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Article

Assessment of Spontaneous Fermentation and Non-*Saccharomyces* Sequential Fermentation in Verdicchio Wine at Winery Scale

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Abstract: The use of non-*Saccharomyces* yeasts in sequential fermentation is a suitable biotechnological process to provide specific oenological characteristics and to increase the complexity of wines. In this work, selected strains of *Lachancea thermotolerans* and *Starmerella bombicola* were used in sequential fermentations with *Saccharomyces cerevisiae* and compared with spontaneous and pure *S. cerevisiae* fermentation trials in Verdicchio grape juice. *Torulaspora delbrueckii* together with the other two non-*Saccharomyces* strains (*L. thermotolerans*, *S. bombicola*) in multi-sequential fermentations was also evaluated. Wines, obtained under winery vinification conditions, were evaluated for their analytical and sensorial profile. The results indicated that each fermentation gave peculiar analytical and aromatic features of the final wine. *L. thermotolerans* trials are characterized by an increase of total acidity, higher alcohols and monoterpenes as well as citric and herbal notes. *S. bombicola* trials showed a general significantly high concentration of phenylethyl acetate and hexyl acetate and a softness sensation while multi-sequential fermentations showed a balanced profile. Spontaneous fermentation was characterized by the production of acetate esters (ethyl acetate and isoamyl acetate), citrus and herbal notes, and tannicity. The overall results indicate that multi-starter fermentations could be a promising tool tailored to the desired features of different Verdicchio wine styles.

Keywords: non-*Saccharomyces*; spontaneous fermentation; sequential fermentation; Verdicchio wine; analytical profile; sensorial profile

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1. Introduction

Non-*Saccharomyces* yeasts are a current and suitable strategy to give a specific analytical and organoleptic profile to wines [1,2]. During mixed fermentation, the metabolic interactions and fermentation behaviors between non-*Saccharomyces* species and *Saccharomyces cerevisiae* can give specific features to final wines [3]. Indeed, specific physiological and technological traits of non-*Saccharomyces* species could be used to modify the structure (total and volatile acidity, glycerol), the aromatic profile through the direct production of aromatic compounds, or to release some volatile compounds from non-volatile precursors as well as biocontrol agents to prevent spoilage yeasts [4–6]. For these reasons, the knowledge obtained by scientific results in this field are an effective support to oenologists in the use of non-*Saccharomyces* to produce distinctive wines with peculiar features [7–11]. Among the non-*Saccharomyces* wine yeasts, *Torulaspora delbrueckii*, *Lachancea thermotolerans* and *Starmerella bombicola* are widely investigated and proposed to be used as starters in mixed wine fermentation. *T. delbrueckii* lead low volatile acidity, high terpenes, thiols and β -phenyl ethanol and an increase of varietal characters when utilized in mixed culture with *S. cerevisiae* [12–15]. *L. thermotolerans* strains produce lactic acid during the alcoholic fermentation causing a decrease of wine pH while reducing its volatile acidity, and in mixed fermentation leads an increase of β-phenyl ethanol, glycerol, and polysaccharides [16–18]. *S. bombicola* (formerly *Candida stellata*) is a widely studied species for its positive contribu-

tions to wine composition including the production of desirable metabolites and the ability to reduce the ethanol level in wine under different fermentation conditions [19–22].

Nevertheless, the recent increase in the number of published works on the use of non-*Saccharomyces* in wine fermentation has highlighted a wide difference, depending on strain used [23–25]. Studies carried out on controlled mixed fermentations in wine have clearly demonstrated the wide intraspecific variability of non-*Saccharomyces* yeasts for the oenological characteristics and their behaviour in co-culture. The oenological variability of non-*Saccharomyces* strains is due to the huge number of different populations with genomic diversity [26–31]. Moreover, in controlled mixed fermentation, physiological and biochemical interactions between non-*Saccharomyces* and *S. cerevisiae* are related to metabolic pathways and influenced by environmental factors [32,33].

Among them, the grape variety strongly influences the fermentation behavior of inoculated yeasts in mixed fermentations affecting the final composition of wine. Verdicchio is a white grape variety, almost exclusively in the Marche region in central Italy, and is used to produce dry, sweet, and sparkling wines, some of which can be aged for ten or more years [34].

The aim of this work was to evaluate spontaneous fermentation and sequential fermentation (non-*Saccharomyces*/*S. cerevisiae*) using three selected strains belonging to *T. delbrueckii*, *L. thermotolerans* and *S. bombicola* in Verdicchio grape juice at winery scale. The impact of non-*Saccharomyces* selected strains on the final product was evaluated through chemical and sensory analyses of the resulting wines.

