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# Pilot Scale Fermentations of Sangiovese: An Overview on the Impact of *Saccharomyces* and Non-*Saccharomyces* Wine Yeasts

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Received: 30 May 2020; Accepted: 22 June 2020; Published: 30 June 2020


**Abstract:** The production of wines with peculiar analytical and sensorial profiles, together with the microbiological control of the winemaking process, has always been one of the main objectives of the wine industry. In this perspective, the use of oenological starters containing non-*Saccharomyces* yeasts can represent a valid tool for achieving these objectives. Here we present the results of seven pilot scale fermentations, each of which was inoculated with a different non-*Saccharomyces* yeast strain and after three days with a commercial *Saccharomyces cerevisiae* starter. The fermentations were carried out in double on 70 L of Sangiovese grape must, the most widely planted red grape variety in Italy and particularly in Tuscany, where it is utilized for the production of more than 80% of red wines. Fermentations were monitored by assessing both the development of the microbial population and the consumption of sugars at the different sampling times. The impact of the different starters was assessed after stabilization through the evaluation of the standard analytical composition of the resulting wines, also taking into account polysaccharides and volatile compounds. Moreover, quantitative descriptive sensory analyses were carried out. Compared to the control wines obtained by inoculating the *S. cerevisiae* starter strain, those inoculated with non-*Saccharomyces*/*Saccharomyces* mixed starters presented a significant differentiation in the chemical-analytical composition. Moreover, sensory analysis revealed differences among wines mainly for intensity of color, astringency, and dryness mouthfeel perception.

**Keywords:** non-*Saccharomyces* yeasts; wine; mixed starter cultures; fermentation; Sangiovese; sensory analysis

## 1. Introduction

Non-*Saccharomyces* yeasts have attracted increasing attention in recent years, with several studies providing evidence of their impact on the organoleptic characteristics and chemical-physical stability of wines. Despite their large intraspecific biodiversity, non-*Saccharomyces* yeasts often show species-specific metabolic features that contribute to the specific imprint of the resulting wines, when inoculated in mixed fermentation with *Saccharomyces cerevisiae* [1]. For instance, among the non-*Saccharomyces* yeasts, those belonging to the species *Torulaspora delbrueckii* result in the production of low volatile acidity, high terpenols, and 2-phenylethanol when utilized in mixed culture with *S. cerevisiae* [2–7]. In addition, the release of higher concentrations of thiols, with consequent increase of varietal

characters, was reported for *T. delbrueckii*/*S. cerevisiae* mixed starters [8,9]. *Starmerella bacillaris* (synonym *Candida zemplinina*) contributes to reduce the amount of acetic acid in mixed fermentation with *S. cerevisiae* [5,10–12]. Moreover, this yeast is usually characterized by high glycerol production [11,13–17] and low ethanol yield [16,18–20] making it an interesting tool to increase the wine sweetness and modulate the ethanol content. *Lachancea thermotolerans* strains produce lactic acid during the alcoholic fermentation causing a decrease of wine pH while reducing its volatile acidity [4,21–24]. Moreover, an increase of 2-phenylethanol, glycerol, and polysaccharides in mixed fermentation *L. thermotolerans*/*S. cerevisiae* was reported [4,21,22,25]. Regarding *Metschnikowia pulcherrima*, some studies showed its high β-glucosidase activity [26–28] with consequent increase of volatile terpene content from glycosylated flavorless precursors present in grapes. Moreover, because of a high β-lyase activity, yeasts belonging to the species *M. pulcherrima* release high quantity of varietal thiols from grape precursors conjugated to cysteine or glutathione [29,30]. In the last few years there has been also a renewed interest in yeasts belonging to the genus *Schizosaccharomyces*. Indeed, besides reducing malic acid in grape juice and/or wine, these yeasts produce high quantities of pyruvic acid [31,32] and polysaccharides during the course of alcoholic fermentation [33–36], positively contributing to the chemical-physical stability of wines. Finally, among yeasts typically considered as potential spoilage, those belonging to the genus *Zygosaccharomyces* have also started to attract attention [37,38]. In particular, the species *Z. florentina* contributes to increase esters and glycerol concentration when used in co-culture with *S. cerevisiae*, thus producing wines with higher floral notes and lower perception of astringency [39].

A Scopus database search with the combination of terms "wine and non-*Saccharomyces*" as query statement to highlight the relevant literature in the last decade, indicates that an increasing number of peer-reviewed publications have considered the use of non-*Saccharomyces* yeasts as starters together with *S. cerevisiae*. In particular, of a total of 458 peer reviewed scientific articles published from 2010 to 2019, the average number of publications/year on this topic was 26 during the period in between 2010 and 2014, and reached 65 in the following years (2015–2019). It is worth pointing out that most of these publications refer to laboratory scale fermentations. In particular, considering publications starting from 2015, 76% of the fermentations were carried out at the laboratory scale (50% in up to 1 L, 15% in 1.2–5 L and 11% in 10–20 L). Instead, pilot plant and industrial scale fermentations, that regarded 24% of the trials, are still quite limited. Of these, 18% were carried out in grape must volumes ranging from 30 to 200 L (pilot plant fermentations) and 5% in 700 to 1000 L (industrial scale fermentations). One fermentation was performed in a 100,000 L vessel.

Moreover, while 12% of the publications starting from 2015 describe fermentations carried out in synthetic media, the majority of works report on the utilization of different grape varieties to evaluate the impact of the non-*Saccharomyces* yeasts on the chemical and physical characteristics of the relevant wine. Among these, Shiraz, Sauvignon Blanc, Barbera, Cabernet Sauvignon, Chardonnay and Merlot were the most frequently utilized. Few articles (4%) describe mixed fermentations in Sangiovese grape must despite the importance of this grape variety that in Italy represents 90% of total world Sangiovese vineyard area (http://www.oiv.int/public/medias/5888/en-distribution-of-the-worlds-grapevine-varieties.pdf). In order to avoid any metabolic interference by other microorganisms, half of the studies evaluated the impact of pure and mixed starters on the final wine by using sterile grape juice.

Indeed, laboratory scale fermentation and synthetic media or sterile grape juice are important conditions to evaluate the specific metabolic traits of the non-*Saccharomyces* yeasts included in the mixed starter and also to establish their possible interactions with *S. cerevisiae*. However, the results obtained under these conditions are likely far away from those obtainable under technological conditions, also due to the unpredictability of the interactions that the inoculated starters may establish with wild grape must microflora.

