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ORIGINAL RESEARCH ARTICLE

Influence of succinic acid on *Oenococcus oeni* and malolactic fermentation

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ABSTRACT

As a consequence of the alcoholic fermentation carried out by yeasts in wine, several compounds can be delivered to the medium, promoting or inhibiting the malolactic fermentation (MLF) and the lactic acid bacteria, mainly *Oenococcus oeni*. Succinic acid is one of these compounds and is an example of the interaction between yeasts, including non-*Saccharomyces* species, and *O. oeni*. However, the influence of succinic acid on the MLF has been researched very little as yet. In this work, we study the influence of succinic acid and pH on *O. oeni* CH11 and PSU-1 strains, both during MLF and in resting cell experiments. Moreover, we analysed the relative expression of some significant genes related to stress and malolactic activity to determine how the *O. oeni* strains were affected by the succinic acid. The results showed that the succinic acid could act as an MLF inhibitor at concentrations higher than 1 g/L, but it can be beneficial at 0.5 g/L. This variable effect also depends on the strains and other winemaking conditions, mainly pH, which influences the dissociated and undissociated forms of both acids. The inhibiting effect of succinic seems clearer when it is at a molar concentration higher than that of L-malic acid. Experiments with resting cells have confirmed that *O. oeni* consumes less L-malic acid when succinic acid is higher than 1 g/L. Genetic expression experiments showed that in the presence of succinic acid (2 g/L), gene *hsp18* encoding stress protein was up-regulated in strain CH11, suggesting a good response and adaptation of this strain to stress. On the other hand, genes *mleA* and *mleP*, which are related to malolactic activity, were not affected by succinic acid, except for strain PSU-1 at pH 4.0. Further research is necessary to understand better the effects of succinic acid on *O. oeni* and MLF.

KEYWORDS: Malolactic fermentation, *Oenococcus oeni*, wine, succinic acid, resting cells, *hsp18*, *mleP*

INTRODUCTION

Alcoholic fermentation (AF) is the main microbiological process in winemaking, as it converts grape must into wine. This fermentation is carried out by the yeast *Saccharomyces cerevisiae*, although during the early stages, non-*Saccharomyces* yeasts are present in the fermenting must (Fleet *et al.*, 1984). There is an increasing interest in these other yeasts (Padilla *et al.*, 2016) due to the production of new aromas (Belda *et al.*, 2017).

The yeast metabolism and the winemaking conditions greatly influence the final composition of the wine. Some compounds produced by yeasts—including non-*Saccharomyces*—during AF have a large impact on the subsequent malolactic fermentation (MLF), carried out mainly by the lactic acid bacterium *Oenococcus oeni* (Lonvaud-Funel, 1999; Bartowsky, 2005; Balmaseda *et al*., 2018; Ferrando *et al.*, 2020; Balmaseda *et al.*, 2021). Some of these compounds are produced by the primary metabolism of yeast, such as ethanol and SO_2 (Arnink and Henick-Kling, 2005), certain organic acids (Lonvaud-Funel and Strasser de Saad, 1982), and medium-chain fatty acids (Guilloux-Benatier *et al.*, 1998; Lonvaud-Funel *et al.*, 1988). Other compounds are delivered by the secondary metabolism as antimicrobial peptides (Branco *et al.*, 2014) and proteinaceous compounds (Osborne and Edwards, 2007). Among the organic acids derived from the yeast metabolism, succinic acid seems to be the most related to MLF inhibition (Caridi and Corte, 1997; Son *et al*., 2009).

Succinic acid is one of the relevant organic acids in wine, whose main role is to confer microbial stability to wines in relation to acidity and pH. They also preserve the colour and sensory properties of wines. Tartaric and malic acids are generally the most prominent acids in wines, while others such as acetic, succinic, citric, lactic, and pyruvic can exist in minor concentrations (Mendes-Ferreira and Mendes-Faia, 2020). Tartaric and malic acids are already present in grape must, but no succinic or lactic acids are found in grapes. Instead, succinic acid is usually the predominant non-volatile organic acid formed by yeasts during AF (Thoukis *et al.*, 1965).

Succinic acid is the 1,4-butanedioic acid (HOOC-CH_2-CH_2-COOH), with an m.w. of 118 g/mol and pK_{a1} of 4.2 and pK_{a2} of 5.6 (ChemIDplus, 2021). Its structure is like that of malic acid (HOOC-CHOH- CH_2-COOH), which has an m.w. of 134 g/mol, and which can also be called hydroxysuccinic acid. The organoleptic threshold of succinic acid in wine is 35 mg/L. It has an unusual bitter-salty taste and excess levels can have a negative impact on the mouthfeel of the wines (Coulter *et al.*, 2004); thus, it may be beneficial to reduce it.

