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Wine Fermentation Performance of Indigenous *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* Strains Isolated in a Piedmont Vineyard

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Abstract: The role of yeast in wine quality is very important. The use of selected autochthonous yeasts is becoming more and more frequent in enology, not only to obtain a diversification of wines, but also as a link between the wine and its territory of origin. The objectives of this work were to test two indigenous yeasts in a cellar on a pilot scale. The yeasts were a strain of *Saccharomyces cerevisiae* and a strain of *Saccharomyces paradoxus* previously isolated in a vineyard in Piedmont (Italy). Studying the oenological characteristics of *S. paradoxus* is of particular interest, as it is rarely found in the cellar–vineyard environment. Molecular biology methods confirmed the predominance of the strain inoculated in the various fermentation tests. Additionally, products of yeast metabolism, including volatile compounds, were quantified at the end of the alcoholic fermentation and sensory profile of wines was tested by a trained panel of tasters. Our results indicated that both strains have good characteristics to be used as starter in winemaking; *S. paradoxus* was characterized by a high production of glycerol and the ability to degrade malic acid, together with a lower production of ethanol and a low volatile acidity, while *S. cerevisiae* conferred to the wine a pleasant smell of rose, as highlighted in the sessions of sensory analysis.

Keywords: wine; indigenous yeasts; alcoholic fermentation; biodiversity; sensory analysis; aromatic compounds

Citation: Costantini, A.; Cravero, M.C.; Panero, L.; Bonello, F.; Vaudano, E.; Pulcini, L.; Garcia-Moruno, E. Wine Fermentation Performance of Indigenous *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* Strains Isolated in a Piedmont Vineyard. *Beverages* **2021**, *7*, 30. https://doi.org/10.3390/beverages7020030

Academic Editors: Antonio Morata, António Manuel Jordão, Fernanda Cosme and Ileana Vigentini

Received: 30 April 2021
Accepted: 26 May 2021
Published: 28 May 2021

1. Introduction

Wine quality depends mainly on the characteristics of the grapes and the diversity of microorganisms (yeasts and bacteria) present during winemaking. During alcoholic fermentation (AF) yeasts of the genus *Saccharomyces* have a predominant role in completing the fermentation of the grape sugars to ethanol. At the end of the fermentation only the species best adapted to the high ethanol content, in many instances *S. cerevisiae* and *S. bayanus*, are found [1].

However, it is known that the grape-wine ecological habitat has a much more complex microbial biodiversity; in fact, wine fermentation is not a 'single species' fermentation, but it is the result of a composite community where the indigenous yeasts also play an important role in the final wine complexity [2,3].

In this contest, these yeasts, *Saccharomyces* and non-*Saccharomyces*, are interesting in oenology, because together with the soil or the microclimate they represent the territory of origin of the wine [4]. Actually, their use is growing in response to the ever-increasing need for wine personalization, which is becoming an important aspect of winemaking [5,6]. Nowadays, wineries are dealing with new challenges due to market demands and climate

change and the selection and the use as starter of non-conventional yeasts can be beneficial since they represent an important resource of biodiversity.

S. cerevisiae is one of the most extensively studied yeast species because it is the main actor of industrial fermentations, such as wine, beer, and bread production [7]. *S. paradoxus* is phylogenetically closed related to *S. cerevisiae*; it is frequently found in association with oak trees [8–10], but rarely with vineyards. *S. paradoxus*, being the subject of studies on ecology and evolution, was the first *Saccharomyces* yeast to be acknowledged as a non-domesticated species [11,12]. Majdak et al. [13] and Orlić et al. [14] reported the possibility of using *S. paradoxus* strains as starter in fermentation because of their contribution to the aroma of the wines. Alonso del Real et al. [15] tested one *S. paradoxus* strain in a mixed co-culture with one S. *cerevisiae* but in their results they found that during the fermentation *S. cerevisiae* dominated over *S. paradoxus*. Due to these divergent data, further studies must be conducted to increase knowledge about the behavior of *S. paradoxus* in wine.

A very important aspect of quality is determined by the aroma components of the wine: Varietal aromas (originating from the grapes) and fermentative aromas (originating during alcoholic and malolactic fermentations). The contribution of yeast fermentation metabolites to the aromatic profile of wine is well documented [16,17], but yeast can also contribute to wine aroma by other mechanisms, the de novo biosynthesis of volatile compounds and the transformation of neutral grape compounds into flavor-active components [18,19].

