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Metschnikowia pulcherrima as biocontrol agent and wine aroma enhancer in combination with a native *Saccharomyces cerevisiae*

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ABSTRACT

One of the main objectives for a sustainable winemaking process is the reduction of the use of sulfur dioxide. In this regard, non-*Saccharomyces* wine yeasts are proposed as biocontrol agent in different steps of wine production chain. Here, a selected strain of *Metschnikowia pulcherrima* (DiSVA 269) and a native *Saccharomyces cerevisiae* low sulfite producer strain (DiSVA 708) were investigated. After preliminary laboratory trials, winemaking process at industrial level showed an effective biocontrol action (reduction of c.a. 1 Log order of wild yeasts) of *M. pulcherrima* inoculated at prefermentative stage in cold clarification (48 h at 10 °C) and during the subsequent fermentation process. The combination of *M. pulcherrima/S. cerevisiae* led a distinctive aromatic profile of wines both in laboratory and winery trials with a significant enhancement of ethyl butyrate, ethyl hexanoate, isoamyl acetate and β-phenyl ethanol. Moreover the use of the two selected strains was the best combination to enhance volatile thiols (3-mercaptohexan-1-ol and 3-mercaptoexil acetate) that well correlate with the sensory analysis (tropical fruits). The overall results indicate that the combined use of *M. pulcherrima* DiSVA 269 and native *S. cerevisiae* DiSVA 708 led a biocontrol action and an improvement of aromatic and sensorial profile of wine with low SO_2 content.

1. Introduction

The use of non-*Saccharomyces* selected strains in sequential fermentation with *Saccharomyces cerevisiae* starter strains is a current well-established winemaking strategy to produce wines with distinctive sensorial properties. Among them, *Metschnikowia* is one of the most investigated genera due to its multiple contribution in winemaking. *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, and *Metschnikowia viticola* are the most species naturally found in wine environments with well-established antimicrobial activities (Belda et al., 2016b; Brysch--Herzberg et al., 2015; Morata et al., 2019; Vicente et al., 2020). *M. pulcherrima* is a well characterized species for several positive features in winemaking: Indeed, it can modulate the synthesis of secondary metabolites to improve the sensorial profile of wine and to act as biocontrol agent. (Varela et al., 2016; Zhang et al., 2018). Recently, a selected strain of *M. pulcherrima* in mixed fermentation with two different *S. cerevisiae* strains determined an impact on the analytical and sensorial profile due to an increase in the levels of the thiol 4-MSP (4-methyl-4-sulfanylpentan-2-one) above the sensory threshold, together with a decrease in higher alcohol production (Ruiz et al., 2018). Another important feature of this non-*Saccharomyces* yeast is the wide possess among the strains of the enzymatic activities such as pectinase, protease, glucanase, lichenase, β-glucosidase, cellulase, xylanase, amylase, sulphite reductase, lipase and β-lyase activity (Barbosa et al., 2018; Vicente et al., 2020). Regarding to the proteolytic activity of *M. pulcherrima* is important feature in mixed fermentation to release amino acids as nutrient for *S. cerevisiae* and act as control of protein haze formation in wines as a biological fining agent. (Marangon et al., 2012). Pectinase activity is strain-dependent on *M. pulcherrima* (Hong et al., 2019; Marangon et al., 2012) while the glucosidase activity promotes the release of varietal aromas from the grape (Belda et al., 2016a). *M. pulcherrima* sequential fermentations seem to increase the final amino acid concentration in wine (Benito et al., 2015).

According to several works, when *M. pulcherrima* is used the reduction of volatile acidity seems to be a trend, with variations estimated between 10% and 75% (Hranilovic et al., 2020; Roca-Mesa et al., 2020).

M. pulcherrima can be also used as biocontrol agent, due to the production of pulcherrimin, a red pigment with antifungal activity (Csutak

Abbreviations: 4-MSP, (4-methyl-4-sulfanylpentan-2-one); DiSVA, Dipartimento Scienze della Vita e dell'Ambiente-Dep. Life Environmental Sciences; OIV, Organizzazione Internazionale della Vigna e del Vino.

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et al., 2013; Kántor & Kacániová, 2015; Oro et al., 2018; Saravanakumar et al., 2008). Based on these attitudes, *M. pulcherrima* species could be used as a strategy to contain spoilage microorganisms and reduce the use of sulfur dioxide particularly in organic wine production.

In this context, the investigations are directed to the reduction of compounds that bind SO_2, the management of pH and towards the search of new compounds of natural origin with antimicrobial activity.

A microbiological approach based on the selection of bio-protective strains can be a useful tool to reduce sulfite concentration in wine-making (Di Gianvito et al., 2022; Escribano-Viana et al., 2022; Windholtz et al., 2021). In this way, the use *S. cerevisiae* strains characterized by the absence or the reduced production of sulfur compounds and which nevertheless highlight the aromatic imprint of the wine is one of the main goals of researchers to satisfy winemakers and consumer requests (Agarbati et al., 2020; Linderholm et al., 2010).

In this work, the combined use of different yeast strains, each with a specific functional role during fermentation and both enhancers of the final wine aroma, could contribute to improve the overall wine quality. Selected strains of *M. pulcherrima* and native *Saccharomyces cerevisiae* have been set up with a dual role: i) play a biocontrol activity with consequent reduction in added sulphites; ii) enhance the final wine aroma profile. The suitability of the studied yeast strains to naturally control the fermentation process will be addressed.

2. Materials and methods

2.1. Yeast strains

M. pulcherrima strain DiSVA 269, already characterized for its biocontrol ability, was used (Oro et al., 2014b). The *S. cerevisiae* native strain DiSVA 708, previously selected and featuring (Agarbati et al., 2020), was used as fermenting yeast. A commercial *S. cerevisiae* starter strain, Lalvin ICV OKAY® (Lallemand Inc., Toulouse, France) were used as control commercial strain. YPD agar medium (yeast extract 1%, peptone 2%, dextrose 2% and agar 1.8%) at 4 °C was used for short-term storage while for long-term storage was used YPD broth supplemented with 80% (w/v) glycerol at −80 °C.

