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Schizosaccharomyces japonicus: A Polysaccharide-Overproducing Yeast to Be Used in Winemaking

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Abstract: Mixed starter cultures made of *Saccharomyces cerevisiae* EC1118 and *Schizosaccharomyces japonicus* #13 were inoculated in commercial grape must, and the impact of different inoculum ratios (1:1; 1:100; 1:10,000) on growth and fermentation kinetics and on the analytical profiles of the experimental wines was here evaluated. Results obtained showed that *S. japonicus* #13 affects *S. cerevisiae* growth and fermentative capability only for *S. cerevisiae*/*S. japonicus* inoculum ratio 1:10,000. The analytical profiles of the wines produced by mixed starter cultures indicated that this non-*Saccharomyces* yeast modulates the concentration of malic and acetic acids and of some of the most important volatile compounds, such as β-phenyl ethanol, in an inoculum-ratio-dependent fashion. Moreover, all experimental wines obtained with *S. japonicus* #13 in mixed cultures reached concentrations of total polysaccharides significantly higher than those obtained with pure cultures of *S. cerevisiae* EC1118, and total polysaccharides increased with the increase of *S. japonicus* #13 cell concentration. Based on these results, *S. japonicus* #13 might be profitably inoculated in combination with *S. cerevisiae* EC1118 to enhance wine complexity and aroma and to improve wine stability by increasing the final concentration of polysaccharides.

Keywords: mixed starter; polysaccharide; *Schizosaccharomyces japonicus*; wine; *Saccharomyces cerevisiae*

1. Introduction

In the last two decades, growing attention has been paid to the possible contributions of non-*Saccharomyces* yeasts to the quality of wine. This is because non-*Saccharomyces* wine-related yeasts may present peculiar oenological characteristics that can positively influence the analytical and sensorial profile of the final wine [1–3]. Indeed, they can be combined with *Saccharomyces cerevisiae* in mixed starter cultures to achieve a number of oenological objectives, including wine biological acidification [4] or deacidification [5–8], reduction of ethanol and/or increase of glycerol concentrations [4,9], increase of the final content of polysaccharides [10], and enhancement of wine complexity and aroma [2]. Accordingly, non-*Saccharomyces* starter cultures have already been commercialized and utilized by winemakers to produce wines with distinct flavor.

Among the non-*Saccharomyces* yeasts, those belonging to the genus *Schizosaccharomyces* have been proposed in winemaking due to their deacidification ability through demalication [5–7,11–17].

In addition, *S. pombe* produces pyruvic acid, which is possibly related to color stability [18] and reduces ethyl carbamate in wine due to its urease activity [19,20].

Recently, Domizio et al. [21] showed that yeasts belonging to the genus *Schizosaccharomyces* release in the media high amounts of cell wall polysaccharides starting from the onset of alcoholic fermentation. These polysaccharides have been characterized, and the results obtained are in agreement with the composition of the cell wall of yeasts related to the genus *Schizosaccharomyces*, the only one having galacto-mannoproteins located in the outer layer of the cell wall [22]. Moreover, Domizio et al. [21] showed that *S. japonicus* releases significant amounts of polysaccharides as compared to *S. pombe*, thus outlining another possible utilization for these non-*Saccharomyces* wine yeasts in the winemaking industry. Indeed, yeast polysaccharides have many positive effects on wine quality, as they contribute to reducing protein and tartrate instability [23–28], increase the 'fullness' sensation [29], and interact with polyphenols aggregates, thus smoothing the perception of astringency [30–32], and retaining aromatic compounds [33,34].

In a previous study, the metabolic characteristics of *S. japonicus* were studied on a synthetic grape juice [21]. Here, the oenological potential of *S. japonicus* #13 is analyzed in association with the commercial starter strain *S. cerevisiae* EC1118 during the fermentation of a natural grape juice.

2. Materials and Methods

2.1. Yeast Strains

Yeast strains used were: *Schizosaccharomyces japonicus* #13, belonging to the yeast culture collection of the Department of Agricultural, Food and Forestry Systems (GESAAF, University of Florence, Florence, Italy) and the commercial starter strain *S. cerevisiae* Lalvin EC1118 (Lallemand Inc., Montreal, QC, Canada).

