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EFFECT OF HYDROXYCINNAMIC ACIDS AND SUCCINIC ACID ON MALOLACTIC FERMENTATION OF WHITE AND RED WINES

Božena Průšová, Karolína Kostelníková, Josef Licek, Jiří Sochor, Mojmír Baroň*

Address(es):
Mendel University in Brno, Faculty of Horticulture, Department of Viticulture and Oenology, Valtická 337, 69144 Lednice, Czech Republic.

*Corresponding author: prusova.bozena@email.cz

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ABSTRACT

This study evaluates the potential of selected organic acids (ferulic, caffeic, coumaric, and succinic) to inhibit malolactic fermentation in white and red wines. Antimicrobial activity against *Oenococcus oeni*, *Lactobacillus plantarum*, and *Lactobacillus brevis* was assessed using growth curves and disk diffusion methods, followed by validation in real wine microsamples. While in vitro growth curves established a baseline inhibitory concentration of 5 $g \cdot L^{-1}$, testing in real wine matrices revealed that synergistic factors (lower pH and ethanol content) significantly reduce the required effective dose. The tested bacteria exhibited distinct sensitivity profiles: *O. oeni* was most effectively suppressed by ferulic and caffeic acids, whereas *L. brevis* was highly susceptible to caffeic and coumaric acids. Conversely, succinic acid provided the strongest inhibition against *L. plantarum*. These species-specific trends were generally confirmed by the disk diffusion method. Furthermore, in white wine microsamples, suppression was successfully demonstrated across all tested concentrations. In contrast, red wine trials exhibited a more significant dose-dependent response, where the inhibition of malolactic activity strictly increased with higher inhibitor concentrations.

Keywords: hydroxycinnamic acids, succinic acid, malolactic fermentation, white wine, red wine, lactic acid bacteria

INTRODUCTION

Malolactic fermentation (MLF), the process of biological de-acidification in winemaking, is based on the L-malic acid decarboxylation to L-lactic acid and CO_2. L-lactic acid is monocarboxylic nature and imparts a more elegant and round taste to wine **(Matthews *et al.*, 2004; Moreno-Arribas and Polo, 2005)**. MLF can occur during or after alcoholic fermentation as a result of the metabolic activity of lactic acid bacteria (LAB), which are present in wine at all stages of winemaking. Four genera were identified as the principal organisms involved in MLF: *Lactobacillus, Leuconostoc, Oenococcus*, and *Pediococcus* **(du Toit *et al.*, 2011)**. *Oenococcus oeni* is the species best adapted to growing in the difficult conditions imposed during winemaking (low pH and high ethanol concentration) **(Davis *et al.*, 1985; Vanvuuren and Dicks, 1993; Lonvaud-Funel, 1999)** and, therefore, the main species of malolactic fermentation (MLF) in wine.

The main influence of other LAB species such as *Lactobacillus hilgardii* and *Pediococcus pentosaceus*, on wine quality is to cause alterations to the wine, including the so-called "lactic disease", and the production of off-flavor compounds **(Chatonnet *et al.*, 1995; Costello and Henschke, 2002)**, and biogenic amines **(Landete *et al.*, 2005; Marcobal *et al.*, 2006)**.

Wine pH has been increasing gradually for the last several years. Red wines with pH values over 3.5–3.6 are more and more frequent. At these pH levels, we can observe very fast growth of various indigenous microorganisms, some of which are spoilage bacteria that can cause loss of wine quality. Among these species, *Lactobacillus plantarum* strains have shown most interesting results for their capacity to induce MLF under high pH conditions, their facultative hetero-fermentative properties that avoid acetic acid production from hexose sugars and their more complex enzymatic profile and different metabolism compared to *O. oeni*, which could play an important role in the modification of wine aromas **(Fugelsang and Edwards, 2007)**.

L.brevis **(Teixeira, 2014)** can cause spoilage of various food products. It is commonly isolated from grapes and wines. It is possible that some strains or species could contribute desirable characteristics to wine quality, although excessive growth could be undesirable. When present in trace amounts, diacetyl enhances wine flavour. However, excessive production of diacetyl from citric acid causes spoilage. *L. brevis* produces mannitol from glucose, which may result in mannite spoilage in wine.

Sulphur dioxide (SO_2) is an additive most frequently employed to control LAB growth and MLF development during winemaking, because of its antioxidant and selective antimicrobial properties, especially against LAB **(Ough and Crowell, 1987)**. The sulfur dioxide concentration required to inhibit LAB is ~30 $mg \cdot L^{-1}$ of free SO_2 for acidic wines (pH between 3.2 and 3.6), or up to 100 $mg \cdot L^{-1}$ for wines with higher pH. However, nowadays there is a growing tendency to reduce the use of SO_2 in wine processing, since high doses can cause organoleptic alterations in the final product, and especially because of the risks to human health of consuming this substance **(Taylor *et al.*, 1986; Fleet, 2002; Ribéreau-Gayon *et al.*, 2006)**. Some alternatives to SO_2 have been introduced based on "natural antimicrobial agents", such as the use of lysozyme, an enzyme obtained from egg white **(Gerbaux *et al.*, 1997; Bartowsky, 2009)**.

Potential alternatives to the use of SO_2 are the phenolic compounds (PCs), which are natural constituents of grapes and wines.

The phenolic composition **(Campos *et al.*, 2003)** of wines is very complex and includes phenolic (hydroxycinnamic and hydroxybenzoic) acids in concentrations ranging from 100 to 200 $mg \cdot L^{-1}$, depending on the grape variety and vinification process **(Reguant *et al.*, 2000)**. In wine, phenolic acids appear mainly in a combined form: hydroxycinnamic acids form esters with tartaric acid (cinnamoyl–tartaric acids) and hydroxybenzoic acids polymerize with other molecules to produce tannins **(Macheix *et al.*, 1990)**. During alcoholic fermentation, free hydroxycinnamic acids are released by hydrolysis of tartaric acid esters, but their proportion is relatively low. Despite the structural similarity between acids from the hydroxycinnamic and hydroxybenzoic families, their effect on growth and survival of lactic acid bacteria can be very different. A previous study showed that some free hydroxybenzoic acids can activate cell growth and reduce the malolactic fermentation rate, whereas others are slightly inhibitory **(Vivas *et al.*, 1997)**. Other authors report that some free hydroxycinnamic acids inhibit growth of a variety of microorganisms, including wine-spoilage strains of *L. collinoides* and *L. brevis* **(Stead, 1993)**, *Escherichia coli, Staphylococcus aureus, Bacillus cereus* **(Herald and Davidson, 1983)** and several yeasts **(Baranowski *et al.*, 1980; Stead, 1993; Campos *et al.*, 2009; Rodríguez *et al.*, 2009; García-Ruiz *et al.*, 2013; Pastorkova *et al.*, 2013; Stivala *et al.*, 2014; Stivala *et al.*, 2015)**.