2.2. Preliminary laboratory scale fermentation trials

The Verdicchio grape juice (vintage 2017) was used for laboratory scale fermentations. The main composition of grape juice was as follows: pH 3.22; initial sugar content 212 g/L; total acidity 4.58 g/L; malic acid 2.7 g/L; nitrogen content YAN (60 mg/L) and total SO_2 27 mg/L. Fermentations were conducted in 250 mL Erlenmeyer flasks locked with a Müller valve containing 200 mL of Verdicchio grape juice at the temperature of 22 °C ± 0.5 under static condition in triplicate. Pre-cultures of strains were carried out using modified YPD (0.5% w/v yeast extract, 2% w/v glucose, and 0.1% w/v peptone) in an orbital shaker (150 rpm) at 25 °C for 24h. The inoculum grape juice was carried out at an initial concentration of approximately 1×10^6 cells/mL. Sequential fermentations were carried out inoculating *M. pulcherrima*, followed after 48 h, by *S. cerevisiae* DiSVA 708 and OKAY®, respectively. Pure cultures of *S. cerevisiae* were used as control trial. The weight loss of the apparatus due to the CO_2 evolution was monitored to evaluate the fermentation kinetics until constant weight (for 2 consecutive days).

2.3. Fermentation trials in winery at industrial level

After the preliminary laboratory trials, *M. pulcherrima* DiSVA 269 strain was inoculated at pre-fermentative stage (during clarification procedures) followed by the inoculation of native *S. cerevisiae* DiSVA 708 or commercial strain OKAY®, or to carry out the fermentation process at industrial level.

2.3.1. Preparation of starter inoculum

To prepare the inoculum all the yeast strains were pre-cultured using a modified YPD medium (0.5% yeast extract, 0.1% peptone and 2% glucose) for 48 h at 25 °C under agitation (150 rpm). 30-L bioreactor (Biostat® C; B. Braun Biotech Int., Goettingen, Germany) containing 25 L of modified YPD was then inoculated (5% vol/vol). Fermentation condition were: 400 rpm/min; air flow of 1 vvm (L/L/min). Yeast biomass production was carried out using a feed batch procedure and, at the end of the process, the cells were collected by centrifugation, and washed three times with sterile distilled water. The inoculum of grape juice was conducted in the form of cream (80% humidity) at a concentration of approximately 1×10^6 cell/mL. This cell concentration was used for *M. pulcherrima* before cold clarification and for both *S. cerevisiae* starter strains for fermentation of the respective vats. The growth kinetics of the yeast strains were monitored during the fermentation at established time.

2.3.2. Winemaking process procedures

Fermentation trials were performed using Verdicchio grape juice coming from vintage 2020. Freshly harvested grapes) were treated following the standard winemaking procedure: soft pneumatic pressing, cold clarification without SO_2 addition at 10 °C for 48 h. The analytical characters of the grape musts were initial sugars 216 g/L, pH 3.34, total acidity 4.37 g/L, malic acid 1.7 g/L, and nitrogen content 90 mg/L. Yeast assimilable nitrogen were adjusted to 250 mg N/L with diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France).

Using two consequential Verdicchio grape juice lots of 600 hL each coming from two consecutive working days (1° and 2° lots), four vats of 300 hL were filled: two of these were inoculated with 1×10^6 cells/mL of *M. pulcherrima* DiSVA 269 strain to assess the potential biocontrol action during cold clarification during 48 h, while the other two vats were not inoculated. After 48 h the four vats were inoculated with *S. cerevisiae* DiSVA 708 and OKAY® strains, respectively with the following scheme:

M. pulcherrima DISVA 269/*S. cerevisiae* OKAY® (1° lot).
S. cerevisiae OKAY® (1° lot).
M. pulcherrima DISVA 269/*S. cerevisiae* DiSVA 708 (2° lot).
S. cerevisiae DiSVA 708 (2° lot).

The fermentations were carried out at 19 ± 1 °C and were monitored by sugar consumption using Baumé (°Bé) densimeter.

2.3.3. Monitoring of yeast population

The evolution of the wild and inoculated yeast strains was followed during the fermentation by viable cell count using lysine agar medium (Oxoid, Hampshire, UK) as selective medium for non-*S. cerevisiae* strains and WL nutrient agar medium (Oxoid, Hampshire, UK) for the differential recognition of form and color diversity of colony. The plates, after incubation at 25 °C for four days, were evaluated for the detection of inoculated and wild yeasts. The combination of the results of lysine agar enumeration and macro- and micro-morphological estimation in WL nutrient agar medium permitted the distinction between inoculated and wild yeasts. The presumptive identities of the yeasts were confirmed by sequencing using ITS 1 and 4 as target region. The ITS1-5.8S rRNA-ITS2 region was amplified by PCR (Polymerase Chain Reaction) using primer pair ITS1 (50-TCCGTAGGTGAACCTCGCG-30) and ITS4 (50-TCCTCCGCTTTATTGATATGC-30), as described by White et al. (1990). The BLAST program and the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) were used to compare the sequences provided with those already in the data library. With the aim to discriminate the inoculated *S. cerevisiae* strains (native or commercial starter) by wild strains, intraspecies characterization of isolates were carried out using primer pairs δ 12/21 as described by Legras & Karst, 2003. The length of the PCR products was estimated by comparing them with 100-bp marker DNA standards (GeneRuler 100-bp DNA Ladder; AB Fermentas).

