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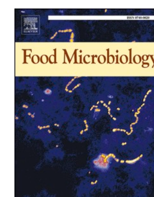

Interaction effects of fumaric acid, pH and ethanol on the growth of lactic and acetic acid bacteria in planktonic and biofilm states

Violeta García-Viñola [a], Jokin Ezenarro [b], Cristina Reguant [c], Nicolas Rozès [a,*], Manuel Malfeito Ferreira [d]

[a] *Universitat Rovira i Virgili, Grup de Biotecnologia Microbiana dels Aliments, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Tarragona, Spain*
[b] *Universitat Rovira i Virgili, ChemoSens Group, Department of Analytical Chemistry and Organic Chemistry, Tarragona, Spain*
[c] *Universitat Rovira i Virgili, Grup de Biotecnologia Enològica, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Tarragona, Spain*
[d] *Universidade de Lisboa, LEAF, Linking Landscape, Environment, Agriculture and Food Research Center, Associate Laboratory TERRA, Instituto Superior de Agronomia, Lisboa, Portugal*

A R T I C L E I N F O

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A B S T R A C T

The microbial stability of wine can be compromised by the presence of lactic acid bacteria (LAB) and acetic acid bacteria (AAB), which can cause spoilage via off flavour production, increased acetic acid production, or biofilm formation. To manage the growth of LAB in winemaking, fumaric acid (FA) has been proposed as an alternative to traditional antimicrobial agents, such as sulfur dioxide (SO_2). This study aimed to evaluate the inhibitory effects of FA on the growth of LAB and AAB based on the influence of pH and ethanol in a synthetic wine-like medium.

The research involved the determination of the individual, 2 × 2 combined, and combined minimum inhibitory concentrations (MICs) of fumaric acid, pH, and ethanol. Specifically, the MIC90 was defined as the concentration required to inhibit the growth of more than 90 % of the initial population, and the MIC50 was defined as the concentration required to inhibit the growth of more than 50 % of the initial population. These thresholds were assessed in 19 bacterial strains (13 LAB and 6 AAB strains) at pH values of 3.5 and 4.0 and ethanol concentrations of 0, 4, 8 and 12 % v/v. Additionally, the impact of FA on biofilm formation was evaluated in the ten bacterial strains that were observed to be most resistant to FA.

The results revealed that the inhibitory effects of FA were enhanced at lower pH values and at higher ethanol concentrations. LAB strains (such as *Oenococcus oeni*) were particularly sensitive to FA, whereas non-*Oenococcus* LAB strains demonstrated resistance to concentrations exceeding 2 g/L under the tested pH (3.5–4.0) and ethanol (0–12 % v/v) conditions. AAB strains (such as *Acetobacter aceti*) tolerated FA concentrations greater than 2 g/L at pH 4.0 in the absence of ethanol; however, the susceptibility increased with increasing ethanol concentrations and decreasing pH. Furthermore, FA significantly inhibited biofilm formation (particularly at a pH of 3.5 and ethanol concentrations greater than 8 % v/v).

In conclusion, when combined with low pH and high ethanol concentrations, FA offers a promising strategy for controlling bacterial growth and biofilm formation in winemaking. This approach has the potential to complement or replace the use of traditional chemical preservatives, such as SO_2.

1. Introduction

The production of wine involves various fermentations performed by microorganisms that influence the flavour, aroma, and spoilage of the wine (Ribéreau-Gayon et al., 2006). The first phase, which involves alcoholic fermentation (AF) and requires the conversion of sugars into alcohol, is well understood and is largely dominated by the yeast *Saccharomyces cerevisiae* (Ruiz-de-Villa et al., 2023). Moreover, lactic acid bacteria (LAB) (such as *Oenococcus oeni*) drive a secondary fermentation process known as malolactic fermentation (MLF). The primary biochemical transformation in MLF involves the decarboxylation of L-malic acid into L-lactic acid and carbon dioxide, thereby reducing the acidity of the wine and creating a smoother mouthfeel (Pardo and Ferrer, 2021). Winemakers may encourage MLF in certain

* Corresponding author.
E-mail address: nicolasrozes@urv.cat (N. Rozès).

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styles of wine, such as red wines or white wines containing high concentrations of L-malic acid or total acidity, in which a rounder, smoother taste is preferred (Pardo and Ferrer, 2021). However, in wines in which an acidic profile is desirable (such as most white and sparkling wines), MLF is typically avoided (Bauer and Dicks, 2004; Pardo and Ferrer, 2021).

Wine spoilage may occur due to the activity of yeasts and bacteria that thrive in the wine environment, thereby leading to undesirable flavours, aromas, and textures (Malfeito-Ferreira, 2011; Mendes Ferreira and Mendes-Faia, 2020). Certain LAB species, including *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, *Oenococcus oeni*, and *Pediococcus damnosus*, can contribute to spoilage by producing unwanted compounds such as acetic acid, diacetyl, exopolysaccharides, tetrahydropyridines, acrolein, ethyl carbamate, or biogenic amines (Bartowsky, 2009; Du Toit and Pretorius, 2000). LAB that become adapted to wine conditions can survive at low pH values (<3.5), high sulfur dioxide levels (50 mg/L), and ethanol concentrations exceeding 10 % (v/v) (Lonvaud-Funel, 1999).

Additionally, acetic acid bacteria (AAB), such as *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans*, are significant factors in winemaking, as they oxidise ethanol into acetic acid, thus resulting in vinegar-like off-flavours; moreover, they can produce acetoin from lactic acid and glycerol (Bartowsky, 2009; Bartowsky and Pretorius, 2009). These AAB species thrive in grape musts and wines with pH values between 3.0 and 4.0, with *G. oxydans* being more commonly documented in grape musts, whereas *A. aceti* predominates in wines (Du Toit and Pretorius, 2002).

An additional challenge for regulating wine microbial stability involves biofilm formation. Biofilms are structured communities of microorganisms that adhere to surfaces and that are encased in a self-produced extracellular matrix (Donlan and Costerton, 2002; Kubota et al., 2008). In winemaking, LAB and AAB can colonise surfaces such as fermentation tanks and barrels (Gosset et al., 2022; Tristezza et al., 2010). These biofilms may include LAB species such as *Lpb. plantarum* or *O. oeni* (Bastard et al., 2016; Gosset et al., 2022; Kubota et al., 2008; Pannella et al., 2020); AAB species such as *Gluconacetobacter xylinus* (Yamada et al., 2012); and spoilage yeasts such as *Brettanomyces* spp. (Tristezza et al., 2010), thus demonstrating that these species could be a persistent threat to the quality of winemaking. Biofilm formation is influenced by nutrient availability, environmental conditions, and the presence of antimicrobial compounds (Donlan and Costerton, 2002; Gosset et al., 2022). Biofilms protect microorganisms from chemical preservatives, which indicates that their persistence can increase in winery environments (Diez et al., 2010; Zgardan et al., 2023). Furthermore, microorganisms in biofilms are often relatively resistant to chemical cleaning and disinfecting agents (Donlan and Costerton, 2002).

To prevent wine spoilage, several physical and chemical methods are employed to remove or inactivate microorganisms (Bartowsky, 2009). Sulfur dioxide is the most commonly used preservative, alongside other agents such as dimethyl dicarbonate (DMDC), chitosan, lysozyme, and bacteriocin (Costa et al., 2008; Tedesco et al., 2022). However, the use of new substitutes for SO_2 is currently being investigated due to the potential health effects of SO_2 (such as allergies), as well as the increasing trend towards producing wines with minimal interventions. Recently, fumaric acid (FA) has been approved for use in winemaking at a maximum concentration of 0.6 g/L to control MLF (OIV-OENO 581A-2021, 2021), based on its utilisation in other foods (Pérez-Díaz, 2011; Tsukatani and Sakata, 2022; Wei et al., 2022). Previous studies have investigated the inhibitory effects of FA on LAB (Cofran and Meyer, 1970; Morata et al., 2020, 2023; Ough and Kunkee, 1974; Pilone et al., 1974). Additionally, the combination of FA with LAB has been demonstrated to exert inhibitory effects on biofilm formation by foodborne bacteria (Ji et al., 2023). These findings provide new avenues for the use of FA to inhibit MLF and AAB growth in winemaking.

In conclusion, the prevention of spoilage by LAB and AAB is crucial for maintaining the organoleptic and hygienic qualities of wine. Advances in the understanding of microbial behaviours, such as biofilm formation and resistance to FA, have provided winemakers with additional strategies to ensure wine stability while reducing the use of sulfur dioxide. Therefore, this study aimed to evaluate i) the effects of FA on the growth of LAB and AAB; ii) the synergistic inhibitory effects of FA combined with changes in pH and ethanol; and iii) the impact of FA on *in vitro* biofilm formation by LAB and AAB.

2. Materials and methods

2.1. Strain maintenance and inoculum conditions

The strains of the different species of LAB and AAB that were used in this study are listed in Table 1. All of the strains were isolated from grape musts, wines, vinegars and other fermenting foods and were selected from the collections of the Microbiology Laboratory at the Instituto Superior de Agronomia (ISA) of Lisbon (Portugal) and from the Oenological Biotechnology Group of the Department of Biochemistry and Biotechnology at the Faculty of Oenology of the Rovira i Virgili University (URV) of Tarragona (Spain). Some of the selected strains belonged to the American Type Culture Collection (ATCC) and the Spanish Type Culture Collection (CECT), whereas some of the other strains are commercial strains of LAB. We used the following microorganisms: 13 strains of LAB, including *Lentilactobacillus hilgardii* (1 strain), *Lpb. plantarum* (2 strains), *Liquorilactobacillus mali* (1 strain), *Lacticaseibacillus casei* (1 strain), *Lvb. brevis* (1 strain), *P parvulus* (1 strain), *L. mesenteroides* (1 strain), and *O. oeni* (4 strains), as well as 6 strains of AAB, including *A. aceti* (3 strains), *Acetobacter cerevisiae* (1 strain), and *G. oxydans* (2 strains).

