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The co-inoculation ratio of *Hanseniaspora vineae-to-Saccharomyces cerevisiae* correlates with aroma metabolic features in wine

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Abstract

Hanseniaspora vineae is a non-*Saccharomyces* yeast used in winemaking to increase the complexity of wines. However, the fermentation rate in sequential inoculations may be challenging, particularly in industrial winemaking settings. This study aimed to assess how different co-inoculation protocols involving *H. vineae* and *S. cerevisiae* affect the fermenting performance and aroma of white and red wines. White and red wines were co-fermented with varying *H. vineae*-to-*S. cerevisiae* ratios (67%, 80%, 90%, 95%, and 98%). Results were compared to sequential and pure *S. cerevisiae* inoculation. Co-inoculation mitigated the inhibitory mechanisms associated with sequential inoculation, resulting in a reduction of 30 days and 6 days of fermentation for white and red wines, respectively. Moreover, the fermentation time in co-inoculation was similar to that of the controls, thereby avoiding the slowdowns typically observed in sequential inoculation. Five yeast-derived metabolic markers, two of which characterizing *H. vineae* metabolism, were studied to evaluate the processes. In white wines, β-phenylethyl acetate and benzyl alcohol were increased by *H. vineae* up to 64-fold and sevenfold, respectively, while ethyl hexanoate was fourfold higher in *S. cerevisiae*. In addition, 2-phenylethanol was up to twofold higher in *S. cerevisiae*. The results for isoamyl acetate varied depending on the co-inoculation ratio. At 67% and 80%, the *H. vineae* protocols showed the highest concentration, even exceeding that of *S. cerevisiae* pure inoculation. All compounds correlated linearly with the *H. vineae*-to-*S. cerevisiae* ratio at inoculum. The same trends were observed in red wines, but to a different extent.

Keywords Hanseniaspora vineae · Mixed fermentation · β-phenylethyl acetate · Benzyl alcohol

Introduction

Hanseniaspora yeasts are among the most important non-*Saccharomyces* genera linked to grapes [1]. In recent years, different strains belonging to this genus, such as *H. uvarum*, *H. osmophila* or *H. vineae* have been isolated and proposed for use in winemaking [2]. *H. vineae* Hv205 was initially isolated from Tannat grapes in Uruguay [3], and its genome has been fully sequenced [4, 5]. Its metabolism was explored under different winemaking conditions [6, 7] before its recent introduction to the market in 2022 [1], and different studies have reported *H. vineae*'s contribution in white, red, and rosé winemaking [8, 9]. Compared to conventional *Saccharomyces* strains, *H. vineae* has been found to increase wine flavor diversity and improve the juice ecosystem, while avoiding contamination by aerobic bacteria and yeasts [6]. In particular, *H. vineae* exhibits an intermediate to low fermentation capability, demonstrating the potential to achieve ethanol levels of up to 10% [10], with a moderate competitive capacity during vinification compared to *Saccharomyces* strains, contributing to increased microbial diversity in the process [9, 10]. Furthermore, *H. vineae* has a complementary secondary metabolism with *Saccharomyces cerevisiae*, which can affect the aromatic profile of the resulting wines [11, 12]. *H. vineae* can lead to the de novo synthesis of

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benzenoids and other phenylpropanoid compounds, including benzyl alcohol, which confers floral and fruity flavors to wines [13]. It has been demonstrated to overproduce up to 90-fold more β-phenylethyl acetate than *S. cerevisiae* [1], thereby improving the aroma profile of wines, particularly in non-aromatic grape varieties [8, 14]. Therefore, the presence of these compounds may indicate the involvement of *H. vineae* in vinification. However, once a particular strain is chosen for its distinctive metabolic traits, its suitability for industrial applications is determined not only by these features but also by its overall capability to produce the desired outcome and meet expectations. The wine industry requires a chosen yeast strain to fulfil fermentation requirements, which include fast fermentation onset, quick sugar consumption kinetics, and complete fermentation of musts containing a high sugar concentration [15, 16].

The oenological attributes of *H. vineae* are intriguing in this industry. Conversely, the flip side of the coin involves the necessity for precise adjustments to ensure the conclusion of fermentation, especially in highly mature white grapes [1, 17]. This improvement was achieved through sequential inoculation with *S. cerevisiae* midway through fermentation. Additionally, compared to *Saccharomyces* strains, *H. vineae* exhibits a slower rate of ethanol and CO_2 production, along with a lower production of fatty acids and branched chain higher alcohols. These characteristics have been proposed to increase yeast diversity during fermentation, allowing native or winery microflora to participate in the fermentation process [6, 18]. Evidence of challenges in achieving satisfactory fermentation performance for industrial purposes under sequential inoculation [17] has led this study to further explore the methods and management of co-inoculated cultures with *H. vineae*. This work aims to evaluate the effect of different co-inoculation protocols of *H. vineae* with *S. cerevisiae* in white and red winemaking in the fermenting kinetic performances while maintaining the aroma features and flavor intensity of the corresponding wines.

Materials and methods

Winemaking

A. White wine vinification.

250 L of grape must were obtained from the pressing (Willmes GmbH, Germany) of Glera grapes under anaerobic conditions, with the use of dry ice, in the de-stemmer and the press, while a continuous free flow of inert gas (Argon) was used in the must collection vessels. To the must, it was then added sulfur dioxide (15 mg/L) as potassium metabisulphite, ascorbic acid (5 mg/L; Dal Cin Gildo S.P.A., Concorrezzo, MB, Italy), and pectolytic enzymes

(10 µL/L; Rapidase Clear Extreme; Oenobrands, Montpellier, France). 24 h later, the must was racked and homogeneously divided into twenty-one 9 L aliquots and then inoculated. At the end of alcoholic fermentations, wines were racked and stabilized with 65 mg/L of sulfur dioxide.

B. Red wine vinification.

About 300 kg of grapes of Termantis variety were randomized manually bunch by bunch to limit the normal compositional variability found in the vineyard, into twenty-one aliquots of about 9.5 kg each. Grapes were destemmed, supplemented with 15 mg/Kg of sulfur dioxide, and inoculated with the same procedure as above. Maceration was managed by hand-punch downs, twice a day. At the end of alcoholic fermentations, wines were racked and stabilized with 65 mg/L of sulfur dioxide as potassium metabisulfite.

Inoculation protocols

Musts were fermented in triplicate with seven different inoculation protocols depending on the active dry yeast species used. The inoculation was made at $5 \cdot 10^6$ cells/ml for each trial. Commercial yeast was used, *S. cerevisiae* (Fermivin® LVCB, Oenobrands, France) and *H. vineae* (Fermivin® VINEAE, Oenobrands, France). In sequential inoculation protocol, the *S. cerevisiae* was added at 1/3 of the alcoholic fermentation. Inoculations and abbreviations used are resumed in Table 1.