2. Materials and Methods

2.1. Yeast Strains

The yeast strains used in this study were *T. delbrueckii* (DiSVA 130), *L. thermotolerans* (DiSVA 322) and *S. bombicola* (DiSVA 66) coming from the Yeast Collection of DiSVA of the Polytechnic University of Marche (Italy) and previously evaluated [16,22,35,36]. *T. delbrueckii* (DiSVA 130) was used only together with *L. thermotolerans* (DiSVA 322) and *S. bombicola* (DiSVA 66) (multi-starter sequential fermentation trial) since it was previously tested in Verdicchio wine in sequential fermentation trials [35,36]. The *S. cerevisiae* commercial strain IOC B 2000 (Institute Enologique De Champagne, Mardeuil, Francia) was used in pure and sequential fermentations. All the yeast strains were maintained on yeast extract–peptone–dextrose (YPD) agar medium (Oxoid, Basingstoke, UK) at 4 °C for short-term storage and in YPD broth supplemented with 80% (*w*/*v*) glycerol at −80 °C for long-term storage.

2.2. Fermentation Trials

The biomass of the non-*Saccharomyces* yeast strains was obtained from pre-cultures in modified YPD medium (0.5% yeast extract, 0.1% peptone and 2% glucose) grown for 48 h at 25 °C in an orbital shaker (150 rpm). After this, each pre-culture, was used to inoculate 2-L bench-top bioreactor (Biostat® C; B. Braun Biotech Int., Goettingen, Germany) containing a 25 L modified YPD medium for *S. cerevisiae* strains and a medium containing 1% yeast extract, 0.5% peptone and 5% of sugar under agitation condition (400 rpm/min) and with air flow (1 L/L/min). A feed batch process was used for the biomass production. Biomass was collected by centrifugation, washed three times with sterile distilled water and inoculated at a 1×10^6 cell/mL initial concentration.

Each fermentation was carried out in 10 hL steel vats containing 8 hL of Verdicchio grape juice at 20 ± 2 °C according to the Table 1. The sugar consumption during the fermentation process was measured by Baumé (°Bé) densimeter. The Verdicchio used in this study come from Ca'Liptra Azienda Agricola s.s. (Cupramontana, Ancona, Italy).

Table 1. The scheme of the fermentation trials of Verdicchio grape juice at vinery scale.

Fermentation Trials	Modality of Inoculum
L. thermotolerans	Sequential inoculation of *S. cerevisiae* after 48 h
S. bombicola	Sequential inoculation of *S. cerevisiae* after 48 h
T. delbrueckii/*L. thermotolerans*/*S. bombicola*	Sequential inoculation of *S. cerevisiae* after 48 h
S. cerevisiae	Single inoculation
Spontaneous fermentation	Un-inoculated

The main analytical parameters of the Verdicchio grape juice used were yeast assimilable nitrogen 85 mg/L, pH 3.3, total acidity 7.2 g/L and density 20.7 °Bé. After 24 h of cold static clarification, the grape juice racking and 20 g/hL of Nutriferm Energy (Enartis, Novara, Italy), and 0.15% thiamine hydrochloride were added. The yeast assimilable nitrogen was adjusted to 250 mg N/L by the addition of diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France). SO_2 (30 mg/L) was only added at the end of fermentation before the storage.

The same batch of grape must was used to carried out all the fermentation trials.

2.3. Biomass Evolution

Samples during fermentation were collected to evaluate the biomass evolution. A viable cell count was carried out using lysine agar medium (Oxoid, Hampshire, UK) as selective medium and WL nutrient agar medium (Oxoid, Hampshire, UK) for the detection of colony diversity. The plates were incubated at 25 °C for four days. The detection of inoculated and wild yeasts was evaluated to combine the results of lysine agar enumeration and macro- and micro-morphological estimation in WL nutrient agar medium. The identities of the representative yeasts were obtained by sequencing. The BLAST program and the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST accessed on 18 April 2019) were used to compare the sequences provided with those already in the data library.

2.4. Analytical Procedures

Volatile acidity and total acidity were measured using the current analytical methods according to the Official European Union Methods [37]. Acetaldehyde, ethyl acetate, n-propanol, isobutanol, amyl and isoamyl alcohols and acetoin were quantified by direct injection into a gas chromatography system (GC-2014; Shimadzu, Kyoto, Japan). Each sample was prepared and analyzed as reported by Canonico et al. [35].

The volatile compounds were extracted using an ether/hexane (1/1, *v*/*v*) extraction technique and evaluated by capillary gas chromatography. For quantification, before their extraction the wines were spiked with a known amount of 3-octanol as the internal standard (1.6 mg/L). A glass 0.25-µm Supelcowax®-10 capillary column was used (Sigma -Aldrich, St. Louis, MO, USA) (length, 60 m; internal diameter, 0.32 mm). One microlitre was injected in split–splitless mode: 60 s splitless; temperature of injection, 220 °C; temperature of detector, 250 °C; carrier gas, nitrogen; and flow rate, 2.5 mL/min. The temperature program was: 50 °C for 5 min, then raised 3 °C/min to 220 °C, and then 220 °C for 20 min. The compounds were identified and quantified by comparisons with external calibration curves for each compound as reported by Canonico et al. [35].

