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RESEARCH ARTICLE

Saccharomyces cerevisiae responds similarly to co-culture or to a fraction enriched in *Metschnikowia pulcherrima* extracellular vesicles

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Abstract

The recent introduction of non-conventional yeast species as companion wine starters has prompted a growing interest in microbial interactions during wine fermentation. There is evidence of interactions through interference and exploitation competition, as well as interactions depending on physical contact. Furthermore, the results of some transcriptomic analyses suggest interspecific communication, but the molecules or biological structures involved in recognition are not well understood. In this work, we explored extracellular vesicles (EVs) as possible mediators of interspecific communication between wine yeasts. The transcriptomic response of *Saccharomyces cerevisiae* after 3 h of contact with a fraction enriched in EVs of *Metschnikowia pulcherrima* was compared with that induced by active *M. pulcherrima* cells. Interestingly, there is a high level of overlap between the transcriptomic profiles of yeast cells challenged by either *M. pulcherrima* whole cells or the EV-enriched fraction. The results indicate an upregulation of yeast metabolism in response to competing species (in line with previous results). This finding points to the presence of a signal, in the EV-enriched fraction, that can be perceived by the yeast cells as a cue for the presence of competitors, even in the absence of metabolically active cells of the other species.

INTRODUCTION

One of the current trends in wine biotechnology is to take advantage of the metabolic diversity of wine yeast species alternative to *Saccharomyces cerevisiae*. This objective can be reached, for example, by using commercial non-*Saccharomyces* starters, local multispecies starters, or controlled spontaneous fermentations (Mas & Portillo, 2022). With these practices, winemakers pursue a wide range of technological and quality advantages, such as those related to the wine's aromatic profile; the management of organic acids (malic, lactic, pyruvic), volatile acidity, final ethanol content, or wine colloids; and the control of spoilage microorganisms (Gonzalez et al., 2021; Mas & Portillo, 2022; Vejarano & Gil-Calderón, 2021). The role of microbial interactions is of particular relevance nowadays, owing to the widespread use of commercial *S. cerevisiae* and non-*Saccharomyces* starters from the first minutes of fermentation. Interactions can take place at different levels, including competition for available resources, release of toxic compounds, or exchange of metabolites; and may involve communication mechanisms relying on volatile or soluble molecules, as well as physical contact

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between cells (Mencher et al., 2021). The impact of microbial interactions in winemaking has been evidenced by several authors by analysing growth or fermentation kinetics (Englezos et al., 2019; Renault et al., 2013), metabolic profiles (Roullier-Gall et al., 2020) including volatile compounds, or gene-expression changes at the single gene (Milanovic et al., 2012) or global transcriptome levels (Curiel et al., 2017; Mencher et al., 2021a; Shekhawat et al., 2019; Tondini et al., 2019; Tronchoni et al., 2017).

To understand the mechanisms of interaction between different wine yeast species, some research groups have focused on the importance of cell-to-cell contact. Several devices have been developed to keep cells from the different species apart, while allowing for the free exchange of soluble metabolites. Depending on the yeast species or strains involved, or the actual experimental conditions, examples of both contact-dependent and contact-independent interactions have been published (Branco et al., 2017; Brou et al., 2018; González et al., 2018; Li & Mira de Orduña, 2017; Pérez-Nevado et al., 2006). Physical contact may play a role in these interactions by different mechanisms (Branco et al., 2017; Pérez-Torrado et al., 2017; Rossouw et al., 2018).

Comparative transcriptomic analysis (single versus mixed cultures) has become an invaluable tool to gain insight into the nature of microbial interactions during wine fermentation (Comitini et al., 2021; Curiel et al., 2017; Shekhawat et al., 2019). The detection of changes in the transcription pattern in mixed cultures depends on the experimental setup and can respond to different categories of stimuli. On the one hand, a variation in the kinetics of consumption of limiting nutrients can alter the activation time of the respective compensatory mechanisms. On the other hand, differences in the kinetics of accumulation of potentially toxic metabolites (e.g., ethanol) may result in the activation of stress responses at different stages. There may also be recognition mechanisms, whereby some yeast species sense the presence of cells of a different species (yeast or bacterial), triggering a specific transcriptional response. The latter interaction mechanism can be difficult to detect in most published transcriptomic datasets, as samples for transcriptomic analysis are usually obtained after at least 24 h of co-culture (i.e. when the concentration of some of the main compounds in the growth medium is already quite different between single and mixed cultures). Transcriptomic analysis after short periods of cell contact seems to be a good approach to uncover this type of interspecific communication. For example, upregulation of sugar utilization genes was observed after 2–3 h of mixed culture in synthetic grape must for *S. cerevisiae* challenged with *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* or other wine yeast species (Curiel et al., 2017; Mencher et al., 2021; Tronchoni et al., 2017). The transcriptional responses in such a short time frame suggest that the challenged cells respond to stimuli other than nutrient consumption or production of major fermentation metabolites (although they cannot be totally excluded).

As mentioned above, inter and intraspecific cell-to-cell communication in yeast might involve cell contact, volatile, and non-volatile compounds (Bardwell, 2004; González et al., 2018; Kemsawasd et al., 2015; Pérez-Torrado et al., 2017; Ramakrishnan et al., 2016). In addition, extracellular vesicles (EVs) are produced by cells from many different organisms (Théry et al., 2018), and have been linked to several biological roles (Stahl & Raposo, 2019). They have been widely studied in pathogenic yeasts (Rodrigues & Janbon, 2021); and have been recently reported for several wine yeast and bacterial species under winemaking conditions (Mencher et al., 2020, 2022). Recently, EVs have also been suggested to play a role in interspecific communication between wine yeast strains (Morales et al., 2021).

The aim of this work was to test this hypothesis by challenging wine yeast cells with EVs produced by a different wine yeast species. One isolate of *S. cerevisiae* was selected as the recipient strain and one strain of *M. pulcherrima* as the source of EVs. This choice responds to previous results, as this combination of strains gave rise to a prominent transcriptomic impact in *S. cerevisiae* (Mencher et al., 2021). Transcriptomic analysis was used to assess the response of *S. cerevisiae* cells to EVs, and this response was compared with the response to *M. pulcherrima* living cells under the same experimental conditions.

MATERIALS AND METHODS

Strains and growth conditions

S. cerevisiae FX10 (Laffort) and *M. pulcherrima* CECT11202 strains were used in this study. Pre-cultures were grown in YPD (20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract). Synthetic must contained (per litre): glucose 100 g, fructose 100 g, DL-malic acid 6 g, citric acid 6 g, YNB w/o aa w/o $(NH_4)_2SO_4$ 1.7 g, nitrogen sources (Asp 29 mg, Glu 80 mg, Ser 52 mg, Gln 333 mg, Hys 31 mg, Gly 12 mg, Thr 50 mg, Arg 296.28 mg, Ala 97 mg, Tyr 13 mg, Cys 18.2 mg, Val 29 mg, Met 21 mg, Trp 116 mg, Phe 25 mg, Ile 22 mg, Leu 32 mg, Lys 13.72 mg, Pro 400 mg, NH_4Cl 306 mg), anaerobiosis factors (ergosterol 15 mg, oleic acid 5 mg, tween 80 0.5 mL), inositol 18 mg; pH adjusted at 3.3 with NaOH. Synthetic must 2x has the same composition with double concentration.

Pre-cultures were grown in 50 mL centrifuge tubes in 20 mL of YPD during 48 h at 25°C without agitation. Prior to inoculation of synthetic must, pre-cultures were centrifuged at 8000 g for 5 min at 10°C with two washing steps with distilled water.

Collection of a fraction rich in EVs from M. pulcherrima

Extracellular vesicle-enriched fractions from *M. pulcherrima* were obtained using protocols modified from previous publications (Gil-Bona et al., 2015; Lobb et al., 2015; Mencher et al., 2020). *M. pulcherrima* cultures for production of EVs were inoculated to an initial optical density (OD_{600}) of 0.2 in 1 L flasks. Each flask contained 350 mL of synthetic must and closed with wide aluminium foil caps allowing aeration. Flasks were incubated at 25°C at 150 rpm for 24 h. Six flasks (2100 mL) were used for this experiment.