Recently, we isolated and characterized the *Saccharomyces* and non-*Saccharomyces* yeasts present in the grapes of a new implantation vineyard of Grignolino in Piedmont (Italy). Grignolino is a red variety cultivated in Piedmont in the northwest of Italy to produce three DOC (Denomination of Controlled Origin) wines. Some of the characterized strains showed good technological basic features such as fermentative vigor and low volatile acidity, as tested in laboratory fermentations [20].

In this work, the specific enological characteristics of two indigenous strains (*S. paradoxus* and *S. cerevisiae*) isolated in this vineyard were determined by comparing their wine fermentation performances in a pilot scale in real cellar conditions. Their dominance was ascertained at the end of alcoholic fermentation (AF) and their impact on the final wines was investigated by quantifying volatile compounds and by performing sensory analysis test.

2. Materials and Methods

2.1. Yeast Strains

Saccharomyces cerevisiae and *Saccharomyces paradoxus* strains were previously isolated in a Grignolino Vineyard in Nord Italy [20]. They are included in the Microbial Culture Collection of Oenological and Viticultural Environment (CREA-CMVE) of the Center with the references: *S. cerevisiae* (ISE1567) and *S. paradoxus* (ISE1618). A yeast pre-inoculum, previously grown in YEPG broth, was prepared in commercial grape juice (Bravo, Rauch, Austria) and then inoculated (5×10^6 cells/mL) in Grignolino must. Each strain was tested in triplicate.

2.2. Vinification

The trials were run during the 2018 vintage. Homogeneous samples of Grignolino grapes were harvested in crates. Grapes were divided into six homogeneous lots of 130 kg each, containing equally distributed grapes of each row of the vineyard. After destemming and crushing, 80 mg/kg of potassium metabisulfite was added to all six trials.

AF was carried out at a temperature of 24–25 °C. In the first four days of fermentation, a pump down was carried out in the morning and a pumping over of about 20% of the volume in the evening. From the fifth day onwards, two pump-overs in the air for half the volume were done twice a day. Racking off was carried out after 20 days of maceration. At the end of AF, approximately 70 L of wine was obtained in each fermentation. Wine fermented with *S. paradoxus* is termed SpW, and the one with *S. cerevisiae* ScW.

2.3. Yeast Dominance Analysis

At the end of fermentation, yeasts were isolated by dilution and spreading on WL (Wallerstein Laboratory) agar. After growth, 24 colonies from each sample were randomly collected from plates. Dominance analysis was performed by microsatellite multiplex PCR (MM-PCR) to distinguish *S. cerevisiae* strains [21].

MM-PCR data were managed using Bionumerics software (Applied Maths, Belgium). The band pattern profile obtained on the colonies isolated at the end of fermentation was compared with the profile of the inoculated strain.

Since microsatellite loci amplification is not possible in *S. paradoxus*, the species was assessed by amplifying the D1–D2 domain with primers NL1–NL4 [22] and sequencing. For this, the NS1/ITS2 primer pair was used to amplify the ITS1 region of the 18S rDNA; PCR products were digested with MspI, and separated by electrophoresis [8]. Gels were processed using Bionumerics software as above.

2.4. Chemical Analysis

Density, volatile acidity, titratable acidity, and ethanol content were analysed according to the methods of the OIV (International Organisation of Vine and Wine). Residual sugar and glycerol were quantified using an HPLC with a refractometric detector using the following conditions: Rezex RCM-Monosaccharide column (300 × 7.8 mm, 8 µm, Phenomenex, Torrance, CA, USA), water as eluent with a flow of 0.35 mL/min, column temperature 85 °C, and injection volume 20 µL. Organic acids were quantified by HPLC Agilent 1100 as described [23], and YAN (Yeast Assimilable Nitrogen) was determined by formol titration [24].

2.5. Sensory Analysis

The wine sensory descriptive analysis (sensory profile) was conducted by a trained panel (6 males and 7 females) following a methodology deriving from the ISO norms [25]—similarly to other procedures [26,27]—using ISO (3591-1977) approved glasses in an ISO (8589-2007) tasting room.

