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Metschnikowia pulcherrima in Cold Clarification: Biocontrol Activity and Aroma Enhancement in Verdicchio Wine

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Abstract: Non-*Saccharomyces* wine yeasts are not only proposed to improve the sensory profile of wine but also for several distinctive promising features. Among them, biocontrol action at different steps of the wine production chain could be a suitable strategy to reduce the use of sulfur dioxide. In this work, the activity of a selected strain of *Metschnikowia pulcherrima* was evaluated as inoculum in cold clarification with the aim to reduce SO_2 and improve the aromatic profile of the wine. Fermentation processes were carried out at the winery level for two consecutive vintages using a *pied de cuve* as the starter inoculum coming from indigenous *Saccharomyces cerevisiae* strains. *M. pulcherrima* revealed an effective bio-protectant action during the pre-fermentative stage even if the timely and appropriate starter inoculum in the two years permitted the effective control of wild yeasts during the fermentation also in the control trials. In general, the main oenological characters did not show differences if compared with an un-inoculated trial, while the inoculum of *M. pucherrima* in cold clarification determined an enhancement of ethyl hexanoate, isobutanol, acetaldehyde, and geraniol even if they are considered in different amounts for each year. Indeed, the analytical and sensory profiles of wines were also influenced by the vintage and variation *pied the cuve* population. Nonetheless, the overall results indicated that *M. pulcherrima* led to biocontrol action and an improvement of the aromatic and sensory profile of the wine.

Keywords: *Metschnikowia pulcherrima*; bioprotectant; aroma profile; Verdicchio wine; winery level

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1. Introduction

Wine consumers are increasingly attentive to the diversification of distinctive styles but also to the negative impact that chemical preservatives have on human health [1]. In the wine industry, the most common chemical additive is sulphur dioxide (SO_2). This additive is considered an essential tool for winemakers due to its low-cost and its combined antioxidant and antimicrobial properties against a wide spectrum of microorganisms [2]. SO_2 is used to decrease undesirable microorganisms, reduce the oxidation of phenolic compounds, and improve the quality and the shelf life of wine during the various stages of the wine production [3]. Sulfites pose a problem for human health, especially for sensitive consumers [4]. Sulphur dioxide is related to headaches, allergic reactions, and breathing difficulties in asthma patients. For these reasons, the use of this additive is strictly controlled by European Union legislation and reductions in all food and beverages products are required.

Several technological approaches were proposed to control wine spoilage microorganisms [5], even if a definitive substitute for SO_2 has not been proposed, especially for wines stored for a long time. In recent years, the use of microorganisms as bioprotective agents or their antimicrobial products was extensively investigated particularly at the prefermentative stage [6–8]. Currently, species such as *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, and *Lachancea thermotolerans* are the most applied in wine protection [9]. This strategy is based on the inoculum of viable antagonist microorganisms or their antimicrobial products

during, at the end, or after the wine fermentation [10–12]. In wine making, bio-protectant strains can be a useful tool to reduce or replace sulfite addition [8,13,14].

In recent years, the increased number of studies has led to a better understanding of the impact of mixed fermentation on overall wine quality. Among non-*Saccharomyces* yeast, *M. pulcherrima* was one of the most investigated species for its positive contribution to wine making, both as a bio-protectant agent and owing to its effect on analytical and sensory wine traits [15–17]. *M. pulcherrima* is a well-characterized species for several positive aspects of wine making. Indeed, its metabolic characteristics are the synthesis of secondary metabolites to improve the volatile profile of the wine and act as a biocontrol agent. In mixed fermentation, *M. pulcherrima* led to a reduction of ethyl acetate compound, favoring the formation of 2-phenylethyl acetate, an increase of acetate esters, β-damascenone, and higher alcohols, particularly isobutanol and 2-phenyl ethanol [18,19]. The action of *M. pulcherrima* manifests as a biocontrol agent due to the production of pulcherrimin, a red pigment with antifungal activity [14] that exhibited wide antimicrobial activity against yeasts such as the *Candida, Brettanomyces/Dekkera*, *Hanseniaspora*, and *Pichia* genera as well as filamentous fungi such as the *Botrytis, Penicillium*, *Alternaria*, and *Monilia* genera [20–23].

In this work a selected *M. pulcherrima* strain previously characterized for its antimicrobial activity [24] was inoculated at cold clarification stage under winery condition for two consecutive vintages. The impact of *M. pulcherrima* toward the wild yeasts and the contribution on the aroma and sensory profile of wine was evaluated.

2. Materials and Methods

2.1. Fermentation Trials at the Winery Level

2.1.1. *M. pulcherrima* Biomass Production

The biomass of the *M. pulcherrima* selected strain was obtained from pre-cultures in a modified YPD medium (0.5% yeast extract, 0.1% peptone and 2% glucose) and grown for 48 h at 25 °C in an orbital shaker (150 rpm). After this, each pre-culture was used to inoculate a 2 L bench-top bioreactor (Biostat® C; B. Braun Biotech Int., Goettingen, Germany) containing 25 L of modified YPD medium with airflow (1 L/L/min). A feed batch process was used for biomass production. Biomass was collected by centrifugation, washed three times with sterile distilled water, and inoculated at a 1×10^6 cell/mL initial concentration on grape juice in cold clarification. This value was determined using the Thoma-Zeiss counting chamber.