Based on these observations, in the present work seven different non-*Saccharomyces*/*S. cerevisiae* mixed starters were inoculated in Sangiovese grape must at the pilot plant scale and their impact on the final product was evaluated through chemical and sensory analyses of the resulting wines after

stabilization. Sangiovese is the most widely planted red grape variety in Italy, particularly in Tuscany where it represents the obligatory variety in the production of wines with a protected and guaranteed designation of origin (DOCG) such as Chianti Classico and Brunello di Montalcino.

## 2. Material and Methods

### 2.1. Yeast Strains

Seven non-*Saccharomyces* strains from the yeast culture collection of the Department of Agriculture, Food, Environment and Forestry (DAGRI, University of Florence, Italy) and of the Department of Life and Environmental Sciences (DiSVA, Polytechnic University of Marche, Ancona, Italy) were used (Table 1). The yeast strains were sub-cultured on YPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) at six months intervals, and maintained at 4 °C.

**Table 1.** Origin of the seven non-*Saccharomyces* strains and the commercial strain of *S. cerevisiae* used in this study.

| Strain | Species | Origin |
|---|---|---|
| # 4 | *Pichia fermentans* | DiSVA [a] |
| # 22 | *Starmerella bacillaris* | DAGRI [b] |
| # 32 | *Hanseniaspora osmophila* | DAGRI [b] |
| # 42 | *Zygotorulaspora florentina* | DAGRI [b] |
| # 46 | *Metschnikowia pulcherrima* | DiSVA [a] |
| # 92 | *Torulaspora delbrueckii* | DAGRI [b] |
| # 103 | *Lachancea thermotolerans* | DiSVA [a] |
| # EC1118 | *Saccharomyces cerevisiae* | Lallemand [c] |

[a] Department of Life and Environmental Sciences of the Polytechnic University of Marche (Italy), [b] Department of Agriculture, Food, Environment and Forestry, University of Florence (Italy). [c] Lallemand Inc. (Montreal, QC, Canada).

The strains reported in Table 1 were isolated from grapes and musts of different origins and characterized for their enological performances in mixed fermentations carried out in grape juice at laboratory scale [4,37,38]. A commercial *S. cerevisiae* starter, Lalvin EC1118 (Lallemand Inc., Montreal, QC, Canada), was used as reference strain and for comparison determination.

### 2.2. Pilot Scale Fermentation

The fermentation trials were carried out in 100 L steel tanks containing 70 L of Sangiovese grape must with the following characteristics: pH 3.66, 234 ±7 g/L sugars, 4.0 g/L total acidity (as tartaric acid), 1.2 g/L malic acid. Non-*Saccharomyces* yeasts were inoculated in 12 L filtered sterilized commercial red grape must, aliquoted within 2 L flasks, each containing 1.5 L and grown for 48-h at 25 °C under shaking conditions (150 rpm). Cell concentration was determined by microscope counting. Each tank was inoculated with $10^7$ cell/mL of the non-*Saccharomyces* yeast strain. After 3 days of fermentation, *S. cerevisiae* EC1118 was inoculated as active dry yeast (ADY) at the final concentration of $10^7$ cell/mL. Control trials were inoculated with $10^7$ cell/mL of *S. cerevisiae* EC1118. Skin cap was punched down twice a day and fermenting must was sampled during the fermentation process immediately after inoculation (T0) and 3, 5, and 10 days after inoculation (T3, T5, T10, respectively) to evaluate the evolution of the yeast populations as viable cell counts and to determine the residual sugars. Alcoholic fermentation was monitored by periodically measuring the density by a double scale hydrometer (density and Baumé). All trials were fermented at 25 °C, in duplicate. After completion of fermentation, the wines were naturally fined by three successive rackings over a month at 16–18 °C and added with $SO_2$ up to 100 mg/L before bottling (0.75 L cork-capped glass bottles).

### 2.3. Analyses

#### 2.3.1. Evaluation of Cell Growth

During the fermentation, cell concentrations were evaluated as viable cell counts and expressed as number of colony forming units (cfu)/mL at the different sampling times. Viable plate count was carried out both on lysine agar (LA medium; Oxoid Unipath, Hampshire, UK), and Wallerstein Laboratory nutrient agar medium (WL medium; Oxoid Unipath, Hampshire, UK) [40] to estimate the non-*Saccharomyces* yeast and the total yeast population, respectively.

#### 2.3.2. Analytical Determinations

Residual sugar, organic acid, total and volatile acidity and pH were determined according to Official EU Methods (EC 2000). Total polysaccharides concentration was evaluated by HPLC [35], using a Varian instrument equipped with auto-sample injector (loop 20 µL) and coupled with refractive index detector. For the separation of total polysaccharides, a column Progel-TSK G-OLIGO PW (Supelco 808031) and a TSK-gel PW (Supelco 808034) precolumn were used with isocratic elution (NaCl 0.2 M; 0.8 mL/min; 40 °C). Before injection, the samples were filtered (1.2 µm) and purified on polyamide SC6 (Macherey-Nagel, Dylan, Germany). Polysaccharides quantification was performed by comparison with an external calibration curve of mannans from *S. cerevisiae* (M7504, Sigma-Aldrich, St. Louis, MO, USA), at concentrations ranging from 50 mg/L to 500 mg/L.

Total polyphenols in wines were determined by the Folin–Ciocalteu method according to Di Stefano [41] and expressed as catechin equivalents in mg of catechin/L, whereas total anthocyanins were determined by direct reading of the absorbance at 540 nm of wine in hydrochloric ethanol solution [42] and expressed in g malvidin/L [43].

Higher alcohols and acetaldehyde were determined by a GC method with flame ionization detector (FID) detection at 250 °C, on a Carlo Erba HRGC 5300 instruments equipped with a glass column (2 m; 2 mm ID) packaged with Carbopack C + 0.2% Carbowax 1500, 80–100 meshes (Supelco). The other chromatographic conditions were as follows: temperature gradient from 45 °C to 160 °C (3 °C/min), held to 160 °C for 20 min; inj. temperature 220 °C; carrier: Helium 2 mL/min; injection volume: 1 µL of distilled sample spiked with 3-methyl-2-butanol as internal standard. The acquisition and elaboration of the FID signal was carried out by means of Galaxy software (Varian Inc., Walnut Creek, CA, USA).

