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Preliminary Characterisation of *Metschnikowia pulcherrima* to Be Used as a Starter Culture in Red Winemaking

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Abstract: In the last decade, the application of non-*Saccharomyces* yeasts in oenology as a natural tool to obtain wine diversification and higher quality has aroused great interest. In this work, three *Metschnikowia pulcherrima* strains, isolated from a vineyard, were characterised through the evaluation of their main oenological properties, antimicrobial activity, and specific enzymatic activities (β-glucosidase, β-lyase, polygalacturonase, and protease). The *M. pulcherrima* strains did not produce any inhibition against *Saccharomyces cerevisiae*, while they were able to exert an antimicrobial action against some unwanted bacteria and yeasts frequently present in grape must and potentially causing the alteration of wines. After this preliminary screening, *M. pulcherrima* AS3C1 has been selected to be used in the winemaking of red grape *Vitis vinifera* cv. Aglianico on a pilot scale. The effect of the sequential inoculation of *M. pulcherrima* AS3C1 with a commercial strain of *S. cerevisiae* was verified using for comparison a single inoculum with *S. cerevisiae* and a spontaneous fermentation. Our results showed a higher concentration of anthocyanins and catechins in wines obtained by the sequential inoculation of *M. pulcherrima* AS3C1 and *S. cerevisiae*. On the basis of the data obtained, *M. pulcherrima* AS3C1 possesses an enzymatic profile and some oenological properties that could contribute positively to the definition of the chemical composition of wines, suggesting its possible use for red winemaking processes.

Keywords: non-*Saccharomyces*; *Metschnikowia pulcherrima*; sequential inoculation; Aglianico wine

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1. Introduction

In winemaking, non-*Saccharomyces* yeasts have long been considered as undesirable microorganisms, responsible for incomplete fermentations and the production of unpleasant compounds. In the last few decades, the focus on these yeasts which are largely predominant in grape berries and in the must at the beginning of alcoholic fermentation [1,2] has changed. In fact, some non-*Saccharomyces* yeasts, including *Metschnikowia pulcherrima*, are now marketed in active dry yeast form [3]. Recent research has focused on the potential of using non-*Saccharomyces* yeasts (*Hanseniaspora spp.*, *Lachancea thermotolerans*, *M. pulcherrima*, *Pichia kluyveri*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *Candida stellata*, etc.) to obtain wines with peculiar flavour profiles or reduced ethanol and acidity content [4,5]. Therefore, the co-inoculation or the sequential inoculation of selected non-*Saccharomyces* and *Saccharomyces cerevisiae* strains is now a consolidated oenological practice.

M. pulcherrima is one of the most investigated species due to its multiple contribution to winemaking [6,7]. Members of this species are characterised by interesting oenological features and present several positive aspects for winemaking, for instance through the modulation and synthesis of secondary metabolites that can improve the sensory profile

of wine [8]. *M. pulcherrima* is generally incapable of independently completing alcohol fermentation. Accordingly, the sequential inoculation of *M. pulcherrima* and *S. cerevisiae* is a strategy that allows the completion of alcoholic fermentation and the improvement of flavour complexity of the resulting wines [9–11]. *M. pulcherrima* produces pulcherriminic acid. The depletion of iron by pulcherriminic acid and the formation of pulcherrimin, an insoluble pigment (red iron chelate), is known for its antioxidant and antimicrobial effects [12–15]. Pulcherrimin-producing yeast species are considered effective antimicrobial agents against various microorganisms, with great potential for biocontrol applications [16]. It has also been observed that the amount of pulcherrimin formed varies greatly within strains and may be a function of environmental conditions [17]. In addition to the bio-protective action, other recent studies have highlighted the impact of *M. pulcherrima* on the compositional and sensory characteristics of wines [18]. *M. pulcherrima* is capable of producing extracellular hydrolytic enzymes that can contribute to the release of aroma compounds in wine, such as β-glucosidase, which promotes the release of varietal aromas from grapes by hydrolysing bound monoterpenes [11,19]. Some *M. pulcherrima* strains were tested in white grape must varieties, like Chardonnay, Verdicchio, Pecorino, and Sauvignon blanc to improve the aromatic profile of wines [7,11,20] and in red grape must varieties, like Pinot noir, Merlot, and Cabernet sauvignon for bio-protection [21–24]. However, to date, there are only a few strains of *M. pulcherrima* on the market, which are commercialised in the form of active dry yeast, and are mainly used as biocontrol agents [3]. Furthermore, it is important to emphasise that to date there are no targeted studies on *M. pulcherrima* aimed at selecting specific strains to be used as starter cultures in red winemaking.

