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Impact of stress combination on *Brettanomyces bruxellensis* growth and volatile phenol production

Paul Bodin[1], Somaya Sachot[1], Aurélie Roland[1], Carole Camarasa[1,*], Fabienne Remize[1]

[1] SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France

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*correspondence:
carole.camarasa@inrae.fr

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ABSTRACT

Brettanomyces bruxellensis is a major wine spoilage yeast responsible for the production of volatile phenols, including 4-ethylphenol (EP) and 4-ethylguaiacol (EG), from grape-derived hydroxycinnamic acids. This study investigated how oenological stress factors, particularly temperature, pH, and *p*-coumaric acid concentration, modulate *B. bruxellensis* growth, viability, *p*-coumaric acid consumption, and conversion into 4-vinylphenol (VP) and EP. Three *B. bruxellensis* strains, 2OT13_02, L0463 and 1961-Mx-M1 E2, representing distinct genetic backgrounds, were grown in synthetic wine at two temperatures with varying *p*-coumaric acid concentrations. Complementarily, strain 2OT13_02 was exposed to pH 3.3 and pH 5.6 across a large range of *p*-coumaric acid concentrations. Cell counts and viability were monitored by flow cytometry, while LC-UV-MS/MS quantified *p*-coumaric acid, VP and EP. Elevated temperature strongly impaired growth and viability in a strain-dependent manner. At 28 °C, 1961-Mx-M1 E2 showed near-zero growth and mortality close to 100 %, whereas 2OT13_02 maintained the highest metabolic activity. At 20 °C, all strains consumed almost all *p*-coumaric acid, but their conversion profiles differed, with 2OT13_02 and 1961-Mx-M1 E2 producing mainly EP and L0463 accumulating both VP and EP. Increasing temperature reduced EP formation and favoured VP accumulation, indicating a limitation of VP reduction under stress. Wine pH also reshaped the pathway: pH 5.6 reduced *p*-coumaric acid consumption but prevented the metabolic block observed at high *p*-coumaric acid concentrations and pH 3.3. These results show that volatile phenol production by *B. bruxellensis* is not solely determined by precursor availability, but by the interaction between strain background, acid stress, and temperature. Taking into account these stress combinations may help winemakers better anticipate *Brettanomyces* spoilage risks and optimise non-chemical control strategies.

KEYWORDS: fermentation, yeast, *Brettanomyces*, phenolic compounds, wine spoilage, pH, temperature, off-flavour

INTRODUCTION

Brettanomyces bruxellensis is widely recognised as a major spoilage yeast in winemaking, particularly affecting red wines during ageing and storage (Chatonnet *et al.*, 1992; Wedral *et al.*, 2010). The primary defect associated with this yeast is its ability to produce volatile phenols, specifically 4-ethylphenol (EP) and 4-ethylguaiacol (EG), which impart undesirable phenolic, medicinal, leathery, stable-like, horse-sweat and smoky off-flavours (Chatonnet *et al.*, 1992; Yang, 2024). These compounds are synthesised from grape-derived hydroxycinnamic acids, mainly *p*-coumaric and ferulic acids (Chatonnet *et al.*, 1992). The underlying metabolic pathway involves an initial decarboxylation step, converting hydroxycinnamic acids into vinyl derivatives, followed by a reduction step that transforms vinylphenols into ethylphenols. This pathway is pivotal to wine alteration as it directly links the natural hydroxycinnamic acid composition of the wine matrix to the synthesis of sensory-active spoilage compounds by *B. bruxellensis* (Chatonnet *et al.*, 1992; Heresztyn, 1986; Kheir *et al.*, 2013; Tchobanov *et al.*, 2008).

The capacity of *B. bruxellensis* to persist in cellar environments, including barrels, tanks, valves and other notoriously difficult-to-clean areas, makes its control especially challenging for wine producers (Le Montagner *et al.*, 2024). Beyond its microbiological relevance, *B. bruxellensis* represents a major economic concern since contaminated wines can suffer from sensory deterioration, reduced commercial value, additional analytical and stabilisation costs, or even complete product loss (Alston *et al.*, 2021).

From a winemaking perspective, the risk of volatile phenol production does not solely depend on the presence of *B. bruxellensis* or the availability of hydroxycinnamic acid precursors. It is also strongly influenced by environmental parameters that affect yeast growth, viability and metabolic activity (Arroyo-López *et al.*, 2009; Barata *et al.*, 2008; Chandra *et al.*, 2014; Ciani *et al.*, 2003). Among these parameters, temperature and pH are particularly relevant because they vary during fermentation, ageing and wine storage, and because they are well documented to modulate yeast physiology and stress tolerance (Barata *et al.*, 2008; Yalcin & Yesim Ozbas, 2008; Zuehlke & Edwards, 2013). In addition, pH is a key determinant of wine microbial stability, notably because it governs the antimicrobial efficiency of sulphur dioxide by modifying the proportion of its active form, molecular SO_2. However, while these parameters are well recognised as important regarding wine microbiology, their combined effects on *B. bruxellensis* growth, viability, and metabolism remain insufficiently understood.

Hydroxycinnamic acids themselves may also act as stress factors for *B. bruxellensis*. We previously showed that increasing *p*-coumaric acid concentration progressively impaired substrate consumption and conversion efficiency, and that the impact of this compound on viability was strongly pH-dependent, with significantly greater tolerance observed at pH 5.6 than at pH 3.3 (Bodin *et al.*, 2026). This suggests that the toxic effect of hydroxycinnamic acids may depend on their ionisation state and on their capacity to interact with or cross the cell membrane. Consequently, pH could influence not only the survival of *B. bruxellensis*, but also the balance between hydroxycinnamic acid consumption, vinylphenol accumulation and ethylphenol production.

Temperature also plays a pivotal role, as fluctuations in cellar and wine storage conditions directly affect microbial growth kinetics, lag phases, membrane properties, and enzymatic activities. Although temperature effects have been widely studied in fermentative yeasts such as *Saccharomyces cerevisiae* (Arroyo-López *et al.*, 2009; Charoenchai *et al.*, 1998; Salvadó *et al.*, 2011; Yalcin & Yesim Ozbas, 2008), fewer data are available on how temperature modulates both *B. bruxellensis* fitness and its ability to convert hydroxycinnamic acids into volatile phenols (Barata *et al.*, 2008; Zuehlke & Edwards, 2013). This knowledge gap is critical because a condition that limits growth may not necessarily suppress metabolic activity; conversely, a condition that supports cell persistence may alter the final volatile phenol profile.