2.1.2. Preparation of Pied de Cuve

In total, 100 kg of undamaged grapes were harvested and soft pneumatic pressed. Thus, the grape juice obtained was subjected to a clarification (10 °C for 24 h) with the addition of enzymes and bentonite (MICROCOL® ALPHA, Laffort) without the addition of SO_2. The clarified grape juice was then separated and left to ferment for two days. After this period, 15 mL/L SO_2 was added and the *pied de cuve* was thus prepared and used to inoculate the steel vessel of the trials.

2.1.3. Inoculation at Cold Clarification Stage and Fermentation

The fermentation trials were carried out for two consecutive vintages (2021–2022). The main analytical parameters of the Verdicchio grape juice used in 2021 were the initial sugar content 265 g/L, pH 3.09, total acidity 5.17 g/L, and yeast assimilable nitrogen (YAN) content 9 mgN/L. In 2022, the main analytical parameters were initial sugar content 270 g/L, pH 3.11, total acidity 5.17 g/L, and yeast assimilable nitrogen (YAN) content 2 mgN/L.

In each vintage, a lot of grape juice (26 hL) was divided into two lots of Verdicchio grape juice to fill two vats of 15 hL. One steel vessel was inoculated with 1×10^6 cell/mL of *M. pulcherrima* and maintained at 10 °C for 24 h in cold static clarification. The other batch was maintained at the same condition without the inoculum of *M. pulcherrima* as a controlled trial. After 24 h of cold static clarification, the YAN was adjusted to 250 mgN/L

by the addition of diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France). After this time, the two batches were inoculated with the same *pied de cuve* previously prepared.

2.2. Biomass Evolution

Samples during fermentation were collected to evaluate biomass evolution. A viable cell count was carried out using lysine agar medium (Oxoid, Hampshire, UK) as a selective medium and WL nutrient agar medium (Oxoid, Hampshire, UK) for the detection of colony diversity. The plates were incubated at 25 °C for 48–72 h. The detection and enumeration of inoculated and wild yeasts were evaluated to combine the results of lysine agar, and the analysis of macro- and micro-morphological colonies in WL nutrient agar medium.

2.3. Saccharomyces cerevisiae Typing

Based on the micro- and macro-morphology of the colonies, presumptive *S. cerevisiae* pure cultures derived from *pied de cuves* and samples from 2/3 of respective inoculated fermentations were isolated. DNA was extracted at 95 °C for 10 min, and then it was amplified by PCR using the primers ITS1 (5′-TCCGTAGGTGAACCTCGCG-3′) and ITS4 (5′-TCCTCCGCTTTATTGATATGC-3′) following the procedure reported by Agarbati et al. [25]. A total of 74 strains obtained from the two years were then subjected to genotyping using pairs delta12/21 (delta12: 5′-TCAACAATGGAATCCCAAC-3′; delta21: 5′-CATCTTAACACCGTATATGA-3′) and were used for interdelta sequence analyses, as described by Legras and Karst [26]. The amplification was performed as per the following program: 3 min at 95 °C; followed by 25 s at 94 °C, 30 s at 45 °C, and 90 s at 72 °C for 9 cycles; and 25 s at 94 °C, 30 s at 50 °C, and 90 s at 72 °C for 21 cycles and a final extension at 72 °C for 10 min.

2.4. Analytical Procedures

The Official European Union Methods (2000) were used to determine the total acidity, volatile acidity, pH, and ethanol content. Enzymatic kits (Megazyme International Ireland) were utilized to determine glucose and fructose (K-FRUGL) and malic acid (K-DMAL) following the manufacturer procedures, while the ammonium content was determined using a specific enzymatic kit (kit no. 112732; Roche Diagnostics, Germany) and the free α-amino acids were evaluated following Dukes and Butzke protocol [27]. Acetaldehyde, ethyl acetate, n-propanol, isobutanol, amyl and isoamyl alcohols, and acetoin were quantified by direct injection into a gas chromatography system (GC-2014; Shimadzu, Kyoto, Japan). Each sample was prepared and analyzed as reported by Canonico et al. [28]. The volatile compounds were determined by the solid phase microextraction (HS-SPME) method, preparing the sample as follows: 5 mL of wine was placed into a vial, 1 g of NaCl and 3-octanol as the internal standard (1.6 mg/L) were added, and the vial was closed with a septum-type cap and placed on a magnetic stirrer for 10 min at 25 °C. Then, the sample was heated to 40 °C and extracted with a fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) for 30 min by insertion into the vial headspace. The compounds were desorbed by inserting the fiber into a Shimadzu gas chromatograph GC injector, in split–splitless modes following the procedure reported by Canonico et al. [29]. The glass capillary column used was 0.25 µm Supelcowax 10, length 60 m, and internal diameter 0.32 mm.