Cells and debris were removed by centrifugation at 5000*g* for 10 min at 4°C. The cell-free supernatant was collected and treated with one tablet per litre of protease inhibitor (complete mini, EDTA-free, Roche) and then filtered through 0.22 μm. Sample concentration was carried out at 4°C with a 100 kDa tangential filter Vivaflow® 200 (Sartorius), recovering the flowthrough until the dead volume was reached. When the filter reached dead volume, the sample was dialyzed for three times by adding 50 mL of PBS (phosphate buffered saline) containing: NaCl 137 mM, KCl 2.7 mM, Na_2HP0_4 10 mM, KH_2P0_4 2 mM, pH 7.4, and running the ultrafiltration until reaching the dead volume again. The tangential filter was flushed with 50 mL of PBS to recover the sample retained in the filter and the PBS was concentrated in the filter until the dead volume was reached. The second volume was added to the initial sample obtaining a final volume of ~45 mL. A second concentration step was performed using Amicon® Ultra-15,100 K Centrifugal Filter Devices (100,000 NMWL) (Merck KGaA) according to the manufacturer's recommendations to a final volume of 6.5 mL. The concentrated culture was centrifuged again at 10,000*g* for 10 min at 4°C to remove debris. At this step, the sample was split into two to perform an ultracentrifugation step and a size-exclusion chromatography step in parallel.

Size-exclusion chromatography was performed in 500 μL Izon qEV original 70 nm columns (Izon Science LTD). Manufacturer purification protocol was followed resulting in a dilution of the previously concentrated sample (3.25 mL of sample were eluted in 21 mL of PBS).

The EV fraction was subjected to ultracentrifugation in 6.0 mL PC Thick-Walled Tubes (16 × 59 mm; ThermoFisher Scientific, Dreieich, Germany) at 100,000*g* (45,000 rpm) for 70 min at 4°C in ultracentrifuge Sorvall™ MTX150 with S80-AT3 fixed angle rotor (ThermoFisher Scientific). Pellets from ultracentrifugation were washed with PBS, ultracentrifuged under the same conditions, and resuspended in 200 μL of PBS. Before use, this volume was increased to 21 mL to match the concentration of the size-exclusion chromatography sample. Considering the samples withdrawn for controls, the final concentration factor was 47-fold. Samples were stored at 4°C overnight to perform the treatments in the day after concentration.

Twenty μl of each sample were withdrawn and stored at −80°C to perform a nanoparticle tracking analysis (NTA).

Treatment of *S. cerevisiae* with EV-enriched fractions

Four main conditions for treatment were tested, involving EV-enriched fractions from ultracentrifuge or size-exclusion chromatography and two different levels: high concentration (4.5 mL of the respective EV-enriched fraction) and low concentration (0.9 mL of EV-enriched fraction). Controls consisted of *S. cerevisiae* pure cultures and co-cultures of *S. cerevisiae* and *M. pulcherrima* (1:1 ratio). All the controls and treatments were performed by triplicate (Figure S1). *S. cerevisiae* and *M. pulcherrima* pre-cultures were prepared 48 h in advance as described above. *S. cerevisiae* cultures for treatment were inoculated to an initial optical density (OD_{600}) of 4 in a 500 mL sterile bottle with 200 mL of synthetic must. The culture was distributed in 15 mL centrifuge tubes with 10 mL of inoculated media per tube and incubated at 25°C for 3 h. Cultures of *M. pulcherrima* for co-culture treatment were prepared with the same protocol. After this adaptation time, cultures were centrifuged at 5000 g for 10 min. Supernatant was discarded and the cultures suspended in the treatment medium. Due to the high volume of each EV-enriched preparation, to achieve the correct concentration of culture medium, 2x synthetic must was used to reach the 1x medium concentration on each treatment (by adding different combinations of PBS or EV-enriched fraction). For co-culture each species was resuspended separately and then combined in a single tube. Tubes were incubated for an additional 3 h at 25°C. After 3 h, they were centrifuged at 5000 g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 mL of sterile water. Cells in suspension were separated into two tubes, centrifuged at 13,000 g for 3 min. The supernatant was discarded, and the cells were frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

Nanoparticle tracking analysis

Vesicle size, and concentration were obtained in the ICTS "NANBIOSIS" on a NanoSight NS300 (Malvern Instruments Ltd.) using NTA 3.4 Build 3.4.4 Nanoparticle Tracking and Analysis software. The size-exclusion chromatography sample was diluted 1:10 in PBS and the ultracentrifuge sample was not diluted. Samples were acquired with camera shutter and gain was optimized for data collection. 10 s videos were taken, and the frame sequences were analyzed under

auto-particle detection and tracking parameters: detection threshold, pixel blur, and minimum track length. All samples were run at room temperature.

RNA extraction and RNAseq analysis

Total RNA extraction was performed with the RNA extraction kit Pure Link RNA mini kit (Invitrogen) and subjected to DNase treatment with PureLink DNase kit (Invitrogen) following the manufacturer's instructions. Library preparation and sequencing of RNA was performed at the Genomics Core Facility in the Center for Biomedical Research of La Rioja (CIBIR). Poly-A filtering step was performed prior to the generation libraries. From the libraries, 100-bp pair-end sequence reads were produced with Illumina HiSeq 1500. Alignment of reads to the S288c R64 *S. cerevisiae* yeast reference genome assembly was carried out using HISAT 2.1.0 (Kim et al., 2019). Alignment quality was assessed with the module rnaseqQC from Qualimap v2.2.1 (Okonechnikov et al., 2015). Integration of results was carried out with MultiQC v1.11 (Ewels et al., 2016).

Each comparison was analyzed with EdgeR (Robinson et al., 2010) to study the differentially expressed genes (DEGs) through the package SARTools (Varet et al., 2016). Functional enrichment of the DEGs was carried out using Gene Set Enrichment Analysis (GSEA) with the online tool WebGestalt 2019 (Liao et al., 2019). Pre-ranked dataset for GSEA was calculated using the $\log_2$Fold Change and *p*-values obtained with EdgeR (Reimand et al., 2019). KEGG pathway for *S. cerevisiae* was selected as the functional database. Parameters used for the analysis were 15 genes as minimum number of genes for a category, 200 as maximum number of genes for a category, an FDR < 0.05 and 1000 permutations. Plots of leading genes obtained by GSEA were performed in KEGG database (Kanehisa, 2000) and SGD database (Cherry et al., 2012).

RESULTS

Overview of differential gene expression

Nanoparticle tracking analysis results of vesicle fractions from ultracentrifuge shows a diameter of 196.6 ±5.1 nm and a concentration of 1.7e10 ± 5.1e9 particles/mL. Vesicles from size-exclusion chromatography show a size of 211.0 ±8.4 nm and a concentration of 3.5e10 ± 1.8e9 particles/mL. According to this quantification, a summary showing concentration of EVs in each treatment is shown in Table S1.

Vesicles purified by size-exclusion chromatography (VAI and VBI) showed a very low impact on the transcriptome of *S. cerevisiae*. VBI treatment contained five times more particles than VAI. But, the number of differentially expressed genes increases just from 1 to 3 (Table 1) between both conditions. A dose-dependent response is more evident for vesicles recovered by ultracentrifugation. In this case, both concentration levels had a clear impact on the transcription profile, but the response of *S. cerevisiae* to VBU treatment (the highest level) was much stronger than to VAU. Furthermore, the transcriptional response is stronger for fractions recovered by ultracentrifugation than for fractions recovered by size-exclusion chromatography (VBU and VBI, respectively, if we take the fraction with the highest concentration in each case). According to both Figure 1 and Table 1, a ranking can be established for the strength of the transcriptomic response induced in *S. cerevisiae* by the different EV-enriched preparations: VAI < VBI < VAU < VBU. The complete set of genes differentially expressed in this RNAseq analysis is listed in Appendix S1.

