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Aroma Features of *Hanseniaspora vineae* Hv205 Wines in Sequential and Co-Inoculation Strategies

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Abstract: *Hanseniaspora vineae* (Hv) is a non-*Saccharomyces* yeast with unique metabolic features, making it appealing for wine production. However, Hv presents high nutritional requirements that may lead to slow fermentation. This study investigated the impact of sequential inoculation of *Saccharomyces cerevisiae* (Sc) in white winemaking at different time points (24, 48, 74, 100 and 200 h) during Hv fermentation and compared them to simultaneous inoculations. The 200 h protocol extended fermentation by an average of 13 days compared to pure Sc, decreasing with earlier sequential inoculation. Sc wines were richer in isoamyl acetate and ethyl hexanoate than Hv wines, with no significant differences among inoculation protocols. β-phenylethyl acetate was increased in Hv wines, particularly in the 24 h protocol. The 2-phenylethanol concentration was negatively correlated with the *S. cerevisiae* inoculation delay. Hv altered the wine aroma features, enhancing the compounds associated with rose-like scents. Reducing the Sc inoculation delay aligned Hv with industrial standards while maintaining increased β-phenylethyl acetate production. However, co-inoculation with Sc seems to better meet the Hv requirement without sacrificing the main aromatic features of Hv, demonstrating faster sugar depletion and higher acetate and ethyl ester contents, suggesting that co-inoculation yields a more modulable wine aroma profile.

Keywords: mixed fermentation; β-phenylethyl acetate; non-*Saccharomyces*

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1. Introduction

In recent years, the use of unconventional yeast in winemaking has increased sharply due to their overexpressed or unique metabolic features that influence wine quality [1]. *Saccharomyces cerevisiae* (Sc) demonstrates its ability to shape the final composition of wine primarily when introduced at the onset of fermentation. However, when added to partially fermented must by apiculate yeasts, its metabolic activity undergoes significant alterations [2]. The robust fermentative capacity of Sc, coupled with its ability to ferment even in the presence of oxygen (known as the Crabtree effect), positions it as an efficient ethanol producer. This strategy allows Sc to dominate the fermentation process by out-competing other microorganisms due to the toxic effects of ethanol [3,4]. The metabolic focus of Sc primarily revolves around ethanol production, emphasizing primary metabolism while secondary metabolism, responsible for aroma production, is often neglected [5]. Conversely, non-*Saccharomyces* yeast species exhibit a high degree of development in secondary metabolism, contributing to increased flavour diversity. Indeed, employing mixed culture inoculum has shown promise in enhancing flavour complexity in wine production.

Recent sensory studies on mixed culture fermentations involving various white and red grape varieties have reported increased complexity, highlighting the potential for sensory enrichment [6–10].

Studies have indicated that *Hanseniaspora vineae* can be introduced as part of a sequential mixed starter, engaging in competition with indigenous yeasts present in non-sterile must [11,12]. Furthermore, the utilization of non-*Saccharomyces* yeasts, including *H. vineae*, in co-inoculated fermentations alongside Sc revealed interesting potential impacts on wine characteristics [11]. *H. vineae* exhibits a complementary secondary metabolism with Sc, the predominant yeast in wine fermentation. This collaboration helps establish an enhanced juice ecosystem, mitigating the risk of contamination by aerobic bacteria and yeasts [13]. Additionally, *H. vineae* has been observed to reduce the synthesis of medium-chain fatty acids and display a robust acetylation capacity of aromatic higher alcohols, which are known inhibitors of many yeasts. Consequently, the mixed inoculation of *H. vineae* with other yeast strains, such as Sc, emerges as a strategic approach to create optimal conditions for flavour complexity and diversity in wine production [13–15]. This strategy is anticipated to identify yeasts capable of sharing fermentation medium nutrients, characterized by reduced nitrogen demand, thereby promoting the development of clean flavours.

This study aims to investigate the adaptability of *H. vineae* to mixed fermentation processes with Sc, specifically examining both sequential and co-inoculation approaches. Our purpose is to determine the optimal conditions for achieving a favourable balance between fermentation duration and the metabolic characteristics of *H. vineae*, with a particular emphasis on assessing the consequences of varying delays before the introduction of *S. cerevisiae* as well as varying ratios of *H. vineae* to *S. cerevisiae* in simultaneous inoculations.

2. Materials and Methods

2.1. Winemaking

250 kg of each variety (Glera and Gewurztraminer) coming from Trentino region (Italy) were destemmed and then pressed (Willmes GmbH, Lorsch, Germany). Dry ice was employed in both the de-stemmer and the press, while a continuous flow of inert gas (Argon) was maintained during the collection of must in vessels. The obtained must was supplemented with sulfur dioxide (15 mg/L), pectolytic enzymes (Rapidase Clear Extreme; Oenobrands, Montpellier, France) and was chemically sterilized with dimethyldicarbonate (16 mL/hL). After 24 h, the must underwent racking and was aliquoted into 10 L demijohns per replicate before the inoculation process. Following the completion of alcoholic fermentations, the wines underwent racking and were stabilized with 65 mg/L of sulfur dioxide. The compositions of grape musts are reported in Table 1.

Table 1. Quality control parameters of grape musts. YAN: yeast assimilable nitrogen.

Grape	Reducing Sugars (g/L)	pH	Titratable Acidity (g/L)	Malic Acid (g/L)	Tartaric Acid (g/L)	Potassium (g/L)	YAN (mg/L)
Gerwürztraminer	230	3.53	3.46	1.58	5.22	1.86	114
Glera	213	3.15	4.90	2.62	3.90	0.98	223

2.2. Inoculation Protocols

Glera and Gewürztraminer must undergo fermentation in triplicate, employing distinct inoculation protocols corresponding to the specific active dry yeast species utilized. In each trial, the inoculation was performed at a concentration of 5×10^6 cells/mL. The yeast strains employed included commercial S. *cerevisiae* (Fermivin® LVCB, Corimpex, Italy) and *H. vineae* (Fermivin® *VINEAE*, Corimpex, Italy). Details of the inoculations and corresponding abbreviations are summarized in Table 2. The yeasts were inoculated after a 20-min rehydration in distilled water at 37 °C, either separately or as a mixture in case of co-inoculum. With the inoculations 300 mg/L of yeast lysate (Natuferm Bright, Oenobrands, Montpellier, France) and 0.3 mg/L of thiamine were added to the musts. In

the case of co-inoculated musts, 48 h into fermentation, a second addition of the same yeast lysates was made (300 mg/L), while for sequential inoculations, the same nutrient additions were performed at the moment of the *S. cerevisiae* inoculations.

Table 2. Inoculation protocols utilized in the different studies.