Strains were maintained in 40 % glycerol (Panreac, Barcelona, Spain) at −80 °C. An inoculating loop was used to inoculate LAB in 10 mL of modified MRS liquid medium (Margalef-Català et al., 2016), which was further incubated at 30 °C for 48 h. AAB were cultured in

Table 1
Classification, names of the species, codes and origin of the strains of lactic acid bacteria and acetic bacteria used in the study.

Group	Species[a]	Code	ISA-URV code or Brand and commercial name or Culture collection code [b]//Origin of isolate
Lactic acid bacteria	*Lentilactobacillus hilgardii*	LH1	ISA4387//Wine
	Lactiplantibacillus plantarum	LP1	ISA4395//Wine
		LP2	Lallemand ML PRIME™//Wine
	Liquorilactobacillus mali	LM1	ISA4403//Grape must
	Lacticaseibacillus casei	LC1	CECT745//Rumen of sheep
	Levilactobacillus brevis	LB1	CECT216//Beer
	Pediococcus parvulus	PP1	ISA4401//Wine
	Leuconostoc mesenteroides	LMES1	CECT219//Fermenting olives
	Oenococcus oeni	OO2	ISA4400//Wine
		OO3	Lallemand Lalvin VP41//Wine
		OO4	ATCC BAA-331//Wine
		OO5	Christian Hansen CH11//Wine
		OO6	URV1PW13//Wine
Acetic acid bacteria	*Acetobacter aceti*	AA1	ISA4201//Vinegar
		AA2	ISA4417//Vinegar
		AA3	CECT298//Vinegar
	Acetobacter cerevisiae	AC1	ISA4723//Vinegar
	Gluconobacter oxydans	GO2	ISA4245//Vinegar
		GO3	URV390//Grape must

[a] Present taxonomic denomination of the species *Lactobacillus hilgardii*, *Lactobacillus plantarum, Lactobacillus mali, Lactobacillus casei* and *Lactobacillus brevis*.

[b] CECT (Spanish Type Culture Collection), ATCC (American Type Culture Collection).

modified GY consisting of 5 % (w/v) glucose, 1 % (w/v) yeast extract and 1 % (w/v) peptone (with all reagents obtained from Panreac) and incubated at 28 °C for 72 h in the presence of oxygen.

To determine the inoculum growth point, growth curves of optical density (OD) versus colony-forming units per millilitre (CFU/mL) were established, and linear regression was performed. The regression line was validated via a coefficient of determination (R^2) exceeding 90 % (Table SD1). The OD was measured at 600 nm via a Chromate Model 4300 spectrophotometer and Chromate Manager 6.3.1.302 software (Awareness Technology, Inc., Florida, USA). Viable cells were chosen after decimal dilutions with a saline solution of 0.9 % NaCl inoculated in solid media. LAB were plated on modified MRS media (Margalef-Català et al., 2016) supplemented with 2 % agar (Scharlau, Barcelona, Spain) and incubated for 7 days at 30 °C. For AAB, counts were performed on GYC (a solid medium consisting of GY supplemented with 1 % calcium carbonate and 2 % agar [Panreac]), which was subsequently incubated for 72 h at 28 °C. The relationship between the OD and viable counts (CFU/mL) was used to determine the inoculum volume that was required to achieve an initial viable count of approximately 10^6 cells/mL.

2.2. Minimum inhibitory concentration (MIC) determination

2.2.1. Culture medium

To evaluate the ability of different bacterial strains to grow in the presence of inhibitors, a wine-like medium (WLM) was prepared following the protocol described by Bordas et al. (2015) (the complete composition of the WLM is provided in Supplementary Table SD1). The WLM was modified to include fumaric acid (FA) at concentrations ranging from 0 to 2 g/L (Ravago Chemicals, Barcelona, Spain), pH levels of 3.5 and 4.0, and ethanol concentrations of 0, 4, 8 and 12 % v/v (AGA - Álcool e Géneros Alimentares, S.A., Vila Nova de Gaia, Portugal). The pH was measured using a HANNA HI5221 pH meter (Hanna Instruments Portugal, Lisbon, Portugal).

2.2.2. Experimental design

Forty distinct WLM conditions were prepared based on a full factorial experimental design (Fig. 1), with considerations for the different combinations of FA concentrations, pH levels, and ethanol concentrations. The following variables and their respective levels were utilised: i) FA: 0, 0.5, 1, 1.5 and 2 g/L; ii) pH: 3.5 and 4.0; and iii) ethanol: 0, 4, 8, or 12 % (v/v). All of the conditions were established in 400 μL 96-well microplates (Wuxi Nest Biotechnology Co., Ltd., Jiangsu, China), with each well containing 200 μL of inoculated WLM. The bacterial strains were inoculated at a concentration of 10^6 cells/mL. Blank wells were prepared for each WLM condition without any bacterial inoculation to serve as controls. The microplates were statically incubated at 25 °C for 7 days. After the mixture was shaken once a day for 30 s to homogenise the suspension, the OD at 600 nm was measured. The Chromate spectrophotometer (Model 4300) and the Chromate Manager 6.3.1.302 software (Awareness Technology) were used for analysis at times of 0 h and every 24 h for seven days, with a preagitation step of 20 s occurring before each measurement (DSG Titertek, Flow Laboratories, Lancashire, United Kingdom). A total of 760 fermentations were conducted (19 strains × 5 FA concentrations × 2 pH levels × 4 ethanol concentrations); additionally, for each condition, four biological replicates (four independent samples treated in the same manner from the same inoculum) were performed, thus resulting in a total of 3040 fermentations.

2.2.3. Determination of MICs

The minimum inhibitory concentrations (MICs) of FA that were required to reduce growth by 50 % (MIC50) and by more than 90 % (MIC90) compared with the control conditions (0 g/L FA, pH 4.0, and 0 % v/v ethanol) were determined by measuring the optical density (OD), which represents total growth, after seven days of incubation.

The MIC50 and MIC90 values were calculated for all of the conditions described in Section 2.1.2. The final total growth under each condition was determined as the difference between the OD value at the end of the 7-day incubation period (ODt_7) and the OD value at the start of incubation on Day 0 (ODt_0), as explained in Equation (1). To evaluate the effect of each condition on bacterial growth, the percentage reduction was calculated by comparing the total growth under each experimental condition to the total growth observed under the control conditions (0 g/L FA, pH 4.0, and 0 % v/v ethanol) for each strain. This calculation was performed using the formula described by (Nagalakshmi et al., 2019) and is detailed in Equation (2). The MIC50 and MIC90 were defined as the lowest concentrations of FA that resulted in a reduction in total growth of approximately 50 % and 90 %, respectively, relative to the control conditions.

$$\text{Total growth} = ODt_7 - ODt_0 \quad \text{Equation 1}$$

$$\%\ \text{of growth} = (\text{Total growth} / \text{Total growthcontrol}) * 100 \quad \text{Equation 2}$$

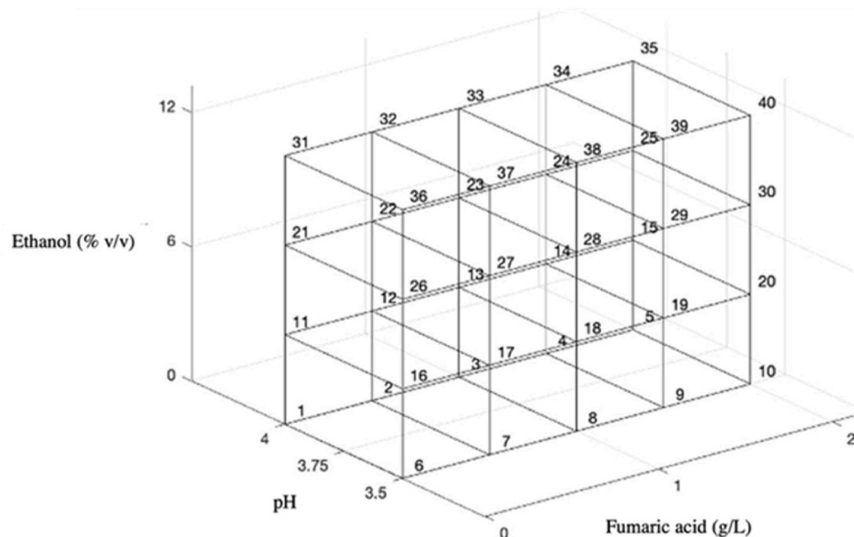

Fig. 1. Illustration of the full factorial experimental design of the studied conditions (1–40) of wine-like medium in the growth during seven days for all the strains of lactic acid bacteria and acetic acid bacteria, where x is pH (3.5 and 4), y is fumaric acid (0, 0.5, 1, 1.5 and 2 g/L), and z is ethanol (0, 4, 8 and 12 % v/v).

2.3. Determination of the effects of fumaric acid, pH and ethanol via response surface methodology (RSM)

The individual and combined effects of FA, pH and ethanol on microbial growth were analysed via response surface methodology (RSM). For this purpose, the experimental factors were normalised and coded into equally spaced levels ranging from −1 to 1 and with a mean value of 0 (rather than using the actual values of FA, pH, and ethanol). This normalisation approach enhances the robustness of the model and ensures that the magnitude of each coefficient directly reflects the importance of the corresponding factor, which is consistent with the interpretation methodology described by Box et al. (2005).