The two experimental conditions inducing the strongest effect on *S. cerevisiae* were co-culture (ScMp), and the highest concentration of EVs recovered by ultracentrifugation (VBU). *S. cerevisiae* responds similarly to both treatments. This is true both in terms of the number of genes showing differential expression over the control (Table 1) and the degree of change observed (Figure 1; panels C and E). A mutual comparison of the *S. cerevisiae* transcriptome in these two growth conditions, VBU and ScMp, shows they are indeed very similar. Only 27 genes are differentially expressed between these two conditions (Table 1). Functional enrichment analysis was performed for the transcriptomic response of *S. cerevisiae* to these two treatments, as detailed below.

Functional enrichment analysis of upregulated genes

According to GSEA analysis, "Ribosome" and "Biosynthesis of amino acids" appear as the two most prominent categories in response of *S. cerevisiae* to co-cultivation with *M. pulcherrima* or to cultivation

TABLE 1 Result of differential expression analysis for the extracellular vesicles treatments and co-culture control.

Test vs. ref	Downregulated genes	Upregulated genes	Total DEG genes
VAI vs. Ctrl	0	1	1
VBI vs. Ctrl	1	2	3
VAU vs. Ctrl	34	25	59
VBU vs. Ctrl	916	702	1618
ScMp vs. Ctrl	718	697	1415
VBU vs. ScMp	16	11	27

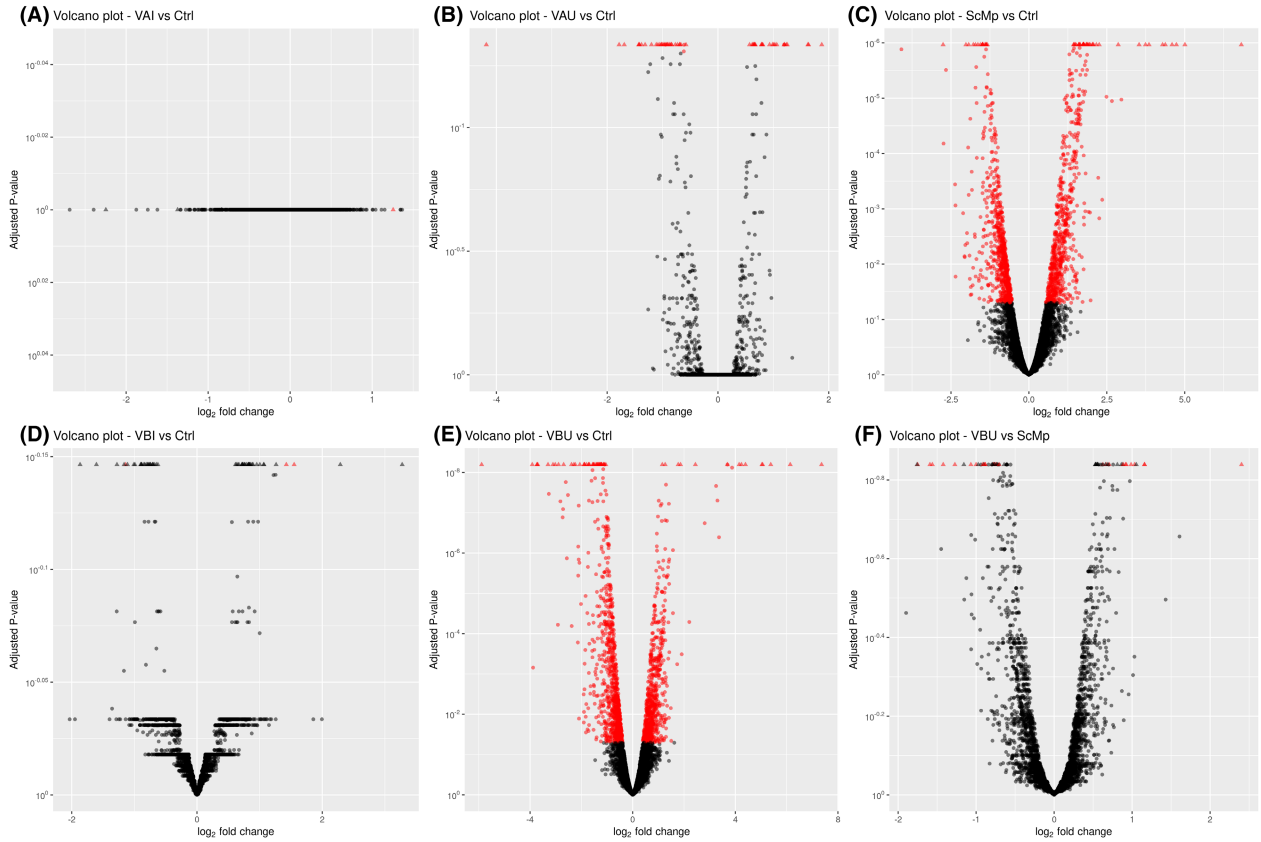

FIGURE 1 Volcano plots of differentially expressed genes for each treatment against control conditions. (A) Treatment of low concentration of EVs-EF concentrated by size-exclusion chromatography (VAI); (B) Treatment of low concentration of EVs-EF concentrated by ultracentrifugation (VAU); (C) Co-culture control (ScMp); (D) Treatment of high concentration of EVs-EF concentrated by size-exclusion chromatography (VBI); (E) Treatment of high concentration of EVs-EF concentrated by ultracentrifugation (VBU); (F) Comparison between VBU and ScMp reads. Transcript showing differential expression above $\log_2$Fold Change 0.5 or below $\log_2$Fold Change −0.5 and *p*-values below 0.05 are highlighted in red.

in the presence of a fraction enriched in EVs from *M. pulcherrima* (VBU treatment) under the experimental conditions used in this work (Figures 2 and 3). Both categories point to the activation of protein biosynthesis probably related with potentiation of cell growth.

Concerning the KEGG pathway labelled "Ribosome" 126 or 135 out of the 174 protein-coding genes appear upregulated in, respectively, VBU or ScMp conditions (Appendix S1). Most of them show $\log_2$Fold Change values around 1. Regarding this pathway, the transcriptional response of *S. cerevisiae* to live *M. pulcherrima* cells overlapped almost perfectly with the EV-enriched fraction, with only 12 genes unique to the response to the cells and the three responding only to the EV-enriched fractions behaving in fact in the same way in both conditions, but just below the thresholds of statistical significance in one of them (Appendix S1). Interestingly, genes coding for mitochondrial ribosomal proteins are mostly excluded of the overexpressed genes, with only six exceptions (YCR046C, YDR115W, YCR003W, YNL284C, YNR036C, and YGL068W), for *M. pulcherrima* living cells, or four exceptions (YCR046C, YDL202W, YDR115W, YGL068W, and YNR036C) for the EV-enriched fraction, among the 35 genes labelled for mitochondrial ribosome (Appendix S1).

As mentioned above, the activation of genes encoding ribosomal proteins is aligned with a corresponding upregulation of genes involved in amino acid biosynthesis. Indeed, transcriptional activation of amino acid biosynthetic pathways involves genes from almost all branches and sub-pathways (Figure S2). Although the percentage of upregulated genes and the degree of change are not as striking as in the case of ribosomal proteins, both data point to the activation of protein biosynthesis in the VBU treatment. The correspondence between the responses induced by the EV-enriched fraction and *M. pulcherrima* cells is again very good. There are 18 differentially expressed genes in this pathway in only one or the other condition, but most of them fall just below the significance threshold in the condition in which they were not highlighted (Appendix S1).

