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Schizosaccharomyces japonicus/Saccharomyces cerevisiae mixed starter cultures: New perspectives for the improvement of Sangiovese aroma, taste, and color stability

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ABSTRACT

Schizosaccharomyces japonicus and *Saccharomyces cerevisiae* were inoculated in Sangiovese grape must and the impact of simultaneous (co-inoculation) and sequential inoculation protocols on growth and fermentation kinetics, and on the analytical and sensory profiles of the experimental wines, was evaluated at the end of the alcoholic fermentation and after aging in bottle.

While *Sch. japonicus* proved to affect *S. cerevisiae* growth and fermentative performances solely in sequential inoculation, the combined activity of the two yeasts always resulted in significantly higher total polysaccharides concentrations ($p < 0.05$), no matter the inoculation protocol utilized. Moreover, *Sch. japonicus* modulated the concentration of some volatile compounds when in mixed culture. In particular, ethyl acetate, that reached high levels at the end of alcoholic fermentation, showed a significant decrease after twenty-four months of bottle aging ($p < 0.05$), when the occurrence of hydrolysis/esterification reactions led to a significant increase of other acetate esters ($p < 0.05$). As a result, sensory analyses showed no significant differences for the ethyl acetate perception in mixed fermentation and control aged wines. Moreover, sequentially inoculated wines showed significantly higher sweetness compared to control wines, possibly due to the higher glycerol and polysaccharide content.

1. Introduction

Wine aroma and color may be strongly influenced by the presence, persistence and metabolic activity of specific microorganisms during the alcoholic fermentation. For example, yeasts belonging to the genus *Schizosaccharomyces* are known to release more polysaccharides than any other microorganism of oenological origin, and to affect wine aroma complexity and stability (Domizio, Lencioni, Calamai, Portaro, & Bisson, 2018; Domizio, Liu, Bisson, & Barile, 2017; Millarini et al., 2020; Romani et al., 2010). These non-*Saccharomyces* yeasts are rather rarely found throughout the whole fermentation process. However, it is possible to exploit their biotechnological potential by inoculating them in mixed fermentation with *Saccharomyces cerevisiae* starter strains. Accordingly, yeasts belonging to the species *Schizosaccharomyces pombe* have found industrial applications thanks to their ability to metabolize malic acid, permitting biological deacidification of juice and/or wine (Ciani, 1995; Dharmadhikari & Wilker, 1998; Gao & Fleet, 1995; Magyar & Panyik, 1989; Munyon & Nagel, 1977; Silva, Ramon-Portugal, Andrade, Texera, & Strehaino, 2003; Snow & Gallander, 1979; Thornton & Rodriguez, 1996; Vilela, 2019; Yokotsuka, Otaky, Naitoh, & Tanaka, 1993). Other potential applications of *Schizosaccharomyces* yeasts in the wine sector have been proposed. Benito, Palomero, Morata, Calderon & Suarez-Lepe (2012) reported that *Sch. pombe* reduces ethyl carbamate and produces high concentrations of pyruvic acid, a compound of particular interest for the color stability of wine (Morata et al., 2003). Moreover, Benito, Calderon, Palomero, and Benito (2015) proposed the inoculation of *Sch. pombe* and *Lachancea thermotolerans* in low acidity musts in order to modulate the chemical composition of wine through the consumption of malic acid and the production of lactic acid, respectively, and reduce the risk of biogenic amines production

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Table 1
Fermentation trials.

Code	Yeast	Inoculum concentration	Inoculum modality
Sc	*S. cerevisiae*	10^6 cell/mL	Pure culture
Sj + Sc - co	*Sch. japonicus*	10^6 cell/mL	Co-inoculum
	S. cerevisiae	10^6 cell/mL	
Sj + Sc - 24h	*Sch. japonicus*	10^6 cell/mL	Sequential inoculum (24 h)
	S. cerevisiae	10^6 cell/mL	

associated to malolactic fermentation.

More recently, other authors showed that the combined inoculation of *Sch. japonicus* and *S. cerevisiae* in mixed starter cultures results in a massive release of cell wall polysaccharides (Domizio et al., 2018; Romani et al., 2018). Yeast polysaccharides have a plethora of positive effects on wine among which: increase of the fullness sensation (Vidal et al., 2004); interaction with polyphenols aggregates and consequent decrease in the astringency perception (Escot, Feuillat, Dulau, & Charpentier, 2001; Poncet-Legrand, Doco, Williams, & Vernhet, 2007; Quijada-Morín, Williams, Rivas-Gonzalo, Doco, & Escribano-Bailon, 2014); retention of wine aroma compounds (Chalier, Angot, Delteil, Doco, & Gunata, 2007; Lubbers, Voilley, Feuillat, & Charpentier, 1994); increase in wine colloidal stability (Waters, Pellerin & Brillouet, 1994; Moine--Ledoux & Dubourdieu, 1999, Dupin et al., 2000; Brown et al., 2007; Gonzalez-Ramos, Cebollero, & Gonzalez, 2008). Besides polysaccharides, *Sch. japonicus* produces color-active and ester compounds during alcoholic fermentation (Domizio et al., 2018).

Here, with the aim of gathering further information on the oenological potential of *Sch. japonicus/S. cerevisiae* mixed starter cultures, the two yeasts were simultaneously (co-inoculated) or sequentially inoculated in Sangiovese grape must and their impact on growth and fermentation kinetics, and on the analytical and sensory profiles of the experimental wines, was evaluated at the end of the alcoholic fermentation and after twenty-four months of bottle aging.

2. Materials and methods

2.1. Yeast strains

Sch. japonicus #13 belonging to the yeast culture collection of the Department of Agriculture, Food, Environment and Forestry (DAGRI, University of Florence, Italy) was used. A commercial strain of *S. cerevisiae*, Lalvin EC1118 (Lallemand Inc.), was used as the reference strain for the species.

2.2. Fermentation trials

Sangiovese grapes from a vineyard located in the Chianti Classico area (Tuscany, Italy) were used for red wine fermentation trials during 2017 vintage. Grapes were destemmed, crushed and the resulting grape must with pomaces (7 L) was placed into 10-L glass fermenters. Grape must chemical characteristics were the following: pH 3.2; 220 g/L of sugar; 4.8 g/L of titratable acidity (as tartaric acid); 2.5 g/L of malic acid. Fermenters were equipped with valves containing sulfuric acid, allowing the CO_2 to escape, and with a pole permitting manual cups punch down twice a day.