2.4. Analytical procedures

Total acidity (OIV-MA-AS313-01), volatile acidity (OIV-MA-AS313-02), pH (OIV-MA-AS313-15), and ethanol content (OIV-MA-AS313-24) were evaluated according to the use the standard methods of OIV (https://www.int/standards/compendium-of-international-methods-of-wine-and-must-analysis, *OIV*). Enzymatic kits (Megazyme International Ireland) were utilized to determine glucose and fructose (K-FRUGL) and malic acid (K-DMAL) following the manufacturer procedures. The ammonium content was determined using a specific enzymatic kit (kit no. 112732; Roche Diagnostics, Germany) while the free α-amino acids were evaluated following Dukes and Butzke protocol (1998). Ethyl acetate, acetaldehyde, and higher alcohols were quantified by direct injection using a gas-chromatograph with flame ionization detector (GC-2014; Shimadzu, Kjoto, Japan). The final wines, prepared following the instruction of Canonico et al. (2018), were analyzed to quantify the main volatile compounds as described by Canonico et al. (2019) using the solid-phase microextraction (HS-SPME) method with the fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Sigma-Aldrich, St. Louis, MO, USA).

Volatile thiols 3-mercaptohexan-1-ol (3-MH), 3-mercaptohexylacetate (3-MHA), were determined by derivatization and SPE online extraction and High-Performance Liquid Chromatography−Tandem Mass Spectrometry (HPLC-MS/MS) following the methodologies of Capone et al. (2015). Internal standard calibration was used to quantify the thiols concentration in the wine samples.

2.5. Sensory analysis

At the end of the fermentation carried out in winery, the wines were transferred into full 750 mL bottles with 30 mg/L of SO_2 closed with the crown cap and maintained at 4 °C until sensory analysis. After 6 months of refinement, the wines were subjected to sensory analysis based on principal sensory category. A group of 15 trained testers, 10 males and 5 females aged 25–45 years and composed by oenologists, sommeliers and wine producers, conducted the sensory analysis. The score scale was from 1 to 10, where 10 was the score that quantitatively represented the best judgment (maximum satisfaction), while 1 was the score to be attribute in case of very poor satisfaction. The sensory analysis was conducted from 10:00 to 12:00 a.m. in the following ways: 30 mL of each wine were served at 22 ± 1 °C (room temperature) in glasses labeled with code and covered to prevent volatile loss. The order of presentation was randomized among judges.

2.6. Statistical analysis

The data of analytical character of wines were elaborated using the analysis of variance (ANOVA). The means were analyzed using the statistical software package JMP® 11. The significant differences were detected using Duncan tests and the experimental data were significant with a p-values <0.05.

3. Results

3.1. Fermentation trials at laboratory scale: biomass evolution and main volatile compounds

The growth kinetics of pure (control) and sequential fermentations carried out at lab scale are reported in Fig. 1. *S. cerevisiae* pure fermentations carried out using native DiSVA 708 strain and starter strain OKAY® as control, respectively are reported in Fig. 1a and b. As expected, native and commercial strains showed a similar behavior between them, as well as the wild yeasts population exhibited an initial comparable trend in both fermentations achieving over 10^7 CFU/mL at 5th day of fermentation. After that, *S. cerevisiae* DiSVA 708 (Fig. 1a) led a slower decrease of wild yeasts from 5th day until the end of fermentation in comparison with *S. cerevisiae* OKAY® (Fig. 1b) that disappear after 8 days.

During sequential fermentations with *M. pulcherrima* DiSVA 269, wild yeasts did not exceed 10^6 CFU/mL disappearing in both cases (Fig. 1c and d) at 8th day of fermentation. Both S. *cerevisiae* strains inoculated after 48 h, maintained the similar trend observed during pure fermentations, while *M. pulcherrima* DiSVA 269 population disappeared after the 8th day either in the presence of *S. cerevisiae* DiSVA 708 and OKAY®.

These preliminary results indicated that *M. pulcherrima* DiSVA 269 with both native *S. cerevisiae* DiSVA 708 and OKAY ®strains determined an effective control on the development of wild yeasts.

However, the analysis of the main volatile compounds of resulting wines (Table 1) showed that *M. pulcherrima* DiSVA 269 in sequential