2.5. Sensory Analysis

At the end of the fermentation, the wines were decanted and after three months were transferred into filled 750 mL bottles, closed with the crown cap and maintained at 4 °C until sensory analysis. After this period of refinement, they were subjected to sensory evaluation. Wines were subjected to sensory analysis based on the principal sensory categories. Regarding smell analysis, the following descriptors were evaluated: ripe fruit, tropical fruit, citrusy, honey, toasted burnt, sweet toasted, spicy, cooked vegetable, aromatic herbs, herbal and phenolic. The taste descriptors were: acidity, alcohol, bitter,

softness, structure, balance and tannicity. A group of 15 testers, 10 males and 5 females aged 25–45 years (80% expert and 20% non-expert), used a score scale of 1 to 10, where 10 was the score that quantitatively represented the best judgment (maximum satisfaction), while 1 was the score to be attributed in case of poor satisfaction. The expert testers were composed of oenologists, sommeliers and wine producers. All evaluations were conducted from 10:00 to 12:00 a.m. Thirty milliliters of each wine were served at 22 $\pm$ 1 °C (room temperature) in glasses labeled with code and covered to prevent volatile loss. The order of presentation was randomized among judges [16].

2.6. Statistical Analysis

All experimental data resulting from the chemical and the sensory analyses were subjected to an analysis of variance (ANOVA). The averages obtained were processed using the statistical software package JMP 11® (statistical discovery from SAS, New York, NY, USA).

Significant differences between the averaged data were determined using the Duncan test. The experimental data were significant with associated p-values < 0.05. The processed data of the sensory analysis were used to construct graphs that provided indications both of the contribution of each descriptor to the overall organoleptic quality of the wine and of the significant differences between wines in relation to each descriptor. The results of the sensory analysis were also subjected to Fisher ANOVA, to determine the significant differences with a p-value < 0.05.

A principal component analysis (PCA) was applied to analyze the means of each volatile compound and the mean data were normalized to neutralize any influence from hidden factors. The PCA was carried out using the statistical software package JMP 11®.

3. Results

3.1. Biomass Evolution

The growth kinetics of biomass was reported in Figure 1. *S. cerevisiae* pure inoculated fermentation (Figure 1a) reached the maximum of biomass concentration at the third day (10^7 cells/mL), maintaining this level until the end of fermentation. The presence of wild yeasts could be detected at 10^6 cells/mL until the third day to disappear on the sixth day.

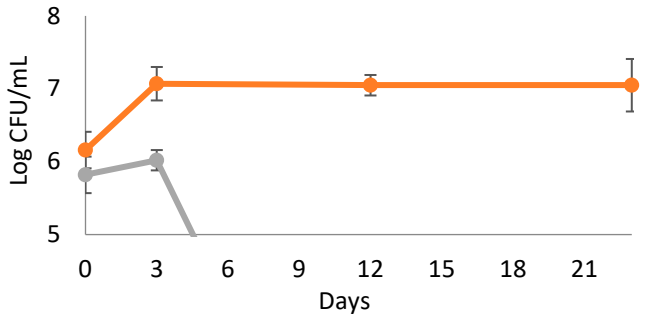
The fermentation inoculated with the sequential inoculation *S. bombicola*/*S. cerevisiae* (Figure 1b) showed the maximum microbial growth of the non-*Saccharomyces* strain at the third day of fermentation, maintaining a concentration of 10^7 cells/mL until the end of the process. *S. cerevisiae* starter strain, after the inoculum at the third day, achieved a similar CFU to *S. bombicola* strain at the eighth day and until the end of fermentation. A similar trend was exhibited for the sequential fermentation inoculated with the *L. thermotolerans* (Figure 1c).

As regards the multi-sequential fermentation with all three non-*Saccharomyces* yeast strains (Figure 1d), *L. thermotolerans* reached the highest cell concentration, while *T. delbrueckii* showed the lowest biomass production. From the third day of fermentation, when *S. cerevisiae* starter strain was inoculated, all three non-*Saccharomyces* yeasts showed a progressive decrease of CFU/mL, while the *S. cerevisiae* starter strain showed after the ninth day a constant dominance over the non-*Saccharomyces* strains with 10^7 cells/mL at the end of fermentation.

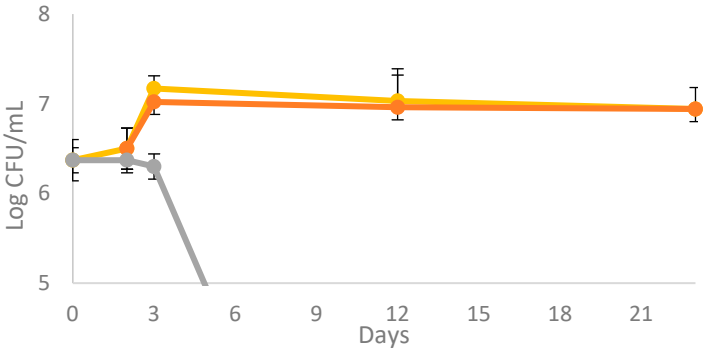
Figure 1e showed the evolution of wild yeast population during spontaneous fermentation. The beginning of fermentation was dominated by wild non-*Saccharomyces* yeasts (mainly *S. bacillaris and H. uvarum*) while from the second to the fourth day of fermentation there was the appearance of *S. cerevisiae* strain that dominated the fermentation process. In all fermentation trials, the occurrence of apiculate yeasts was quite constant and above 10^6 cell/mL until the fourth day of fermentation to disappear then on the fifth day.