Yeasts were inoculated after 20 min of rehydration in distilled water at 37 °C (separately or as a mixture). Concurrently with inoculation, 300 mg/L yeast lysates (Natuferm® Bright, Oenobrands, France) and 0.3 mg/L thiamine were added to the musts. After about 48 h of fermentation, the second addition of the same yeast lysates was made at a dose of 300 mg/L, except for the Hv processes, which were supplemented with the yeast autolysates at the sequential inoculation of *S. cerevisiae*, at 1/3 of the alcoholic fermentation.

Table 1 Summary of the inoculation protocols, with percentages indicating the proportion of each yeast species in relation to the total number of viable cells ($5 \cdot 10^6$ CFU/mL) inoculated for each trial

Abbreviation	Percentage *S. cerevisiae*	Percentage *H. vineae*	Inoculation modality
Sc	100%	0%	Pure
Hv	100%	100%	Sequential inoculation
C67	33%	67%	Co-inoculation
C80	20%	80%	Co-inoculation
C90	10%	90%	Co-inoculation
C95	5%	95%	Co-inoculation
C98	2%	98%	Co-inoculation

Fermentation kinetics

The fermentation kinetics of the wines were evaluated by measuring the density with a portable density meter (DMA 35, Anton Paar, GmbH, Austria). Data were collected twice a day after must homogenization. For each thesis and replicate, the measurement was made in triplicate. The parameterization of the kinetics was performed by comparing kinetics on the percentage of the fermentative course. Percentage points were then chosen that were useful for characterizing fermentation performance. Specifically, the times required to reach 3%, 5%, and 10% were calculated for the evaluation of the beginning of the alcoholic fermentation; 90%, 95%, and 97% for the evaluation of the last steps of fermentation, along with 25%, 50% and 75% for the control of exponential and stationary phases. Values were obtained by interpolation between the two closest experimental points, assuming a linear behavior between them.

Cell count

For verification of the viable cell concentration of the dry yeasts and, consequently, calculations of the corresponding inoculum dose, a cell count was performed on the active dry yeasts used. The diluted samples were spread on a WL Nutrient Agar medium (OXOID, Oxford, UK). Petri dishes were incubated at 25 °C for 4 days, after which yeast cells were quantified according to the OIV-MA-AS4-01: 2010 method.

Yeast evolution monitoring

The evolution of yeast population, and, in particular of *H. vineae,* was followed by plate and microscopical yeast count, according to the OIV methods (OIV, 2010). More in detail, a microscopic yeast count was performed by diluting 1:10 the sample with peptone-water (mycological peptone, 1 g/L in distilled water. Oxoid, UK). 1 mL of diluted sample was stained, adding 1 mL of methylene blue solution (OIV, 2022), and incubating for 10 min at room temperature in dark conditions. The stained sample was counted utilizing a Burcher chamber and an optic microscope ZL-N400T CF (Optika Science, I) equipped with a 400× objective and a phase contrast apparatus. Plate count was performed utilizing different synthetic media to discriminate among the yeast species utilized in this work. The total yeast population was enumerated onto WL agar medium (Oxoid, UK), after incubation at 25 ± 2 °C for 3 days. *H. vineae* was discriminated by plating samples onto Lysine Agar (Oxoid, UK), and incubated for 5 days at 25 ± 2 °C. The attribution of species was based on morphological characters of different yeast isolated, according to the description reported on the OIV methods (OIV, 2010).

Fourier-transform infrared spectroscopy (FTIR) measurement of must and wine basic chemical parameters

50 mL of juice, previously centrifuged (5000 rpm, 5 min) and filtered (25 mm × 0.45 μm cellulose acetate syringe cartridge; Alltech, Deerfield, IL, USA), were analyzed for °Brix, pH, and titratable acidity with a WineScan™ FT 120 Type 77,310 (Foss Electric A/S, Hillerød, Denmark), calibrated with the official methods. Wines were prepared in the same way for analysis of ethanol, pH, and titratable acidity.

GC–MS/MS analysis of volatile compounds

The method reported by [19] was used to analyze the volatile organic compounds (VOCs) in wine. Briefly, 50 mL of wine was diluted with Milli-Q water to 100 mL after adding 100 μL of internal standard (mixture of 1-heptanol at 230 mg/L and ethyl-3-hydroxybutyrate at 1000 mg/L in ethanol/water 50:50), the volatile compounds were then extracted by solid-phase extraction (SPE) and finally analyzed by GC–MS/MS. The gas chromatographic system used was a GC Agilent Intuvo 9000 coupled with a Triple Quadrupole MS Agilent 7000 equipped with an electron ionization source operating at 70 eV. Separation was obtained by injecting 2 μL in split mode (1:5) into a DB-Wax Ultra Inert (20 m × 0.18 mm id × 0.18 μm film thickness) capillary column with constant He flow of 0.8 mL/min. The injector temperature was set at 250 °C. The oven temperature was programmed starting at 40 °C for 2 min, raised to 55 °C by 10 °C/min, then raised to 165 °C by 20 °C/min, and finally raised to 240 °C by 40 °C/min and held at this temperature for 5 min. The mass spectrum was acquired in dMRM (dynamic multiple reaction monitoring) mode. The transfer line and source temperatures were set at 250 °C, and 230 °C, respectively. To identify and quantify the VOCs, the pure standard of each selected compound was injected at different concentration levels.

Results

Fermentation kinetics

The fermentation kinetics of Glera and Termantis are depicted in Supplementary Material S1, where it can be observed how management with sequential inoculation of *H. vineae* and *S. cerevisiae* (Hv) delayed the completion of alcoholic fermentation in both white and red wine vinification. Regarding Glera, Hv prolonged fermentation (~ 40 d) not only compared to Sc (~ 7d) but also to all co-inoculated trials with a mixture of both yeasts. The same differences, but to a different extent, were observed in Termantis, for which the time required for the completion of fermentation

in Hv processes (~ 16 d) almost doubled Sc (~ 8 d). Although inhibitory mechanisms cannot be ruled out, this speculation is supported when parametrized kinetics are analyzed (Fig. 1); every Glera process inoculated with *H. vineae* accelerates sugar consumption up to the middle of fermentation. In addition, at 25% fermentation, the Hv sequential protocol was faster than that for C67. Interestingly, despite the better performance during the first stages, the time needed to achieve 97% sugar consumption significantly increased with the *H. vineae* ratio and led to an average delay compared to Sc of ~ 72 h, ~ 61 h, ~ 46 h, ~ 31 h, and ~ 24 h for the C98, C95, C90, C80, and C67 processes, respectively. The same trend can be observed in Termantis (Fig. 1), for which Sc was the slowest process, up to 50% of the alcoholic fermentation, even if not significantly differentiated from all of them. The kinetics tended to improve with a lower proportion of *H. vineae*, leading to a mean delay to achieve 97% of fermentation of ~ 24 h, ~ 33 h, ~ 11 h, and ~ 18 h for C98, C95, C90, and C80 processes, respectively, concerning Sc, with C67 comparable, on average anticipating by ~ 4 h.