Control strategies against *B. bruxellensis* mainly rely on preventive hygiene (Guzzon *et al.*, 2013; Tristezza *et al.*, 2010), sulphur dioxide management and, when necessary, curative treatments such as chitosan, dimethyl dicarbonate or physical stabilisation (Arriagada-Carrazana *et al.*, 2005; Goy *et al.*, 2009; Ough, 1975; Vernhet, 2019). However, the efficiency of these strategies depends on the wine matrix, pH, strain variability, and physiological state of the yeast (Agnolucci *et al.*, 2014; Vigentini *et al.*, 2008). A better understanding of the environmental conditions that promote or limit both growth and volatile phenol production is therefore essential to improve risk assessment and support decision-making in wineries.

Therefore, the objective of this study was to investigate how two key oenological parameters, namely temperature and pH, combined with hydroxycinnamic acid concentration, modulate *B. bruxellensis* growth, viability, substrate consumption and volatile phenol production. The experimental conditions were deliberately selected to generate marked contrasts and to clarify the underlying physiological and metabolic mechanisms, rather than to reproduce the range of conditions typically encountered in wine strictly. By linking microbial fitness to metabolic output, this work aimed to provide new insights into the factors that favour *Brettanomyces* spoilage and to generate practical knowledge that may help wine professionals better anticipate and manage the risk of volatile phenol formation during wine production and ageing.

MATERIALS AND METHODS

1. Reagents and standards

Yeast extract, glucose and peptone for YPD broth were purchased from Fisher BioReagent™ (Thermo Fisher Scientific, Waltham, MA, USA). *p*-Coumaric acid and all analytical standards (analytical grade, purity > 99 %) used in LC-MS/MS analysis were purchased from Biosolve (Dieuze, France).

2. Strains and storage conditions

Saccharomyces cerevisiae EC1118 (Lallemand, Blagnac, France) was used to prepare synthetic wine (SW).

Three *B. bruxellensis* strains were selected to represent contrasting genetic backgrounds and volatile phenol conversion profiles (Table 1). Based on previous phenotyping, 2OT13_02 displayed an EP-dominant profile (21 % VP/79 % EP), 1961-Mx-M1 E2 showed an intermediate profile with similar proportions of VP and EP (54 %/46 %), whereas L0463 displayed a strongly VP-dominant profile (94 %/6 %) (Bodin *et al.*, submitted). The strains also differed in their sensitivity to *p*-coumaric acid, with 2OT13_02 being the most tolerant, 1961-Mx-M1 E2 showing an intermediate response, and L0463 being the most sensitive under previously tested conditions. These strains therefore represented contrasting phenotypes in terms of both VP-to-EP conversion and hydroxycinnamic acid tolerance. In addition, they belonged to different genetic groups and ploidy backgrounds, allowing the study to encompass part of the known intraspecific diversity of *B. bruxellensis* (Avramova *et al.*, 2018a; Harrouard *et al.*, 2023; Loegler *et al.*, 2025).

Strains were routinely cultivated in YPD broth (20 g/L D-glucose, 20 g/L peptone, and 10 g/L yeast extract) at 28 °C for 72 h and preserved at −70 °C in YPD supplemented with 20 % (*v/v*) glycerol.

3. Synthetic wine preparation

To replicate wine-like conditions favourable for *B. bruxellensis* growth between alcoholic and malolactic fermentations, SW was prepared using a synthetic must (SM) following the method described by Bodin *et al.* (2026). In brief, the SM composition, adapted from Bely *et al.* (1990) and Rollero *et al.* (2015), mimics that of grape must and includes glucose, fructose, organic acids, mineral salts, vitamins, and yeast assimilable nitrogen (YAN). For pH-related experiments, the medium pH was adjusted to either 3.3 or 5.6 using 10 M NaOH.

Alcoholic fermentation was performed with *S. cerevisiae* EC1118 at 20 °C until residual sugar concentrations dropped below 2 g/L, as measured using an enzymatic assay (Enzytec™ Liquid D-Glucose/D-Fructose, R-Biopharm®, Darmstadt, Germany). The resulting SW was filtered through a 0.2 µm membrane (SteriCap PLUS, Millipore®, Merck KGaA, Darmstadt, Germany) to remove yeast cells and sterilise the medium. Organic acids, sugars, and alcohols were quantified by high-performance liquid chromatography (HPLC) according to previously published methods (Bodin *et al.*, 2026; Deroite *et al.*, 2018). Free SO_2 levels were determined enzymatically using a multiparameter analyser (Gallery™, Thermo Scientific™, Waltham, MA, USA). The final chemical composition of SW is presented in Table S1.

4. Cultivation of *B. bruxellensis*

B. bruxellensis strains were progressively acclimated to SW according to the protocol previously described by Bodin *et al.* (2026). In brief, adaptation was achieved through successive precultures in YPD:SW:SM media containing gradually increasing proportions of SW. Cells from fresh YPD cultures were initially inoculated into a YPD:SW:SM mixture (25:25:50, *v/v/v*) and incubated at 20 °C for 3 days. The cultures were then transferred into a SW:SM mixture (50:50, *v/v*) and incubated for an additional 3 days. Subsequently, cells were collected by centrifugation, the supernatant was discarded, and the pellet was inoculated into fresh SW at an initial concentration of 1×10^6 cells/mL. All experiments were carried out in hermetically sealed 50 mL flasks containing 30 mL of SW. Cultures were then incubated at either 20 °C or 28 °C. The temperature of 28 °C was selected to represent warm conditions that may transiently occur during red wine alcoholic fermentation or during the early post-fermentation period, rather than typical long-term wine-ageing conditions. Each condition was performed in triplicate and monitored over a 25-day cultivation period (Table S2).

TABLE 1. Characteristics of the three *B. bruxellensis* strains selected for this study. Strains were chosen to represent contrasting genetic backgrounds and volatile phenol conversion phenotypes, including EP-dominant, intermediate VP/EP, and VP-dominant profiles.