Abbreviation	Inoculation Strategy	Starter	Time span for *S. cerevisiae* Sequential Inoculation	Glera Fermentation	Gewürztraminer Fermentation
Sc	Pure	*S. cereviae* 100%		•	•
S.24	Sequential	*H.vineae* 100%	24 h	•	•
S.48	Sequential	*H.vineae* 100%	48 h	•	•
S.68	Sequential	*H.vineae* 100%	68 h	•	
S.74	Sequential	*H.vineae* 100%	74 h	•	
S.100	Sequential	*H.vineae* 100%	100 h	•	•
S.200	Sequential	*H.vineae* 100%	200 h	•	
C80	Coinoculation	*H.vineae* 80%: *S. cerevisiae* 20%			•
C98	Coinoculation	*H.vineae* 98%: *S. cerevisiae* 2%			•

2.3. Fermentation Kinetics

The fermentation kinetics of the wines were assessed using a digital density meter (DMA 35, Anton Paar, GmbH, Austria), measuring density after must homogenization twice daily. Each measurement, for every thesis and replicate, was conducted in triplicate. To parameterize the kinetics, the fermentative course percentages were compared, and specific points were chosen to characterize performance. The times to reach 3%, 5%, and 10% were calculated for the onset of alcoholic fermentation, while 90%, 95%, and 97% were determined for the final stages. Additionally, 25%, 50%, and 75% were considered for controlling exponential and stationary phases. Values were interpolated between the nearest experimental points, assuming a linear behaviour.

2.4. Fourier-Transform Infrared Spectroscopy (FTIR) Measurement of Must and Wine Basic Chemical Parameters

Analysis of the primary control parameters in must and wine was performed on 50 mL of juice using Fourier-transformed infrared spectroscopy (WineScanTM FT 120 Type, 77310 Foss Electric A/S, Hillerød, Denmark), calibrated with official methods of the Office International de la Vigne et du Vin [16].

2.5. GC-MS/MS Analysis of Volatile Compounds

The method outlined by Paolini et al. (2018) [17] was utilized for the analysis of volatile organic compounds (VOCs) in wine. In summary, 50 mL of wine was diluted to 100 mL with Milli-Q water, and 100 µL of internal standard (n-heptanol) was added. The volatile compounds were subsequently extracted through solid-phase extraction (SPE) and analyzed using GC-MS/MS. The gas chromatographic system employed was a GC Agilent Intuvo 9000 coupled with a Triple Quadrupole MS Agilent 7000, featuring an electron ionization source operating at 70 eV. A DB-Wax Ultra Inert capillary column (20 m × 0.18 mm id × 0.18 µm film thickness) was used, with a constant helium flow of 0.8 mL/min and an injection volume of 2 µL in split mode (1:5). The injector temperature was set at 250 °C. The oven temperature program initiated at 40 °C for 2 min, increased to 55 °C at 10 °C/min, further raised to 165 °C at 20 °C/min, and finally elevated to 240 °C at 40 °C/min, maintaining this temperature for 5 min. Mass spectrum acquisition occurred in dMRM (dynamic multiple reaction monitoring) mode. The transfer line and source temperatures were set at 250 °C and 230 °C, respectively. Identification and quantification of VOCs involved injecting pure standards of each selected compound at various concentration levels.

2.6. Statistical Analysis

All experiments were conducted in biological triplicate, and data analysis was performed using R version 4.0.3 in RStudio. Principal component analysis was performed using the R package ggplot2. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons tests ($\alpha = 0.05$) were conducted using the package stats and agricolae.

3. Results and Discussion

The results of the parametrized kinetics in sequential inoculation are reported in Figure 1. The findings revealed that the longer the delay for *S. cerevisiae* inoculation, the longer the fermentation process (97% of the alcoholic fermentation). The pure inoculum with *S. cerevisiae* performed best, with differences appearing after 50% of alcoholic fermentation. However, *H. vineae* processes were comparable, or in some cases faster (S.48) than the pure culture with *S. cerevisiae* until the 10% of the AF, confirming the good performances during the initial stages of fermentation already reported [18]. Differences in *H. vineae* metabolism are wide with respect to *S. cerevisiae* [19], particularly in the uptake profile of nitrogen compounds, which affects fermentation [20] and metabolic outcomes [21]. These nutritional requirements influence the growth of *S. cerevisiae* during fermentation [20], which can lead to undesirable stuck and sluggish fermentations [22,23]. In this research, employing *H. vineae* in consecutive inoculations did not result in stuck fermentation (Table 3). Nevertheless, as the time interval for *S. cerevisiae* inoculation increased, the overall fermentation performance deteriorated, and each combined procedure prolonged the fermentation process. The time needed to achieve 97% of fermentation was positively ($t_{97\%}$ (h) = 4.4 time span $_{S.\ cerevisiae}$ (h) + 185.8) and statistically ($R^2 = 0.98$; $p < 0.05$) correlated with the time span of *S. cerevisiae* inoculation in sequential processes with up to a 74 h delay (Figure 2). From this point on, the fermentation time plateaued regardless of the sequential inoculation delay. These results likely indicate a saturation point in terms of initial population size and rate growth, suggesting a high level of competition between the two yeast species. Although *S. cerevisiae* has been recognized for negatively impacting the proliferation and survival of non-*Saccharomyces* species [5,10,20], recent studies have demonstrated the competitive capabilities of *H. vineae* towards *S. cerevisiae* in co-inoculated mixed industrial fermentations [13], probably as a consequence of the suboptimal environment for *S. cerevisiae* caused by *H. vineae* [20], as previously observed for other non-*Saccharomyces* [23,24]. Moreover, despite *H. vineae* being recognized as a non-toxin-producing yeast [13], acclimatizing *S. cerevisiae* in mixed fermentations with *H. vineae* can be challenging primarily due to the apiculate yeast's high competitiveness for nutrients [11,20,25].

Nevertheless, results are aligned with studies that have demonstrated favourable kinetics of *H. vineae* during the first stages of fermentation at winemaking conditions [25]. Along with the competition for nutrients between species, yeast interactions in fermentation are known to be dynamic [1,23,24,26,27].

Among the main quality control parameters of wines, *H. vineae* processes were richer in ethanol than Sc (Table 3). This difference likely resulted from the lower production of glycerol, the primary by-product of alcoholic fermentation [28]. *H. vineae* and other *Hanseniaspora* spp. Have been also reported to degrade malic acid during alcoholic fermentation [18,29,30]; this reduction has been found to be more important with a higher number of viable cells inoculated [31]. At the fermenting conditions, this feature seems to be more yeast-dependent, than time span inoculum-dependent, and only S.200 slightly reduced malic acid with respect to other *H. vineae* processes (Table 3). Results suggest that malate could be mainly metabolized during the first stages of cell development. The malic-acid-degrading activity of *H. vineae* is probably at the base of the small but statistically relevant differences in pH values, higher in all the mixed fermentations except for S.24. Additionally, tartaric acid was found to be preserved at higher concentrations in *H. vineae* wines, compared to *S. cerevisiae*. These results, along with the absence of a potassium variation among processes, may refer to a possible inhibition in bitartrate precipitation,

probably as a consequence of the faster and increased release of *H. vineae* mannoproteins already reported to occur during fermentation [32].