Viable cells of *H. vineae* and *S. cerevisiae* were monitored on days three and six after the initial inoculum, the former before the *S. cerevisiae* sequential inoculation of the Hv processes (Fig. 2). No *H. vineae* was found in Sc processes either at day three or at day six, while *S. cerevisiae* was present in Hv processes only at day six, with a limited number of viable cells (~ 7.7·10^4 CFU/mL), even following

S. cerevisiae inoculation with 5·10^6 CFU/mL. At the first monitoring point, the number of *H. vineae* cells was comparable among the co-inoculated trials, but also to Hv and the viable cells of *S. cerevisiae* in Sc, which was approximately 2 10^7 CFU/mL. After three days, the ratio between *H. vineae* and *S. cerevisiae* was approximately between 80% (C67) and 98% (C98), slightly increasing the proportion of *H. vineae* in the lower ratio protocols (C67, C80, and C90) compared to the initial inoculum. This behavior confirms the respect of *H. vineae* towards *S. cerevisiae* [1] but also a higher colonization rate of the apiculate yeast [20]. At day six, when the alcoholic fermentation exceeded 50% in all processes, *H. vineae* was still the main species in Hv and in those protocols in which *H. vineae* was inoculated with over 90% of the cells (Fig. 2) however, with fewer viable cells in all protocols respect the first sampling point. At this second point, *H. vineae* cells showed an upward trend with the initial dose employed in the co-inoculated trials, reflecting the initial ratio of *H. vineae* inoculum. On the other hand, the number of viable *S. cerevisiae* cells was consistent at both sampling points with the initial number of cells inoculated in the different protocols. Between both species, *H. vineae* represented ~ 35%, ~ 40%, ~ 60%, ~ 70%, and ~ 82% in C67, C80, C90, C95, and C98, respectively on day six. Despite the higher rate of *H. vineae* in the first stages, these results indicate a sort of maintenance of the original proportions employed, with no preferential colonization of one species

Fig. 1 Parameterization of the fermentation kinetics of **a** Glera and **b** Termantis according to the type of inoculum of *Saccharomyces cerevisiae* and *Hanseniaspora vineae*. ■ Hv: inoculation of Sc upon reaching 1/3 of fermentation; ■ Sc: fermentation managed only with *Saccharomyces cerevisiae*; The numbers of the remaining theses correspond to the percentage of *Hanseniaspora vineae* for co-inoculation with *Saccharomyces cerevisiae* at the start of fermentation: ■ 98%, ■ 95%, ■ 90%, ■ 80%, ■ 67%. Data are compared with one-way ANOVA followed by Tukey HSD multiple comparison ($p < 0.05$). Different letters indicate values statistically differentiated. The x-axis represents time in hours (h), while the y-axis represents the percentage of the fermentation process (%)
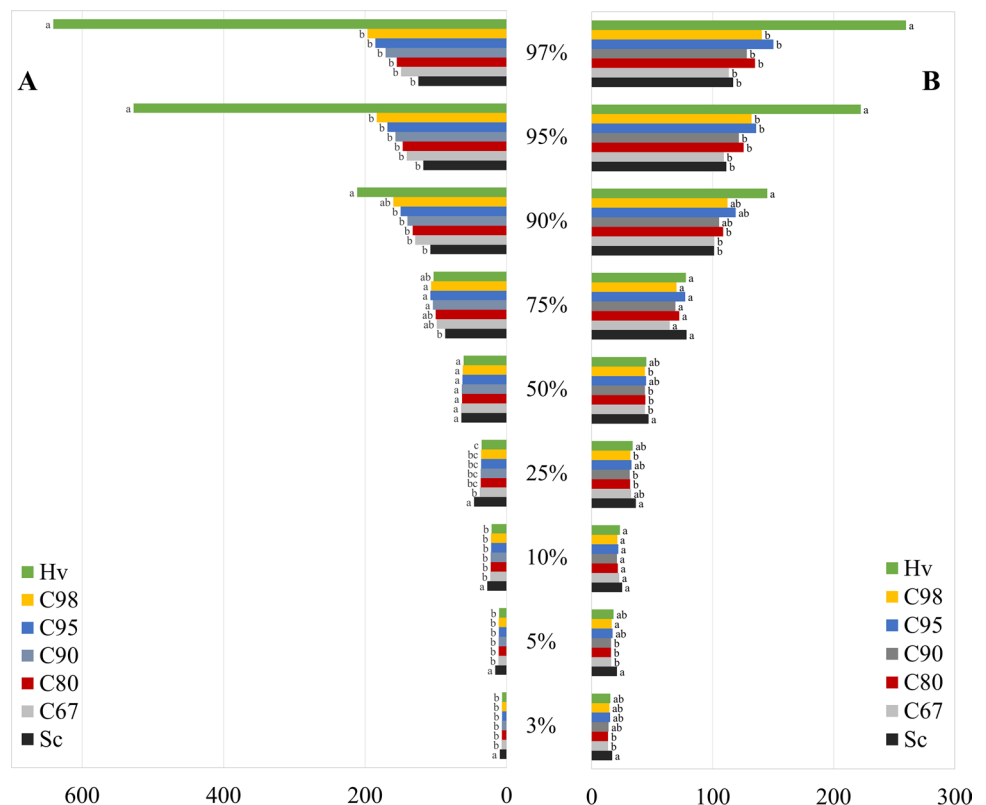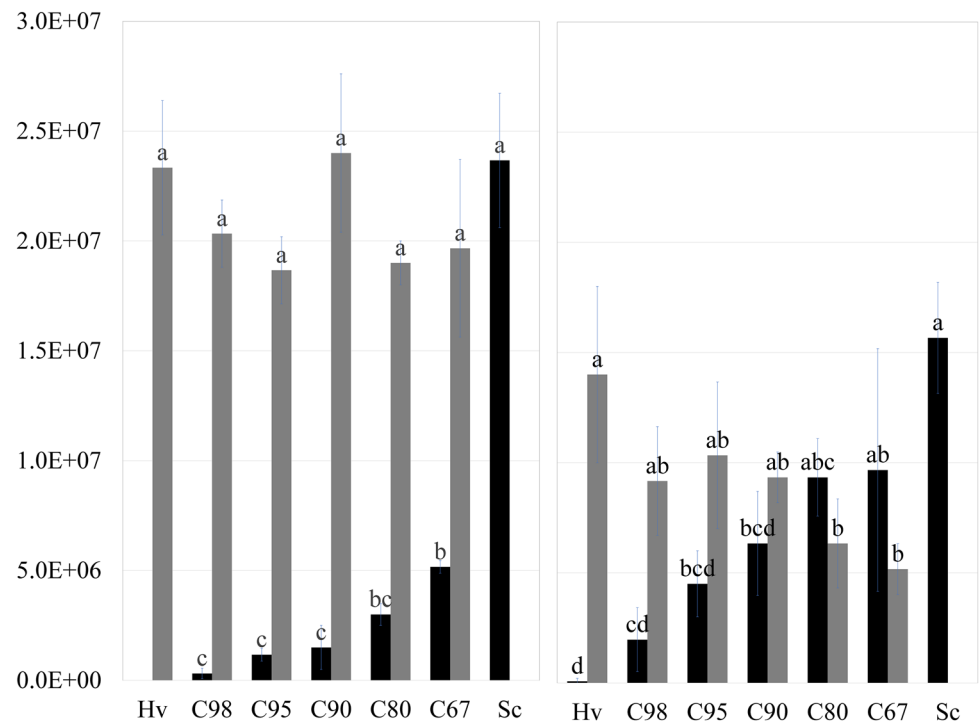
Fig. 2 Mean values (n = 3) ± standard deviation of viable cells (CFU/mL) at day three (left) and day six (right) of alcoholic fermentation of (gray) *H. vineae* and (black) *S. cerevisiae* in the fermenting must of Glera. Data are compared with one-way ANOVA followed by Tukey HSD multiple comparison (p < 0.05). Different letters indicate values statistically differentiated