Sánchez-Maldonado *et al.* (2011) studied the structure-function relationships of the anti-bacterial activity of phenolic acids on LAB and demonstrated that the presence of the double bond of the hydroxycinnamic acids plays an important role in their antibacterial activity. In addition, the PC concentration required for antibacterial effects against LAB are lower in a medium like wine **(Stead, 1993)**. To date, scientific studies have focused on assessment of the inhibitory activity of PCs in optimal bacterial growth.

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 **(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 **(Torres-Guardado *et al.*, 2022)** 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 g-amino butyric acid (GABA) bypass, glyoxylic acid bypass and the methyl citric 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 **(Torres-Guardado *et al.*, 2024)**. The organoleptic threshold of succinic acid in wine is 35 $mg{\cdot}L^{-1}$. It has an unusual bitter-salty taste and excess levels can have a negative impact on the mouthfeel of the wines **(Coulter, 2004)**; thus, it may be beneficial to reduce it.
S. cerevisiae strains are known to produce succinic acid, with values from 200 $mg.L^{-1}$ to more than 1 $g.L^{-1}$ **(Heerde and Radler, 1978; Coulter, 2004; Zhu *et al.*, 2020; Torres-Guardado *et al.*, 2024)**, 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 **(Lonvaudfunel *et al.*, 1988; Caridi and Corte, 1997)**. 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 $g{\cdot}L^{-1}$. Moreover, in sequential fermentation of *S. cerevisiae-T. delbrueckii*, succinic acid was produced up to 0.95 $g{\cdot}L^{-1}$ **(Zhu *et al.*, 2020)**. Moreover, **Contreras *et al.* (2014)** found productions of 1 to 2 $g{\cdot}L^{-1}$ of this acid by strains of *Metschnikowia pulcherrima*, *Schizosaccharomyces malidevorans* and *Candida stellata*.

MATERIAL AND METHODS

Biological material

Grapes and wines

Souvignier Gris white wine was used for this experiment (initial must parameters: 210 $g{\cdot}L^{-1}$ of fermentable sugars, pH 3.1, titratable acids 9.2 $g{\cdot}L^{-1}$, YAN 152 $mg{\cdot}L^{-1}$), and red wine of the Pinot Noir variety (initial must parameters: 220 $g{\cdot}L^{-1}$ of fermentable sugars pH 3.4, titratable acids 8.8 $g{\cdot}L^{-1}$; YAN 150 $mg{\cdot}L^{-1}$). The grapes were de-stemmed, crushed, maceration took place in the case of the red wine variant. After alcoholic fermentation, which was supported by active dry yeast (R9002) and FermControl nutrition (FermControl BIO, Germany), the wine was bottled in a 30 L container for the purpose of the experiment. For the inhibition experiment, the wine was heat sterilized using an autoclave. Then, according to the measured values, the content of malic acid was adjusted to a value of 1.5 $g{\cdot}L^{-1}$.

Microorganisms

The bacterial species are referred to using the traditional nomenclature commonly used in oenological literature (Lactobacillus plantarum and Lactobacillus brevis); according to the current taxonomy, these species are classified as *Lactiplantibacillus plantarum* and *Levilactobacillus brevis, respectively*.
Oenococcus oeni – CCM 2834. The strain CCM 2834, a synonym of ML 34, originates from red wine.
Lactobacillus brevis – CCM 1815. The strain CCM 1815 was isolated from fermenting green olives of the Sevillano variety. Another designation for this strain is ATCC 8287.
Lactobacillus plantarum – CCM 7039. The synonym of this strain is ATCC 14917 and it is isolated from sauerkraut.

Inhibitors

Four hydroxycinnamic acids in crystalline solid form were used as inhibitors for this research. For each experiment, acids were diluted to specific concentrations or weighed and dissolved in wine samples. Ferulic acid (Sigma Aldrich, Germany), Succinic acid (Carl Roth, Germany), Caffeic acid (Apollo Scientific, UK), Coumaric acid (Carl Roth, Germany).

Culture media

Mueller Hinton agar - MHA (HiMedia Laboratories, USA); MRS (Sigma Aldrich, Germany); Nutrient Broth No. 1 (Sigma Aldrich, Germany).

Microbiological analysis

Cultivation of bacteria

To prepare a culture of *Oenococcus* and *Lactobacillus* bacteria, bacteria in the form of gelatine discs were used, which were transferred to Erlenmayer flasks with a sterile loop at the same time as the prepared broth and cultivated for 24 hours in an Orbital Shaker-Incubator ES-20. Indications of an increase were easy to spot. The previously clear broth was heavily turbid after culture, indicating a massive increase in the microbiological population.

Disc diffusion method

The disk diffusion method is one of the oldest methods of testing the sensitivity of bacteria to antibiotics. Nevertheless, it still remains one of the most widely used methods in routine clinical laboratories. It is suitable for testing most bacterial pathogens, including pathogens that are more difficult to cultivate. It allows testing a wide range of antimicrobial drugs and does not require any special equipment **(Matuschek *et al.*, 2014)**.
400 µL of the prepared bacterial suspension was applied to each Petri dish with solidified MH agar in the laminar box using an automatic pipette and spread evenly over the entire surface with a sterile loop. Four wells were then made into the medium using a sterilized corkscrew. Subsequently, 40 µl of inhibitor with concentrations of 10; 5; 2.5; 1 $g{\cdot}L^{-1}$ were dosed into each of the wells. The experiment was performed in triplicate for each acid.
The dishes were then cultured upside down for 24 hours. *Oenococcus oeni* was cultured at 25°C *and Lactobacillus brevis* and *plantarum* were cultured at 37°C. After incubation, inhibition zones were calculated.

Growth curves

The growth characteristics of the three strains of lactic bacteria were generated using a Multiskan™ FC automatic microplate photometer (ThermoFisher Scientific, USA). Individual hydroxycinnamic acids were diluted to concentrations of 10; 5; 2.5 and 1 $g{\cdot}L^{-1}$. The prepared bacterial culture was diluted using Nutrient Broth No. 1 (Sigma Aldrich, Germany). It was then pipetted in a laminar box into sterile 96-well microtitre plates.
270 µl bacterial suspension. As a blank, the suspension well was diluted with 30 µl of distilled water. In the other wells, 30 µl of tested hydroxycinnamic acids of different concentrations were added by pipette. The microplate with the prepared samples was placed in a MultiskanTM FC photometer. Absorbance was measured at 15-minute intervals for 24 hours. The wavelength of the light for the measurement was set to 595 nm. Each variant was subjected to triplicate measurements. The obtained data were processed and graphically interpreted in the Microsoft® Excel® Pro Office 365 program.