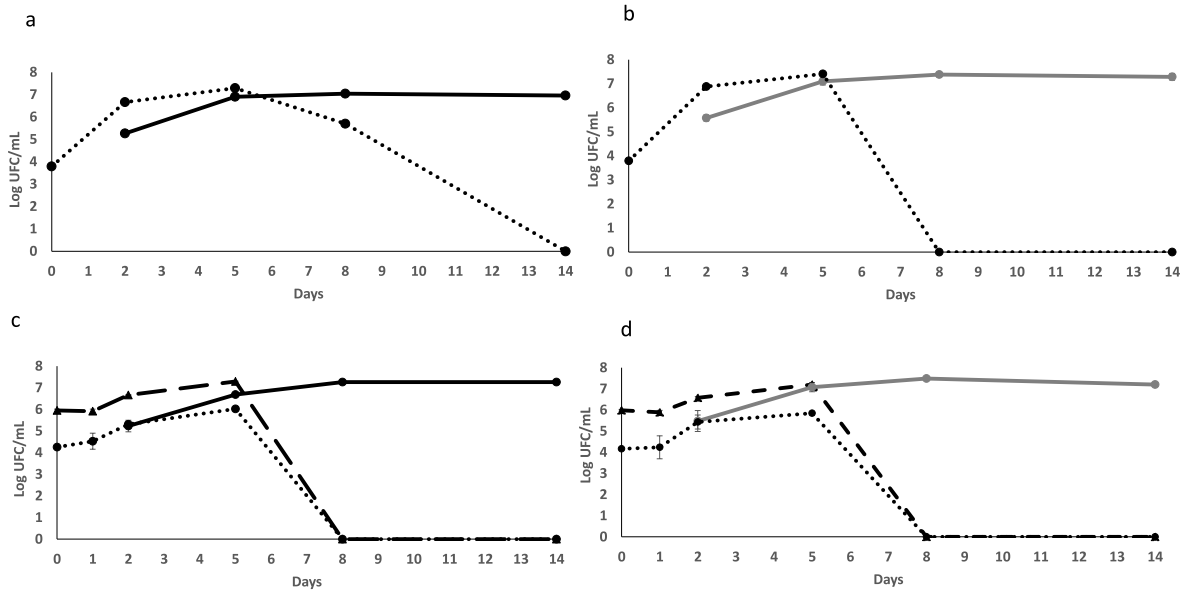

Fig. 1. Growth kinetics of pure and sequential fermentations carried out at lab scale. a) *S. cerevisiae* DiSVA 708 pure fermentation and b) *S. cerevisiae* OKAY® pure fermentation; c) *M. pucherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* DiSVA 708; d) *M. pucherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* OKAY®. *S. cerevisiae* D (—●—); OKAY® (—●—); *M. pulckerrima* (—▲—) and Wild yeasts (••●••).

Table 1
Some main volatile compounds (mg/L) of the fermentation trials carried out at laboratory scale. Data are means ± standard deviations. Values displaying different superscript letters (^{a,b,c,d}) within each line are significantly different according to Duncan tests ($p < 0.05$).

	S. cerevisiae DiSVA708	*M. pulcherrima*/*S. cerevisiae* DiSVA708	*S. cerevisiae* OKAY	*M. pulcherrima*/*S. cerevisiae* OKAY
Ethyl butyrate	0.121 ± 0.016^{b}	0.410 ± 0.033^{a}	0.429 ± 0.016^{a}	0.453 ± 0.07^{a}
Isoamyl acetate	0.867 ± 0.172^{d}	1.08 ± 0.23^{c}	1.630 ± 0.031^{b}	2.493 ± 0.13^{a}
Ethyl exanoate	0.107 ± 0.020^{b}	0.147 ± 0.006^{a}	0.063 ± 0.012^{c}	0.048 ± 0.00^{c}
Hexanol	0.012 ± 0.001^{c}	0.013 ± 0.006^{c}	0.054 ± 0.009^{a}	0.038 ± 0.00^{b}
Linalol	0.079 ± 0.044^{b}	0.117 ± 0.055^{a}	0.043 ± 0.007^{b}	0.062 ± 0.01^{b}
β-Phenyl Ethanol	33.4 ± 0.05^{b}	57.8 ± 0.072^{a}	42.2 ± 0.019^{b}	32.5 ± 0.010^{b}

fermentations with both *S. cerevisiae* strains differently influenced the main volatile compounds of the final product. Indeed, sequential fermentation *M. pulcherrima*/OKAY® led only significant increase of isoamyl acetate, while sequential fermentation *M. pulcherrima/S. cerevisiae* DiSVA 708 determined a significant enhancement of several volatile compounds as ethyl butyrate, isoamyl acetate, ethyl hexanoate, β-phenyl ethanol and linalool, indicating a possible positive interaction in the formation of these compounds.

3.2. Fermentation trials in winery at industrial level

3.2.1. M. pulcherrima DiSVA 269 as biocontrol agent during clarification procedures

Based on the results obtained at laboratory scale, the selected strain *M. pulcherrima* was used at pre-fermentative stage in cold clarification and then inoculated with *S. cerevisiae* as reported above.

Results reported in Fig. 2 showed that the presence of *M. pulcherrima* determined a significant reduction (approximately 1 log, 90% of reduction) of wild yeast population, mainly represented by *H. uvarum* (data not shown), in both inoculated vats, while in the vats without the inoculum of *M. pulcherrima,* no wild yeast population reduction was shown.

3.2.2. Biomass evolution and sugar consumption of fermentation processes

Growth kinetics of fermentations inoculated and uninoculated with *M. pulcherrima* strain are reported in Fig. 3. The results showed that *S. cerevisiae* DiSVA 708 exhibited a similar trend in comparison to OKAY®. Indeed, the two *S. cerevisiae* strains (Fig. 3 a, b) exhibited the maximum cell concentration at 7th day of fermentation (c.a 10^8 cell/ml) to remain constant until the end of fermentation. The results showed that *S. cerevisiae* starter strain OKAY® exhibited a more effective control on the wild yeasts in comparison to DiSVA 708. However, in both fermentation trials the wild yeasts disappear a 7th day. *M. pulcherrima* sequential fermentation with OKAY® (Fig. 3d) showed a decrease of wild yeasts to disappear at 3rd day of fermentation. Moreover, the biomass evolution of OKAY® did not affect by *M. pulcherrima*. The inoculum of *M. pulcherrima* DiSVA 269 improved the control on wild yeasts in both inoculated fermentations even though in *M. pulcherrima/S. cerevisiae* DiSVA 708 fermentation showed a lower control on wild yeasts if compared to *M. pulcherrima*/OKAY® fermentation.

Regarding the sugar consumption (Fig. 4), all fermentations exhibited a similar trend in fermentation kinetics with the only exception of *S. cerevisiae* DiSVA 708 pure culture that exhibited a slower sugar consumption that other trials. All fermentations showed a complete sugar consumption at the end of fermentation. Moreover, the results highlighted a positive interaction on fermentation kinetics of *M. pulcherrima* when used in sequential fermentation with *S. cerevisiae* DiSVA 708.