Succinic acid is an intermediate of the tricarboxylic acid cycle (TCA), and it, therefore, is produced by yeasts during the AF in the early fermentation stages (Conway and Brady, 1950), but also during the stationary phase (Lamikanra, 1997; Arikawa *et al*., 1999). However, it acts as an intermediary in other metabolic pathways such as γ-amino butyric acid (GABA) bypass, glyoxylic acid bypass and the methylcitric acid cycle. Therefore, due to mechanical stimuli and maceration of red grapes, the GABA concentration in must can increase and could encourage yeast to produce succinic acid (De Klerk, 2010).

S. cerevisiae strains are known to produce succinic acid, with values from 200 mg/L to more than 1 g/L (Heerde and Radler, 1978; Coulter *et al*., 2004; De Klerk, 2010; Zhu *et al*., 2020), thus explaining the increases of this acid in winemaking. This production of succinic acid can influence MLF, which has been shown with a cryotolerant *S. cerevisiae* strain that inhibits MLF (Caridi and Corte, 1997; Son *et al*., 2009). This organic acid has been described as a possible competitive inhibitor of MLF due to its similarity with L-malic acid (Lonvaud-Funel *et al*., 1988; Caridi and Corte, 1997).

In addition, non-*Saccharomyces* yeasts can also significantly produce succinic acid. For example, Ciani and Maccarelli (1998) found that strains of *Torulaspora delbrueckii* produced it within the range of 0.3 to 0.8 g/L. Moreover, in sequential fermentation of *S. cerevisiae–T. delbrueckii,* succinic acid was produced up to 0.95 g/L (Zhu *et al*., 2020). Moreover, Contreras *et al*. (2014) found productions of 1 to 2 g/L of this acid by strains of *Metschnikowia pulcherrima*, *Schizosaccharomyces malidevorans* and *Candida stellata*.

Besides the above-mentioned studies of MLF inhibition by succinic-producing yeasts, the influence of succinic acid *per se* upon MLF and on *O. oeni* has been studied very little (Lonvaud-Funel and Strasser de Saad, 1982). Interestingly, it seems that succinic acid concentration can decrease during MLF (Yilmaz and Gökmen, 2021) and especially in induced simultaneous MLF with AF (Taniasuri *et al.*, 2016). Consequently, its real influence on MLF remains unclear. Due to the lack of information and to clarify the impact of this compound in winemaking conditions, the main aim of this work was to evaluate for the first time the effect of different succinic acid concentrations on *O. oeni* strains in MLF. Keeping in mind the different dissociated or undissociated forms of succinic acid in the function of pH, assays at different pH—3.5 and 4.0—were included in the evaluation of the impact of succinic acid. To better understand the physiological response to succinic acid, the gene expressions related to stress and malolactic activity were also analysed.

MATERIALS AND METHODS

1. Strains and culture conditions

Four strains of *Oenococcus oeni* of diverse origins were initially used: VP41 (Lalvin VP41) from Lallemand Inc. (Montréal, Canada); 1Pw13 (CECT 8893) from our own collection; PSU-1 (ATCC BAA-331), a reference strain with the genome fully annotated; and CH11 (Viniflora CH11), from Chr. Hansen Holding A/S (Hoersholm, Denmark). Before the fermentation assays, cells of these strains were precultured at 28 ºC in an incubator with 10 % (v/v) CO_2 in MRS broth supplemented with D, L-malic acid (4 g/L) and

fructose (5 g/L) at pH 5.0 for 72 h at least two times prior to experimental use.

2. Influence of succinic acid concentration on the growth of *O. oeni* strains

In order to see the influence on *O. oeni* growth, the above-mentioned precultures in supplemented MRS broth were also assayed with three different succinic acid concentrations (0.5, 1 and 2 g/L), and bacterial populations were quantified indirectly by measuring the Abs at 600 nm in a spectrophotometer Polarstar Omega (Biogen, Madrid, Spain).

3. Malolactic fermentation assays with the addition of succinic acid

MLF were carried out in wine-like media (WLM) (Bordas *et al.*, 2013) containing 2 g/L of L-malic acid, 12 % (*v/v*) ethanol, and three different succinic acid concentrations (0.5, 1 and 2 g/L), as well as the control without succinic acid, at pH 3.5 and 4.0, adjusted with NaOH1N. All fermentations were performed in triplicate in 250 mL bottles at 20 ºC, inoculated with *O. oeni* strains at 2×10^7 CFU/mL. The samples were taken every 24 h to evaluate the L-malic acid consumption using an enzymatic kit (BioSystems, Barcelona, Spain) and the cells were harvested by centrifugation (6000 × *g*, 10 min). They were then frozen in liquid nitrogen and kept at –80 ºC until the RNA extraction.