Mixed fermentation trials were inoculated with 10^6 cell/mL of *Sch. japonicus* #13 after 48 h growth in filtered sterilized commercial white grape juice. Control and mixed fermentation trials were inoculated with 10^6 cell/mL of *S. cerevisiae* EC1118 (Lallemand) (Domizio et al., 2018). For that, active dry yeast (ADY) was rehydrated according to the manufacturer. For simultaneous (co-inoculation) and sequential inoculation protocols, *S. cerevisiae* was inoculated together with, or 24 h after, *Sch. japonicus,* respectively. Cell concentration was determined by microscope counting using a Thoma chamber. Immediately after inoculation, grape must was supplemented with 30 g/hL of NutrientVit Start (Lallemand). Fermentations were carried out at 25 °C. Three biological replicates were carried out for each trial for a total of nine biological samples, as reported in Table 1.

Grape must was sampled every day throughout the fermentation process to evaluate yeast growth. Briefly, 100 μL aliquots of grape must serial dilutions were plated onto differential Wallerstein Laboratory nutrient agar medium (WL) (Oxoid Unipath, Basingstoke, England) for yeast viable plate count (Pallmann et al., 2001). Sugar concentration was evaluated daily till the end of fermentation (residual sugar <2.0 g/L) after which wines were pressed and the control trials were inoculated with commercial malolactic bacteria (Oeno 1, LaMothe Abiet, Canejan/Bordeaux, France). Malic acid concentration was monitored at the end of alcoholic and malolactic fermentation, just before bottling and after twenty-four months of bottle aging. Wines were added with 50 mg/L SO_2, left to settle, and bottled.

2.3. Analytical determinations of the fermentation products

For organic acid, residual sugars, and ethanol determinations, samples were filtered through 0.45 μm nitrocellulose membranes and 20 μL were injected into a high-performance liquid chromatography (HPLC) apparatus (Varian Inc., equipped with a 410 series autosampler, a 210 series pump, a 356-LC refractive index detector, and a 335-LC diode array detector, set at 210 nm). Isocratic separation was performed at 75 °C on a ((300 + 150) cm × 7.8 mm) Phenomenex column (Rezex-ROA Organic Acids, sulfonated styrene divinyl-benzene matrix in H+ form). The mobile phase was 10.5 mmol/L sulfuric acid at a flow rate of 0.6 mL/min. Each compound was quantified by comparison with its relevant external calibration curve (from 0.5 g/L to 20 g/L), and the areas of the peaks were recorded and integrated using Galaxie Chromatography Data System version 1.9.302.530 (Varian Inc.).

Total polysaccharide content was evaluated by HPLC according to Domizio, Liu, Bisson, and Barile (2014). Isocratic separation of the polysaccharides was performed at 65 °C on a Supelco TSK G-OLIGO-PW (808031) column (30 cm × 7.8 mm i.d.) equipped with a Supelco TSKGEL OLIGO (808034) guard column (4 cm × 6 mm i.d.). A mobile phase of 0.2 mol/L sodium chloride at a flow rate of 0.8 mL/min was used. Quantification was performed by comparison with a calibration curve of mannan from *S. cerevisiae* (M7504, Sigma-Aldrich), at concentrations ranging from 50 to 500 mg/L, and the area of the mannan peak was recorded and integrated by using the same software as above.

2.4. Wine pigment and tannin analyses

Phenolic Analysis by RP-HPLC DAD. Monomeric anthocyanins, polymeric pigments and tannins were quantified by HPLC (Peng, Iland, Oberholster, Sefton, & Waters, 2002). The analysis was carried out on a Perkin Elmer Series 200 LC equipped with an autosampler and a diode-array detector (DAD series 200) (Perkin Elmer). Chromatograms were acquired at 280 and 520 nm, recorded and processed using Total Chrome Navigator software (Perkin Elmer). A polystyrene divinylbenzene column (250 mm × 4.6 mm PLRP-S 100A 5 μm, Polymer Laboratories) was used with a guard cartridge (10 × 4.6 mm) packed with the same material (both from Lab Service Analytica Srl.Bologna, Italy). Both

columns were held at 28 °C. Wines were clarified by centrifugation at 12,000 g in a Mikro 12–24 centrifuge (Hettich, Tuttlingen, Germany) for 10 min and filtered at 0.22 μm with syringe filter before injection. One mL of sample was collected in 2 mL HPLC vials with an addiction of 10 μL of formic acid. The volume injected was 20 μL with the binary pump flow set on 1 mL/min using the following eluents: (A) water solution of 1.5% (w/w) of *ortho*-phosphoric acid (85%), (B) 20% of (A) in acetonitrile. Eluent gradients were set as follows: for the first 55 min, from 92% to 73% of eluent A, maintaining the isocratic conditions of 73% from minute 55 to 59, reduction from 73% to 30% between 59 and 64 min, maintaining at 30% from the minute 64 to 69 and increasing to 92% from 70 to 76 min. Acetonitrile of HPLC grade was from Panreac (Barcelona, Spain). Orthophosphoric acid and ethanol of analytical reagent grade were from Sigma-Aldrich (Steinheim, Germany). . Malvidin-3-O-glucoside ≥99% and (+)–catechin were purchased from Sigma Aldrich.

Color indexes. Color intensity (CI) and hue were evaluated according to the method of Glories (1984). CI and wine hue were measured using a 1 mm path length quartz cell. CI was expressed as the sum of absorbances at 420 (A420), 520 (A520), and 620 nm (A620). Wine hue was expressed as the ratio between absorbance at 420 (A420) and 520 nm (A520).

Total Phenolic index. Total phenolic index (TPI) was determined as described by Ribereau-Gayon (1970) and measured as absorbance at 280 nm using a 1 mm path length quartz cell. Samples were diluted 1:10 with ultrapure water (Elix 5 System, Millipore, Billerica, MA, USA). Ultrapure water was used as a reference. Wine samples were centrifuged before analysis (10,000 rpm per 10 min). All analyses were performed in triplicate.

CIEL*a*b* Coordinates. CIE (Commission Internationale de l'Eclairage) L*, a* and b* color coordinates were also measured. Visible spectra were recorded at 400–700 nm reflectance (RSA-PE-20 Integrating Sphere,Labsphere, North Sutton, NH). UV WinLab Software (version 2.85.04, Perkin Elmer) was used to record the spectra and CIE L*a*b* color coordinates were calculated using Color software (version 3.00, 2001, Perkin Elmer). A Lambda 35 (Perkin Elmer) UV–visible spectrophotometer Color indexes was used for total phenolic index and CIEL*a*b* Coordinates.