on the other after six days. However, these differences could plausibly be related to the ethanol produced at this stage of fermentation. The number of viable *H. vineae* cells by day six was considerable, as the alcohol content in wines (8% to 9%) approached the levels of ethanol tolerance reported under laboratory conditions[14, 18].

The quality control parameters of wine at the end of fermentation are reported in the supplementary material (S2a, S2b). Despite the differences observed for some of them, it will be focused on those associated with the acid profile (Fig. 3a, b), which has already been reported to be modified by *H. vineae* under winemaking conditions [17]. Processes inoculated with *H. vineae* resulted in lower titratable acidity of wines, in both Glera and Termantis, with a negative correlation with the initial *H. vineae* ratio inoculated. The lowering of titratable acidity was mostly related to the decrease in malic acid concentration, which showed the same downward trend in both varieties. This statement becomes even more evident when evaluating Hv processes, for which malic acid is about half that of Sc in Glera wines and ~0.4 g/L lower in Termantis. Contrary to malic acid, tartaric acid presented an unclear trend, and the sole protocol to be influenced in both wine styles is Hv, probably as a consequence of an increased potassium bitartrate precipitation because of the slower fermenting kinetics.

Volatile acidity was higher in Sc processes in the white winemaking of Glera and, in contrast, much lower than Hv in Termantis. Within *H. vineae* co-inoculated protocols, this parameter showed the same upward trend for both wine styles, with the *H. vineae* ratio being clearer and statistically

significant in the red wine vinification of Termantis. *H. vineae* produces high concentrations of ethyl acetate [23], which, along with acetic acid, represents the volatile acidity of the wines. Nonetheless, ethyl acetate imparts fruity characteristics [24], and wines produced with *H. vineae* are perceived as fruitier [8, 18].

Regarding aroma compounds, the volatilome of the resulting wines is reported in supplementary material S3a and S3b, respectively for Glera and Termantis. To evince better the effect of yeast species and inoculum protocols, some metabolic markers known to be influenced by yeast species or directly produced due to lipid, nitrogen, and central carbon metabolisms have been selected (Table 2).

Ethyl hexanoate

Ethyl hexanoate in wines is formed by yeast from the esterification of ethanol with hexanoic acid, resulting mainly from lipid metabolism in the production of long-chain fatty acids [25, 26]. It confers wine-ripe fruit notes and presents an odor threshold of only 14 µg/L [27]. These features, along with the higher concentration of this ester normally reported in wines compared to others, convey its importance to wine quality. Ethyl hexanoate concentration was the highest among the main ethyl esters in both Glera and Termantis (Supplementary material S3a, S3b), and values were at least 44% and 55% higher than any other ethyl ester in Sc wines. Between species, Sc was richer than Hv in both white and red wine vinifications: ~ eightfold higher in Glera, and ~ 2.5-fold