In all the sensory sessions, 4 wines were served (50 mL) in a randomized order and identified with a three-digit code. All the wines were tasted in a preliminary tasting session to define the odor descriptors with the help of a predefined odor list [28]. The choice of descriptors was made on the identification frequencies. The second-level descriptors (fresh herbaceous, dry herbaceous, and balsamic/resinous) were chosen when their frequency of identification was higher than 39 (13 assessors × 6 wines/2), and the third level descriptors (e.g., rose, geranium flower, pepper, cloves, raspberry, cherry and jam/marmalade) when their frequency was higher than 19.5 (13 assessors × 6 wines/4). The taste and mouth-feel attributes evaluated were acidity, bitterness, astringency, body (structure) and taste-olfactory persistence. The chosen attributes were confirmed by presenting to the panel appropriate standards, and measured twice in the wines in two different tasting sessions. Qualitative and quantitative sensory analyses were performed by FIZZ (Biosystems, Couternon, France). The intensity of the wine sensory attributes measures was acquired in two repetitions using a non-structured scale (0–100).

The sensory profile of each wine obtained from the average of the two-tasting session of each of the 3 wines produced with the same yeast is represented with radar diagrams. The quantitative sensory results (sensory profiles) were processed with ANOVA and Tukey's test (p = 95%).

2.6. Free Volatile Compounds Analysis

The volatile compounds were extracted by solid-phase extraction (SPE) as follows: 30 mL wine was diluted threefold with water and 300 µL and added with 1-heptanol (51.43 mg/L) as internal standard; samples were loaded onto a 1 g C18-EC cartridge (Biotage AB, Uppsala, Sweden), extracted with 5 mL dichloromethane and concentrated to 100 µL under a weak nitrogen flow. After the addition of 1-pentanol and stirring, the

aqueous phase was extracted with dichloromethane and concentrated to 100 µL as before. The GC-MS analysis was performed with an Agilent 7890 Series gas chromatograph with an Agilent 5975 N Mass Selective Detector. The chromatographic conditions were: Helium carrier gas with a flow of 1 mL/min; the sample (1 µL) was injected in splitless mode on a Zebron ZB-WAX column (60 m × 0.25 mm, 0.25 µm, Phenomenex, Torrance, CA, USA); the source and the transfer line were kept at 230 °C and the injector at 250 °C [29]. Data were acquired in TIC mode (Total Ion Current) and processed with the ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The identifications were achieved by comparing the retention times with those of pure reference compounds (when available), or comparing the LRI (linear retention index) to those reported in the literature. All high purity standards were purchased from Sigma–Aldrich (Milan, Italy).

2.7. Statistical Analysis

ANOVA, Tukey's test (p = 95%) and PCA were performed using XLSTAT (Addinsoft, France).

3. Results and Discussion

3.1. Enological Characteristics of S. paradoxus and S. cerevisiae Strains

To overcome any inhomogeneity during ripening of the grapes that could result in differences in acidity, alcohol, color or polyphenol content of wines, the initial grape mass was collected and randomized distributed in boxes. The musts had the following mean chemical parameters: Brix 23.44°; titrable acidity (expressed as tartaric acid) 6.4 g/L; pH 3.49; YAN 287.7 mg/L.

Microbiological and biomolecular analyses were performed to evaluate the dominance of the yeasts at the end of fermentation. In *S. cerevisiae* wines, microsatellite analysis showed that the inoculated *S. cerevisiae* strain dominated the fermentations (Figure 1).