3.2.3. Frequency and dominance of S. cerevisiae starter strains

The results using of interdelta sequences indicated that, *S. cerevisiae* DiSVA 708 showed a lower ability to dominate the fermentation process carried out at industrial level (Table 2). Indeed, *S. cerevisiae* DiSVA 708 was 60% in both pure and sequential fermentation while the commercial starter strain OKAY® showed a percentage of was 80 and 90% in pure

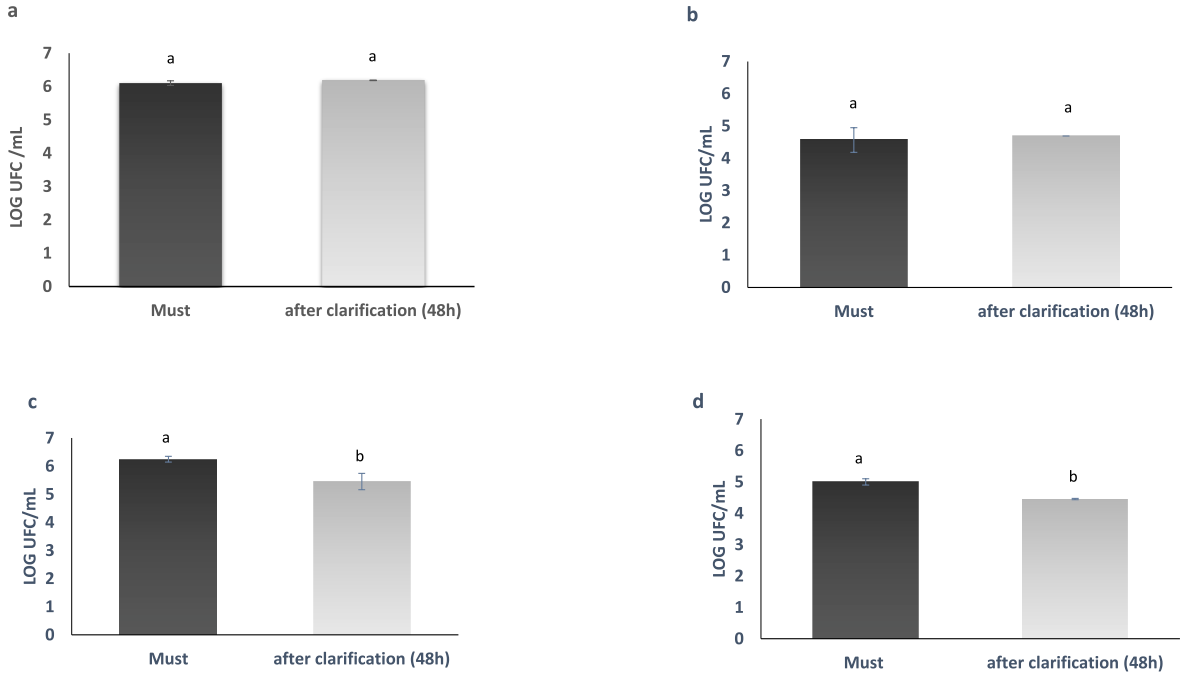

Fig. 2. Effect of *M. pulcherrima* DiSVA 269 on wild yeasts after clarification (48h). 1° Lot: a) with *M. pulcherrima* inoculum and b) without inoculum respectively; 2 Lot c) with *M. pulcherrima* inoculum and d) without inoculum respectively.

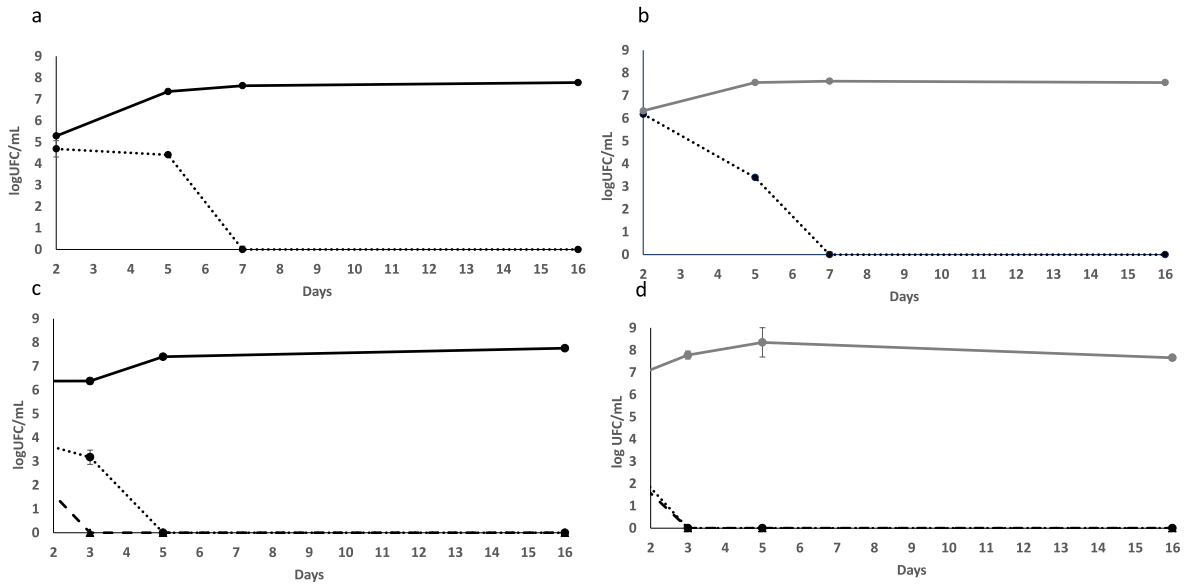

Fig. 3. Growth kinetics of pure and sequential fermentations carried out at industrial level. a) *S. cerevisiae* DiSVA 708 pure fermentation and b) *S. cerevisiae* OKAY® pure fermentation; c) *M. pucherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* DiSVA 708; d) *M. pucherrima* sequential fermentation with *S. cerevisiae* OKAY®. *S. cerevisiae* DiSVA 708 (—●—); OKAY® (—●—); *M. pulcherrima* DiSVA 269 (—▲ -) and Wild yeasts (••●••).