Minor volatile compounds were evaluated by capillary gas-liquid chromatography as previously reported [36]. In particular, the analyses were carried out on a Carlo Erba HRGC 5300 instrument, injecting an ether/hexane extracts (1/1, *v*/*v*) of the wine samples previously spiked with 3-octanol as internal standard. The chromatographic conditions were as follow: glass capillary column 0.25 µm Supelcowax 10 (60 m length, 0.32 mm internal diameter, 0.25 µm film thickness). One µL was injected in split-splitless mode (60 s splitless); carrier gas: helium at 2.5 mL/min flow rate; injection temperature: 220 °C; elution temperature gradient: from 50 °C (held 5 min) to 220 °C (3 °C/min); detection by flame ionization detector (FID) at 250 °C. The acquisition and integration of the FID signals were carried out using the Galaxy software (Varian Inc., Walnut Creek, CA, USA) and the content of each compound was evaluated in respect to an external standard curve. All analyses were carried out in double from each fermentation tank.

#### 2.3.3. Sensory Evaluation of Wines

The wines were left to mellow for about four months after bottling, before sensory evaluation. Wine tasting was performed by an 11 member trained and formed panel, in two sessions. Organoleptic evaluations were conducted by quantitative descriptive analysis, using a pre-defined protocol and descriptive terminology, previously developed by the tasting group. In particular, every sample was tasted twice by each taster, within completely randomized blocks, and the panelists were asked to express their judgment by quantification of each sensory descriptor (color intensity, floral, fruity,

preserved fruit, spicy, candy, sulfur, chemical, earthy, mouthfeel volume, acidity, tannic intensity, astringency, dryness, bitterness) on the basis of a four point structured scale, from 0 to 3 for the olfactory descriptors (no presence of the perception to high intensity), or from 1 to 4 (low to high intensity) for color intensity and mouthfeel descriptors. The olfactive descriptors were chosen previously in a round table session of the panel basing on a free profile description of the same wines and the judges were subsequently trained on them.

For evaluation of gustative descriptors, the method used was that developed by ICV (Institute Cooperative du Vin, Lattes, France) for the Quantitative Descriptive Analysis of red wine [44,45].

### *2.4. Statistical Analysis*

Data from chemical analysis of the wines were subjected to one-way ANOVA using STATISTICA 7 (Statsoft, Tulsa, OK, USA) software. Duncan test was carried out to compare mean values and evaluate significant differences. Mean values of volatile compounds were analyzed by principal component analysis (PCA), using JMP® 11 statistical software.

The sensory scores were statistically analyzed and compared according to analysis of variance (ANOVA) using a mixed effect model considering as fixed factors those related to the experimental thesis and as random factors the deviations due to the effect of the judge from the general average of each parameter.

## 3. Results and Discussion

Growth kinetics of *S. cerevisiae* EC1118 pure culture (control fermentation) and of non-*Saccharomyces*/*S. cerevisiae* mixed cultures are reported in Figure 1. In all mixed cultures the initial concentration of the non-*Saccharomyces* yeasts ranged from $10^6$ to $10^7$ cell/mL. *S. cerevisiae* was able to dominate in most of the mixed fermentations and showed, in mixed culture, a growth kinetics that was similar to that of control. In particular, in spite of the presence of the non-*Saccharomyces* yeasts, *S. cerevisiae* reached a cell concentration of about $10^8$ CFU/mL (T5) and maintained it until the end of the fermentation (T10). When in mixed culture with *H. osmophila*, *S. cerevisiae* reached a lower cell concentration ($2.5 \times 10^7$ cell/mL at T5) which remained unvaried for 5 further days of fermentation (T10). Similarly, *L. thermotolerans*, even if to a lower extent, affected the growth of *S. cerevisiae*, which reached a cell concentration of $4.8 \times 10^7$ cell/mL and $6.7 \times 10^7$ cell/mL at T5 and T10, respectively. Conversely, and contrary to that found by Englezos et al. [20], *S. bacillaris* did not affect *S. cerevisiae* growth. Similar to *H. osmophila* and *L. thermotolerans*, *Z. florentina* and *T. delbrueckii* showed a higher level of competitiveness being still present at concentrations ranging from $1 \times 10^4$ to $5 \times 10^5$ cells/mL at T10. On the contrary, *P. fermentans*, *S. bacillaris*, and *M. pulcherrima* persisted at high concentration up to T5 and they almost disappeared at T10.

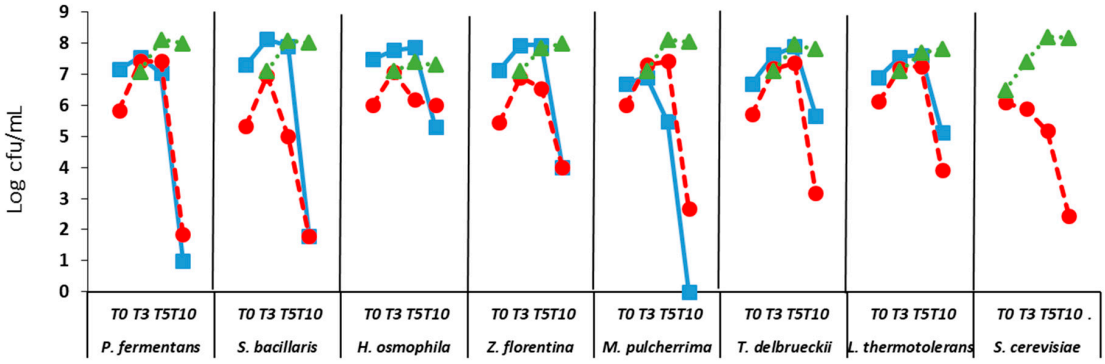


**Figure 1.** Growth of inoculated non-*Saccharomyces* (■), other non-*Saccharomyces* yeasts (●) and *S. cerevisiae* (▲). Viable plate counts were done immediately after (T0), and 3, 5, and 10 days after inoculation (T3, T5, and T10, respectively). Data are means ±SD ($n$ = 2).

Sugar consumption was consistent with growth kinetics (Figure 2). As expected, in control fermentations (*S. cerevisiae* pure culture), it started soon after grape crushing and proceeded faster compared to that observed in all mixed fermentation trials. Among these, the combination *H. osmophila*/*S. cerevisiae* showed an impairment of sugar consumption with 7.50 g/L residual sugar at T10, while the other mixed cultures left from 1 to 1.4 g/L residual sugar.

