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Ecological Distribution and Oenological Characterization of Native *Saccharomyces cerevisiae* in an Organic Winery

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Abstract: The relation between regional yeast biota and the organoleptic characteristics of wines has attracted growing attention among winemakers. In this work, the dynamics of a native *Saccharomyces cerevisiae* population was investigated in an organic winery. In this regard, the occurrence and the persistence of native *S. cerevisiae* were evaluated in the vineyard and winery and during spontaneous fermentation of two nonconsecutive vintages. From a total of 98 strains, nine different *S. cerevisiae* biotypes were identified that were distributed through the whole winemaking process, and five of them persisted in both vintages. The results of the oenological characterization of the dominant biotypes (I and II) show a fermentation behavior comparable to that exhibited by three common commercial starter strains, exhibiting specific aromatic profiles. Biotype I was characterized by some fruity aroma compounds, such as isoamyl acetate and ethyl octanoate, while biotype II was differentiated by ethyl hexanoate, nerol, and β-damascenone production also in relation to the fermentation temperature. These results indicate that the specificity of these resident strains should be used as starter cultures to obtain wines with distinctive aromatic profiles.

Keywords: native *S. cerevisiae*; *S. cerevisiae* biodiversity; spontaneous fermentation; molecular characterization; winery environment

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1. Introduction

For thousands of years, *Saccharomycetes* have been used by humans to produce fermented foods and beverages. Over the past decades, modern industrial winemaking has been based on the use of starter cultures of specialized and selected wine strains [1,2]. Although the use of selected *Saccharomyces cerevisiae* strains guarantees the control of fermentation, their widespread utilization can lead to a double consequence: the reduction of indigenous microbiota and the standardization of the wine aroma complexity, which is often linked to the involvement of native yeasts during the fermentation [3–8].

To overcome this trend, in recent decades, several winemakers have carried out spontaneous fermentations exploiting indigenous yeasts coming from the vineyard or vinery equipment, with the aim to improve the analytical and aroma complexity and to give peculiar and recognizable flavors to the final wine [5,9]. Indeed, recent studies demonstrated that specific grape varieties and the climate of a specific geographical area seem to influence the yeast community [10–14], indicating a variation of the microbial community of grapes in the function of regional distribution [15–19]. Recently, the correlation between the regional microbial community and the organoleptic characteristics of wine has attracted growing attention [11,20,21]. The understanding of the mechanisms and factors that stabilize a specific epiphytic population in different grape varieties could be useful to establish a correlation with the influence of regional yeast microbiota in the analytical and aromatic characters of the final wine [11,20–22]. However, controversies exist regarding the origin of native *S. cerevisiae* strains. Several studies showed that the direct isolation of *S. cerevisiae* strains in undamaged grapes and vineyards is very difficult [8,23–25]. Mortimer and Polsinelli [26] already showed that damaged grapes represent a depository habitat

of *Saccharomyces* yeasts. Several authors claimed that the detection of *S. cerevisiae* strains in both vineyards and grapes, is obtained only after grape autoenrichment, due to their deficiency [27–29]. On the other hand, it is well known that *S. cerevisiae* is widely spread in the winery environment and on equipment [30–33]. The ability of *S. cerevisiae* to dominate the fermentation process and other competitive advantages allow this species to survive and reproduce in stressful winery conditions.

Knowledge of the occurrence of *S. cerevisiae* and their colonization on grapes or in the cellar could be of help to preserve the indigenous population and to establish a possible correlation between them and the final wine quality.

In the present study, the occurrence, and the persistence of native *S. cerevisiae* in the vineyard and winery and in uninoculated wine fermentation was investigated. The organic winery under investigation, which began its activity in 2013 and had never used commercial starter strains, represents an ideal winemaking environment to investigate the dynamics of grape-related and/or winery-related native yeast populations. Within the population of *Saccharomyces* strains, the dominant biotypes were evaluated and characterized for their oenological aptitude and aromatic profile with the aim to use these native strains as starter cultures.

2. Materials and Methods

2.1. Sampling Campaign

The sampling campaign was carried out in an organic winery (Azienda olivitivinicola Giulia Fiorentini, located in the town of Cupramontana, Ancona, Italy, geographical coordinates: 43°26′48″ N, 13°6′59″ E; 505 m altitude) that has never used commercial starter strains.

Thirty-four samples were collected in the 2016 vintage: (i) 10 samples of the Montepulciano grape variety and 10 samples after autoenrichment; (ii) 12 items from the winery environment and pieces of equipment (3 walls, 1 floor, 2 wooden barrels; 4 destemmers, and 2 steel fermentation tanks); and (iii) 2 Verdicchio spontaneous fermentation.

Twenty-four samples were collected in the 2019 vintage: (i) 6 samples of the Verdicchio grape variety and 6 samples after autoenrichment; (ii) 9 items from the winery environment and pieces of equipment (3 walls, 2 floors, 2 destemmers, 2 steel fermentation tanks); and (iii) 2 Verdicchio spontaneous fermentation. Details are reported in Table S1.

The two vineyards (Montepulciano and Verdicchio) each have two hectares and are located close to the winery. The grapes were randomly collected from randomized blocks.

Grape samples (about 1 Kg of undamaged ripe grape bunches) were picked in sterile plastic bags. The related vineyards are located close to each other and close to the cellar (distance of about 300 m). Both vineyards were subjected to the same fungicide treatment used for organic wine production (copper and sulfur compounds). Winery environment and equipment samples were sampled using sterile swabs streaked on random environmental surfaces (10 cm^2). Furthermore, samples of spontaneous fermentation were collected through the middle valve of the vat under sterile conditions. All the samples were stored on ice during sampling and immediately transported to the laboratory and processed.