2.2. Fermentation Trials

Fermentations were carried out at 25 °C in 500 mL Erlenmeyer flasks containing 450 mL of commercial grape juice (231 g/L reducing sugars; 1.6 g/L malic acid), pasteurized for 15 min at 80 °C. Fermentations trials were inoculated with 72 h precultures grown in the same medium at 25 °C. Inocula cell concentrations were determined as total cell count with a Thoma Zeiss chamber under a light microscope. The flasks were equipped with Müller valves containing sulfuric acid, allowing the CO_2 to escape, and weighed every day until the end of fermentation to monitor the fermentation kinetics. During fermentation, samples were taken for analytical determination and viable cell counts. All fermentations were carried out as two independent experiments, each carried out in duplicate, under static conditions.

2.3. Growth Kinetics

One hundred µL aliquots of serial dilutions of each sample were subjected to viable plate count on Wallerstein Laboratory nutrient agar medium (WL) (Oxoid Unipath Ltd., Hampshire, UK) [35]. WL is a differential medium that leads to a presumptive yeast identification based on both color and morphology of the colonies, and was used for the viable count of *S. cerevisiae* and *S. japonicus*.

2.4. Analytical Determinations of the Fermentation Products

After filtration (0.45 µm nitrocellulose membranes) and appropriate dilution in water, twenty µL of the samples were injected on an HPLC apparatus (Varian Inc., Palo Alto, CA, USA) consisting of a 210 series pump, a 410 series autosampler, a Rezex-ROA Organic Acids column ((300 + 150) cm × 7.8 mm; Phenomenex, Torrance, CA, USA) containing sulfonated styrene divinyl-benzene matrix in H^+ form, a 335-LC Diode Array detector (set at 210 nm), and a 356-LC refractive index detector. Chromatographic separation was carried out at 75 °C, with 10.5 mM H_2SO_4 at 0.6 mL/min. Ethanol, residual sugars and organic acids were quantified in comparison with the relevant external calibration

curve (from 0.5 to 20 g/L), and the areas of the related peaks were recorded and integrated using Galaxie Chromatography Data System version 1.9.302.530 (Varian Inc.).

The polysaccharide concentration was evaluated using a HPLC method, on a Varian system (Varian Inc.) coupled with a refractive index detector, according to Domizio et al. [10]. In particular, a TSKGEL G-Oligo-PW (Supelco 808031) column, and a TSK-GEL Oligo (Supelco 808034) guard column (Bellefonte, PA, USA), were used, with isocratic elution (0.2 M NaCl; 0.8 mL/min; 65 °C). The polysaccharide content was evaluated on the basis of a calibration curve using a commercial preparation of mannan from *S. cerevisiae* (M7504 Sigma-Aldrich, Milan, Italy), at concentrations from 50 to 500 mg/L. The area of the mannan peak was recorded and integrated using the same Galaxie software as above. For each wine, the values are reported as increments with respect to the initial amounts of total polysaccharides in the must. All analyses were carried out in duplicate.

2.5. Volatile Compound Analysis

The quantification of higher alcohols, ethyl acetate and acetaldehyde was performed by gas chromatography with a flame ionization detector at 250 °C on a Carlo Erba HRGC 5160 instrument, under the following chromatographic conditions: a glass column (length, 2 m; internal diameter, 2 mm) packed with Carbopack C + 0.2% Carbowax 1500, 80–100 mesh (Supelco); injection volume 1 µL of pre-distilled sample spiked with 3-methyl 2-butanol as internal standard; injector temperature 220 °C; elution by temperature gradient from 45 to 160 °C (3 °C/min), and then held at 160 °C for 20 min; carrier gas helium at 2 mL/min; detection by flame ionization detector at 250 °C.

The acquisition and integration of the flame ionization detector signal were carried out using the Galaxy software (Varian Inc.).

The minor volatile compounds were evaluated by capillary gas-liquid chromatography on a Carlo Erba HRGC 5300 instrument; using the ether/hexane extracts (1/1, *v*/*v*). The wine samples were spiked with 3-octanol as internal standard. The chromatographic conditions were as follows: glass capillary column 0.25 µm Supelcowax 10 (60 m length, 0.32 mm internal diameter, 0.25 µm film thickness); sample injection 1µL (split-splitless mode, 60 s splitless); injection temperature 220 °C; elution by temperature gradient from 50 °C (held 5 min) to 220 °C (3 °C/min) and maintained at 220 °C for 20 min; detection by flame ionization detector at 250 °C. The acquisition and integration of the flame ionization detector signal were carried out using the Galaxy software (Varian Inc.).