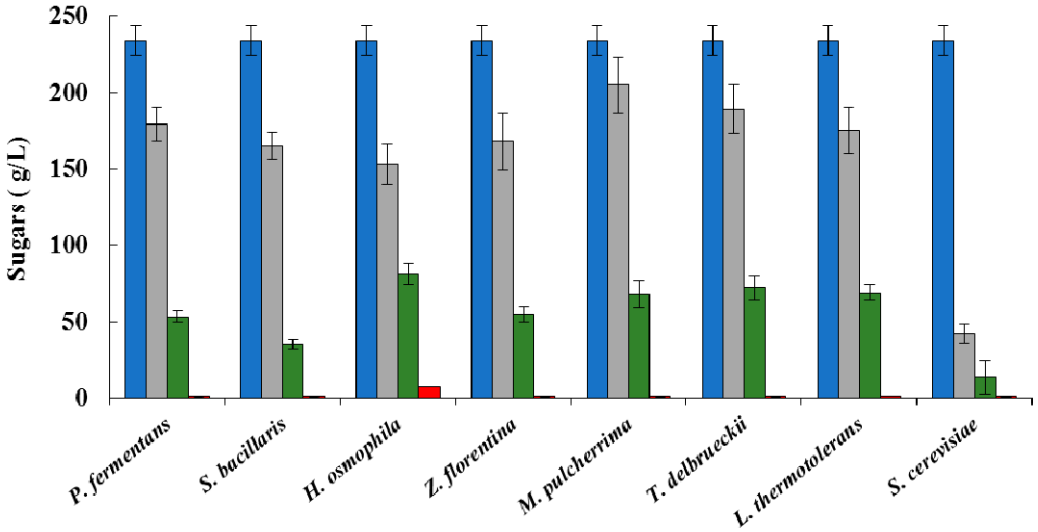


**Figure 2.** Non-*Saccharomyces* and *S. cerevisiae* sugar consumption in each fermentation trial. Different colors indicate different sampling times; T0 (■), T3 (■), T5 (■), T10 (■). Data are means ±SD ($n$ = 2).

In most of the mixed cultures ethanol concentrations were comparable to that of the control (Table 2). Mixed starters involving *Z. florentina* and *T. delbrueckii* were exceptions and produced less ethanol than the control. Lencioni et al. [39], also found slightly lower ethanol concentrations in *Z. florentina*/*S. cerevisiae* fermentations performed at laboratory scale in white grape must, with respect to the pure *S. cerevisiae* culture. Regarding the mixed starter *T. delbrueckii*/*S. cerevisiae*, decreases in ethanol concentrations, ranging from 0.3% to 0.5%, were also reported by other authors at the end of pilot-scale fermentations [46,47]. Non-*Saccharomyces*/*S. cerevisiae* mixed starters have already been proposed as a tool for the possible reduction of ethanol content in wine [19,48–54]. Indeed, the lower ethanol concentration is a consequence of some features of non-*Saccharomyces* yeasts, such as reduced ethanol yield, low fermentation efficiency, and respiro-fermentative metabolism.

**Table 2.** Main analytical parameters of the wines evaluated before bottling.

| | pH | Ethanol% (*v*/*v*) | Volatile Acidity (g/L) | Total Acidity (g/L) | Malic Acid (g/L) | Lactic Acid (g/L) | Free $SO_2$ (mg/L) | Total Polyphenols (mg catechin/L) | Total Anthocyanins (mg malvidin/L) |
|---|---|---|---|---|---|---|---|---|---|
| *P. fermentans* | 3.37 ± 0.03 $^{ab}$ | 12.80 ± 0.01 $^{abc}$ | 0.34 ± 0.02 $^{bc}$ | 5.50 ± 0.05 $^{bc}$ | 1.08 ± 0.02 $^{abc}$ | 0.11 ± 0.03 $^{cd}$ | 18 ± 0.8 $^{bc}$ | 1536 ± 27.32 $^{a}$ | 194 ± 3.55 $^{ab}$ |
| *S. bacillaris* | 3.34 ± 0.02 $^{ab}$ | 12.85 ± 0.00 $^{bc}$ | 0.37 ± 0.02 $^{b}$ | 5.80 ± 0.06 $^{ab}$ | 1.06 ± 0.02 $^{bc}$ | 0.11 ± 0.00 $^{d}$ | 13 ± 2.50 $^{c}$ | 1556 ± 30.50 $^{a}$ | 204 ± 0.50 $^{a}$ |
| *H. osmophila* | 3.41 ± 0.05 $^{a}$ | 12.88 ± 0.13 $^{ab}$ | 0.53 ± 0.02 $^{a}$ | 5.50 ± 0.33 $^{bc}$ | 0.96 ± 0.23 $^{cd}$ | 0.24 ± 0.09 $^{b}$ | 16 ± 1.00 $^{bc}$ | 1381 ± 66.00 $^{ab}$ | 173 ± 12.50 $^{bc}$ |
| *Z. florentina* | 3.34 ± 0.01 $^{ab}$ | 12.63 ± 0.03 $^{bc}$ | 0.26 ± 0.01 $^{c}$ | 6.15 ± 0.00 $^{a}$ | 1.34 ± 0.01 $^{a}$ | 0.22 ± 0.01 $^{bc}$ | 14 ± 0.00 $^{bc}$ | 1394 ± 44.50 $^{ab}$ | 172 ± 2.00 $^{bc}$ |
| *M. pulcherrima* | 3.34 ± 0.02 $^{ab}$ | 12.78 ± 0.03 $^{abc}$ | 0.25 ± 0.04 $^{c}$ | 5.87 ± 0.06 $^{ab}$ | 1.24 ± 0.01 $^{ab}$ | 0.15 ± 0.00 $^{bcd}$ | 12 ± 3.00 $^{c}$ | 1518 ± 53.50 $^{ab}$ | 187 ± 0.50 $^{abc}$ |
| *T. delbrueckii* | 3.32 ± 0.00 $^{b}$ | 12.70 ± 0.15 $^{c}$ | 0.30 ± 0.03 $^{bc}$ | 6.07 ± 0.02 $^{a}$ | 1.13 ± 0.02 $^{bc}$ | 0.17 ± 0.02 $^{bcd}$ | 14 ± 0.50 $^{bc}$ | 1350 ± 42.50 $^{ab}$ | 181 ± 11.50 $^{abc}$ |
| *L. thermotolerans* | 3.40 ± 0.02 $^{a}$ | 12.98 ± 0.03 $^{a}$ | 0.37 ± 0.02 $^{b}$ | 5.58 ± 0.07 $^{bc}$ | 1.04 ± 0.02 $^{d}$ | 0.78 ± 0.01 $^{a}$ | 19 ± 1.00 $^{ab}$ | 1421 ± 7.50 $^{ab}$ | 188 ± 2.00 $^{ab}$ |
| *S. cerevisiae* | 3.40 ± 0.02 $^{ab}$ | 13.00 ± 0.00 $^{a}$ | 0.28 ± 0.06 $^{bc}$ | 5.36 ± 0.10 $^{c}$ | 1.15 ± 0.09 $^{abc}$ | 0.16 ± 0.01 $^{bcd}$ | 24 ± 3.00 $^{a}$ | 1300 ± 92.00 $^{b}$ | 163 ± 12.50 $^{c}$ |

Data are media ± semi-difference of two independent experiments. Data with different superscript letters within each column are significantly different (Duncan test; $p \leq 0.05$).