4. MLF assays with different proportions of L-malic and succinic acids

To evaluate the influence of the ratio of the two acids on MLF, experiments were carried out in WLM with different concentrations of L-malic acid (0.5, 1, 2 and 3 g/L) and succinic acid (0.5, 1 and 2 g/L). All assays were carried out at pH 3.5 and 4.0, at 20 ºC. Control assays without succinic acid were included for each L-malic acid concentration. All experiments were performed in 50 mL Falcon tubes inoculated with 2×10^7 CFU/mL. *O. oeni* and samples were taken every 24 h to evaluate L-malic consumption using an enzymatic kit (BioSystems).

5. Experiments with resting cells

O. oeni cells were grown in 250 mL of MRS broth at 28 ºC to the early stationary phase. They were then harvested by centrifugation at 6000 × *g* for 5 min at room temperature, following Mira de Orduña *et al.* (2000). Cells were resuspended in resting-cell buffer, which contained per litre of deionised water 7.5 g tartaric acid and 1 mL of a mineral solution with 200 g/L $MgSO_4 \cdot 7H_2O$ and 50 g/L $MnSO_2 \cdot 4H_2O$). Then, resting cells were transferred to 50 mL Falcon tubes containing 2 g/L of L-malic acid (control assay) and 12 % (*v/v*) of ethanol. The other conditions had the same molar amount of 2 g/L of L-malic acid (14.9 mM) and different proportional molar succinic acid concentrations (3.7 mM, 7.4 mM, 14.9 mM and 29.8 mM) at pH 3.5 and 4.2. This last pH was chosen because it is the pK_{a1} for succinic acid. The Falcon tubes were placed in a water bath at 25 ºC and stirred gently. Samples were taken periodically and centrifuged at 6000 *g* for 5 min and the supernatants were kept at 4 ºC until the L-malic was analysed with an enzymatic kit (BioSystems).

6. Analysis of gene expression

The *O. oeni* RNA extractions were performed as described by Chomczynski and Sacchi (2006) and then purified with a Roche RNeasy kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

cDNA was synthesised from RNA (10 ng/µL) using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) as recommended. To analyse the expression of four genes related to stress and malolactic activity, four pairs of primers (Table 1) were taken from previous works (Beltramo *et al.*, 2006; Desroche *et al.*, 2005; Olguín *et al.*, 2010). They were about 18-22 bp long, contained 50 % G/C and had a melting temperature (T_M) above 60 ºC. The *O. oeni gyrA* and *gyrB* genes were used as housekeeping genes (internal control), using the primers described by Desroche *et al.* (2005).

Real-time qPCR was performed following Olguín *et al.* (2010) using a QuantStudio 5 real-time PCR instrument (Thermo Fisher). The results were analysed using the comparative critical threshold (ΔΔCT) method in which the amount of target RNA was adjusted to a reference (internal target RNA) described by Livak and Schmittgen (2001). The relative expression value (RE) was calculated using the Ct values of *gyrA* and *gyrB* and the result is the mean of the two results. The analysis was made from biologically duplicated independent assays and for each sample technical triplicates were analysed by qPCR.

TABLE 1. Gene description and primer sequences used in this work.

Target gene	Description	Forward primer (5´→ 3´)	Reverse primer (5´→ 3´)	Amplicon length (bp)	Reference
gyrA	Gyrase subunit A	CGCCCGACAAACCGCATAAA	CAAGGACTCATAGATTGCCGAA	95	(Desroche *et al.*, 2005)
gyrB	Gyrase subunit B	GAGGATGTCCGAGAAGGAATTA	GCCTGCTGGGCATCTGTATTA	107	(Desroche *et al.*, 2005)
mleP	Malate permease	GTGCTGACTTATTTGACCCGC	ATGTTCCACGACGACCAACC	141	(Augagneur *et al.*, 2007)
mleA	Malolactic enzyme	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	156	(Beltramo *et al.*, 2006)
hsp18	Heat shock protein	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102	(Beltramo *et al.*, 2006)
atpB	ATPase F_1 F_0 β-subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	93	(Beltramo *et al.*, 2006)