2.5. Sensory Analysis

At the end of the fermentation, the wines were decanted and after three months, were transferred into filled 750 mL bottles, closed with the crown cap, and maintained at 4 °C until sensory analysis. After this period of refinement, they were subjected to sensory evaluation. A group of 15 testers, 10 males and 5 females aged 25–45 years (80% expert and 20% non-expert), used a score scale of 1 to 10, where 10 was the score that quantitatively represented the best judgment (maximum satisfaction) and 1 was the score to be attributed

in case of poor satisfaction. The expert testers were composed of oenologists, sommeliers, and wine producers.

2.6. Data Analyses

The analysis of variance (ANOVA) using the statistical software package JMP 11® (statistical discovery from SAS, New York, NY, USA) was used to process all experimental data. The averages obtained were processed significant differences between the averaged data and were determined using the Duncan test. The experimental data were significant with associated p-values < 0.05. The results of the sensory analysis were also subjected to Fisher ANOVA to determine the significant differences with a p-value < 0.05.

3. Results

3.1. Effect of M. pulcherrima Addition in Cold Clarification

The results of the viable wild yeasts (WY) population before and after cold clarification with and without *M. pulcherrima* are reported in Figure 1a,b. Vintages from 2021 (a) and 2022 (b) were considered. In the first year, the initial wild yeast population in grape juice was about 10^5 CFU/mL in both trials. Without the use of *M. pulcherrima* there was an increase of about one log CFU/mL, while the inoculated trial showed a containment of the wild yeast population, which remained almost constant.

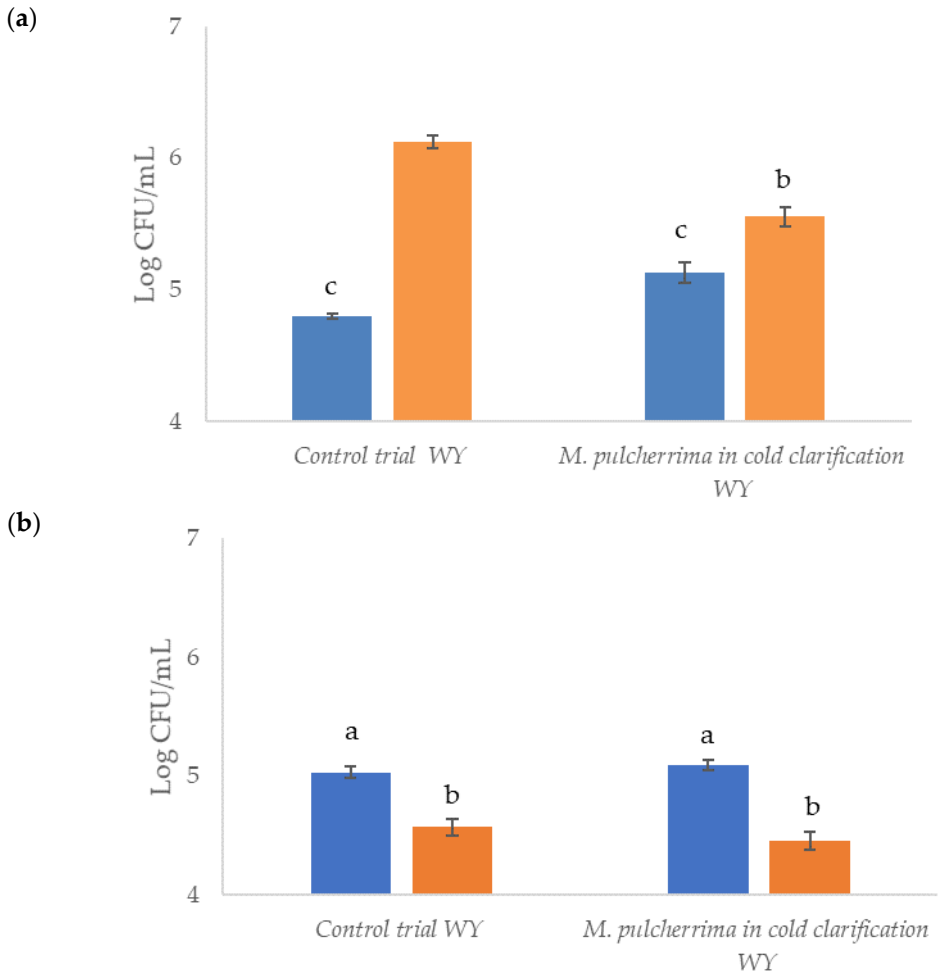

Figure 1. Effect of *M. pulcherrima* addition in cold clarification on wild yeasts (WY) in comparison with control fermentation: (**a**) 2021 vintage and (**b**) 2022 vintage. ■ before clarification; ■ after clarification. Data (n = 3) are the means ± standard deviation. Data with different superscript letters (^{a,b,c}) are significantly different (Duncan tests; $p < 0.05$).