Specie	Strain	Supplier	Isolation source	Geographical origin	Genetic group*	Ploidy	Total volatile phenol production (µM)**	VP/EP distribution (%)**
B. bruxellensis	2OT13_02	ISVV	Wine	France	Wine 1 A2	Allotriploid	540.2	21/79
B. bruxellensis	L0463	CRBO	Wine	France	Kombucha 1 D2	Diploid	221.1	94/6
B. bruxellensis	1961-Mx-M1 E2	ISVV	Wine	France	Wine 3.1 D1	Diploid	362.1	54/46

* Genetic-group assignments were based on Avramova *et al.* (2018a), Harrouard *et al.* (2023), and Loegler *et al.* (2025). The first designation corresponds to the ecological/genetic group used in previous studies, while the second corresponds to the genomic cluster defined by Loegler *et al.* (2025).

** The VP/EP ratio represents the relative proportion of vinylphenol (VP) to ethylphenol (EP), expressed as a percentage. Data were obtained from Bodin *et al.* (submitted) under the following conditions: 700 µM *p*-coumaric acid, pH 3.3, and 20 °C.

ISVV, Institute of Vine and Wine Sciences, Bordeaux, France; CRBO, Microbiological Resources Center Oenology, Bordeaux, France.

To investigate volatile phenol production in interaction with temperature, *p*-coumaric acid was added at a concentration of 170 µM or 370 µM. Additional concentrations of 680 µM and 1350 µM were specifically tested in the pH variation experiment. A 140 mM *p*-coumaric acid stock solution was prepared in a 50:50 (*v/v*) water-ethanol mixture. Control experiments were performed without the addition of *p*-coumaric acid.

5. Enumeration and viability determination

B. bruxellensis population and viability were monitored by flow cytometry. Culture samples were diluted 10-fold in phosphate-buffered saline (PBS) containing propidium iodide (1.5 µM) and analysed using an Attune NxT Acoustic Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Dead cells were detected based on fluorescence measured in the YL2 channel (561 nm excitation, 615/20 nm emission filter, 400 mV), whereas total cell populations were identified using forward scatter (FSC; 488 nm laser, 488/10 nm filter, 200 mV) and side scatter (SSC; 488 nm laser, 488/10 nm filter, 220 mV).

6. Phenolic compound analysis by LC-UV-MS/MS

p-coumaric acid, 4-vinylphenol, and 4-ethylphenol were quantified by LC-UV-MS/MS according to the method previously described (Bodin *et al.*, 2026).

Quantification was carried out using a stable isotope dilution assay (SIDA) for *p*-coumaric acid and 4-vinylphenol; 4-ethylphenol was quantified by internal calibration using 3-phenylphenol as an internal standard. Chromatographic separation was performed using reversed-phase UHPLC coupled to UV detection and triple quadrupole MS/MS operating in positive electrospray ionisation mode with dynamic multiple reaction monitoring (dMRM). The analytical method was previously validated for linearity, accuracy, repeatability, intermediate precision, limit of detection (LOD), and limit of quantification (LOQ). Detailed calibration ranges and validation parameters are available in Bodin *et al.* (2026).

7. Statistical analysis

All statistical analyses were conducted using R version 4.6.1 in RStudio (2026.04.0+526, Posit Software PBC, Boston, MA, USA). A significance level of $\alpha = 0.05$ was applied for all analyses.

Data normality and homogeneity of variances were evaluated using the Shapiro–Wilk and Bartlett tests, respectively. When both assumptions were satisfied, differences between groups were assessed using one-way ANOVA. In cases where these assumptions were not met, non-parametric tests were applied: the Wilcoxon–Mann–Whitney test for comparisons between two groups and the Kruskal–Wallis test for comparisons involving more than two groups (R package stats v4.3.2).

Post hoc pairwise comparisons following ANOVA were performed using Tukey's HSD test implemented in the agricolae package (v1.3-7). Differences were considered statistically significant when the *p*-value was less than 0.05.

RESULTS AND DISCUSSION

1. Temperature-dependent modulation of growth, viability, and *p*-coumaric acid bioconversion

1.1. Effect of temperature on the growth and viability of three *B. bruxellensis* strains

To investigate the impact of temperature on *B. bruxellensis,* the growth of three strains (2OT13_02, L0463, and 1961-Mx-M1 E2) was compared at either 20 °C or 28 °C in SW supplemented with varying concentrations of *p*-coumaric acid (0 µM, 170 µM, and 370 µM).

The growth parameters were calculated from the growth dynamics over time (Figures S1–S3) and summarised in Figure 1 and Table 2, along with the physiological state of the cells at the end of the experiment.

The *B. bruxellensis* strains displayed variable responses to temperature, with strain-dependent differences in growth and mortality under the tested conditions (Figure 1 and Table 2).

When focusing on strain 2OT13_02 in the absence of *p*-coumaric acid, reduced growth rate was observed at 28 °C compared with 20 °C, with values decreasing from 0.32 d^{-1} to 0.19 d^{-1} (Figures 1 and S1, Table 2). Similarly, for strain L0463, the growth rate dropped from 0.27 d^{-1} at 20 °C to 0.18 d^{-1} at 28 °C, while the lag phase was substantially prolonged by 211 h (Table 2).

Under the 170 µM *p*-coumaric acid condition, strains 2OT13_02 and L0463 exhibited reduced growth rates as temperature increased; rates fell from 0.30 d^{-1} and 0.26 d^{-1} at 20 °C to 0.11 d^{-1} and 0.10 d^{-1} at 28 °C, respectively. Lag times were also affected, increasing by 21 h and 282 h for strains 2OT13_02 and L0463, respectively (Table 2).

Under the 370 µM *p*-coumaric acid condition, the temperature effect on growth rate was further exacerbated, with values decreasing from 0.23 d^{-1} and 0.20 d^{-1} to 0.10 d^{-1} and 0.05 d^{-1} for strains 2OT13_02 and L0463, respectively. The data also showed marked increases in lag times, which rose by 209 h and 337 h, respectively (Table 2).

Analysis of mortality percentages at the end of the experiment revealed a substantial effect of the temperature increase (Figure 1). At 20 °C, final mortality rates in the absence of *p*-coumaric acid were 27.2 %, 10.2 %, and 19.1 % for strains 2OT13_02, L0463, and 1961-Mx-M1 E2, respectively. At 28 °C, mortality increased significantly, reaching 82.8 %, 74.9 %, and 99.7 %, respectively, for the same strains at the end of the experiment (p-value < 0.001). This significant increase in mortality between the two temperature conditions was also observed in the presence of *p*-coumaric acid, at both initial concentrations tested, 170 µM and 370 µM, and for all strains.