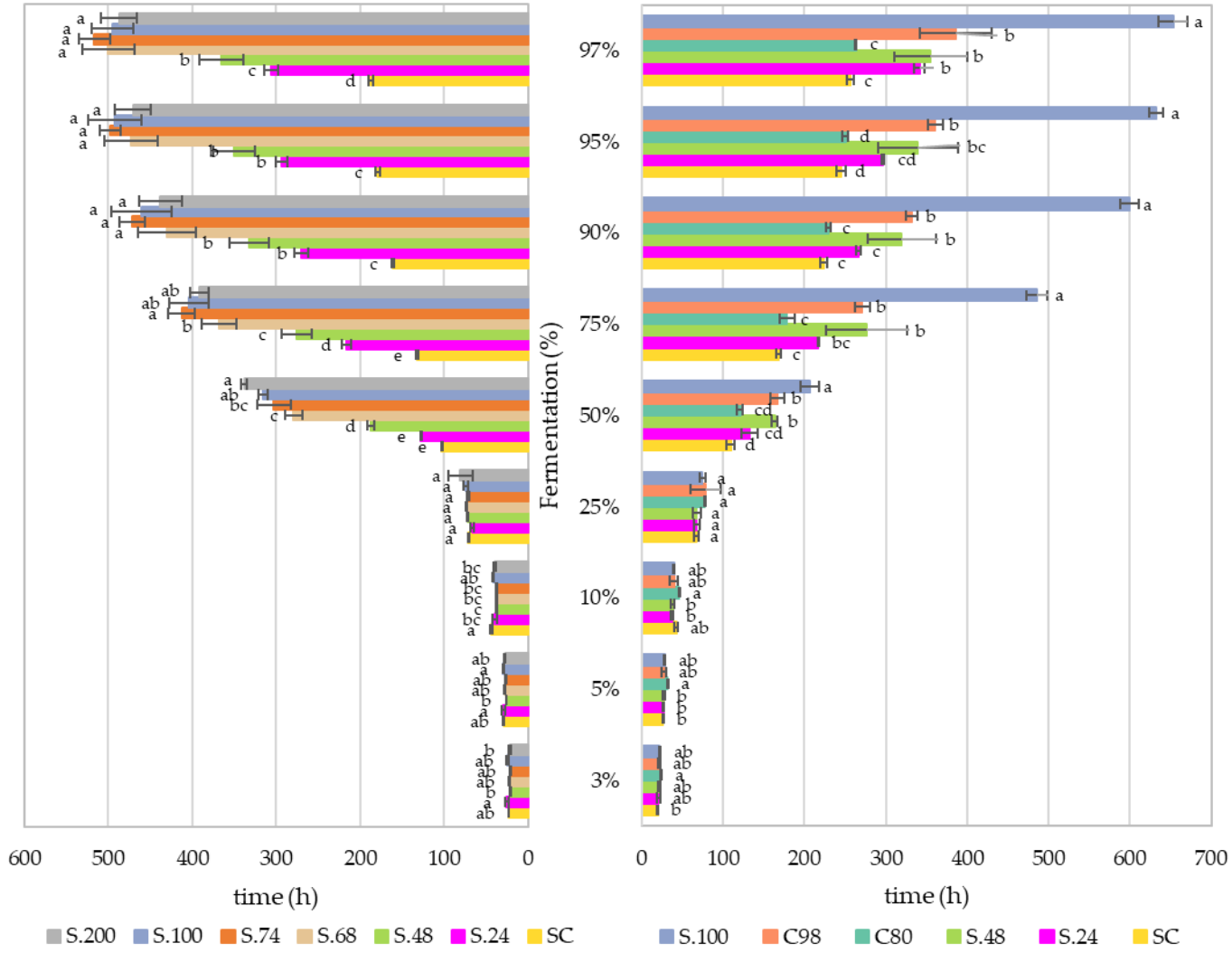

Figure 1. Parameterization of the fermentation kinetics of Glera (**left**) and Gewürztraminer (**right**) according to the type of inoculum of *S. cerevisiae* and *H. vineae*. Results were compared with one-way ANOVA followed by Tukey HSD multiple comparisons ($p < 0.05$; $n = 3$). Different letters indicate values statistically differentiated.

Table 3. Mean concentration ($n = 3$) ± standard deviation of the main quality control parameters of wines at the end of the fermentation in Glera. Data are compared with ANOVA followed by Tukey HSD multiple comparisons ($p < 0.05$). Different letters indicate values statistically differentiated.

	SC	S.24	S.48	S.68	S.74	S.100	S.200
Ethanol (%vol)	12.40 ± 0.05 ^{b}	12.88 ± 0.29 ^{a}	13.00 ± 0.07 ^{a}	12.86 ± 0.08 ^{a}	12.93 ± 0.08 ^{a}	12.85 ± 0.12 ^{a}	12.90 ± 0.09 ^{a}
Glucose + Fructose (g/L)	<1.0 ^{a}	<1.0 ^{a}	<1.0 ^{a}	2.53 ± 1.59 ^{a}	2.16 ± 0.86 ^{a}	2.06 ± 1.79 ^{a}	0.66 ± 1.15 ^{a}
pH	3.45 ± 0 ^{c}	3.45 ± 0 ^{c}	3.48 ± 0.01 ^{b}	3.5 ± 0.01 ^{ab}	3.51 ± 0 ^{a}	3.5 ± 0 ^{ab}	3.51 ± 0 ^{a}
Titratable acidity (g/L)	6.36 ± 0.05 ^{a}	5.46 ± 0.05 ^{b}	5.3 ± 0 ^{c}	5.23 ± 0.05 ^{cd}	5.06 ± 0.05 ^{e}	5.13 ± 0.05 ^{de}	5.03 ± 0.05 ^{e}
Volatile acidity (g/L)	0.51 ± 0.01 ^{a}	0.33 ± 0.02 ^{b}	0.28 ± 0 ^{c}	0.28 ± 0 ^{c}	0.29 ± 0 ^{c}	0.27 ± 0.01 ^{c}	0.25 ± 0 ^{c}
Total dry extract (g/L)	23.1 ± 0.17 ^{ab}	21.23 ± 0.11 ^{b}	22.13 ± 0.4 ^{ab}	23.8 ± 1.3 ^{a}	23.1 ± 0.62 ^{ab}	23.2 ± 1.3 ^{ab}	22.36 ± 0.92 ^{ab}
Malic acid (g/L)	2.86 ± 0.03 ^{a}	2.33 ± 0.03 ^{b}	2.3 ± 0 ^{b}	2.23 ± 0.04 ^{bc}	2.25 ± 0.06 ^{bc}	2.24 ± 0.03 ^{bc}	2.19 ± 0.01 ^{c}
Lactic acid (g/L)	<Lod	<Lod	<Lod	<Lod	<Lod	<Lod	<Lod
Tartaric acid (g/L)	1.72 ± 0.02 ^{c}	1.81 ± 0.04 ^{b}	1.9 ± 0.02 ^{a}	1.81 ± 0.03 ^{b}	1.83 ± 0.03 ^{ab}	1.81 ± 0.02 ^{b}	1.86 ± 0.01 ^{ab}
Ashes (g/L)	2.03 ± 0.05 ^{b}	2.03 ± 0.05 ^{b}	2.13 ± 0.05 ^{ab}	2.16 ± 0.05 ^{ab}	2.16 ± 0.05 ^{ab}	2.16 ± 0.05 ^{ab}	2.2 ± 0 ^{a}
Glycerol (g/L)	8.73 ± 0.05 ^{a}	7.26 ± 0.15 ^{b}	7.13 ± 0.05 ^{bc}	6.9 ± 0.17 ^{c}	6.8 ± 0.2 ^{c}	6.86 ± 0.05 ^{c}	6.96 ± 0.05 ^{bc}
Potassium (g/L)	0.73 ± 0.01 ^{a}	0.67 ± 0.04 ^{a}	0.72 ± 0.05 ^{a}	0.74 ± 0.02 ^{a}	0.71 ± 0.02 ^{a}	0.73 ± 0 ^{a}	0.75 ± 0.02 ^{a}