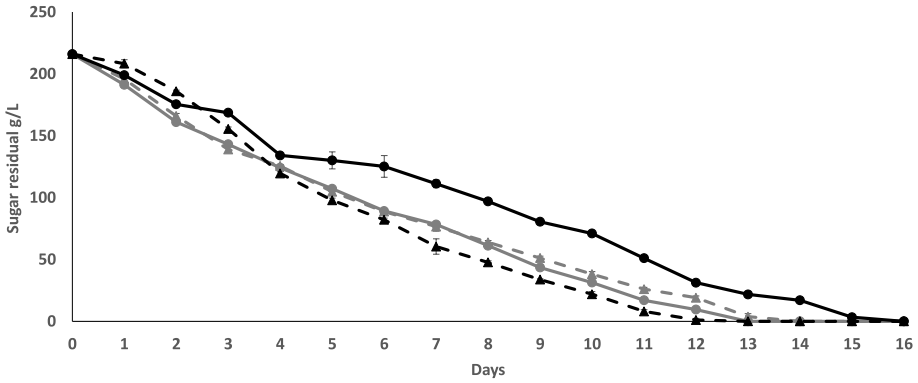

Fig. 4. Kinetics of sugar consumption of the pure and sequential fermentation carried out at industrial level. *S. cerevisiae* Disva 708 (—●—); OKAY® (—●—); *M. pulcherrima* DiSVA 269/*S. cerevisiae* Disva 708 (—▲ -) and *M. pulcherrima*/OKAY® (- -▲- -).

Table 2
Percentage values of isolates of *S. cerevisiae* detected close to the end of fermentation process in the fermentation assayed.

Fermentation trials	% of isolates
M. pulcherrima DISVA 269/*S. cerevisiae OKAY®*	90
S. cerevisiae OKAY®	80
M. pulcherrima DISVA 269/*S. cerevisiae* DiSVA 708	60
S. cerevisiae DiSVA 708	60

and sequential fermentation, respectively.

3.2.4. Main oenological characters of wine

The results of the main analytical characters of wines are shown in Table 3. The presence of *M. pulcherrima* during cold clarification showed a significant reduction of total and volatile acidity indicating its influence in both *S. cerevisiae* starter strains. The production of malic acid is comparable in both pure *S. cerevisiae* fermentations, while the *M. pulcherrima*/OKAY® showed a significant lower malic acid content.

3.2.5. Volatile compounds of wine

In Table 4 are shown the results concerning the principal volatile

Table 3
Chemical characterization of resulting wine. The analytical characters of the grape musts were initial sugars 216 g/L, pH 3.34, total acidity 4.37 g/L, malic acid 1.7 g/L, and nitrogen content 90 mg/L.
Data are means ± standard deviations. Values displaying different superscript letters (^{a,b,c}) within each column are significantly different according to Duncan tests ($p < 0.05$).

	Ethanol (%v/v)	Total Acidity (Tartatic Acid g/L)	Volatile Acidity (Acetic Acid g/L)	Malic Acid (g/L)
M. pulcherrima/ OKAY	13.90 ± 0.02^{a}	5.07 ± 0.04^{c}	0.24 ± 0.01^{b}	1.25 ± 0.07^{b}
OKAY	14.06 ± 0.09^{a}	5.41 ± 0.03^{b}	0.25 ± 0.01^{ab}	1.55 ± 0.07^{a}
M. pulcherrima/*S. cerevisiae* DiSVA 708	13.92 ± 0.04^{a}	5.12 ± 0.014^{c}	0.21 ± 0.02^{b}	1.5 ± 0.14^{a}
S. cerevisiae DiSVA	13.93 ± 0.06^{a}	6.28 ± 0.01^{a}	0.29 ± 0.014^{a}	1.55 ± 0.07^{a}

Table 4
The main by-products and volatile compounds in final wines in presence and absence of *M. pulcherrima.*
MP: *M. pulcherrima*; OAV: Odor Activity value
Data are the means ± standard deviation. Data with different superscript letters (^{a,b,c,d}) within each row are significantly different (Duncan tests; $p < 0.05$).