Fig. 3 (**a**) Mean values (n=3) ±standard deviation of the acidic parameters of wines at the end of the alcoholic fermentation of Glera. Titratable acidity (gray); Malic acid (red); Tartaric acid (black) and Volatile acidity (blue). Data are compared with one-way ANOVA followed by Tukey HSD multiple comparison (p < 0.05). Different letters indicate values statistically differentiated. (**b**) Mean values (n=3) ±standard deviation of the acidic parameters of wines at the end of the alcoholic fermentation. Titratable acidity (gray); Malic acid (red); Tartaric acid (black) and Volatile acidity (blue). Data are compared with one-way ANOVA followed by Tukey HSD multiple comparison (p < 0.05). Different letters indicate values statistically differentiated
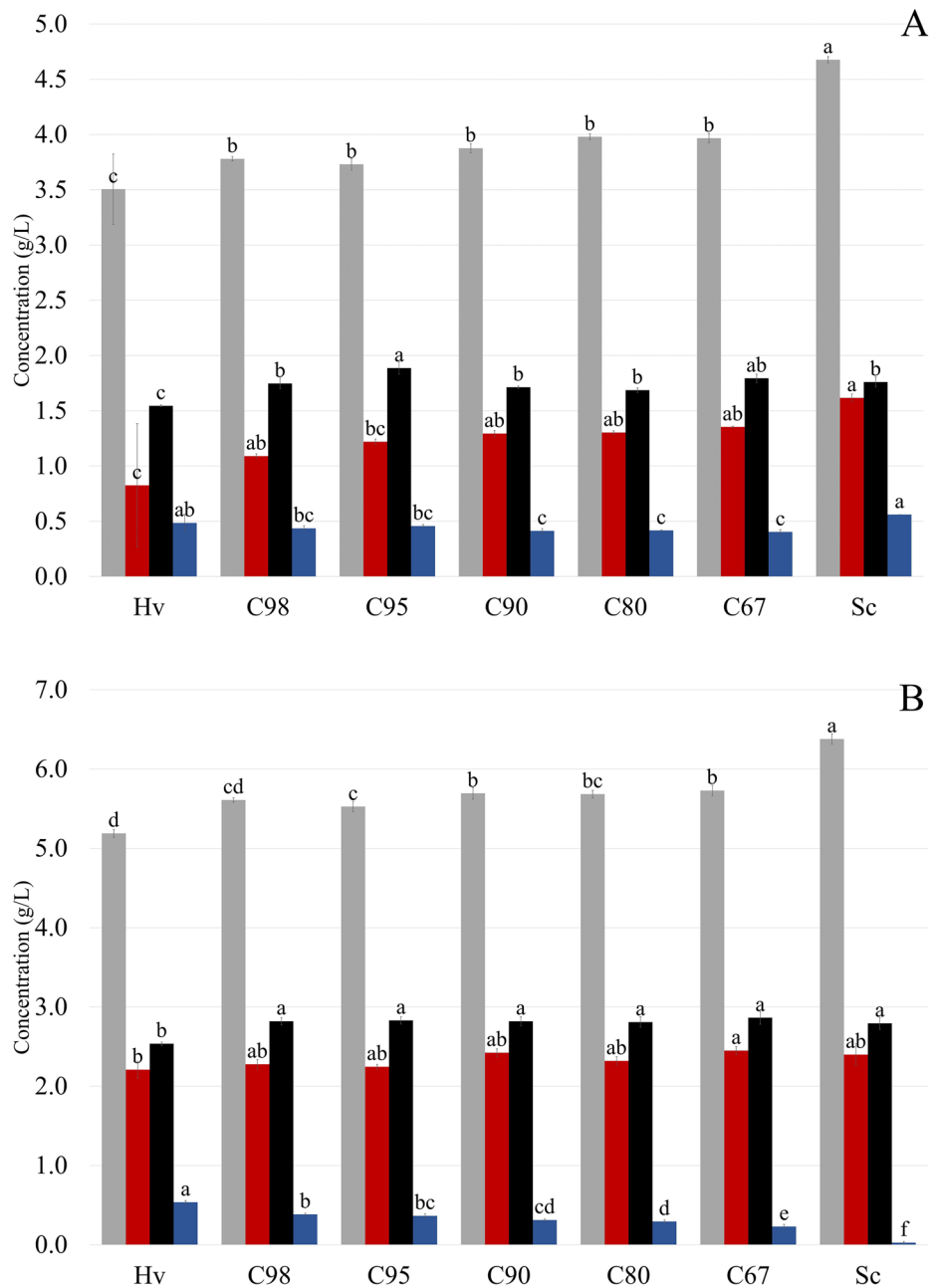
higher in Termantis. These differences have been reported in several studies that compared the metabolic features of *H. vineae* in sequential or pure inoculation [17, 28]. Within the co-inoculated processes, a downward trend was observed with a higher *H. vineae* ratio in both white and red wine vinifications (Fig. 4a and b, respectively), which allowed differentiating C67 from the rest of the processes, C80 from Hv in Glera wines, and C67 from C95 in Termantis.

Isoamyl acetate

Among the higher alcohol acetate esters, isoamyl acetate was selected as it is largely the main yeast-derived compound of this family of volatile molecules in *S. cerevisiae* [29, 30]. It is formed as a result of the esterification of isoamyl alcohol by acetyltransferases, and its content in wine is highly dependent on the nitrogen metabolism of yeast [31, 32]. It characterizes the aroma of young wines with its typical hint

Table 2 Mean values (n = 3) ± standard deviation of the concentration of aroma compounds in white wine (Glera) and red wine (Termantis) vinifications

Cultivar	Compound (mg/L)	Sc	C67	C80	C90	C95	C98	Hv
Glera	Ethyl hexanoate	0.24 ± 0.03^a	0.10 ± 0.00^b	0.07 ± 0.00^{bc}	0.06 ± 0.01^{cd}	0.05 ± 0.01^{cd}	0.04 ± 0.01^{cd}	0.03 ± 0.00^d
	Isoamyl acetate	0.72 ± 0.08^b	1.02 ± 0.02^a	0.99 ± 0.01^a	0.84 ± 0.16^{ab}	0.68 ± 0.26^{ab}	0.54 ± 0.17^{bc}	0.22 ± 0.01^c
	2-phenylethanol	20.08 ± 0.90^a	10.54 ± 0.29^b	9.17 ± 0.38^{bcd}	8.88 ± 0.5^{cd}	8.55 ± 0.47^{cd}	8.42 ± 0.15^d	10.07 ± 0.75^{bc}
	β-phenylethyl acetate	0.12 ± 0.01^c	4.91 ± 0.05^b	5.45 ± 0.45^b	6.73 ± 0.69^a	7.17 ± 0.36^a	7.6 ± 0.22^a	6.70 ± 0.56^a
	Benzyl alcohol	0.007 ± 0.000^b	0.008 ± 0.000^b	0.008 ± 0.001^b	0.008 ± 0.001^b	0.009 ± 0.001^b	0.009 ± 0.000^b	0.045 ± 0.030^a
Termantis	Ethyl hexanoate	0.10 ± 0.01^a	0.06 ± 0.01^b	0.05 ± 0.00^{bc}	0.05 ± 0.00^{bc}	0.04 ± 0.01^c	0.04 ± 0.00^{bc}	0.05 ± 0.00^{bc}
	Isoamyl acetate	0.35 ± 0.01^a	0.32 ± 0.04^a	0.34 ± 0.03^a	0.31 ± 0.02^a	0.30 ± 0.03^a	0.30 ± 0.02^a	0.29 ± 0.02^a
	2-phenylethanol	55.7 ± 2.0^a	45.9 ± 2.1^b	45.4 ± 2.5^{bc}	44.0 ± 0.7^{bc}	38.9 ± 3.3^{cd}	39.5 ± 2.1^{bcd}	35.9 ± 3.1^d
	β-phenylethyl acetate	0.05 ± 0.00^d	0.13 ± 0.03^{cd}	0.20 ± 0.03^{bcd}	0.16 ± 0.01^{bc}	0.21 ± 0.02^{bcd}	0.26 ± 0.06^a	0.24 ± 0.06^{ab}
	Benzyl alcohol	0.24 ± 0.00^a	0.26 ± 0.02^a	0.26 ± 0.02^a	0.26 ± 0.03^a	0.27 ± 0.02^a	0.27 ± 0.00^a	0.27 ± 0.02^a