In this study, three *M. pulcherrima* strains isolated from the vineyards of Aglianico cultivar located in Southern Italy [25] were preliminarily characterised for their main oenological characteristics, specifically enzymatic and antimicrobial activities. Finally, *M. pulcherrima* AS3C1 was tested in the fermentation of red grape must (*Vitis vinifera* cv. Aglianico) on a pilot scale in order to verify its effect on the main oenological parameters of the obtained wine.

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions

In this study, *M. pulcherrima* AS3C1, ASB3R, and 14AS, (accession number to GenBank OM038321, OM038324, and OM038320), isolated from a vineyard and belonging to the Di.A.A.A. (Department of Agricultural, Environmental and Food Sciences; Campobasso, Italy) culture collection of the University of Molise, were used. For oenological properties determination and pilot-scale winemaking, the commercial strain *S. cerevisiae* Actiflore® F33 (Laffort, Bordeaux, France) was used as a reference. For each trial described below, yeast strains were cultured aerobically at 28 °C in YEPD broth (Merck Millipore, Darmstadt, Germany). After 48 h of incubation, the broth cultures were centrifuged at 5000 rpm for 10 min at 4 °C, and the cell pellets were washed twice with saline solution (0.9% NaCl). For the subsequent testing, an assessment of initial cell density was performed using a Thoma hemocytometer chamber (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Pre-Selection Trials

2.2.1. Determination of Oenological Properties

Fermentative vigour, sulphite tolerance, alcohol, and volatile acidity production were evaluated using red grape must (*Vitis vinifera* cv. Aglianico) having the following chemical composition: sugars 197 g/L, total acidity 8.0 g/L, and pH 3.35. The above-mentioned properties were evaluated using flasks (working volume 250 mL) plugged with a Müller valve (Alamo, Madrid) containing sulfuric acid to avoid the microbial contamination and release of carbon dioxide (CO_2). Before use, the must was clarified by centrifugation (10 min at 8000 rpm) and then sterilised by a Millipore® Stericap™ Plus Vacuum Filter pore size 0.22 μm (Merck KGaA, Darmstadt, Germany). The fermentation tests were performed at

24.0 ± 1.0 °C using 100 mL of grape must and each individual yeast was inoculated in order to have an initial concentration of about 10^6 CFU/mL.

The experimental conditions were the following: test A, must without potassium metabisulphite (Esseco Srl, Trecate, Italy); test B, must added with 50 mg/L potassium metabisulphite; and test C, must added with 100 mg/L potassium metabisulphite. Fermentation vigour (test A) and sulphite tolerance (tests B and C) were determined by calculating the weight loss caused by the CO_2 release after 72 h from inoculation [26] and the values were expressed as g CO_2/100 mL of must. Alcohol (% *v*/*v*) and volatile acidity (g/L as acetic acid) were determined according to the OIV method [27].

2.2.2. Methods for In Vitro Evaluating Antimicrobial Activity

Producers are defined as microorganisms capable of exerting (producing) an inhibitory action against other specific microorganisms defined as indicators. The antimicrobial activity of *M. pulcherrima* AS3C1, ASB3R, and 14AS (producers) was evaluated against the following indicator microorganisms: *Hanseniaspora guilliermondii* (ATCC 10630); *Pichia terricola* (ATCC 58068); *Schizosaccharomyces pombe* (ATCC 24843); *S. cerevisiae* (ATCC 9763); *Lactiplantibacillus plantarum* (ATCC 14917); *Levilactobacillus brevis* (ATCC 14869); *Pediococcus acidilactici* (ATCC 8042), belonging to the American Type Culture Collection (ATCC, Manassas, VA, USA); and against the commercial strain *S. cerevisiae* Actiflore® F33 (Laffort, Bordeaux, France).

The agar well diffusion assay following the protocol of Testa. et al. [28] was applied using YEPD agar for the yeasts and MRS agar (Merck Millipore, Darmstadt, Germany) for the lactic acid bacteria (LAB). A total of 100 µL of broth culture (10^5 CFU/mL) of each indicator strain were inoculated into Petri plates (Ø 90 mm) containing 20 mL of soft agar medium (0.7% wt/vol agar). Finally, 70 µL of broth culture of each producer strain (10^7 CFU/mL) was added into single 5 mm diameter wells [28]. Sterile water as the negative control was used, while chloramphenicol (100 µg/mL) against bacteria and cycloheximide (100 µg/mL) against yeasts as the positive control were used. All the reagents used were supplied by Merck KGaA (Darmstadt, Germany). After incubation at 28 °C for 48–72 h, antibacterial activity was reported as the diameter (mm) of the clear zone of inhibition (ZOI) around the inoculated wells.