3.2. The Main Analytical Characters

The data of the main analytical characters are reported in Table 2. The pH parameter seemed not to be affected by the presence of non-*Saccharomyces* yeast strains since the results of sequential fermentations and spontaneous fermentation did not differ by inoculated fermentation with *S. cerevisiae* starter culture. As expected, the wine inoculated by *L. thermotolerans* led a higher total acidity and lower volatile acidity values in comparison with the other fermentations. *S. bombicola* sequential and multi-sequential fermentation, led a significantly high value of volatile acidity.



(**a**)



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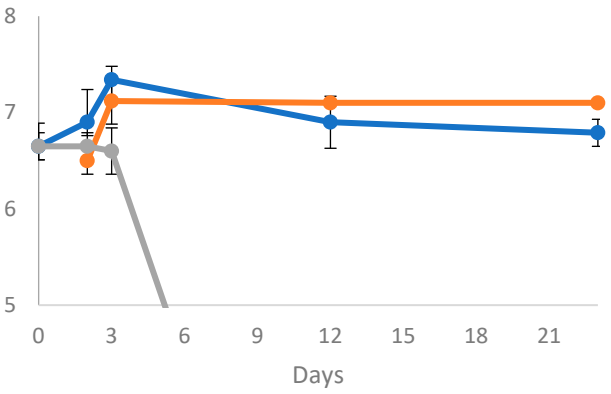
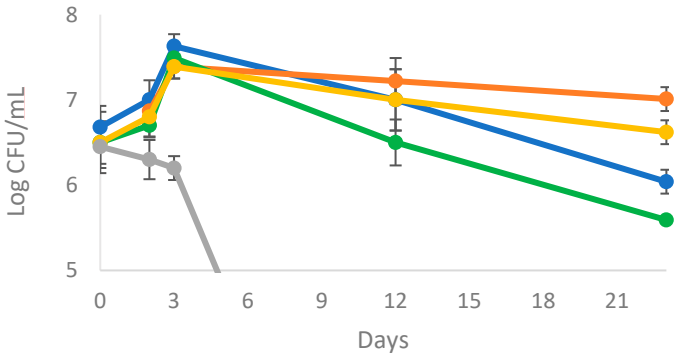
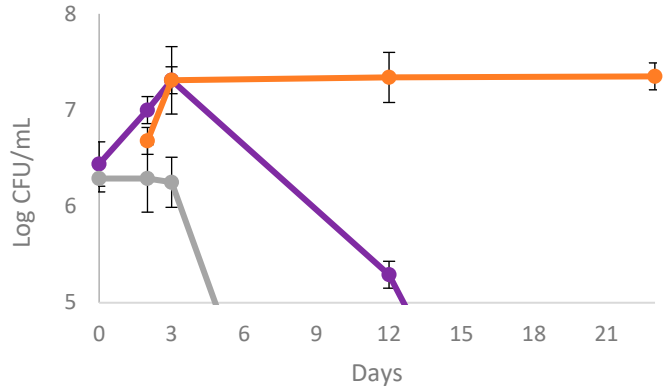


(**c**)

Figure 1. *Cont.*



(**d**)



(**e**)

Figure 1. Yeast growth kinetics for *S. cerevisiae* (—●—) in pure inoculation (**a**); in sequential fermentation with *S. bombicola* (—●—) (**b**) and with *L. thermotolerans* (—●—) (**c**). Sequential fermentation with *S. bombicola* (—●—) *L. thermotolerans* (—●—) and *T. delbrueckii* (—●—) (**d**); Spontaneous fermentation with evolution of whole wild non-*Saccharomyces* yeasts (—●—) (excluded apiculate yeasts) (**e**). In all figures apiculate yeasts (—●—).

There are no significant differences regarding the ethanol content in all resulting wines. A slight reduction of the ethanol content from 13.5% *v*/*v* of *S. cerevisiae* to 12.8% *v*/*v* of the multi-sequential fermentation inoculated with the three non-*Saccharomyces* strains was detected.

