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SHORT COMMUNICATION

# Succinic acid significantly affects the global expression of *Oenococcus oeni* PSU-1

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## ABSTRACT

*Oenococcus oeni* is the predominant lactic acid bacterium performing malolactic fermentation (MLF) in wine. Succinic acid, produced by yeasts during alcoholic fermentation, can improve the organoleptic properties of wine, but it can have a negative impact on *O. oeni* and MLF. The levels of succinic acid in wine can increase with the current use of non-*Saccharomyces* yeasts. In this work, transcriptomic analysis by RNA-seq was performed in wine-like medium supplemented with 2 g/L succinic acid, and a control medium without it, during MLF performed by *O. oeni* PSU-1. Approximately 25 % of the 1,638 detected transcripts were downregulated, exhibiting half the expression of those in the control or less, and other 29 % were upregulated, with double expression or more. We found that genes of clusters of orthologous groups related to the metabolism of nucleotides, translation, and membrane transport were predominantly downregulated by succinic acid, while those related to the transport and metabolism of carbohydrates, transcription, inorganic ion metabolism and defense mechanisms were predominantly upregulated. Considering the greater upregulation of carbohydrate metabolism genes, we analysed those involved in the phosphotransferase system. In this first transcriptomic study of the effect of succinic acid on *O. oeni,* we observed a global cell response with many changes in gene expression related to the observed MLF delay by succinic acid.

**KEYWORDS:** *Oenococcus oeni*, RNA-seq, succinic acid, transcriptomics, wine

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## INTRODUCTION

*Oenococcus oeni*, the lactic acid bacterium (LAB) mostly found in wine environments, is the predominant species performing the malolactic fermentation (MLF) by decarboxylating L-malic acid to L-lactic acid and improving wine quality and microbial stability (Bartowsky, 2017; Betteridge *et al*., 2015; Lorentzen and Lucas, 2019).

Succinic acid is produced by yeasts during the early stages of alcoholic fermentation (De Klerk, 2010). *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts are known to produce succinic acid at concentrations ranging from 200 mg/L to 2 g/L (Benito, 2018; Contreras *et al*., 2014; Zhu *et al*., 2020). The flavour of succinic acid is a complex mixture of sour, salty and bitter tastes, and it is responsible for the special taste characterising all fermented beverages (Chidi *et al*., 2018; Conde *et al*., 2007). This acid can have a positive impact on the sensory quality of wine due to the increase in fruity aromatic esters, such as ethyl and diethyl succinates (Bartowsky and Pretorius, 2009; De Klerk, 2010; Vicente *et al*., 2022).

By contrast, succinic acid can have a negative impact on *O. oeni* development and on MLF performance (Caridi and Corte, 1997; Son *et al*., 2009). There is a clear MLF inhibition at concentrations of succinic acid higher than 1 g/L or generally when the molar concentration of succinic is higher than that of L-malic acid (Torres-Guardado *et al*., 2022).

For some years, there has been an increase in the use of non-*Saccharomyces* strains in order to improve the aroma and complexity of wines (Benito *et al*., 2019; Padilla *et al*., 2016). They are generally used in sequential fermentations before *S. cerevisiae*. Nevertheless, the inoculation strategy can affect MLF (du Plessis *et al*., 2017) and if both non-*Saccharomyces* and *S. cerevisiae* produce some succinic acid, its level in wine can be increased (Torres-Guardado *et al*., 2024), with the consequent risk of suppressing MLF. The aim of this work was to evaluate the effect of succinic acid on *O. oeni* cells via transcriptomics, performed by NGS (next generation sequencing) of total RNA (RNA-seq), to determine the global gene expression profile and to analyse which genes were most affected. A relatively high concentration (2 g/L) of succinic acid was used to determine the most important effects.

