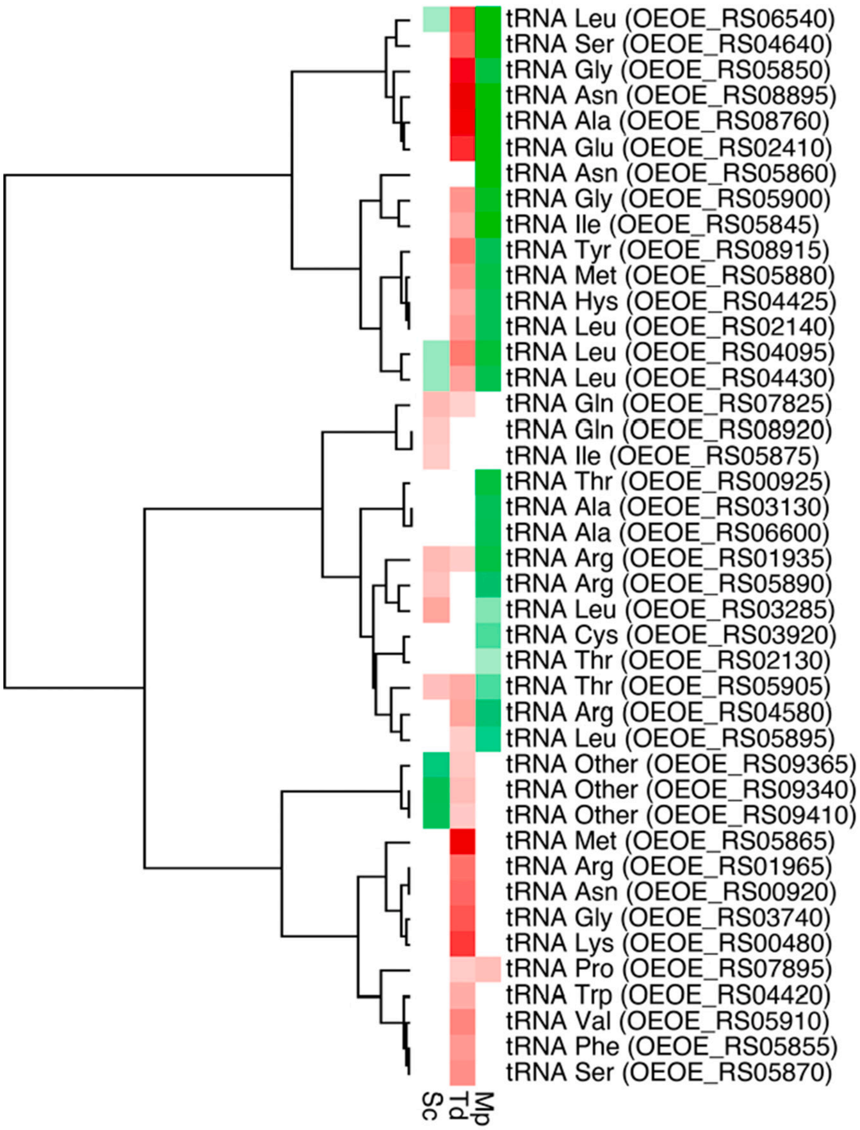


Fig. 6. Heat map and clusterization of the differentially expressed genes (DEG) corresponding to tRNA locus tags in *O. oeni* PSU-1 fermenting in the three different wines. Sc, Td and Mp refer to wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation and *M. pulcherrima* sequential inoculation, respectively. Total length of the dendrogram represents 100% similarity. Green (increased) and red (decreased) intensity represents increasing fold change for each DEG. No colour means no DEG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

O. oeni molecular mechanisms for adapting to wine. The results highlight the relevance of yeast-bacteria interaction and compatibility in MLF performance. The non-*Saccharomyces* yeast promoted a faster MLF. The abundance of malolactic enzyme was similar in all the conditions and does not explain the differences in MLF duration. The expression of the stress protein Hsp20 was confirmed as a reliable marker of stressful conditions and its increase could be correlated to slower MLF. Within the complex *O. oeni* molecular response, the specificity of peptide utilization might have played a key positive role in *O. oeni* adaptation to Mp and Td wines. This points to the need for further research on the characterization of peptides released by different yeast species and their use by *O. oeni* to enable us to better understand microbial interactions in wine and their effect on MLF. The study of more yeast and bacteria strains will be necessary to elucidate if the observed effects are species or strain dependent.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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