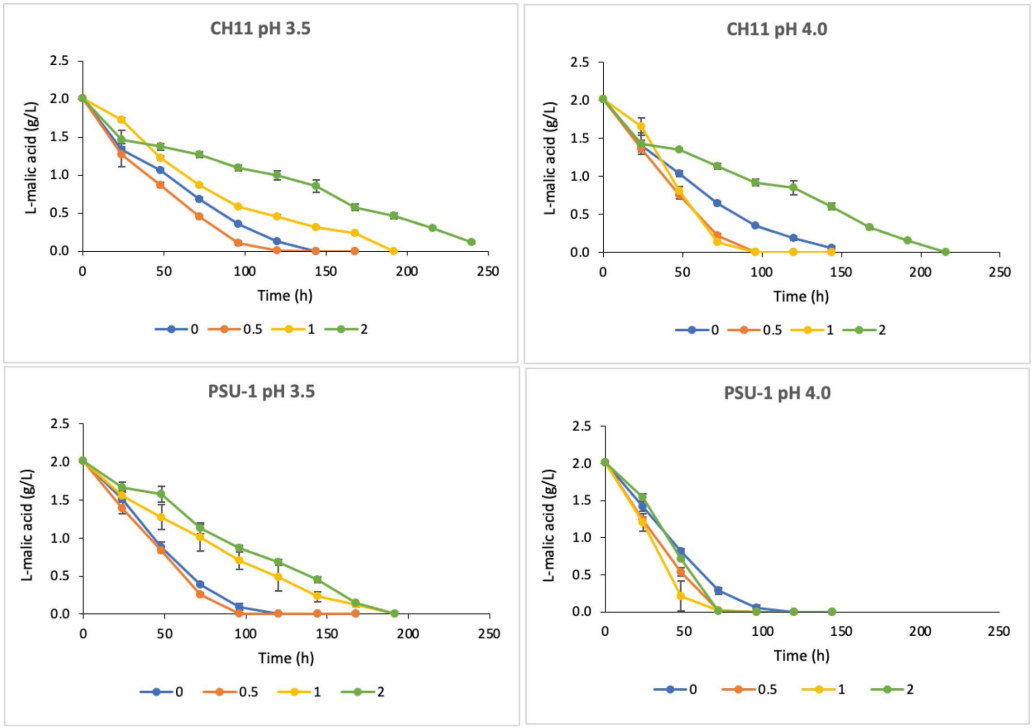

FIGURE 1. L-malic consumption in WLM with 12 % ethanol (v/v) at pH 3.5 and 4.0 by *O. oeni* strains CH11 and PSU-1, in the presence of different concentrations of succinic acid: 0 g/L (control, blue), 0.5 g/L (red), 1 g/L (yellow), and 2 g/L (green). The values are the means of triplicates and the error bars represent the SD values.

7. Chemical analyses

The organic acid contents (acetic, citric, succinic, L-malic and L-lactic acids) in the final samples of MLF trials were determined by high-performance liquid chromatography (HPLC) following Zhu *et al.* (2020). All samples were filtered previously by injecting them through 0.2 µm Captiva filters (Agilent Technologies). The chromatograms were analysed using the Agilent ChemStation Plus software.

8. Statistical analyses

The data obtained were submitted to one-way ANOVA using the Tukey test, with a confidence intewrval of 95 %, obtaining significant results with a p-value of ≤ 0.05, using the XLSTAT 2021 software (Addinsoft, Paris, France).

RESULTS AND DISCUSSION

1. Effect of succinic acid on cells growth

To see the effect of succinic acid concentration on the growth of *O. oeni* strains, previous assays were carried out in the same MRS broth used in precultures of fermentation assays. There was a clear lower growth when succinic acid was present for both strains assayed (Supplementary Figure 1), and this effect was progressively significantly higher with increasing concentration from 0.5 g/L to 2 g/L of succinic acid. As seen, with 1 g/L of succinic acid, the maximum population reached was about 50 % (strain PSU-1) or 70 % (CH11) of the control, and with 2 g/L it was about 30 % for PSU-1 and a mere 20 % of the control for strain CH11.

2. Effect of succinic acid on malolactic fermentation

After the first experiments for comparing four different *O.oeni* strains, PSU-1 and CH11 were selected and used exclusively throughout the entire study since they were representative of the two behaviours observed in response to adding succinic acid. *O. oeni* CH11 showed a stronger inhibition of MLF in the presence of 2 g/L of succinic acid than strain PSU-1. This agrees with the above commented stronger growth inhibition of CH11 at 2 g/L of succinic acid added to the MRS medium. The other *O. oeni* strains VP41 and 1Pw13 showed similar behaviour to CH11 but with longer and slower MLF in all assays (Supplementary Figure 2). Control fermentations in WLM with strain PSU-1, at both pH 3.5 and 4.0, were completed in 120 h, and those with strain CH11 finished in 144 h (Figure 1). The bacterial population in the samples of these WLM fermentations was followed, but no increase was detected in any of them nor in controls (data not shown). It must be taken into account that growth is usually very difficult in the harsh conditions of WLM, similar to wine, with ethanol and low pH, as observed previously (Bordas *et al.,* 2015; Jiang *et al.,* 2018). Nonetheless, the inoculated population can survive and finish the MLF.