In all the GC analysis, the compounds were identified and quantified by comparisons with external calibration curves.

2.6. Data Analysis

Analysis of variance (ANOVA) was applied to the experimental data for the analytical characteristics of samples. The means were analyzed using the STATISTICA 7 software (Stat-soft, Tulsa, OK, USA). The significant differences were determined using Duncan tests, and the data were considered significant if the associated p values were <0.05.

3. Results and Discussion

3.1. Cell Growth and Fermentation Kinetics

Fermentation trials were inoculated with mixed and pure cultures. For mixed cultures, three different inoculum ratios of *S. cerevisiae* EC1118/*S. japonicus* #13 were utilized (1:1; 1:100; 1:10,000). Pure cultures of *S. cerevisiae* EC1118 were inoculated individually at the same level of inoculum utilized for mixed cultures (10^7, 10^5 and 10^3 cell/mL) as control of growth and fermentation kinetics.

When inoculum ratios of 1:1 and 1:100 were utilized, *S. japonicus* #13 displayed no negative effect on *S. cerevisiae* EC1118, which showed growth kinetics similar to those in pure cultures (Figure 1A,B). In contrast, with inoculum ratio 1:10,000 *S. cerevisiae* EC1118 exhibited a marked reduction in growth rate (Figure 1C). Here, *S. cerevisiae* took 10 days to reach the highest cell concentration, while in pure

culture it needed 3 days to achieve similar results. Accordingly, the fermentation kinetics of pure cultures of *S. cerevisiae* (evaluated in terms of g of CO_2 produced) were comparable to those of the mixed starter cultures with inoculum ratios 1:1 and 1:100, but were slower with inoculum ratio 1:10,000. Thus, a correct calibration of the inoculum ratio is necessary in order to avoid the inhibitory effect of *S. japonicus* #13 on *S. cerevisiae* EC1118. According to Taillandier et al. [36], *Saccharomyces* growth is inhibited by *S. pombe*, and this inhibition is proportional to the *Schizosaccharomyces* concentration. This behavior was proposed as an amensalistic effect similar to that due to the production of a killer toxin, although that study did not confirm this hypothesis.