Mixed starters, including *P. fermentans*, *S. bacillaris*, and *L. thermotolerans*, determined an increase of volatile acidity of about 0.1 g/L, as compared to the control (Table 2). Although non-significant, *H. osmophila*/*S. cerevisiae* resulted in a more marked increase in volatile acidity which reached values of 0.5 g/L. This is in contrast with previous results obtained with the same yeast strain in co-fermentation with *S. cerevisiae*, but at laboratory scale and with a commercial white grape must [38]. Instead, the increase in volatile acidity observed in the fermentation inoculated with *S. bacillaris*/*S. cerevisiae* is in agreement with the results obtained by Whitener et al. [55] but in contrast with previously published works showing *C. zemplinina* (synonym *S. bacillaris*) able to reduce the amount of acetic acid when in mixed culture with *S. cerevisiae* [5,10–12]. Nisiotou et al. [56] found lower acetic acid concentration in sequential fermentation *S. bacillaris*/*S. cerevisiae* carried out as a pilot plant as compared to those performed at laboratory scale fermentation. These discrepancies might be due to the significant strain diversity within this species, as already observed by Englezos et al. [11], but also to a strain specific response to the different fermentation conditions, including the grape variety utilized. In our experimental trials, the presence of other microorganisms, starting from the beginning of the alcoholic fermentations performed at pilot scale, might have interfered with the metabolic activity of the *S. bacillaris* yeast strain. Similar observations can be extended to the mixed fermentation conducted with *L. thermotolerans* that is usually recognized for low volatile acidity production in wine [57].

The utilization of *P. fermentans* and *S. bacillaris* resulted also in a significantly higher amount of both total polyphenols and anthocyanins, with respect to the control (Table 2). Recent works indicate that wine color and anthocyanin composition may benefit from the fermentative activity of non-*Saccharomyces* yeasts [58,59]. In particular, it was shown that the inoculation of non-*Saccharomyces*/*Saccharomyces* mixed starters results in higher acetaldehyde production, with effects on anthocyanin-derived pigments [60]. Here, no differences in acetaldehyde content were observed at T10 although its increase at T3 and T5 cannot be excluded in fermentations carried out by mixed starters including *P. fermentans* and *S. bacillaris*.

Analyses of total polysaccharides, glycerol and volatile compounds, together with the sensory analyses were performed four months after bottling.

Polysaccharides, in particular mannoproteins, impact wine sensorial features by decreasing astringency, improving the mouthfeel and fullness, adding complexity and aromatic persistence, and increasing roundness and sweetness [61–64]. With the exception of those including *H. osmophila* and *Z. florentina*, all mixed starters produced significantly higher polysaccharides concentrations in respect to the control (Figure 3). In particular, the increase ranged from 2.5% to 33%. In this respect, the most interesting association was *L. thermotolerans*/*S. cerevisiae* with a final content of total polysaccharides of 732 mg/L versus 550 mg/L of the control, in agreement with the result obtained by Gobbi et al. [22] with a different *L. thermotolerans* strain. The release of polysaccharides by non-*Saccharomyces* yeasts is not new and a wide intraspecific biodiversity for this characteristic was observed in *Hanseniaspora*, *Zygosaccharomyces* [4,35,37,38] and *Schizosaccharomyces* yeasts [34].

The concentration of glycerol, responsible for the sweetness of red and white wines [65,66], was significantly higher in most of the wines produced by mixed starters, apart from those including *H. osmophila*, *P. fermentans* and *Z. florentina* (Figure 3). As expected, the association *S. bacillaris*/*S. cerevisiae* resulted in the highest glycerol concentration (11.4 g/L), in accordance with that already observed for the species *S. bacillaris* [25,67,68].

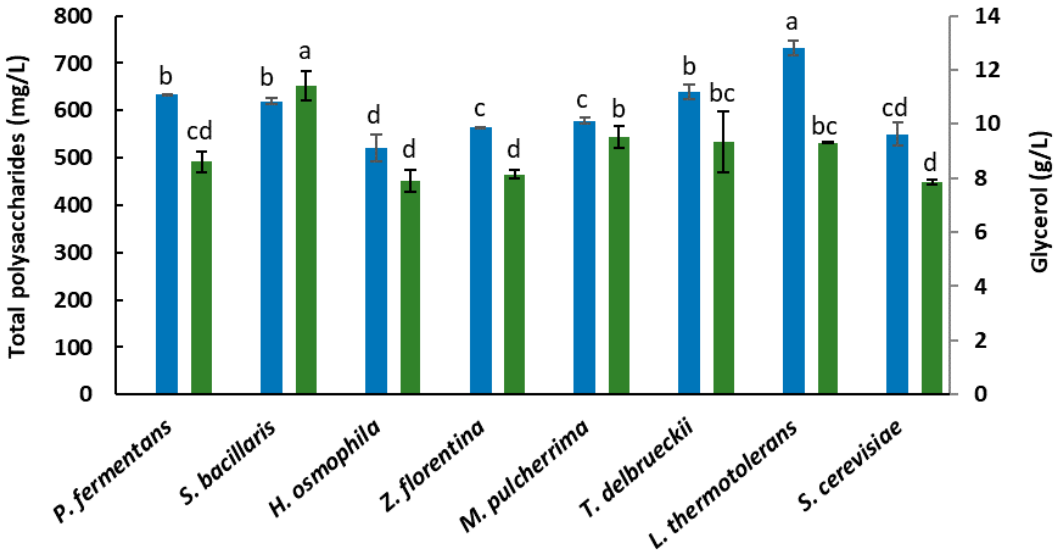


**Figure 3.** Total polysaccharides (■) and glycerol (■) in wines obtained four months after bottling. Data are means ±SD ($n$ = 2). Values displaying different letters (a, b, c, d) are significantly different according to the Duncan test ($p \leq 0.05$).