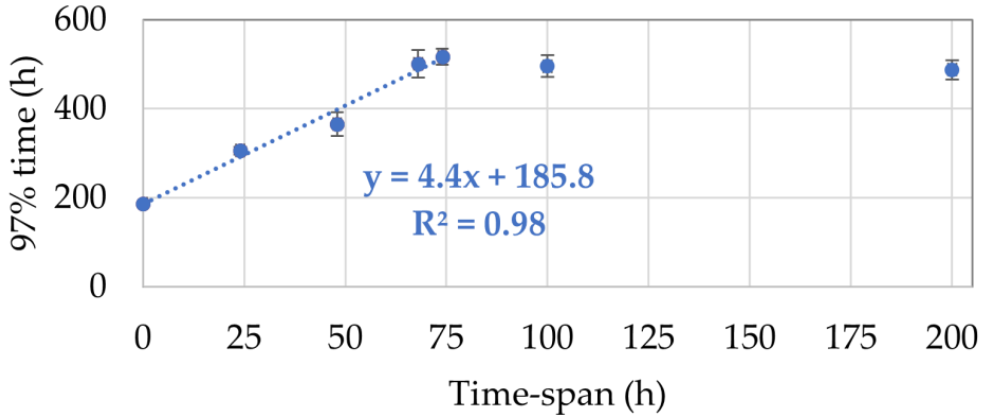

Figure 2. Correlation between the delay in *S. cerevisiae* sequential inoculation and the time needed for the completion of 97% of the alcoholic fermentation.

When unconventional yeasts are used under industrial conditions, it is mandatory to attain the desired metabolic features without compromising the overall quality of wines and production requirements. This risk is heightened when nitrogen compounds are restricted in the fermenting must, which is also associated with the production of yeast-derived aroma compounds [33,34]. Among the interesting features influencing wine quality [32,35,36], *H. vineae* stands out, for the overproduction of specific aroma compounds, and precisely those derived from the metabolism of aromatic amino acids [11,36,37]. Table 4 details the volatile profile of wines at the end of fermentation, revealing β-phenylethyl acetate as the primary compound affected by *H. vineae*. This compound that emanates a distinct fragrance reminiscent of roses [38] can enhance yeast-derived scents in wines and differentiate them from the fruity aromas typically associated with *S. cerevisiae* fermentations. The different time span mixed inoculations increased the concentration of β-phenylethyl acetate from 8- (S.200) up to 12-fold (S.24) compared with *S. cerevisiae.* Several studies have reported the overproduction of this acetate ester both in terms of concentration and aroma traits [11,36,37,39,40]. Unexpectedly, the shorter pure fermentation protocols of *H. vineae* (S.24 and S.48) exceeded the production of this metabolite compared to S.100 and S.200, despite previous studies with other non-*Saccharomyces* species reporting greater metabolic contribution with the initial absence of *S. cerevisiae* [11]. The results could be the consequence of *H. vineae's* control over the initial stages of alcoholic fermentation, even when co-inoculated with *S. cerevisiae* [31], and the consequent depletion of nutrients that have retarded fermentation. This has led to a 60% increase in the time needed to complete fermentation in the most delayed sequential inoculations, probably resulting in a higher hydrolysis of the ester. Nevertheless, it cannot be excluded a synergistic effect between both yeasts. In any case, it is interesting to note that *H. vineae*, differently from other non-*Saccharomyces* [41–44], is able to produce its distinctive metabolites with a short delay in sequential inoculation. The overproduction of β-phenylethyl acetate reported is mainly due to the increased acetylation ratio of 2-phenylethanol in *H. vineae* processes, which ranged from ~23% and up to 39% with respect to *S. cerevisiae*'s (~3%).

The overproduction of β-phenylethyl acetate was associated with a decrease in the content of isoamyl acetate, as documented in previous studies [11,45]. Isoamyl acetate is the primary acetate ester in young white wines and is characterized by fruity banana aromas. Overall, the production of acetate esters in *H. vineae* processes was higher than in Sc. Notwithstanding, isoamyl acetate accounted for 86% of the total acetate esters analyzed in Sc, while in mixed fermentations, it ranged from 19% to 25%. In contrast, β-phenylethyl acetate accounted for 10% of the total acetates in Sc and 72% to 80% in *H. vineae* processes, resulting in a modified profile. This modulation along with the increased total acetate production up to 50%, contributes to the distinctiveness of *H. vineae* wines [36].

Table 4. Concentration of volatile compounds at the end of the fermentation in Glera wines. Values represent the means of three replicates ± SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. Different superscript letters denote significant differences ($p < 0.05$) among yeast treatments within the same grape variety.