During the 2022 vintage (Figure 1b), although the wild population was quantitatively comparable to that of the first year, a significant reduction of WY was shown in both trials with and without the inoculation of *M. pulcherrima* (a reduction of WY of c.a. 0.6 Log CFU/mL and 0.3 Log CFU/mL, respectively). The major containment of WY in the 2022 vintage could also be explained by the presence of wild *M. pulcherrima* (about 10^4 cell/mL) in grape juice.

3.2. Pied de Cuve Inoculum and S. cerevisiae Strains Typing

The two lots of clarified grape juice in both vintages (2021–2022) were inoculated with *pied de cuves* coming from spontaneous fermentation described in the material and methods section. Both *pied de cuves* before the inoculum of vats showed the exclusive presence of *S. cerevisiae* with a viable cell count of 5×10^8 cell/mL. The results of the characterization at the strain level of *S. cerevisiae* population are reported in Table 1. Biotyping results revealed the presence of a total of nine different profiles. The biotypes present in the *pied de cuves* almost dominated the fermentation processes with 90% and 58.3% in the 2021 vintage control and inoculated *M. pulcherrima* trials, respectively, (biotypes I, II, III, and IV) and 60% and 50% in the 2022 vintage control and inoculated *M. pulcherrima* trials, respectively (biotypes II, III, and VIII). Interestingly, 65% of the *S. cerevisiae* population showed the same biotype profile (biotypes I, II, and III) contributing to both 2021 and 2022 vintages.

Table 1. *S. cerevisiae* biotypes found in the spontaneous fermentation of *pied de cuves* and in the fermentation processes.

	2021			**2022**		
	Byotype	**%**	**n. Strains**	**Byotype**	**%**	**Strains**
Pied de cuve	I	12.5	2	II	67.0	12
	II	37.5	6	III	11.0	2
	III	12.5	2	VII	11.0	2
	IV	12.5	2	VIII	11.0	2
	V	25.0	4	-	-	-
Fermentation control	VI	10.0	1	II	40.0	4
	I	20.0	2	III	-	-
	II	30.0	3	VIII	20.0	2
	III	-	-	I	20.0	2
	IV	40.0	4	IX	20.0	2
M. pulcherrima in cold clarification	VI	42.0	5	I	50.0	4
	I	17.0	2	II	50.0	4
	II	17.0	2	III	-	-
	III	8.0	1	-	-	-
	IV	17.0	2	-	-	

3.3. Biomass Evolution and Saccharomyces cerevisiae Characterization

The biomass evolution after 24 h of cold clarification and the inoculum of *pied de cuve* in the vintage 2021 is reported in Figure 2a. The *S. cerevisiae* evolution in the two fermentations showed a similar trend, therefore the presence of *M. pulcherrima* did not affect the development of *S. cerevisiae*. The initial cell concentration was 10^6 cell/mL, achieving the maximum of growth kinetics at the 5th day of fermentation to remain constant until the end of fermentation. WY in both fermentations exhibited a similar trend: they started with the different amounts found in Figure 1 and disappeared at the 4th of fermentation. After cold clarification, the inoculated *M. pulcherrima* maintained a cell concentration of 10^3 cell/mL until the 8th day and then disappeared on the 12th day. The growth kinetics of trials of the vintage 2022 are reported in Figure 2b. The biomass evolution of *S. cerevisiae* also showed a similar trend to the vintage 2022, although there was a more abundant inoculum (10^7 cell/mL) achieving the maximum biomass on the 3rd day of fermentation (10^8 cell/mL) to remain constant at the end of fermentation. Regarding wild yeast evolution,

the two fermentations did not show differences in terms of the cell concentration. The overall results indicated that an adequate starter inoculum is also relevant in the control of WY with and without *M. pulcherrima*.