2.2.3. Cryotolerance

The cryotolerance was evaluated by inoculating the yeast cultures at a concentration of about 10^6 CFU/mL in YEPD broth. The test was conducted at 4 °C under agitation (150 rpm) using an orbital shaker (Heathrow Scientific, Vernon Hills, IL, USA). The growth capacity was determined visually after 24 h of incubation [29].

2.2.4. Hydrogen Sulphide (H_2S) Production

H_2S production was evaluated on BIGGY agar (Bismuth Sulphite Glucose Glycine Yeast; Thermo Fisher Scientific, Waltham, MA, USA) as reported by Iorizzo et al. [29]. Each yeast strain was spread on the surface of BIGGY agar medium and the plates were incubated at 25 °C for 5 days. After incubation, the H_2S-negative strains showed white colonies, while the H_2S-producing strains showed brown or dark brown colonies. The following chromatic scale was used for the results: 1, white colour (no production); 2, light brown; 3, brown; 4, dark brown; and 5, dark brown/black [29].

2.2.5. Pulcherrimin Production

Pulcherrimin production was evaluated using the YEPD agar medium (Merck Millipore, Darmstadt, Germany) supplemented with 0.05% (wt/vol) ferric chloride as reported by Pawlikowska et al. [30]. Briefly, 10 µL of broth cultures (cellular density 10^6 CFU/mL) from each *M. pulcherrima* strains, cultured for 24 h at 28 °C in YEPD broth, were spotted onto agar plates. After incubation for 5 days at 25 °C, the colonies surrounded by reddish halos were recorded as positive results.

2.2.6. Biogenic Amine Detection

The qualitative detection of biogenic amines was performed as described by Granchi et al. [31] with some modifications. Briefly, the *M. pulcherrima* strains precultured in YEPD broth at 28 °C for 48 h were spotted on plates containing a medium having the following composition: 3% (wt/vol) yeast extract, 2% (wt/vol) of each amino acid precursors (histidine, tyrosine, phenylalanine, and ornithine), 1,5% (wt/vol) agar, and 1% (wt/vol) glucose and bromocresol purple (0.015 g/L). The pH of the medium was adjusted to 5.2. The inoculated Petri dishes were incubated at 28 °C for 72 h. The decarboxylation of the amino acid to the corresponding biogenic amine were registered as an increase in pH, detected by the change in the colour of the medium. Histamine-, putrescine-, and phenylethylamine-producing strains were identified by purple colouration, and tyramine production was detected by the decolourisation of the medium. The same medium without amino acid precursor was used as the negative control. All the reagents used in the experiment were purchased from Merck KGaA (Darmstadt, Germany).

2.3. Evaluation of Enzymatic Activities

2.3.1. β-Lyase Activity

The β-lyase activity was evaluated using a medium having the following composition: 0.1% (wt/vol) S-methyl-L-cysteine, 0.01% (wt/vol) pyridoxal-5-phosphate, 1.2% (wt/vol) Yeast Carbon Base, and 2% (wt/vol) agar, pH adjusted to 3.5 [32]. The broth culture of each yeast strain precultured in YEPD broth at 28 °C for 48 h was spotted onto the medium and the plates were incubated at 28 °C for 48–72 h. The growth of the colonies after the incubation indicated the presence of β-lyase activity. *Torulaspora delbrueckii* ATCC 36,240 and *Rhodotorula glutinis* ATCC 2527, belonging to the American Type Culture Collection (ATCC, Manassas, VA, USA), were used as the positive and negative controls, respectively. All the reagents used in the experiment were purchased from Merck KGaA (Darmstadt, Germany).

2.3.2. β-Glucosidase Activity

The β-glucosidase activity was determined as reported by Mateo et al. [33] using an esculin agar medium having the following composition: 2 g/L glucose, 1 g/L peptone, 1 g/L yeast extract, 0.3 g/L esculin, 0.01 g/L ferric ammonium citrate, and 15 g/L agar (Merck KGaA, Darmstadt, Germany). The yeast broth cultures (in YEPD) of 48 h growth at 28 °C were spotted onto the surface of the medium and the plates were incubated at 28 °C for 72 h. The presence of the enzymatic activity was visualised as a dark halo around the yeast colonies. *R. glutinis* ATCC 2527 and *T. delbrueckii* ATCC 36240, both from the American Type Culture Collection (ATCC Manassas, VA, USA), were used as the positive and negative controls, respectively.