After the experimental conditions for each strain were normalised, a first-order polynomial equation was employed to calculate the individual effects of FA, pH, and ethanol on the growths of various LAB and AAB strains. Using this approach, the polynomial equation was fitted by the least squares method to model the observed percentage of total growth as a function of the primary effects of the three factors (FA, pH, and ethanol), as well as their secondary (two-factor) and tertiary (three-factor) interactions. This relationship is described in Equation (3) (Box et al., 2005):

$$\%\text{Total growth} = \beta_0 + \beta_1\cdot[\text{FA}] + \beta_2\cdot\text{pH} + \beta_3\cdot[\text{Ethanol}] + \beta_4\cdot[\text{FA}]\cdot\text{pH} + \beta_5\cdot[\text{FA}]\cdot[\text{Ethanol}] + \beta_6\cdot\text{pH}\cdot[\text{Ethanol}] + \beta_7\cdot[\text{FA}]\cdot\text{pH}\cdot[\text{Ethanol}] + \varepsilon \quad \text{Equation 3}$$

β_0: intercept ([FA], pH and [Ethanol] = 0)

$\beta_1, \beta_2, \ldots, \beta_k$: regression coefficients

ε: error term.

For the regression coefficients corresponding to each effect or interaction, positive values indicated a decrease in growth when the value of the factor decreased, as was expected for pH. Conversely, negative values indicated a decrease in growth when the value of the factor increased, as was expected for FA and ethanol. All of these effects suggested an inhibition of growth, with the magnitude of the coefficient as an absolute value directly reflecting the strength of the effect. For interactions between two or three factors, the signs could vary depending on the involved factors and their respective directions (either + or -). This analysis was performed using the MATLAB code provided by Dr. Marko Laine from the Finnish Meteorological Institute, Helsinki, Finland.

2.4. Effects of fumaric acid, pH and ethanol on biofilm formation

The assessment of the effects of FA on biofilms involved a multistep experimental approach adapted from Salamandane et al. (2023). In that study, the experimental conditions were varied to test the influence of different environmental factors on the robustness of biofilm formation and its response to FA under controlled conditions. This study was conducted in two stages. In the first stage, the ability of the strains to form biofilms was tested using nutrient-rich media (both MRS-modified and YPD media). In the second stage, the formation of biofilms was performed in liquid media to evaluate the cells in two states: the planktonic (individual cells growing freely in the liquid medium) and biofilm (cells in an adherent state) states. Moreover, biofilm formation was assessed in WLMs with different concentrations of FA and ethanol, as well as two different levels of pH.

Initially, in the first phase, the 10 selected bacterial strains (including 7 strains of LAB and 3 strains of AAB) exhibiting the highest MIC90 values (which were most resistant to FA) were selected for the first experiment. These strains were tested to determine whether they had the ability to form biofilms via MRS-modified medium for LAB and YPD growth medium for AAB. For testing, an inoculum of 10^6 CFU/mL of these selected strains was prepared in liquid media, as detailed in Section 2.1.2, followed by vortex mixing to ensure homogeneity. Subsequently, 1 mL of the inoculum was transferred into each well of a sterile 24-well microplate (P24) (maximum volume of 3 mL, Wuxi Nest Biotechnology Co., Ltd.), where a sterile stainless-steel plate coupon (1 cm × 1 cm and 1 mm thick, type 304, 2B finish; Metalurgica Quinacorte, Lda, Lousa, Portugal) was placed on the same well to facilitate biofilm formation. This procedure was conducted three times for each strain. In the empty wells, 1 mL of distilled water or Ringer's solution (Biokar Diagnostics, Beauvais, France) was added to maintain consistent environmental conditions. The microplates were incubated at 30 °C for LAB and 27 °C for AAB for 72 h. After incubation, a new microplate was utilised for the subsequent step, where a 3 mm layer of glass beads (Witeg Labortechnik GmbH, Wertheim, Germany) was placed at the bottom of each well. Sterile tweezers were employed to carefully handle and wash a steel coupon with 2 mL of Ringer's solution (1 mL per side), thereby ensuring gentle washing to remove any loosely attached cells. Following this washing step, the coupon was inserted into the well containing the beads, and additional beads (along with 1 mL of Ringer's solution) were added to the top side of the coupon. The microplate was placed on a plate shaker and agitated at maximum speed for 2 min to promote interaction between the coupon and the beads. Finally, serial dilutions of up to 10-fold were performed for each well containing a coupon, and 100 μL of each dilution was plated onto the appropriate medium for the respective microorganism. After the incubation period was completed, the colonies were counted, and strains that formed biofilms exceeding 10^4 CFU/mL after 72 h were selected for final testing with WLM to determine the effect of FA on the formation of biofilms.

The second step involved the preparation of the WLM, where both planktonic growth without coupons and biofilm formation were tested for each condition. Two FA concentrations (null or 0.6 g/L) were used, with the latter concentration representing the maximum concentration allowed in wine (OIV-OENO 581A-2021, 2021), with two pH values (3.5 and 4.0) and four ethanol concentrations (0, 4, 8, and 12 % v/v) also being utilised. The same FA, pH, and ethanol conditions were tested as controls without coupons to evaluate the growth of the strains in liquid containing WLM under planktonic conditions. To assess growth capacity, serial dilutions in solid media were performed after 7 days. A total of 960 conditions were analysed (10 strains × 2 FA conditions × 2 pH levels × 4 ethanol concentrations × 2 liquid and biofilm growth conditions × 3 biological replicates).

2.5. Statistical analysis

The data were processed and analysed using Excel 2024, version 16.89 (Microsoft Corporation, Redmond, WA, USA). To investigate the inhibitory effects of pH, ethanol, and formic acid (FA) on microbial growth, a MATLAB code provided by Professor Marko Laine from the Finnish Meteorological Institute in Helsinki was used. This code integrates linear regression analysis with the design of the experiments and response surface methodology (RSM). Additionally, ANOVA-simultaneous component analysis (ASCA) was conducted to explore the interaction effects and patterns within the data. This analysis was performed using the PLS Toolbox (Eigenvector Research, Inc.) within MATLAB (The MathWorks, Inc.). Finally, a dendrogram of RSM values was generated using agglomerative hierarchical clustering (AHC), and the similarity between the bacteria was calculated via the Pearson correlation coefficient, which was performed with XLSTAT version 2022.5.1.1388.

3. Results and discussion

3.1. MIC50 and MIC90 determinations

The MIC50 and MIC90 effects induced by FA during bacterial growth with different combinations of pH and ethanol are listed in Table 2.

For LAB, most of the specified *Lactobacillus* species exhibited greater resistance to FA at pH 4, with MIC values often exceeding 2.0 g/L.

Table 2
Fumaric acid concentration (g/L) from which Minimum Inhibitory Concentration (MIC50 and MIC90) was observed for all strains of lactic acid bacteria and acetic acid bacteria under different pH (3.5 and 4.0) and ethanol conditions (0, 4, 8 and 12 % v/v). (–) no observed growth.

Species	Strain	pH	MIC50				MIC90			
			Ethanol (% v/v)				Ethanol (% v/v)			
			0	4	8	12	0	4	8	12
Lentilactobacillus hilgardii	LH1	4.0	>2.0	1.5	1.5	0.5	>2.0	>2.0	>2.0	2.0
		3.5	1.5	1.5	1.0	–	>2.0	>2.0	1.5	–
Lactiplantibacillus plantarum	LP1	4.0	>2.0	>2.0	0.5	–	>2.0	>2.0	>2.0	2.0
		3.5	1.5	0.5	–	–	>2.0	>2.0	1.0	–
	LP2	4.0	>2.0	>2.0	0.5	–	>2.0	>2.0	2.0	1.0
		3.5	2.0	1.5	0.5	–	>2.0	>2.0	1.0	–
Liquorilactobacillus mali	LM1	4.0	2.0	1.5	0.5	–	>2.0	1.5	1.0	–
		3.5	1.0	0.5	–	–	1.5	1.0	0.5	–
Lacticaseibacillus casei	LC1	4.0	1.0	0.5	0.5	–	1.0	0.5	0.5	0.5
		3.5	–	–	–	–	0.5	0.5	–	–
Levilactobacillus brevis	LB1	4.0	2.0	1.0	–	–	>2.0	1.5	1.5	1.5
		3.5	–	–	–	–	0.5	0.5	0.5	–
Pediococcus parvulus	PP1	4.0	0.5	0.5	–	–	1.0	0.5	0.5	0.5
		3.5	0.5	0.5	–	–	0.5	0.5	–	–
Leuconostoc mesenteroides	LMES1	4.0	0.5	0.5	0.5	–	>2.0	0.5	0.5	0.5
		3.5	0.5	0.5	–	–	0.5	0.5	–	–
Oenococcus oeni	OO2	4.0	0.5	0.5	–	–	0.5	0.5	0.5	0.5
		3.5	0.5	–	–	–	0.5	0.5	0.5	–
	OO3	4.0	0.5	0.5	0.5	–	1.0	1.0	0.5	0.5
		3.5	0.5	0.5	–	–	0.5	0.5	0.5	–
	OO4	4.0	1.0	1.0	–	–	1.5	1.5	1.5	–
		3.5	1.0	–	–	–	1.0	0.5	–	–
	OO5	4.0	0.5	–	–	–	1.0	1.0	1.0	0.5
		3.5	–	–	–	–	0.5	0.5	0.5	–
	OO6	4.0	1.0	0.5	0.5	–	1.5	1.5	1.5	1.0
		3.5	0.5	–	–	–	0.5	0.5	0.5	–
Acetobacter aceti	AA1	4.0	>2.0	>2.0	0.5	0.5	>2.0	>2.0	>2.0	1.5
		3.5	1.0	–	–	–	2.0	2.0	0.5	–
	AA2	4.0	>2.0	>2.0	2.0	–	>2.0	>2.0	2.0	–
		3.5	0.5	–	–	–	>2.0	1.5	–	–
	AA3	4.0	>2.0	–	–	–	>2.0	0.5	0.5	0.5
		3.5	>2.0	–	–	–	>2.0	–	–	–
Acetobacter cerevisiae	AC1	4.0	1.5	1.5	1.0	1.0	2.0	2.0	1.5	1.5
		3.5	1.5	1.5	1.0	–	2.0	1.5	1.5	–
Gluconobacter oxydans	GO2	4.0	>2.0	>2.0	1.5	–	>2.0	>2.0	>2.0	0.5
		3.5	1.5	–	–	–	>2.0	>2.0	–	–
	GO3	4.0	2.0	2.0	0.5	–	>2.0	>2.0	>2.0	–
		3.5	–	–	–	–	2.0	2.0	–	–