Regarding the sulfur dioxide that was not added before the fermentation, the spontaneous fermentation showed the highest concentration (35 mg/L) if compared with all the other trials. This behavior could be due to the acetaldehyde production by yeasts during the first and middle stage of fermentation and the re-metabolization in the final phase. Another possible explanation could be the higher SO_2 production by the native *S. cerevisiae.*

3.3. The Main Volatile Compounds

The data of the main volatile compounds are reported in Table 3. The results show that Verdicchio wine produced by *S. cerevisiae* starter strain and spontaneous fermentation exhibited a significantly lower acetaldehyde content in comparison with sequential fermentations, even if the final amounts of this compound did not negatively affect these wines. Acetaldehyde production by yeasts during the first and middle phase of fermentation may be re-metabolized in the final stages by *S. cerevisiae.*

Table 2. Main analytical parameters of the Verdicchio wine obtained by different trials at the end of fermentation.

Fermentation Trials	pH	Total Acidity (g/L)	Volatile Acidity (g/L)	Glycerol (g/L)	Ethanol (%*v*/*v*)	SO_2 Total (mg/L)
S. cerevisiae	3.34 ± 0.00 ^{a}	6.7 ± 0.07 ^{c}	0.69 ± 0.01 ^{b}	5.65 ± 0.12 ^{b}	13.5 ± 0.1 ^{a}	9.6 ± 0.2 ^{b}
S. bombicola/S. cerevisiae	3.31 ± 0.00 ^{ab}	6.7 ± 0.14 ^{c}	0.85 ± 0.02 ^{a}	6.28 ± 0.30 ^{a}	13.4 ± 0.1 ^{a}	6.4 ± 0.1 ^{c}
L. thermotolerans/S. cerevisiae	3.25 ± 0.002 ^{b}	7.7 ± 0.14 ^{a}	0.59 ± 0.02 ^{c}	6.25 ± 0.01 ^{a}	13.0 ± 0.02 ^{b}	6.4 ± 0.2 ^{c}
T. delbrueckii— S. Bombicola— L. thermotolerans/ S. cerevisiae	3.29 ± 0.00 ^{ab}	7.0 ± 0.07 ^{b}	0.83 ± 0.02 ^{a}	6.20 ± 0.12 ^{a}	12.8 ± 0.1 ^{b}	6.4 ± 0.2 ^{c}
Spontaneous fermentation	3.30 ± 0.01 ^{ab}	7.3 ± 0.14 ^{ab}	0.61 ± 0.01 ^{c}	5.84 ± 0.13 ^{a}	13.5 ± 0.2 ^{a}	35 ± 0.3 ^{a}

Data are means ± standard deviations. Values displaying different superscript letters ($^{a–c}$) within each column are significantly different according to Duncan tests ($p < 0.05$).

Table 3. The main by-products of fermentation and volatile compounds of the wines. Data are means ± standard deviations. Values displaying different superscript letters ($^{a–e}$) within each line are significantly different according to Duncan tests ($p < 0.05$).

mg/L	*S. cerevisiae*	*S.bombicola/S. cerevisiae*	*L. thermotolerans/ S. cerevisiae*	*L. thermotolerans— T.delbrueckii— S. bombicola/ S. cerevisiae*	Spontaneous Fermentation
ESTERS					
Ethyl butyrate	0.034 ± 0.010 ^{a}	0.028 ± 0.007 ^{a}	0.020 ± 0.010 ^{a}	0.020 ± 0.004 ^{a}	0.034 ± 0.013 ^{a}
Ethyl acetate	97.25 ± 0.29 ^{c}	106.48 ± 0.79 ^{b}	95.30 ± 1.40 ^{c}	97.13 ± 0.72 ^{c}	109.62 ± 0.36 ^{a}
Ethyl hexanoate	0.062 ± 0.030 ^{a}	0 ± 0 ^{c}	0.021 ± 0.004 ^{bc}	0.008 ± 0.001 ^{bc}	0.041 ± 0.014 ^{ab}
Ethyl octanoate	0.059 ± 0.005 ^{a}	0.053 ± 0.003 ^{a}	0.023 ± 0.001 ^{c}	0.031 ± 0.001 ^{c}	0.039 ± 0.001 ^{b}
Phenyl ethyl acetate	0.010 ± 0.001 ^{ab}	0.012 ± 0.003 ^{a}	0.007 ± 0.001 ^{bc}	0.010 ± 0.001 ^{abc}	0.005 ± 0.006 ^{c}
Hexyl acetate	0.005 ± 0.003 ^{b}	0.015 ± 0.007 ^{a}	0 ± 0 ^{b}	0.003 ± 0.001 ^{b}	0 ± 0 ^{b}
Isoamyl acetate	0.261 ± 0.020 ^{a}	0.383 ± 0.233 ^{a}	0.382 ± 0.160 ^{a}	0.563 ± 0.231 ^{a}	0.653 ± 0.174 ^{a}
ALCOHOLS					
n-propanol	24.33 ± 0.18 ^{c}	29.55 ± 0.04 ^{b}	31.81 ± 0.27 ^{a}	28.35 ± 0.40 ^{b}	27.66 ± 1.86 ^{b}
Isobutanol	74.71 ± 1.05 ^{e}	98.68 ± 0.01 ^{b}	92.72 ± 0.16 ^{c}	85.32 ± 0.05 ^{d}	111.63 ± 0.19 ^{a}
Amyl alcohol	28.41 ± 2.13 ^{a}	19.57 ± 1.41 ^{b}	26.91 ± 2.13 ^{a}	19.46 ± 0.94 ^{b}	24.76 ± 0.11 ^{a}
Isoamyl alcohol	204.54 ± 0.43 ^{c}	205.54 ± 0.13 ^{c}	216.15 ± 0.13 ^{b}	216.53 ± 1.08 ^{b}	256.75 ± 1.74 ^{a}
β-Phenyl ethanol	46.19 ± 8.64 ^{ab}	46.24 ± 4.32 ^{ab}	41.89 ± 2.53 ^{b}	56.860 ± 6.75 ^{a}	55.915 ± 5.95 ^{a}
CARBONYL COMPOUNDS					
Acetaldehyde	12.59 ± 0.54 ^{d}	30.67 ± 0.93 ^{b}	40.22 ± 0.97 ^{a}	25.11 ± 0.12 ^{c}	12.28 ± 1.57 ^{d}
MONOTERPENES					
Linalool	0.007 ± 0.001 ^{a}	0.008 ± 0.001 ^{a}	0.006 ± 0.001 ^{a}	0 ± 0 ^{b}	0.010 ± 0.003 ^{a}
Geraniol	0.004 ± 0.002 ^{ab}	0.004 ± 0.001 ^{ab}	0 ± 0 ^{b}	0.005 ± 0.002 ^{a}	0.007 ± 0.001 ^{a}
Nerol	0.017 ± 0.001 ^{b}	0 ± 0 ^{c}	0.096 ± 0.013 ^{a}	0.004 ± 0.002 ^{bc}	0.004 ± 0.001 ^{bc}