Activation of "Pyrimidine metabolism", or "Purine metabolism", is highlighted again, although with different levels of signification for both VBU and ScMp samples (Figures 2 and 3). This also suggests that

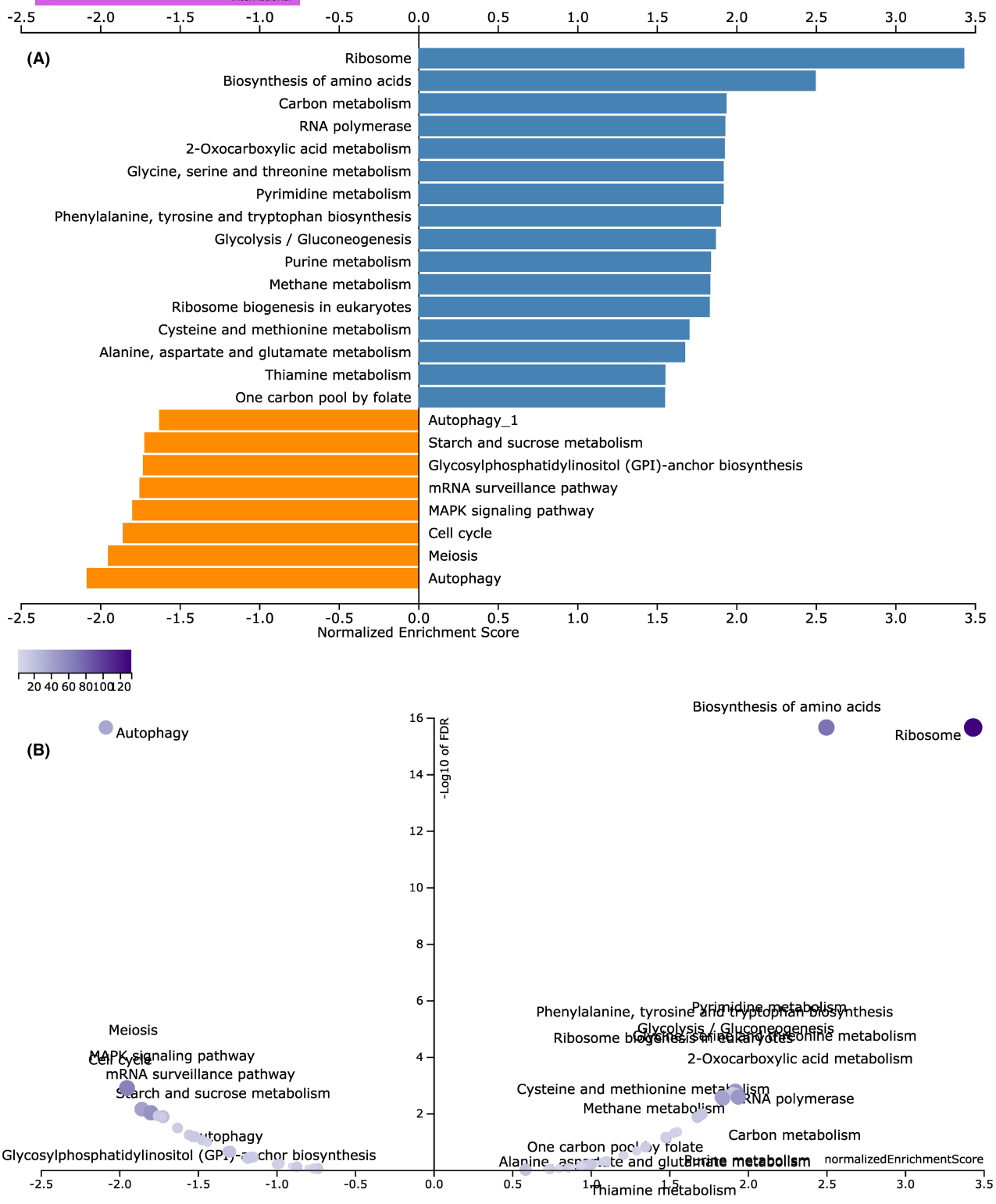

FIGURE 2 (A) Bar chart of categories overexpressed (blue bars) and repressed (orange bars) in co-culture treatment. All shown categories have an FDR below 0.05. (B) Categories from bar chart represented with the $-\text{Log}_{10}$ of FDR value and the Normalized Enrichment Score.

cell proliferation is the main target of transcriptional remodelling induced in *S. cerevisiae* by this EV-enriched fraction.

Stimulation of protein biosynthesis appears to be coordinated with the activation of energy-generating reactions, highlighted by the transcriptional activation under the two experimental conditions of pathways like "Glycolysis/Gluconeogenesis" or "Carbon metabolism", together with related categories like "2-oxocarboxyilic acid metabolism", "Pentose phosphate pathway", or

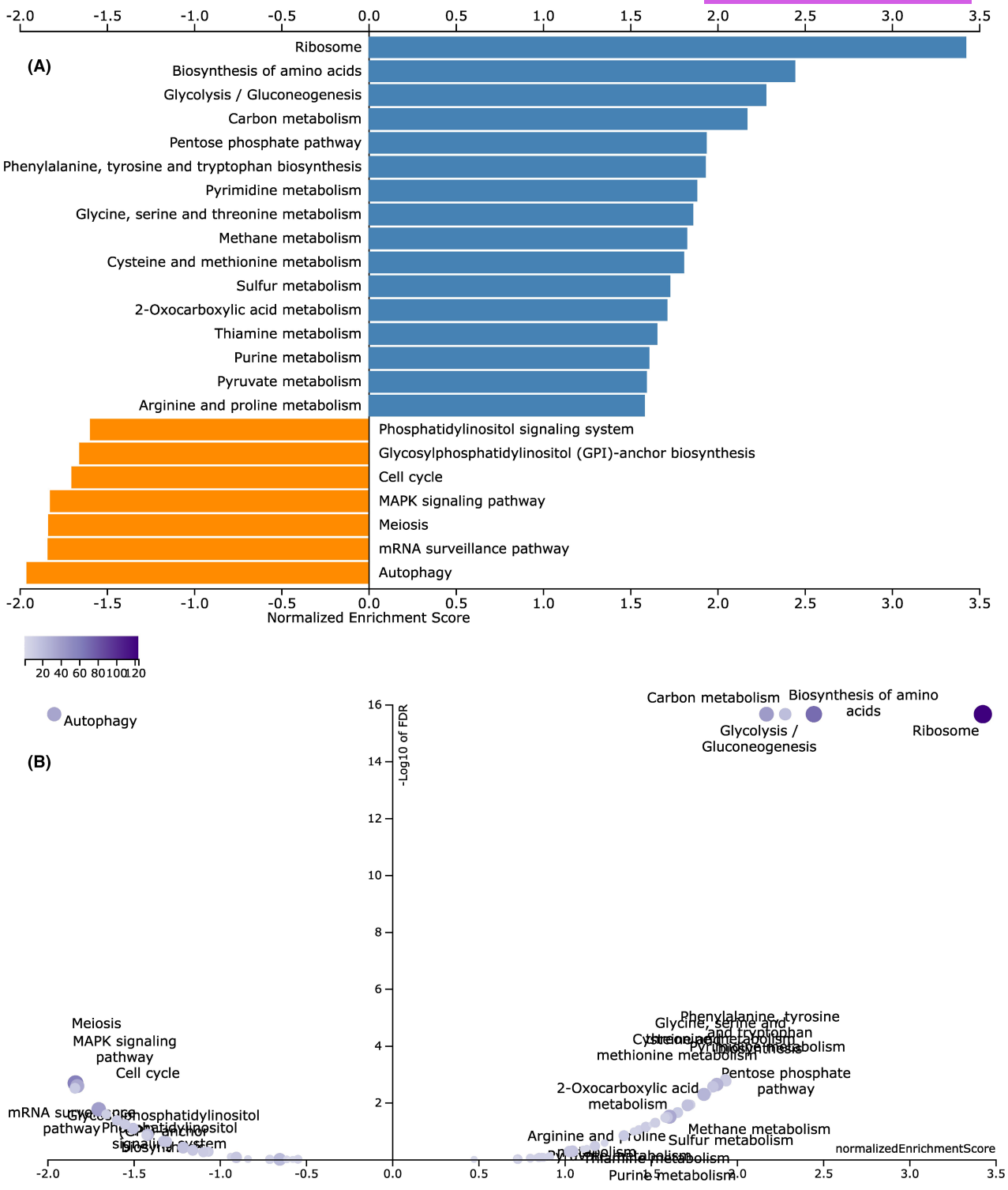

FIGURE 3 (A) Bar chart of categories overexpressed (blue bars) and repressed (orange bars) in VBU treatment. All the categories have an FDR below 0.05. (B) Categories from bar chart represented with the $-\text{Log}_{10}$ of FDR value and the Normalized Enrichment Score.

"Pyruvate metabolism" (the last two categories just for VBU) (Figures 2 and 3).