Inhibition test on a microsample of wine

For the preparation of the bacterial suspension, commercial strains of Viniflora Oenos bacteria were used, which were transferred sterilely into the previously prepared NB broth with a volume of 50 ml in Erlenmayer flasks. Subsequently, the flasks were cultured for 24 hours in an Orbital Shaker-Incubator ES-20. After 24 hours, turbidity was visible, indicating bacterial growth. 0.05 was weighed on an analytical balance; 0.025 and 0.0125 g of caffeic, ferulic, succinic and coumaric acids to give concentrations of 1; 0.5 and 0.25 $g{\cdot}L^{-1}$. The acids were mixed in sterile Erlenmeyer flasks together with 50 mL of prepared wine and 500 mg of bacterial suspension. At the same time, a control was created that did not contain any of the acids, but only a combination of wine and bacteria. In other flasks, potassium disulfite at a concentration of 50 $mg{\cdot}L^{-1}$ was added to the wine and bacteria. Everything was done in three repetitions. Each of the flasks was described and thoroughly covered with aluminium foil. During the experiment, samples were taken and the contents of malic and lactic acids were measured by HPLC. Once the malic acid concentration was zero, the malolactic fermentation was finished and 1 mL of sample from each flask was plated on a petri dish with MRS agar. Accordingly, the microbial stability could be determined.

Chemical analysis

HPLC analysis

The organic acid content was determined using the HPLC method (high-pressure liquid chromatography). The Shimadzu LC-10A instrument (Shimadzu, Japan) was used. Before the measurement itself, the wine samples were centrifuged (3000 x g; 6 min) and diluted 10x with demineralized water. This was followed by injection of the diluted sample in a volume of 20 µl. A 1.5 mM H_2SO_4 solution was used as the mobile phase, the flow rate was 0.75 $mL{\cdot}min^{-1}$ and the separation temperature was 60 °C. Carbohydrates were determined at 190 nm, organic acids at 210 nm. The determination of individual analytes was performed based on external calibration.
Instrumentation: Shimadzu LC-10A binary high-pressure system, system controller: SCL-10Avp, 2 pumps: LC-10Advp, column thermostat with manual injection valve Rheodyne: CTO-10Acvp, DAD detector: SPD-M10Avp, Software: Lcsolution, Separation conditions: column: Watrex Polymer IEX H form 10µm; 250x8 mm + 10x8 mm, separation temperature: 60 °C, sample injection volume: 20 µL, mobile phase flow rate: 0.75 $mL{\cdot}min^{-1}$, isocratic elution.

Statistical analysis

All experiments were performed in triplicate to ensure correct statistical evaluation. Data were statistically evaluated using Statistica 14 software. ANOVA analysis was performed and mean values (n=3) were divided into homogeneous groups (labelled a, b, c, d, e, f, g, h…) based on Fisher LSD test (α= 0.05).

RESULTS AND DISCUSSION

Growth curves results

Oenococcus oeni

The effects of individual acids at concentrations of 10, 5, 2.5, and 1 g·L⁻¹ on the growth of *Oenococcus oeni* are presented in Figure 1. Overall, all tested acids induced a prolonged lag phase of approximately 3 hours. After this initial period, absorbance increased markedly in most variants. The highest absorbance values were generally recorded at the lowest inhibitor concentration (0.25 g·L⁻¹), reaching a maximum value of 0.664. The strongest inhibitory activity was observed at 10 g·L⁻¹, confirming that inhibition increased with increasing inhibitor concentration. At 5 g·L⁻¹, ferulic acid exhibited the most pronounced inhibitory effect against *O. oeni*, whereas coumaric acid showed the weakest activity. Lower concentrations produced absorbance values close to those of the untreated bacterial culture, indicating only limited inhibition.

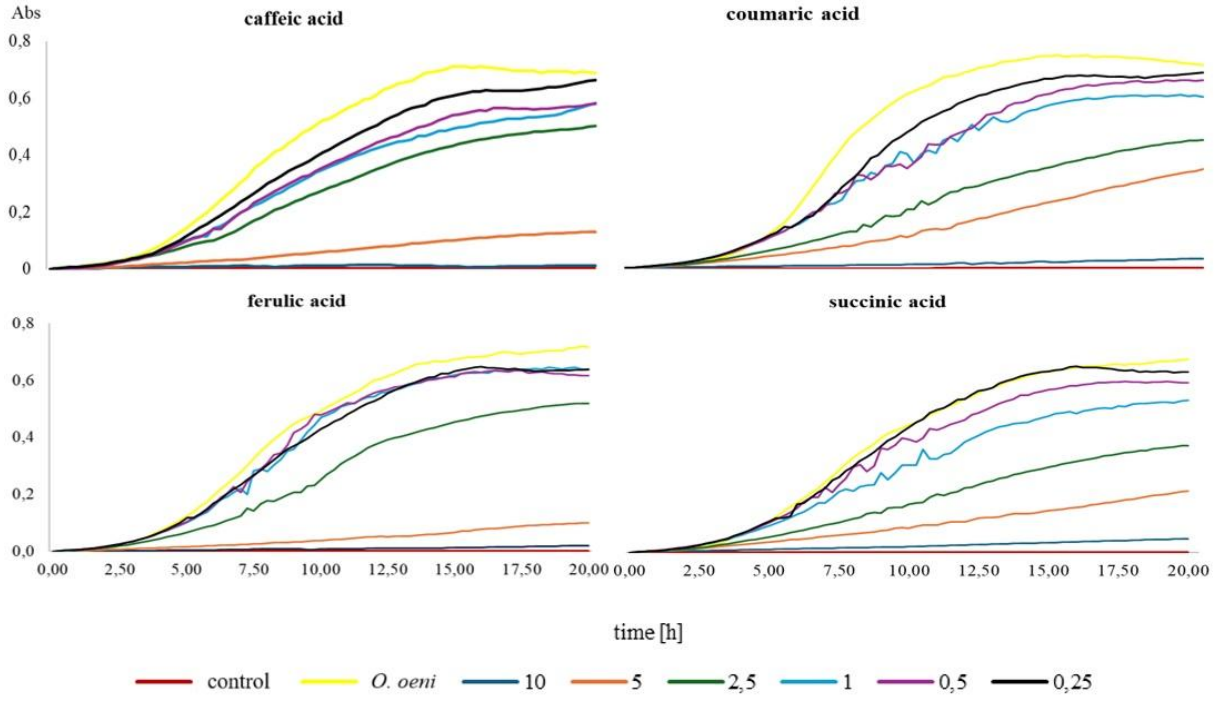

Figure 1 Graphic representation of *Oenococcus oeni* growth curves
Legend: control = distilled water; *O.oeni* = culture without inhibitor; numbers (0,25-10) indicate inhibitor concentration in g·L⁻¹