Hv: inoculation of Sc upon reaching 1/3 of alcoholic fermentation; Sc: Saccharomyces cerevisiae; The numbers of the rest correspond to the percentage of Hanseniaspora vineae used for co-inoculation with Saccharomyces for a total starting inoculum of 510^6 UFC/mL: C98: 98%, C95: 95%, C90: 90%, C80: 80%, C67: 67%. Data are compared with one-way ANOVA followed by Tukey HSD multiple comparison (p < 0.05). Different letters indicate values statistically differentiated

of banana and "yellow" fruit [33, 34] because of its low odor threshold of 30 µg/L [23]. Compared with Sc, sequential inoculations of *H. vineae* (Hv) in Glera and Termantis wines resulted in a lower concentration of this acetate. This difference was more evident in Glera wines (> threefold) than in the red wine vinification of Termantis, where the difference was not statistically significant. A lower production with sequential inoculation has been reported previously [17, 35] nevertheless, the C67 and C80 Glera processes were richer in isoamyl acetate than in Sc. Among the co-inoculation processes, a negative correlation was observed with higher *H. vineae* ratios in Glera (Fig. 4a), and even if not differentiated, mean values showed the same tendency in Termantis wines (Fig. 4b).

2-phenylethanol

2-phenylethanol in wines is derived from phenylalanine, which is metabolized or produced by yeasts via the Erlich and Shikimate pathways [36]. This alcohol can actively participate in the flavor profile, developing honey-like and rose-like notes when present at concentrations greater than 14 mg/L [37]. It is also a necessary precursor for the production of β-phenylethyl acetate by yeast. This compound was consequently considered as *H. vineae* presents specific features in the biosynthetic pathways of aromatic amino acids [1], influencing the volatile yeast derivatives. The concentration of 2-phenylethanol was lower in Hv compared to the other processes, both in white and red winemaking. Differences were noticeable in white wines, for which Sc concentrations were approximately twice as high as in Hv

however, the absolute values were higher in red wines. These results have already been observed in both pure [18, 38] and sequential *H. vineae* inoculation in winemaking [8], probably because *S. cerevisiae* can activate the phenylpyruvate pathway under nutritional starvation conditions, overproducing this alcohol in YAN-limited fermenting musts [12]. The YAN of Glera and Termantis musts was low (30 mg/L and 61 mg/L, respectively), probably explaining the exceeding of the odor threshold in Sc wines. For the inoculated processes, two statistically differentiated groups were observed: C67 was separated from the rest by its higher concentration in Glera wines, and C95 and C98 in Termantis. For both wine styles, 2 phenylethanol showed the same downward trend with the increase in the *H. vineae* ratio as for the previous metabolites (Fig. 4a, b, respectively). Some authors have explained the lower concentration of 2-phenylethanol in wines through the overproduction of the corresponding ester by yeasts [5], which is a compound that characterizes *H. vineae* wines [38]. However, this explanation fails to fully explain the difference in concentration in molar terms [17] and may be the result of the different competing metabolic pathways employed by *H. vineae* in the catabolism and metabolism of aromatic amino acids [1].

β-Phenylethyl acetate

Among the metabolic yeast markers studied, β-phenylethyl acetate is probably the most important feature of *H. vineae* [21, 38], in terms of both concentration and aroma characteristics [1]. This compound exhibits a marked rose-like scent [39], for which wines produced with *S. cerevisiae*

Fig. 4 (**a**) Correlation between the concentration of a) 2-phenylethanol; b) β-phenylethyl acetate; c) ethyl hexanoate; and d) isoamyl acetate and the *H. vineae* ratio in co-inoculated fermentations of Glera. The dotted lines represent the 95% confidence interval. (**b**) Correlation between the concentration of a) 2-phenylethanol; b) β-phenylethyl acetate; c) ethyl hexanoate; and d) isoamylacetate and the *H. vineae* ratio in co-inoculated fermentations of Termantis. The dotted lines represent the 95% confidence interval
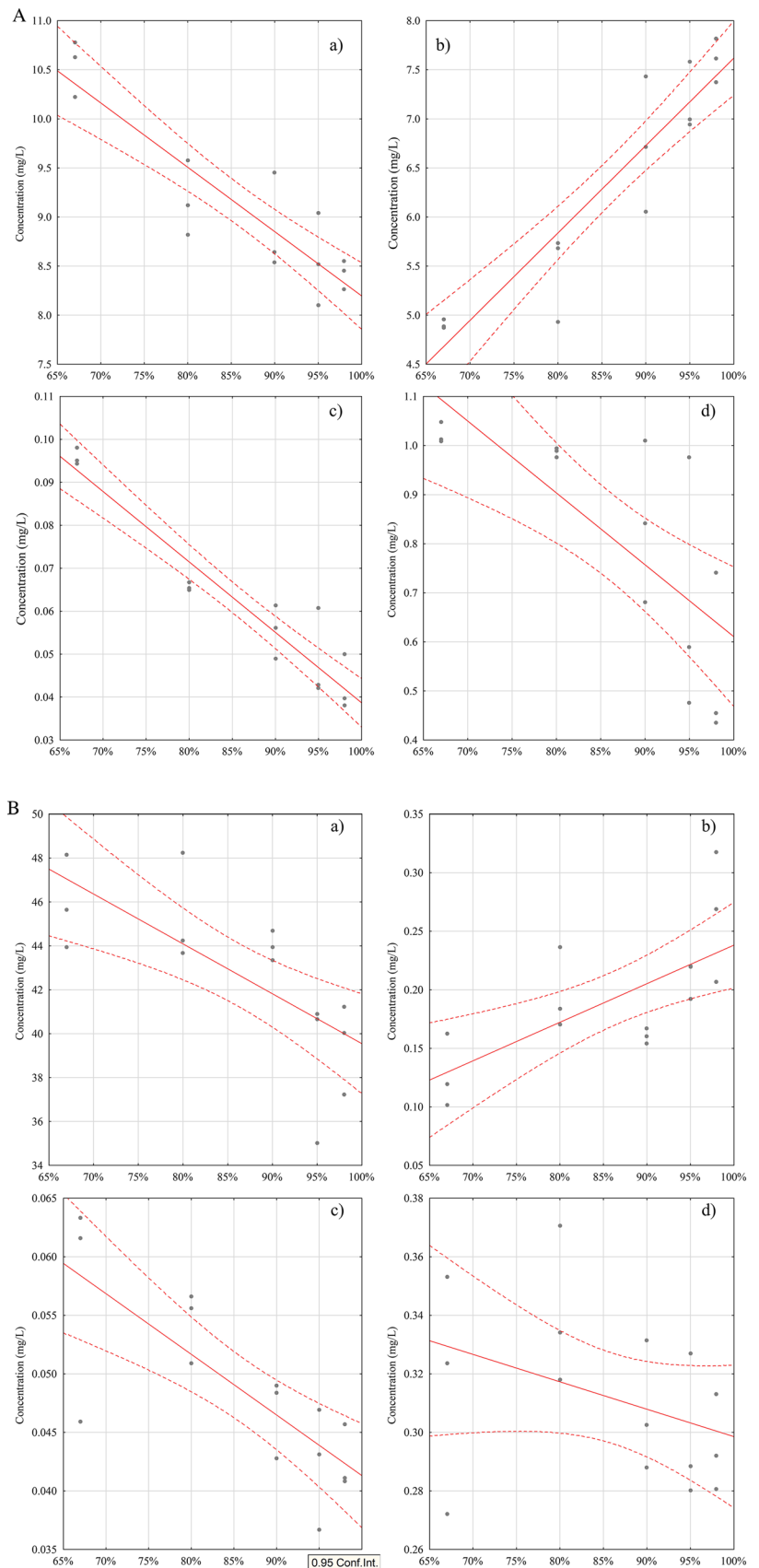
rarely exceed the odor threshold [40]. The results confirm this assertion, and Sc wines did not exceed 0.25 mg/L of this acetate ester, both in white and red wines. Hv processes were characterized by increased amounts of β-phenylethyl acetate compared to Sc, more clearly in white wines, for which the concentration was up to64-fold higher. These results confirm the remarkable production of this ester by *H. vineae*, as previously reported by several authors [14, 18, 21]. β-phenylethyl acetate values were consistent with those of previous studies on red wine production with sequential inoculation with *H. vineae* [41]. Regardless of the absolute values and diversly of the previous metabolites, the concentration of β-phenylethyl acetate showed an upward trend with the *H. vineae* ratio (Fig. 4a and b).