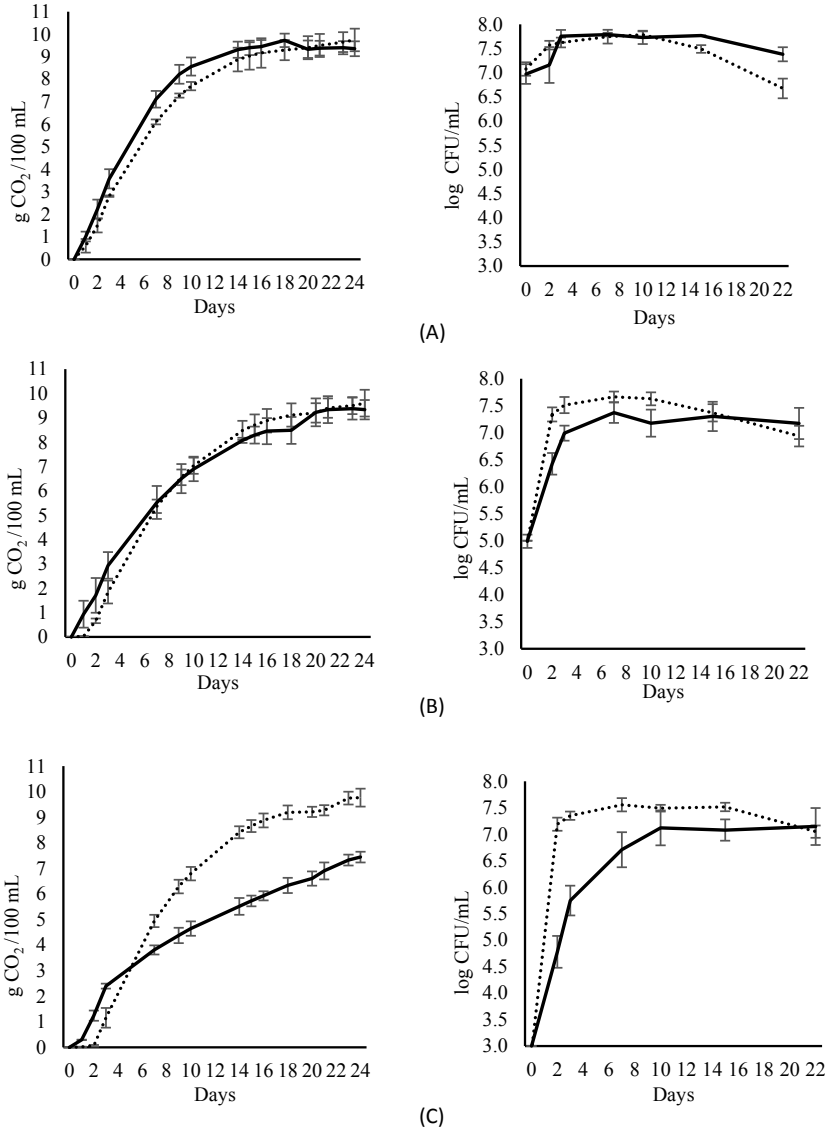

Figure 1. Fermentation kinetics (left panels) and cell growth (right panels) of *S. cerevisiae* EC1118 in pure (dot line) and mixed culture (full line) with *S. japonicus* #13. Results relative to the three *S. cerevisiae*/*S. japonicus* inoculum ratios are reported. ((**A**) ratio 1:1; (**B**) ratio 1:100; (**C**) ratio 1:10,000). Data are means ± standard deviations of two independent experiments, each carried out in duplicate. Where not seen, bars lay within the symbols.

3.2. Metabolic Profile of Fermentations

The main fermentation parameters and compounds were analyzed on the experimental wines obtained with mixed and pure starter cultures (Table 1). Mixed starter cultures generally produced wines with ethanol concentrations that were comparable to those produced by *S. cerevisiae* EC1118 pure cultures, although, when the inoculum ratio 1:10,000 was utilized, sugar fermentation was slackened and a higher amount of residual sugar was present after 24 days. This could be due to the lower fermentation power of *S. japonicus* #13 (Figure 1), but also to the reduced growth of *S. cerevisiae* EC1118, during the first 10 days in mixed culture (Figure 1C). In general, mixed starter cultures produced lower volatile acidity in comparison to *S. cerevisiae* pure cultures, no matter the inoculum ratio (Table 1), but here no statistically significant differences were found. The ability to metabolize malic acid, typical of *S. pombe*, was here also confirmed for the species *S. japonicus. S. japonicus* #13 was able to metabolize from a minimum of 29 to a maximum of 83% malic acid in mixed culture, depending on the inoculum ratio. Indeed, malic acid degradation seemed adversely affected by *S. cerevisiae* when inoculated at the same cell concentration as *S. japonicus* #13. In fact, at the inoculum ratio 1:1, the amount of malic acid consumed was comparable to that of pure cultures of *S. cerevisiae* EC1118. The reduction of malic acid and total acidity and the increase of pH observed in wines fermented by *S. japonicus* #13 in pure and mixed cultures were compatible with the occurrence of malo-alcoholic fermentation.

Mixed starter cultures produced inoculum-ratio-dependent increases in glycerol and reached concentrations that were significantly higher than those produced by pure cultures of *S. cerevisiae*.

Regarding the polysaccharides released in the experimental wines, those fermented by the mixed starter cultures showed concentrations of polysaccharides significantly higher than those fermented by the pure cultures of *S. cerevisiae*. In addition, polysaccharide concentration increased in an inoculum-ratio-dependent fashion. Similar to those observed for glycerol production, these results reflect the contribution of *S. japonicus* to grape must fermentation. The release of polysaccharides during fermentation is not new for *S. japonicus* yeasts [21].