## MATERIALS AND METHODS

### 1. Strain, culture growth, malolactic fermentation conditions and L-malic acid quantification

*O. oeni* PSU-1 (ATCC BAA-331) was cultured at 27 °C in a $CO_2$ (10 %) incubator in MRS broth supplemented with D, L-malic acid (4 g/L) and fructose (5 g/L) at a pH of 5.0. When the cultures reached the late exponential phase ($OD_{600nm}$ = 1.6), they were inoculated at a concentration of $2 \cdot 10^7$ cells/mL in a wine-like medium (Bordas *et al*., 2015) at pH 3.4 with 2 g/L of succinic acid (WLMS) or without it (WLM). Fermentation was carried out at 20 °C under static conditions in triplicate. The population of *O. oeni* was determined by viable counting on solid medium plates using the same medium used for growth (supplemented MRS) with 20 g/L of agar. L-Malic acid consumption was measured by the enzymatic method (McCloskey, 1980) approved by OIV, using a Y15 Analyzer (BioSystems, Barcelona, Spain).

### 2. Sample preparation

WLM and WLMS samples were taken in the middle of MLF when the L-malic acid concentration was around 1 g/L. Fifty millilitres of each sample were centrifuged at 4,600 × *g* for 20 min at 4 °C. The pellet was washed with 10 mM Tris-HCl at pH 8, prepared with diethyl pyrocarbonate (DEPC)-treated water, frozen in liquid nitrogen and kept at –80 °C until RNA extraction.

### 3. RNA extraction from *O. oeni* cells and RNA quality assessment

Following Margalef-Català *et al*. (2016), the cell pellet was defrosted and washed again with 10 mM Tris-HCl at pH 8, prepared with diethyl pyrocarbonate (DEPC)-treated water. A High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used for extraction following the manufacturer's instructions. Total nucleic acid concentrations were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). The extracted RNA was stored at –80 °C until RNA analysis. RNA quality was assessed using the Agilent RNA ScreenTape Assay.

### 4. Transcriptomic analysis

The transcriptomic analysis by RNA-seq was performed by the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili–Eurecat, following optimised protocols. The sequencing libraries were created using Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus (20040525, Illumina) following the manufacturer's instructions. As a result, a sample DNA library was obtained that contained the retrotranscribed and modified RNA fragments to be sequenced. To this end, the NextSeq 2000 equipment of the Illumina platform was used, generating up to 50 million 2 x 76 pb reads per sample.

The data analysis included mapping against the reference genome GenBank [CP000411.1, *Oenococcus oeni* strain PSU-1] (NCBI, 2023) using HISAT2 (2.2.1); annotating and quantifying the aligned reads with StringTie (2.1.4); and comparing the gene expression levels using the DESeq2 R package (1.30.0). The samples were normalised by the relative log expression method.

### 5. Interpretation of the differentially expressed genes found via transcriptomic analysis

For each detected RNA the statistical comparison between WLMS samples and WLM ones was done calculating the logarithm of the fold change in base 2 (log2FC) of expression values, so that log2FC = 1 meant duplicate expression, log2FC = 2 meant quadruple and so on. Differentially expressed genes (DEGs) were considered significant when

the p-value was < 0.05 and the log2FC was < -1 (less than half expression) for downregulated genes, and log2FC was > 1 (more than double expression) for upregulated genes.

The significant DEGs were classified into clusters of orthologous groups (COGs). The FUNAGE-Pro web server (De Jong *et al*., 2022) was used to perform the COG enrichment analysis of the DEGs. The COGs with a greater number of significant DEGs, such as genes coding for the phosphotransferase system (PTS) related to carbohydrate metabolism (Cibrario *et al*. 2016), were studied in more detail.

## RESULTS AND DISCUSSION

### 1. Impact of succinic acid on MLF

As previously observed (Torres-Guardado *et al*., 2022), and thus as expected, MLF took longer in the presence of succinic acid (17 days) than under the control conditions without this acid (6 days) (Figure S1). Besides that, no significant changes in the viable population were detected during MLF in WLM. The intermediate and final populations were similar to the initial ones, $2 \cdot 10^7$ cells/mL, indicating the survival of the cells as well as the lack of growth during MLF.

### 2. Results of differential expression analysis

A total of 1,638 transcripts were detected in the transcriptomic analysis by RNA-seq, that is 88 % of 1,862, the total known genes of *O. oeni* PSU-1 (NCBI, 2023). RNA was not found for the other 224 genes. The relative expression results for cells grown with succinic acid were globally different to those of the control, since 873 of detected transcripts were found to correspond to identified DEGs, which represented 49 % of the total genes of *O. oeni*. A total of 405 of these genes were downregulated with a log2FC < -1 (25 % of detected genes), and 468 genes were upregulated with a log2FC > 1 (29% of detected genes). We considered the other 765 found transcripts as non DEGs, because their log2FC values were between 1 and -1.