2.3.3. Protease Activity

Protease activity was evaluated using 10% (wt/vol) skim milk (Merck KGaA, Darmstadt, Germany) added with 1.5% (wt/vol) agar. The yeast broth cultures (in YEPD) of 48 h growth at 28 °C were spotted across the surface of the medium. The Petri dishes were incubated at 28 °C for 72 h. The presence of the enzymatic activity was indicated by the presence of a clear zone surrounding the spot [34].

2.3.4. Polygalacturonase Activity

The polygalacturonase activity was determined as reported by Napa-Almeyda et al. [35] with some modification. The yeast strains were grown for 24 h at 28 °C in yeast malt broth (YM, Merck KGaA, Darmstadt, Germany) supplemented with 1.5% (wt/vol) citrus pectin (Spectrum Chemical, Gardena, CA, USA) to induce the production of pectinolytic enzymes. The cultures were maintained under stirring using a digital orbital shaker (Heathrow Scientific, Vernon Hills, IL, USA) set at 200 rpm. Polygalacturonase activity was determined using the agar spot assay. For this purpose, the broth culture (10 µL) of each yeast strain (10^6 CFU/mL) was spotted onto the surface of a YM agar medium

supplemented with 1.5% (wt/vol) citrus pectin. The same medium without inoculum was used as the negative control. The plates were incubated overnight at 28 °C for 3 days. The presence of the enzymatic activity was evaluated by the presence of a clear zone surrounding the spot. *M. pulcherrima* ATCC 18406, belonging to the American Type Culture Collection (ATCC Manassas, VA, USA), and *L. thermotolerans* NCYC 412, belonging to the National Collection of Yeast Cultures (NCYC, Norwich, UK) were used as the positive and negative controls, respectively.

2.4. Winemaking Trials

After the pre-selection tests, *M. pulcherrima* AS3C1 was chosen as the starter for the winemaking trials. For this purpose, red grapes (*Vitis vinifera* cv. Aglianico) were harvested during the 2023 vintage upon reaching ripeness level, and transported to the laboratory of the Department of Agricultural, Environmental, and Food Sciences of the University of Molise. The grapes were destemmed and crushed without the addition of adjuvants. The resulting must had the following chemical composition: pH 3.21, sugars 228.5 g/L, total acidity 9.57 g/L, and yeast assimilable nitrogen (YAN) 145.0 g/L. Before use, 80 mg/L of potassium metabisulphite was added to the grape must. For winemaking, the following three different tests were performed: test AG-1, must inoculated with *S. cerevisiae* F33 alone; test AG-2, must inoculated with *M. pulcherrima* AS3C1 and, after 48 h, with *S. cerevisiae* F33; and test AG-3, spontaneous fermentation. Each test was carried out in triplicate using stainless steel tanks (working volume 1 hL) containing 80 L of grape must with skins. Fulling was carried out three times a day until the end of the alcoholic fermentation. The starter yeasts were inoculated to have an initial cell density of about 10^6 CFU/mL and the fermentations were conducted at 24.0 ± 1.0 °C.

2.5. Fermentative Kinetics Parameters

The fermentation progress was monitored assessing the yeast viability and the ethanol production. Yeast viable cell counts were evaluated by the plate-counting technique using WL agar (Merck KGaA, Darmstadt, Germany) containing 100 mg/L chloramphenicol (Merck KGaA, Darmstadt, Germany) for bacterial growth inhibition. The plates were incubated at 28 °C for 72 h in aerobic conditions. The colony colour and colony topography parameters were adopted to differentiate *S. cerevisiae*, *M. pulcherrima*, and other yeasts [36].

The validation of the taxonomic collocation of the yeasts species was carried out by molecular identification. About that, the D1/D2 domain of the 26S rDNA gene was amplified using the primers NL1 (5′-GCA TAT CAATAA GCG GAG GAA AAG-3′) and NL4 (3′-GGT CCG TGT TTC AAG ACGG-5′) as reported by Iturritxa et al. [37].