Table 1 Results of end of *Oenococcus oeni* growth curves development

Variant	Caffeic acid	Ferulic acid	Coumaric acid	Succinic acid
Control	0.006 ± 0.000 a	0.001 ± 0.000 b	0.001 ± 0.000 a	0.001 ± 0.000 a
O. oeni	0.692 ± 0.001 f	0.718 ± 0.001 g	0.660 ± 0.001 h	0.664 ± 0.002 h
10	0.012 ± 0.000 a	0.020 ± 0.000 c	0.031 ± 0.000 b	0.047 ± 0.000 b
5	0.129 ± 0.000 c	0.099 ± 0.001 d	0.326 ± 0.003 c	0.209 ± 0.002 c
2.5	0.500 ± 0.002 d	0.518 ± 0.000 e	0.427 ± 0.001 d	0.367 ± 0.001 d
1	0.576 ± 0.004 b	0.640 ± 0.002 a	0.575 ± 0.001 e	0.521 ± 0.003 e
0.5	0.578 ± 0.002 b	0.619 ± 0.001 f	0.628 ± 0.001 f	0.586 ± 0.001 f
0.25	0.660 ± 0.003 e	0.638 ± 0.001 a	0.652 ± 0.001 g	0.622 ± 0.000 g

Legend: The means were obtained from the last three absorbance values of each curve. The results are shown in format: Mean±SE. Mean values (n=3) were divided into homogeneous groups (marked by letters a, b, c, d, e, f, g, h) based on Fisher's LSD test (α= 0.05).

We can confirm our results with a study by **Campos *et al.* (2003)**, who investigated the effect of several phenolic acids associated with wine on the growth and vitality of *O. oeni* and *L. hilgardii* strains. Their study demonstrated that hydroxycinnamic acids exerted stronger inhibitory effects on *O. oeni* than hydroxybenzoic acids. Although ferulic acid inhibited bacterial growth, its effect was weaker than that of caffeic and p-coumaric acids. Furthermore, ferulic acid at 500 mg·L⁻¹ did not significantly inhibit *O. oeni*, while caffeic acid was already effective at concentrations as low as 200 mg·L⁻¹.

Another study examined the effects of ferulic acid on the growth of different strains of *Brettanomyces* yeast. Ferulic acid showed the most significant inhibitory effect in comparison with other tested substances **(Lentz and Harris, 2015)**.
The study of **Torres-Guardado *et al.* (2022)** also demonstrated the inhibitory effect of succinic acid on *O. oeni* strains PSU-1 and CH11. Increasing the succinic acid concentration from 0.5 to 2 g·L⁻¹ resulted in a progressive reduction in bacterial growth. At 1 g·L⁻¹, the population of strain PSU-1 reached approximately 50% of the maximum growth level, whereas strain CH11 reached around 70%. When the concentration was increased to 2 g·L⁻¹, growth declined further to approximately 30% and 20% for PSU-1 and CH11, respectively. In addition, succinic acid at concentrations of 1 and 2 g·L⁻¹ inhibited malolactic fermentation in both strains at pH 3.5. Strain CH11 displayed greater sensitivity to succinic acid than PSU-1. At pH 4.0, inhibition was observed only in the treatment containing 2 g·L⁻¹ succinic acid with strain CH11 when compared with the control.

Lactobacillus brevis

Figure 2 illustrates the effects of individual acids at concentrations of 10, 5, 2.5, and 1 g·L⁻¹ on the growth of *Lactobacillus brevis*. As anticipated, the highest tested concentration (10 g·L⁻¹) produced the most substantial inhibition, confirming a clear concentration-dependent response. At a concentration of 5 g·L⁻¹, caffeic and coumaric acids were the most effective inhibitors of *L. brevis* growth, while succinic and ferulic acids showed the lowest inhibitory activity. Similar to the observations for *O. oeni*, lower concentrations generated values close to those of the untreated culture, indicating only minor inhibitory effects.
The results correspond well with those reported by (Stead, 1993), who demonstrated that hydroxycinnamic acids, including caffeic, coumaric, and ferulic acids, can influence the growth of lactic acid bacteria in wine. At concentrations of 0.5 and 1 g·L⁻¹, all three acids significantly inhibited bacterial growth, with coumaric and ferulic acids exerting stronger effects than caffeic acid. In contrast,

a concentration of 0.1 g·L⁻¹ stimulated bacterial growth. The study further revealed that *L. collinoides* was more susceptible to hydroxycinnamic acid inhibition than *L. brevis*. Among the tested compounds, coumaric acid displayed the strongest inhibitory effect.