All these detected expressed genes are displayed in Supplementary Table S1, as are the log2FC values of the WLMS samples compared to those of the WLM samples. The statistics for the significant results is shown in the "padj" column with the adjusted p values, confirming that the changes were significant (padj < 0.05). Other genes not detected in this analysis but included in GenBank for *O. oeni* PSU-1 are also shown in Table S1 (locus number typed in orange). According to the GenBank database, almost all the identified DEGs encoded for proteins (93 %).

### 3. Global analysis of the functions affected by succinic acid in *O. oeni* PSU-1

The 873 DEGs were classified into COGs, whose names can be seen in legend of Figure 1. Using the FUNAGE-Pro web server for *O. oeni* PSU-1, we obtained COG descriptions for

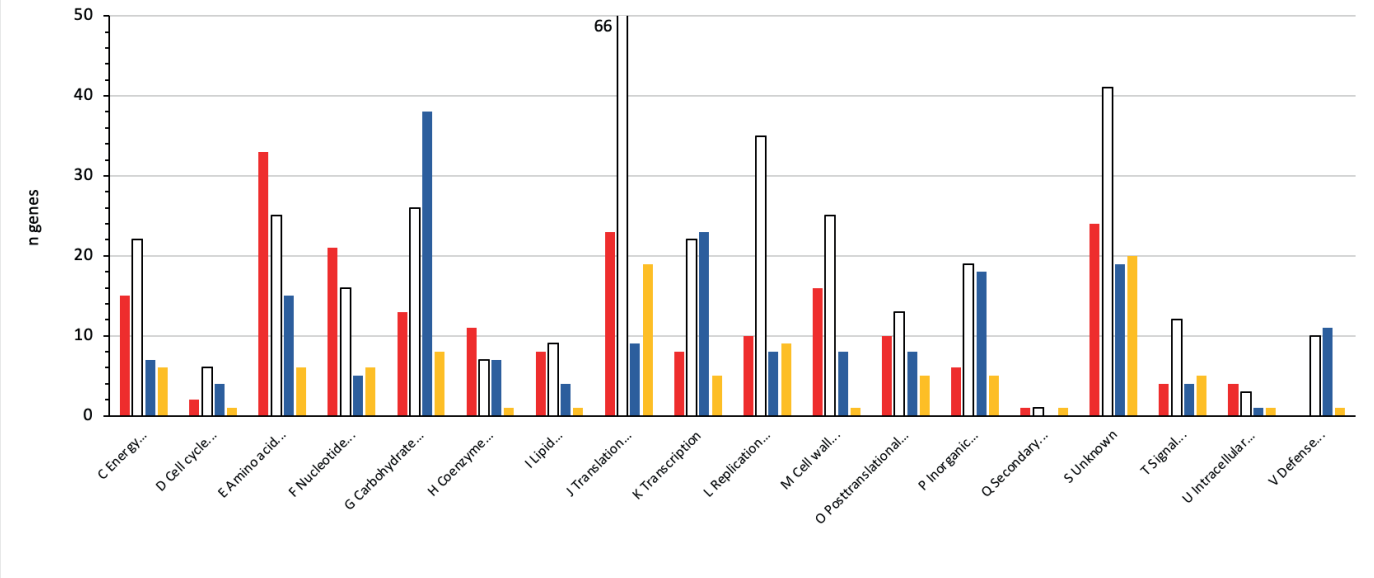


**FIGURE 1.** Number of genes of *O. oeni* PSU-1 for each representative COG, significantly underexpressed (red) or overexpressed (blue) in WLM + 2 g/L succinic acid, according to transcriptomic RNA-seq analysis. Differentially expressed genes (white) genes that were not significant and those for which RNA was not detected (yellow) are also shown. COG full names according to last update (NCBI, 2022) are: C Energy production and conversion; D Cell cycle control, cell division, chromosome partitioning; E Amino acid transport and metabolism; F Nucleotide transport and metabolism; G Carbohydrate transport and metabolism; H Coenzyme transport and metabolism; I Lipid transport and metabolism; J Translation, ribosomal structure and biogenesis; K Transcription; L Replication, recombination and repair; M Cell wall/membrane/envelope biogenesis; O Post-translational modification, protein turnover, and chaperones; P Inorganic ion transport and metabolism; Q Secondary metabolites biosynthesis, transport, and catabolism; S Function unknown; T Signal transduction mechanisms; U Intracellular trafficking, secretion, and vesicular transport; V Defense mechanisms.