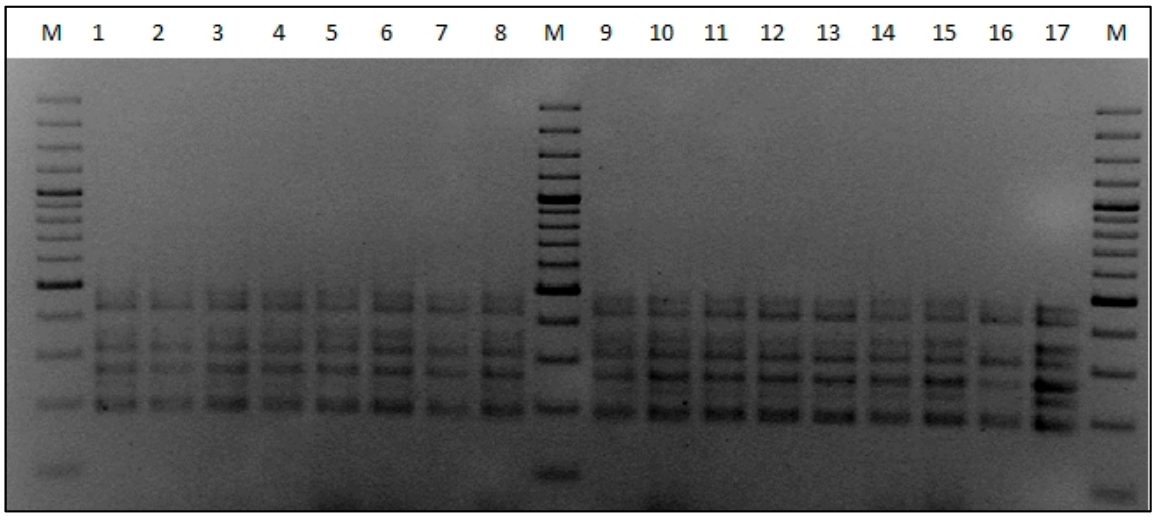

Figure 1. Electrophoretic profile obtained by MM-PCR of 16 colonies of *S. cerevisiae* strains showing the same profile of the inoculated strain (Lane 17). Lanes M: Molecular marker 100 bp.

As expected, no amplification was achieved in *S. paradoxus* wine isolated colonies by MM-PCR because this method is *S. cerevisiae* species-specific; thus, ARDRA analysis was conducted, confirming that they belonged to the *S. paradoxus* species (Figure 2).

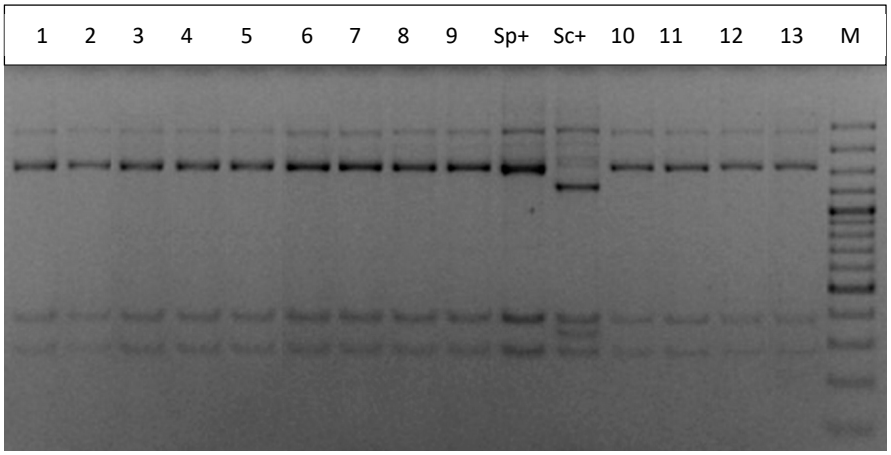

Figure 2. Electrophoretic profile obtained by ARDRA analysis enzymatic digestion with MspI of the colonies isolated in wine inoculated with *S. paradoxus*. M: Molecular marker 100 bp; lanes 1–13: colonies isolated; Lane Sp+: *S. paradoxus* positive control; Lane Sc+: *S. cerevisiae* positive control.

The progress of alcoholic fermentation (13 days) in the different trials was similar. At the end of the AF, significant differences ($p \leq 0.05$) in glycerol (6.70 vs 8.62 g/L) and malic acid (3.27 vs 2.50 g/L) content between *S. cerevisiae* and *S. paradoxus* were recorded (Table 1).

Table 1. Chemical parameters of the wines at the end of the alcoholic fermentation.

	SpW	ScW
Density 20/20	0.99456	0.99368
Ethanol % *v*/*v*	12.73	13.29
Residual sugars g/L	≤1	≤1
Titratable acidity g/L	6.32	6.35
Volatile acidity g/L	0.19	0.25
Malic acid g/L	2.50 [a]	3.27 [b]
pH	3.54	3.52
Glycerol g/L	8.62 [a]	6.70 [b]

Results are the average of three independent fermentations; titratable acidity was expressed as tartaric acid. Volatile acidity was expressed as acetic acid. SpW: *S. paradoxus* wines; ScW: *S. cerevisiae* wines. Different letters "a" and "b" mean statistical differences at ANOVA and Tukey's test (p = 95%).