	Compounds (μg/L)	SC	S.24	S.48	S.68	S.74	S.100	S.200
Acetates	β-phenylethyl acetate	385.23 ± 153.33 ^{d}	4642.56 ± 153.52 ^{a}	4158.53 ± 198.77 ^{ab}	3823.03 ± 193.24 ^{bc}	3945.8 ± 426.84 ^{abc}	3788.5 ± 532.17 ^{bc}	3206.1 ± 80.12 ^{c}
	ethylphenyl acetate	0.46 ± 0.05 ^{a}	0.26 ± 0.05 ^{b}	0.33 ± 0.05 ^{ab}	0.43 ± 0.11 ^{ab}	0.3 ± 0 ^{ab}	0.33 ± 0.05 ^{ab}	0.3 ± 0 ^{ab}
	isobutyl acetate	35.16 ± 0.81 ^{a}	13.2 ± 1.38 ^{b}	11.46 ± 0.55 ^{b}	11.46 ± 1.48 ^{b}	11.03 ± 1 ^{b}	11 ± 0.5 ^{b}	6.83 ± 1.02 ^{c}
	isopentyl acetate	3282.16 ± 114.21 ^{a}	1181.16 ± 177.04 ^{b}	1179.86 ± 6.64 ^{b}	1317.36 ± 259.7 ^{b}	1233.43 ± 107.5 ^{b}	1366.96 ± 108.96 ^{b}	774.26 ± 78.04 ^{c}
	n-butyl acetate	75.3 ± 3.26 ^{a}	28.73 ± 4.74 ^{bc}	27.36 ± 1.25 ^{bc}	32.2 ± 7.3 ^{b}	29.06 ± 2.94 ^{bc}	33.06 ± 5.31 ^{b}	18.16 ± 2.92 ^{c}
	n-hexyl acetate	25.86 ± 1.25 ^{d}	26.06 ± 1.62 ^{d}	29.73 ± 1.3 ^{bc}	34.03 ± 1.64 ^{a}	35.7 ± 0.43 ^{a}	33 ± 0.85 ^{ab}	27.23 ± 1.44 ^{cd}
Alcohols	1-hexanol	308.63 ± 18.72 ^{c}	456.93 ± 4.72 ^{b}	462.43 ± 11.79 ^{ab}	482.13 ± 25.53 ^{ab}	477.83 ± 9.86 ^{ab}	478.1 ± 3.78 ^{ab}	497.33 ± 12.84 ^{a}
	3-methylthio-1-propanol	259.33 ± 15.59 ^{d}	390.33 ± 2.3 ^{b}	387.56 ± 6.57 ^{b}	437.33 ± 32.81 ^{a}	437.33 ± 4.58 ^{a}	409.63 ± 11.9 ^{ab}	340.43 ± 16.23 ^{c}
	2-phenyl ethanol	13,617.43 ± 754.13 ^{a}	11,896.46 ± 181.11 ^{c}	12,028.33 ± 109.2 ^{bc}	13,364.16 ± 531.06 ^{a}	13,130.1 ± 185.2 ^{ab}	13,434.73 ± 321.76 ^{a}	14,030.13 ± 409.26 ^{a}
	benzyl alcohol	2.43 ± 0.7 ^{a}	3.3 ± 0.81 ^{a}	8.83 ± 7.48 ^{a}	4.1 ± 1.44 ^{a}	1.86 ± 0.25 ^{a}	9.26 ± 8.51 ^{a}	5.4 ± 4.47 ^{a}
	cis-3-hexen-1-ol	20.3 ± 1.12 ^{b}	22.46 ± 0.2 ^{ab}	23.16 ± 1.87 ^{a}	23.16 ± 0.56 ^{a}	22.36 ± 0.41 ^{ab}	22.3 ± 0.55 ^{ab}	22.73 ± 0.75 ^{ab}
	trans-3-hexen-1-ol	5.3 ± 0.91 ^{a}	5.13 ± 0.51 ^{a}	6.46 ± 0.55 ^{a}	6.5 ± 0.34 ^{a}	5.86 ± 0.4 ^{a}	5.9 ± 1.15 ^{a}	5.9 ± 0.52 ^{a}
Ethyl esters	diethyl succinate	38 ± 0.69 ^{a}	25.03 ± 0.92 ^{de}	26.66 ± 1.2 ^{cd}	31.06 ± 2.05 ^{b}	28.66 ± 0.83 ^{bc}	28.36 ± 0.9 ^{bc}	22.9 ± 0.43 ^{e}
	ethyl-2-methylbuthyrate	0.6 ± 0 ^{a}	0.33 ± 0.05 ^{b}	0.33 ± 0.05 ^{b}	0.33 ± 0.05 ^{b}	0.36 ± 0.05 ^{b}	0.33 ± 0.05 ^{b}	0.23 ± 0.05 ^{b}
	ethyl butyrate	201.63 ± 7 ^{a}	94.63 ± 8.3 ^{b}	95.3 ± 8.27 ^{b}	100.3 ± 4.15 ^{b}	95.63 ± 4.01 ^{b}	86.83 ± 2.65 ^{b}	59.13 ± 2.6 ^{b}
	ethyl decanoate	160.36 ± 31.94 ^{d}	317.63 ± 9.12 ^{c}	462.23 ± 73.11 ^{ab}	511.23 ± 36.44 ^{a}	429.06 ± 6.63 ^{abc}	403.06 ± 57.18 ^{abc}	385.9 ± 12.11 ^{bc}
	ethyl dodecanoate	8.26 ± 1.5 ^{b}	9.5 ± 0.65 ^{b}	14.13 ± 2.77 ^{ab}	18.13 ± 3.36 ^{a}	13.26 ± 2.45 ^{ab}	12.06 ± 2.95 ^{ab}	8.96 ± 0.55 ^{b}
	ethyl hexanoate	414.33 ± 44.56 ^{a}	136.86 ± 13.19 ^{b}	132.16 ± 12.26 ^{b}	145.46 ± 4.8 ^{b}	128.96 ± 9.57 ^{b}	124.83 ± 13.82 ^{b}	100.7 ± 1.04 ^{b}
	ethyl isovalerate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	ethyl lactate	932.36 ± 76.31 ^{a}	470.2 ± 32.61 ^{b}	445.06 ± 28.25 ^{bc}	378.06 ± 26.2 ^{bcd}	343.23 ± 28.78 ^{cd}	327.03 ± 31.04 ^{d}	289.96 ± 13.95 ^{d}
	ethyl octanoate	424.86 ± 128.97 ^{a}	175.13 ± 10.92 ^{b}	183.96 ± 36.25 ^{b}	233.66 ± 4.3 ^{b}	163.73 ± 11.04 ^{b}	152.56 ± 37.04 ^{b}	142.73 ± 6.03 ^{b}
	methyl salicilate	0 ± 0 ^{b}	0.3 ± 0 ^{a}	0.23 ± 0.05 ^{a}	0.26 ± 0.05 ^{a}	0.23 ± 0.05 ^{a}	0.23 ± 0.05 ^{a}	0.2 ± 0 ^{a}
Fatty acids	butanoic acid	459.33 ± 17.35 ^{a}	236.23 ± 11.95 ^{b}	222.13 ± 6.36 ^{bc}	227.53 ± 4.37 ^{bc}	218.63 ± 6.94 ^{bc}	204.56 ± 4.16 ^{cd}	185 ± 8.21 ^{d}
	decanoic acid	726.03 ± 120.83 ^{c}	1374.63 ± 151.64 ^{b}	1955.46 ± 102.26 ^{a}	1900.2 ± 133.15 ^{a}	1735.16 ± 270.48 ^{ab}	1854.43 ± 284.79 ^{ab}	1844.2 ± 150.23 ^{ab}
	hexanoic acid	1572.93 ± 37.16 ^{a}	424.43 ± 34.81 ^{b}	384.06 ± 16.7 ^{bc}	390.53 ± 12.1 ^{bc}	365.23 ± 25.23 ^{bc}	334.36 ± 13.82 ^{cd}	292.93 ± 7.19 ^{d}
	isobutyric acid	181.26 ± 3.1 ^{a}	91.3 ± 13.35 ^{b}	77.8 ± 6.5 ^{bcd}	67.1 ± 7.2 ^{cd}	59.7 ± 4.3 ^{d}	68.8 ± 9.4 ^{cd}	84.7 ± 2.92 ^{bc}
	isovaleric acid	244.73 ± 9.07 ^{a}	171.46 ± 1.2 ^{bc}	167.3 ± 4.23 ^{bc}	176.46 ± 17.86 ^{b}	166.03 ± 5.16 ^{bc}	149.53 ± 9.76 ^{cd}	139.63 ± 7.84 ^{d}
	nonanoic acid	5.6 ± 0.95 ^{a}	5.36 ± 0.32 ^{a}	5.2 ± 0.26 ^{a}	5.03 ± 0.57 ^{a}	4.83 ± 0.6 ^{a}	5.16 ± 0.2 ^{a}	4.76 ± 0.49 ^{a}
	octanoic acid	2629.2 ± 77.17 ^{a}	881.2 ± 103.87 ^{b}	863.1 ± 17.54 ^{b}	859.8 ± 51.23 ^{b}	876 ± 93.6 ^{b}	840.73 ± 31.54 ^{b}	718.23 ± 54.7 ^{b}
	valeric acid	30.9 ± 0.79 ^{a}	13.86 ± 0.8 ^{b}	12.53 ± 1.1 ^{bc}	10.73 ± 0.65 ^{cd}	12.8 ± 0.87 ^{bc}	10.13 ± 1.17 ^{cd}	8.03 ± 1.88 ^{d}
Terpenes	alpha terpineol	11.76 ± 0.92 ^{d}	15.36 ± 1.44 ^{cd}	17 ± 1.99 ^{bc}	20.73 ± 2.33 ^{ab}	23.23 ± 1.53 ^{a}	20.83 ± 2.41 ^{ab}	20.46 ± 1.06 ^{ab}
	beta citronellol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	geranic acid	2.33 ± 0.5 ^{c}	4.9 ± 0.78 ^{bc}	4.96 ± 0.76 ^{bc}	6.66 ± 1.81 ^{ab}	9.16 ± 0.81 ^{a}	8.43 ± 0.7 ^{a}	7.33 ± 0.95 ^{ab}
	geraniol	16.36 ± 2.01 ^{a}	16.03 ± 0.92 ^{a}	22.06 ± 5 ^{a}	17.46 ± 4.68 ^{a}	13.73 ± 1.81 ^{a}	14.96 ± 3.54 ^{a}	13.83 ± 1.91 ^{a}
	linalol oxide A	22.03 ± 1.05 ^{a}	21.33 ± 1.11 ^{a}	20.5 ± 1.47 ^{a}	20.73 ± 0.56 ^{a}	20.66 ± 1.06 ^{a}	20.5 ± 0.7 ^{a}	21.33 ± 0.49 ^{a}