2.2. Samples Processing and S. cerevisiae Isolation

Grape samples were processed as described by Agarbati et al. [23–25]. Briefly, ten grape samples from the 2016 vintage and six grape samples from the 2019 vintage were hand-crushed and subjected to shaking at 120 rpm for 30 min to allow the transfer of yeasts from the grape surface to the must. Then, 1 mL of fresh must was used for decimal serial dilution and spread on WL nutrient agar (Wallerstain Laboratories, Oxoid, Hampshire, UK) supplemented with 0.005% chloramphenicol (Sigma-Aldrich, Saint Louis, MI, USA) to suppress bacteria growth. The remaining fresh must was transferred into a 250 mL sterile Erlenmeyer flask closed with Pasteur bung and set up for autoenrichment at room temperature with the aim to promote *S. cerevisiae* yeast development. The eventual development of this yeast was monitored after 7 and 15 days of autoenrichment through viable cell counts

using the same media. Samples were collected by the winery's equipment, and the cotton swab was placed into 10 mL of sterile water and shaken for 20 min at 120 rpm on a MAXQ 4450 shaker (Thermo Fisher Scientific, Waltham, MA, USA) to allow the transfer of the microorganisms from the swab to the solution. Serial decimal dilutions were carried out and spread on the same media described for grape samples. In the same way, samples collected during must fermentation were diluted and spread on WL nutrient agar (Wallerstain Laboratories, Oxoid, Hampshire, UK) with 0.005% chloramphenicol added. All plates were incubated at 25 °C for 5 days then presumptive *S. cerevisiae* colonies were isolated on the basis on its typical macro and micromorphology. Each presumptive colony of *S. cerevisiae* was purified on YPD agar (yeast extract 1%, peptone 2%, D-glucose 2%, and agar 1.8%) medium and then collected for short time at 4 °C using the same medium.

2.3. Identification and Genotyping Characterization of S. cerevisiae Strains

Based on the micro and macromorphology of the colonies, 150 presumptive *S. cerevisiae* pure cultures were then assayed by genomic DNA analyses. DNA was extracted at 95 °C for 10 min, and then it was amplified by PCR using primers ITS1 (5′-TCCGTAGGTGAACCTCGCG-3′) and ITS4 (5′-TCCTCCGCTTTATTGATATGC-3′) following the procedure reported by Agarbati et al. [23]. A total of 98 strains, the resulting *S. cerevisiae* strains, were then subjected to genotyping using different primers: firstly, the primer pairs delta12/21 (delta12: 5′-TCAACAATGGAATCCCAAC-3′; delta21: 5′-CATCTTAACACCGTATATGA-3′) were used for interdelta sequence analyses, as described by Legras and Karst [34]. The amplification was performed as per the following program: 3 min at 95 °C, followed by 25 s at 94 °C, 30 s at 45 °C, and 90 s at 72 °C for 9 cycles and 25 s at 94 °C, 30 s at 50 °C, and 90 s at 72 °C for 21 cycles and a final extension at 72 °C for 10 min.

A second genotyped of *S. cerevisiae* strains was carried out by a PCR reaction using the random amplified microsatellites technique inter-single sequence repeats (ISSR). The primer used was 5′-ARRTYCAGCAGCAGCAG-3′, where R (A or G) and Y (C or T) indicate degenerate sites [35,36]. Amplification was performed as reported: 5 min at 94 °C, followed by 40 cycles of 60 s at 94 °C, 60 s at 55 °C, and 120 s at 72 °C and a final extension at 72 °C for 5 min.

Amplification products from both PCR reactions were separated by electrophoresis on 1.5% (*w*/*v*) and 2% (*w*/*v*) agarose gels, respectively, submitted to 66 V for 1.5 h in 0.5 × TBE buffer. Before PCR reactions, the DNA of yeasts was extracted at 95 °C for 10 min using a Biorad Thermal Cycler [23].

2.4. Fermentation Trials

Two dominant biotypes (I and II) were used to carry out microfermentation trials to evaluate their fermentative aptitudes at 22 °C and 16 °C fermentation temperatures using fresh Verdicchio must composed as follows: initial sugar content 201 g/L, pH 3.25, total acidity 6.34 g/L, and yeast assimilable nitrogen (YAN) content 98 mgN/L. The YAN was adjusted to 250 mgN/L by the addition of diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France). Before its use, the must was treated with 0.2 mL/L of dimethyl dicarbonate overnight at 4 °C to suppress the wild yeasts, confirmed through viable cell counts using WL nutrient agar (Wallerstain Laboratories, Oxoid, Hampshire, UK).

The strains used were precultured in modified YPD (0.5% *w*/*v* yeast extract, 2% *w*/*v* glucose, and 0.1% *w*/*v* peptone) for 24 h at 25 °C in an orbital shaker (rotation 150 rpm). The cells were collected, washed twice with sterile water, and used to inoculate flasks containing 70 mL of must, at an initial concentration of approximately 1×10^6 cells/mL for each yeast. The flasks were locked with Müller valves containing sulfuric acid to allow CO_2 to escape from the system and to avoid contamination. The trials were incubated at 22 °C and 16 °C under static conditions, and the weight loss of the flasks, due to the CO_2 evolution, was monitored until a constant weight (for two consecutive days).