A detailed view of the impact of the EV-enriched fraction on the expression of genes in the "Glycolysis/Gluconeogenesis" pathway for the VBU condition is shown in Figure 4. Among the 19 genes showing differential expression (VBU vs. Control) belonging to this KEGG pathway, 17 of them appear overexpressed while only two of them, that are not an integral part of the pathway (YLR446W and YMR110C), appear downregulated. Although this pathway is labelled as "Glycolysis/Gluconeogenesis" the results indicate that mostly the glycolytic pathway is upregulated. Certainly, *MDH2* (YOL126C) coding for the enzyme

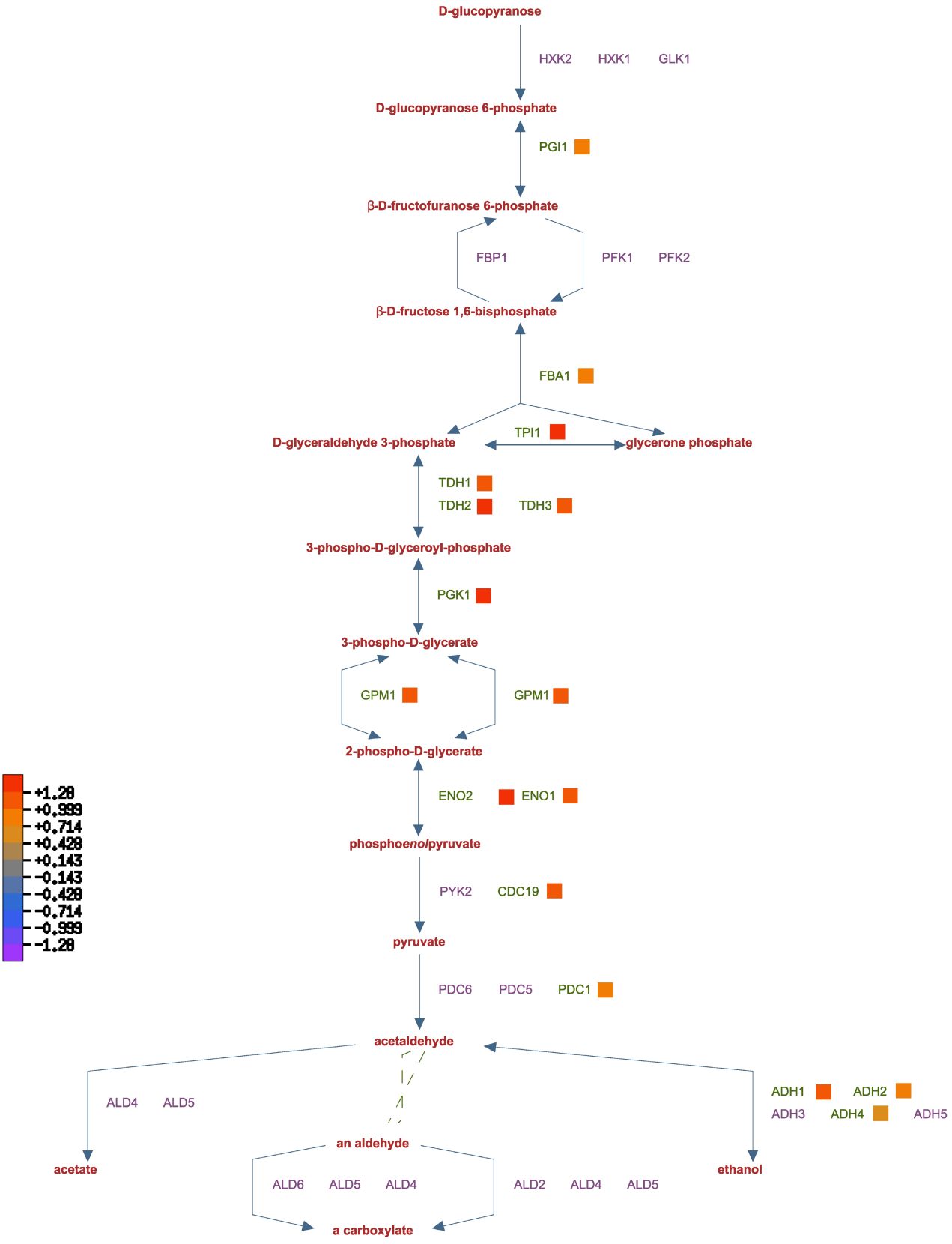

FIGURE 4 Superpathway of glucose fermentation from SGD database. Genes in purple are not statistically significant. Genes in green show statistically significant differential expression against control in VBU treatment. Colour box indicates the level of expression in $\log_2$Fold Change.

catalysing the first irreversible step in gluconeogenesis appears overexpressed in VBU (Appendix S1), although not significantly in the case of ScMp. However, this enzyme is not exclusive for gluconeogenesis, as it is also required for the glyoxylate shunt. The overlapping between the transcriptional responses triggered by whole cells and an EV-enriched fraction is again striking (Appendix S1).

Functional enrichment analysis of downregulated genes

"Autophagy" appears as the most significant category enriched among genes repressed in both the VBU and ScMp treatments (Figure 2). Since autophagy is critical for cell survival under stationary phase (Alvers et al., 2009; Davey et al., 2012; De Virgilio, 2012) this result suggests that cells treated with the EV-enriched fraction of *M. pulcherrima* (or confronted to living cells of *M. pulcherrima*) exit the lag phase faster than untreated cells (but this hypothesis should be confirmed by ad hoc experiments). More than 32% of the genes with this tag for *S. cerevisiae* were found to be repressed in VBU, while only below 5% of "Autophagy" genes were overexpressed in each condition (Figure 5). A similar picture arises by analysing the transcriptional response to *M. pulcherrima* whole cells (Appendix S1), with 28% of the genes in the category overexpressed and a high degree of overlapping between both datasets. Although autophagy is mostly regulated at other levels of control, the transcriptional control of autophagy is also well established (Di Malta et al., 2019; Shu et al., 2020). This result suggests that autophagic processes are less active in cells challenged with the EV-enriched fractions than in the control condition. The inclusion of *TOR1* (YJR066W) among the downregulated genes in both conditions (Appendix S1) is somewhat paradoxical, as this is a negative effector of autophagy. However, TORC1 is mainly regulated at the post-translational level (Morozumi & Shiozaki, 2021), and changes in transcription levels might fail to reflect the actual kinase activity. Within this pathway we also found reduced expression of two transcription factors required for the expression of several stress-induced genes, *MSN2* and *MSN4*; albeit the second one falls below the significance threshold for ScMp (Martínez-Pastor et al., 1996). This is also in agreement with a general downregulation of autophagy, since *MSN2/4* are required for autophagic flux under some environmental conditions (He & Klionsky, 2009; Vlahakis et al., 2017).

DISCUSSION

The results described above confirm prior indications that *S. cerevisiae* adapts its transcriptional programme to the presence of competing yeast strains, even before nutrient consumption or production of key metabolites becomes apparent (Curiel et al., 2017; Mencher et al., 2021; Tronchoni et al., 2017). To minimize the ambiguity created by changes in the composition of the medium, previous evidence for direct yeast communication relied to a great extent on using short contact time between cells of different species. However, results obtained in this work with EV-enriched fractions, which are free of active cells, point even more strongly to the existence of direct biotic interactions between wine yeasts.