L. thermotolerans/*S. cerevisiae* sequential fermentation showed a significant increase in n-propanol than the other fermentations.

High values of isobutanol and isoamyl alcohol were observed in wine produced by spontaneous fermentation, while low values were observed in wine inoculated with *S. cerevisiae* starter strain. Moreover, spontaneous fermentation showed a comparable amount of amyl alcohol to *L. thermotolerans*/*S. cerevisiae* and *S. cerevisiae* starter strain. The fermentation carried out with muti-starter sequential fermentation showed the highest amount of β-phenyl ethanol.

Regarding to esters content, spontaneous fermentation produced the highest content of ethyl acetate and isoamyl acetate while the other fermentations showed a comparable amount (multi-sequential, sequential and pure fermentations).

S. bombicola/*S. cerevisiae* sequential fermentation was characterized by a general significantly higher concentration of phenylethyl acetate and hexyl acetate. Moreover, this sequential fermentation showed a comparable amount to *S. cerevisiae* in ethyl octanoate content while ethyl hexanoate was not detected.

Finally, no significant differences were detected for the ethyl butyrate and isoamyl acetate content. Regarding monoterpenes, a significant increase was detected in *L. thermotolerans*/*S. cerevisiae* sequential fermentation, particularly in the nerol content.

The data of the main volatile compounds were elaborated by the PCA analysis to assess the comprehensive effect of the aromatic compounds on the different fermentation trials (Figure 2). The overall variance explained was 63.4% (PC1 36.3%; PC2 27.1%). The graphical representation showed a clear separation between spontaneous fermentation (upper right quadrant) and *S. cerevisiae* pure culture (lower right quadrant), highlighting a different aromatic characterization of the resulting wines. Regarding the sequential fermentation, *L. thermotolerans* was in the upper left quadrant and *S. bombicola* in the lower left quadrant. The sequential fermentation carried out with the three non-*Saccharomyces*, is positioned between the two sequential fermentations indicating the contribution of each non-*Saccharomyces* on the volatile profile.