The trials were carried out in triplicate, and three commercial starter strains widely used in Verdicchio grape juice fermentation were used as controls: Lalvin EC1118, Lalvin ICV OKAY® (Lallemand Inc., Toulouse, France), and VIN13 (Anchor Wine Yeast, Cape Town, South Africa).

2.5. Analytical Determinations

The resulting wines were analyzed for the main analytical determinations. The fermentation rate was calculated as g CO_2/day over the 3rd day of fermentation. Volatile acidity and total SO_2 content were obtained following the current analytical methods, according to the Official European Union Methods [37]. Acetaldehyde was determined using a specific enzyme kit (Roche Diagnostics S.p.A., Milano, Italy), while the free α-amino acids were quantified using the o-phthaldialdehyde/N-acetyl-l-cysteine spectrophotometric assay, as described by Dukes and Butzke [38]. Ethanol production evaluated at the end of fermentation was measured according to the Association of Official Analytical Chemists [39]. Specific enzymatic kit (Megazyme, Ireland) was used to determine the concentrations of glucose and fructose, according to the manufacturer instructions.

The volatile compounds were determined by solid-phase microextraction (HS-SPME) method, preparing the sample as follows: 5 mL of wine was placed into a vial; 1 g of NaCl and 3-octanol as the internal standard (1.6 mg/L) were added, and the vial was closed with a septum-type cap and placed on a magnetic stirrer for 10 min at 25 °C. Then the sample was heated to 40 °C and extracted with a fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) for 30 min by insertion into the vial headspace. The compounds were desorbed by inserting the fiber into Shimadzu gas chromatograph GC injector, in split–splitless modes following the procedure reported by Canonico et al. [40]. The glass capillary column used was 0.25 µm Supelcowax 10, length 60 m, and internal diameter 0.32 mm.

2.6. Data Analyses

The experimental data were analyzed by one-way ANOVA (analysis of variance) using the software STATISTICA 7 (Statsoft inc., Tulsa, OK, USA). The data were considered significantly different with an associated p-value < 0.05 (Duncan tests). The mean data of the main analytical and volatile compounds, normalized to neutralize hidden factors, were used to carry out Principal Component Analysis (PCA) using the package JMP 11® software.

3. Results

3.1. Occurrence of S. cerevisiae on Grapes in Winery Environment and in Uninoculated Fermentations

The source of isolation and number of *S. cerevisiae* isolated are reported in Table 1.

Table 1. Source and number of *S. cerevisiae* isolates during 2016 and 2019 vintages.

Sampling Year	Source of *S. cerevisiae*	Number of Samples	Number of Samples with *S. cerevisiae*	Number of *S. cerevisiae* Isolated
2016	Grapes (direct isolation)	10	0	0
	Grapes (after autoenrichment)	10	2	10
	Winery equipment and environment	12	4	13
	Spontaneous fermentation	2	2	20
2019	Grapes (direct isolation)	6	0	0
	Grapes (after autoenrichment)	6	2	27
	Winery equipment and environment	9	1	8
	Spontaneous fermentation	2	2	20

No *S. cerevisiae* were found on grape surfaces using the direct isolation procedures. *S. cerevisiae* were isolated only after the autoenrichment of fresh grape juice (spontaneous fermentation of pressed harvested grapes, under sterile conditions).

A total of 98 *S. cerevisiae* colonies were isolated in the two years: 37 colonies from grapes after enrichment (10 in the 2016 vintage and 27 in the 2019 vintage), 21 colonies from winery equipment and the environment (13 in the 2016 vintage and 8 in the 2019 vintage) (the specific source are reported in Table S1, Supplementary Material), and 40 strains from spontaneous wine fermentation (half in the 2016 vintage and half in the 2019 vintage). Overall, we isolated a total of 43 and 55 *S. cerevisiae* in the 2016 and 2019 vintages, respectively.

3.2. Biotypes of S. cerevisiae: Frequency and Distribution

The combination of biotype analyses, interdelta sequences, and ISSR polymorphism, revealed eight different biotypes among 98 *S. cerevisiae* isolates from 13 samples out of the 57 total samples evaluated (Figure S1, Supplementary Materials). The distribution and the frequency of each biotype, expressed as number of isolates and percentage, are reported in Table 2.

Out of the eight biotypes identified, biotypes I and II dominated in both years. Biotype I represented 54%, while biotype II represented 32% of all isolates. In the 2016 vintage, the frequency of biotypes I and II was 68% and 19%, respectively. Biotype I was detected in all samples, mainly during must fermentation and in the winery environment (26% and 28%, respectively). Biotype II was mainly detected during must fermentation (16%), poorly in the winery environment (2%), and not detected on grapes.

In the 2019 vintage, the two biotypes showed a very similar frequency (biotype I: 43%; biotype II: 42%) and were present in all samples, mainly on grapes (both biotypes 24%) then in wine fermentation (biotype I: 11%; biotype II: 13%), while the lowest percentage was found in the winery environment (biotype I: 9%; biotype II: 5%).

The other biotypes (III, IV, V, VI, VII, and VIII) were randomly detected in the different samples of the two vintages. Biotypes III, IV, and V were detected in both vintages, while biotype VIII was only detected in the 2016 vintage and biotypes VI and VII only in the 2019 vintage.

3.3. Oenological Characterization of Predominant Native S. cerevisiae Isolated Strains

3.3.1. Fermentation Kinetics

The oenological characteristics of *S. cerevisiae* biotypes I and II (the most dominant biotypes) were evaluated and compared with three commercial starter strains, widely used in the Verdicchio winemaking area.