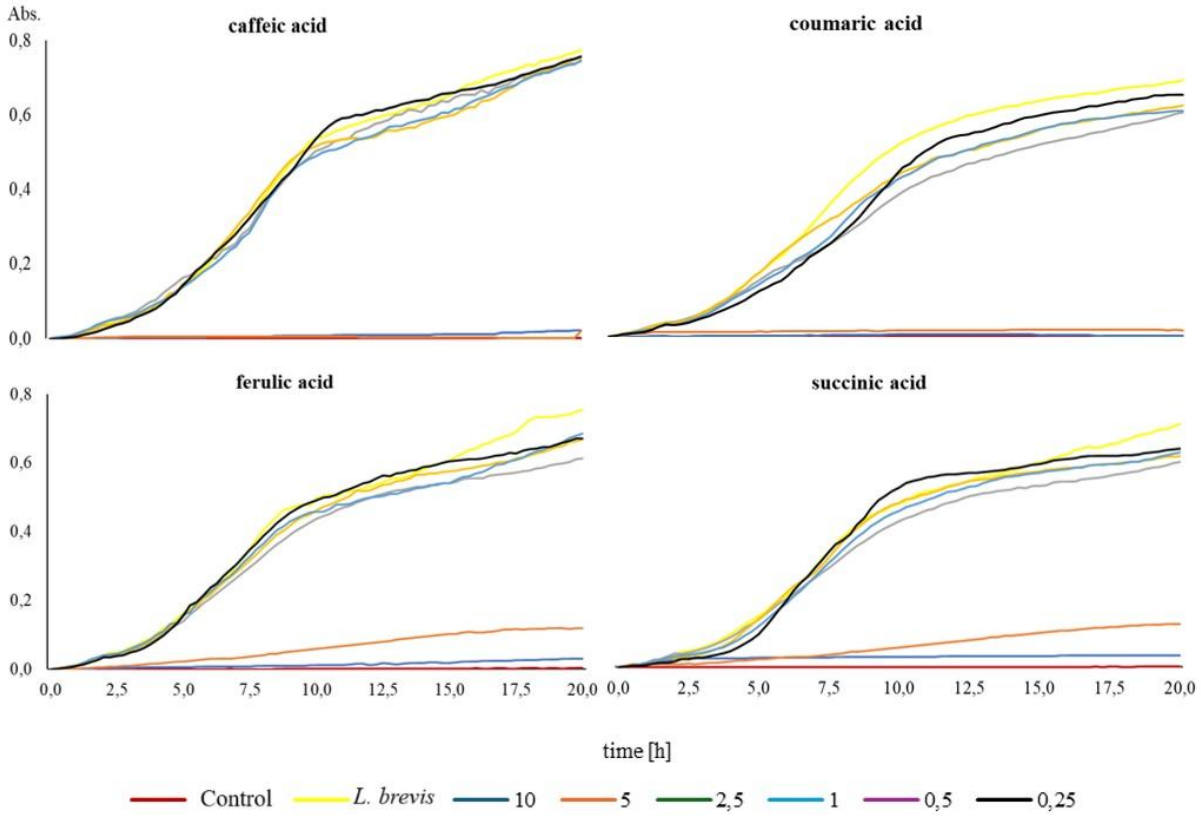

Figure 2 Graphic representation of *Lactobacillus brevis* growth curves
Legend: control = distilled water*; L. brevis* = culture without inhibitor; numbers (0,25-10) indicate inhibitor concentration in g·L⁻¹

Table 2 Results of end of *Lactobacillus brevis* growth curves development

Variant	Caffeic acid	Ferulic acid	Coumaric acid	Succinic acid
Control	0.001 ± 0.000 a	0.002 ± 0.000 a	0.002 ± 0.000 a	0.002 ± 0.000 a
L. brevis	0.860 ± 0.001 g	0.691 ± 0.001 d	0.692 ± 0.001 d	0.656 ± 0.004 c
10	0.010 ± 0.000 b	0.05 ± 0.000 a	0.003 ± 0.000 a	0.005 ± 0.000 a
5	0.007 ± 0.000 a,b	0.151 ± 0.003 c	0.538 ± 0.001 b	0.011 ± 0.000 a
2.5	0.739 ± 0.005 d	0.746 ± 0.006 e	0.675 ± 0.004 c	0.598 ± 0.001 b
1	0.696 ± 0.001 c	0.794 ± 0.006 f	0.745 ± 0.003 f	0.682 ± 0.006 e
0.5	0.750 ± 0.003 e	0.776 ± 0.005 b	0.709 ± 0.007 e	0.670 ± 0.005 d
0.25	0.833 ± 0.003 f	0.772 ± 0.004 b	0.767 ± 0.005 g	0.793 ± 0.005 f

Legend: The means were obtained from the last three absorbance values of each curve. The results are shown in format: Mean±SE. Mean values (n=3) were divided into homogeneous groups (marked by letters a, b, c, d, e, f, g, h) based on Fisher's LSD test (α= 0.05).

Lactobacillus plantarum

Effects of individual acids at concentrations of 10; 5; 2.5 and 1 g·L⁻¹ on the growth of *Lactobacillus plantarum* are shown in the Figure 3. In general, all tested acids were characterized by a shorter lag phase, compared to the other *O.oeni* and *L.brevis*. Subsequently, there was a significant increase in absorbance for most variants, which showed a different inhibitory effect. At a concentration of 5 g·L⁻¹, succinic acid had the highest inhibitory effect on *L.plantarum* bacteria, and caffeic acid the lowest.
Similar observations were reported by **Rozès and Peres (1998)**, who investigated the impact of caffeic and ferulic acids on the growth of *L. plantarum*. Both compounds inhibited bacterial growth, although tannins exhibited an even stronger inhibitory effect than the tested acids. The observed inhibition was associated with progressive changes in the composition of specific fatty acids.

Landete *et al.* (2007) obtained comparable results when assessing the antimicrobial activity of caffeic, ferulic, and coumaric acids against *L. plantarum*. Their findings confirmed the inhibitory potential of these compounds and showed that complete suppression of bacterial growth required relatively high concentrations. Specifically, complete inhibition was achieved at 12.5–25 mM for coumaric acid, 50–100 mM for caffeic acid, and 25–50 mM for ferulic acid. These results highlight the potential of hydroxycinnamic acids as antimicrobial agents against *L. plantarum*.

Table3 Results of end of *Lactobacillus plantarum* growth curves development

Variant	Caffeic acid	Ferulic acid	Coumaric acid	Succinic acid
Control	0.001 ± 0.000 a	0.001 ± 0.000 a	0.002 ± 0.000 a	0.001 ± 0.000 b
L. plantarum	0.707 ± 0.000 f	0.666 ± 0.001 e	0.656 ± 0.002 e	0.707 ± 0.000 f
10	0.657 ± 0.000 e	0.035 ± 0.001 b	0.044 ± 0.000 b	0.047 ± 0.000 c
5	0.347 ± 0.000 b	0.376 ± 0.000 c	0.266 ± 0.001 c	0.127 ± 0.000 d
2.5	0.427 ± 0.000 c	0.494 ± 0.004 d	0.373 ± 0.001 d	0.580 ± 0.004 e
1	0.580 ± 0.004 d	0.679 ± 0.002 f	0.668 ± 0.001 g	0.774 ± 0.000 h
0.5	0.774 ± 0.000 h	0.712 ± 0.000 g	0.664 ± 0.001 f	0.713 ± 0.000 a
0.25	0.713 ± 0.001 g	0.777 ± 0.001 h	0.707 ± 0.000 h	0.713 ± 0.001 a

Legend: The means were obtained from the last three absorbance values of each curve. The results are shown in format: Mean±SE. Mean values (n=3) were divided into homogeneous groups (marked by letters a, b, c, d, e, f, g, h) based on Fisher's LSD test (α= 0.05).

Disc diffusion method results

The inhibition zone diameters are expressed in millimetres. A zone diameter of 4 mm corresponds to the diameter of the well itself, indicating that no bacterial growth inhibition occurred. Values exceeding 4 mm represent inhibition caused by the tested acids. The obtained results are summarized in Table 4.