2.5. Volatile compounds analysis

Higher alcohols, acetaldehyde, ethyl acetate and ethyl lactate were determined using an AutoSystem XL gas chromatograph equipped with FID (flame ionization detector) (Romani et al., 2020). Total free volatile profile was determined by HS-SPME GCMS method developed by Canuti et al. (2009). The analytical system for the determination of the volatile compounds comprised an AutoSystem XL gas chromatograph (Perkin Elmer) paired with a Turbomass Gold mass selective detector (Perkin Elmer). The software used was TurboMass v.5.1.0. An Innowax column (30 m × 0.25 mm o.d., 0.25 μm film thickness, Agilent Technology) was used. Volatile compounds were identified and quantified by using the reference standards (≥99% purity) (Sigma -Aldrich, Saint Louise, MO, USA). In the absence of the reference standards, volatile compounds were identified by matching the NIST MS library spectra and quantified based on the relative response to the octan-2-ol internal standard.

2.6. Sensory analysis

Sensory analyses were carried out following the Quantitative Descriptive Analysis (QDA) method (Gacula, 2008). Seventeen trained judges (11 males and 6 females), recruited from students and staff of the Department of Agriculture, Environment and Forestry of the University of Florence (DAGRI) formed the panel.

All the sensory evaluations were performed in isolated, ventilated sensory booths under red lights, to eliminate bias attributed to color differences. The presentation was monadic with a balanced presentation order for carry-over effect, according to a complete block design, with three wines per session evaluated in three replicates, for a total of three sessions.

The wine samples (30 mL) were poured at room temperature (around 19 °C) and presented in standard tasting glasses (ISO-3591, 1977) covered with plastic lids and identified by random three-digit codes. The sensory data from the three session's descriptive analyses were combined using the shared or synonymous attributes and standardized to mean zero for each sensory attribute within each descriptive analysis.

Every evaluation session lasted about 7 min. The panelists answered on a 10-point category scale (one scale per sample), anchored with 1 (absent) on the left end and 10 on the right end (very strong). The reference standards corresponded to 6 on the intensity scale (medium intensity).

For sensory analyses performed on wines sampled after 6 and twenty-four months of bottle aging, the score card was compiled with the taste and tactile descriptors (Sweetness, Acidity, Bitterness, Astringency) and the odor attribute ethyl acetate.

All the sensory data were collected using FIZZ software (Version 2.00L, Biosystemes, Couternon, France).

2.7. Data analysis

Each fermentation trial was carried out in triplicate and two technical replicates were obtained for each biological sample. Resulting data were subjected to one-way analysis of variance (ANOVA, general linear model). The differences between data were tested with Tukey's honest significant difference test at the 0.05 significance level. The means and the standard deviation of the mean (mean ± SD) are also reported. The data were analysed using the Statgraphics Centurion software (Ver.XV, StatPoint Technologies, Warrenton, VA).

Fisher's LSD post hoc test was used to determine the significant differences between group means (p-value = 0.05).

3. Results and discussion

3.1. Yeast cell growth and fermentative performances

In co-inoculated fermentations (Sj + Sc-co) *Sch. japonicus* did not affect the development of *S. cerevisiae* which showed growth kinetics similar to that of the control (Sc) (Fig. 1). In both trials, 2 days after the inoculum, *S. cerevisiae* reached about 5 x 10^7 CFU/mL and maintained comparable cell concentrations until the end of the fermentation. In contrast, *S. cerevisiae* growth and fermentation kinetics were affected by *Sch. japonicus* in sequential inoculation trials (Sj + Sc-24h) (Fig.1; Fig. 2). Here, *S. cerevisiae* showed a marked reduction in growth and reached the concentration of 2.1 × 10^7 cell/mL, 4 days after the inoculum. In agreement, sugar consumption was comparable in co-inoculated and control trials (Fig. 2) and slower in sequential inoculation trials where seven extra days were required to take it to completion. At the end of alcoholic fermentation residual sugar was below 2.0 g/L in all trials. However, it was significantly higher ($p < 0.05$) in sequential inoculations (Table 2) possibly due to the lower cell density of *S. cerevisiae*. Accordingly, also ethanol yield was lower in sequentially inoculated wines in respect to control and co-inoculated wines (Table 2). These results further support that already reported by other authors. In particular, Romani et al. (2018) clearly showed that growth and fermentation kinetics of *S. cerevisiae* were comparable in pure and co-inoculated *Sch. japonicus/S. cerevisiae* trials, when the inoculum ratio was 1:1, but slower when the inoculum ratio was 10,000:1, a condition comparable to that here observed after 1 day in sequential culture (Fig. 1). Similarly, Taillandier, Gilis, and Strehaino (1995) remarked that *Saccharomyces* growth was inhibited by *Sch. pombe*, and this inhibition was proportional to the *Schizosaccharomyces* cell concentration. Interestingly, while *Sch. japonicus* growth kinetics could not be assessed

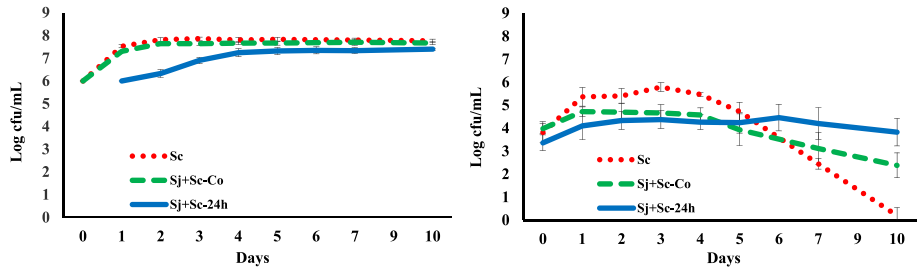

Fig. 1. Growth of *S. cerevisiae* (left panel) and non-*Saccharomyces* yeasts (right panel) in the control (Sc) (dot line) and in the mixed cultures: co-inoculum (Sj + Sc-co) (dashed line) and sequential imoculum (Sj + Sc-24h) (full line). Error bars show standard deviations of three independent experiments.

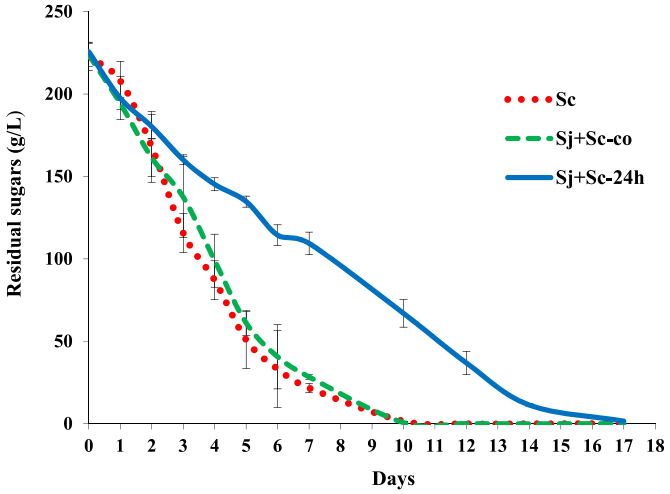

Fig. 2. Sugar consumption in control (Sc) (dot line) and mixed fermentation trials: co-inoculated culture (Sj + Sc-co) (dashed line) and sequential culture (Sj + Sc-24h) (full line). Error bars show standard deviations of three independent experiments, each analysed in duplicate.