2.6. Basic Parameters of Wines

At the end of alcoholic fermentation, the wines obtained were subjected to chemical analysis. The pH, total acidity (g/L as tartaric acid), volatile acidity (g/L as acetic acid), alcohol content (% *v*/*v*), reducing sugar (g/L), colour intensity (CI), and tonality (T) were determined according to the OIV method [27]. DL-malic acid (g/L), L-lactic acid (g/L), D-lactic acid (g/L), glycerols (g/L), anthocyanins (mg/L), catechins (mg/L), and acetaldehydes (mg/L) were determined using enzymatic and colourimetric kits (Steroglass, Perugia, Italy) according to the manufacturer's instructions.

2.7. Statistical Analysis

All the data are expressed as the mean ± standard deviation (±SD) obtained from three biological replicates. The yeast viable cell counts and chemical parameters were analysed by a General Linear Model based on ANOVA (IBM SPSS Statistics 21). Statistical significance was attributed to the values of $p \leq 0.05$.

3. Results and Discussion

3.1. Oenological Properties

The fermentative vigour and sulphite tolerance results of the *M. pulcherrima* strains tested are shown in Table 1. The values of alcohol and volatile acidity are shown in Table 2. In test A, all the strains showed a moderate fermentative vigour, ranging between 0.89 and 0.91 g CO_2/100 mL of must. In comparison, *S. cerevisiae* F33 showed a value of 2.11 g CO_2/100 mL. The alcohol amount produced by *M. pulcherrima* strains ranged between 4.0 and 4.8% (*v*/*v*), as expected lower than that produced by *S. cerevisiae* F33 (11.6% *v*/*v*). These results confirm the low alcohol tolerance of *M. pulcherrima* [18], and why its use is recommended together with other yeasts with high fermentative power, such as *S. cerevisiae* [19], in order to avoid incomplete alcoholic fermentation, unless it is a technological choice aimed at obtaining low-alcohol wines [38]. The tested *M. pulcherrima* strains produced very low amounts of volatile acidity (between 0.16 and 0.19 g/L), confirming what is reported in the literature regarding the scarce contribution to the increase in volatile acidity content in wines [39,40].

Table 1. Fermentation vigour and sulphite tolerance of *M. pulcherrima* AS3C1, 14AS, ASB3R, and *S. cerevisiae* F33 tested in Aglianico grape must. Test A, without potassium metabisulphite; test B, added with 50 mg/L potassium metabisulphite; and test C, added with 100 mg/L potassium metabisulphite. All values are expressed as mean ± standard deviation (n = 3).

Yeast Strains	Test A	Test B	Test C
AS3C1	0.89 ± 0.05 ^{a}	0.89 ± 0.05 ^{a}	0.73 ± 0.02 ^{b}
14AS	0.88 ± 0.07 ^{a}	0.86 ± 0.01 ^{a}	0.51 ± 0.06 ^{b}
ASB3R	0.91 ± 0.05 ^{a}	0.90 ± 0.02 ^{a}	0.75 ± 0.05 ^{b}
S. cerevisiae F33	2.11 ± 0.04 ^{a}	2.13 ± 0.10 ^{a}	1.98 ± 0.02 ^{a}

Different superscript letters in each row indicate significant differences. ($p < 0.05$). Fermentation vigour (test A) and sulphite tolerance (test B and test C) were determined by calculating the weight loss caused by the CO_2 release after 72 h from inoculation and the values were expressed as g CO_2/100 mL of must.

Table 2. Alcohol (% *v*/*v*) and volatile acidity (g/L acetic acid) produced by *M. pulcherrima* AS3C1, ASB3R, and 14AS in Aglianico wines. Test A, without potassium metabisulphite; test B, added with 50 mg/L potassium metabisulphite; and test C, added with 100 mg/L potassium metabisulphite.

	Yeast Strains	Test A	Test B	Test C
Alcohol (% *v*/*v*)	AS3C1	4.2 ± 0.1 ^{a}	4.2 ± 0.2 ^{a}	4.0 ± 0.1 ^{a}
	14AS	4.0 ± 0.1 ^{a}	3.8 ± 0.1 ^{a}	3.7 ± 0.1 ^{b}
	ASB3R	4.8 ± 0.2 ^{a}	4.4 ± 0.1 ^{a}	4.0 ± 0.2 ^{b}
	S. cerevisiae F33	11.6 ± 0.2 ^{a}	11.4 ± 0.1 ^{a}	11.2 ± 0.1 ^{b}
Volatile acidity (g/L acetic acid)	AS3C1	0.16 ± 0.01 ^{a}	0.12 ± 0.01 ^{b}	0.12 ± 0.02 ^{b}
	14AS	0.16 ± 0.01 ^{a}	0.15 ± 0.02 ^{a}	0.12 ± 0.01 ^{b}
	ASB3R	0.19 ± 0.01 ^{a}	0.15 ± 0.01 ^{b}	0.15 ± 0.02 ^{b}
	S. cerevisiae F33	0.46 ± 0.02 ^{a}	0.41 ± 0.01 ^{b}	0.42 ± 0.02 ^{b}

Different superscript letters in each row indicate significant differences ($p < 0.05$).