However, their sensitivity to FA increased as the pH decreased to 3.5, particularly at ethanol concentrations above 4 %, where the MIC values decreased to approximately 1.0 g/L or lower. Certain strains (such as *Llb. hilgardii* and *Lpb. plantarum*) exhibited significant resistance, whereas other strains (such as *Lbs. casei*) were more sensitive and exhibited a marked decline in growth at pH 3.5 and higher ethanol levels. *Llb. hilgardii* is commonly found in wines with high ethanol contents (Jin et al., 2022). Some strains of *Lpb. plantarum* are well adapted to acidic and high-ethanol environments in wine (Brizuela et al., 2019), where resistance to FA may be attributed to its conversion to succinic acid via the reductive tricarboxylic acid cycle (Dudley and Steele, 2005; Tsuji et al., 2013). Indeed, Romero-Gil et al. (2016) reported that 2 g/L FA did not affect the growth of *Lpb. plantarum* at pH 4. The resistance of *Lvb. brevis* has also been observed throughout the fermentation process, thereby suggesting that it can withstand increasing ethanol concentrations (Maluleke et al., 2023). In the present study, the MIC values of FA for *P. parvulus* PP1 and *L. mesenteroides* LMES1 followed similar patterns, with increased sensitivity being observed at pH 3.5. The resistance of *P. parvulus* PP1 to relatively high ethanol concentrations may be due to the production of exopolysaccharides, which play a significant role in adhesion and biofilm formation (Dols-Lafargue, 2018). Among the different strains of *O. oeni*, the MIC values were generally low, especially at pH 3.5, where a concentration of 0.5 g/L FA inhibited growth. This finding indicates the greater vulnerability of *O. oeni* to FA under wine-related conditions compared with other LAB species, as described by Morata et al. (2020, 2023). Other studies have demonstrated that FA concentrations below 300 mg/L can effectively inhibit several strains of *O. oeni* in the presence of ethanol (Manca de Nadra and Strasser de Saad, 1991). Across all of the tested strains, the antimicrobial activity of FA significantly increased under more acidic conditions (lower pH) and higher ethanol concentrations. Lower MIC values at decreasing pH levels are associated with a greater proportion of undissociated acid, as determined by the pKa1 and pKa2 values of 3.03 and 4.44, respectively (Gancel et al., 2022). For example, at pH 3.5, the proportions of undissociated acid were $\alpha1 = 25.3$ % and $\alpha2 = 89.7$ %, whereas the proportions of undissociated acid were $\alpha1 = 9.7$ % and $\alpha2 = 73.4$ % at pH 4.0, according to the Henderson-Hasselbalch equation (Henry and Senozan, 2001). Thus, at pH 3.5, there is approximately 2.6 times more undissociated acid than at pH 4.0, thereby allowing the acid to more freely penetrate the membrane, lower the intracellular pH, disrupt metabolism, and reduce viability (Shah et al., 2016). Additionally, previous studies have shown that FA can inhibit the glutamate decarboxylase (GAD) system, which regulates the intracellular pH under acidic conditions. This system transforms glutamate into GABA, which is a reaction that consumes protons and increases the intracellular pH to prevent intracellular acidification (Barnes and Karatzas, 2020). In general, the resistance to FA at pH 4 can be attributed to the maintenance of cellular integrity and metabolic function in the presence of weak organic acids, which is a common feature of many LAB species (Zapaśnik et al., 2022). Other studies have demonstrated that most LAB possess genes that encode the enzyme fumarate reductase (which is involved in the conversion of fumarate into succinic acid); moreover, this enzyme

activity explained the metabolic capacity of FA and the resistance features of these microorganisms (Kaneuchi et al., 1988; Verce et al., 2019). However, the expression of this gene varies according to species and strain, which could explain the observed variability in the metabolic capacities of these bacteria (Verce et al., 2019). This variability extends to acid stress resistance mechanisms, thereby further explaining the differences observed across species and strains (Barnes and Karatzas, 2020). Increased ethanol levels also affect the cytoplasmic membrane, thus altering its structure and function. Specifically, ethanol increases membrane fluidity, which enhances intracellular acid transport and leads to intracellular acidification. This process depends on the lipid composition of the membrane, as ethanol reduces interactions between fatty acid chains, thus increasing phospholipid mobility and organic acid entry (Bonomo et al., 2018). The results of this study were obtained by using a WLM, which enabled the estimation of the actual behaviour of LAB in wines, where the maximum authorised dose of FA is 0.6 g/L. This concentration was not effective against the strains *Llb. hilgardii* LH1, *Lpb. plantarum* LP1 and LP2, *Lqb. mali* LM1 and *Lvb. brevis* LB1 under the investigated conditions. In contrast, this concentration demonstrated effectiveness against *Lbs. casei*, *P. parvulus*, and *O. oeni* at ethanol concentrations greater than 4 % (v/v) at pH levels of 3.5 and 4.0.

Similar patterns were observed for the AAB strains (including *Acetobacter* and *Gluconobacter* species). At pH 4.0, most of the strains exhibited MIC values exceeding 2.0 g/L FA, which decreased with increasing ethanol concentrations and decreasing pH. *Acetobacter aceti* demonstrated high resistance to FA, with MIC values decreasing as ethanol levels increased, particularly at 12 % (v/v) (where no growth was observed). These results align with the ability of this strain to metabolise ethanol into acetic acid (Sakurai et al., 2011) and its ability to adapt to low-pH environments (Du Toit and Pretorius, 2002). Additionally, some studies have shown that some strains of *A. aceti* possess two types of fumarate hydratases that provide the ability to metabolise FA (Sakurai et al., 2011), which consequently induces tolerance to acetic acid and other organic acids (Lynch et al., 2019). *A. cerevisiae* is a typical AAB found in grape must that is known for its tolerance to wine conditions (Navarro et al., 2013) and resistance to FA; moreover, it grows well below pH 4 (Song et al., 2016). In this study, *G. oxydans* (another common AAB strain documented in winemaking) demonstrated greater resistance to FA compared to *Acetobacter*, although its growth decreased at higher ethanol concentrations and lower pH values. *G. oxydans* can grow in high ethanol concentrations but is more prevalent in grape must, wherein it thrives at pH 3.5 (Deppenmeier et al., 2002; Mamlouk and Gullo, 2013).

Overall, the results demonstrate that the antimicrobial activity of FA is significantly influenced by pH and ethanol concentration, as has been widely reported (Peh et al., 2020; Bonomo et al., 2018; Drysdale and Fleet, 1989; Guerzoni et al., 1995; Navarro et al., 2013; Sumby et al., 2019). By comparing bacterial behaviours under similar conditions, this study highlights the variable resistance of species and strains to FA at concentrations exceeding the maximum permitted concentration of 0.6 g/L under challenging pH and ethanol conditions.

3.2. Determination of the individual and combined effects of fumaric acid, pH and ethanol via response surface methodology (RSM)

The interaction of FA with pH and ethanol was assessed via the RSM approach. The growth percentages of the 19 strains of LAB and AAB were analysed by quantifying the significance of the effect and interaction of each factor affecting growth. To interpret the results, coefficient values (cv) with positive or negative signs were used for the individual factors, and absolute coefficient values were used for the combined factors. This inhibitory effect was reflected in the individual coefficients of the evaluated ranges, where the coefficient values were negative for FA and ethanol (thus indicating that higher concentrations resulted in reduced growth), and positive coefficient values were observed for pH (thus indicating that higher pH promoted growth). For interactions, the signs of the coefficients were dependent on the influencing factors. The significance of the calculated coefficients was determined using p values.