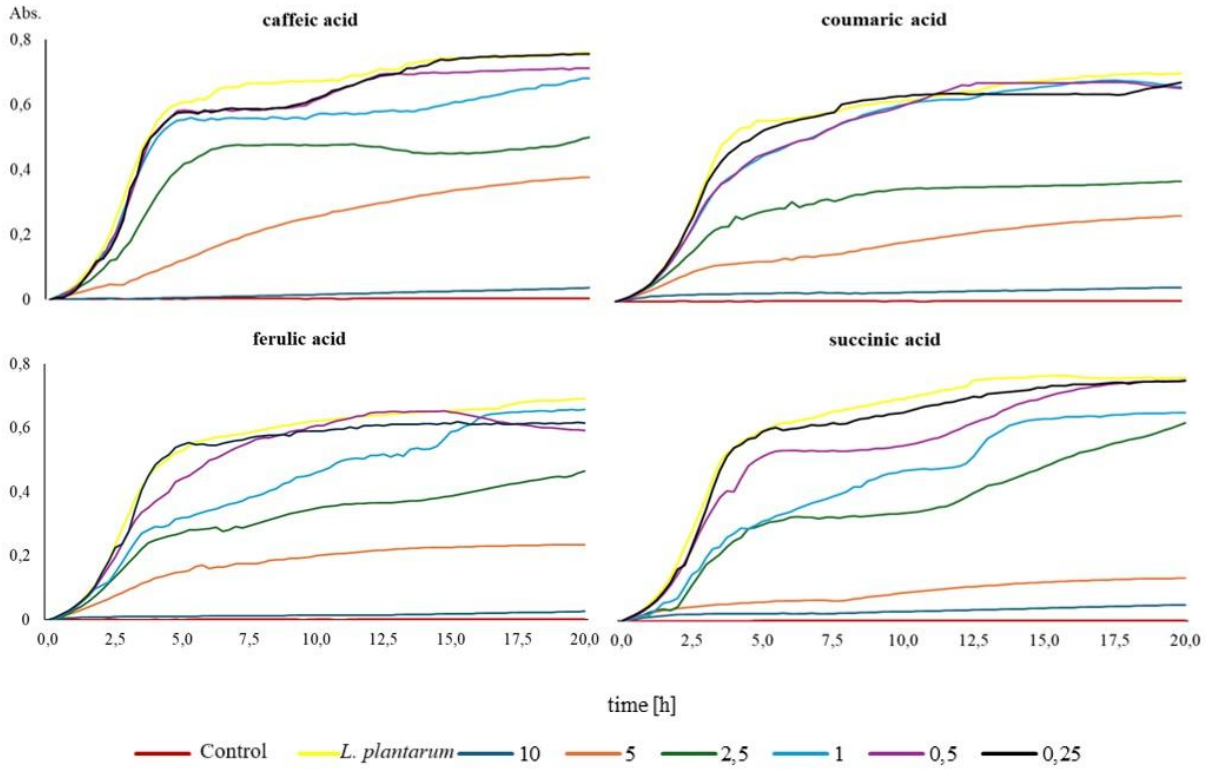

Figure 3 Graphic representation of *Lactobacillus plantarum* growth curves
Legend: control = distilled water*; L. plantarum* = culture without inhibitor; numbers (0,25-10) indicate inhibitor concentration in g·L^{-1}

Table 4 Results of disc diffusion method

Microorganism	Concentration (g·L^{-1})	Caffeic acid	Ferulic acid	Coumaric acid	Succinic acid
O. oeni	**10**	10.00 ± 1.15 d	10.33 ± 0.67 c,d	11.00 ± 1.00 c,d	10.33 ± 0.33 b
	5	6.33 ± 0.33 a,b,c	7.33 ± 0.89 a,b	4.67 ± 0.67 a,b	4.00 ± 0.00 a
	2.5	4.00 ± 0.00 a	4.00 ± 0.00 a	4.00 ± 0.00 a	4.00 ± 0.00 a
	1	4.00 ± 0.00 a	4.00 ± 0.00 a	4.00 ± 0.00 a	4.00 ± 0.00 a
L. brevis	**10**	10.67 ± 1.20 d	12.33 ± 0.33 d	12.00 ± 0.58 d	10.00 ± 0.00 b
	5	8.00 ± 0.58 c,e	6.67 ± 0.33 a,b	8.33 ± 0.33 b,c	6.67 ± 0.33 c
	2.5	6.00 ± 0.58 a,b	4.33 ± 0.33 a	6.33 ± 0.33 a,b	4.33 ± 0.33 a
	1	4.00 ± 0.00 a	4.00 ± 0.00 a	4.00 ± 0.00 a	4.00 ± 0.00 a
L. plantarum	**10**	9.67 ± 0.89 d,e	8.33 ± 1.76 b,c	10.33 ± 2.73 c,d	11.33 ± 0.33 d
	5	7.33 ± 0.33 b,c	6.67 ± 1.67 a,b	6.67 ± 0.88 a,b	8.67 ± 0.33 d
	2.5	4.00 ± 0.00 a	6.00 ± 1.00 a,b	4.67 ± 0.67 a,b	4.67 ± 0.67 a
	1	4.00 ± 0.00 a	4.00 ± 0.00 a	4.67 ± 0.67 a,b	4.00 ± 0.00 a

Legend: The values are shown in format: Mean±SE. Mean values (n=3) were divided into homogeneous groups (marked by letters a, b, c, d, e, f, g, h) based on Fisher's LSD test (α= 0.05)

Bacterial growth inhibition was observed only at concentrations higher than 2.5 g·L^{-1}. In general, larger inhibition zones were associated with increasing acid concentrations. The strongest inhibitory activity was recorded for *L. brevis*, where caffeic acid at 10 g·L^{-1} produced an inhibition zone with a diameter of 13 mm. In comparison, the inhibitory effect of caffeic acid against *O. oeni* and *L. plantarum* was less pronounced, and the inhibition zone boundaries appeared less distinct. Nevertheless, a substantial reduction in bacterial growth was still evident at the highest tested concentration of 10 g·L^{-1}.

No inhibition zones were detected at lower concentrations. Similar findings were reported by **Yuan *et al.* (2018)**, who evaluated the antimicrobial properties of hydroxycinnamic acids present in ethyl acetate extracts obtained from enzymatic hydrolysates. Among the tested compounds was caffeic acid, which was assessed against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*. Their results demonstrated a considerable antibacterial effect of caffeic acid compared with the positive control, confirming its ability to suppress bacterial growth.

The effects of phenolic compounds can be specific to individual bacterial species, as shown by research **García-Ruiz *et al.* (2008)**, where caffeic acid showed a significant inhibitory effect on *O. oeni*, while it did not show the same effect on *L. hilgardii*.

Succinic acid was another compound evaluated in this study. At a concentration of 10 g·L^{-1}, it inhibited the growth of all tested bacterial species. The most pronounced effect was observed in *L. plantarum*, where the inhibition zone was completely sterile and exhibited sharp, well-defined edges. In contrast, the inhibition zones of the remaining species contained occasional bacterial colonies or less clearly defined boundaries. At 5 g·L^{-1}, succinic acid was capable of restricting the growth of *L. brevis* and *L. plantarum* only. As with the other acids, its inhibitory activity increased with increasing concentration. No significant inhibition was detected at concentrations of 2.5 or 1 g·L^{-1}.

Ferulic acid also exhibited strong antimicrobial activity at 10 g·L^{-1} against all monitored bacterial strains. A gradual decline in inhibition was observed as the concentration decreased. Unlike *L. brevis* and *L. plantarum*, *O. oeni* did not develop an inhibition zone at 2.5 g·L^{-1}.