However, within each temperature condition, *p*-coumaric acid concentration had a limited effect on mortality. At 28 °C, strain 2OT13_02 showed some variability among the three *p*-coumaric acid conditions, with mortality rates of 82.8 %, 81.3 %, and 72.6 %, respectively. These differences

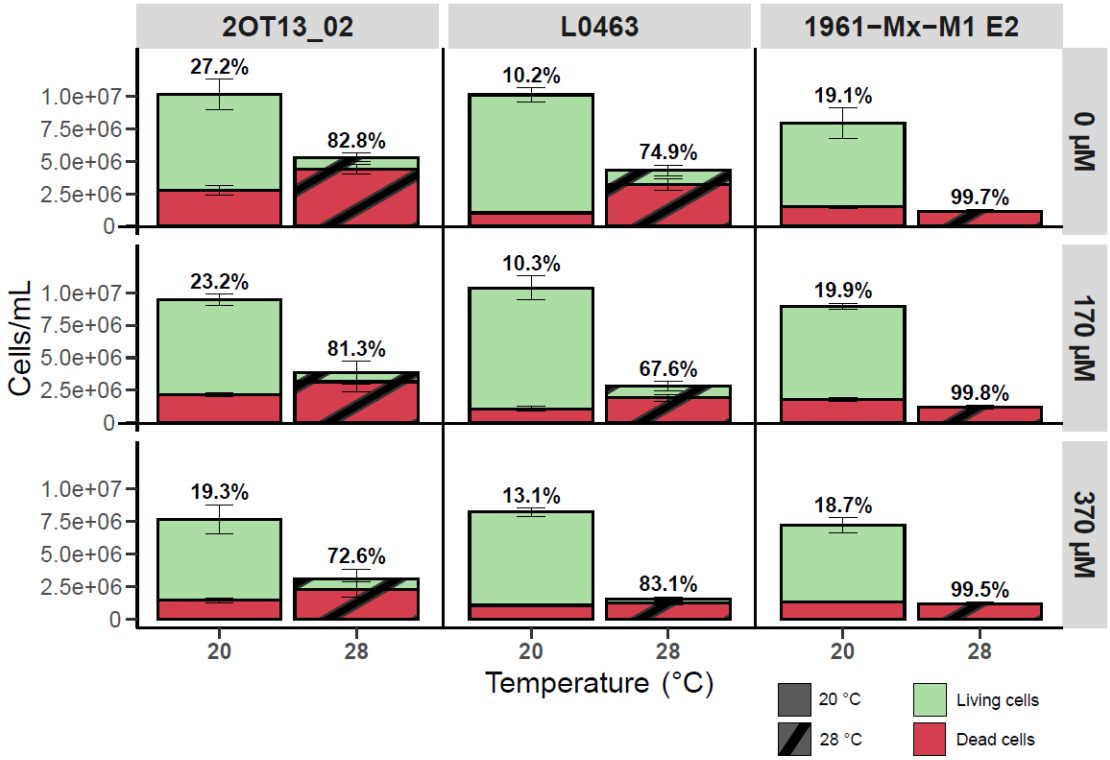


FIGURE 1. End-point evaluation (t = 25 days) of living cells (green bars) and dead cells (red bars) for three *B. bruxellensis* strains grown under three initial *p*-coumaric acid conditions: 0 µM, 170 µM, and 370 µM. The percentages displayed above each bar represent the mortality ratio measured at the end of the experiment. Hatched bars correspond to results obtained at 28 °C. Each bar represents the mean of three biological replicates, and error bars indicate the standard deviation.

TABLE 2. Growth parameters of three strains of *B. bruxellensis* under three *p*-coumaric acid supplementation conditions at 20 °C or 28 °C. The maximum specific growth rate (µ) is expressed in day^{-1} and the lag phase duration (λ) is expressed in hours. Values are expressed as mean ± standard deviation. Different letters indicate significant differences in the maximum specific growth rate among the tested *p*-coumaric acid concentrations.

Strains	Temperature	*p*-Coumaric acid					
		0 µM		170 µM		370 µM	
		µ (d^{-1})	λ (h)	µ (d^{-1})	λ (h)	µ (d^{-1})	λ (h)
2OT13_02	20 °C	0.32 ± 0.03a	12	0.30 ± 0.01a	13	0.23 ± 0.01b	20
	28 °C	0.19 ± 0.01a	13	0.11 ± 0.02b	34	0.10 ± 0.03b	229
	Statistical comparison	**		***		**	
L0463	20 °C	0.27 ± 0.01a	30	0.26 ± 0.01a	48	0.20 ± 0.01b	101
	28 °C	0.18 ± 0.02a	241	0.10 ± 0.02b	330	0.05 ± 0.02b	438
	Statistical comparison	**		***		***	
1961-Mx-M1 E2	20 °C	0.29 ± 0.03a	25	0.27 ± 0.02a	31	0.21 ± 0.02b	36
	28 °C	0.03 ± 0.01a	nc	0.05 ± 0.04a	nc	0.04 ± 0.03a	nc
	Statistical comparison	***		***		**	

nc, not calculated; ***, *p*-value < 0.001; **, *p*-value < 0.01; *, *p*-value < 0.05.

were statistically significant (p-value < 0.05) (Figure 1). Similarly, mortality rates for strain L0463 ranged from 67.6 % to 83.1 % across the three conditions, with significant differences observed (p-value < 0.01) (Figure 1).

At 20 °C, the growth of strain 1961-Mx-M1 E2 was similar regardless of the *p*-coumaric acid concentration, though an increase in the lag phase was observed. The impact of the 28 °C condition was particularly pronounced for this strain, which exhibited a near-zero growth rate under all tested conditions (Figures 1 and S3). Consequently, the lag phase could not be estimated under these conditions. At 28 °C, strain 1961-Mx-M1 E2 exhibited very high mortality rates at the end of the experiment across all tested conditions, reaching 99.7 %, 99.8 %, and 99.5 % under the control, 170 µM, and 370 µM conditions, respectively (Figure 1).