FA strongly inhibited the growth of all of the LAB strains (Table 3, Fig. 2 and Supplementary Figure SD1). Among these strains, *O. oeni* strains demonstrated the highest sensitivity to FA, followed by *Llb. hilgardii* LH1 (cv = −21.21) and *P. parvulus* PP1 (cv = −21.18). These results align with those of previous studies demonstrating the ability of FA to inhibit *O. oeni* (Cofran and Meyer, 1970; Morata et al., 2020, 2023). In contrast, the effect of pH varied among the strains, although increased pH consistently promoted growth. *Lvb. brevis* LB1 (cv = 16.10) exhibited the greatest positive response to increased pH, followed by *Llb. hilgardii* LH1 (cv = 11.82), thereby suggesting that these strains were more affected by lower pH levels. However, pH was the factor exhibiting the least overall impact and significance. Compared with other species, *O. oeni* and *P. parvulus* strains generally demonstrated greater tolerance to low pH, as evidenced by their minimum cv values of 2.31 and the lower coefficients for the combined effects of FA and pH. These findings were consistent with previous studies indicating that LAB are naturally adapted to thrive in acidic environments, where rapid decreases in external pH do not constitute acid shock (Siegumfeldt et al., 2000). With respect to the individual effects of ethanol, the cv values ranged from −15.99 to 8.61, with a coefficient of variation of approximately 54 % being noted, thus indicating significant disparity between species. The strains that were most affected by ethanol were *Lpb. plantarum* LP1 and LP2 (cv = −25.63 and −33.25, respectively), followed by *Llb. hilgardii* LH1 (cv = −25.98). These results align with studies showing that ethanol significantly limits LAB growth, especially at concentrations greater than 6 % (Diez et al., 2010; Pielech-Przybylska et al., 2019). Conversely, *O. oeni* exhibits greater resilience to ethanol, which is consistent with its ability to adapt to wine-related stress conditions (Bonomo et al., 2018; Tourdot-Marechal et al., 2000). In terms of the interactions between the inhibitors, the combination of FA and pH exerted a significant negative effect on the growth of some strains, with the strongest impacts being observed in *Lbs. casei* LC1 (cv = −14.94), *Lvb. brevis* LB1 (cv = −10.65), and *O. oeni* OO6 (cv = −10.02). The combined effect of FA and ethanol generally had a more pronounced impact across all of the strains, particularly on *P. parvulus* PP1 (cv = −19.16), *O. oeni* OO2 (cv = −17.36), and *O. oeni* OO4 (cv = −19.66). Moreover, the interaction between pH and ethanol elicited a significant effect on *Lqb. mali* LM1 (cv = −8.82) and *Lvb. brevis* LB1 (cv = −13.42). However, for the remaining strains, the combination of pH and ethanol did not play a critical role in growth inhibition. The three-way interaction between FA, pH, and ethanol significantly affected only *Llb. hilgardii* LH1 (cv = −8.75) and *O. oeni* OO4 (cv = 8.67), thereby suggesting that these strains are particularly vulnerable to the combined stress of all three factors. Most of the strains demonstrated high R^2 values, thus indicating that the inhibitors explained most of the observed growth variability. However, *P. parvulus* PP1 (57.21 %), *O. oeni* OO3 (54.51 %), and *O. oeni* OO5 (65.58 %) exhibited lower R^2 values, thereby suggesting that other factors (such as experimental error) may have influenced their growth (Box et al., 2005; Hwang and Lee, 2018).

With respect to the inhibitory effect on AAB strains (Table 4, Fig. 3 and SD1), FA exhibited a highly significant inhibitory effect on all of the strains, with the strongest impact being observed in *A. aceti* strains (cv = −22.69, −10.44, and −11.06) and *A. cerevisiae* AC1 (cv = −39.49). The *G. oxydans* strains were less sensitive, thereby indicating a milder inhibitory effect, which is consistent with previous studies that highlighted the ability of *G. oxydans* to synthesise FA (Neffe-Skocińska et al., 2024). An increase in pH positively influenced growth in all of the strains, although it had the least impact on growth compared to the other factors. *A. aceti* AA1 (cv = 20.38) and *G. oxydans* GO3 (cv = 15.85) were the most responsive strains to increasing pH levels. AAB generally grow within a broader pH range (3.0–8.0), thereby demonstrating their adaptability to acidic conditions (Sengun and Karabiyikli, 2011); however, optimal growth occurs between pH levels of 5.4 and 6.3 (Navarro

Table 3
Individual and combined effects of fumaric acid ([FA] 0–2 g/L), pH (3.5–4), and ethanol ([Ethanol] 0–12 % v/v) expressed as coefficients of regression on the growth of lactic acid bacteria calculated by Response Surface Methodology. The table shows the coefficients for the individual factors ([FA], pH, and [Ethanol]), their pairwise interactions ([FA] * pH, [FA] * [Ethanol] and pH * [Ethanol]), and their three-way interaction ([FA] * pH * [Ethanol]), as well as the corresponding values of the coefficient of determination (R^2) for each RSM model. Statistical significance: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

Species	*Lentilactobacillus hilgardii*	*Lactiplantibacillus plantarum*		*Liquorilactobacillus mali*	*Lacticaseibacillus casei*	*Levilactobacillus brevis*	*Pediococcus parvulus*	*Leuconostoc mesenteroides*	*Oenococcus oeni*				
Strain	LH1	LP1	LP2	LM1	LC1	LB1	PP1	LMES1	OO2	OO3	OO4	OO5	OO6
Intercept	47.01	31.20	40.61	20.42	12.35	20.64	11.03	12.47	10.23	11.52	14.87	8.89	14.93
[FA]	−21.21***	−14.97***	−19.23***	−19.02***	−20.09***	−19.58***	−21.18***	−19.79***	−20.33***	−22.04***	−19.14***	−16.07***	−22.33***
pH	11.82***	7.48***	7.94***	7.74***	9.78***	16.1***	2.31	5.40	2.31	3.54	9.61***	4.09	8.78***
[Ethanol]	−25.98***	−26.63***	−33.25***	−23.15***	−8.66**	−17.88***	−10.26*	−11.76**	−8.8*	−8.6*	−15.47***	−6.95*	−10.45***
[FA] * pH	0.74	−5.01**	0.68	−0.33	−14.94***	−10.65**	−3.74	−5.63	−4.49	−6.08	−9.32***	−6.45*	−10.02***
[FA]* [Ethanol]	2.37	4.65	1.01	16.11***	12.85**	8.93*	19.16**	15.21**	17.36**	15.73**	19.66***	12.74**	14.64***
pH * [Ethanol]	4.98*	−2.55	−0.73	−8.82**	−5.32	−13.42***	0.92	−2.91	−0.61	−1.87	−9.35***	−2.47	−5.12*
[FA] * pH * [Ethanol]	−8.75**	2.31	−6.09	−1.99	6.16	0.3	−3.19	−2.48	0.99	2.28	8.67**	3.78	3.99
R^2	0.9216	0.9215	0.8984	0.8538	0.7538	0.8325	0.5721	0.8538	0.5451	0.5779	0.8975	0.6558	0.8435

et al., 2013). Ethanol acted as a potent inhibitor for all of the strains, with *A. aceti* AA3 (cv = −39.86) exhibiting the highest sensitivity. Most AAB strains can tolerate ethanol concentrations of up to 5–10 % (v/v) in similar proportions (Gullo et al., 2014). Additionally, the interaction between FA and pH was not significant, except for *G. oxydans* GO3 (cv = −3.61), which was possibly due to the capacity of AAB to resist organic acids in lower pH environments (Lynch et al., 2019; Zgardan et al., 2023). The interaction between FA and ethanol was more pronounced, particularly regarding *A. aceti* AA2 (cv = 9.35) and *A. cerevisiae* AC1 (cv = 10.35), thereby suggesting that ethanol may reduce the inhibitory effect of FA on these strains. *G. oxydans* GO3 also exhibited a positive interaction (cv = 7.98), thus reinforcing the strain-specific nature of these interactions. Finally, the three-way interaction between FA, pH, and ethanol had a significant inhibitory effect on *A. aceti* AA1 (cv = −19.05) and *A. cerevisiae* AC1 (cv = −15.88), which indicated that the combination of all three factors creates a highly inhibitory environment for these strains. The R^2 values for AAB indicated that the proposed model was very accurate for most of the strains, except for *G. oxydans* GO2 (R^2 = 72.11 %), thereby suggesting the need for further investigations into other growth-influencing factors (Box et al., 2005). Overall, FA, pH, and ethanol individually exerted significant inhibitory effects on LAB and AAB, with notable variations being observed in the sensitivities across the strains. The interaction effects highlighted the strain-specific nature of growth inhibition under multifactorial stress conditions. The results indicated that the primary determinants of FA inhibition were FA concentration and ethanol level, whereas pH had less significance, whereby it influenced the proportion of undissociated acid. In conclusion, FA may be more effective at lower pH values; however, its inhibitory efficacy can improve with higher ethanol concentrations.

3.3. ASCA analysis and dendrogram

To obtain a comprehensive interpretation of the growth effects of FA, pH and ethanol for all strains of LAB and AAB, an ASCA (Bertinetto et al., 2020) was conducted, as previously described by Ezenarro et al. (2024). The absolute values of the regression coefficients from the RSM models (Tables 3 and 4) were used to assess the extent of the inhibition of growth, as depicted in Fig. 4. In the right panel (Fig. 3B), the influence of the three variables (FA, pH, and ethanol) on the principal component (PC1) for both the LAB and AAB strains is shown, with a p value of 0.017. The results indicated that the inhibition caused by pH and ethanol had the strongest influence on PC1, thus suggesting that these variables were the most significant factors in differentiating LAB and AAB strains. In the left panel (Fig. 3A), the distributions of the LAB and AAB strains in relation to all of the investigated factors are shown. When focusing on the LAB strains (represented as red points in the left panel), most of the strains (including *Lbs. casei*, *P. parvulus*, *L. mesenteroides*, and *O. oeni*) were positioned below the dotted line on the Y-axis, which corresponded to the strains that were most sensitive to FA, thereby confirming the findings of Table 2. This distribution aligned with the broader tolerance to these inhibitors that has been experimentally observed in other studies (Morata et al., 2023; Pérez-Díaz, 2011), where the strains demonstrated less sensitivity to pH and more sensitivity to ethanol. In contrast, *Llb. hilgardii*, *Lpb. plantarum*, and *Lvb. brevis* were positioned in the positive PC1 area, thus indicating that these strains were more strongly affected by variations in pH (decreased levels) and ethanol (increased levels), with less of an influence being exerted by FA. These results were consistent with previous findings showing that LAB species (particularly *Lpb. plantarum* and *Llb. hilgardii*) exhibited sensitivity to ethanol concentrations greater than 6 % (Diez et al., 2010) but demonstrated relatively less inhibition due to FA. The green points in the left panel represent AAB strains, which were primarily located in the upper right region of the PC1 axis. Except for *A. cerevisiae* AC1, these strains were more affected by decreased pH and increased ethanol levels compared to FA, thereby suggesting that AAB were well adapted to organic acids in the medium, as has been previously described in the