Table 2
Main analytical parameters of the experimental wines at the end of the alcoholic fermentation and after twenty-four months of bottle aging.

	End of alcoholic fermentation			24 months after bottling		
	Sc	Sj + Sc-co	Sj + Sc-24h	Sc	Sj + Sc-co	Sj + Sc-24h
Residual sugars (%, w/v)	0.20 ± 0.006 a	0.27 ± 0.003 a	0.90 ± 0.005 b	0.21 ± 0.008 b	0.28 ± 0.004 c	0.17 ± 0.005 a
Ethanol (%, v/v)	13.95 ± 0.580 c	13.48 ± 0.270 b	12.76 ± 0.260 a	13.53 ± 0.570 c	13.35 ± 0.270 b	12.8 ± 0.271 a
Volatile acidity[a] (g/L)	0.24 ± 0.020 b	0.17 ± 0.080 a	0.15 ± 0.020 a	0.45 ± 0.003 a	0.67 ± 0.003 c	0.59 ± 0.004 b
Malic acid (g/L)	2.46 ± 0.210 c	1.09 ± 0.080 b	0.46 ± 0.060 a	0.00 ± 0.000 a	0.00 ± 0.000 a	0.25 ± 0.020 b
Glycerol (g/L)	9.22 ± 0.360 a	13.12 ± 0.498 b	15.11 ± 0.919 c	9.33 ± 0.248 a	12.87 ± 0.570 b	15.21 ± 0.462 c
pH	3.32 ± 0.065 a	3.40 ± 0.015 b	3.32 ± 0.022 a	3.34 ± 0.010 a	3.51 ± 0.010 c	3.46 ± 0.010 b

Legend: Same letter within the same row indicates no significant difference (LSD - least significant difference test, significance at $p \leq 0.05$).

[a] Volatile acidity as g/L of acetic acid. Data are means of three independent experiments, each analysed in duplicate.

due to cell flocculation after the second day, viable plate count on WL differential medium highlighted the inhibitory effect of this yeast on the non-*Saccharomyces* yeasts naturally occurring in Sangiovese grape must. These achieved a maximum concentration of 1×10^4 UFC/mL and 5×10^4 UFC/mL in co-inoculated and in sequential fermentation trials, respectively (Fig. 1) while reaching 6×10^5 UFC/mL in the control. After 10 days the non-*Saccharomyces* yeasts were still present at concentration of 2.4×10^2 UFC/mL (Sj + Sc-co) and of 6×10^3 UFC/mL (Sj + Sc-24h) while disappearing in the control. Thus, the inoculation protocol and the relative abundance of *Sch. japonicus* and *S. cerevisiae*, seemed to modulate the composition of the grape must microbiota and play an important role for the success of the alcoholic fermentation. In particular, the simultaneous inoculation of *Sch. japonicus/S. cerevisiae* might represent an interesting tool to control the development of non-*Saccharomyces* yeasts in the first stages of the alcoholic fermentation with no side effects on the success of the fermentative process. However, further studies are necessary to evaluate the specificity of the inhibitory effect of *Sch. japonicus* and how different inoculation protocols may affect growth and fermentative activity of grape must resident microflora.

3.2. Analytical profiles of wines

The main analytical parameters of the experimental wines are reported in Table 2. Volatile acidity that, similar to that reported by Romani et al. (2018), at the end of the alcoholic fermentation, was slightly lower in the mixed fermentation trials (0.17 ± 0.02 g/L co-inoculum and 0.15 ± 0.01 g/L sequential inoculum), as compared to the control (0.24 ± 0.02 g/L), rose significantly in all trials after bottle aging, although never exceeding the acceptability threshold of 0.7 g/L.

At the end of alcoholic fermentation, malic acid concentration of the control wines (2.46 ± 0.21 g/L) was comparable to that of grape must (2.5 ± 0.20 g/L) (Table 2) while 57% and 84% malic acid reductions were observed in co-inoculated and sequentially inoculated trials, respectively. Malic acid was completely consumed in the control wines at the end of malo-lactic fermentation. Analytical determinations of malic acid, carried out just before bottling, showed its complete consumption in the coinoculated wines while 0.25 ± 0.02 g/L was still present in the sequentially inoculated wines. Thus, the capability to convert malic acid into ethanol, typical of *Sch. pombe*, was here confirmed also for the species *Sch. japonicus*. Moreover, and according to that already observed by Domizio et al. (2018), *Sch. japonicus* malic acid consumption was negatively affected by *S. cerevisiae* when the two yeasts were sequentially inoculated.

Glycerol concentration at the end of the fermentation was higher in mixed fermentation trials and remained substantially unvaried at the end of bottle aging (Table 2). This is in accordance with that already observed by Domizio et al. (2018) in Trebbiano grape must and, considering that glycerol results in wine sweetness (Noble & Bursick, 1984), the rise of this compound could represent an additional advantage of the inoculation of *Sch. japonicus/S. cerevisiae* starter cultures.

Regarding polysaccharides, mixed fermentations reached concentrations that were significantly higher than that of the control, starting from the beginning of alcoholic fermentation. Moreover, while in the control the maximum level of polysaccharides (282 ± 14 mg/L) was reached after seven days (Fig. 3), in mixed fermentation trials the

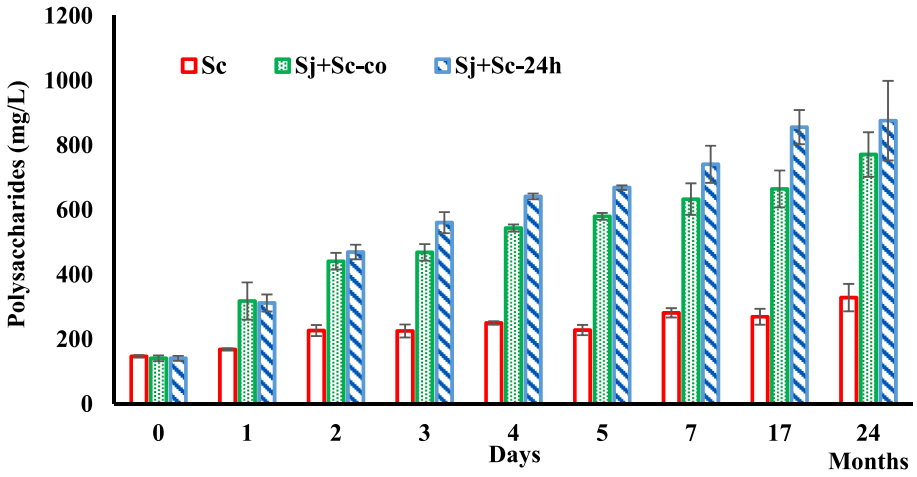

Fig. 3. Total polysaccharides throughout the alcoholic fermentation and after twenty-four months of bottle aging in control (□), co-inoculated (⊞) and sequential (⬔) fermentation trials wines. Error bars show standard deviations of three independent experiments, each analysed in duplicate.