**TABLE 1.** Different expression (log2FoldChange, significant negative values in red and positive in blue) of *O. oeni* PSU-1 genes related to the PTS system–according to Jamal *et al.* (2013)–in cells grown in WLMS (succinic acid 2 g/L) compared to control in WLM.

| Locus tag | Product (GenBank) | log2FoldChange | Description | Symbol |
|---|---|---|---|---|
| OEOE_RS03075 | HPr his-protein | -1.15 | phosphocarrier protein HPr | hpr |
| OEOE_RS01045 | IIC cellobiose | 1.28 | PTS cellobiose transporter subunit IIC | celA |
| OEOE_RS01050 | IIA cellobiose | 1.46 | PTS lactose/cellobiose transporter subunit IIA | celA |
| OEOE_RS01055 | IIB cellobiose | 1.72 | PTS sugar transporter subunit IIB | celA |
| OEOE_RS01120 | IIC galacticol | 1.00 | PTS transporter subunit IIC | galA |
| OEOE_RS01350 | IIC cellobiose | 2.18 | PTS sugar transporter subunit IIC | |
| OEOE_RS01415 | IIA glucose | 1.66 | PTS glucose transporter subunit IIA | celB |
| OEOE_RS01420 | IIBC β-glucoside | 1.34 | PTS transporter subunit EIIB | celB |
| OEOE_RS01620 | IIB cellobiose | 2.40 | PTS sugar transporter subunit IIB | celC |
| OEOE_RS01625 | IIA cellobiose | 4.17 | PTS lactose/cellobiose transporter subunit IIA | celC |
| OEOE_RS01630 | 6P- β-glucosidase | 4.00 | 6-phospho-beta-glucosidase | celC |
| OEOE_RS01635 | 6P- β-glucosidase | 3.03 | 6-phospho-beta-glucosidase | celC |
| OEOE_RS01645 | IIC cellobiose | 1.57 | PTS cellobiose transporter subunit IIC | celC |
| OEOE_RS01740 | IIBC fructose | 2.75 | PTS sugar transporter subunit IIC | |
| OEOE_RS01830 | IID mannose | 1.15 | PTS system mannose/fructose/sorbose family transporter subunit IID | manA |
| OEOE_RS01835 | IIA mannose | 1.31 | PTS sugar transporter subunit IIA | manA |
| OEOE_RS02230 | IIAB mannose | 1.51 | PTS sugar transporter subunit IIB | manB |
| OEOE_RS02235 | IIC mannose | 1.69 | PTS mannose/fructose/sorbose transporter subunit IIC | manB |
| OEOE_RS02240 | IID mannose | 1.93 | PTS system mannose/fructose/sorbose family transporter subunit IID | manB |
| OEOE_RS05815 | IIA cellobiose | 1.62 | PTS lactose/cellobiose transporter subunit IIA | celD |
| OEOE_RS05820 | IIB cellobiose | 1.86 | PTS sugar transporter subunit IIB | celD |
| OEOE_RS05825 | IIC cellobiose / pseudogene | 3.12 | PTS transporter subunit EIIC | celD |
| OEOE_RS05830 | 6P- β-glucosidase | 2.24 | glycoside hydrolase family 1 protein | celD |
| OEOE_RS06445 | P-trehalase | 4.00 | alpha,alpha-phosphotrehalase | treA |
| OEOE_RS06450 | IIBC trehalose | 3.48 | PTS transporter subunit EIIC | treA |
| OEOE_RS06455 | IIA glucose/trehalose | 2.23 | PTS glucose transporter subunit IIA | treA |
| OEOE_RS07145 | IIC ascorbate/ pseudogene | 2.20 | PTS ascorbate transporter subunit IIC | ascA |
| OEOE_RS07150 | IIB ascorbate | 1.93 | PTS sugar transporter subunit IIB | ascA |

398 genes (46 % of DEGs), of which 209 were downregulated and 189 were upregulated (Table S1). Figure 1 shows the number of genes that were under- or overexpressed for each representative COG.