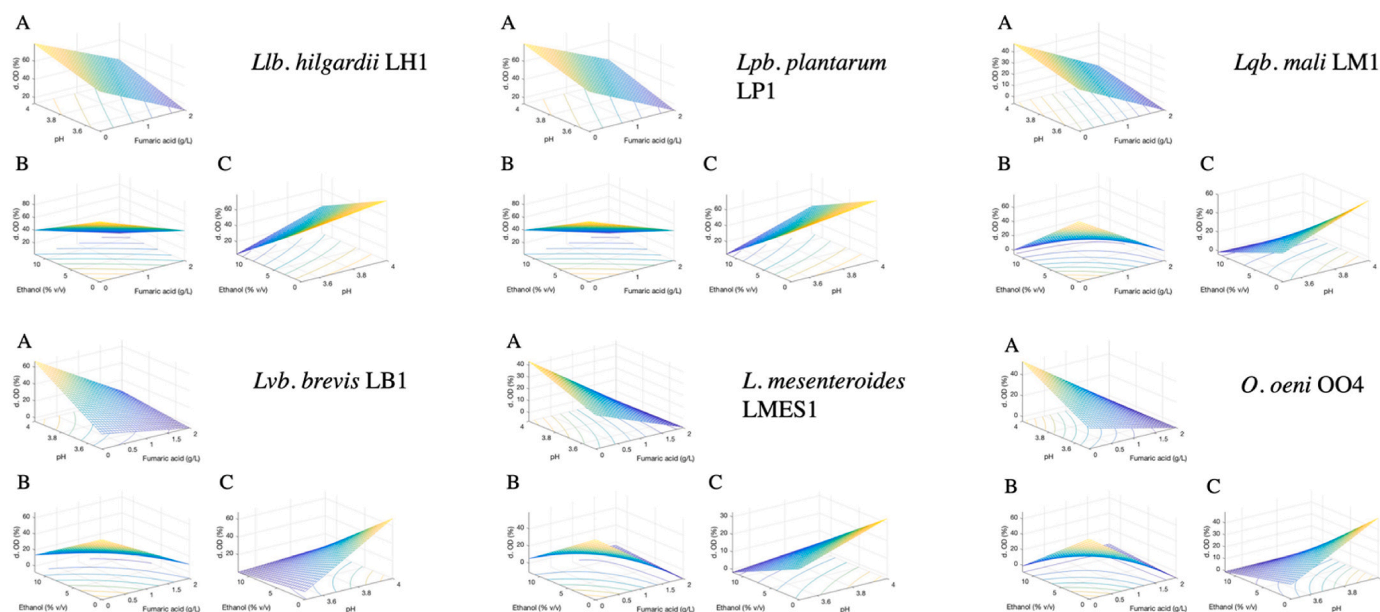

Fig. 2. The Response Surface Methodology (RSM) of strains *Lentilactobacillus hilgardii* LH1, *Lactiplantibacillus plantarum* LP1, *Liquorilactobacillus mali* LM1, *Levilactobacillus brevis* LB1, *Leuconostoc mesenteroides* LMES1, *Oenococcus oeni* OO4, under the influence of fumaric acid (0–2 g/L), pH (3.5 and 4.0), and ethanol (0–12 % v/v) on their growth expressed in % of optical density at 600 nm. The RSM was represented in three graphs: Graph A shows the relationship between pH and fumaric acid (x: pH, y: fumaric acid, z: % OD); Graph B shows the relationship between ethanol and fumaric acid (x: ethanol, y: fumaric acid, z: % OD) and Graph C shows the relationship between ethanol and pH (x: ethanol, y: pH, z: % OD).

Table 4
Individual and combined effects of fumaric acid ([FA] 0–2 g/L), pH (3.5–4), and ethanol ([Ethanol] 0–12 % v/v) expressed as significance coefficients of regression on the growth of acetic bacteria calculated by Response Surface Methodology. The table shows the coefficients for the individual factors ([FA], pH, and [Ethanol]), their pairwise interactions ([FA] * pH, [FA] * [Ethanol] and pH * [Ethanol]), and their three-way interaction ([FA] * pH * [Ethanol]), as well as the corresponding values of the coefficient of determination (R^2) for each RSM model. Statistical significance: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

Species	*Acetobacter aceti*			*Acetobacter cerevisiae*	*Gluconobacter oxydans*	
Factors/Strain	AA1	AA2	AA3	AC1	GO2	GO3
Intercept	41.76	22.81	41.35	44.05	18.06	24.47
[FA]	−22.69***	−10.44***	−11.06**	−39.49***	−10.97**	−8.49***
pH	20.37***	8.28***	13.58***	8.53**	6.43*	15.85***
[Ethanol]	−25.94***	−26.80***	−39.86***	−27.69***	−26.76***	−21.34***
[FA] * pH	−2.22	−2.96	−6.76	−4.22	−5.23	−3.61*
[FA]* [Ethanol]	2.62	9.35***	2.03	10.35*	10.05	7.98**
pH * [Ethanol]	−3.14	−5.73**	1.15	1.47	−5.83	−10.17***
[FA] * pH * [Ethanol]	−19.05***	−0.32	−1.72	−15.88**	−0.29	1.92
R^2	0.9480	0.9217	0.8573	0.8833	0.7211	0.9267

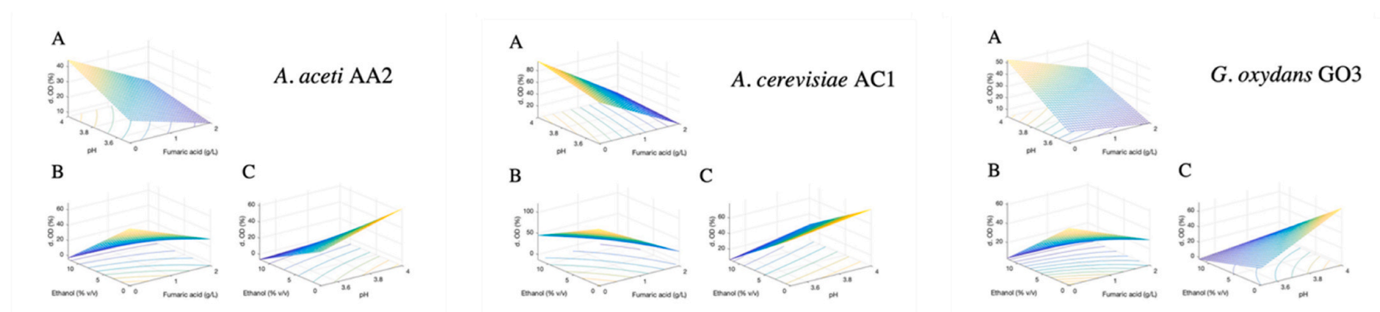

Fig. 3. The Response Surface Methodology (RSM) of strains *Acetobacter aceti* AA2, *Acetobacter cerevisiae* AC1 and *Gluconobacter oxydans* GO3, under the influence of fumaric acid (0–2 g/L), pH (3.5 and 4.0), and ethanol (0–12 % v/v) on their growth expressed in % of optical density at 600 nm. The RSM was represented in three graphs: Graph A shows the relationship between pH and fumaric acid (x: pH, y: fumaric acid, z: % OD); Graph B shows the relationship between ethanol and fumaric acid (x: ethanol, y: fumaric acid, z: % OD) and Graph C shows the relationship between ethanol and pH (x: ethanol, y: pH, z: % OD).

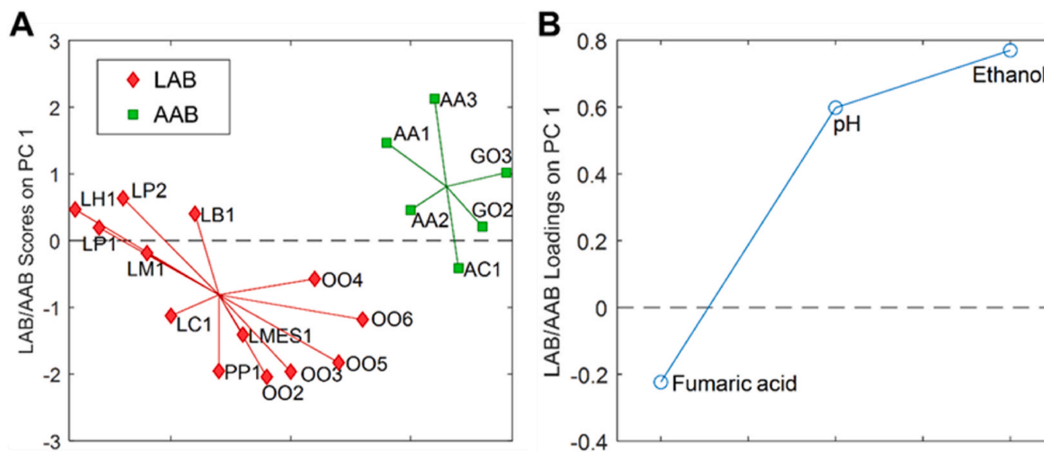

Fig. 4. ASCA (ANOVA-Simultaneous Component Analysis) plot depicting the effects of fumaric acid, pH, and ethanol on lactic acid bacteria and acetic acid bacteria strains for the absolute values of the regression coefficients of RSM models. The panel A shows the "Strain Scores on PC1", where LAB strains are represented in red and acetic acid bacteria strains in green. The panel B displays the "Strain Loadings on PC1," indicating the contributions of each inhibitor to the principal component. Strain code as described in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

literature (Drysdale and Fleet, 1989; Mamlouk and Gullo, 2013). The separation between the AAB and LAB strains highlighted their different sensitivities to the inhibitors. Although LAB strains generally exhibited greater sensitivity to FA, and AAB strains generally exhibited greater sensitivities to pH and ethanol, there were exceptions. For example, certain *Lactobacillus* strains in the LAB group exhibited a response similar to that of the AAB strains, whereas *A. cerevisiae* in the AAB group demonstrated a distinct response pattern.