Table 2 reports the concentrations of the major volatile compounds produced in fermentation. Mixed starter cultures showed a general enhancement of higher alcohol production as compared to pure cultures of *S. cerevisiae* EC1118. In particular, significant inoculum-ratio-dependent increases of isobutanol, amylic and isoamylic alcohols were observed, while n-propanol decreased. Acetaldehyde concentration decreased in an inoculum-ratio-dependent fashion, as did ethyl lactate concentration. On the contrary, ethyl acetate concentrations produced by mixed starters were significantly higher than those produced by pure cultures of *S. cerevisiae* EC1118, and were definitely above the detection level of 150 mg/L [37] with the exception of the trial inoculated with the inoculum ratio 1:1 (162.22 mg/L).

In Figure 2, the minor volatile compounds that characterize the aromatic profile of wines are reported. Fermentation trials inoculated with different concentrations of *S. cerevisiae* EC1118 in pure culture did not show significant variations in the volatile compounds analyzed. On the other hand, the production of isoamyl acetate, β-phenyl ethanol, and phenyl ethyl acetate was significantly higher than that obtained with pure cultures of *S. cerevisiae*. It is worth underlining that acetate ester compounds, such as isoamyl acetate, phenyl ethyl acetate and alcohols such as β-phenyl ethanol, provide fruity and floral aromas. In all of the mixed fermentations, they were produced at concentrations that were above their threshold values (0.03 mg/L, 0.25 mg/L and 14 mg/L, respectively) [38,39] and, therefore, they may positively influence the final sensory characteristics of wine.

For ethyl esters (ethyl hexanoate, ethyl octanoate, and ethyl decanoate) their amount in the fermentation trials inoculated with mixed starter cultures was related to the inoculum ratio, showing a progressive decrease with the increase of *S. japonicus* #13 cell concentration. Indeed, at an inoculum ratio of 1:1, ethyl octanoate and ethyl decanoate showed no significant differences from pure cultures of *S. cerevisiae* EC1118, while at inoculum ratio 1:10,000, they were both found at significantly lower concentrations. Similar to the other ethyl esters, the concentration of ethyl hexanoate also decreased in an inoculum-ratio-dependent fashion, but its concentration was consistently higher in fermentations inoculated with mixed cultures. The concentration of ethyl esters such as ethyl hexanoate (fruity:

strawberry, anise), ethyl octanoate (pineapple, pear) and ethyl decanoate (fruity, fatty, pleasant) in mixed fermentation trials were, in general, above their threshold levels (0.005 mg/L, 0.002 mg/L, 0.2 mg/L, respectively) [39].