Importantly, these results support the hypothesis that EVs play a role in interspecific communication between different wine yeast species. The high similarity in the transcriptional responses of *S. cerevisiae* to whole *M. pulcherrima* cells and to the highest concentration of EV-enriched fraction recovered by ultracentrifugation suggest that this fraction contains molecules or biological structures (e.g. EVs) required for *S. cerevisiae* to sense the presence of *M. pulcherrima*. It should be noted that a transcriptomic response similar to that induced by living cells was only obtained with the highest concentration of EVs (i.e. EVs from about 20 times the culture volume in the experiment). This fact can be interpreted in several ways. In mixed cultures, EVs are continuously produced (and probably taken up or adsorbed on cell surfaces). This constant delivery can be expected to be more effective in triggering a biological response than a single dose of EVs. It is also possible that a combination of effectors is more effective in inducing a transcriptomic response. This would be supported by the lower response triggered by fractions purified by size-exclusion chromatography (VAI and VBI), which are expected to show a lower protein content (Monguió-Tortajada et al., 2019). Alternatively, cell-to-cell contact might be a most effective way of inducing a biological response in *S. cerevisiae*, while EVs need to reach high concentrations to mimic whole cells. Several contact-dependent interactions have been described for wine yeasts. For example, accumulation of GAPDH-derived peptides in the cell surface of *S. cerevisiae* to induce death of non-*Saccharomyces* yeasts (Branco et al., 2017) or the regulation of the yeast ecosystem dynamics in an oenological context by the modification of the *S. cerevisiae* adhesion properties (Rossouw et al., 2018). Among the mechanisms of contact-independent communication, the production of [GAR^+] prion induced by acid and lactic bacteria (Ramakrishnan et al., 2016) and the production of aromatic amino acid compounds by *S. cerevisiae* (González et al., 2018) are two clear examples. The large difference in the transcriptional response triggered by vesicles recovered by size-exclusion chromatography or ultracentrifugation suggests that other components of the EV-enriched fractions could

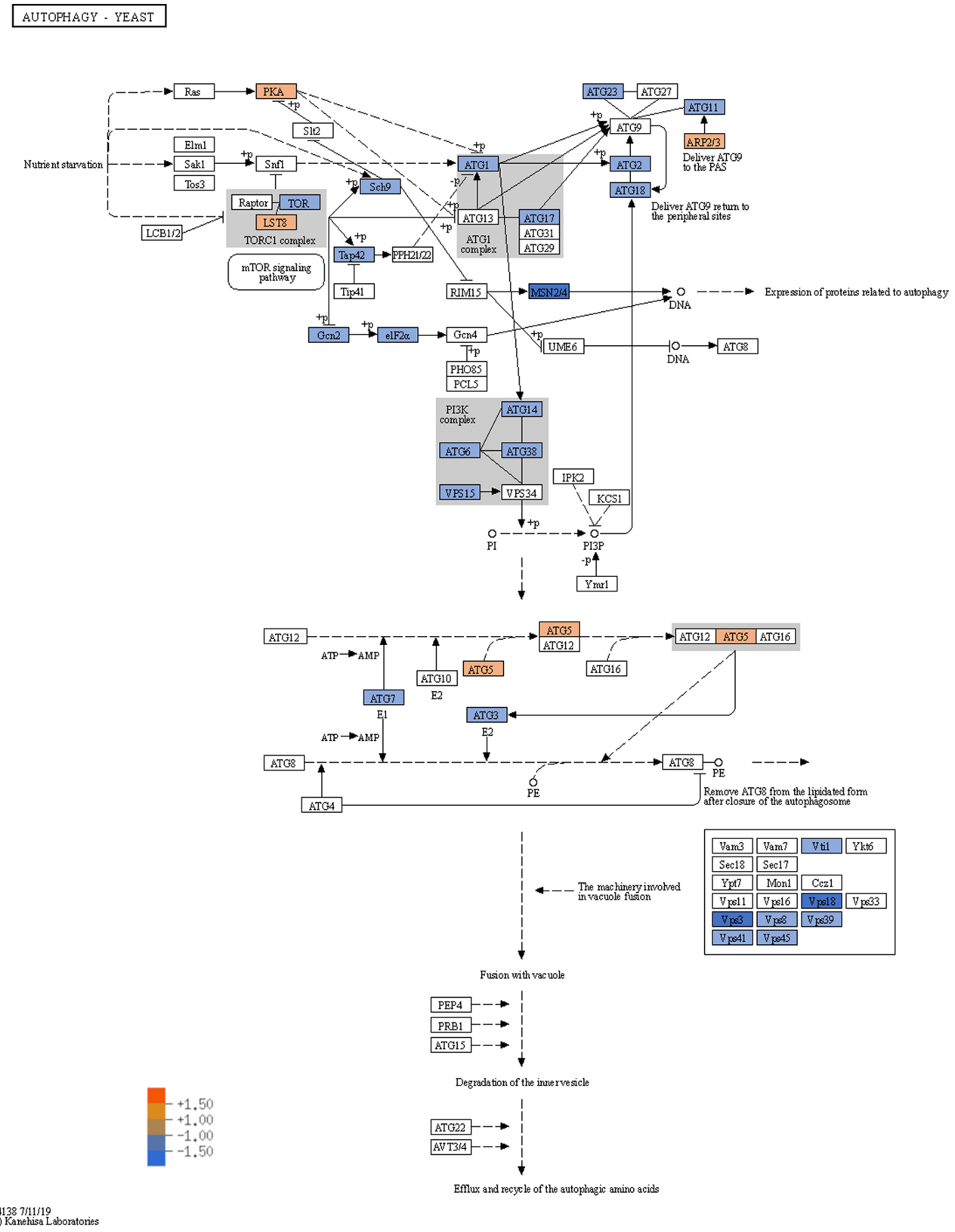

FIGURE 5 Autophagy pathway from KEGG database. Genes showing statistically significant differential expression against control in VBU treatment are highlighted. In orange, genes with $\log_2$Fold Change over 0.5 and *p*-value under 0.05. In blue, genes with $\log_2$Fold Change under −0.5 and *p*-value under 0.05.

be responsible for the observed effects. Alternatively, size-exclusion chromatography, under the conditions used in this work, could be stripping EVs of surface-associated proteins, or otherwise altering their structure, so that their biological function is disrupted. Further research is needed to unequivocally assign biological effects to the EVs and not to other possible components of the EV-enriched fraction (in fact, the two options are not mutually exclusive), and to determine whether the components of the EV-enriched fractions are the main natural effectors of this interaction or just a minor contributor.

The transcriptional response of *S. cerevisiae*, when challenged with whole *M. pulcherrima* cells or with the highest level of the EV-enriched fraction, is consistent with an accelerated exit from lag phase, induced by the competing yeast species. Some cell functions important for growth appear as upregulated, including synthesis of ribosomal proteins and amino acids, required for biomass production; and glycolysis, providing energy for growth. On the other side, cell functions required to survive under harsh conditions are downregulated. Notably, this involves autophagy, required for cell survival under stationary phase conditions (Suzuki et al., 2011), or *MSN2/4*, required for the expression of stress-responsive genes (Sadeh et al., 2011). So, under conditions permissive for yeast growth, the presence of a potential competitor seems to stimulate the change from a "resist" to a "proliferate" developmental programme in *S. cerevisiae*.

The generalized transcriptional activation observed for amino acid biosynthesis genes is probably just responding to components of the EV-enriched fraction, and not to a difference in amino acid availability between the control and any of the two tested conditions. The growth medium used in the assays described in this work is synthetic grape must. It contains amino acids in amounts mimicking a nitrogen-sufficient grape must (Beltran et al., 2005; Bely et al., 1990) and had been refreshed just 3 h before sample collection. In the case of whole *M. pulcherrima* cells some ambiguity on the impact of amino acid consumption might subsist, despite the short incubation time; but this is not the case for the EV-enriched fraction.

The observed upregulation of genes coding for cytoplasmic ribosomal proteins is not paralleled by genes coding for mitochondrial ribosomal proteins. It is not clear whether this responds to a sequential activation pattern, with mitochondria being awaked later in this programme, or to the nearly anaerobic conditions used in this assay. In support of anaerobic conditions masking some of the possible transcriptional responses, it can be mentioned that the downregulation of the TCA cycle observed in a previous work under aerated conditions (Mencher et al., 2021), did not reach significant values in the current assays.

CONCLUSIONS

In summary, this work shows evidence that the constituents of an EV-enriched fraction of *M. pulcherrima* can induce a transcriptional response in *S. cerevisiae*. This response is very similar to that induced by metabolically active cells of *M. pulcherrima* and seems to pursue a faster resume of growth in the experimental conditions used in this work. This result is compatible with the hypothesis that EVs might play a role in interspecies interactions among wine yeast species (Morales et al., 2021), but further research is necessary to unequivocally identify all the chemical signals and mechanisms involved. With this work, the growing body of knowledge on yeast EVs (in a variety of biological processes, such as pathogenesis) can now be extended to the biotechnological context. The involvement of extracellular signals, beyond metabolic interactions, in modulating yeast physiology in wine could have implications for the design of mixed cultures and their use in winemaking. It also adds a layer of complexity to our understanding of ecological interactions during wine fermentation, whether inoculated or spontaneous. However, we are just beginning to discover the occurrence of these interactions. We need to increase our knowledge of the mechanisms, the degree of generality, and the strain and species dependence, before we can use this information for practical purposes.