Furthermore, none of the tested bacterial species showed measurable inhibition at 1 g·L^{-1}. Comparable results were described by **García-Ruiz *et al.* (2011)**, who investigated the effects of ferulic acid on *L. hilgardii*, *Pediococcus pentosaceus*, and *O. oeni*. Their study demonstrated that ferulic acid possessed a greater capacity to inhibit the growth of lactic acid bacteria than the other tested hydroxycinnamic acids. These findings suggest that ferulic acid may represent a useful tool for controlling lactic acid bacteria in oenological applications and could potentially serve as an alternative to conventional antimicrobial agents. Such growth-suppressing properties are particularly relevant for fermentation management and wine quality preservation.

The obtained results further demonstrate that coumaric acid inhibited the growth of all tested bacterial species. As the concentration increased, the diameter of the bacteria-free zone increased accordingly. *L. brevis* was the most susceptible species, followed by *L. plantarum*, whereas *O. oeni* showed the highest tolerance. Concentrations of 1 and 2.5 g·L^{-1} were insufficient to inhibit *O. oeni*, as shown in Table 4, while slight inhibition was still observed in both *L. brevis* and *L.*

plantarum. The greatest inhibitory effects were achieved at concentrations of 5 and 10 g·L⁻¹, where all bacterial species displayed clearly visible inhibition zones. Antimicrobial activity of coumaric acid against a bacterial strain *S. dysenteriae* was confirmed in a study **Lou *et al.* (2012)**. The acid showed the highest inhibitory activity against bacteria with a minimum inhibitory concentration of 10 g·L^{-1}.

Results of inhibition test on a microsample of wine

0.5 g·L^{-1} to wine most inhibited the activity of lactic acid bacteria, and the content of malic acid is the closest to the content with the use of sulphur. The highest concentration of 1 g·L^{-1} was an equally good inhibitor, but also the lowest concentration of 0.25 g·L^{-1}, which was also able to suppress the growth of bacteria very well. We can state that in almost all variants and doses statistically significantly higher values of malic acid content were observed compared to the control sample, where MLF was performed. The exception is the addition of succinic acid to red wine in doses of 0.25 and 0.5 g·L^{-1}, where the values were statistically identical to the control sample. On the other hand, it should be noted that only the variant treated with a safe dose of SO_2 showed a completely no decrease in malic acid content. All other measurements showed significantly lower values of malic acid content.

Similar findings have been reported by**(Stivala *et al.*, 2017),** who investigated the influence of various wine-derived phenolic compounds on the growth of two spoilage-associated bacterial species isolated from Argentine red wines. Their study demonstrated that caffeic and coumaric acids exerted the strongest inhibitory effects at specific concentrations and incubation periods. The greatest suppression of *L. hilgardii* 6F, *L. hilgardii* X1B, and *P. pentosaceus* 12p was observed following treatment with 400 mg·L⁻¹ of caffeic or coumaric acid for 96 h. Among the tested microorganisms, *P. pentosaceus* was identified as the most sensitive, whereas *L. hilgardii* displayed greater resistance. The authors further highlighted a close relationship between the chemical structure of phenolic compounds and their antibacterial activity.

All concentrations presented in Table 5 demonstrated the ability to inhibit bacterial growth. As the inhibitor concentration decreased, a gradual decline in malic acid preservation was observed. Ferulic acid showed the strongest inhibitory effect at a concentration of 1 g·L⁻¹, where the final malic acid concentration was closest to that recorded in the sulphur dioxide treatment.

The results of the study **Wasko *et al.* (2014)** also indicate the potential of phenolic acids and their derivatives as effective growth inhibitors of two *Lactobacillus helveticus* bacteria, T103 and T159. Ferulic acid and its derivatives were found to inhibit the growth of strain T159 more effectively than strain T103. In many cases, ferulic acid derivatives had a lower minimum inhibitory concentration than free ferulic acid. These results suggest that ferulic acid and its derivatives could be promising candidates for natural antibacterial agents to control the growth of *Lactobacillus helveticus* bacteria, especially strain T159.

Table 5 Results of malic acid content

Inhibitor	Concentration (g·L^{-1})	Malic acid (g·L^{-1})	
		White wine	Red wine
SO_2	**0.05**	1.60 ± 0.03 d	1.28 ± 0.04 e
MLF	**no added**	0.48 ± 0.07 c	0.50 ± 0.01 a
Caffeic acid	**1**	1.40 ± 0.02 a,b	0.87 ± 0.01 c
	0.5	1.41 ± 0.01 b	0.86 ± 0.01 c
	0.25	1.28 ± 0.03 a	0.76 ± 0.11 b
Ferulic acid	**1**	1.45 ± 0.01 a	1.05 ± 0.04 d
	0.5	1.33 ± 0.02 a	0.83 ± 0.02 c
	0.25	1.38 ± 0.03 a	0.60 ± 0.02 b
Coumaric acid	**1**	1.56 ± 0.03 b	1.01 ± 0.01 d
	0.5	1.42 ± 0.02 a	0.82 ± 0.04 c
	0.25	1.35 ± 0.01 a	0.61 ± 0.00 b
Succinic acid	**1**	1.33 ± 0.04 b	0.75 ± 0.03 b
	0.5	1.15 ± 0.13 a,b	0.56 ± 0.04 a
	0.25	0.98 ± 0.01 a	0.51 ± 0.02 a

Legend: The values are shown in format: Mean±SE. Mean values (n=3) were divided into homogeneous groups (marked by letters a, b, c, d, e, f, g, h) based on Fisher's LSD test (α= 0.05).

The use of sulphites in winemaking remains widespread due to their preservative, antioxidant, and antimicrobial properties. However, consumer concerns regarding potential health risks have stimulated interest in alternative compounds. **Ledesma *et al.* (2024)** investigated the combined effects of p-coumaric acid and sodium bisulphite against *Lacticaseibacillus paracasei* AT45 in wine. Their results revealed a synergistic antibacterial interaction between these compounds, leading to a substantial reduction in bacterial viability compared with untreated controls. Such findings suggest that combining phenolic acids with reduced sulphite doses may provide an effective strategy for controlling undesirable microorganisms while simultaneously limiting the formation of biogenic amines.

Further evidence for the antimicrobial activity of hydroxycinnamic acids in wine was provided by **García-Ruiz *et al.* (2008)**, who examined the effects of ferulic and coumaric acids on the growth and metabolism of microorganisms, particularly *Lactobacillus plantarum* and selected yeast species. Their results demonstrated that ferulic acid exerted a stronger inhibitory effect than coumaric acid. Moreover, the sensitivity to these compounds differed among microbial species. In contrast, esterified forms of these acids did not affect the growth of *L. plantarum*. These observations underline the importance of considering the specific biological activity of individual phenolic compounds when evaluating their antimicrobial potential.