In conclusion, the LAB and AAB strains exhibited different trends in sensitivities to FA, pH, and ethanol, with FA generally being more effective in inhibiting the growth of LAB compared with AAB.

Agglomerative hierarchical clustering (AHC) was performed to examine whether similarities existed among all of the LAB and AAB strains based on their responses to FA, pH, and ethanol (Fig. 5). To construct the dendrogram, the absolute values of the regression coefficients derived from RSM were used. This visualisation allowed for the identification of strain clusters with similar behaviours. The analysis revealed three main clusters. Cluster C1 (green) included *Llb. hilgardii* LH1, *Lpb. plantarum* LP1 and LP2, *Lqb. mali* LM1, *Lvb. brevis* LB1, *A. aceti* AA2 and AA3, and *G. oxydans* GO2 and GO3. These strains of the genus *Lactobacillus* (with the exception of *Lacticaseibacillus casei, Acetobacter*, and *Gluconobacter*) exhibited a homogeneous response to the investigated conditions, with a similarity greater than 65 % being observed. Cluster C2 (blue) consisted of *Lbs. casei* LC1, *P. parvulus* PP1, *L. mesenteroides* LMES1, and *O. oeni* strains (OO2, OO3, OO4, OO5, and OO6). These strains exhibited highly homogeneous responses to the analysed conditions, with nearly 80 % similarity being observed; moreover, they were grouped together as bacterial cocci, with a response demonstrating less sensitivity. Cluster C3 (red) comprised *A. cerevisiae* AC1 and *A. aceti* AA1, which are predominantly acetic acid bacteria (AAB); moreover, these strains exhibited strong similarity (above 75 %) and nearly identical responses to the investigated factors. From a taxonomic perspective, this clustering revealed that *Leuconostoc* and *Oenococcus* species were closely grouped, whereas *Lactiplantibacillus* formed a distinct group on its own. The genera *Levilactobacillus*, *Lacticaseibacillus*, and *Pediococcus* were more distantly related to *Lactiplantibacillus* in the taxonomic clusters (Dicks and Endo, 2009).

3.4. Effects of fumaric acid, pH and ethanol on the formation of biofilms

To evaluate the effects of FA on biofilm formation, 7 strains of LAB (*Llb. hilgardii* LH1, *Lpb. plantarum* LP1, *Lqb. mali* LM1, *Lvb. brevis* LB1, *L. mesenteroides* LMES1, *O. oeni* OO4 and *O. oeni* OO5) and 3 strains of AAB (*A. aceti* AA2, *A. cerevisiae* AC1 and *G. oxydans* GO3) were selected

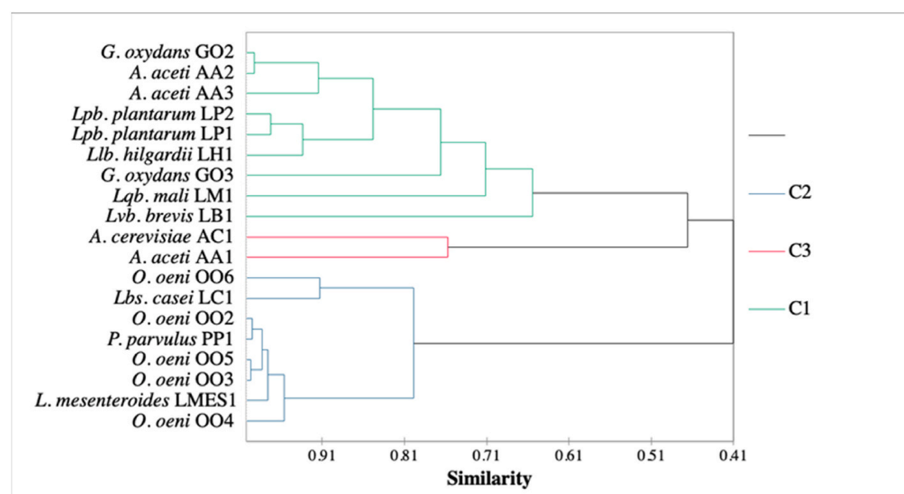

Fig. 5. Dendrogram representing the hierarchical relationships among all strains of lactic acid bacteria and acetic acid bacteria based on their response to the effects of fumaric acid, pH, and ethanol, using the absolute values of the regression coefficients derived from the Response Surface Methodology.

due to their higher MIC90 values. The inhibitory effects of FA at 0 and 0.6 g/L (the maximum legal dose) on growth and biofilm formation were assessed under different ethanol concentrations (0, 4, 8, and 12 % v/v) and pH levels (3.5 and 4.0).

The results revealed that, in the case of LAB (Table 5), *Llb. hilgardii* LH1 grew at pH 4 under all of the ethanol concentrations in both planktonic and biofilm conditions. Moreover, FA did not exhibit an inhibitory effect on biofilm formation at ethanol concentrations of 0 % and 4 % v/v. However, at 8 % v/v ethanol, FA began to inhibit biofilm formation, and this inhibition persisted at 12 % v/v ethanol. At pH 3.5, the strain grew at ethanol concentrations of 0 % and 4 % v/v; however, when present, FA inhibited growth at both concentrations. No growth was observed at ethanol concentrations of 8 % and 12 %. For *Lpb. plantarum* LP1, at pH levels of 4 and 3.5, growth was detected at ethanol concentrations of 0 % and 4 % v/v, with FA consistently demonstrating an inhibitory effect in all cases. However, no growth occurred at ethanol concentrations of 8 % and 12 % v/v at pH 3.5, which prevented the assessment of the inhibitory effects of FA at these higher ethanol concentrations. In *Lqb. mali* LM1, at pH 4, growth was observed at ethanol concentrations of 0, 4 and 8 % v/v, with FA consistently inhibiting growth and preventing biofilm formation in all cases. At pH 3.5, the growth pattern was similar, with growth being detected at ethanol concentrations of 0 %, 4 %, and 8 % v/v and inhibition by FA being observed in both the planktonic and biofilm states. At 12 % v/v ethanol, no growth was observed under any condition. For *Lvb. brevis* LB1, growth was recorded at all of the ethanol concentrations at pH 4 (both in liquid and biofilm cultures). However, FA did not inhibit biofilm formation in the presence of concentrations of 0 %, 4 %, or 12 % v/v ethanol, whereas inhibition was observed in liquid cultures under the same ethanol conditions. At pH 3.5, growth occurred at concentrations of 0 % and 4 % v/v ethanol, with FA inhibiting growth in both cases. At higher ethanol concentrations, no growth was observed, except in the liquid culture at 4 % v/v ethanol, where FA inhibited growth. These findings suggest that biofilm formation may provide some protection, which is a phenomenon that has been previously documented in *Lvb. brevis* (a common biofilm producer associated with wine spoilage) (Tristezza et al., 2010). For L. *mesenteroides* LMES1, growth was observed at concentrations of 0 %, 4 % and 8 % v/v ethanol at pH 4 in liquid cultures, whereas biofilm formation only occurred at a concentration of 0 % v/v ethanol. Additionally, FA consistently inhibited planktonic growth. Moreover, no growth was observed at 12 % v/v ethanol. At pH 3.5, growth was only detected in liquid ethanol at ethanol concentrations of 0 % and 4 % v/v, with FA inhibiting growth in both cases, thereby indicating that *L. mesenteroides* LMES1 lacked the ability to form biofilms.

For *O. oeni* OO4, growth was observed at all of the ethanol concentrations (0, 4, 8and 12 % v/v) at pH 4 in liquid media. Biofilm formation occurred at 0 % and 4 % v/v ethanol, with FA consistently inhibiting growth in all of the samples. At pH 3.5, growth in liquid cultures occurred only at ethanol concentrations of 0 % v/v and 4 % v/v, and biofilm formation was restricted to 0 % v/v ethanol, with FA exhibiting inhibitory effects under all of the conditions. Similarly, for *O. oeni* OO5, growth in liquid cultures was observed at all of the ethanol concentrations at pH 4, and FA inhibited growth in all of the cases.