The results obtained in the presence of succinic acid varied depending on the pH and strains. Succinic acid inhibited the MLF of the two *O. oeni* strains at concentrations of 1 and 2 g/L at pH 3.5, whereas at pH 4 only the MLF with 2 g/L of succinic acid with *O. oeni* CH11 was inhibited in comparison to the control assay (0 g/L succinic acid). At pH 3.5 with 2 g/L

of succinic acid, 43 % and 34 % of the L-malic acid was not consumed by CH11 and PSU-1, respectively, at the time that the control had exhausted all the L-malic acid. This shows that *O. oeni* CH11 was more sensitive to this concentration of succinic acid than PSU-1. The results with 1 and 2 g/L of succinic acid showed that the pH has a relevant influence on the MLF and the inhibitory effect of this organic acid. Meanwhile, at pH 3.5, succinic acid reduced the L-malic acid consumption rate in CH11 and PSU-1; however, at pH 4.0, both strains were able not only to carry out MLF but also to do so in less time than the control assay. Therefore, pH is an important factor because this physicochemical condition affects the dissociated and undissociated forms of succinic and malic acids, as observed by Augagneur *et al*. (2007).

Nevertheless, both strains CH11 and PSU-1 showed similar results in the 0.5 g/L succinic acid and in all cases, MLF finished about 24 h earlier than in the control assay. This best MLF performance in the presence of 0.5 g/L than without succinic acid was also found for the other two strains, 1Pw13 and VP41 (Supplementary Figure 1). Therefore, we have found that this low concentration of succinic acid could be slightly beneficial for *O. oeni* and the MLF, while levels of 1 g/L succinic acid or higher are clearly inhibitory. This probable benefit could be related to the above-commented decrease in succinic acid in some MLFs (Taniasuri *et al*., 2016; Yilmaz and Gökmen, 2021); however, we did not find significant variation in the succinic acid concentration in any case (data not shown).

Anyway, it is clear that MLF is inhibited by a concentration of 1 g/L or higher of succinic acid for both strains and pHs. This inhibition is probably related to intracellular acidification by this acid, which would lead to a decrease in cell viability, affecting, in consequence, the MLF. Although, as commented above, we had not detected variation—neither decrease nor decrease—in biomass in these assays with WLM, the inhibition effect on cell growth was clear in the previous assays with MRS medium.

Regarding the important role that pH played in the performance of the MLF with the two strains, the results obtained with 1 and 2 g/L of succinic acid could be related to the dissociated and undissociated forms of succinic and L-malic acids. At pH 3.5, L-malic acid would be present at almost 50 % in dissociated monoanionic form (L-malic acid pK_{a1} = 3.4) (Tourdot-Maréchal *et al*., 1993), whereas a large fraction of succinic acid (more than 70 %) is in its undissociated form (succinic acid pK_{a1} = 4.2) (Jansen and van Gulik, 2014). However, at pH 4.0, only around 50 % of succinic acid would be undissociated (Jansen and van Gulik, 2014). At pH values above 4.5, the passive diffusion of L-malic acid into *O. oeni* cells would be negligible (Tourdot-Maréchal *et al*. (1993). However, at pH 3.2, the permeability of the cells to the undissociated acid by simple diffusion could represent more than 50 % of the total L-malic acid uptake. Considering that succinic acid and L-malic acid have a very similar chemical structure, it would be feasible that undissociated succinic acid could enter the *O. oeni* cell at pH 3.5, producing intracellular acidification due to the complete deprotonation of succinic acid at the cytosolic pH (*O. oeni* intracellular pH = 5.8–6.1; succinic acid pk_{a1} = 4.2 pk_{a2} = 5.6). Internal acidification has been associated with the inhibition of MLF by other acidic compounds at pH values close to 3, such as octanoic acid and some phenolic acids (Capucho and San Romao, 1994; Campos *et al*., 2003). The cytosolic acidification is known to inhibit the enzymatic activities of *O. oeni* in general and it affects the viability of the cells, as commented. However, moreover, the malolactic activity could be specifically affected due to the changes in the L-malate anionic state inside the cell. According to Acevedo *et al.* (2020), the optimal substrate for the malolactic enzyme in *O. oeni* is dianionic L-malate, which is the form present at the standard intracellular *O. oeni* pH (5.8–6.1). The internal acidification produced by succinic acid could convert dianionic L-malate into the monoanionic form, negatively affecting the malolactic enzyme efficiency. Altogether, the internal acidification of *O. oeni*, favoured at a low pH, could explain the stronger inhibition of succinic acid at pH 3.5 than at pH 4 observed in this work.

In regard to the composition of organic acids after MLF, there were no differences in L-lactic acid production (data not shown) because it was in accordance with the L-malic acid consumption. In addition, the amounts of acetic acid in each treatment were in accordance with the citric acid consumption and no statistical differences were found (data not shown). Neither one was above the acetic acid threshold (0.7 g/L) (Drysdale and Fleet, 1989).