The functional categories of genes (*id est*, the COGs) related to the nucleotides (F), translation (J) and amino acid transport and metabolism (E) were predominantly downregulated, while those related to carbohydrate transport and metabolism (G), transcription (K), inorganic ion transport and metabolism (P) and defense mechanisms (V) were predominantly upregulated. The other COGs were neither predominantly downregulated nor upregulated; these were related to energy (C), cell cycle control and cell division (D), coenzymes transport and metabolism (H), lipids transport and metabolism (I), replication, recombination and repair (L), cell wall/membrane/envelope biogenesis (M), post translational mechanisms (O), secondary metabolism (Q), signal transduction mechanisms (T) and intracellular trafficking and secretion (U).

Several previous transcriptomic studies on *O. oeni* have evaluated different types of stress associated with wine, including the response to acidic conditions (Liu *et al*., 2017; Margalef-Català *et al*., 2016; Olguín *et al*., 2015). In the present study, the pH was the same (3.4) as that of the control

fermentations with added succinic acid to uniquely evaluate the possible inhibitory effect of succinic acid. However, the internal acidification of the *O. oeni* cytosol due to succinic acid dissociation could be one of the possible mechanisms of inhibition.

Among the significantly underexpressed genes of different cellular functions were those of nucleotide transport and metabolism category (F). Seven genes in the form of an operon that encodes key enzymes in DNA synthesis (from OEOE_RS01235 to OEOE_RS01265) were the most downregulated of all the DEGs found here, with a 16-fold reduction in expression (log2F < -4) due to the presence of succinic acid (Table S1). The functions of these genes, such as dihydroorotase and carbamoyl phosphate synthase, are associated with the pyrimidine biosynthetic pathway (Kilstrup *et al*., 2005). In addition to their roles as precursors for RNA and DNA, pyrimidine nucleotides play important roles in the biosynthesis of components of the cell envelope, including peptidoglycan and exopolysaccharides (EPSs). Other essential functions negatively affected by this acid were translation (J) and amino acid transport and metabolism (E). Among the genes most affected by these functions were those involved in ribosomal assembly and those encoding peptidases and amino acid transporters (Table S1).

Many of the transcriptionally activated genes classified in the metabolism and transport of inorganic ions (P) category were phosphate permeases, such as two genes associated with the transport of spermidine/putrescine by ATPase (OEOE_RS07075 and OEOE_RS7080) [Table S1]. Spermidine/putrescine uptake has been associated with an energy-producing state/membrane potential in *E. coli* (Kashiwagi *et al*., 1997), and these amines protect against oxidative stress (Tkachenko *et al*., 2001). In another study, adaptation to WLM conditions resulted in the overexpression of six out of the eight transporters of these polyamines annotated in the PSU-1 genome (Margalef-Català *et al*., 2016).

Transcription (K) is a functional category that is clearly activated in response to succinic acid. The most activated genes were transcriptional regulators involved in the complex response to the inhibition produced by succinic acid, such as genes encoding for GntR (OEOE_RS06440) and TetR (OEOE_RS00850) of transcriptional regulators families (Table S1). In spite of the fact that regulatory elements for the transcription of stress-related *O. oeni* genes have been well described (Beltramo *et al*., 2004; Guzzo *et al*., 2000), further research is needed to determine the specific function of these two genes activated by succinic acid.

Another clearly activated COG by succinic acid was defense mechanisms (V), with 11 genes being overexpressed, and none underexpressed. Interestingly, nine of these 11 genes were annotated as ABC transporter ATP-binding protein: OEOE_RS00945, 00950, 00975, 03525, 03510, 06465, 06635, 07885 and 07980. They probably they have the function of increasing tolerance to succinic acid, as found in similar ABC membrane transporters, such as *omrA* gene of *O. oeni*, which protects from ethanol and other stress factors (Bourdineaud *et al*., 2004), and also other genes of *Lactococcus lactis*, shown to increase specifically acid-stress tolerance (Zhu *et al*., 2022).