Table 4. *Cont.*

	Compounds (μg/L)	SC	S.24	S.48	S.68	S.74	S.100	S.200
	linalol oxide B	9.93 ± 0.63 [a]	9.96 ± 0.96 [a]	9.86 ± 0.85 [a]	10.36 ± 0.3 [a]	10.16 ± 0.55 [a]	9.76 ± 0.55 [a]	10.93 ± 0.37 [a]
	linalool	99.36 ± 3.3 [a]	103.03 ± 3.58 [a]	102.6 ± 2.95 [a]	99.53 ± 2.73 [a]	98.73 ± 9.84 [a]	104.46 ± 5.81 [a]	97.33 ± 3.5 [a]
	nerol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	rose oxide I	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	rose oxide II	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	terpinen 4 ol	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Others	benzaldehyde	8.56 ± 0.23 [b]	19.1 ± 1.03 [a]	18.2 ± 6.21 [ab]	25.06 ± 1.42 [a]	26.23 ± 1.43 [a]	20.33 ± 4.74 [a]	20.33 ± 4.55 [a]
	benzothiazole	1.73 ± 0.2 [a]	1.53 ± 0.05 [a]	1.73 ± 0.4 [a]	1.9 ± 0.17 [a]	1.56 ± 0.32 [a]	1.33 ± 0.11 [a]	1.86 ± 0.4 [a]
	beta damascenone	1.43 ± 0.2 [b]	1.8 ± 0.34 [ab]	1.83 ± 0.15 [ab]	2.13 ± 0.2 [a]	1.76 ± 0.2 [ab]	2.03 ± 0.35 [ab]	1.76 ± 0.05 [ab]
	beta damascone	30.4 ± 5.34 [b]	34.56 ± 2.17 [ab]	42.56 ± 1.55 [ab]	46.13 ± 5.85 [a]	37.7 ± 3.48 [ab]	45.43 ± 7.26 [a]	37.33 ± 2.32 [ab]
	guaiacol	1.3 ± 0.1 [a]	1.23 ± 0.32 [a]	1.06 ± 0.25 [a]	1.16 ± 0.15 [a]	1.13 ± 0.05 [a]	1.4 ± 0.36 [a]	1.46 ± 0.3 [a]
	zingerone	0.5 ± 0 [a]	0.2 ± 0 [b]	0.23 ± 0.05 [b]	0.23 ± 0.05 [b]	0.2 ± 0 [b]	0.26 ± 0.05 [b]	0.2 ± 0 [b]

But *H. vineae's* also influenced the concentration of ethyl esters in wines. The study's outcomes corroborated the specific feature of *H. vineae*, which produced a higher quantity (up to 3-fold) of ethyl decanoate compared to *S. cerevisiae* at the expense of shorter esters, however with no clear trend among inoculation protocols. Despite the lower overall content, ethyl decanoate accounted for circa 50% of ethyl esters in *H. vineae* processes with no differences between protocols, while in *S. cerevisiae* processes its contribution was limited to ~13%. Conversely, in these latter processes, both ethyl hexanoate and octanoate represented ~35% of the total esters, while in *H. vineae* only ~15% and ~20% respectively. These findings are consistent with the fatty acid profile of wines and are in line with previous studies that report lower levels of fatty acids in *H. vineae* compared to *Saccharomyces* strains [35].

Benzyl alcohol was not differentiated between treatments, diversely to what previously reported [46] in which *H. vineae* is capable of producing levels up to dozens of times higher than *S. cerevisiae*. Nonetheless, the level of its oxidized form, benzaldehyde, increased more than 2-fold in *H. vineae* processes and reached up to four times the concentration. There was no discernible pattern concerning the duration of the inoculum, indicating the more pronounced expression of the phenylpropanoid pathway. However, benzyl alcohol and benzaldehyde are also present in grapes as glycosides, and the release of these compounds during fermentation could be the result of the glycosidase activity of yeasts. Certain strains of *Hanseniaspora* spp., including *H. vineae*, have been found to possess active β-glucosidases, contributing to enhanced aromas in Muscat wines [47–49]. This activity could be the basis of the increased concentration found for some terpenes, like α-terpineol, norisoprenoids and methyl salicylate, despite the modest concentrations of them found in wines. Nevertheless, it cannot be excluded the biochemical origin from other precursors. Whatever the pathway, there is no clear trend between time span of sequential inoculation in mixed fermentations.