Previous studies have also shown that phenolic extracts obtained from white and red grape varieties possess antimicrobial activity against pathogenic bacteria in culture media**(Papadopoulou *et al.*, 2005; Vaquero *et al.*, 2007).** Owing to their high phenolic acid content, these extracts appear to be more effective against bacteria than against yeasts, indicating a higher level of yeast resistance. Considerable effort has therefore been devoted to developing environmentally friendly extraction methods capable of producing broad-spectrum phenolic fractions suitable for industrial applications. It should be noted, however, that these earlier investigations were performed in culture media specifically designed to support bacterial growth. Under more stressful conditions, such as those present in wine, lower inhibitor concentrations may be sufficient. While our in vitro experiments required 5–10 g·L⁻¹ to establish baseline inhibition, the wine microsample trials demonstrated clear antimicrobial activity at technologically relevant concentrations ranging from 0.25 to 1 g·L⁻¹. Furthermore, synergistic interactions among phenolic compounds or between phenolics and SO_2 may allow a reduction in the concentration of individual antimicrobial agents. The antimicrobial efficacy of phenolic compounds is therefore strongly influenced by both their chemical structure and concentration. Nevertheless, the concentrations required for substantial inhibition often exceed the levels naturally occurring in wine. The application of phenolic extracts must also be considered carefully because of their potential impact on wine physicochemical properties and sensory attributes, including colour and aroma **(Palma *et al.*, 1999)**.

Furthermore, this part of study is important for demonstrating of naturally higher efficiency due to the structure-activity relationships (SAR) between additives and the matrix of wines. The low pH of wine (~3–4) shifts the dissociation equilibrium of weak acids towards the undissociated (neutral) form, which is more lipophilic and more easily passively crosses the cytoplasmic membrane; after entry into the cell (higher intracellular pH), dissociation, intracellular acidification, anion stress and energetics disruption occur – the basic "weak-acid" mechanism in bacteria **(Hirshfield *et al*., 2003)**. In addition, in the oenological context, hydroxycinnamic acids (caffeic, p-coumaric, ferulic) themselves function as weak acids (pKa ~4.2) and their antimicrobial effect is pH-dependent; in wine LAB (*Oenococcus oeni, Lactobacillus hilgardii*) it has been shown that hydroxycinnamates induce ion leakage, proton influx and a decrease in viability, i.e. direct membrane action and SAR related to lipophilicity/structure **(Campos *et al.,* 2009b)**. The natural occurrence of these compounds in wine (free forms in the m g·L^{-1} range, but technologically variable) demonstrates that they are actually present matrix components; e.g. caffeic acid has been reported to be approximately 0.87–33.48 mg·L^{-1} in commercial wines **(Proestos *et al.,* 2012)** and low mg·L^{-1} units for caffeic/p-coumaric/ferulic acid have been reported in white wines **(Lampíř and Pavloušek, 2013).**

Ethanol also acts as a key "matrix enhancer": in O. oeni it has been shown in detail that ethanol alters membrane fluidity and that adaptation involves membrane modifications directly linked to tolerance/leakage **(Da Silveira *et al.*, 2003)**. The study of combined stresses further shows that the order and combination of ethanol + acidity + cold significantly changes membrane fluidity and can progress to states correlating with a total loss of viability in ethanol-acid shock **(Chu-Ky *et al*., 2005)**. Analogously, in wine lactobacilli (e.g. *Lactobacillus hilgardii*), ethanol induces changes in fatty acid composition and membrane fluidity that are related to ethanol tolerance – which supports the mechanistic link "ethanol → membrane → sensitivity to other stressors" **(Couto *et al.*, 1996)**. In this matrix, therefore, a dose of 0.25 g·L^{-1} for individual hydroxycinnamates makes technological sense as targeting low millimolar levels, for which inhibition at higher hundreds of mg/L has been repeatedly described in model systems for wine LAB. Classically, it has been shown in spoilage *Lactobacillus* that hydroxycinnamates at 500–1000 mg·L^{-1} significantly inhibit growth in acidic medium with ethanol **(Stead, 1993)**, and recently study demonstrated strong inhibition of MLF by combinations including p-coumaric and ferulic acid (0.4 g·L^{-1}) in conjunction with other inhibitors **(Kostelníková *et al*., 2025)**. For succinic acid, the upper limit of addition of up to 1 g·L^{-1} is also in line with the natural variability in wine (often about 0.5–1.5 g·L^{-1}) and its impact on MLF is modulated by strain and conditions (pH/ethanol) **(Chidi *et al.*, 2018; Torres-Guardado *et al.*, 2022)**. The relationship to SO_2 can

be summarized as follows: the effective antimicrobial fraction is pH-dependent (molecular SO_2) and the real activity may be reduced in wine by binding in the matrix; therefore, the barrier combination (pH + ethanol + phenolic acids + SO_2) is technologically rational **(Howe et al., 2018; OIV, 2021).**

CONCLUSION

In this research, we evaluated the capacity of specific hydroxycinnamic acids and succinic acid to suppress malolactic fermentation. Our findings indicate that a concentration of 5 $g \cdot L^{-1}$ is sufficient to achieve baseline inhibition in growth curve assays, whereas real wine environments require lower effective doses owing to acidic conditions and ethanol presence. The tested organic compounds exhibited distinct inhibitory profiles across the various lactic acid bacteria strains. For *O. oeni*, ferulic and caffeic acids emerged as the most potent suppressors, while coumaric acid displayed the weakest impact. In contrast, *L. brevis* growth was heavily restricted by caffeic and coumaric acids, but showed high resistance to ferulic and succinic acids. Succinic acid exerted the most pronounced inhibition on *L. plantarum*, whereas caffeic acid was the least effective against this species.
These trends were generally mirrored in the disk diffusion assays; however, *L. plantarum* deviated slightly, showing peak sensitivity to 5 $g \cdot L^{-1}$ of succinic and caffeic acids, and the highest tolerance toward ferulic and coumaric acids. During trials with wine microsamples, where the matrix's low pH and ethanol acted synergistically, all tested acids effectively suppressed bacterial activity in white wine even at minimal concentrations, showing minimal statistical variation. Conversely, the red wine matrix revealed more pronounced statistical differences among the tested concentration levels. The differences in malic acid preservation between the application of caffeic acid and succinic acid were statistically negligible. A clear dose-dependent relationship was confirmed for ferulic and coumaric acids, where increasing concentrations led to higher inhibition and lower malic acid consumption. Considering these results alongside natural occurrence levels, we suggest a technological application of 0.25 $g \cdot L^{-1}$ for individual hydroxycinnamic acids and 1 $g \cdot L^{-1}$ for succinic acid to boost the antimicrobial properties of wines.

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