The fermentation kinetics did not show differences among the native *S. cerevisiae* biotype I, biotype II, and commercial starters at fermentation temperatures of both 16 °C and 22 °C (Figure 1).

3.3.2. Analytical Characteristics

The main oenological characteristics of wines carried out at 16 °C are reported in Table 3. The results obtained show that biotype I exhibited the significant lowest value of all parameters tested apart from volatile acidity that was significantly higher than that shown by the other strains. Regarding the ethanol content, biotype I showed the highest value of 12.31% *v*/*v*, comparable with those produced by the starters OKAY and EC1118, while biotype II revealed the lowest ethanol content.

Table 2. Occurrence of *S. cerevisiae* biotypes in different years and samples. Percentage values were calculated on the basis of the number of isolates of the same year. For both vintages (2016/2019), the percentage values were calculated considering the sum of the isolates of both sampling years. * No presence of *S. cerevisiae*.

Sampling Year	Number of Isolates	Biotype	Origin of Sampling			Total of Yeast for Each Biotype
			Grape	Winery Environment	Wine Fermentation	
2016	43	I	6 (14%)	12 (28%)	11 (26%)	29 (68%)
		II	/ *	1 (2%)	7 (16%)	8 (19%)
		III	2 (5%)	/	/	2 (5%)
		IV	1 (2%)	/	/	1 (2%)
		V	/	/	1 (2%)	1 (2%)
		VI	1 (2%)	/	/	1 (2%)
		VII	/	/	1 (2%)	1 (2%)
		VIII	/	/	/	/
		IX	/	/	/	/
2019	55	I	13 (24%)	5 (9%)	6 (11%)	24 (43%)
		II	13 (24%)	3 (5%)	7 (13%)	23 (42%)
		III	1 (2%)	/	2 (4%)	3 (5%)
		IV	/	/	1 (2%)	1 (2%)
		V	/	/	2 (4%)	2 (4%)
		VI	/	/	/	/
		VII	/	/	/	/
		VIII	/	/	1 (2%)	1 (2%)
		IX	/	/	1 (2%)	1 (2%)
2016/2019	98	I	19 (19%)	17 (17%)	17 (17%)	53 (54%)
		II	13 (13%)	4 (4%)	14 (14%)	31 (32%)
		III	3 (3%)	/	2 (2%)	5 (5%)
		IV	1 (1%)	/	1 (1%)	2 (2%)
		V	/	/	3 (3%)	3 (3%)
		VI	1 (1%)	/	/	1 (1%)
		VII	/	/	1 (1%)	1 (1%)
		VIII	/	/	1 (1%)	1 (1%)
		IX	/	/	1 (1%)	1 (1%)

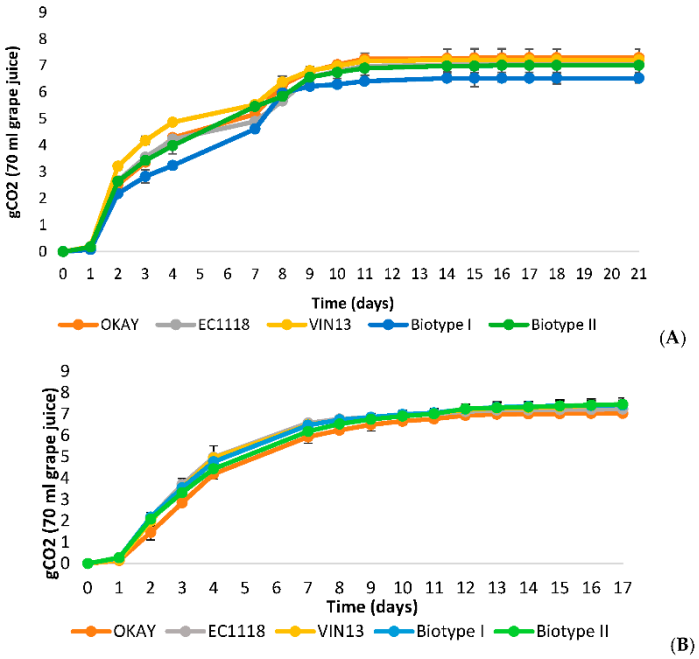

Figure 1. Fermentation kinetic of *S. cerevisiae* biotypes I and II in comparison with three commercial starters. (**A**) Fermentation carried out at 16 °C. (**B**) Fermentation carried out at 22 °C.

Table 3. Main analytical characteristics of wines obtained by the two dominant biotypes compared with commercial starter strains, fermented at 16 °C. Data are means ± standard deviations, and those with different superscript letters ($^{a–e}$) within each column are significantly different, according to Duncan's test (p-value < 0.05).