concentration of polysaccharides constantly increased with time reaching 665 ± 57 mg/L (co-inoculation) and 856 ± 53 mg/L (sequential inoculation). Twenty-four months after bottling the concentration of polysaccharides showed no relevant variations in the control and in mixed fermentation trials (Fig. 3). These results clearly reflect the role of *Sch. japonicus* in the release of polysaccharides and agree with previous studies that reported the impact of this yeast on polysaccharides content in both synthetic and natural grape musts (Romani et al., 2018; Domizio et al., 2017; 2018). Moreover, they endorse the utilization of *Sch. japonicus* in fermentation to compensate the low production of polysaccharides by *Saccharomyces* yeasts, normally ranging from 50 to 150 mg/L (Rosi, Gheri, Domizio, & Fia, 2000), thus avoiding the utilization of more expensive exogenous yeast polysaccharides.

The phenolic compounds and colorimetrical indexes of the experimental wines analysed at the end of the alcoholic fermentation and after twenty-four months of bottle aging are reported in Table 3. Interestingly, an increase of the polymeric pigments (PP) was observed in mixed inoculated wines as compared to the control wines. In sequentially inoculated wines the increase was significantly higher at the end of the alcoholic fermentation as well as twenty-four months after bottling ($p < 0.05$). Similarly, at the end of bottle aging, the content of tannins was significantly higher in sequentially inoculated as compared to the control wines ($p < 0.05$). Instead, Total Phenolic Index (TPI) was similar in all the trials at the end of the alcoholic fermentation and twenty-four months after bottling. However, at the end of bottle aging, sequentially inoculated wines showed a smaller decrease of TPI values as compared to the control and the co-inoculated wines and colorimetrical indexes were consistent with the content of the phenolic compounds. At the end of the alcoholic fermentation, parameter a* of the CIEL*a*b* (direction of the wine colour towards the redness (+) or greenness (−)) was significantly higher in the sequentially inoculated wines as compared to the control and the co-inoculated wines ($p < 0.05$) while there were no differences among trials for the parameter L*, indicating the same luminosity. On the contrary, after twenty-four months of bottle aging, the sequentially inoculated wines showed a significant higher value of parameter a* ($p < 0.05$) and were lighter (albeit not significantly) as compared to the control and the co-inoculated wines.

Overall, results regarding phenolic compounds and colorimetrical indexes might be correlated to the higher polysaccharides content in both mixed fermentations as compared to the control wines. Indeed, polymers of flavan-3-ol, the most abundant macromolecule present in wine, tend to aggregate, and eventually precipitate during wine aging, with a consequent decrease of their concentration. Instead, hydrophobic interactions and hydrogen bonds occurring between polysaccharides and tannins, might stabilize the condensed tannins, decreasing the

Table 3
Phenolic compounds (mg/L) and colorimetrical indexes of the experimental wines analysed at the end of alcoholic fermentation and after twenty-four months of bottle aging.

	End of alcoholic fermentation			24 months after bottling		
	Sc	**Sj + Sc–Co**	**Sj + Sc-24h**	**Sc**	**Sj + Sc–Co**	**Sj + Sc-24h**
Delphinidin-3-*O*-glucoside[a]	17.91 ± 2.46 a	19.33 ± 2.38 ab	20.54 ± 4.19 b	2.65 ± 0.13 a	2.89 ± 0.39 a	2.56 ± 0.32 a
Cyanidin-3-*O*-glucoside[a]	13.45 ± 3.19 a	18.06 ± 1.61 b	24.29 ± 6.90 c	5.82 ± 0.99 a	5.65 ± 0.73 a	6.12 ± 0.61 a
Petunidin-3-*O*-glucoside[a]	33.13 ± 3.91 ab	35.02 ± 3.12 b	32.73 ± 5.17 a	2.89 ± 0.94 a	3.02 ± 0.59 a	2.15 ± 0.42 a
Peonidin-3-*O*-glucoside[a]	32.96 ± 3.13 a	39.85 ± 1.17 b	38.54 ± 7.01 b	5.44 ± 1.24 a	6.62 ± 0.36 a	4.81 ± 1.91 a
Malvidin-3-*O*-glucoside	110.64 ± 14.65 a	127.08 ± 7.37 b	105.69 ± 15.47 a	20.59 ± 2.43 b	15.55 ± 0.71 a	15.45 ± 2.26 a
Total monomeric anthocyanins[a]	208.15 ± 18.47 a	239.35 ± 14.31 a	248.66 ± 12.58 a	37.64 ± 5.48 a	33.49 ± 1.64 a	31.10 ± 6.24 a
Polymeric pigments[a]	29.99 ± 8.29 a	33.08 ± 7.83 a	47.92 ± 11.09 b	44.85 ± 9.11 a	69.88 ± 12.83 a	123.52 ± 14.39 b
Tannins[b]	653.48 ± 79.45 a	655.12 ± 85.43 a	772.26 ± 83.01 a	557.15 ± 65.59 a	767.15 ± 166.40 ab	888.15 ± 199.16 b
Quercetin (mg/L)	40.63 ± 12.11a	39.13 ± 10.58 a	34.13 ± 10.08 a	57.86 ± 12.73 a	52.47 ± 12.52 a	48.60 ± 13.22 a
Color intensity	8.50 ± 1.85 ab	7.61 ± 1.05 b	8.56 ± 0.14 a	7.42 ± 1.64 a	7.98 ± 0.01 b	9.25 ± 0.21 c
Total phenols Index	42.84 ± 4.53 a	43.06 ± 6.23 a	39.02 ± 0.99 a	37.83 ± 5.05 a	36.71 ± 1.54 a	37.11 ± 0.99 a
Hue	0.51 ± 0.03 a	0.56 ± 0.01 b	0.57 ± 0.03 b	9.42 ± 0.44 b	10.00 ± 0.69 b	8.60 ± 0.51 a
L*	71.74 ± 4.24 a	76.58 ± 2.95 a	71.92 ± 0.59 a	78.13 ± 3.47 ab	79.52 ± 2.22 b	75.82 ± 0.05 a
a*	39.57 ± 5.71 b	32.19 ± 3.64 a	33.05 ± 1.72 a	19.45 ± 2.99 a	17.39 ± 2.36 a	23.15 ± 1.41 b
b*	0.10 ± 0.03 b	−0.47 ± 0.16 a	−0.38 ± 0.03 a	9.85 ± 2.59 a	9.43 ± 1.50 a	9.90 ± 1.13 a

Legend: Same letter within the same row indicates no significant difference (LSD - least significant difference test, significance at $p \leq 0.05$).
[a] Expressed as mg/L of malvidin-3-*O*-glucoside.
[b] Expressed as mg/L of (+)-catechin. Data are means of three independent experiments, each analysed in duplicate.