Overall, these results highlight the combined effect of temperature and *p*-coumaric acid toxicity on growth impairment, as shown by both reduced growth rates and increased lag times (p-value < 0.01) (Table 2). These findings are consistent with previous observations showing the toxicity of *p*-coumaric acid towards these strains under wine-like conditions (Bodin *et al.*, 2026). However, the data suggest that temperature had a stronger influence than *p*-coumaric acid under these conditions. In this experimental context, the increase in temperature had a greater impact on yeast physiology, particularly on total cell populations, than *p*-coumaric acid concentration. For strain 2OT13_02, total cell populations decreased by 24 % in response to *p*-coumaric acid, whereas temperature alone induced a 59 % reduction. A similar pattern was observed for strains 1961-Mx-M1 E2 and L0463, with reductions of 9 % and 18 % linked to *p*-coumaric acid, respectively, compared with stronger decreases of 85 % and 57 % associated with temperature alone. However, this interpretation should be considered within the limits of the experimental conditions tested. A smaller increase in temperature combined with a higher *p*-coumaric acid concentration could lead to different effects and potentially reveal a stronger impact of *p*-coumaric acid stress than temperature on yeast physiology. This suggests that the relative contribution of each stress factor may depend on their respective intensity and on the balance between thermal stress and hydroxycinnamic acid-induced toxicity.

1.2. Temperature-dependent modulation of *p*-coumaric acid metabolism and volatile phenol formation in three *B. bruxellensis* strains

The consumption of *p*-coumaric acid and the production of VP and EP were investigated for the three strains at the two temperatures and the two levels of *p*-coumaric addition (Figure 2).

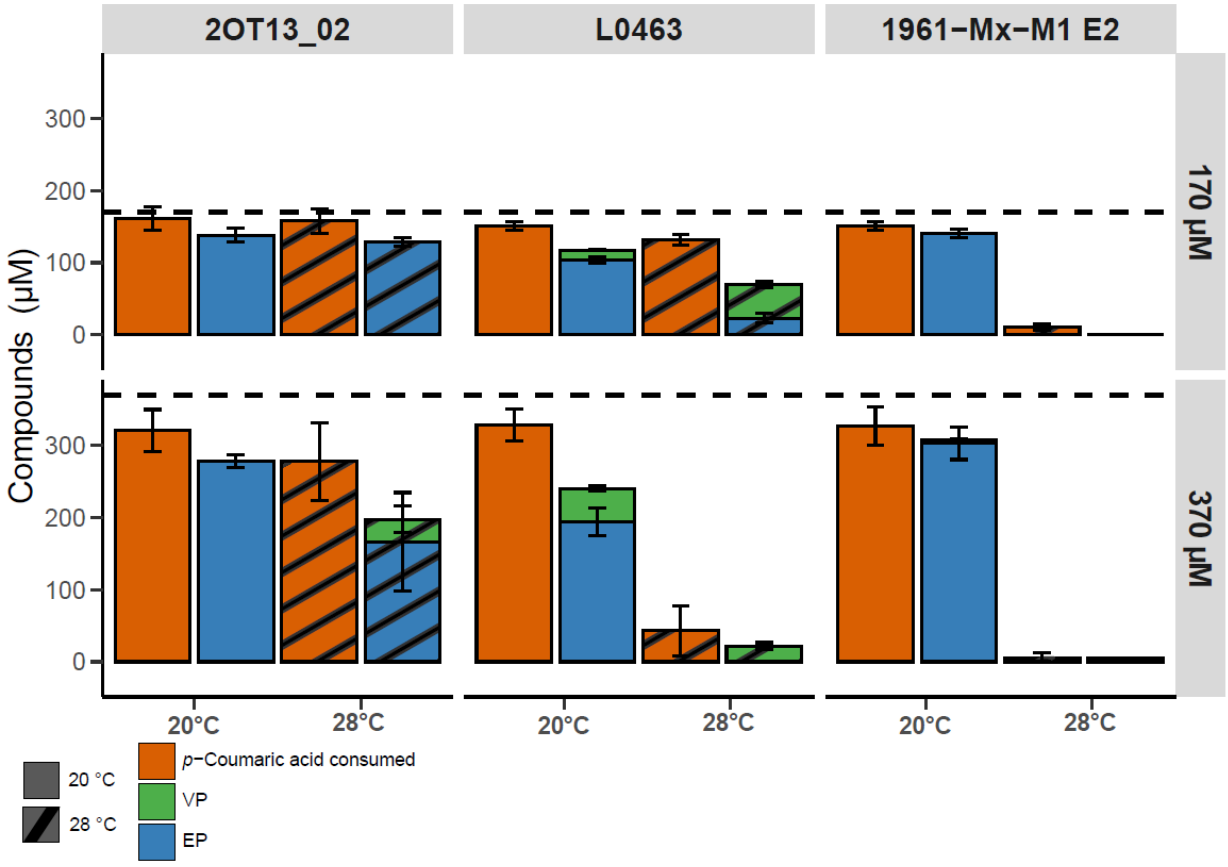


FIGURE 2. Amount of *p*-coumaric acid consumed (orange bars) and volatile phenols produced, including vinylphenol (green bars) and ethylphenol (blue bars), by three *B. bruxellensis* strains grown under two initial *p*-coumaric acid conditions: 170 µM and 370 µM (dashed lines). Hatched bars correspond to results obtained at 28 °C. Each bar represents the mean of three biological replicates, and error bars indicate the standard deviation.

At 20 °C, all strains consumed nearly all of the initial *p*-coumaric acid, regardless of the concentration tested (*p*-value > 0.05). However, the fate of the consumed acid differed between strains. Strains 2OT13_02 and 1961-Mx-M1 E2 converted *p*-coumaric acid almost exclusively into EP (81.4 % and 93.2 %, respectively), whereas strain L0463 displayed a mixed conversion profile, with both EP and VP accumulation (68.2 % and 13.8 %, respectively). In this latter strain, VP reached 14.25 µM and 45.87 µM under the 170 µM and 370 µM conditions, respectively, corresponding to a higher proportion of VP at 370 µM than at 170 µM (Figure 2). For L0463, the sum of VP and EP concentrations was lower than the amount of *p*-coumaric acid consumed, indicating that approximately 18 % of the consumed *p*-coumaric acid was not recovered as VP or EP.

Overall, the temperature increase affected *p*-coumaric acid consumption and conversion, although the magnitude of this effect was strain-dependent. The conversion of *p*-coumaric acid by 1961-Mx-M1 E2 was almost completely abolished, consistent with the strong sensitivity of this strain to 28 °C (Figure 2). In contrast, 2OT13_02 appeared to be the least affected strain, especially at 170 µM, where the impact of temperature on *p*-coumaric acid consumption and metabolite production remained limited (*p*-value > 0.05) (Figure 2). However, at 370 µM, the temperature effect was more pronounced, with a 40 % decrease in EP production at 28 °C compared with 20 °C. This was also associated with the detection of VP at 28 °C, reaching 31.1 µM and representing 15.7 % of the total volatile phenols produced, suggesting a partial limitation of the VP-to-EP conversion step under this condition. Strain L0463 showed an intermediate but clearly temperature-sensitive profile, as EP production was significantly reduced at 28 °C for both *p*-coumaric acid concentrations (*p*-value < 0.001). At 170 µM, EP production decreased by 77.8 %, while VP accumulation increased from 14.2 µM at 20 °C to 46.5 µM at 28 °C, indicating a marked impairment of VP reduction into EP while *p*-coumaric acid consumption was maintained. At 28 °C and 370 µM *p*-coumaric acid, the response was further amplified: *p*-coumaric acid consumption was drastically decreased by 86.9 %, while EP production was completely abolished.