Samples	Fermentation Rate (gCO_2/Day) *	Volatile Acidity (g/L)	Total SO_2 (mg/L)	Acetaldehyde (mg/L)	Free α-Amino Acids (mgN/L)	Ethanol (% *v*/*v*)
Biotype I	0.9 ± 0.1^{c}	0.7 ± 0.1^{a}	4.0 ± 0.0^{e}	9.5 ± 0.7^{d}	35.8 ± 0.7^{d}	12.3 ± 0.1^{a}
Biotype II	1.1 ± 0.0^{b}	0.3 ± 0.0^{bc}	41.0 ± 1.4^{a}	62.5 ± 0.76^{a}	32.2 ± 0.6^{e}	11.7 ± 0.4^{b}
OKAY	1.1 ± 0.1^{bc}	0.3 ± 0.0^{c}	11.0 ± 0.7^{d}	40.1 ± 0.3^{c}	43.6 ± 0.9^{b}	12.2 ± 0.2^{a}
EC1118	1.2 ± 0.0^{b}	0.4 ± 0.1^{b}	23.0 ± 0.7^{b}	48.4 ± 0.9^{b}	38.8 ± 0.6^{c}	12.3 ± 0.2^{a}
VIN13	1.4 ± 0.1^{a}	0.2 ± 0.0^{d}	16.0 ± 0.7^{c}	47.3 ± 0.5^{b}	46.0 ± 0.7^{a}	11.9 ± 0.1^{ab}

* Fermentation rate calculated over the 3rd day of fermentation. Sugars (glucose/fructose) at the end of the fermentation were completely consumed.

Furthermore, biotype II exhibited a significant increase in acetaldehyde and total SO_2 content in comparison with the other strains tested. Among the *S. cerevisiae* starter strains, VIN13 exhibited a significantly higher fermentation rate (g CO_2/day over the 3rd day of fermentation) and free amino acid content in comparison with other fermentation trials.

The results of the main analytical characters of the fermentation carried out at 22 °C are reported in Table 4. The data obtained show a different trend from those obtained at 16 °C (Table 3). Indeed, the fermentation rate did not display significant differences among the strains tested, with the only exception being the *S. cerevisiae* starter strain OKAY® that showed a significantly lower fermentation rate. About other characteristics, significant differences were found. The commercial starter strains VIN13 and OKAY® showed a significant increase in volatile acidity. Total SO_2 was significantly higher in biotype II confirming the behavior shown at 16 °C. All fermentation led to a low acetaldehyde content with the only exception being VIN13 that showed the highest value, but it was below the threshold. The value of free α-amino acids was generally comparable among the strains tested. The same ethanol content was detected at 22 and 16 °C for biotype I with slight variations for the OKAY and VIN13 starters. Conversely, biotype II as well as the starter

EC1118 showed an increase in the ethanol content of about 0.4% *v*/*v* during fermentation trials carried out at 22 °C.

Table 4. Main analytical characteristics of wines obtained by the two dominant biotypes compared with commercial starter strains, fermented at 22 °C. Data are means ± standard deviations, and those with different superscript letters ($^{a–e}$) within each column are significantly different, according to Duncan's test (p-value < 0.05).

Samples	Fermentation Rate (gCO_2/Day) *	Volatile Acidity (g/L)	Total SO_2 (mg/L)	Acetaldehyde (mg/L)	Free α-Amino Acids (mgN/L)	Ethanol (% *v*/*v*)
Biotype I	1.2 ± 0.1^{a}	0.3 ± 0.1^{bc}	2.0 ± 0.0^{c}	45.7 ± 0.6^{b}	40.3 ± 0.5^{b}	12.3 ± 0.1^{b}
Biotype II	1.1 ± 0.1^{ab}	0.2 ± 0.0^{c}	24.0 ± 0.7^{a}	19.2 ± 1.0^{e}	45.5 ± 0.2^{a}	12.1 ± 0.3^{b}
OKAY	1.0 ± 0.1^{b}	0.4 ± 0.1^{ab}	3.0 ± 0.0^{c}	25.3 ± 0.5^{c}	45.9 ± 0.3^{a}	12.3 ± 0.0^{b}
EC1118	1.2 ± 0.0^{a}	0.3 ± 0.0^{bc}	10.0 ± 0.7^{b}	21.3 ± 0.9^{d}	39.6 ± 0.9^{b}	12.7 ± 0.1^{a}
VIN13	1.2 ± 0.0^{a}	0.5 ± 0.1^{a}	2.0 ± 0.0^{c}	74.6 ± 0.6^{a}	45.9 ± 0.9^{a}	12.2 ± 0.0^{b}

* Fermentation rate calculated over the 3rd day of fermentation. The residual sugars (glucose/fructose) at the end of the fermentation were totally consumed.

3.3.3. Volatile Compounds

The results of the main volatile compounds of wines fermented at 16 °C are reported in Table 5. The data show that strains belonging to different biotypes were characterized by the production of ester compounds. Biotype I exhibited a significant increase in isoamyl acetate (banana aroma) and ethyl octanoate (apple, pear, and peach aroma), while biotype II showed a significantly increased amount in ethyl hexanoate (apple aroma). *S. cerevisiae* starter strains showed different aromatic profiles: OKAY® was characterized by a significant increase in ethyl butyrate content; EC1118 was characterized by relevant amounts of β-phenyl ethanol, and VIN13 showed a significantly high production of phenylethyl acetate. About hexanol, linalool, and geraniol, the results did not show a significant difference among the strains.

The volatile compounds produced at 22 °C are reported in Table 6. The data show a different behavior of the starter strains in comparison with the 16 °C fermentation trials. At 22 °C, biotype I showed a significant increase in isoamyl acetate, while biotype II was characterized by a significantly high production of nerol and β-damascenone. Among the starter strains tested, OKAY® confirmed at 22 °C the high production of ethyl butyrate together with a relevant production of terpens, β-phenyl ethanol, and diethyl succinate. EC1118 was distinguished by the other strains by the production of phenylethyl acetate and ethyl hexanoate, while VIN13 in this fermentation condition did not show any relevant production of a particular compound.