The concentrations of the main volatile compounds are reported in Table 3. The concentrations of acetaldehyde, propanol, and hexanol produced by mixed starter cultures were comparable to that of the control. In contrast, mixed starters resulted in higher production of some of the higher alcohols. In particular, significant increases of 2-methyl-1-propanol (isobutanol) were observed in respect to control fermentation (47 ± 2 mg/L). This compound ranged from a minimum of 66 mg/L (for the associations including *H. osmophila* and *Z. florentina*) to a maximum of 123 ± 8 mg/L in the wine produced by *S. bacillaris*/*S. cerevisiae*. However, the sum of amylic alcohols (i.e., 2-methyl-1-butanol, 3-methyl-1-butanol) was significantly higher in wines produced with the mixed starters including *M. pulcherrima* (328 mg/L), *T. delbrueckii* (299 mg/L) and *L. thermotolerans* (294 mg/L) than in the control wine (266 mg/L). In agreement with that reported by other authors [69–72], mixed starters including *Hanseniaspora, Pichia* and *Zygosaccharomyces* showed lower production of higher alcohols. Interestingly, all the wines obtained with mixed starters presented significantly higher concentrations of 2-phenylethanol (8–9.5 mg/L) (which provides a rose-like flavor) compared to the control (6.1 mg/L). In particular, the highest concentrations of 2-phenylethanol were reached in mixed fermentations including *M. pulcherrima* (9.2 mg/L) and *T. delbrueckii* (9.4 mg/L). These results agree with those found by other authors showing that *M. pulcherrima* and *T. delbrueckii* produce high level of 2-phenylethanol [3,73,74]. Ethyl acetate was the main ester produced. At high concentrations (>100–150 mg/L) ethyl acetate determines a solvent-like aroma. Interestingly, with the exception of the associations including *M. pulcherrima* and *H. osmophila* that nearly doubled the amount produced by *S. cerevisiae* starter culture (54 mg/L and 51 mg/L, respectively), the other mixed starters determined slight increases in ethyl acetate in respect to the control. In any case, ethyl acetate concentration was always below the perception threshold (Table 3). These findings confirm those already observed in other studies [37,38], where some non-*Saccharomyces* yeasts, generally considered spoilage yeasts, produced in mixed culture ethyl acetate concentrations below those normally produced by the relevant pure culture. On the other hand, many studies report that most non-*Saccharomyces* yeasts can produce high amounts of ethyl acetate [71,75]. However, this discrepancy may be due to the wide inter-generic and intra-generic variability observed for the production of this ester compound. Accordingly, Domizio et al. [38] found, by analyzing eleven yeast strains of *Hanseniaspora* (belonging to four different species), that ethyl-acetate production ranged from 27 to 333 mg/L. It is also worth underlining that this compound, at low concentration, might contribute to wine fruity aroma.

**Table 3.** Volatile compounds (mg/L) of wines four months after bottling.

| | *P. fermentans* | *S. bacillaris* | *H. osmophila* | *Z. florentina* | *M. pulcherrima* | *T. delbrueckii* | *L. thermotolerans* | *S. cerevisiae* |
|---|---|---|---|---|---|---|---|---|
| **Acetaldehyde** | 71 ± 2 $^{a}$ | 66 ± 0 $^{a}$ | 61 ± 3 $^{a}$ | 62 ± 1 $^{a}$ | 72 ± 8 $^{a}$ | 61 ± 3 $^{a}$ | 62 ± 5 $^{a}$ | 64 ± 7 $^{a}$ |
| **1-propanol** | 34 ± 2 $^{b}$ | 38 ± 1 $^{ab}$ | 33 ± 3 $^{b}$ | 41 ± 2 $^{a}$ | 35 ± 2 $^{b}$ | 33 ± 1 $^{b}$ | 41 ± 2 $^{a}$ | 36 ± 2 $^{ab}$ |
| **2-Methyl-1-propanol** | 72 ± 2 $^{d}$ | 123 ± 8 $^{a}$ | 66 ± 3 $^{d}$ | 66 ± 1 $^{d}$ | 92 ± 1 $^{b}$ | 74 ± 3 $^{cd}$ | 84 ± 1 $^{bc}$ | 47 ± 2 $^{e}$ |
| **2-Methyl-1-butanol** | 54 ± 0 $^{bc}$ | 42 ± 2 $^{d}$ | 45 ± 5 $^{cd}$ | 53 ± 1 $^{bcd}$ | 68 ± 7 $^{a}$ | 58 ± 1 $^{ab}$ | 63 ± 2 $^{ab}$ | 61 ± 3 $^{ab}$ |
| **3-Methyl-1-butanol** | 212 ± 9 $^{bcd}$ | 184 ± 5 $^{d}$ | 213 ± 6 $^{bcd}$ | 187 ± 1 $^{d}$ | 260 ± 14 $^{a}$ | 241 ± 7 $^{ab}$ | 231 ± 5 $^{abc}$ | 205 ± 3 $^{cd}$ |
| **Hexanol** | 0.108 ± 0.012 $^{bc}$ | 0.096 ± 0.001 $^{c}$ | 0.109 ± 0.001 $^{bc}$ | 0.113 ± 0.003 $^{b}$ | 0.111 ± 0.007 $^{bc}$ | 0.115 ± 0.002 $^{ab}$ | 0.129 ± 0.002 $^{a}$ | 0.117 ± 0.001 $^{ab}$ |
| **2-Phenylethanol** | 7.355 ± 0.140 $^{ab}$ | 7.995 ± 0.015 $^{ab}$ | 8.920 ± 0.5 $^{a}$ | 6.995 ± 0.045 $^{ab}$ | 9.240 ± 0.250 $^{a}$ | 9.455 ± 0.355 $^{a}$ | 8.440 ± 0.430 | 6.125 ± 0.095 $^{b}$ |
| **Ethyl acetate** | 35 ± 0 $^{bc}$ | 39 ± 2 $^{b}$ | 54 ± 1 $^{a}$ | 31 ± 0 $^{cd}$ | 51 ± 3 $^{a}$ | 38 ± 2 $^{b}$ | 39 ± 1 $^{b}$ | 28 ± 2 $^{d}$ |
| **Isoamyl acetate** | 0.027 ± 0.005 $^{b}$ | 0.020 ± 0.001 $^{b}$ | 0.042 ± 0.003 $^{a}$ | 0.028 ± 0.001 $^{ab}$ | 0.034 ± 0.002 $^{ab}$ | 0.034 ± 0.003 $^{ab}$ | 0.030 ± 0.001 $^{ab}$ | 0.040 ± 0.001 $^{a}$ |
| **Phenylethyl acetate** | 0.003 ± 0.001 $^{b}$ | 0.003 ± 0.001 $^{b}$ | 0.016 ± 0.005 $^{a}$ | 0.004 ± 0 $^{b}$ | 0.005 ± 0.001 $^{b}$ | 0.006 ± 0.001 $^{b}$ | 0.004 ± 0 $^{b}$ | 0.003 ± 0 $^{b}$ |
| **Ethyl lactate** | 3.4 ± 0.6 $^{b}$ | 3.7 ± 0 $^{b}$ | 5.5 ± 2.1 $^{b}$ | 5.6 ± 0.4 $^{b}$ | 5.5 ± 0.2 $^{b}$ | 4.4 ± 0.2 $^{b}$ | 16.5 ± 1 $^{a}$ | 4.7 ± 0.6 $^{b}$ |
| **Ethyl butyrate** | 0.181 ± 0.01 $^{abc}$ | 0.192 ± 0.006 $^{ab}$ | 0.149 ± 0.009 $^{c}$ | 0.205 ± 0.009 $^{a}$ | 0.166 ± 0.01 $^{bc}$ | 0.171 ± 0.021 $^{bc}$ | 0.194 ± 0.001 $^{ab}$ | 0.172 ± 0.001 $^{abc}$ |
| **Ethyl hexanoate** | 0.013 ± 0.002 $^{bc}$ | 0.008 ± 0.001 $^{d}$ | 0.007 ± 0.001 $^{d}$ | 0.010 ± 0.001 $^{cd}$ | 0.014 ± 0.001 $^{b}$ | 0.014 ± 0.001 $^{b}$ | 0.014 ± 0.001 $^{b}$ | 0.023 ± 0.002 $^{a}$ |
| **Ethyl octanoate** | 0.013 ± 0.003 $^{b}$ | 0.008 ± 0.001 $^{cd}$ | 0.003 ± 0 $^{e}$ | 0.011 ± 0.001 $^{bc}$ | 0.013 ± 0 $^{b}$ | 0.010 ± 0.001 $^{bcd}$ | 0.007 ± 0.001 $^{de}$ | 0.024 ± 0.001 $^{a}$ |