Table 4
Main volatile compounds (mg/L) of the experimental wines analysed at the end of alcoholic fermentation, determined by gas chromatography.

	End of alcoholic fermentation		
	Sc	**Sj + Sc–Co**	**Sj + Sc-24h**
Acetaldehyde	18.56 ± 0.21 a	23.99 ± 0.25 b	36.92 ± 0.93 c
1-Propanol	21.74 ± 1.45 c	18.16 ± 1.05 a	20.59 ± 2.82 b
2-Methyl-1-propanol	43.87 ± 5.23 a	71.31 ± 3.12 b	88.44 ± 4.34 c
2-Methyl-1-butanol	103.63 ± 1.62 a	125.04 ± 12.76 b	127.75 ± 10.22 b
3-Methyl-1-butanol	289.90 ± 12.54 a	355.51 ± 22.62 b	460.51 ± 24.30 c
Ethyl acetate	26.74 ± 6.59 a	677.54 ± 10.30 b	691.81 ± 7.37 b
Ethyl lactate	113.72 ± 6.79 a	155.65 ± 17.84 b	156.16 ± 13.44 b

Legend: Same letter within the same row indicates no significant difference (LSD - least significant difference test, significance at $p \leq 0.05$). Data are means of three independent experiments, each analysed in duplicate.

Table 5
Main volatile compounds of the experimental wines analysed after twenty-four months of bottle aging.

	24 months after bottling		
	Sc	**Sj + Sc–Co**	**Sj + Sc-24h**
Higher alcohols			
[b]1-Hexanol (mg/L)	0.27 ± 0.12 a	0.45 ± 0.12 a	0.40 ± 0.04 a
[b]1-Eptanol (mg/L)[5]	0.31 ± 0.05 a	0.25 ± 0.04 a	0.20 ± 0.03 a
[b]β-Phenyl ethanol (mg/L)[5]	63.33 ± 10.49 a	49.68 ± 2.38 ab	39.55 ± 1.68 b
[a]1-Propanol (mg/L)	21.03 ± 1.56 ab	19.05 ± 1.03 a	24.19 ± 4.58 b
[a]2-Methyl-1-propanol (mg/L)	44.49 ± 3.27 a	75.25 ± 3.88 b	95.16 ± 15.72 c
[a]2-Methyl-1-butanol (mg/L)	96.88 ± 2.39 a	126.10 ± 12.22 b	127.04 ± 21.33 b
[a]3-Methyl-1-butanol (mg/L)	281.24 ± 11.99 a	368.76 ± 21.35 b	476.44 ± 78.47 c
Total (mg/L)	**507.55**	**639.54**	**762.98**
Oxigenated compounds			
[a]Acetaldehyde (mg/L)	7.92 ±0.48 b	7.14± 0.44 a	7.64± 0.85 ab
Acetates ester			
[b]Ethyl acetate (mg/L)	61.30 ± 11.02 a	165.60 ± 15.12 c	144.9 ± 9.87 b
[b]Isobuthyl acetate (μg/L)	0.23 ± 0.02 a	1.50 ± 0.26 b	1.40 ± 0.49 b
[b]Isoamyl acetate (μg/L)	30.00 ± 5.00 a	150.00 ± 20.00 b	140.00 ± 10.00 b
[b]Hexyl acetate (μg/L)	0.25 ± 0.09 a	1.80 ± 0.27 b	3.60 ± 0.52 c
[b]2-Phenylethyl acetate (μg/L)	1.36 ± 0.09 a	25.30 ± 2.62 c	18.00 ± 2.34 b
Total (mg/L)	**61.33**	**165.75**	**145.06**
Ethyl esters			
[b]Ethyl isovalerate (μg/L)	30.00 ± 5.00 a	70.00 ± 20.0 b	100.00 ± 40.00 b
[b]Ethyl butyrate (μg/L)	9.95 ± 2.20 a	38.90 ± 3.30 b	54.00 ± 14.40 b
[b]Ethyl hexanoate (mg/L)[1]	1.29 ± 0.28 a	2.48 ± 0.15 c	1.90 ± 0.17 b
[b]Ethyl nonanoate (μg/L)[2]	0.31 ± 0.08 a	0.33 ± 0.16 a	0.39 ± 0.04 a
[b]Ethyl decanoate (μg/L)[2]	36.30 ± 7.65 a	35.20 ± 1.26 a	39.00 ± 4.55 a
[b]Ethyl dodecanoate (μg/L)[2]	5.10 ± 0.80 a	6.00 ± 0.71 a	5.70 ± 0.82 a
[a]Ethyl lactate (mg/L)	151.78 ± 12.45 c	47.88 ± 9.56 a	68.47 ± 4.78 b
[b]Diethyl succinate (mg/L)	6.80 ± 2.95 a	9.51 ± 2.79 a	8.04 ± 2.05 a
[b]Isoamyl butanoate (μg/L)[1]	6.70 ± 0.93 a	22.00 ± 3.80 b	33.00 ± 5.41 c
Total (mg/L)	**248.23**	**232.30**	**310.50**
Terpens			
[b]β-Linalool (μg/L)	0.35 ± 0.10 a	0.42 ± 0.04 a	0.34 ± 0.12 a
[b]4-Terpineol (μg/L)[3]	0.55 ± 0.09 a	0.56 ± 0.02 a	0.46 ± 0.04 a
[b]β-Citronellol (μg/L)	1.30 ± 0.09 a	1.60 ± 0.17 a	1.50 ± 0.18 a
Total (μg/L)	**2.20**	**2.58**	**2.30**
Norisoprenoids			
[b]Riesling acetal (μg/L)[4]	0.15 ± 0.04 a	0.13 ± 0.04 a	0.15 ± 0.05 a
[b]TDN (μg/L)[4]	0.13 ± 0.06 a	0.10 ± 0.04 a	0.16 ± 0.08 a
[b]Vitispirane I (μg/L)[4]	0.74 ± 0.30 a	0.49 ± 0.22 a	0.73 ± 0.21 a
[b]Vitispirane II (μg/L)[4]	0.27 ± 0.10 a	0.19 ± 0.09 a	0.29 ± 0.07 a
Total (μg/L)	**1.29**	**0.91**	**1.33**
Fatty acids			
[b]Pentadecanoic acid (μg/L)[6]	3.60 ± 0.24 b	2.60 ± 0.36 a	2.50 ± 0.33 a
[b]Octanoic acid (mg/L)[6]	0.21 ± 0.03 b	0.02± 00 a	0.01± 00 a
[b]Nonanoic acid (mg/L)[6]	0.09 ± 0.02 a	0.08 ± 0.02 a	0.07 ± 0.02 a
Total (μg/L)	**3.90**	**2.70**	**2.58**