Biofilm formation occurred only at 0 % v/v ethanol, with FA demonstrating inhibitory effects. At pH 3.5, growth occurred at ethanol concentrations of 0 % v/v and 4 % v/v, with FA inhibiting growth; moreover, no biofilm formation was observed under any condition. *O. oeni* is capable of producing exopolysaccharides under specific conditions (Dimopoulou et al., 2016), and the production of exopolysaccharides in *O. oeni* depends on both glucose and fructose availabilities, ethanol levels, and nitrogen content in the medium; moreover, this production is strain dependent (Ibarburu et al., 2007). Across all of the strains, with the exception of *Llb. hilgardii* LH1 and *Lvb. brevis* LB1 (a common biofilm former in wine), a general trend was observed where higher ethanol concentrations and lower pH levels reduced bacterial growth and biofilm formation. FA consistently demonstrated inhibitory effects at 0.6 g/L, particularly in terms of biofilm formation, with the impact being more pronounced at lower pH values (3.5). Furthermore, Ji et al. (2023) and Tsukatani & Sakata (2022) reported that the combination of FA with lactic and ferulic acids (a hydroxycinnamic acid found in wine) significantly reduced biofilm formation in foodborne pathogens, thereby suggesting a synergistic effect that enhances microbial safety in food products.

Table 5
Effect of fumaric acid (0 and 0.6 g/L), pH (3.5 and 4) and ethanol (0, 4, 8 and 12 % v/v) in the growth after seven days in planktonic (growth free in wine-like medium) and in the formation of biofilms (in stainless-steel plate coupon in wine-like medium) on different strains of lactic and acetic acid bacteria. Letters "g" indicates growth, "ng" indicates no growth, inhibition was recorded as positive (+) if 0.6 g/L of fumaric acid reduced growth in liquid or biofilm formation, and negative (−) if no inhibition in liquid or biofilm formation was observed. Biological replicates = 4.

	Ethanol (% v/v)		0				4				8				12			
			Planktonic		Biofilm		Planktonic		Biofilm		Planktonic		Biofilm		Planktonic		Biofilm	
	Strain	pH/ Fumaric acid	0 g/L	0.6 g/L	0 g/L	0.6 g/L	0 g/L	0.6 g/L	0 g/L	0.6 g/L	0 g/L	0.6 g/L	0 g/L	0.6 g/L	0 g/L	0.6 g/L	0 g/L	0.6 g/L
LACTIC ACID BACTERIA	*Llb. hilgardii* LH1	4.0	g	−	g	−	g	−	g	−	g	−	g	+	g	+	g	+
		3.5	g	+	g	+	g	+	ng	ng	g	+	ng	ng	ng	ng	ng	ng
	Lpb. plantarum LP1	4.0	g	+	g	+	g	+	g	+	g	+	g	+	g	+	g	+
		3.5	g	+	g	+	g	+	ng	ng	g	+	ng	ng	ng	ng	ng	ng
	Lqb. mali LM1	4.0	g	+	g	+	g	+	g	+	g	+	g	+	ng	ng	ng	ng
		3.5	g	+	g	+	g	+	g	+	g	+	ng	ng	ng	ng	ng	ng
	Lvb. brevis LB1	4.0	g	+	g	−	g	+	g	−	g	+	g	−	g	+	g	−
		3.5	g	+	g	+	g	+	g	+	ng	ng	g	+	ng	−	ng	ng
	L. mesenteroides LMES1	4.0	g	+	g	+	g	+	ng	ng	g	+	ng	ng	g	+	ng	ng
		3.5	g	+	ng	ng	g	+	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng
	O. oeni OO4	4.0	g	+	g	+	g	+	g	+	g	+	ng	ng	g	+	ng	ng
		3.5	g	+	g	+	g	+	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng
	O. oeni OO5	4.0	g	+	g	+	g	+	ng	ng	g	+	ng	ng	g	+	ng	ng
		3.5	g	+	ng	ng	g	+	ng	ng	g	+	ng	ng	ng	ng	ng	ng
ACETIC ACID BACTERIA	*A. aceti* AA2	4.0	g	−	g	−	g	−	g	−	g	+	g	+	g	+	g	+
		3.5	g	−	g	−	g	−	g	−	g	+	g	+	ng	ng	g	+
	A. cerevisiae AC1	4.0	g	−	g	−	g	−	ng	ng	g	+	ng	ng	g	+	ng	ng
		3.5	g	−	g	−	g	−	ng	ng	ng	+	ng	ng	ng	ng	ng	ng
	G. oxydans GO3	4.0	g	−	g	−	g	−	g	−	g	+	ng	ng	ng	ng	ng	ng
		3.5	g	−	g	−	g	−	g	−	ng	ng	ng	ng	ng	ng	ng	ng

The results for the AAB strains are shown in Table 5. For *A. aceti* AA2, growth was observed at all of the ethanol concentrations at pH 4 under both liquid and biofilm conditions. FA had no inhibitory effect at ethanol concentrations of 0 % and 4 % v/v. Additionally, at pH 3.5, growth in liquid cultures occurred at ethanol concentrations of 0 %, 4 %, and 8 % v/v, with FA exhibiting inhibitory effects only at 8 % v/v. With 12 % v/v ethanol, no growth occurred in the liquid cultures; however, biofilm formation was observed with FA inhibition. These results revealed the capacity of *A. aceti* to form biofilms in wine under stress conditions (Bartowsky and Henschke, 2008; Tristezza et al., 2010). For *A. cerevisiae* AC1, growth in liquid cultures was observed under all of the conditions at pH 4, with no inhibition by FA being observed at ethanol concentrations greater than 8 % v/v. Biofilm formation occurred only at 0 % v/v ethanol, and FA exerted no inhibitory effect at pH 3.5. A similar trend was observed; specifically, growth occurred at ethanol concentrations of 0 % and 4 % without inhibition by FA; however, no growth was detected at higher ethanol concentrations. Thus, *A. cerevisiae* lacks the ability to form biofilms under these stress conditions. For *G. oxydans* GO3 at pH 4, growth in liquid medium was observed at ethanol concentrations of 0 %, 4 %, and 8 %, with inhibition by FA occurring only at 8 % ethanol. Biofilm formation occurred at concentrations of 0 % and 4 % ethanol without inhibition by FA. At pH 3.5, growth in liquid medium and biofilm formation were observed at ethanol concentrations of 0 % and 4 %, with no inhibitory effects of FA being observed on either growth or biofilm formation.

In summary, the combined effects of pH, ethanol, and FA on biofilm formation were evident. At pH 4, the strains exhibited greater growth capacity, even at higher ethanol concentrations. In contrast, at pH 3.5, growth was more restricted. Moreover, FA exhibited minimal inhibitory effects, particularly at lower ethanol concentrations, which was likely due to the relatively low concentration of FA that was used (0.6 g/L). However, with 12 % (v/v) ethanol, the inhibitory effect of FA became more pronounced in some strains, particularly concerning biofilm formation. At the highest ethanol concentration (12 %), growth was significantly limited, thus making it difficult to fully assess the inhibitory potential of FA under these conditions.

4. Conclusions

These results demonstrate the effectiveness of FA in inhibiting the growth of LAB and AAB under wine-like conditions with pH values of 3.5 and 4 and ethanol concentrations of 0 %, 4 %, 8 %, and 12 % (v/v). In terms of the MIC values, the concentration of FA required to reduce bacterial populations by 50 % (MIC50) and 90 % (MIC90) was influenced by pH and ethanol concentration in most cases. Specifically, a lower pH (3.5) and higher ethanol concentration ($\geq$8 % v/v) enhanced the inhibitory effects of FA, thereby reducing the MIC required to inhibit growth. This trend was consistently demonstrated across most of the LAB and AAB strains.

The inhibitory effects of FA on LAB strains were most pronounced in *O. oeni*, which exhibited the lowest MIC values under the investigated pH and ethanol ranges. Conversely, *Lactobacillus* species demonstrated greater resistance, requiring higher FA concentrations (MIC90 values of up to 2.0 g/L) for effective inhibition under these conditions. Similarly, the AAB strains also exhibited notable resistance to FA, with MIC values being observed near 2 g/L under favourable conditions (a high pH level of 4 and 0 % ethanol). However, their growth was significantly reduced at higher ethanol concentrations, with *G. oxydans* emerging as the most resistant AAB species to FA compared with *A. aceti* and *A. cerevisiae*. Overall, the MIC50 and MIC90 values were highly strain dependent among the investigated species.

The regression coefficients that were calculated using RSM revealed that all three factors (pH, ethanol, and FA) significantly affected the growth of individual LAB and AAB strains. In general, LAB strains were more sensitive to FA, whereas AAB strains were more sensitive to pH and ethanol. Notable exceptions were observed, as many *Lactobacillus* strains exhibited behaviour similar to that of AAB strains. Additionally, *A. cerevisiae* was observed to be more sensitive to FA than to pH and ethanol.

Hierarchical clustering analysis grouped all of the strains into three distinct clusters. The first cluster predominantly included *Lactobacillus*, *Acetobacter*, and *Gluconobacter* strains. The second cluster consisted of coccus-shaped LAB strains such as *P. parvulus*, *L. mesenteroides*, and *O. oeni*. The third cluster contained only two AAB species.

With respect to biofilm formation, the combination of FA, low pH, and high ethanol concentrations effectively minimised biofilm formation, particularly in LAB strains.

These findings highlight the high efficacy of FA against *O. oeni*, which is the primary LAB responsible for MLF in wine, although its effectiveness was reduced against certain *Lactobacillus* species. These results provide a solid foundation for optimising FA use in winemaking, thereby demonstrating its potential as a viable alternative to sulfur dioxide for controlling bacterial spoilage, preventing MLF, and enhancing the microbiological stability and overall quality of wine.

CRediT authorship contribution statement

Violeta García-Viñola: Writing – original draft, Methodology, Investigation. **Jokin Ezenarro:** Supervision, Data curation. **Cristina Reguant:** Writing – original draft, Supervision, Funding acquisition. **Nicolas Rozès:** Writing – review & editing, Supervision, Funding acquisition. **Manuel Malfeito Ferreira:** Writing – original draft, Visualization, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

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