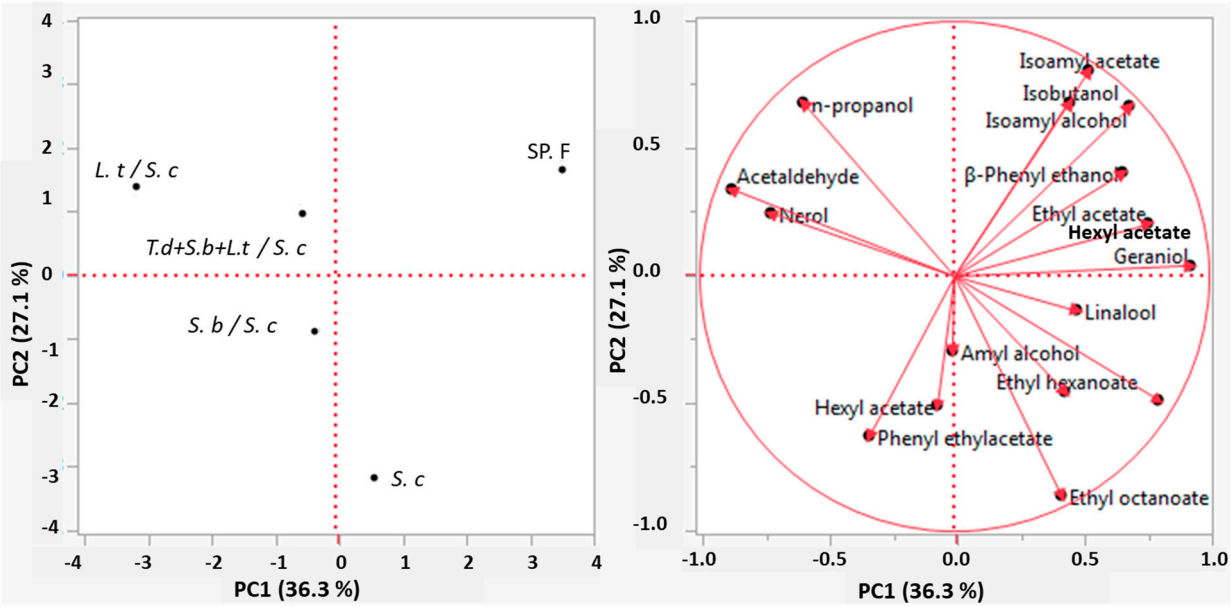


Figure 2. Principal component analysis based on the data regarding the main volatile compounds of wines obtained by *S. cerevisiae* (*S. c*); *S. bombicola*/*S. cerevisiae* (*S. b*/*S. c*); *L. thermotolerans*/*S. cerevisiae* (*L. t*/*S. c*); *T. delbrueckii*—*S. bombicola*—*L. thermotolerans*/*S. cerevisiae* (*T. d* + *S. b* + *L. t*/*S. c*); and spontaneous fermentation (SP.F).

3.4. Sensorial Analysis

After a period of 6 months of refinement, the wines underwent a sensory analysis (Figure 3). Regarding the olfactory component (Figure 3a), *S. cerevisiae* pure culture showed a significant increase in tropical fruit, honey and sweet toasted notes as compared to all other fermentations. These results also seemed to match the data relating to the volatile profile. Indeed, the amount of esters compounds was higher in these fermentation trials.

The *L. thermotolerans*/*S. cerevisiae* sequential fermentation exhibited the least sensation of tropical fruit, while the wines were distinguished by spicy and herbal notes.

Verdicchio wine made with S. *bombicola*/*S. cerevisiae* was significantly characterized by citrus notes, while the honey note was significantly lower than that detected in the other wines. The only fermentation that was not characterized by significant differences was the test carried out with the three non-*Saccharomyces* yeasts in sequential fermentation,

highlighting a balance between the different aromatic notes (Figure 3b). Citrus and aromatic herbs were significantly increased in spontaneous fermentation than the other wines.

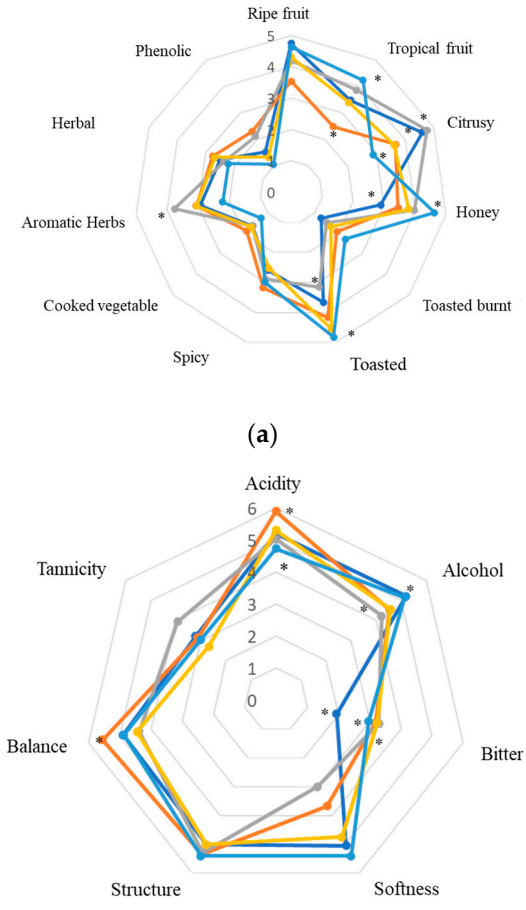


Figure 3. Sensory analysis of Verdicchio wine fermented by *S. bombicola* (—); *L. thermotolerans* (—); Spontaneous fermentation (—); *T. delbrueckii*/*L. thermotolerans*/*S. bombicola* (—) and *S. cerevisiae* (—). (**a**) smell analysis, (**b**) taste analysis. *, Significantly different (Fisher ANOVA; p-value < 0.05).

As regarding the gustative analysis (Figure 3b), Verdicchio wine produced by *L. thermotolerans* showed particularly emphasized acid and bitter notes but also more balanced notes than other wines.