To further analyze differences among yeast inoculation protocols, the aroma compositions were explored using PCA (Figure 3), with the result being that the first two principal components, PC1 and PC2, are 64.91% of the cumulative variance. The individuals of wine samples for the seven protocols showed separation that largely reflected the use of Hv species from left to right along PC1 (56.56% of the total variance). According to variable loadings for PC1, increasing of isopentyl acetate, isobutyl acetate, most short, medium chain fatty acids and their ethyl esters positively correlated with the Sc treatment; while β-phenylethyl acetate, methyl salicylate, decanoic acid and its ester, benzaldehyde and C6 alcohols positively correlated with the presence of *H. vineae* in the inoculum. Differences among sequential inoculation time span were more subtle compared to differences between pure culture and mixed inoculum, and their separation was mostly explained along PC2. due to the decrease in norisoprenoids and ethyl decanoate and dodecanoate.

Based on these results and on previous studies that have demonstrated the positive correlation between the metabolic characteristics and fermentation performance of *H. vineae* with the number of initially viable cells co-inoculated with S. *cerevisiae* in mixed fermentations [13,31], the features of *H. vineae* in different sequential (S.24, S.48, S.100) and co-inoculation processes (C80 and C98) were compared with those of pure S. *cerevisiae* fermentation of a Gewürztraminer grape must. The parametrized fermentation kinetics reported in Figure 1 showed that the time needed for fermentation in C98 (~16 d) was comparable to that of S.48 (~15 d), which was slower than that of S.24 (~14 d) but faster than that of S.100 (~27 d). Interestingly, C80 displayed a comparable time to pure Sc, finishing fermentation in 10 d, consistently with [31].

As for the Glera trial, the yeast-derived volatile profile was clearly affected by *H. vineae*, and 39 out of the 47 volatiles analyzed were significantly influenced (Table S1). The results confirmed the specific features reported above, and β-phenylethyl acetate, its acetylation ratio, decanoic acid and its ester, and some of the grape-derived compounds found in musts as glycosides were higher in *H. vineae* wines (Supplementary Table S2). Interestingly, some *H. vineae* processes were richer in isoamyl acetate than in Sc, diversly to what was previously reported for Glera fermentation. Grouping results based on the main

yeast metabolic pathway involved in their production (Figure 4), three out of the five *H. vineae* processes (C.80, C.98, and S.24) showed higher amounts of total acetate esters and comparable concentrations of total ethyl esters. Only S.100 was consistently lower in both families, with a different contribution between compounds depending on the process.

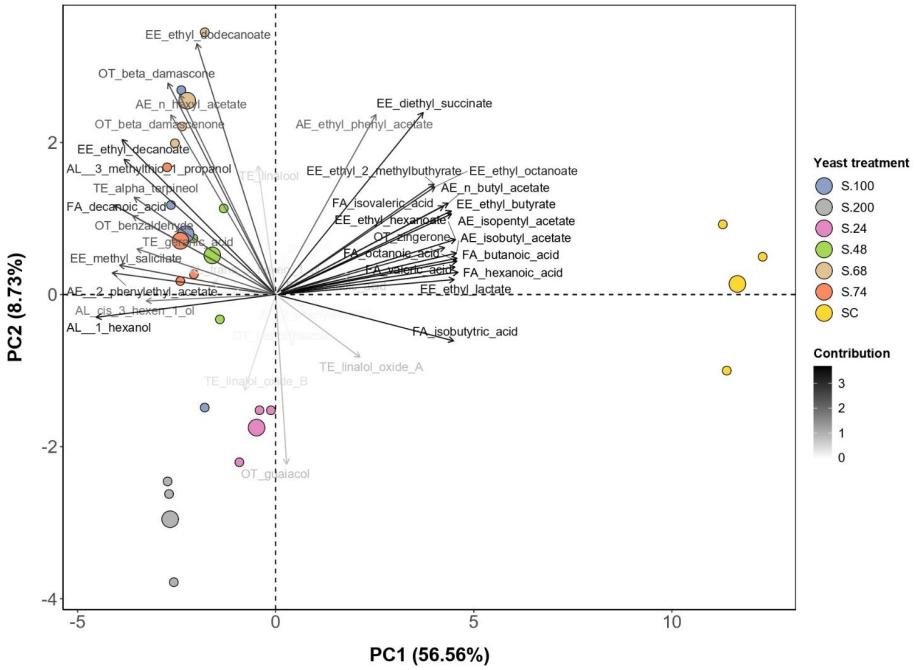

Figure 3. Principal components analysis (PCA) of the volatile composition of Glera wines produced as a result of different inoculation strategies.

As shown in the PCA biplot in Figure 5, the differences in yeast inoculation effects were delineated by both PC1 and PC2, with a cumulative explained variance of 61.9%. In fact, differences in aroma components were driven by both the yeast species and inoculum modality. The separation of individuals of wine samples along PC1 was mainly due to an increase in β-phenylethyl acetate, benzyl alcohol, and terpenes, and a decrease in 2-phenyl ethanol and fatty acids, correlated with all the *H. vineae* starting inocula (S.24, S.48, and C98) except for C80. The latter exhibited a negative correlation with the previously mentioned compounds and was positioned closest to pure Sc along the horizontal axis. In contrast, among the Hv-inoculated samples, S.100 samples were situated distinctly apart from others in the upper left quadrant, and their separation on PC2 was associated with benzaldehyde and terpenes.

The yeast inoculation method yielded wine samples with highly diverse aroma profiles. Co-inoculation resulted in wines with higher concentrations of acetates and esters in the final wine (Figure 5), with C80 showing a closer resemblance to the outcomes achieved with Sc. This similarity was observed in terms of the fermentation process, comparable to that of Sc (Figure 1), and in terms of the aromatic profile, as evident in the PCA. Interestingly, C80 exhibited the highest aromatic concentration in terms of acetates and esters (Figure 4). In contrast, C98, while maintaining the specific features of *H. vineae* (Supplementary Table S1, Figure 5), experienced a slowdown in the completion of fermentation compared to Sc or C80. A similar behaviour was observed for S.24, where an increase in the delay in sequential inoculation resulted in a deceleration of the fermentation process and a slight loss of overall aromatic intensity, eventually leading to a completely different profile from the sequential inoculation delay at 1/3 of fermentation (S.100). This delay caused a significant slowdown in sugar consumption and, unexpectedly, a decrease in acetates in general, particularly in β-phenylethyl acetate, although it remained well above the perception threshold, unlike the pure Sc-fermented wines.