## 4. Upregulation of genes associated with carbohydrate metabolism

COG of carbohydrate metabolism (G) showed a predominance of upregulated genes. Among those, many belong to the PTS (Table 1), which is associated with sugar transport and was described by Jamal *et al*. (2013) in *O. oeni* PSU-1. In this way, sugar metabolism would have a significant role in the response to the stress caused by succinic acid. The upregulated PTS genes (Table 1, in blue) belong mainly to the permeases associated with the transport of cellobiose (*celA, celB, celC, celD*), mannose (*manA, manB*) and trehalose (*treA*).

Trehalose is one of the sugars in the medium (0.25 g/L trehalose and 0.4 g/L fructose), but WLM does not contain sources of cellobiose or mannose. Jamal *et al*. (2013) described the difficulty in predicting the substrate specificity of PTS permeases from sequence comparisons and observed the transcriptional activation of some genes in the presence of sugars, which is not supposed to be specific to the studied permease. For example, at the *celA* locus, Jamal *et al*. (2013) reported that this gene was highly overexpressed in the presence of cellobiose, as well as, to a lesser extent, in the presence of trehalose. Similary, Cibrario *et al*. (2016) found that *manA* and *manB* were also overexpressed in response to the stress conditions of wine in the same PSU-1 strain.

Additionally, the PTS may comprise other substrates that are different from sugars, since most PTS permeases have been shown to phosphorylate several substrates in different bacteria (Postma *et al*., 1993). In other LAB, transcriptomic and proteomic studies have shown enhanced levels of glycolytic enzymes under acid, thermal, and osmotic stresses, but without increasing the synthesis of lactic acid (Papadimitrou *et al*., 2016). LAB such as *Lactiplantibacillus plantarum* and *Lactococcus lactis* modify pyruvate metabolism at the expense of lactic acid, increasing the synthesis of basic compounds (*e.g*., lysine and diacetyl/acetoin), exopolysaccharides (EPS), and/or glycogen (Heunis *et al*., 2014; Zuljan *et al*., 2014). Here, we did not observe an increase in D-lactic acid produced by *O. oeni* in sugar metabolism (data not shown); therefore, some of these mechanisms could be involved in the cellular response to succinic acid. In the case of *O. oeni*, the biosynthesis of EPS has been associated with biofilm formation as a mechanism for cell survival under stressful conditions (Dimopoulou *et al*., 2018). The upregulation of genes encoding the glycosyltransferases OEOE_RS07010, OEOE_RS07115 and OEOE_ RS07295 was observed in response to succinic acid (Table S1). This enzymatic activity has been associated with biofilm formation and the bacterial stress response in LAB (Schwab *et al*., 2007). However, further research is needed to understand the role of PTS activation under wine stress conditions.

## CONCLUSION

The MLF delay of more than 10 days compared to that of the control in the presence of 2 g/L succinic acid confirmed the known negative effect of relatively high levels of succinic acid in wine on *O. oeni*. Here, we related this delay to several variations in global gene expression revealed by transcriptomic analysis by RNA-seq. A total of 1,638 transcripts were detected, of which 873 were found to be DEGs. Gene expression was downregulated in approximately 25 % of the genes, and it was upregulated in another 29 %.

Gene expression of functional categories related to the metabolism of nucleotides (F), translation (J), and amino acid transport and metabolism (E) were predominantly downregulated, while those related to the transport and metabolism of carbohydrates (G), transcription (K), inorganic ion metabolism (P) and defense mechanisms (V) were predominantly upregulated.

The greater downregulation of gene expression related to pyrimidine metabolism could be related to the observed negative impact of succinic acid, including the role of pyrimidine in the synthesis of cell envelope components. On the other hand, considering the greater upregulation of carbohydrate metabolism genes expression, we analysed those belonging to the PTS, and found that sugar permeases were the most overexpressed.

In conclusion, in this first transcriptomic study of the effect of succinic acid on *O. oeni*, we observed a global cellular response with many changes in gene expression. We suggest that all these stress-*ƒ*related changes caused by succinic acid are related to previously reported *O. oeni* and other LAB damage caused by inhibiting compounds, such as ethanol.

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