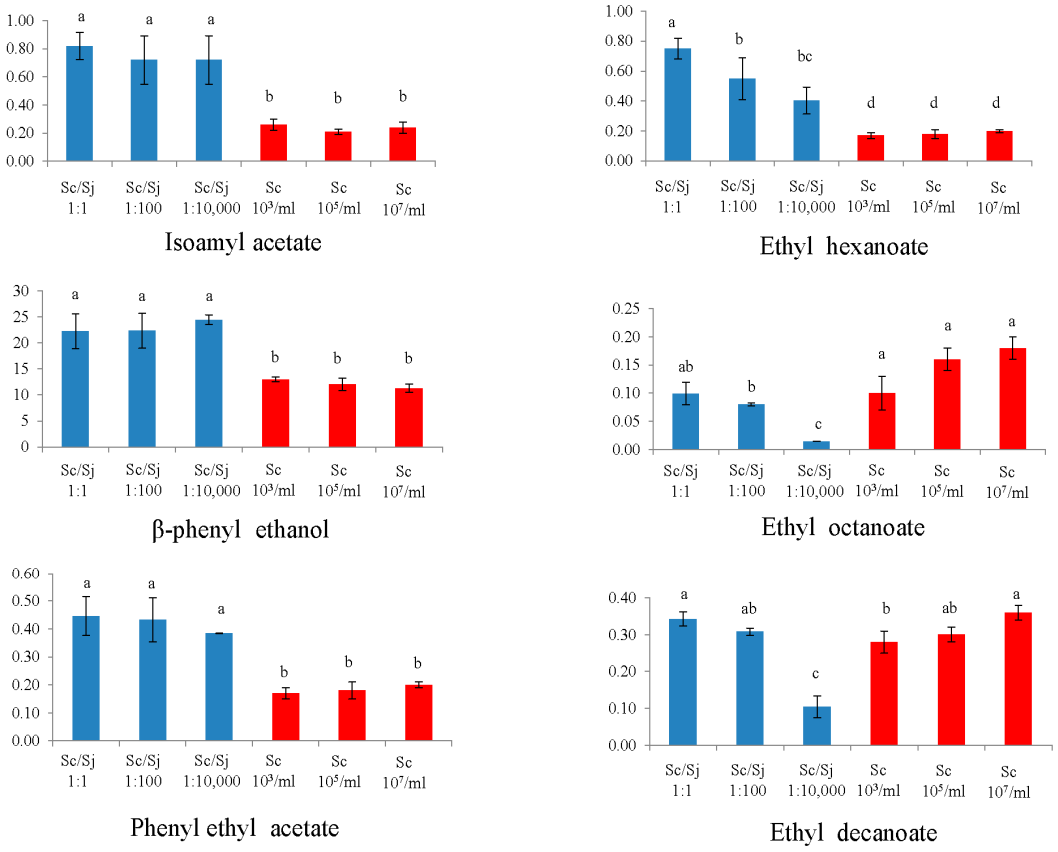

Figure 2. Volatile compounds of wines obtained with *S. cerevisiae* EC1118 in pure cultures (Sc) and mixed cultures with *S. japonicus* #13 (Sc/Sj). Data are means ± standard deviations of two independent experiments, each carried out in duplicate. Values displaying different letters (a, b, c, d) are significantly different according to the Duncan test (0.05%).

Table 1. Analytical profile of wines obtained with *S. cerevisiae* EC1118 in pure cultures and in mixed cultures with *S. japonicus* #13.

Yeasts	Inoculum Cell Concentration	pH	Total Acidity (g/L, Tartaric Acid)	Volatile Acidity (g/L, Acetic Acid)	Ethanol (%, *v*/*v*)	Sugar (%, *w*/*v*)	Malic Acid (g/L)	Glycerol (g/L)	Polysaccharides (mg/L)
S. cerevisiae	(10^7 cell/mL)	3.35 ± 0.02^{b}	6.60 ± 0.13^{a}	0.36 ± 0.01^{a}	13.78 ± 0.28^{a}	0.19 ± 0.01^{b}	1.30 ± 0.03^{a}	6.57 ± 0.12^{b}	88 ± 13.0^{d}
S. cerevisiae	(10^5 cell/mL)	3.37 ± 0.02^{b}	6.68 ± 0.00^{a}	0.41 ± 0.03^{a}	13.73 ± 0.26^{a}	0.21 ± 001^{b}	1.29 ± 0.05^{a}	6.73 ± 0.35^{b}	84 ± 3.0^{d}
S. cerevisiae	(10^3 cell/mL)	3.33 ± 0.02^{b}	6.62 ± 0.16^{a}	0.44 ± 0.06^{a}	13.62 ± 0.39^{a}	0.21 ± 0.01^{b}	1.20 ± 0.06^{b}	7.21 ± 0.11^{b}	80 ± 1.4^{d}
S. cerevisiae/*S. japonicus*	(10^7 cell/mL)/(10^7 cell/mL) Ratio 1:1	3.39 ± 0.04^{b}	6.31 ± 0.04^{a}	0.37 ± 0.04^{a}	13.80 ± 0.24^{a}	0.20 ± 0.01^{b}	1.14 ± 0.03^{b}	8.60 ± 0.83^{b}	481 ± 26^{c}
S. cerevisiae/*S. japonicus*	(10^5 cell/mL)/(10^7 cell/mL) Ratio 1:100	3.49 ± 0.01^{a}	5.40 ± 0.49^{b}	0.36 ± 0.06^{a}	13.75 ± 0.33^{a}	$0.28 \pm 0.14^{a,b}$	0.27 ± 0.03^{c}	12.12 ± 1.19^{a}	1160 ± 72^{b}
S. cerevisiae/*S. japonicus*	(10^3 cell/mL)/(10^7 cell/mL) Ratio1:10,000	3.41 ± 0.13^{a}	5.30 ± 0.35^{b}	0.37 ± 0.08^{a}	13.34 ± 0.27^{a}	0.58 ± 0.32^{a}	0.31 ± 0.02^{c}	13.08 ± 1.39^{a}	1427 ± 40^{a}

Data are means ± standard deviations of two independent experiments, each carried out in duplicate. Values displaying different superscript letters (^{a}, ^{b}, ^{c}, ^{d}) within each column are significantly different according to the Duncan test (0.05%).