Remarkably, the *S. paradoxus* strain production of glycerol, a very important compound for wine quality that provides sweetness and fullness [30], was high, thereby confirming previous data indicating that *S. paradoxus* produces a higher amount of glycerol than *S. cerevisiae* [31]. The observed ability of *S. paradoxus* to degrade malic acid is an interesting property in fermentation of musts with a high acidity; several studies demonstrated that fermentations with this species lead to a degradation of malic acid [10,32,33]; therefore, our results are in agreement with these previous works. In particular, Bovo et al. [32] affirmed that *S. paradoxus* strains were able to degrade high amounts of malic acid in ripe grape must, i.e., high glucose and low malic acid concentration.

No significant differences ($p \leq 0.05$) were found for the other parameters analyzed, even though the quantity of ethanol produced was slightly lower in the fermentations of *S. paradoxus* ($\bar{x}$ = 12.73 g/L) than in those of *S. cerevisiae* ($\bar{x}$ = 13.29 g/L). This result agrees with Orlic et al. [31] who reported that *S. paradoxus* always produced lower ethanol concentrations than *S. cerevisiae*.

It is important to note that this lower ethanol production was not accompanied by a higher volatile acidity. At the same time, as mentioned before, glycerol content shows significantly higher values in *S. paradoxus* than in *S. cerevisiae* fermentations. These data lead to hypothesize that in the *S. paradoxus* strain a carbon flow balance shifted towards glycerol

to a greater extent than *S. cerevisiae*, thus showing interesting technological prospects for natural reduction of alcohol content in wines [32,33].

3.2. Free Volatile Compounds and Sensory Profile of the Wines

Grignolino variety is cultivated in Piedmont, northwest Italy, to produce DOC wines that are generally described as dry and slightly tannic, with a moderately bitter taste and a persistent aftertaste. In a previous study [34] carried out on a suitable number (n = 36) of commercial Grignolino wines, the olfactory descriptors were violet-rose, geranium, pepper, raspberry, straw-hay. These attributes were also present in this study, even though the odor complexity was higher, which could be due to various reasons (grape quality, evolution of grape growing and winemaking techniques, different yeasts).

Regarding the organoleptic characteristics of the under-study wines, the sensory profiles obtained with the two yeasts (Figure 3) were different only for the odor attribute rose, which was significantly higher in the ScW wines (ANOVA and Tukey's test, p = 95%). Raspberry odor, cherry and dry herbaceous were also slightly higher in these wines, but all wines were very similar for taste and mouth-feel attributes.

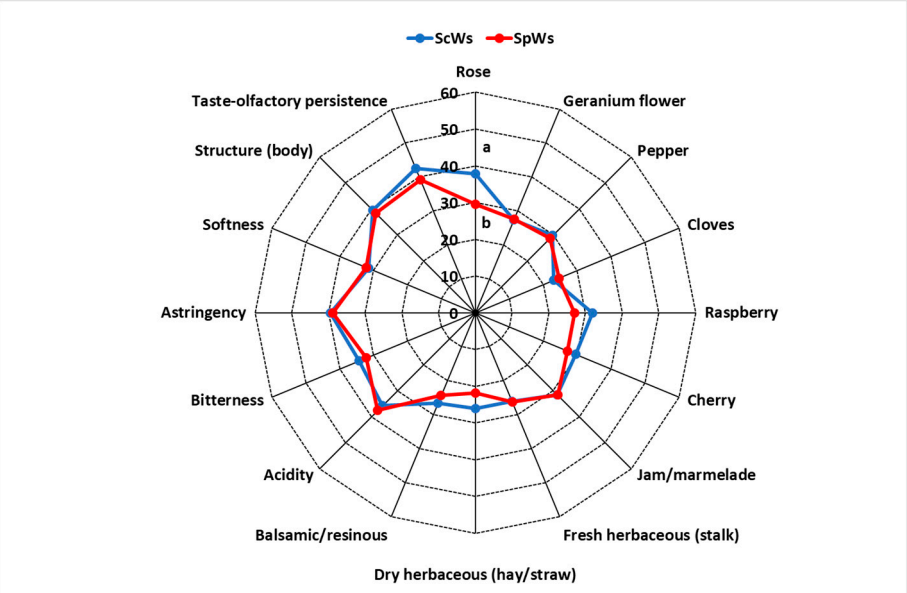

Figure 3. Sensory profile of Grignolino wines obtained by the two autochthonous strains. Different letters indicate significant statistical differences with ANOVA and Tukey's test (p = 95%). (ScWs: Average profile of wines produced with *S. cerevisiae*; SpWs: Average profile of wines produced with *S. paradoxus*).