AUTHOR CONTRIBUTIONS

Miguel Mejías Ortiz: Investigation (equal); writing–original draft (equal). **Ana Mencher:** Investigation (equal); writing – review and editing (equal). **Pilar Morales:** Conceptualization (equal); funding acquisition (equal); investigation (equal); project administration (equal); supervision (equal); writing – review and editing (equal). **Jordi Tronchoni:** Supervision (equal); writing – review and editing (equal). **Ramon Gonzalez:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

RNA-seq raw data (bam files) related to this study were submitted to the NCBI repository under the Bioproject PRJNA856460.

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REFERENCES

Alvers, A.L., Fishwick, L.K., Wood, M.S., Hu, D., Chung, H.S., Dunn, W.A., Jr. et al. (2009) Autophagy and amino acid homeostasis are required for chronological longevity in *Saccharomyces cerevisiae*. *Aging Cell*, 8, 353–369. Available from: https://doi.org/10.1111/j.1474-9726.2009.00469.x

Bardwell, L. (2004) A walk-through of the yeast mating pheromone response pathway. *Peptides*, 25, 1465–1476. Available from: https://doi.org/10.1016/j.peptides.2003.10.022

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A. & Guillamón, J.M. (2005) Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry*, 53, 996–1002. Available from: https://doi.org/10.1021/jf0487001

Bely, M., Sablayrolles, J.-M. & Barre, P. (1990) Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *Journal of Fermentation and Bioengineering*, 70, 246–252. Available from: https://doi.org/10.1016/0922-338X(90)90057-4

Branco, P., Kemsawasd, V., Santos, L., Diniz, M., Caldeira, J., Almeida, M.G. et al. (2017) *Saccharomyces cerevisiae* accumulates GAPDH-derived peptides on its cell surface that induce death of non-*saccharomyces* yeasts by cell-to-cell contact. *FEMS Microbiology Ecology*, 93, fix055. Available from: https://doi.org/10.1093/femsec/fix055

Brou, P., Taillandier, P., Beaufort, S. & Brandam, C. (2018) Mixed culture fermentation using *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* with direct and indirect contact: impact of anaerobic growth factors. *European Food Research and Technology*, 244, 1699–1710. Available from: https://doi.org/10.1007/s00217-018-3095-3

Cherry, J.M., Hong, E.L., Amundsen, C., Balakrishnan, R., Binkley, G., Chan, E.T. et al. (2012) *Saccharomyces* genome database: the genomics resource of budding yeast. *Nucleic Acids Research*, 40, D700–D705. Available from: https://doi.org/10.1093/nar/gkr1029

Comitini, F., Agarbati, A., Canonico, L. & Ciani, M. (2021) Yeast interactions and molecular mechanisms in wine fermentation: a comprehensive review. *International Journal of Molecular Sciences*, 22, 7754. Available from: https://doi.org/10.3390/ijms22147754

Curiel, J.A., Morales, P., Gonzalez, R. & Tronchoni, J. (2017) Different non-*saccharomyces* yeast species stimulate nutrient consumption in *S. cerevisiae* mixed cultures. *Frontiers in Microbiology*, 8, 2121. Available from: https://doi.org/10.3389/fmicb.2017.02121

Davey, H.M., Cross, E.J.M., Davey, C.L., Gkargkas, K., Delneri, D., Hoyle, D.C. et al. (2012) Genome-wide analysis of longevity in nutrient-deprived *Saccharomyces cerevisiae* reveals importance of recycling in maintaining cell viability. *Environmental Microbiology*, 14, 1249–1260. Available from: https://doi.org/10.1111/j.1462-2920.2012.02705.x

De Virgilio, C. (2012) The essence of yeast quiescence. *FEMS Microbiology Reviews*, 36, 306–339. Available from: https://doi.org/10.1111/j.1574-6976.2011.00287.x

Di Malta, C., Cinque, L. & Settembre, C. (2019) Transcriptional regulation of autophagy: mechanisms and diseases. *Frontiers in Cell and Developmental Biology*, 7, 114. Available from: https://doi.org/10.3389/fcell.2019.00114

Englezos, V., Rantsiou, K., Giacosa, S., Río Segade, S., Rolle, L. & Cocolin, L. (2019) Cell-to-cell contact mechanism modulates *Starmerella bacillaris* death in mixed culture fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology*, 289, 106–114. Available from: https://doi.org/10.1016/j.ijfoodmicro.2018.09.009

Ewels, P., Magnusson, M., Lundin, S. & Käller, M. (2016) MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, 32, 3047–3048. Available from: https://doi.org/10.1093/bioinformatics/btw354

Gil-Bona, A., Llama-Palacios, A., Parra, C.M., Vivanco, F., Nombela, C., Monteoliva, L. et al. (2015) Proteomics unravles extracellular vesicles as carriers of classical cytoplasmic proteins in *Candida albicans*. *Journal of Proteome Research*, 14, 142–153. Available from: https://doi.org/10.1021/pr5007944

González, B., Vázquez, J., Cullen, P.J., Mas, A., Beltran, G. & Torija, M.J. (2018) Aromatic amino acid-derived compounds induce morphological changes and modulate the cell growth of wine yeast species. *Frontiers in Microbiology*, 9, 670. Available from: https://doi.org/10.3389/fmicb.2018.00670

Gonzalez, R., Guindal, A.M., Tronchoni, J. & Morales, P. (2021) Biotechnological approaches to lowering the ethanol yield during wine fermentation. *Biomolecules*, 11, 1569. Available from: https://doi.org/10.3390/biom11111569

He, C. & Klionsky, D.J. (2009) Regulation mechanisms and signaling pathways of autophagy. *Annual Review of Genetics*, 43, 67–93. Available from: https://doi.org/10.1146/annurev-genet-102808-114910

Kanehisa, M. (2000) KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, 28, 27–30. Available from: https://doi.org/10.1093/nar/28.1.27

Kemsawasd, V., Branco, P., Almeida, M.G., Caldeira, J., Albergaria, H. & Arneborg, N. (2015) Cell-to-cell contact and antimicrobial peptides play a combined role in the death of *Lachancea thermotolerans* during mixed-culture alcoholic fermentation with *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, 362, fnv103. Available from: https://doi.org/10.1093/femsle/fnv103

Kim, D., Paggi, J.M., Park, C., Bennett, C. & Salzberg, S.L. (2019) Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology*, 37, 907–915. Available from: https://doi.org/10.1038/s41587-019-0201-4

Li, E. & Mira de Orduña, R. (2017) Acetaldehyde kinetics of enological yeast during alcoholic fermentation in grape must. *Journal of Industrial Microbiology and Biotechnology*, 44, 229–236. Available from: https://doi.org/10.1007/S10295-016-1879-7

Liao, Y., Wang, J., Jaehnig, E.J., Shi, Z. & Zhang, B. (2019) WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Research*, 47, W199–W205. Available from: https://doi.org/10.1093/nar/gkz401

Lobb, R.J., Becker, M., Wen, S.W., Wong, C.S.F., Wiegmans, A.P., Leimgruber, A. et al. (2015) Optimized exosome isolation protocol for cell culture supernatant and human plasma. *Journal*

of Extracellular Vesicles, 4, 27031. Available from: https://doi.org/10.3402/jev.v4.27031

Martínez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *The EMBO Journal*, 15, 2227–2235. Available from: https://doi.org/10.1002/j.1460-2075.1996.tb00576.x

Mas, A. & Portillo, M.C. (2022) Strategies for microbiological control of the alcoholic fermentation in wines by exploiting the microbial terroir complexity: a mini-review. *International Journal of Food Microbiology*, 367, 109592. Available from: https://doi.org/10.1016/j.ijfoodmicro.2022.109592