Together, these results show that the effect of increasing temperature was amplified by the initial hydroxycinnamic acid concentration, particularly in strain L0463. Temperature and *p*-coumaric acid did not appear to act as independent constraints, but rather as combined stress factors affecting *B. bruxellensis* physiology. From an applied perspective, this cumulative effect is particularly relevant, as wine represents a multi-stress environment in which low pH, ethanol, nutrient limitation, phenolic compounds, and temperature may jointly impair yeast performance (Smith & Divol, 2016). Here, the first detectable impact of this stress combination was observed in growth dynamics, through increased lag times and reduced viability. This suggests that cell adaptation and survival are the primary targets of the combined stress, before any marked effect on *p*-coumaric acid catabolism can be observed. This hierarchy of responses may explain why volatile phenol production was more affected than *p*-coumaric acid consumption under the harshest conditions (370 µM). Indeed, while the three strains remained able to consume *p*-coumaric acid at 20 °C, the increase to 28 °C strongly impaired growth and viability, which likely limited the metabolic capacity required for efficient conversion into volatile phenols. Thus, under cumulative stress, cellular physiology appears to be affected progressively: first through altered latency and survival, then through reduced conversion efficiency, and finally through impaired substrate consumption only when physiological stress becomes more severe.

The strain-dependent response observed in this work supports this interpretation. The three strains belong to distinct genetic backgrounds and differ in ploidy, which may contribute to differences in stress tolerance and metabolic behaviour, as previously reported for SO_2 resistance (Avramova *et al.*, 2018b). However, the genetic background alone does not appear sufficient to predict the response to temperature or *p*-coumaric acid. For example, strain 2OT13_02 was also previously identified as one of the most tolerant strains to *p*-coumaric acid under wine-like conditions (Bodin *et al.*, 2026), which may partly explain its enhanced ability to maintain growth and volatile phenol production under combined stresses. Conversely, strains that performed well at 20 °C did not necessarily show higher resistance at 28 °C, indicating that tolerance to wine-like conditions at moderate temperatures does not directly predict tolerance to higher temperatures.

Compared with previous results for *p*-coumaric acid consumption obtained at 20 °C with an initial concentration of 700 µM (Bodin *et al.*, submitted), the three strains remained strong *p*-coumaric acid consumers and volatile phenol producers under permissive conditions (≤ 370 µM). However, differences in VP and EP profiles, particularly the higher EP accumulation observed for the strains 2OT13_02 and 1961-Mx-M1 E2, may reflect the combined influence of temperature, strain physiology, and the lower initial *p*-coumaric acid concentration used in this experiment. One possible explanation is that lower substrate concentrations may favour a more complete conversion towards EP when growth is maintained. Nevertheless, this hypothesis should be considered with caution, as temperature-induced growth inhibition can also restrict pathway activity, and similar mortality levels did not always lead to comparable volatile phenol production (Bodin *et al.*, submitted).

From an oenological perspective, 28 °C should not be considered as a thermal treatment, but rather as a model of transient warm conditions that may occur during wine alcoholic fermentation or the early post-fermentation period. Indeed, 28 °C has previously been used as a warm fermentation regime in red winemaking studies and remains below the temperatures required for reliable thermal inactivation of *B. bruxellensis* (Couto *et al.*, 2005; Fabrizio *et al.*, 2015). Previous studies showed that *B. bruxellensis* can grow and produce volatile phenols in wine at 15, 20, and 25 °C, whereas complete loss of viability was observed at 36 °C for the strains tested (Barata *et al.*, 2008). Under otherwise

identical conditions, lower temperatures also appear to favour higher final concentrations of 4-ethylphenol. Therefore, the inhibition observed at 28 °C in the present study should be interpreted as a strain- and environment-dependent response resulting from the interaction between temperature and *p*-coumaric acid, rather than as a general inhibitory effect of moderate heating.

Studies conducted under wine-ageing conditions have shown that very low storage temperatures, generally below 12 °C, may be required to substantially limit the development of *B. bruxellensis*, often in combination with oenological preservatives such as SO_2 (Cibrario *et al.*, 2020; Oswald & Edwards, 2017; Zuehlke & Edwards, 2013). Nevertheless, such conditions do not necessarily prevent volatile phenol production or ensure complete population elimination, as strain-dependent persistence and subsequent recovery may remain possible. Low temperatures appear to act primarily by extending the lag phase and slowing cellular activity, rather than by immediately suppressing metabolism. This observation is consistent with the present results obtained at higher temperatures, in which changes in growth dynamics and viability preceded the reduction in volatile phenol production. In both cases, temperature mainly modulated the kinetics of *B. bruxellensis* development, while complete inhibition of volatile phenol production was rarely achieved. However, temperatures below 12 °C are seldom maintained during active fermentation and the early post-fermentation stages, notably in meridional areas.

The results obtained at 28 °C are therefore particularly relevant to the post-alcoholic fermentation and pre-malolactic fermentation stages of red winemaking, during which *B. bruxellensis* may develop when the activity of other microbial populations is limited. The temperatures reached during these stages may contribute to reducing volatile phenol production without completely preventing it. Early population monitoring during warm post-fermentation periods, followed by controlled low-temperature storage, may therefore help to restrict subsequent proliferation. However, the magnitude of this effect remains dependent on several interacting factors, including the contaminating strain, pH, and hydroxycinnamic acid concentration.

Overall, the key outcome is not a direct relationship between mortality and substrate conversion, but rather a strain-dependent physiological response to cumulative stresses. Under the conditions tested, the lag phase and cell viability appeared to be affected first, followed by volatile phenol production and, under more severe stress conditions, hydroxycinnamic acid consumption.