Legend: Same letter within the same row indicates no significant difference (LSD - least significant difference test, significance at $p \leq 0.05$). Data are means of three independent experiments, each analysed in duplicate. [1]expressed as ethyl butyrate, [2] expressed as ethyl octanoate, [3] expressed as β-linalool, [4] expressed as β-damascenone, [5] expressed as 1-hexanol, [6]espressed as 2-octanol (IS).

[a] Determined by gas chromatograph.

[b] Determined by HS-SPME-GC-MS.

Table 6
Mean value and significance of the wine sensory attributes evaluated at the end of malo-lactic fermentation and after twenty-four months of bottle aging.

	After malo-lactic fermentation					24 months after bottling				
	Sc	Sj + Sc-co	Sj + Sc-24h	F	LSD	Sc	Sj + Sc-co	Sj + Sc-24h	F	LSD
Ethyl acetate	2.47 a	4.95 c	3.40 b	33.44***	0.61	1.95	2.66	2.75	1.55 NS	–
Sweetness	5.47	5.38	5.66	0.91 NS	–	3.04 a	3.41 ab	4.00 b	3.90*	0.71
Acidity	3.97	3.80	4.19	1.02 NS	–	3.95	4.04	4.04	0.03 NS	–
Bitterness	2.52 a	3.54 b	2.38 a	10.06***	0.57	2.71	2.41	2.58	0.35 NS	–
Astringency	4.57 b	3.30 a	3.61 a	12.14***	0.53	4.66	3.95	4.54	2.32 NS	–

Fisher values F with significative levels and groups; * = $p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

astringency and improving the color (Fuster & Escot, 2002; Watrelot, Schulz, & Kennedy, 2017). This is particularly important for Sangiovese wine that, being rich in unstable and oxidizable phenols (Canuti et al., 2018), is characterized by limited color stability. Accordingly, Rinaldi, Coppola, and Moio (2019) observed that commercial mannoproteins added to a Sangiovese wine promoted the pigmented polymer formation with consequent increased stability of wine color during time.

Moreover, various studies have also shown that anthocyanin-tannin and tannin-tannin condensation reactions might be mediated by acetaldehyde, permitting the generation of stable pigments in wine (Bakker & Timberlake, 1997; Eglinton et al., 2004). In this context, the higher content of acetaldehyde found in mixed fermentation wines as compared to the control (Table 4), might have contributed to the higher content of polymeric pigments observed in *Sch. japonicus/S. cerevisiae* wines. In particular, in co-inoculated and sequentially inoculated wines acetaldehyde content was 1.2 and 2 fold higher respectively than in the control (18.6 ± 0.21 mg/L) but in any case below the reported perception threshold in wine (100 mg/L) (Lambrechts & Pretorius, 2000). Moreover, no more significant differences were observed after twenty-four months of bottle aging.

In addition, Domizio et al. (2017, 2018) reported that *Sch. japonicus* produces high amounts of pyruvic acid within the first week of the alcoholic fermentation. This compound might have reacted with grape anthocyanins to produce pyranoanthocyanin adducts, such as vitisin A, responsible of the stabilization of anthocyanins during wine aging (Morata, Calderón, González, Gómez-Cordovés, & Suárez, 2007). Finally, a different absorption of polyphenols by *S. cerevisiae* and *Sch. japonicus* cell walls might have occurred, with consequent impact on color changing (Caridi et al., 2017; Morata et al., 2003).

In Tables 4 and 5, the concentrations of the main volatile compounds are reported. At the end of the alcoholic fermentation, all wines presented high concentrations of aliphatic alcohols (propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol) (Table 4). In particular, these were higher in mixed fermentation wines and remained unvaried in all trials even after twenty-four months of bottle aging (Table 5). According to De-La-Fuente-Blanco et al. (2016) these higher alcohols may play a negative role on red wine aroma, by suppressing fruity and woody notes depending on the aromatic context, and are reported to be responsible for a pungent smell and taste when present at concentration higher than 300 mg/L (Eteviant, 1991). Interestingly, twenty-four months after bottling, β-phenyl ethanol (flowery, honey like odor), the major phenolic alcohol in wine, was present, at significantly higher concentration in the control than in the two mixed fermentation wines ($p < 0.05$). However, it is worth to highlight here that the concentration of the corresponding acetate, the ester phenethyl acetate, responsible for floral aroma (such as roses), was 13-fold and 18-fold higher in the co-inoculated and the sequentially inoculated wines, respectively, than in the control (1.36 ± 0.09 μg/L).