	MP OKAY	OAV	OKAY	OAV	MP DiSVA 708	OAV	DiSVA 708	OAV
Esters (mg/L)								
Ethyl butyrate	0.126 ± 0.00^{b}	0.31	0.064 ± 0.035^{b}	0.16	0.303 ± 0.075^{a}	0.757	0.148 ± 0.01^{b}	0.37
Ethyl acetate	27.01 ± 0.39^{b}	2.25	42.96 ± 0.36^{a}	3.58	25.67 ± 0.83^{b}	2.13	12.28 ± 0.97^{c}	1.02
Phenyl ethyl acetate	0.038 ± 0.00^{c}	0.52	ND		0.10 ± 0.01^{a}	1.36	0.049 ± 0.02^{b}	0.067
Ethyl exanoate	0.194 ± 0.011^{a}	2.42	0.041 ± 0.003^{b}	0.51	0.130 ± 0.020^{a}	1.625	0.037 ± 0.001^{b}	0.462
Ethyl octanoate	0.005 ± 0.00^{a}	0.008	0.006 ± 0.000^{a}	0.01	0.005 ± 0.001^{a}	0.0086	0.002 ± 0.000^{a}	0.003
Isoamyl acetate	0.914 ± 0.28^{ab}	5.71	0.517 ± 0.171^{ab}	3.23	1.029 ± 0.314^{a}	6.43	0.307 ± 0.001^{b}	1.91
Alcohols (mg/L)								
n- propanol	75.83 ± 0.33^{b}	0.247	104.79 ± 5.04^{a}	0.342	31.83 ± 0.15^{c}	0.104	13.98 ± 0.18^{d}	0.045
Isobutanol	11.54 ± 0.76^{b}	0.288	13.83 ± 0.57^{a}	0.345	13.12 ± 0.52^{ab}	0.328	13.79 ± 0.83^{a}	0.344
Amyl alcohol	6.41 ± 0.90^{b}	0.1	9.76 ± 0.09^{a}	0.15	9.87 ± 3.41^{a}	0.15	19.50 ± 1.35^{b}	0.304
Isoamyl alcohol	89.49 ± 1.08^{b}	1.41	110.18 ± 0.01^{a}	1.83	13.99 ± 7.09^{c}	0.23	94.73 ± 3.08^{b}	1.57
β-Phenyl Ethanol	13.12 ± 0.33^{ab}	0.92	16.05 ± 0.20^{a}	1.14	19.04 ± 0.27^{a}	1.36	8.08 ± 0.23^{b}	0.57
Carbonyl Compounds (mg/L)								
Acetaldehyde	1.40 ± 0.24^{c}	2.8	3.80 ± 0.93^{c}	7.6	7.97 ± 1.16^{b}	15.94	13.98 ± 1.36^{a}	27.96
Monoterpenes (mg/L)								
Linalool	0.18 ± 0.100^{ab}	3.2	0.371 ± 0.147^{a}	14.84	0.221 ± 0.054^{ab}	8.84	0.028 ± 0.008^{b}	1.12
Geraniol	0.025 ± 0.01^{a}	0.83	0.036 ± 0.015^{a}	1.2	0.038 ± 0.012^{a}	1.26	0.014 ± 0.008^{a}	0.466
Nerol	0.074 ± 0.05^{a}	4.93	0.202 ± 0.140^{a}	13.46	0.136 ± 0.022^{a}	9.06	0.028 ± 0.008^{a}	1.86
Thiols (ng/L)								
3-mercaptohexan-1-ol	367.1 ± 0.0^{b}	6.11	35.7 ± 0.0^{d}	0.59	1215.1 ± 0.0^{a}	20.25	190.6 ± 0.00^{c}	3.17
3-mercaptoexil acetate	388.9 ± 0.0^{a}	92.56	52.8 ± 0.0^{c}	12.57	181.8 ± 0.0^{b}	43.28	17.4 ± 0.00^{d}	4.14

compounds. Regarding to the esters content, the presence of *M. pulcherrima* (inoculation at cold clarification stage) led an increase in ethyl butyrate, ethyl hexanoate, phenyl ethyl acetate and isoamyl acetate content that resulted significant with the starter *S. cerevisiae* DiSVA 708. *M. pulcherrima/S. cerevisiae* OKAY® showed the only appearance of phenyl ethyl acetate. Regarding to the higher alcohols both *S. cerevisiae* fermentations without the inoculation of *M pulcherrima* were characterized by a high final content of amylic alcohols that were strongly reduced in inoculated fermentations with *M. pulcherrima. S. cerevisiae* OKAY® was characterized by a high production of n-propanol (in both fermentations) while *M. pulcherrima* determined a significant increase in β-phenyl ethanol but only with DiSVA708. Regarding to the monoterpenes a relevant high content was detected for linalool in OKAY® fermentation trials and in presence of *M. pulcherrima*/DiSVA708 in comparison with DiSVA708 pure culture. while no significant differences were shown for the other terpenes production. The acetaldehyde was significant higher in wine fermented by DiSVA708. The presence of *M. pulcherrima* led a significant increase of 3-mercaptohexan-1-ol and 3-mercaptoexil acetate particularly in *M. pulcherrima*/DiSVA708 trial.

The enhancement of volatile compounds found with *M. pulcherrima* in the laboratory trials were substantially confirmed by the results obtained in the winery, particularly with the DiSVA708 starter strain. These results, confirming the positive role on fruity characters and wine complexity of *M. pulcherrima*, also indicated differences in the interactions with *S. cerevisiae* starter strain.

3.2.6. Sensory analysis

To establish a further role of *M. pulcherrima* in aroma complexity. the wines produced with and without it in cold clarification, were undergo to sensory analysis. Results reported in Fig. 5 highlighted a general positive appreciation by the tasters of the wines, each distinguished by specific aromatic notes and without defects. Wines obtained with pure *S. cerevisiae* DiSVA708 were perceived more balanced and structured and significantly characterized by citrusy, and softness note, with a low perception of bitter notes. Instead, *M. pulcherrima/S. cerevisiae* DiSVA 708 led a wine with tropical fruit notes. This result matches the results for the main volatile compounds evaluated. Indeed, the fermentation carried out with *M. pulcherrima/S. cerevisiae* DiSVA708 showed a

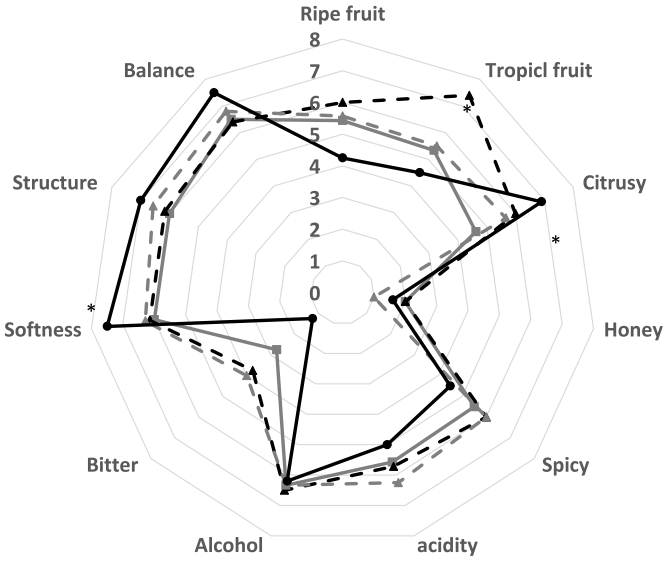

Fig. 5. Sensory analysis of Verdicchio wine fermented by *S. cerevisiae* DiSVA 708 (—●—)and OKAY® (—■—) pure fermentation; *M. pulcherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* DiSVA 708 (—▲—)and OKAY®.

significant increase in 3-mercaptohexan-1-ol and 3-mercaptoexil acetate responsible of passion fruit and grapefruits notes.