2. Influence of pH on *p*-coumaric acid conversion into volatile phenols

It has been previously demonstrated that pH is a key factor in modulating the toxicity of *p*-coumaric acid for the strain 2OT13_02, with greater tolerance observed at pH 5.6 than at pH 3.3 (Bodin *et al.*, 2026). At pH 5.6, approximately one pH unit above the pKa of *p*-coumaric acid (~4.6), the compound is predominantly present in its dissociated form. For a given *p*-coumaric acid concentration, mortality rates were higher at pH 3.3 than at pH 5.6. For instance, at the highest concentration tested (1350 µM), mortality reached 99.7 % at pH 3.3, whereas it remained at 10.5 % at pH 5.6.

Previous work also showed that, at pH 3.3, increasing concentrations progressively impaired substrate consumption and conversion efficiency in this strain. This physiological shift is associated with a metabolic redirection from exclusive EP production toward VP accumulation and incomplete carbon recovery (Bodin *et al.*, 2026).

Building on these findings, the present study investigates the combined effect of pH and initial *p*-coumaric acid concentration on the volatile phenol production pathway in strain 2OT13_02. Specifically, we evaluated two pH levels (3.3 and 5.6) across five initial *p*-coumaric acid concentrations (0 µM, 170 µM, 370 µM, 680 µM, or 1350 µM) (Figure 3).

At pH 5.6, *p*-coumaric acid consumption was lower than at pH 3.3 for initial concentrations of 170 µM, 370 µM, and 680 µM, with decreases of 71.5 %, 72.6 %, and 69.2 %, respectively (Figure 3). The consistency of these reductions suggests that the impact of pH on substrate consumption remained relatively constant within this concentration range. However, at the highest concentration tested (1350 µM), *p*-coumaric acid consumption was severely impaired at pH 3.3, dropping to 200 µM (15 % of the initial amount). In contrast, consumption reached 322 µM at pH 5.6, representing approximately 24 % of the initial substrate, a proportion comparable to that observed at lower *p*-coumaric acid concentrations at this same pH. These results indicate that the inhibition of *p*-coumaric acid consumption at high concentrations is strongly pH-dependent.

Volatile phenol profiles also differed markedly between the two pH conditions (Figure 3). At pH 5.6, VP production remained low across all tested concentrations, peaking at only 21.6 µM. Compared with pH 3.3, this represents a significant reduction in VP accumulation, particularly at the 680 µM and 1350 µM *p*-coumaric acid concentrations, where production decreased by 96.2 % and 81.8 %, respectively.

In contrast, EP production increased progressively with the initial *p*-coumaric acid concentration at pH 5.6, reaching 271 µM at the 1350 µM *p*-coumaric acid concentration (Figure 3). Up to an initial *p*-coumaric acid concentration of 680 µM, EP production remained lower at pH 5.6 than at pH 3.3, with decreases of 81.6 %, 70.3 %, and 36.0 % for the 170 µM, 370 µM, and 680 µM *p*-coumaric acid concentrations, respectively. However, EP production increased with *p*-coumaric acid concentration under both pH conditions, suggesting that the difference between the two pH levels was primarily quantitative within this concentration range. The most notable difference emerged at the highest *p*-coumaric acid concentration. At pH 3.3, EP production ceased to increase and declined sharply when *p*-coumaric acid exceeded 370–680 µM. In contrast, at pH 5.6, EP production continued to rise, reaching a level nearly 9 times higher than at pH 3.3 under the 1350 µM condition.. The decrease at pH 3.3 was likely associated with the

substantial loss of viability observed at the highest *p*-coumaric acid concentration, supporting the hypothesis that cell stress initially limits metabolic activity before ultimately impairing substrate consumption (Bodin *et al.*, 2026).

This pH-dependent effect may be partly explained by changes in the ionisation state of *p*-coumaric acid (Ong & Nagel, 1978). At low pH, a higher proportion of the molecule exists in its protonated, undissociated form, facilitating its passive diffusion across the plasma membrane (Frazier & Westhoff, 1977). Once inside the cell, dissociation in the near-neutral cytoplasm likely leads to intracellular acidification and increased energetic costs to restore pH homeostasis (Campolongo *et al.,* 2014). Conversely, at pH 5.6, a larger fraction of *p*-coumaric acid is deprotonated, which restricts passive membrane diffusion and mitigates intracellular stress. The low VP accumulation observed at pH 5.6 suggests that the reduction of VP into EP is not the rate-limiting step under these conditions and that the conversion pathway is less saturated than at pH 3.3. Specifically, the reduced cellular uptake of *p*-coumaric acid at pH 5.6 likely allows the phenolic acid decarboxylase (PAD) and vinylphenol reductase (VPR) to metabolise, or detoxify efficiently, the incoming *p*-coumaric acid flux into EP, without causing a significant disruption of acid-related metabolism. Beyond its influence on cell entry and toxicity, pH may also directly modulate the activity or catalytic efficiency of the enzymes involved in the pathway, including VPR (Godoy *et al.*, 2008; Tchobanov *et al.*, 2008). Therefore, the lower VP accumulation and sustained EP production observed at pH 5.6 likely result from a combination of reduced intracellular acid stress and a more favourable physiological and enzymatic environment for VP reduction into EP.

Taken together, these results indicate that increases in pH and in *p*-coumaric acid concentration had opposite effects on the pathway. Increasing the *p*-coumaric acid concentration enhanced metabolic stress and progressively limited EP production at pH 3.3, whereas increasing the pH reduced this stress and prevented the metabolic block observed at high *p*-coumaric acid concentrations. From a physiological perspective, this suggests that the decrease in EP production at pH 3.3 was not only due to the substrate concentration itself, but also to the heightened stress state of the cells under acidic conditions.