3. Influence of succinic acid on MLF at different ratios of L-malic and succinic acid

As seen above, the increasing amounts of succinic acid exerted an inhibition effect on the L-malic acid consumption. To understand this effect in more depth, we carried out assays in the same WLM with different ratios of L-malic and succinic acids. In addition, we aimed to determine whether there was an inhibition threshold of succinic acid depending on the initial L-malic acid concentration. At the same time, these assays simulated the real situation of the diversity of initial L-malic acid levels (from 0.5 to 3 g/L in these assays) in different climatic zones of winemaking.

To determine this influence, L-malic acid consumption rates were calculated for the different assays, shown in Supplementary Table 1. As seen, the main significant inhibition of succinic acid was when L-malic acid was 0.5 or 1 g/L. To visualise these results better, they are summarised in Figure 2.

There is a significantly bigger inhibition of the consumption rate for most conditions when 2 g/L of succinic acid is present, in agreement with the inhibition of MLF kinetics we found and discussed above. In terms of the pH, a slightly greater L-malic acid consumption can be observed at pH 4.0 than at 3.5, as expected since the higher pH is less stressful for *O. oeni*. The consumption tendencies are similar for both the strains and the succinic concentrations for the two pHs (Figure 2).

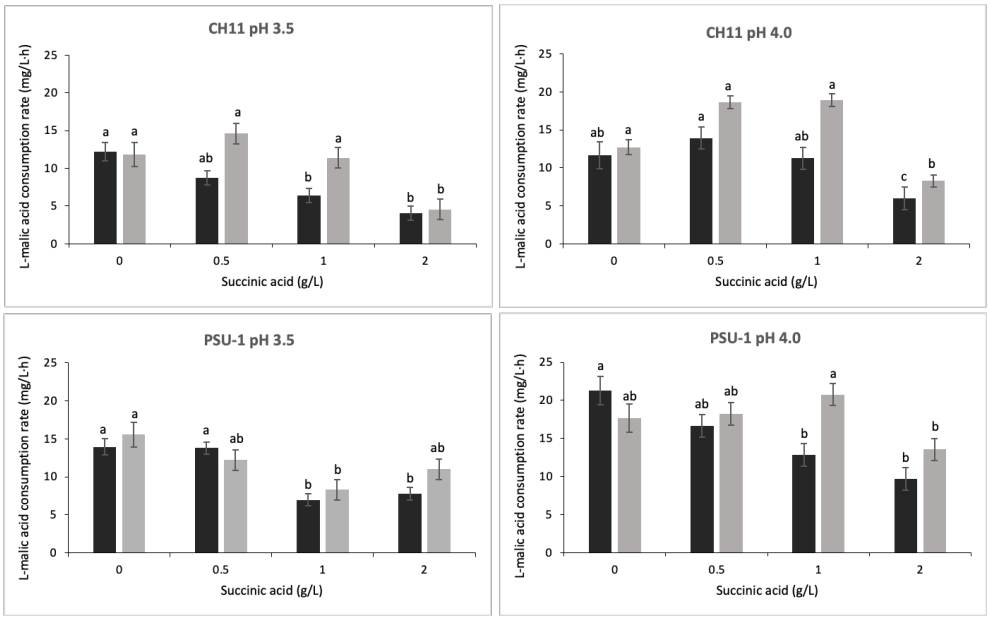

FIGURE 2. L-malic acid consumption rate (mg/L·h) by *O. oeni* strains CH11 and PSU-1 in WLM with 12 % ethanol (v/v) at pH 3.5 and 4.0 with L-malic acid (0.5 g/L [black], and 1 g/L [grey]), at different concentrations of succinic acid. $^{a–c}$ Values of columns for each strain and pH are significantly different at $p \leq 0.05$ according to the Tukey test.

The most relevant result here is that there is generally more inhibition of the consumption rate at 0.5 g/L of L-malic acid than at 1 g/L, which is clearer for strain CH11 (Figure 2). It seems that succinic acid has an inhibiting effect on the consumption rate of L-malic acid when this is in a lower ratio with respect to succinic acid. That is, succinic acid has a clear inhibition effect on MLF when the concentration of this acid is higher than that of L-malic acid. In addition to the inhibition of MLF due to the already mentioned intracellular acidification caused by succinic acid, there could be competition between the succinic and L-malic acids for the malolactic enzyme due to their similar chemical structures, as suggested by Lonvaud-Funel and Strasser de Saad (1982).

It is particularly important to take this effect into account nowadays, when it is usual to have a low L-malic acid content in wine, mainly due to climate change that leads to less acidic grapes (Mira de Orduña, 2010).

4. The effect of succinic acid on the consumption of L-malic acid by resting cells

Unlike the WLM, where *O. oeni* can grow, the resting cell buffer provides a way to monitor the malolactic enzymatic activity without interference from nutrients and metabolic changes related to growth. Therefore, in resting cell assays, there is no adaptation phase of growing cells and the effect of succinic is more direct. Cells number was about $5 \cdot 10^9$/mL as they were harvested from 250 mL at the early stationary phase (10^9/mL) and resuspended to a final volume of 50 mL, as said in Methods.