Among volatile compounds, ethyl acetate was the main acetate ester produced. This compound, when present at concentrations above 150 mg/L, produces a solvent-like aroma in wine (Lambrechts & Pretorius, 2000). At the end of the alcoholic fermentation this threshold was abundantly surpassed in mixed fermentation wines that showed ethyl acetate concentrations about 25-fold the amount found in the control wine (26.74 ± 6.59 mg/L). Thus, *Sch. japonicus* attitude towards the production of this volatile compound was here confirmed (Domizio et al., 2018). At the end of bottle aging ethyl acetate doubled in the control wine, while showing 4- and 5-fold reductions in co-inoculated and sequentially inoculated wines, respectively. In these last it reached concentrations that were close to the threshold value of 150 mg/L. Similar results were observed for the ethyl lactate (buttery aroma). Its concentration, at the end of the alcoholic fermentation, was significantly lower in the control (113 ± 6.79 mg/L) as compared to mixed fermentation wines (~156 mg/L). However, at the end of bottle aging ethyl lactate rose in the control (151.78 ± 12.45 mg/L) while drastically declining in co-inoculated (47.88 ± 9.56 mg/L) and sequentially inoculated (68.47 ± 4.78 mg/L) wines. Moreover, isobutyl acetate (banana), isoamyl acetate (banana and apple), and hexyl acetate (pear apple, cherry), were present, in mixed fermentation wines, at concentrations far above their perception thresholds (1.6 mg/L, 0.16 mg/L and 0.6 mg/L, respectively) (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004), and at significantly higher concentrations as compared to the control wine. In particular, isobutyl acetate content was 6.1- and 6.5-fold higher in the co-inoculated and sequentially inoculated wines, respectively, as compared to the control (0.23 ± 0.02 mg/L). Isoamyl acetate concentrations exceeded by ~5 times that of the control (30 ± 5 μg/L). Hexyl acetate amounts were 7.2 (co-inoculated) and 14.4 (sequentially inoculated) times that of the control (0.25 ± 0.09 mg/L) (Table 5). Similarly, most of the ethyl ester compounds such as ethyl isovalerate (apple), ethyl butyrate (strawberry, apple, and banana), ethyl hexanoate (green apple, banana, and violet) and diethyl succinate (melon) were significantly higher in both mixed fermentation wines as compared to the control ($p < 0.05$) (Table 5). Accordingly, Domizio et al. (2018), when evaluating the impact of mixed fermentation *Sch. japonicus/S.cerevisiae* in Trebbiano grape must, found significant higher concentrations of various esters in the mixed wines reflecting the additive contribution of *Sch. japonicus*. This aromatic profile is compatible with ethyl acetate undergoing hydrolysis during bottle aging (Lilly, Lambrechts, & Pretorius, 2000; Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008). Indeed, in the course of wine aging chemical reactions of hydrolysis and esterification normally occur, and converge to the levels of ester equilibrium (Shinohara, Shimizu, & Shimazu, 1979; Blake, Kotseridis, Brindle, Inglis, & Pickering, 2010; Ferreira, Escudero, Fernández, & Cacho, 1997; Gallo, Beltran, Heredia, González-Miret & Hernanz, 2011; Garde-Cerdán et al., 2008; Oliveira, J. M., Oliveira, P., Baumes & Maia, 2008; Pérez-Coello, González-Viñas, Garcıa-Romero, Dıaz-Maroto, & Cabezudo, 2003; Robinson et al., 2010, Roussis, Lambropoulos, & Papadopoulou, 2005). Thus, in mixed fermentation wines, the hydrolysis of ethyl acetate might have determined an increase of acetate and ethyl esters and of the volatile acidity. In any case volatile acidity never exceeded unacceptable levels in these wines (Table 2).

3.3. Sensory evaluation

Sensory evaluation carried out after malolactic fermentation and after bottle aging revealed significant differences among the wines obtained with the different inoculation protocols. In particular, three

attributes resulted significantly different after malolactic fermentation: one of taste (bitterness), the tactile descriptor astringency, and the odor descriptor ethyl acetate (Table 6). The two mixed fermentation wines were less astringent than the control, while the co-inoculated wine was perceived more bitter than the other two. The ethyl acetate attribute significantly distinguished the three wines, with the lowest intensity value in the control and the highest in the co-inoculated wine.

After twenty-four months of bottle aging, only the sweetness attribute differed among the trials with the sequential inoculum producing significantly sweeter wine than the control ($p < 0.05$). Sweetness intensity might be ascribed to a higher perceived softness which in turn was possibly due to the higher glycerol and polysaccharides content in mixed fermentation wines. In fact, polysaccharides-polyphenols interaction could prevent precipitation of tannins (Guadalupe & Ayestarán, 2008) or polyphenol aggregation (Riou, Vernhet, Doco, & Moutounet, 2002), resulting in a masking effect on bitterness and astringency. Rinaldi et al. (2019) showed an increase of the perception of volume and roundness of Sangiovese wines after being treated with three different commercial *S. cerevisiae* mannoproteins. According to Escot et al. (2001) mannoproteinis could compete with tannins for interaction with salivary proteins, resulting in reduced precipitation. A positive effect of mannoproteins in reducing astringency was also previously observed (Rinaldi, Gambuti, & Moio, 2012). Other authors have also shown that wine sweetness was related to the release of polysaccharides and of the hydrolysis products of the stress protein Hsp12p in red wines fermented by *S. cerevisiae* (Marchal, Marullo, Moine, & Dubourdieu, 2011). Indeed, the impact of *Sch. japonicus* galacto-mannoproteins on sweetness attribute and wine aroma volatility needs further investigation. O ther authors have observed an increase in the release of some aroma compounds due to a salting out effect (Mitropoulou, Hatzidimitriou, & Paraskevopoulou, 2011). In this context, it is worth pointing out that Rinaldi et al. (2019) found an enhancement of floral and fruity aromas of Sangiovese wines treated with commercial mannoproteins.

4. Conclusions

Based on the results here presented, the inoculum of *Sch. japonicus/S. cerevisiae* starter cultures appears a promising tool for the improvement of Sangiovese wine aroma, taste and color stability. Mixed fermentation wines were perceived as having lower mouthfeel sensation of astringency compared to those obtained with *S. cerevisiae*. Moreover, *Sch. japonicus* in combination with *S. cerevisiae* increased the sweetness perception and color stability and modulated the concentration of some of the most important volatile compounds. This was particularly true for ethyl acetate. Its high concentration at the end of alcoholic fermentation seems to serve as a reservoir for the evolution of volatile ester compounds during wine aging.

Indeed, the *Sch. japonicus/S. cerevisiae* ratio in mixed starter cultures and the timing of inoculation with *S. cerevisiae* have to be optimized. However, the results here presented, although needing to be confirmed in different grape musts, open a new scenario for the exploitation of *Sch. japonicus* and other high ethyl acetate producers yeasts in the wine industry.

CRediT authorship contribution statement

Lorenzo Portaro: Formal analysis, Data curation, interpretation of the data, revising it critically for important intellectual content, Writing – original draft. **Francesco Maioli:** Formal analysis, interpretation of the data, revising it critically for important intellectual content, Writing – original draft. **Valentina Canuti:** Formal analysis, Data curation, interpretation of the data, revising it critically for important intellectual content, Writing – original draft, Writing - review & editing. **Monica Picchi:** Formal analysis, Data curation, interpretation of the data, revising it critically for important intellectual content, Writing – original draft. **Livio Lencioni:** Formal analysis, Data curation, interpretation of the data, revising it critically for important intellectual content, Writing – original draft. **Ilaria Mannazzu:** Formal analysis, Data curation, interpretation of the data, revising it critically for important intellectual content, Writing – original draft, Writing - review & editing. **Paola Domizio:** Conceptualization, Formal analysis, Supervision, interpretation of the data, revising it critically for important intellectual content, Writing – original draft, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare:

✓no conflict of interest.

✓ This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

✓ The following authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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