To assess the overall effects of yeast strains under different fermentation temperature, the main analytical characteristics and volatile compounds were analyzed by Principal Component Analyses (PCA) reported in Figure 2. The PCA analysis related to the fermentation carried out at 16 °C (Figure 2a) and showed that all *S. cerevisiae* starter strains were grouped in the lower left quadrant, while the two dominant biotypes I and II were placed separate with the starter strains between them. Biotype I was in the lower right quadrant, while biotype II was in the upper right quadrant. Under 16° C of fermentation temperature, a greater differentiation of the analytical and volatile compounds was found. Otherwise, data obtained after fermentation carried out at 22 °C reported in Figure 2b showed a different distribution of the strains in comparison with fermentation carried out at 16 °C. Biotype I was located in the lower left quadrant with *S. cerevisiae* VIN13. The other three strains were each in a different quadrant.

Table 5. Main volatile compounds of wines obtained by the two native *S. cerevisiae* strains compared with commercial starters, fermented at 16 °C. Data are means ± standard deviations, and those with different superscript letters ($^{a–d}$) within each row are significantly different, according to Duncan's tests (p-value < 0.05).

Volatile Compounds	Biotype I	Biotype II	OKAY	EC1118	VIN13
Alcohols					
Hexanol (μg/L)	10.0 ± 0.0^{b}	20.0 ± 0.0^{a}	20.0 ± 0.0^{a}	20.0 ± 0.0^{a}	$20.0 \pm 0.0^{a\,b}$
β-Phenyl ethanol (mg/L)	29.1 ± 0.4^{b}	24.8 ± 0.4^{d}	20.2 ± 0.9^{e}	50.5 ± 0.1^{a}	27.2 ± 1.1^{c}
Esters					
Isoamyl acetate (mg/L)	2.5 ± 0.1^{a}	2.2 ± 0.0^{b}	1.2 ± 0.1^{e}	1.6 ± 0.1^{c}	1.3 ± 0.1^{d}
Phenylethyl acetate (mg/L)	0.3 ± 0.0^{c}	0.3 ± 0.0^{c}	0.2 ± 0.0^{d}	0.6 ± 0.0^{b}	0.7 ± 0.0^{a}
Ethyl hexanoate (mg/L)	0.2 ± 0.0^{d}	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{cd}	0.2 ± 0.0^{c}
Ethyl butyrate (mg/L)	0.2 ± 0.0^{d}	0.3 ± 0.0^{c}	1.4 ± 0.0^{a}	0.7 ± 0.0^{b}	0.7 ± 0.0^{b}
Ethyl octanoate (μg/L)	50.0 ± 0.1^{a}	30.0 ± 0.1^{b}	10.0 ± 0.0^{b}	20.0 ± 0.0^{b}	10.0 ± 0.0^{b}
Diethyl succinate (μg/L)	10.0 ± 0.1^{a}	20.0 ± 0.0^{a}	10.0 ± 0.1^{a}	10.0 ± 0.0^{a}	10.0 ± 0.0^{a}
Terpenes					
Linalool (μg/L)	40.0 ± 0.1^{a}	50.0 ± 0.1^{a}	60.0 ± 0.1^{a}	50.0 ± 0.1^{a}	10.0 ± 0.0^{b}
Nerol (μg/L)	30.0 ± 0.1^{a}	20.0 ± 0.1^{a}	20.0 ± 0.1^{a}	20.0 ± 0.0^{a}	20.0 ± 0.0^{a}
Geraniol (μg/L)	30.0 ± 0.0^{a}	40.0 ± 0.1^{a}	10.0 ± 0.0^{a}	0.0 ± 0.0^{a}	10.0 ± 0.0^{a}
Enones					
β-Damascenone (μg/L)	10.0 ± 0.0^{ab}	30.0 ± 0.1^{a}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.1^{b}

Table 6. Main volatile compounds of wines carried out at 22 °C. Data are means ± standard deviations, and those with different superscript letters ($^{a–d}$) within each column are significantly different, according to Duncan's tests (p-value < 0.05).

Volatile Compounds	Biotype I	Biotype II	OKAY	EC1118	VIN13
Alcohols					
Hexanol (μg/L)	20.0 ± 0.0^{a}	10.0 ± 0.0^{a}	20.0 ± 0.1^{a}	10.0 ± 0.0^{a}	20.0 ± 0.1^{a}
β-Phenyl ethanol (mg/L)	30.7 ± 3.1^{c}	12.9 ± 2.9^{e}	35.2 ± 0.7^{a}	32.7 ± 2.9^{b}	16.3 ± 2.1^{d}
Esters					
Isoamyl acetate (mg/L)	3.8 ± 0.3^{a}	1.8 ± 0.1^{b}	1.3 ± 0.0^{c}	1.1 ± 0.1^{c}	1.5 ± 0.0^{bc}
Phenylethyl acetate (mg/L)	0.2 ± 0.0^{b}	0.1 ± 0.0^{c}	0.1 ± 0.0^{bc}	0.4 ± 0.1^{a}	0.1 ± 0.0^{c}
Ethyl hexanoate (mg/L)	0.2 ± 0.1^{c}	0.4 ± 0.2^{b}	0.36 ± 0.04^{b}	0.43 ± 0.04^{a}	0.21 ± 0.03^{c}
Ethyl butyrate (mg/L)	0.3 ± 0.0^{c}	0.4 ± 0.0^{c}	1.3 ± 0.2^{a}	0.5 ± 0.1^{bc}	0.5 ± 0.0^{b}
Ethyl octanoate (μg/L)	40.0 ± 0.1^{a}	40.0 ± 0.1^{a}	40.0 ± 0.2^{a}	10.0 ± 0.0^{b}	30.0 ± 0.1^{a}
Diethyl succinate μg/L)	20.0 ± 0.0^{b}	10.0 ± 0.0^{c}	60.0 ± 0.1^{a}	10.0 ± 0.0^{c}	10.0 ± 0.0^{c}
Terpenes					
Linalool (μg/L)	40.0 ± 0.01^{b}	30.0 ± 0.1^{b}	60.0 ± 0.2^{a}	40.0 ± 0.1^{b}	20.0 ± 0.1^{b}
Nerol (μg/L)	10.0 ± 0.01^{b}	30.0 ± 0.0^{a}	30.0 ± 0.1^{a}	20.0 ± 0.1^{b}	10.0 ± 0.0^{b}
Geraniol (μg/L)	10.0 ± 0.0^{ab}	0.0 ± 0.0^{b}	30.0 ± 0.1^{a}	20.0 ± 0.1^{ab}	$10.0 \pm 0.0^{a\,b}$
Enones					
β-Damascenone (μg/L)	10.0 ± 0.0^{b}	20.0 ± 0.1^{a}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}