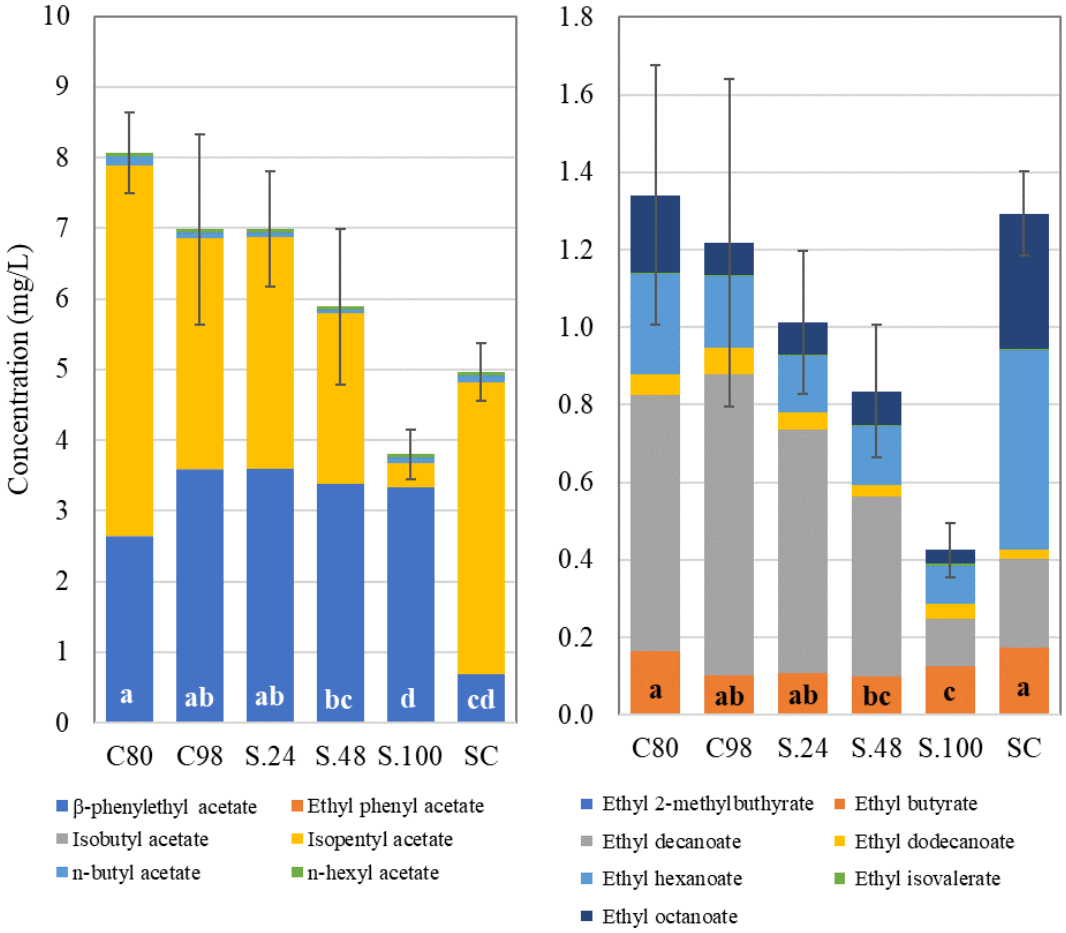

Figure 4. Sum of acetate esters (**left**) and ethyl ester (**right**) in Gewürztraminer wines. The values represent means of triplicates and letters denote significance groups (ANOVA; Tukey's post-hoc $\alpha = 5\%$).

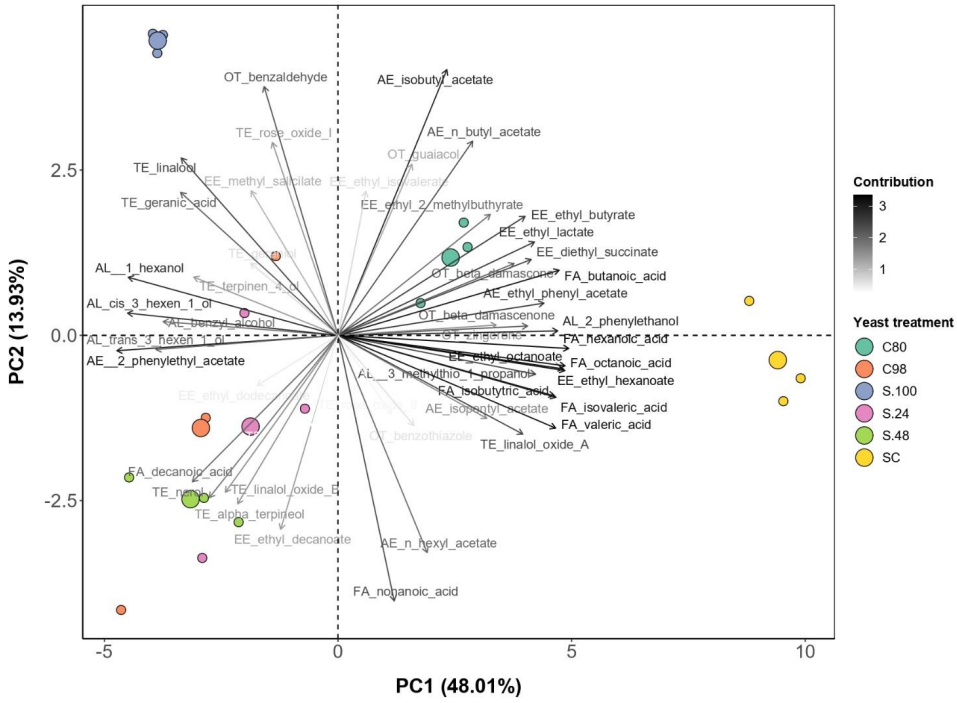

Figure 5. Principal components analysis (PCA) of the volatile composition of Gewürztraminer wines produced as a result of different inoculation strategies.

4. Conclusions

In conclusion, our findings demonstrate that *H. vineae* exhibits adaptability to mixed fermentation processes, whether applied sequentially or through co-inoculation. In the case of sequential inoculation with varying time spans between *H. vineae* and *S. cerevisiae*, distinct sugar consumption kinetics and aroma profiles in wines were observed. A shorter delay before the introduction of *S. cerevisiae* resulted in a more favourable balance between fermentation duration and the metabolic characteristics of *H. vineae*. Conversely, the longest delays were counterproductive, leading to undesirable outcomes both in terms of fermentation duration and aromatic characteristics, even causing a loss of typical yeast traits.

On the other hand, co-inoculation evidenced more advantages in terms of fermentation kinetics, with C80 exhibiting a comparable performance to the single-strain control and demonstrating superior aromatic characteristics, including higher concentrations of both acetates and esters. However, all the final wine profiles varied, influenced by the specific inoculation method. The selection of the inoculum regime, taking into account factors such as matrix profile and nutritional requirements during fermentation, emerges as a fundamental tool to regulate yeast metabolism and shape the aroma of the resulting wines. This biotechnological modulation provides the wine industry with a valuable means to tailor the fermentation process according to their oenological objectives.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10040191/s1, Table S1: Mean concentration (n = 3) ± standard deviation of the main quality control parameters of wines at the end of the fermentation in Gewürztraminer. Table S2: Volatile compounds analyzed at the end of the alcoholic fermentation in Gewürztraminer wines. Values are means of three replicates ± SD.

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