Data with different superscript letters (a, b, c, d) within each line are significantly different (Duncan test; $p \leq 0.05$).

Among other acetates analyzed, 2-phenylethyl acetate (with a fruity and flowery flavor) was significantly higher only in wines fermented by *H. osmophila*/*S. cerevisiae* (0.016 mg/L) in comparison with the control wine (0.003 mg/L). This result is in agreement with the known capacity of this yeast species to release high levels of 2-phenylethyl acetate [70–72,76].

Other ethyl esters compounds, such as ethyl lactate, ethyl butyrate, ethyl hexanoate and ethyl octanoate, were present in all the wines with similar or slightly lower concentrations in comparison to those present in the control wine. An exception, regarding ethyl lactate, was made for wines produced by the association of *L. thermotolerans*/*S. cerevisiae* that reached a concentration about 3-fold higher than that measured in the control wine (4.7 mg/L). This result is in accordance with that observed in previous studies [22,58,77], and is compatible with lactic acid production by *L. thermotholerans* [78].

PCA analysis showed evident differences among the strains tested as a function of volatile compounds production and this reflects the ability of each strain to give a specific aromatic imprint to the final wines (Figure 4). Based on volatile compounds content in the resulting wines, *H. osmophila* and *M. pulcherrima* were positioned in the upper left quadrant and characterized by acetate esters and 2-phenyl ethanol. *S. bacillaris* and *P. fermentans* were placed in bottom left quadrant characterized by the production of isobutanol. *T. delbrueckii* and *L. thermotolerans* were placed in the upper right quadrant due to the production of isoamyl alcohol and isoamyl acetate, while *Z. florentina* and *S. cerevisiae* control strain were positioned in the right bottom quadrant and were characterized by the production of ethyl esters.

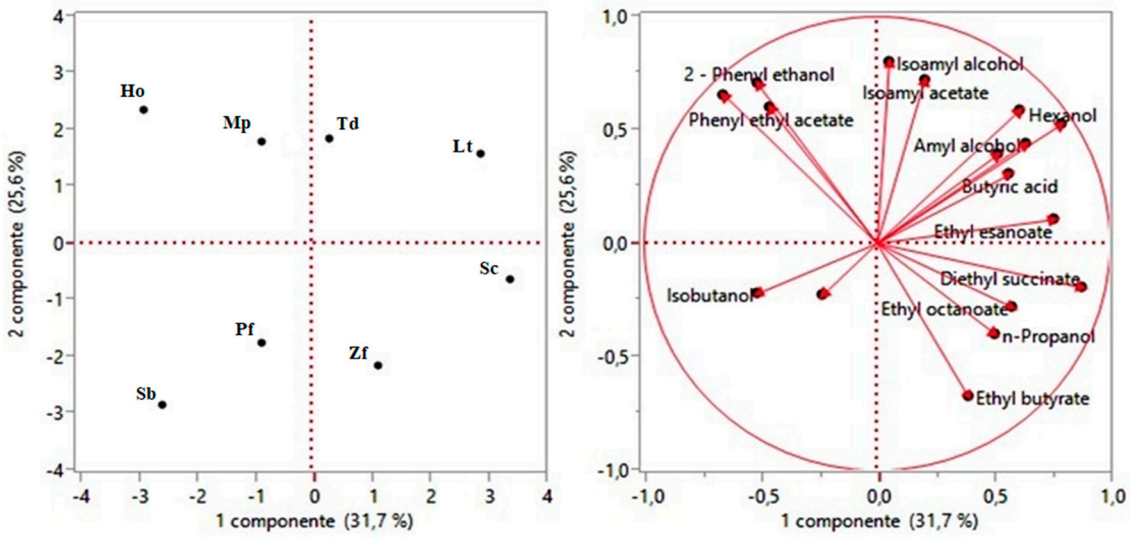


**Figure 4.** Principal component analysis (PCA) based on the production of volatile compounds.

According to the results of sensory analysis carried out four months after bottling, all the wines obtained with mixed fermentation starters were perceived as significantly more provided in color intensity, in respect to the control wine. This was particularly true for wines obtained with associations including *S. bacillaris* and *M. pulcherrima* (Figure 5). This result is in accordance with the higher amounts of total polyphenols and anthocyanins found in the relevant wines, and in respect to the control. Moreover, it agrees with the findings of other authors pointing out that many non-*Saccharomyces* yeasts in sequential fermentation with *S. cerevisiae* may enhance color intensity of wines, promoting the formation of derivatives with more stable color than anthocyanins [79–82]. This is particularly important for Sangiovese wine that, being rich in unstable and oxidizable phenols, is characterized by limited color stability [83] and suggests that the utilization of mixed starters, including non-*Saccharomyces* yeasts, might represent an option for the management of Sangiovese color stability.