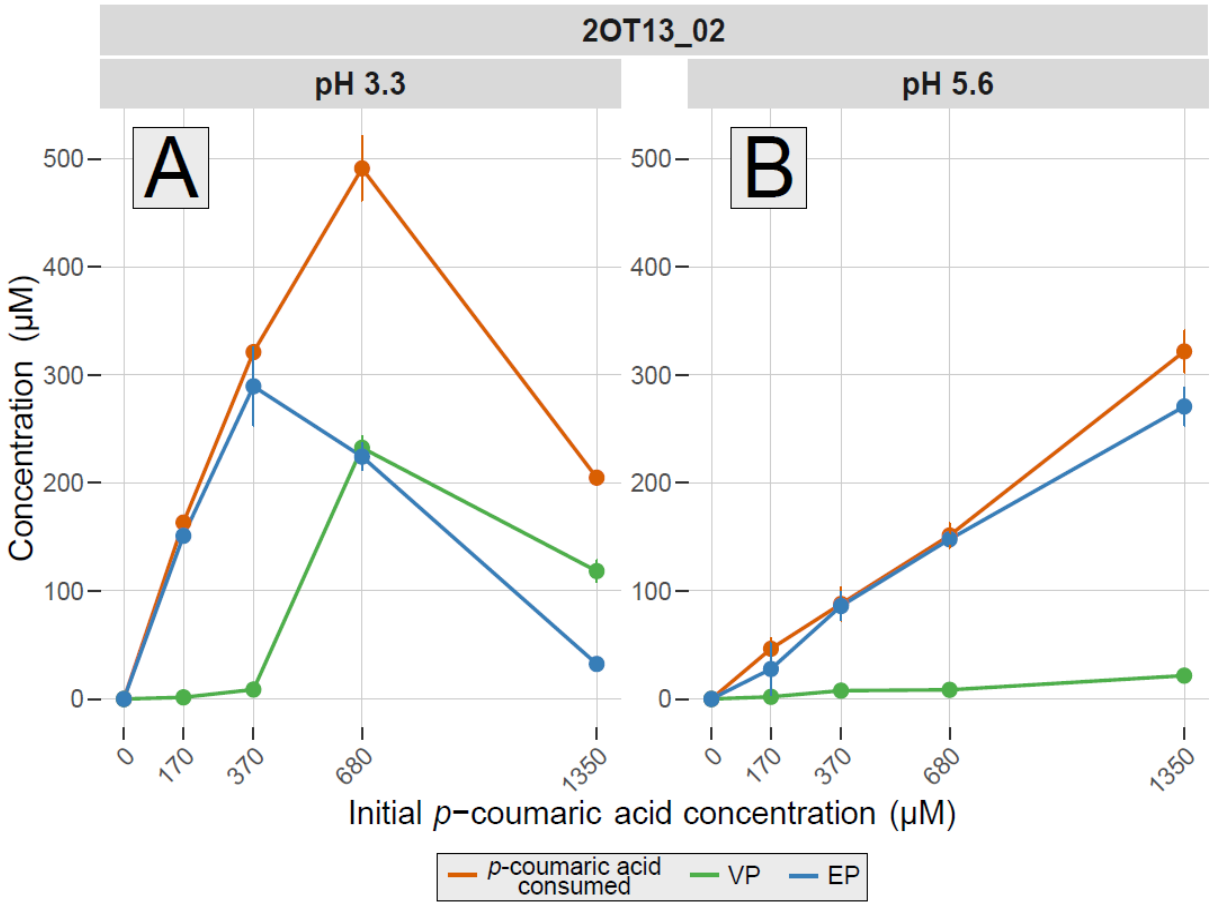


FIGURE 3. Effect of pH and initial *p*-coumaric acid concentration on *p*-coumaric acid consumption and volatile phenol production by *B. bruxellensis* strain 2OT13_02: pH 3.3 (A) and pH 5.6 (B). *p*-Coumaric acid consumption (orange), VP production (green), and EP production (blue) were measured across increasing initial *p*-coumaric acid concentrations. Each point represents the mean of three biological replicates, and error bars indicate the standard deviation.

The pH conditions used in this study were deliberately selected to accentuate the effect of pH on *p*-coumaric acid toxicity and metabolism. Notably, pH 5.6 corresponds approximately to one pH unit above the pKa of *p*-coumaric acid (~4.6), where more than 90 % of the compound is expected to be present in its dissociated form. This condition therefore provided a strong mechanistic contrast with pH 3.3, where *p*-coumaric acid is predominantly undissociated. In particular, pH 5.6 lies well outside the range currently encountered in red wines and should therefore be regarded as a mechanistic condition rather than as a realistic oenological treatment scenario. Moreover, because intermediate pH values were not investigated, no direct conclusion can be drawn regarding the influence of variations within the usual red wine range, approximately pH 3.3–3.9. Within this range, *p*-coumaric acid remains predominantly undissociated, and moderate pH variations may affect its cellular uptake, toxicity and conversion differently from the marked contrast observed between pH 3.3 and 5.6.

Nevertheless, studying the effect of increasing pH remains relevant in the current context of declining must acidity and increasing pH associated with climate change (Mira de Orduña, 2010; Payan *et al.*, 2023). This issue may be particularly important for hydroxycinnamic acids, as their ionisation state and biological effects are strongly influenced by pH. Further experiments using narrower pH increments, for example between pH 3.3 and 4.5, would therefore be required to directly quantify the influence of oenologically relevant pH variations on the physiology of *B. bruxellensis*, hydroxycinnamic acid consumption and volatile phenol production.

From an applied perspective, these findings should not be interpreted as supporting an increase in wine pH to limit volatile phenol formation. Although increasing pH may shift the ionisation equilibrium of hydroxycinnamic acids and reduce their apparent toxicity, it also decreases wine microbiological stability and the antimicrobial efficiency of SO_2, potentially favouring the persistence and proliferation of *B. bruxellensis*. As observed for temperature, pH should therefore be considered as a parameter to monitor within a broader multi-stress environment that also includes ethanol, SO_2, nutrient availability, temperature, *p*-coumaric acid concentration, and strain-specific tolerance. In the context of increasing must and wine pH, the present results indicate that reduced hydroxycinnamic acid toxicity does not necessarily prevent their conversion into volatile phenols. Consequently, pH management alone cannot inhibit volatile phenol production and should be integrated into a broader strategy for assessing and controlling the risk associated with *B. bruxellensis*.

CONCLUSION

This study demonstrates, in three *B. bruxellensis* strains, that temperature and pH are key factors influencing yeast growth, viability, and volatile phenol production. These results improve our understanding of how *B. bruxellensis* adapts to the stressful wine environment in which it can persist and develop. From an oenological perspective, monitoring temperature and applying pH-adapted preservation strategies may help to limit *B. bruxellensis* proliferation and the formation of its characteristic off-flavours, although pH and temperature ranges in wine are naturally more restricted. Ultimately, such approaches could help reduce reliance on chemical preservatives, including SO_2 and DMDC, particularly in light of growing consumer demand for reduced chemical inputs in winemaking. Beyond temperature and pH, oxygen availability should also be considered as a key environmental parameter, as it may further influence *B. bruxellensis* development and its ability to produce volatile phenols under oenological conditions.

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