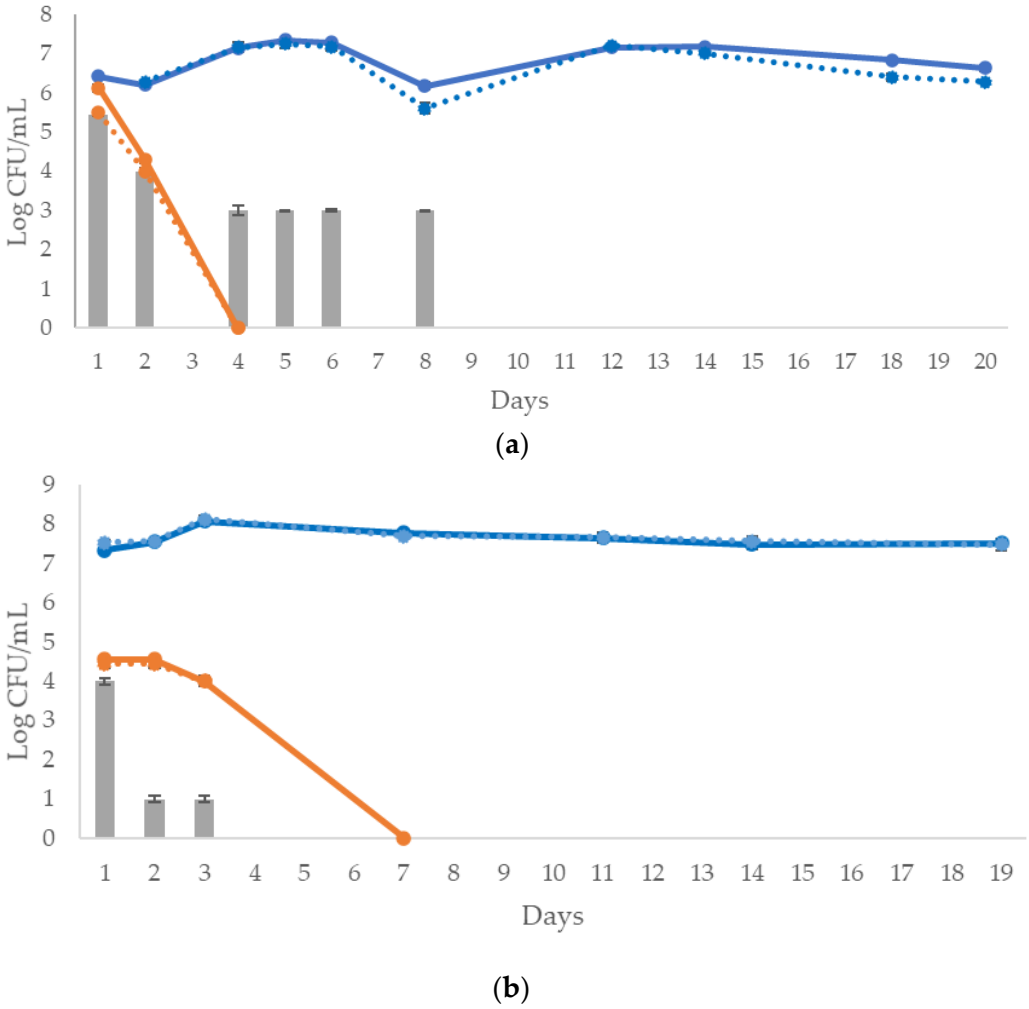

Figure 2. Growth kinetics of the yeasts population in both (**a**) 2021 vintage and (**b**) 2022 vintage wines. *S. cerevisiae* in the control trial; *S. cerevisiae* in *M. pulcherrima* cold clarification; WY in the control trial; WY in *M. pulcherrima* cold clarification; *M. pulcherrima* biomass in the trial with *M. pulcherrima* cold clarification.

3.4. Main Oenological Characters of Wine

The results of the main analytical characters of wines obtained in 2021 and 2022 are shown in Table 2. The presence of *M. pulcherrima* during cold clarification did not generally significantly affect the main parameters analyzed in both vintages 2021 and 2022. Indeed, the resulting wines coming from the two vintages exhibited a similar analytical profile. The only exception was the residual sugar content: the vintage 2021 was significantly higher in the control trial, while in 2022 the data showed an opposite result. Even comparing the wines in the two vintages, the results did not show any differences, except for the higher volatile acidity value in both fermentations in the 2021 vintage.

Table 2. The main analytical characteristics of resulting wine coming from 2021 and 2022 vintages.

	***M. pulcherrima* in Cold Clarification (2021)**	**Control Trial (2021)**	***M. pulcherrima* in Cold Clarification (2022)**	**Control Trial (2022)**
Ethanol %*v*/*v*	15.05 ± 0.02 [a]	14.49 ± 0.01 [b]	14.81 ± 0.15 [a]	15.08 ± 0.02 [a]
Total acidity (g/L tartaric acid)	6.26 ± 0.09 [a]	6.52 ± 0.01 [a]	7.56 ± 0.12 [a]	7.84 ± 0.14 [a]
Sugar residue (g/L)	3.7 ± 0.02 [b]	14.0 ± 0.03 [a]	6.6 ± 0.7 [a]	1.5 ± 0.1 [b]
pH	3.28 ± 0.02 [a]	3.25 ± 0.00 [a]	3.08 ± 0.00 [a]	3.09 ± 0.00 [a]
Volatile acidity (g/L acetic acid)	0.64 ± 0.01 [a]	0.56 ± 0.02 [b]	0.34 ± 0.02 [a]	0.36 ± 0.03 [a]
Total SO_2 (mg/L)	25 ± 0.9 [a]	26 ± 0.8 [a]	21 ± 0.9 [a]	21 ± 0.9 [a]
Malic acid (g/L)	0.55 ± 0.03 [a]	0.49 ± 0.02 [a]	0.54 ± 0.05 [a]	0.55 ± 0.05 [a]
Ethanol yield (g/g)	0.45 ± 0.07 [a]	0.45 ± 0.06 [a]	0.44 ± 0.09 [a]	0.44 ± 0.09 [a]

Data (n = 3) are the means ± standard deviation. Data with different superscript letters ([a,b]) within each row and vintage are significantly different (Duncan tests; $p < 0.05$).