The consumption of 2 g/L (14.9 mM) of L-malic acid by resting cells was very quick (around 80–120 min) for both strains and pH levels and at the different succinic acid concentrations (data not shown). Hence, the high number of resting cells present can consume L-malic rapidly. These short times are similar to those found in similar works (Mira de Orduña *et al.*, 2000).

To compare the L-malic acid consumption rate under the different conditions, we considered the amount of L-malic acid for assays with different succinic acid concentrations at the time when control assays had consumed about half of the L-malic acid (Table 2). This middle point of MLF (i.e., about 1 g/L (7.5 mM)) was at about 20 min for the control without succinic acid. Similarly, the data shown are for other sample conditions also taken at about 20 min. The different values of L-malic acid shown for the control at mid-MLF (from 2.08 to 6.82 mM) can be explained because samples were taken every 20 min and the L-malic consumption was very quick. Therefore, these are approximative values for mid-MLF.

We can observe (Table 2) that for most conditions, when succinic is present, the remaining L-malic acid is significantly higher than the control. Hence, succinic acid slows down the consumption of L-malic acid. Nevertheless, when succinic acid is 3.7 mM (0.5 g/L), the quantity of L-malic acid is significantly lower than the control at mid-MLF, indicating a quicker consumption rate at the lowest concentration of succinic acid. This tendency was not shown for PSU-1 at pH 4.2.

Overall, these results agree with those obtained previously in WLM (Figure 2), where the succinic acid inhibition of MLF was higher for concentrations higher than 1 g/L, that is, more than 7.4 mM of succinic acid.

5. Relative expression of genes related to stress and malolactic activity

To evaluate the possible inhibition of succinic acid in relation to the transcription of relevant genes associated with

TABLE 2. L-malic acid amount (mM) for different succinic acid concentrations of the assays with resting cells, when control assay (0 mM succinic acid) has consumed about half of L-malic, *id est*, at middle MLF.

pH	Succinic acid (mM)	CH11	PSU-1
3.5	0	2.08 ± 0.226 ^{bc}	6.82 ± 0.197 ^{d}
	3.7	0.29 ± 0.185 ^{d}	4.59 ± 0.161 ^{e}
	7.4	1.54 ± 0.185 ^{c}	8.05 ± 0.161 ^{c}
	14.9	2.71 ± 0.185 ^{b}	9.00 ± 0.161 ^{b}
	29.8	4.55 ± 0.185 ^{a}	10.74 ± 0.161 ^{a}
4.2	0	4.28 ± 0.583 ^{cd}	3.73 ± 0.248 ^{d}
	3.7	1.98 ± 0.476 ^{d}	5.24 ± 0.203 ^{c}
	7.4	5.54 ± 0.476 ^{bc}	8.67± 0.203 ^{ab}
	14.9	7.01 ± 0.476 ^{ab}	8.97 ± 0.203 ^{a}
	29.8	8.57 ± 0.476 ^{a}	9.30 ± 0.203 ^{b}

$^{a–e}$ Values for different succinic acid concentrations for the same pH and strain are significantly different at $p \leq 0.05$, according to the Tukey test.