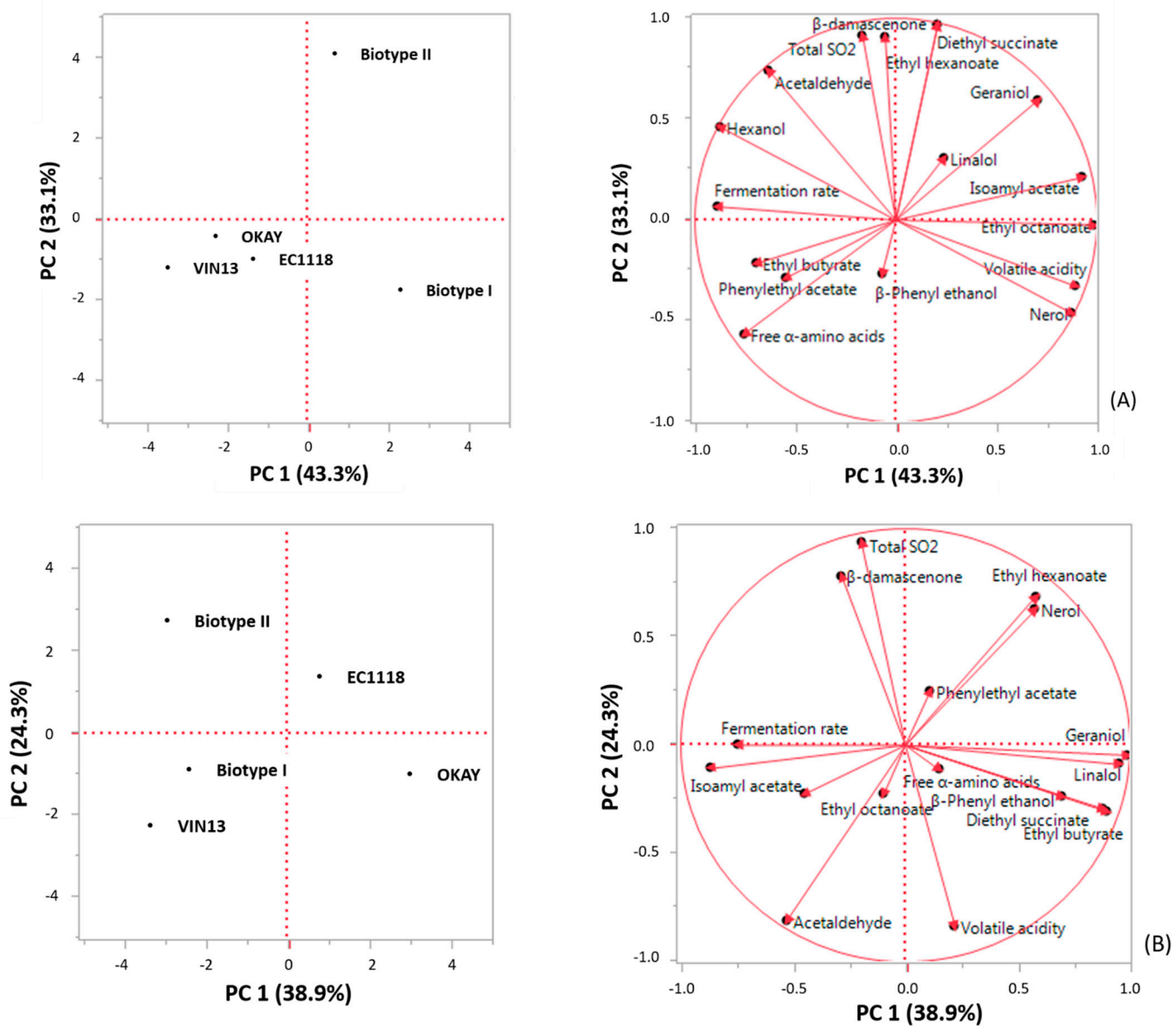

Figure 2. Principal Component Analyses (PCA) for the main analytical characteristics and volatile compounds of wines obtained by two biotypes at 16 °C (**A**) and 22 °C (**B**) Fermentation temperatures.