Analysis of the aromatic compounds of wines at the end of the alcoholic fermentation (Table 2) showed no statistical differences in alcohols and fermentation esters between the two yeasts, but statistical differences in the concentration of terpene compounds were recorded, which can explain the sensory difference for the descriptor "rose". Terpenes are characterized by floral, muscatel or fruity aromas, and their concentrations in grapes and wines depend on various factors, including cultivar, region, wine-making techniques and yeasts.

Table 2. Volatile compounds in the wines at the end of the alcoholic fermentation (μg/L).

	SpW	ScW
Alcohols		
Isoamylalcohol	12,261 ± 1447	13,140 ± 2888
Cis-3-hexenol	73 ± 6	57 ± 21
1- hexanol	1359 ± 66	1402 ± 298
benzylalcohol	7 ± 2	7 ± 1
2 phenylethanol	14,337 ± 1000	17,465 ± 5000
total alcohol	*28,037*	*32,071*
Esters		
ethylhexanoate	279 ± 60	185 ± 31
isoamilacetatate	406 ± 151	418 ± 27
ethyllactate	1284 ± 118	585 ± 42
ethyloctanoate	267 ± 62	177 ± 13
ethylhexanoate	271 ± 46	185 ± 31
ethyldecanoate	55 ± 20	37 ± 4
diethylsuccinate	1187 ± 360	1351 ± 293
ethylpalmitate	12 ± 1	16 ± 4
total esters	*3761*	*2954*
Acids		
isovaleric acid	150 ± 11	99 ± 24
octanoic acid	1869 ± 242	1559 ± 342
decanoic acid	444 ± 172	377 ± 57
lauric acid	37 ± 13	29 ± 4
total acids	*2500*	*2064*
Aldehydes ketones		
benzaldehyde	6 ± 2	8 ± 2
butyrolactone	36 ± 20	40 ± 27
methoxyacetophenone	0	248 ± 95
vanillin	0	14 ± 1
β−damascenone	11 ± 5	8 ± 1
total Aldehydes Ketones	*53* [a]	*318* [b]
Terpenic compounds		
linalool	0 [a]	16 ± 2 [b]
cis-linalooloxide	4 ± 1	5 ± 1
citronellol	14 ± 0	24 ± 2
HO trienol	0	7 ± 5
alpha terpineol	10 ± 1	11 ± 2
geranic acid	40 ± 13	43 ± 4
Total terpenes	*68* [a]	*106* [b]

Different letters "a" and "b" indicate significant statistical differences with ANOVA and Tukey's test (p = 95%).

In this study, particularly significant was the difference between the linalool content in both strains, as linalool (16 μg/L) was only present in the *S. cerevisiae* fermentation (Table 2). The ability of *S. cerevisiae* yeast to synthesize terpenes has already been reported [35,36]. The threshold level for linalool in wine is 50 μg/L, but it can be detected in lower concentrations (10–20 μg/L) when similar aroma-based chemicals are also present [37]. The individual terpenes quantified in this study are not present at levels close to their sensory limits, but they should nevertheless contribute collectively to the floral aspect of the wine aroma descriptor. Rose aroma contribution should collectively include linalool, citronellol and HO-trienol, which are aromatic compounds characterized by floral notes, and the sum of these terpenes supports the difference perceived by the panel in the sensory analysis.

Significant differences were also found in aldehyde and ketone compounds, the total amount of these compounds was higher in ScW than in SpW, in particular for vanillin and for methoxyacetophenone. These compounds increase wine complexity, conferring vanilla, nutty and floral notes.

In general, wines obtained in this study have good enological properties and distinctive characteristics; this is also shown by PCA analysis (Figure 4): Wines obtained by *S. cerevisiae* have higher malic acid, total aldehydes and ketones and ethanol; *S. paradoxus* wines are mainly characterized by a higher content of glycerol.