3.5. Volatile Compounds of Wine

The resulting volatile compounds of vintage wines coming from 2021 and 2022 are reported in Table 3. The inoculation of *M. pulcherrima* at the start of cold clarification generally led to wine with a different volatile profile. Indeed, a significant increase in ethyl hexanoate with relevant high OAV values in comparison with the control was observed. Moreover, the trials with *M. pulcherrima* exhibited a significant increase in monoterpenes content, in particular with respect to geraniol and nerol (1.5 OAV), and two higher alcohols (n-propanol and isobutanol) in comparison with control trials. The results showed a significant increase in the acetaldehyde content with *M. pulcherrima* but within the limits of the negative threshold. On the contrary, the control trial exhibited a significant increase in the isoamyl acetate and β-phenyl ethanol.

Table 3. The main by-products and volatile compounds in final wines during two vintages 2021–2022.

	***M. pulcherrima* in Cold Clarification 2021**	**OAV (Odor Activity Value)**	**Control Trial 2021**	**OAV (Odor Activity Value)**	***M. pulcherrima* in Cold Clarification 2022**	**OAV (Odor Activity Value)**	**Control Trial 2022**	**OAV (Odor Activity Value)**
Esters (mg/L)								
Ethyl butyrate	0.13 ± 0.014 [a]	0.325	0.13 ± 0.00 [a]	0.325	0.187 ± 0.007 [a]	0.467	0.148 ± 0.006 [b]	0.445
Ethyl acetate	35.75 ± 0.41 [a]	2.97	35.55 ± 0.36 [a]	2.96	30.12 ± 0.18 [b]	2.51	31.91 ± 0.35 [a]	2.65
Phenyl ethyl acetate	0.89 ± 0.042 [a]	12.19	0.88 ± 0.034 [a]	12.05	0.821 ± 0.032 [a]	11.24	0.773 ± 0.045 [a]	10.58
Ethyl hexanoate	1.80 ± 0.121 [a]	22.5	0.63 ± 0.142 [b]	7.87	1.470 ± 0.050 [a]	18.37	0.612 ± 0.043 [b]	7.65
Ethyl octanoate	0.00 ± 0.00 [a]	0	0.00 ± 0.00 [a]	0	0.002 ± 0.000 [a]	0.003	0.003 ± 0.001 [a]	0.005
Isoamyl acetate	1.27 ± 0.026 [b]	7.93	2.22 ± 0.147 [a]	13.87	1.484 ± 0.112 [a]	9.27	1.404 ± 0.024 [a]	8.77
Alcohols (mg/L)								
n- propanol	30.88 ± 0.36 [a]	0.100	26.08 ± 0.13 [b]	0.08	36.44 ± 4.92 [a]	0.119	31.64 ± 0.05 [a]	0.103
Isobutanol	20.48 ± 0.29 [a]	0.512	18.30 ± 0.10 [b]	0.45	14.09 ± 0.34 [a]	2.83	11.41 ± 0.24 [b]	0.285
Amyl alcohol	13.00 ± 0.55 [a]	0.203	11.69 ± 0.41 [a]	0.18	10.03 ± 0.29 [a]	0.156	8.64 ± 0.48 [a]	0.135
Isoamyl alcohol	113.94 ± 0.05 [a]	1.89	114.16 ± 0.24 [a]	1.90	89.94 ± 0.12 [a]	1.49	80.95 ± 0.52 [b]	1.34
β-Phenyl Ethanol	55.9 ± 0.130 [b]	3.99	64.5 ± 0.152 [a]	4.60	14.01 ± 0.022 [a]	1	13.32 ± 0.045 [a]	0.951

Table 3. *Cont.*

	M. pulcherrima in Cold Clarification 2021	OAV (Odor Activity Value)	Control Trial 2021	OAV (Odor Activity Value)	*M. pulcherrima* in Cold Clarification 2022	OAV (Odor Activity Value)	Control Trial 2022	OAV (Odor Activity Value)
Carbonyl Compounds (mg/L)								
Acetaldehyde	63.16 ± 0.28 [a]	126.32	25.35 ± 0.37 [b]	70	22.39 ± 0.59 [a]	44.78	16.57 ± 0.1 [b]	33.14
Monoterpenes (mg/L)								
Linalool	0.015 ± 0.001 [a]	0.60	0.012 ± 0.002 [a]	0.48	0.010 ± 0.00 [a]	0.40	0.009 ± 0.00 [a]	0.36
Geraniol	0.010 ± 0.002 [a]	0.33	0.002 ± 0.000 [b]	0.06	0.016 ± 0.00 [a]	0.53	0.005 ± 0.001 [b]	0.17
Nerol	0.023 ± 0.001 [a]	1.53	0.005 ± 0.000 [b]	0.33	0.001 ± 0.000 [a]	0.06	0.001 ± 0.00 [a]	0.06

Data (n = 3) are the means ± standard deviation. Data with different superscript letters ([a,b]) within each row and vintage are significantly different (Duncan tests; $p < 0.05$).