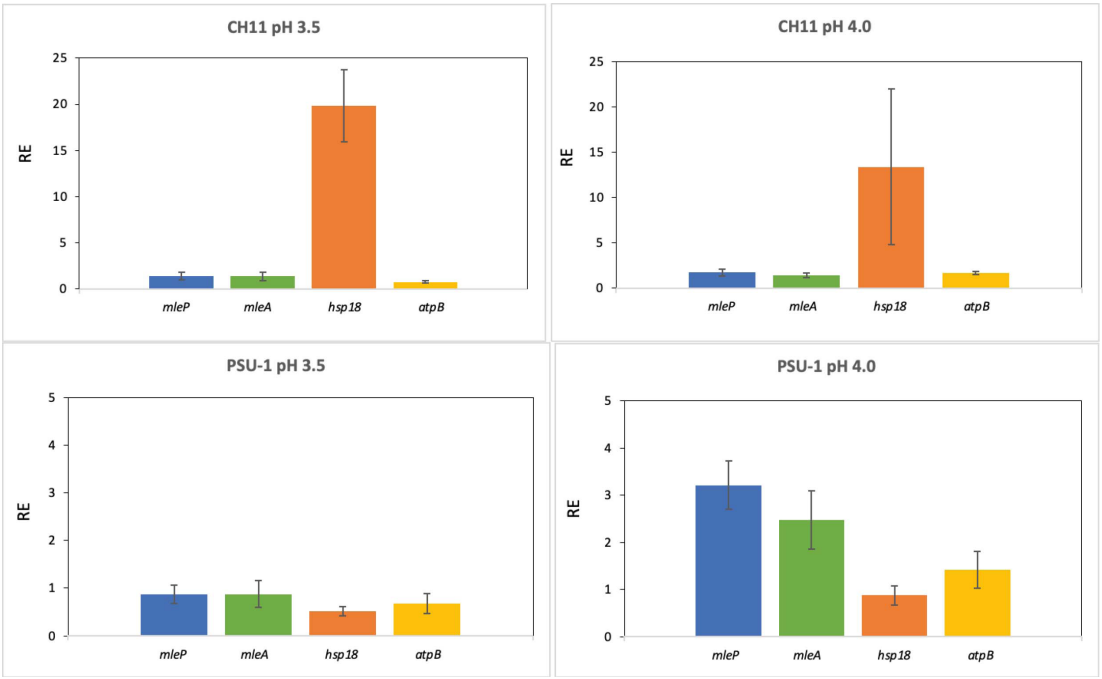

FIGURE 3. Relative expression (RE) of four genes related to stress (*hsp18* and *atpB*) and malolactic activity (*mleP* and *mleA*) of strains CH11 and PSU-1 of *O. oeni* at middle MLF (approx. 1 g/L L-malic acid) in the presence of 2 g/L of succinic acid. The calibrator condition (RE = 1) was the absence of succinic acid. The data shown are mean values between both RE obtained with gene controls *gyrA* and *gyrB* with error bars representing SD values (n = 3).

MLF development, we measured the relative expression of the following genes: i) *mleA*, encoding the malolactic enzyme, and *mleP*, encoding the L-malic acid transporter; ii) *atpB*, encoding the β-subunit of the F_1F_0 ATPase, associated with MLF activity and acid stress response (Cox and Henick-Kling, 1989; Fortier *et al*., 2003) and iii) *hsp18*, encoding for one of the most relevant stress proteins of *O. oeni* with a chaperone function, this gene is known to be activated in response to multiple stresses and has been proposed to be a stress adaptation indicator (Coucheney *et al*., 2005; Beltramo *et al*., 2006). The analysis was carried out with samples from the assay performed in WLM with the most inhibitory concentration of succinic acid (2 g/L). All samples were taken when the L-malic acid concentration was approximately 1 g/L in each case, at mid-MLF. Samples from cultures without added succinic acid were also analysed as control conditions.

In *O. oeni* CH11, the strain that showed the highest sensitivity to succinic acid, the gene *hsp18* was clearly up-regulated at pH 3.5 and 4.0 (Figure 3). In contrast, the PSU-1 strain did

not show overexpression of the *hsp18* gene in any of the assayed conditions. These data suggest that the CH11 strain adopted this strategy to outcome the succinic acid inhibitory effect. However, *O. oeni* PSU-1, the strain least affected by succinic acid, might respond to stress differently, as seen in previous studies (Olguín *et al*. 2010).

No changes in expression were observed for *atpB* in any of the conditions studied. The transient activation of this gene has been described in response to acid shock (Fortier *et al*., 2003). In this study, there was no difference in the transcriptional levels of *atpB* at mid-MLF with or without succinic acid.

Surprisingly, *O. oeni* PSU-1 showed a 3.5 and 2.5-fold up-regulation of the genes *mleP* and *mleA* at pH 4 (Figure 3). This could be related to the faster MLF observed under these conditions (2 g/L succinic acid) with respect to the control condition at the same pH. However, the reason for the enhanced MLF due to the presence of succinic acid remains unclear and needs further research.

The main conclusion of the transcriptional study is that succinic acid does not inhibit the transcription of the *mleA* and *mleP* genes in any of the conditions. Therefore, the inhibition of MLF caused by this compound might be due to the intracellular acidification and possible substrate competition with L-malic acid at the enzymatic level, as previously discussed.

CONCLUSION

In this work, the effect of succinic acid on *O. oeni* and MLF has been evaluated for the first time. It shows that succinic acid can inhibit MLF at concentrations higher than 1 g/L, but it could be beneficial at a lower concentration, near 0.5 g/L. The inhibition is clear when L-malic acid is at a lower ratio with respect to succinic acid. As succinic acid is produced by yeasts, including non-*Saccharomyces* species, this variable effect is an example of an interaction between yeasts and LAB that can be negative or positive depending on the succinic concentration but also on the strains and other winemaking conditions. Therefore, one of the most important factors is the pH because this physicochemical condition influences the dissociated and undissociated forms of both acids. Consequently, the strain-dependent effect can lead to inhibition or promotion of MLF due to the cell response and adaptation. Further research is necessary to understand the molecular mechanisms of the influence of succinic acid on *O. oeni* and possibly the competence of succinic acid against L-malic acid at the enzymatic and transport levels.

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