4. Discussion

In recent years, several investigations claimed different geographical distributions of wine microbioma [10,11]. This relationship between wine microbiota and regional areas has gained relevance in the wine industry [41]. Indeed, the microbiota from a determined region could be able to confer distinctive characteristics to the wine [20,42].

The concept of native yeasts is linked to the conservation of microorganisms naturally present in a specific viticultural area. The knowledge of distribution and the persistence of native *S. cerevisiae* strains, together with their fermentation aptitudes and stress resistance, could provide important information that contributes to promoting their use in winemaking.

In this work, the occurrence of *S. cerevisiae* in different winemaking ecological habitats (grape surfaces, winery environment, and must fermentation) was confirmed. As already demonstrated by several authors, the isolation of *S. cerevisiae* from the surfaces of grapes was only possible after the autoenrichment procedures [23,28,29], supporting the evidence that grapes represent an unfavorable habitat for *S. cerevisiae*. The DNA fingerprinting of native

S. cerevisiae, following the trend previously reported by other authors [9,43–45], showed a high level of DNA polymorphism within the population. This elevated polymorphism in both nonconsecutive vintages indicated a high persistence over the years in this specific organic winery where starter yeasts have never been used. Similar results were reported also by Torija et al. [46] and Granchi et al. [45] that described the persistence of recurrent and predominant *S. cerevisiae* strains during spontaneous wine fermentations of a specific wine-producing area. In this regard, Granchi et al. [45] found that native *S. cerevisiae* strains are representative of a specific oenological ecosystem and that some of them termed "dominant" persisted in different fermentations in the same winery from one year to another, and they seemed to be representative of a single winery rather than of an oenological area.

In agreement with the results of Granchi et al. [45], we found that among nine different biotypes, only biotypes I and II were dominant and recurrent in the grapes, winery environment, and uninoculated fermentation. Biotype I was found in both Verdicchio and Montepulciano grapes, suggesting a nonspecific correlation between yeast strains and grape variety, according to Ganucci et al. [47]. On the contrary, other investigations found specific yeast strains for specific grape varieties [48–50]. To limit the reproducibility problems of interdelta analyses reported by Franco-Duarte et al. [51], a second genotyped (ISSR) was used. However, further investigations could be necessary to validate the different *S. cerevisiae* biotypes detected.

Effectively, the concept of the origin of the yeasts is very complex. Alexandre [52] reported that the wine yeast terroir concept is based on the assumption that wines are produced by spontaneous fermentation carried out by the yeast biota naturally present on grape. Actually, the yeast biota present in must is a mix of different yeast species coming from grapes and winery (equipment, tanks . . .) in which the anthropic management practices (both in vineyard and in the cellar) play a decisive role on the microbiome stability and therefore on its influence in the wine.

In the specific environment considered in this study, it was found that the *S. cerevisiae* population was stable as almost all biotypes were present throughout the production chain, from the vineyard to the cellar and in the finished product [47,53]. This picture was found in two nonconsecutive years (three-year interval), supporting the thesis that the colonization is strictly dependent on the management of the entire vineyard–cellar "system". The agronomical and oenological practices used, such as organic open field treatments and spontaneous fermentation, could have determined the stability of the microbiota in the various ecological habitats (grape surface, cellar, equipment, and fermenting must).

The two native dominant biotypes, evaluated for the main oenological aptitude and the aromatic profile and compared with the most used *S. cerevisiae* starter strains, showed that they can confer a distinctively analytical and volatile profile to wines. Here, the two *S. cerevisiae* biotypes I and II produced specific ester profiles related to a peculiar aromatic note also depending on the fermentation temperature. The ester production, responsible for the fruity character of wines, is a key characteristic of yeast metabolism [54–56]. Different studies reported that the high variability in ester production is strictly related to the yeast strain conferring sensorial divergence to wines [57–59]. In this regard, the characterization of the phenometabolomic fermentative profile could be useful to further investigate on these native strains [60].

In addition, these native yeasts resulted in being better adapted to the specific environment condition and substrate composition as previously indicated by other works [61,62].

For these reasons, the use of native *S. cerevisiae* yeasts could be proposed as starter strains to provide significant diversification to analytical and aroma profiles in line with recent observations that native yeasts may confer peculiar characteristics to wines [11,61,63].

Overall, the results show that the fermentation behavior of these native yeasts was comparable with those exhibited by the commercial starters strains used as the control. Moreover, their occurrence in different habitats of the winemaking process, their temporal persistence in different years, and the fermentation aptitude at different temperatures suggest a suitable application in winemaking.

5. Conclusions

In this specific winemaking environment (vineyard and winery where commercial yeasts have not been used for a long time), a broad and specific *S. cerevisiae* population was found during two nonconsecutive vintages. Among the native *S. cerevisiae*, two strains resulted in the dominant biotypes. The evaluation of the oenological characteristics of these two dominant biotypes highlighted specific differences when compared with commercial starter strains. The knowledge of the specific traits of the native strains could be useful to characterize and enhance the distinctive features of a specific winemaking area. This could be a strategy to improve the quality of the fermentation industry maintaining a strict link between products and the environment, as required by consumers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8050224/s1, Figure S1: Electrophoresis gels of native *S. cerevisiae* strains. (a) represents the different biotypes (I, II, III, IV, V, and VI) obtained by δ12–21 analyses. (b) represents the different biotypes (I, II, III, and IV) obtained by ISSR–PCR. In both agarose gels, the lane indicated as 100 bp represents the molecular weight marker; Table S1: Isolates of *S. cerevisiae*: source and biotype.

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