The other volatile compounds did not show significant differences. The results of the wines coming from 2022 vintages showed an enhancement of ethyl butyrate and ethyl hexanoate content in the presence of *M. pulcherrima*, while control trials showed a significant increase in the ethyl acetate content.

Considering the two vintages, the results confirmed that the use of *M. pulcherrima* in cold clarification affected the volatile compounds in terms of the terpens, alcohols, and some esters compounds.

The main volatile compounds of the vintage wines obtained from 2021 and 2022 with and without the inoculum of *M. pulcherrima* were elaborated using the Principal Component Analysis (PCA). The total variance explained was 86.6% (PC1 = 66.0%; PC 2 = 26.6%). Figure 3 reports the distribution of fermentations to assess the effect of the yeast and the vintage in the function of the volatile compounds. The fermentation trials were in four different quadrants: PC1 distinguished the trials on the base of the vintage while PC 2 separated the trials in the function of the presence of *M. pulcherrima* during the cold clarification stage. The production of ethyl hexanoate and geraniol is characterized more specifically by the *M. pulcherrima* metabolism of volatile compounds imparting a specific aromatic imprint to the wine.

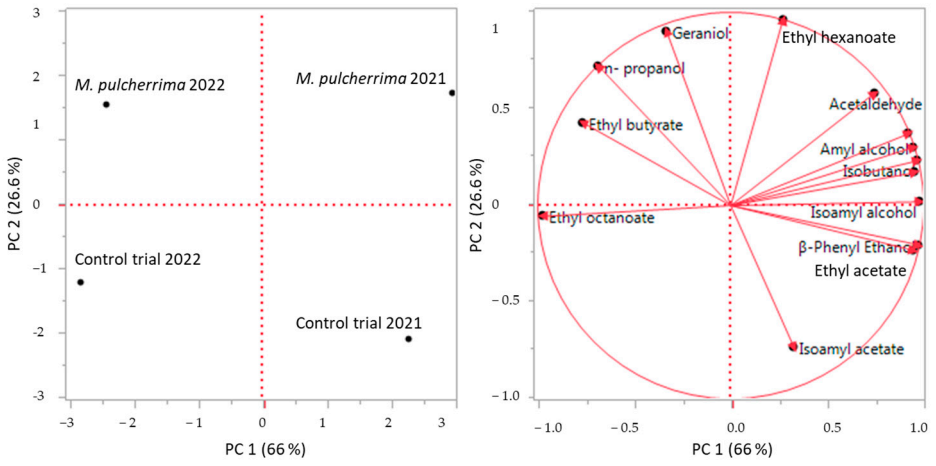

Figure 3. Principal component analysis of the main volatile compounds in wine coming from two consecutive vintages. The variance explained by principal component analysis (PCA) is PC 1 66% X-axis and PC 2 26.6% Y-axis.

3.6. Sensory Analysis

To evaluate the impact of *M. pulcherrima* inoculated in cold clarification on the aroma complexity, the final wines were subjected to sensory analysis. The sensory analysis of wines obtained by the 2021vintage are reported in Figure 4a. The results highlighted a positive judgement of testers regarding each wine, characterized by specific aromatic notes and without defects. The final wine from the control trial exhibited a more pronounced persistence and bitterness. The use of *M. pulcherrima* led to wines with emphasized notes of tropical fruits, sweetness, and more structure. No significant differences were shown regarding the other aromatic descriptors. As for the wines of 2022 (Figure 4b), the use of *M. pulcherrima* led to wine characterized by distinctive descriptors as structure, persistence, herbs, and tropical fruits, in comparison with the control wine. The sensory analysis highlighted the influence of the use of *M. Pulcherrima* in cold clarification on the sensory profile of aroma wine.