No significant differences were shown regarding to the other aromatic descriptors.

4. Discussion

Nowadays great attention is focused on the concept of bioprotection, consisting of the inoculation viable antagonist microorganisms (bacteria, yeasts, or a mixture of them) or the addition of their antimicrobial products in purified form, during, at the end or after the production chain of food and beverages (Comitini et al., 2017; Di Gianvito et al., 2022; Oro et al., 2014a; Simonin et al., 2020). Biological

control implies the reduction or even the elimination of chemical compounds such as sulfur dioxide (SO_2) the most common chemical additive used by winemakers (zhang et al., 2013). leading to the production of high-quality wines with higher add value. In this regard, bio-protection is a tool in fast development, and several formulations based on viable microorganisms or on their antimicrobial compounds have recently started to be proposed in agriculture and food industry (Di Gianvito et al., 2022). To protect grapes, strawberry, and tomato against *Penicillium, Botrytis* and *Monilinia,* a commercial preparation with *Aureobasidium pullulans* has been set up, while a product based on *M. fructicola i*s employed to protect strawberry, blueberry, grape, stone fruit, and pome against *Botrytis, Penicillium, Rhizopus, Aspergillus* and *Monilinia* spp. (Mukherjee et al., 2020; Zhang et al., 2020).

In the winemaking sector several technological alternatives were set up able to control microorganisms such as ultrasound, ultraviolet radiation, pulsed electric field, electrolyzed water, high hydrostatic pressure pre-treatments, or the addition of lysozyme, sorbic acid, dimethyl dicarbonate and chitosan (Guerrero & Cantos-Villar, 2015), a valid and complete substitute for SO_2 has not been found, particularly during prefermentative stage (Giacosa et al., 2019).

The biocontrol action by using non-*Saccharomyces* yeasts have been proposed as a possible alternative to sulphite addition (Comitini et al., 2011). In this regard, be going to appear recent studies carried out by using selected strains of *M. pulcherrima* or a mix of *M. pulcherrima* and *T. delbrueckii* in the red winemaking process at the prefermentative stage (Chacon-Rodriguez et al., 2020; Simonin et al., 2020; Windholtz et al., 2021).

Here, after the preliminary sequential fermentations carried out at laboratory level the biocontrol action found in *M. pulcherrima* DiSVA 269 were confirmed under winery condition, where the biocontrol was exerted at prefermentative stage of a white winemaking process of Verdicchio grape juice determining an improvement of the control on wild yeasts during the subsequent fermentation process.

Other important feature of this oenological practice is the effects on analytical and volatile compounds. The combined use of *M. pulcherrima* DiSVA 269 and native *S. cerevisiae* DiSVA 708 showed a relevant impact on the aromatic profile of wines in both laboratory and winery trials. Indeed, the combined use of the yeasts led a significant enhancement of ethyl hexanoate, isoamyl acetate, phenyl ethyl acetate and β-phenyl ethanol with an OAV higher than 1 (calculated with OTH reported in Table1s supplementary materials). Higher alcohols also contribute to define the overall sensory characteristics of wines. Although the higher alcohols contribute positively to the overall wine flavors, low levels (below 300 mg/L) increased the perception of the varietal aroma of grapes (Escribano et al., 2018). In our study the presence of *M. pulcherrima,* reducing the higher alcohols, may contribute to emphasize the specificity of Verdicchio grapes.

Another relevant result of the investigation regarding to the yeast-yeast interactions in metabolome profiling of wines. *M. pulcherrima* DiSVA 269/*S. cerevisiae* DiSVA 708 turned out the best combination led and enhancement of volatile thiols (3-mercaptohexan-1-ol and 3-mercaptoexil acetate) that good correlate with the sensory analysis of tropical fruit note (Ruiz et al., 2018; Vicente et al., 2020; Zott et al., 2011). *M. pulcherrima* was indicated in several works to improve the concentration of volatile compounds (Zott et al., 2011; Ruiz et al., 2018, Vicente et al., 2020). Indeed, this species positively contribute to volatile thiol release in wines, especially during the pre-fermentation stage in winemaking, (Zott et al., 2011). Intriguingly, it is needed to underline the different results of *M. pulcherrima* strain with the two different *S. cerevisiae* starters strains indicating that different interaction between the inoculated yeasts can take place. In this regard, native *S. cerevisiae* DiSVA 708 was specifically improved and selected for specific characters such as low sulfites production and valuable analytical a sensory profile (Agarbati et al., 2020). The combined use of *M. pulcherrima* DiSVA 269 an *S. cerevisiae* DiSVA 708 specifically selected for the sulfites reduction showed a valuable result.

In conclusion, the results indicate that, under winery condition, the combined use of *M. pulcherrima* DiSVA 269 at the prefermentative stage during cold clarification exerted an effective biocontrol toward wild yeast population. The combination with the native *S. cerevisiae* DiSVA 708 enhanced some aromatic and sensorial characters producing a wine with distinctive features and low SO_2 content.

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Laura Canonico: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – original draft, Writing – review & editing, Supervision. **Alice Agarbati:** Data curation, Validation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Edoardo Galli:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. **Francesca Comitini:** Conceptualization, Investigation, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Maurizio Ciani:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114758.

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