Mencher, A., Mejias-Ortiz, M., Morales, P., Tronchoni, J. & Gonzalez, R. (2022) Protein content of the *Oenococcus oeni* extracellular vesicles-enriched fraction. *Food Microbiology*, 106, 104038. Available from: https://doi.org/10.1016/j.fm.2022.104038

Mencher, A., Morales, P., Curiel, J.A., Gonzalez, R. & Tronchoni, J. (2021) *Metschnikowia pulcherrima* represses aerobic respiration in *Saccharomyces cerevisiae* suggesting a direct response to co-cultivation. *Food Microbiology*, 94, 103670. Available from: https://doi.org/10.1016/j.fm.2020.103670

Mencher, A., Morales, P., Tronchoni, J. & Gonzalez, R. (2021a) Mechanisms involved in interspecific communication between wine yeasts. *Food*, 10, 1734. Available from: https://doi.org/10.3390/foods10081734

Mencher, A., Morales, P., Valero, E., Tronchoni, J., Patil, K.R. & Gonzalez, R. (2020) Proteomic characterization of extracellular vesicles produced by several wine yeast species. *Microbial Biotechnology*, 13, 1581–1596. Available from: https://doi.org/10.1111/1751-7915.13614

Milanovic, V., Ciani, M., Oro, L. & Comitini, F. (2012) *Starmerella bombicola* influences the metabolism of *Saccharomyces cerevisiae* at pyruvate decarboxylase and alcohol dehydrogenase level during mixed wine fermentation. *Microbial Cell Factories*, 11, 18. Available from: https://doi.org/10.1186/1475-2859-11-18

Monguió-Tortajada, M., Gálvez-Montón, C., Bayes-Genis, A., Roura, S. & Borràs, F.E. (2019) Extracellular vesicle isolation methods: rising impact of size-exclusion chromatography. *Cellular and Molecular Life Sciences*, 76, 2369–2382. Available from: https://doi.org/10.1007/s00018-019-03071-y

Morales, P., Mencher, A., Tronchoni, J. & Gonzalez, R. (2021) Extracellular vesicles in food biotechnology. *Microbial Biotechnology*, 14, 8–11. Available from: https://doi.org/10.1111/1751-7915.13657

Morozumi, Y. & Shiozaki, K. (2021) Conserved and divergent mechanisms that control TORC1 in yeasts and mammals. *Genes*, 12, 88. Available from: https://doi.org/10.3390/genes12010088

Okonechnikov, K., Conesa, A. & García-Alcalde, F. (2015) Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics*, 32, btv566. Available from: https://doi.org/10.1093/bioinformatics/btv566

Pérez-Nevado, F., Albergaria, H., Hogg, T. & Girio, F. (2006) Cellular death of two non-*saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology*, 108, 336–345. Available from: https://doi.org/10.1016/j.ijfoodmicro.2005.12.012

Pérez-Torrado, R., Rantsiou, K., Perrone, B., Navarro-Tapia, E., Querol, A. & Cocolin, L. (2017) Ecological interactions among *Saccharomyces cerevisiae* strains: insight into the dominance phenomenon. *Scientific Reports*, 7, 1–10. Available from: https://doi.org/10.1038/srep43603

Ramakrishnan, V., Walker, G.A., Fan, Q., Ogawa, M., Luo, Y., Luong, P. et al. (2016) Inter-kingdom modification of metabolic behavior: [GAR^+] prion induction in *Saccharomyces cerevisiae* mediated by wine ecosystem bacteria. *Frontiers in Ecology and Evolution*, 4, 137. Available from: https://doi.org/10.3389/fevo.2016.00137

Reimand, J., Isserlin, R., Voisin, V., Kucera, M., Tannus-Lopes, C., Rostamianfar, A. et al. (2019) Pathway enrichment analysis and visualization of omics data using g:profiler, GSEA, Cytoscape and EnrichmentMap. *Nature Protocols*, 14, 482–517. Available from: https://doi.org/10.1038/s41596-018-0103-9

Renault, P.E., Albertin, W. & Bely, M. (2013) An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Applied Microbiology and Biotechnology*, 97, 4105–4119. Available from: https://doi.org/10.1007/s00253-012-4660-5

Robinson, M.D., McCarthy, D.J. & Smyth, G.K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26, 139–140. Available from: https://doi.org/10.1093/bioinformatics/btp616

Rodrigues, M. & Janbon, G. (Eds.). (2021) *Fungal Extracellular Vesicles*, Vol. 432, 1st edition. Cham: Springer International Publishing. Available from: https://doi.org/10.1007/978-3-030-83391-6

Rossouw, D., Meiring, S.P. & Bauer, F.F. (2018) Modifying *Saccharomyces cerevisiae* adhesion properties regulates yeast ecosystem dynamics. *MSphere*, 3, e00383–e00318. Available from: https://doi.org/10.1128/mSphere.00383-18

Roullier-Gall, C., David, V., Hemmler, D., Schmitt-Kopplin, P. & Alexandre, H. (2020) Exploring yeast interactions through metabolic profiling. *Scientific Reports*, 10, 6073. Available from: https://doi.org/10.1038/s41598-020-63182-6

Sadeh, A., Movshovich, N., Volokh, M., Gheber, L. & Aharoni, A. (2011) Fine-tuning of the Msn2/4–mediated yeast stress responses as revealed by systematic deletion of Msn2/4 partners. *Molecular Biology of the Cell*, 22, 3127–3138. Available from: https://doi.org/10.1091/mbc.e10-12-1007

Shekhawat, K., Patterton, H., Bauer, F.F. & Setati, M.E. (2019) RNA-seq based transcriptional analysis of *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed-culture fermentations under anaerobic conditions. *BMC Genomics*, 20, 145. Available from: https://doi.org/10.1186/s12864-019-5511-x

Shu, W.-J., Zhao, M.-J., Klionsky, D.J. & Du, H.-N. (2020) Old factors, new players: transcriptional regulation of autophagy. *Autophagy*, 16, 956–958. Available from: https://doi.org/10.1080/15548627.2020.1728611

Stahl, P.D. & Raposo, G. (2019) Extracellular vesicles: exosomes and microvesicles, integrators of homeostasis. *Physiology*, 34, 169–177. Available from: https://doi.org/10.1152/physiol.00045.2018

Suzuki, S.W., Onodera, J. & Ohsumi, Y. (2011) Starvation induced cell death in autophagy-defective yeast mutants is caused by mitochondria dysfunction. *PLoS One*, 6, e17412. Available from: https://doi.org/10.1371/journal.pone.0017412

Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R. et al. (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles*, 7, 1535750. Available from: https://doi.org/10.1080/20013078.2018.1535750

Tondini, F., Lang, T., Chen, L., Herderich, M. & Jiranek, V. (2019) Linking gene expression and oenological traits: comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains. *International Journal of Food Microbiology*, 294, 42–49. Available from: https://doi.org/10.1016/j.ijfoodmicro.2019.01.014

Tronchoni, J., Curiel, J.A., Morales, P., Torres-Pérez, R. & Gonzalez, R. (2017) Early transcriptional response to biotic stress in mixed starter fermentations involving *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. *International Journal of*

Food Microbiology, 241, 60–68. Available from: https://doi.org/10.1016/j.ijfoodmicro.2016.10.017

Varet, H., Brillet-Guéguen, L., Coppée, J.-Y. & Dillies, M.-A. (2016) SARTools: a DESeq2- and EdgeR-based R pipeline for comprehensive differential analysis of RNA-Seq data. *PLoS One*, 11(6), e0157022. Available from: https://doi.org/10.1371/journal.pone.0157022

Vejarano, R. & Gil-Calderón, A. (2021) Commercially available non-*saccharomyces* yeasts for winemaking: current market, advantages over *saccharomyces*, biocompatibility, and safety. *Fermentation*, 7, 171. Available from: https://doi.org/10.3390/fermentation7030171

Vlahakis, A., Lopez Muniozguren, N. & Powers, T. (2017) Stress-response transcription factors Msn2 and Msn4 couple TORC2-Ypk1 signaling and mitochondrial respiration to *ATG8* gene expression and autophagy. *Autophagy*, 13, 1804–1812. Available from: https://doi.org/10.1080/15548627.2017.1356949

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