Benzyl alcohol

Benzyl alcohol is considered a metabolic marker of *H. vineae*, as it can be also de novo synthesized by this yeast in the absence of grape precursors [13]. In Termantis wines, the benzyl alcohol levels were comparable among processes (Table 2). In Glera, the sole differences appeared between Hv and Sc, for which the former was ~ sevenfold higher, according to previous studies on pure fermentation with *H. vineae* [18]. Despite the low content, an upward trend was observed with increasing *H. vineae* ratio, as previously observed for β-phenylethyl acetate.

Discussion

Indigenous non-*Saccharomyces* species have often been associated with the appearance of sensory faults in wines [42, 43]. Besides, non-*Saccharomyces* yeasts present a lower tolerance to ethanol, which does not allow normally to complete fermentation independently [44, 45]. Thus, under industrial conditions, the completion is normally accomplished by *S. cerevisiae*. The increased knowledge carried out during the last 30 years has led non-*Saccharomyces* to be in the spotlight of researchers and winemakers, owing to some specific, overexpressed, or silenced features to be exploited in wine production [46–48]. In the case of *H. vineae*, some strains have been selected because of their ethanol tolerance thanks to the higher glycolytic activity [49] and copy number of alcohol dehydrogenase genes [5], that allows to reach up to 10% v/v under controlled conditions [10]. Along with the alcohol tolerance [12, 22], the Hv205 strain used was further selected from other *H. vineae* strains because of the specific aroma features that impart in wines [50]. Once selected, suitability in industrial contexts depends not only on these attributes but rather on the overall attitude to result in the desired product and fulfill expectations. These also include the possibility of foreseeing

the final characteristics of wines and scheduling the production. This is particularly important in winemaking, as fermentation is condensed in just a few weeks. In addition, the aroma features given by yeasts must comply with varietal aspects that play an important role in the perception of wine typicality.

In our study, the use of *H. vineae* in sequential inoculation resulted in slower fermentation kinetics compared to *S. cerevisiae* in both white and red wine vinification, confirming this feature in semi-industrial wines [17]. Regarding red wine vinification, Hv doubled the time required for the completion of fermentation, and in white wine vinification, needed over five times the time employed by *S. cerevisiae*. Usually, winemakers that use non-*Saccharomyces* species assume different fermentation kinetics only if the metabolic features that they are looking for emerge. In the case of *H. vineae*, its use has been proposed to increase the aroma complexity of wines through the overproduction of some compounds that are mainly involved in the metabolism of aromatic amino acids [5]. However, it is not limited to these compounds and its features allow for increased terpenes and some norisoprenoids in wines [8, 51], probably as a consequence of the exocellular activity of β-glycosidases secreted by *H. vineae*. Recently, it has also been reported the production of safranal [51] during the aging of Albillo produced with *H. vineae* or the enhanced concentration of yeast mannoproteins [8, 51], as a consequence of its faster autolysis [52, 53]. Co-inoculation of *H. vineae* with *S. cerevisiae* in Glerashortened fermentation compared to Hv, and even if with slower kinetics than Sc, some protocols could reasonably comply with most industrial needs during harvest. The shortened time showed a downward trend with an increasing *H. vineae* inoculum ratio. At day three, the number of viable *H. vineae* cells was comparable among the different protocols and with *S. cerevisiae* in Sc. However, at day six, differences were substantial but coherent with the initial inoculum -both *H. vineae* and *S. cerevisiae*- increasing the viable cell ratio in those processes initially poorer than the apiculate yeast (C67, C80, and C90). These results might be interpreted as an advantage of *H. vineae* development during the first stages of the alcoholic fermentation towards *S. cerevisiae*. To date, *H. vineae* has not been reported to produce toxins against *S. cerevisiae* [1], similar to other *Hanseniaspora* spp. [54], supporting previous speculations regarding the vantage of nutrient assimilation [17].

Some species of the genus *Hanseniaspora* have been proposed for the degradation of malic acid during winemaking [55] and the degradation of malic acid by Hv205 was previously noted in sparkling wine production [17]. Although malate dehydrogenases are present in a vast number of *Hanseniaspora* spp., *H. vineae* lacks most malate cell transporters found in *S. cerevisiae* [1]. However, *H. vineae* contains JEN family genes that can metabolize fumarate and

succinate, which are in turn involved in malate degradation by other microorganisms, such as *Pichia kudriavzevii* [56]. The decreasing content of malic acid with an increasing number of viable cells inoculated with *H. vineae* in white and red winemaking provides further evidence of its ability to degrade malate. Nonetheless, further studies are required to elucidate the malate degradation mechanisms of *H. vineae*.