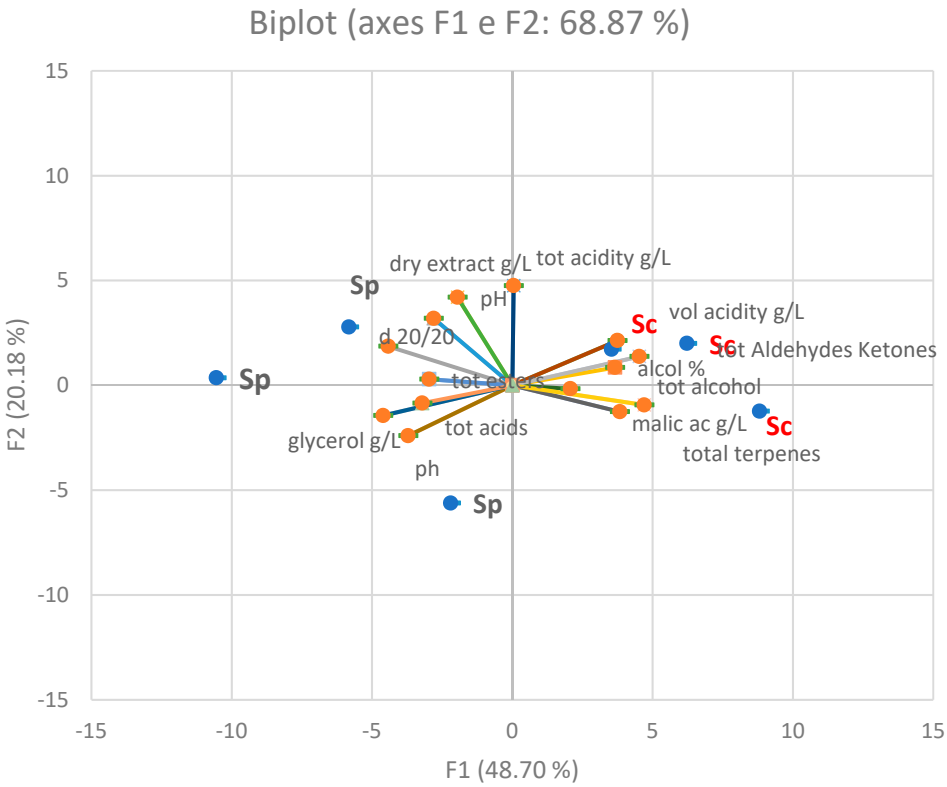

Figure 4. Bipot obtained by PCA analysis on data of the three repetitions.

4. Conclusions

In conclusion, both indigenous *S. cerevisiae* and *S. paradoxus* strains isolated from vineyard possess oenological properties of interest for the wine industry. *S. paradoxus* is characterized by a high production of glycerol and the ability to degrade malic acid. This, together with a lower production of ethanol and a low volatile acidity, makes this *S. paradoxus* strain a very interesting starter from an oenological point of view, in particular for the production of low alcohol content wines. On the other hand, the strain of *S. cerevisiae* gives the wine a pleasant smell of rose, as highlighted in the sessions of sensory analysis. Grignolino is a neutral variety with a very low content of terpene compounds, and thus, in these kinds of varieties, the role of yeast in the production of aromatic compounds is even more important.

Further studies should be conducted to better investigate the use of *S. paradoxus*, in particular its use in mixed cultures with *S. cerevisiae* or other yeast species, or in sequential inoculation, in order to obtain specific results in terms of ethanol content and glycerol production.

Author Contributions: Conceptualization, E.G.-M.; methodology A.C., L.P. (Loretta Panero), M.C.C. and F.B.; writing E.G.-M., A.C., L.P. (Loretta Panero), M.C.C. and F.B.; review and editing, E.G.-M, A.C., L.P. (Loretta Panero), M.C.C., F.B., E.V. and L.P. (Laura Pulcini); supervision, E.G.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All of the data is contained within the article.

Acknowledgments: We thank Valter Pierini of the Portacomaro (Asti, Italy) municipality, Riccardo Durando and Carlo Cerrato responsible for the project "Vigna del Papa" for the permission to sample their vineyard.

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

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