Table 2. Main byproducts of wines obtained with *S. cerevisiae* EC1118 in pure cultures and in mixed cultures with *S. japonicus* #13.

Yeasts	Inoculum Cell Concentration	Acetaldehyde (mg/L)	Ethyl Acetate (mg/L)	Ethyl lactate (mg/L)	Propanol (mg/L)	Isobutanol (mg/L)	Amylic Alcohol (mg/L)	Isoamylic Alcohol (mg/L)
S. cerevisiae	(10^7 cell/mL)	31.61 ± 2.8^{b}	30.74 ± 2.1^{c}	8.95 ± 0.7^{a}	36.63 ± 4.0^{a}	$69.55 \pm 2.2^{c,d}$	12.33 ± 0.4^{c}	113.21 ± 1.9^{c}
S. cerevisiae	(10^5 cell/mL)	33.34 ± 2.3^{b}	32.45 ± 1.0^{c}	$8.73 \pm 0.2^{a,b}$	37.88 ± 3.9^{a}	77.17 ± 2.5^{c}	11.54 ± 0.5^{c}	108.42 ± 2.8^{c}
S. cerevisiae	(10^3 cell/mL)	27.37 ± 0.0^{c}	34.34 ± 2.6^{c}	8.37 ± 0.3^{b}	36.85 ± 2.8^{a}	$72.59 \pm 2.0^{c,d}$	11.74 ± 0.1^{c}	105.32 ± 8.2^{c}
S. cerevisiae/*S. japonicus*	(10^7 cell/mL)/(10^7 cell/mL) (Ratio 1:1)	49.40 ± 2.1^{a}	156.60 ± 2.8^{b}	$8.30 \pm 0.4^{a,b}$	28.10 ± 2.7^{b}	159.60 ± 0.5^{b}	57.30 ± 4.7^{b}	348.80 ± 5.7^{b}
S. cerevisiae/*S. japonicus*	(10^5 cell/mL)/(10^7 cell/mL) (Ratio 1:100)	32.90 ± 4.1^{b}	213.70 ± 6.4^{a}	$7.48 \pm 0.3^{b,c}$	18.90 ± 1.5^{c}	202.80 ± 4.2^{a}	59.10 ± 4.9^{a}	395.70 ± 21.2^{a}
S. cerevisiae/*S. japonicus*	(10^3 cell/mL)/(10^7 cell/mL) (Ratio1:10,000)	25.40 ± 3.7^{c}	216.70 ± 8.5^{a}	3.86 ± 0.2^{c}	18.80 ± 1.5^{c}	203.20 ± 8.5^{a}	59.10 ± 1.4^{a}	401.10 ± 24.4^{a}

Data are means ± standard deviations of two independent experiments, each carried out in duplicate. Values displaying different superscript letters (^{a}, ^{b}, ^{c}, ^{d}) within each column are significantly different according to the Duncan test (0.05%).

4. Conclusions

Yeasts belonging to the genus *Schizosaccharomyces* have often been associated with the production of high levels of acetic acid and of off-flavors such as ethyl acetate and acetaldehyde in wine. The results here presented for *S. japonicus* #13 indicate that when this yeast is co-inoculated with *S. cerevisiae* EC1118, the wine volatile acidity is comparable to that obtained with *S. cerevisiae* in pure culture while the levels of acetaldehyde never exceed the acceptable threshold levels for wine. With respect to the final concentrations of ethyl acetate, these are definitely above the threshold level of perception in wine for inoculum ratios 1:100 and 1:10,000, but not for inoculum ratio 1:1. Based on these results, *S. japonicus* #13 shows promising characteristics for wine production due to its contribution to final ethanol concentration, reduction of total acidity and increase of the concentrations of volatile compounds and polysaccharides. However, the calibration of the inoculum ratio is compulsory for a successful fermentation and the achievement of a balanced wine.

Author Contributions: Cristina Romani and Mirko Gobbi carried out the experimental work, Livio Lencioni, Ilaria Mannazzu, Maurizio Ciani and Paola Domizio set up the experimental design, analyzed the data, and wrote the paper.

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

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