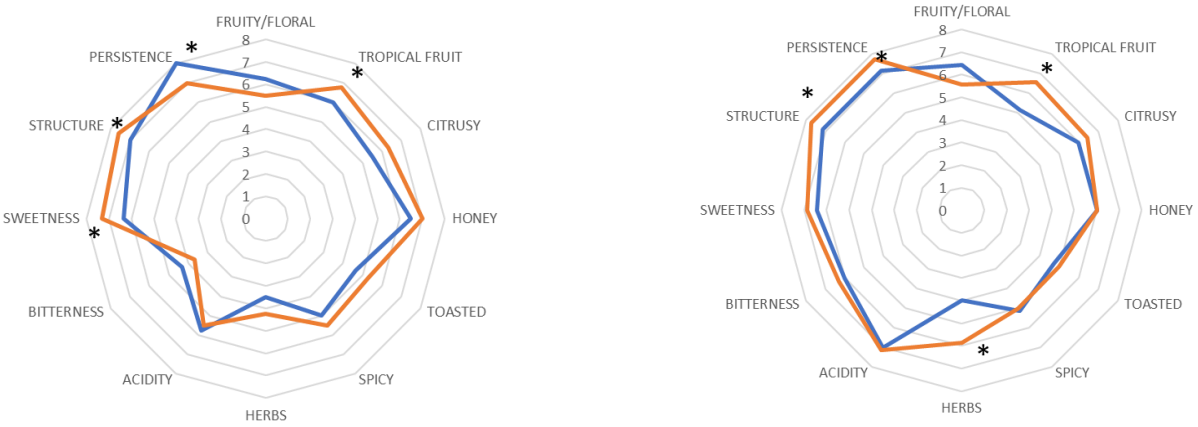

Figure 4. Sensory analysis of Verdicchio wine coming from 2021 vintage (**a**) and 2022 vintage (**b**). control trial; *M. pulcherrima* in cold clarification. *, significantly different (Fischer ANOVA; p-value < 0.05).

4. Discussion

The term "biocontrol" related to the use of microorganisms as biocontrol agents was defined by Baker and Cook [30] as the reduction of a pathogen or disease activities through an organism. In agri-food, this concept is related to an alternative strategy to the use of chemical products and the use of a microorganism with antagonist action against other microorganisms reducing the use of pesticides and boosting food quality and safety [19,30–32]. The increased consumer demands for safe food and beverages invigorated research on the development of safe and ecofriendly wine, where the protection against undesirable microorganisms before, during, after, or at the end of the fermentation process is required [6,7,14]. Effectively, the use of non-*Saccharomyces* yeasts could be a valid strategy as potential antagonists against phytopathogenic fungi of the genera due to their ability to produce a wide spectrum of secondary metabolites. One of the yeast species best known for its biocontrol potential is *M. pulcherrima*. This characteristic is also linked to the production of the pulcherrimin pigment, which in turn is linked to the availability of iron in the medium which would lead to the deprivation of different yeast species as *Brettanomyces/Dekkera*, *Pichia*, *Hanseniaspora*, spp. *Saccharomycodes ludwigii*, and *Candida* spp. [33]. *M. pulcherrima* can be compatible with the main yeast used for wine production, for example in mixed fermentations, thus resulting in a reduction in the SO_2 dose and hence were usually used as an antimicrobial agent [34]. Moreover, recent investigations conducted using *M. pulcherrima* strains or a mix of *M. pulcherrima* and *T. delbrueckii* in the red wine making process at the prefermentative stage [7,8,14].

In this work carried out at winery conditions, the efficacy of *M. pulcherrima* to control the wild yeast population was found in cold clarification. On the other hand, the timely and appropriate starter inoculum in the two years determined an effective control of wild yeast during fermentation with and without *M. pulcherrima* inoculum. The WY control was particularly evident in the 2022 vintage, where a high concentration of wild *M. pulcherrima* was already present in grape juice before the clarification stage, influencing the control trial. These data corroborate the results reported by other recent studies regarding the use of *M. pulcherrima* in wine making as a bioprotectant agent [6–8]. In particular, the inoculum of a selected *M. pulcherrima* strain would ensure the biocontrol activity and attempt to impart specific aromatic traits to the final wine.

The bioprotectant action of *M. pulcherrima* used in cold clarification was displayed without the addition of SO_2 in Verdicchio grape juice with an inoculum of a *pied de cuve* with indigenous *S. cerevisiae* strains.

The molecular monitoring of biotype profiles that led to the fermentation, including those present in the *pied de cuve*, revealed a rather stable yeast strain consortium in the presence and absence of *M. pulcherrima* (80 %) as well as from a vintage to another (50%), even if same differences can be found. This reflected the results of the PCA analysis indicating that the impact on volatile profile was related not only to the effect of *M. pulcherrima* but also to the different vintages and *S. cerevisiae* yeast strain variation. The presence of *M. pulcherrima* increased ethyl hexanoate, isobutanol, acetaldehyde, and geraniol although with different concentrations in both vintages.

Generally, the use of *M. pulcherrima* affected the esters, alcohol, and monoterpens compounds that contribute to defining the overall sensory characteristics of wines and increasing the perception of the varietal aroma of grapes [1]. Indeed, *M. pulcherrima* was indicated in several works to improve the concentration of volatile compounds [35–37]. This species positively contributes to volatile thiol release in wines, especially during the pre-fermentation stage in wine making [36]. The sensory analysis confirmed the results of main analytical characters and volatile compounds, particularly regarding the descriptors of tropical fruit and structure.

In this work, the multifactorial role of *M. pulcherrima* in wine making was highlighted again. As recently reported [38,39], mixed fermentation with *M. pulcherrima* enhanced the ester profile and increased the final quality of the wine, such as the sensory characteristics and color parameters.

In conclusion, the results obtained indicate that the use of *M. pulcherrima* in the cold clarification stage plays an effective biocontrol action against the wild yeast population. This effect could be a strategy to reduce the use of sulfur dioxide in wine fermentation. Moreover, *M. pulcherrima* was able to enhance the aromatic and sensory profile of the wine.

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