Regarding the production of aroma compounds, every volatile metabolic marker studied in white and red wines was significantly correlated with the *H. vineae* ratio in the co-inoculated wines (Table 3), positively only with β-phenylethyl acetate and benzyl alcohol. The linearity of the results with the *H. vineae* ratio at inoculum allowed for modulation of the overall volatile profile in wines, increasing the main *H. vineae* feature (β-phenylethyl acetate production) compared to *S. cerevisiae*, even at the lowest ratio (C67), both in Glera and Termantis wines. This has resulted in a 44-fold to 64-fold increase compared to Sc, confirming the remarkable production of this ester by *H. vineae* [18, 50]. The concentration of β-phenylethyl acetate in mixed fermentations was moreover comparable to that of Hv from C90 in Glera and C67 in Termantis. Thus, even if significantly lower, the aroma characteristics of *H. vineae* emerged even at the lower co-inoculation ratio in mixed fermentations. These results are in agreement with previous studies that have highlighted this metabolic feature in pure and sequential inoculations [1, 57]. However, comparison with Hv processes might be misleading, as acetate esters in wines are subject to acid hydrolysis [58] arising in 2-phenylethanol. Thus, the longer fermentation kinetics of Hv may have increased β-phenylethyl acetate degradation. Considering the sum of β-phenylethyl acetate and 2-phenylethyl alcohol, both of which are mainly derived from the same metabolic pathways, protocols managed with *H. vineae* were not differentiated. Sc was the sole showing different amounts of the sum of phenylalanine derivatives, higher than any other protocol. Differences may be then remitable to the acetylation ratio, for which that found in Sc was much lower (barely 0.01) than in any co-inoculated protocol. This parameter increased linearly with the *H. vineae* ratio: from 0.46 ± 0.01 in C67 to 0.90 ± 0.04 in C98, indicating its dependence on the metabolic activity of *H. vineae* during fermentation. Differences in absolute values between wine styles could be partially attributable to the inhibition of the expression of alcohol acetyltransferases in aerated media [59], such as red winemaking, which could have resulted in only Termantis C98 exceeding the odor threshold in red wines. However, these differences could be also the result of different population dynamics during fermentation. *H. vineae* has been demonstrated to not impede *S. cerevisiae* growth [1], but under anaerobic conditions, it can limit its development during the first stages of fermentation. However, *H. vineae* growth benefits from moderate microaerobic conditions [60], largely exceeding the red winemaking of Termantis, for which aeration conditions are known to increase cell growth [61]. The absence of differences from sequential inoculation suggests that aerobic management applied during maceration may have affected *H. vineae* metabolism, as already observed in Sc [62]. The increased expression of the acetyltransferases of *H. vineae* [38] seems to be somehow specific for aromatic higher alcohols as confirmed by the overproduction of β-phenylethyl acetate, its positive correlation with the increasing inoculum ratio, and the negative correlation with the same found for isoamyl acetate. Not only, but the increased concentration of isoamyl acetate in C67 and C80 white wines suggests a sort of synergy that the presence of *S. cerevisiae* would determine in the formation of this compound. This speculation is supported by the negative correlation found with the *H. vineae* inoculation ratio and the lower concentration found in Hv wines, that may have varied the kinetics of 2-phenylethanol release in the fermenting media, acting as a substrate for acetylation, as previously reported for other non-*Saccharomyces* multistarters used in winemaking [63].

Overall, the results reported for white wines are comparable to those of red wine vinification protocols, but to different extents. These differences might be explained by the concurrent features of wine style production that are intrinsically linked to the aeration conditions in red winemaking. Aeration is known to affect yeast metabolism, and *H. vineae* prefers moderate aeration conditions to best express its enological features [60]. *S. cerevisiae* is a facultative anaerobe that could increase competition against *H. vineae* during the population dynamics of the first steps of alcoholic fermentation [18, 64], thus limiting the effect on the metabolic profile of wines. Lastly, the expression of acetyltransferases is inhibited by oxygen [59, 61], further limiting acetate ester production.

Table 3 Correlation coefficients and p-value between volatile compounds and the initial ratio between *H. vineae* and *S. cerevisiae*

Compound	Glera			Termantis		
	r	r²	*p*	r	r²	*p*
Ethyl hexanoate	− 0.9520	0.9063	0.0000	− 0.7798	0.608	0.0006
Isopenthyl acetate	− 0.7402	0.5479	0.0016	− 0.3799	0.1444	0.1624
2-phenylethanol	− 0.9003	0.8105	0.0000	− 0.7299	0.5327	0.0020
β-phenylethyl acetate	0.9294	0.8638	0.0000	0.6931	0.4804	0.0042
Benzyl alcohol	0.2468	0.069	0.3753	0.2634	0.0694	0.3428

Regarding benzyl alcohol, despite its low content, an upward trend was observed with increasing *H. vineae* ratio as for β-phenylethyl acetate. The increased production of phenylpropanoid derivatives by *H. vineae* is dependent on the nutritional status of musts [65]. In Glera, a slowdown of the alcoholic fermentation kinetics reported in Hv processes during the second part of alcoholic fermentation could have activated the mandelate and 4-hydroxy mandelate pathways[65], leading to a higher production of this aromatic amino acid derivative, not observed co-inoculated trials.No difference were found between processes for any of the terpenes or norisoprenoids analyzed (Supplementary material S3a, S3b); however, compounds belonging to these families were present only in limited concentrations in wines.

Conclusions

This work has demonstrated that different co-inoculation strategies of *H. vineae* with *S. cerevisiae* might be a promising alternative for the use of this apiculate yeast in winemaking industrial conditions. Every parameter studied was correlated with the initial *H. vineae* inoculum ratio, starting from the fermentation kinetics, for which the time needed to complete the alcoholic fermentation was reduced with a lower ratio in both winemaking styles and significantly diminished with respect to the sequential protocol. Interestingly, despite the presence of *S. cerevisiae* at the onset of alcoholic fermentation, *H. vineae* was the main species in the first stages of fermentation and did not limit *S. cerevisiae* contribution.

The metabolic features of *H. vineae* were clearly expressed in co-fermentation, resulting in increased production of β-phenylethyl acetate compared to *S. cerevisiae*, while it remained comparable to the sequential inoculation with the highest studied ratios. The lower ratios resulted in the amelioration of other aroma compounds, better than *S. cerevisiae* for isoamyl acetate in Glera wine. Regarding the acidic parameters, the co-inoculation strategies limited the natural degradation of malic acid, and even if white wines could not be sought, this feature could be exploited in red winemaking in cool climates or vinification of early harvested grapes.

These results underscore the potential impact of strategically managing the co-inoculation ratio to achieve industrial requirements, demonstrating its ability to influence wine quality and increase aroma complexity and product diversity.

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Data availability I don't have any research data outside the submitted manuscript file.

Declarations

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