The wine produced by *S. bombicola* showed a significantly low value in terms of bitterness and acidity, and a greater alcoholic sensation than all the other theses. Unlike what was found for the olfactory component, the thesis with the three non-*Saccharomyces* yeasts in sequential fermentation differed significantly in taste with a more pronounced bitterness and little balance. More marked notes of softness characterized the Verdicchio wine produced by *S. cerevisiae* pure culture and *S. bombicola* sequential fermentations.

4. Discussion

Several non-*Saccharomyces* yeasts are characterized by a significant production of aroma compounds such as esters, higher alcohols, acids, and monoterpenes [38,39], contributing to the flavor complexity of wine. A recent trend in winemaking in small wineries is the practice of un-inoculated must fermentation. This fermentation allows to exploit the potential of indigenous non-*Saccharomyces* yeasts species on grape surfaces to improve the aromatic profile of wine [40–42]. However, the spontaneous fermentations were characterized from the non-repeatability of the result. The practice of mixed/sequential fermentation

aims to conjugate the control of fermentation with the achievement of a peculiar profile as well as an enhancement of the aroma complexity of wine [43–45]. In this work, the impact of sequential inoculations of different non-*Saccharomyces* yeasts was compared with pure and spontaneous fermentation. These fermentations, carried out in Verdicchio grape juice at winery level, had the aim to investigate the different contributions of each non-*Saccharomyces*.

L. thermotolerans was investigated in different wines exhibiting a different behavior depending on the variety of grape juice. In this work, conducted in Verdicchio grape juice, a general behavior (enhancement of total acidity and a reduction of volatile acidity) was shown as reported in different grape varieties such as Sangiovese, Pinot gris, Merlot and Airen wines [10,46–48], indicating that these enological traits are specific to this species.

Regarding the production of higher alcohols, here *L. thermotolerans* sequential fermentation showed an increase in n-propanol, but the trend can be variable. Indeed, in Chardonnay and Airen wines *L. thermotolerans* sequential fermentation produced lower concentrations of these compounds [17,49], while in another work it showed an opposite trend [50]. These data confirmed that the grape variety is crucial to determine the effect of this yeast on the production of higher alcohols. Another positive feature was the production of terpenes previously detected, and mainly depending on the glucosidase activity of the strain used [43].

The positive fermentative features of *S. bombicola* such as ethanol reduction and the enhancement of glycerol was only in part detected in this work. The different fermentative conditions used at laboratory or pilot scale could have affected the results and thus, further investigations are needed.

T. delbrueckii DiSVA 130 here was only tested in multi- sequential fermentation with the other two non-*Saccharomyces* selected strains (*L. thermotolerans* and *S. bombicola*), to improve the complexity of resulting wine since this strain was extensively investigated in Verdicchio wine, mostly during different vintages and in sequential fermentation with various *S. cerevisiae* starter strains [35,36]. The concurrent inoculation of the three non-*Saccharomyces* yeasts in sequential fermentation with *S. cerevisiae* showed a complex analytical composition and sensorial profile indicating an effect that should be further investigated.

Different researchers highlighted that uninoculated wines have a better mouthfeel and consistency, and more complex aromatic profiles than wines inoculated with a commercial starter strain [51–53]. In this work, spontaneous fermentation carried out on Verdicchio wine determined an analytical and aromatic profile with a peculiar characteristic, as citrus and aromatic herbs notes made the wine well characterized. On the other hand, the poor reproducibility of spontaneous fermentation which concerns the analytical and microbiological composition of grapes/must can result in a variable and sometimes an undesirable final product.

Further investigations could be necessary to better understand the correlation of the impact of spontaneous fermentation and non-*Saccharomyces* yeasts on the aroma profile of the resulting wines. Indeed, several factors interfere on the perception of aromatic notes such as the interaction with other compounds but also the perception of panelists.

However, despite being an indispensable element of wine, it is considered difficult for naive consumers to understand.

The aroma of wine is one of the main factors contributing to the quality and allows to distinguish a variety of wines. A multi-component blend flavor can be integrated and considered as a single concept, described as “complex”. Among the various characteristics of wine, “complexity” is one of its most important aspects to understand the wine itself. The analysis of the complexity of wine is a cognitive and multi-dimensional process, which requires a detailed sensory analysis to separate the various aromatic and gustatory components. Complexity is therefore considered a positive aspect and a desirable characteristic of the wine [54].

In conclusion, the results highlighted that each of the non-*Saccharomyces* yeasts give a peculiar analytical and aromatic feature in the resulting wine. For this reason, the practice

of the use of mixed fermentation carried out with more selected non-*Saccharomyces* yeasts species could be a strategy to further characterize and differentiate the final wine. The results indicated that some peculiar enological characteristics of each non-*Saccharomyces* species can be expressed in different varieties while other features specifically interact with the varietal characteristics.

The oenologists will choose the yeast to be used in mixed fermentations according to the desired features of different Verdicchio wine styles.

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