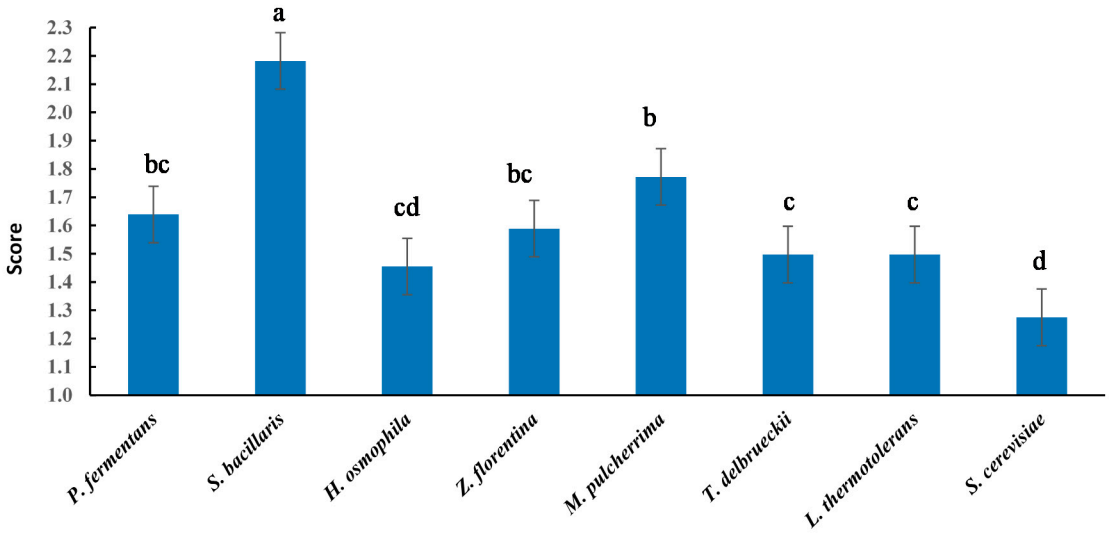


**Figure 5.** Sensory perception of color in wines 4 months after bottling (QDA score: scale 1–4). Values displaying different letters (a, b, c, d) are significantly different according to the Duncan test ($p \leq 0.001$).

Despite the differences found in the relevant volatile compounds profile, no significant differences among the wines were found in the aromatic profile (descriptors: floral, fruity, canned fruits, spicy, candy, chemical, earthy).

Concerning the taste descriptors used in the organoleptic assessment of wines, significant differences resulted only regarding astringency ($p \leq 0.01$) and mouth dryness ($p \leq 0.001$) perception (Figure 6). In particular, while the association including *P. fermentans* resulted in a more astringent wine, in respect to the control, that including *T. delbrueckii* emerged as less astringent with respect to the control wine and all the other wines.

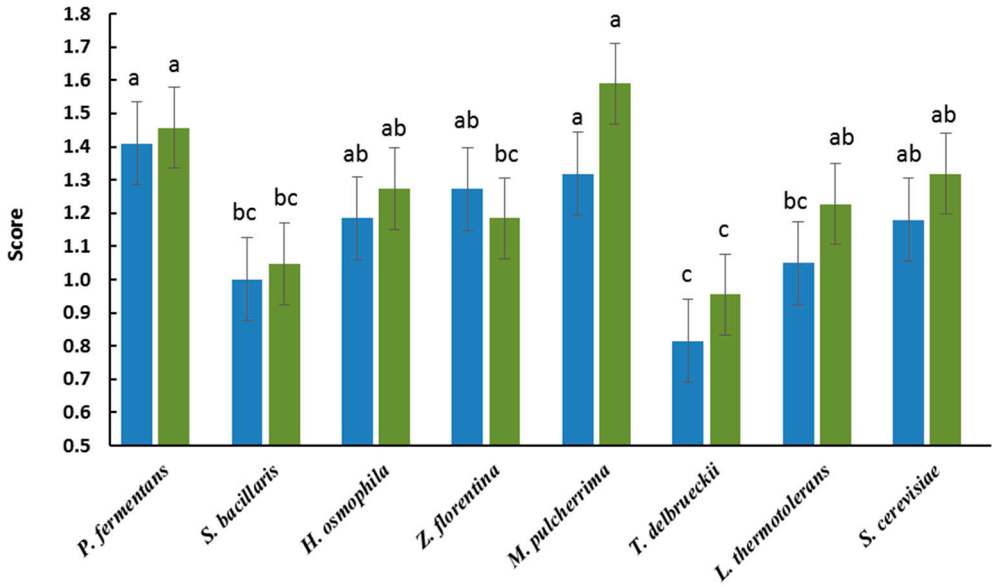


**Figure 6.** Sensory perception of astringency (■) and dryness (■) in wines 4 months after bottling (QDA: scale 1–4) Values displaying different letters (a, b, c, d) are significantly different according to the Duncan test (for astringency at $p \leq 0.01$ and for dryness at $p \leq 0.001$).

The perception of mouth dryness was higher in wine deriving from the mixed starter including *M. pulcherrima*, while the association *T. delbrueckii*/*S. cerevisiae* proved the most effective in reducing this sensation in the mouth.

## 4. Conclusions

The utilization of wine starters containing non-*Saccharomyces* yeasts in association with *S. cerevisiae* represents a valid tool for the achievement of different oenological objectives. Non-*Saccharomyces* yeasts modify the chemical-analytical profile of wines and through their impact on taste descriptors they may be utilized to modulate wine sensory properties. Accordingly, wines inoculated with non-*Saccharomyces*/*Saccharomyces* mixed starters presented a significant differentiation in the chemical-analytical composition, astringency, dryness perception and intensity of color. In particular, while the association *P. fermentans*/*S. cerevisiae* resulted in a more astringent wine, *T. delbrueckii*/*S. cerevisiae* emerged as the less astringent. Moreover, all associations exerted a positive effect on color intensity and wine produced by *S. bacillaris*/*S.cerevisiae* obtained the highest score. These last results also suggest the utilization of non-*Saccharomyces*/*S.cerevisiae* mixed starters for the management of Sangiovese color stability.

**Author Contributions:** Conceptualization, P.D., M.C., I.M., L.L.; methodology, P.D., M.C., I.M., L.L.; investigation, P.D., M.C., I.M., L.L., C.R., A.B.B.; formal analysis, L.L., C.R., A.B.B.; writing—original draft preparation, P.D., I.M.; writing—review and editing, all authors. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was part of a research project funded by the Italian Ministry of Economic Development and Consorzio Tuscania, Florence, Italy (http://www.ricercatuscania.it).

**Conflicts of Interest:** The authors declare no conflict of interest.

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