Sulphite tolerance is an important technological property to be considered for the selection of yeast starters in winemaking [39], considering that sulphur dioxide is widely used in vinification processes mainly to control the growth of spoilage microorganisms and to prevent the oxidation of wine. In our study, *M. pulcherrima* strains were able to resist at 50 mg/L of potassium metabisulphite with a sulphite tolerance ranging between 0.86 and 0.90 expressed as g CO_2/100 mL of must. These values were not significantly different from those detected in test A (without potassium metabisulphite addition). Significantly lower values, between 0.51 and 0.75 g CO_2/100 mL of must, were found with the addition of 100 mg/L of potassium metabisulphite. In comparison, *S. cerevisiae* F33 showed values of 2.13 and 1.98 g CO_2/100 mL of must when 50 and 100 mg/L of potassium metabisulphite were used, respectively, denoting the high resistance of this strain to sulphites. Our results

confirm the sulphite resistance of *M. pulcherrima* highlighted in previous studies [9,39]. This resistance could be functional to the use of *M. pulcherrima* as a starter in winemaking that involves the use of low doses of sulphur dioxide capable of inhibiting other non-*Saccharomyces* yeasts present in the must in the initial stages of fermentation.

3.2. Antimicrobial Activity

The use of sulphites in winemaking together with the synergic action of yeast strains with antimicrobial activity can amplify the inhibition activity against undesirable indigenous microorganisms initially present in musts [41]. For this reason, our pre-selection tests included this feature. The data of the antimicrobial activity, estimated as the clear zones of inhibition (ZOI, mm) exhibited by *M. pulcherrima* strains against various indicator microorganisms, are presented in Table 3. Figure 1 shows, by way of example, the inhibitory effects, measured on YEPD agar, of the *M. pulcherrima* strains against *H. guilliermondii* ATCC 10630.

Table 3. Antimicrobial activity, estimated as the diameter (mm) of the clear zone of inhibition ZOI, exerted by *M. pulcherrima* AS3C1, ASB3R, and 14AS against various indicator microorganisms.

	Producer Strains		
Indicator Strains	**ASB3R**	**AS3C1**	**14AS**
H. guilliermondii	8.0 ± 0.3^{b}	10.1 ± 0.3^{a}	10.1 ± 0.5^{a}
S. pombe	7.9 ± 0.2^{a}	4.0 ± 0.3^{c}	6.0 ± 0.2^{b}
S. cerevisiae	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
S. cerevisiae F33	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
P. terricola	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
L. brevis	19.0 ± 0.5^{a}	13.0 ± 0.3^{b}	0.0 ± 0.0^{c}
P. acidilactici	19.0 ± 0.4^{a}	11.9 ± 0.4^{b}	0.0 ± 0.0^{c}
Lp. plantarum	18.9 ± 0.3^{a}	10.1 ± 0.2^{b}	0.0 ± 0.0^{c}

Different superscript letters in each row indicate significant differences ($p < 0.05$).

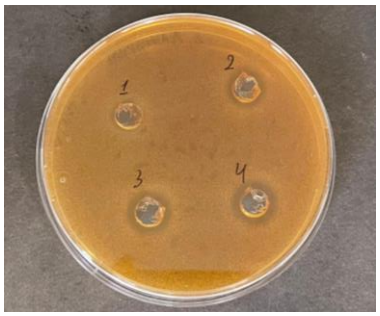

Figure 1. Inhibitory effects of *M. pulcherrima* strains against *H. guilliermondii* ATCC 10630: (**1**) negative control; (**2**) inhibitory activity of *M. pulcherrima* ASB3R; (**3**) inhibitory activity of *M. pulcherrima* AS3C1; (**4**) inhibitory activity of *M. pulcherrima* 14AS.

Among the indicator yeasts, the antagonistic activity was found against *H. guilliermondii* (inhibition zone diameter was 8.0 ± 0.3 mm for ASB3R, 10.1 ± 0.3 mm for AS3C1 and 10.1 ± 0.5 mm for 14AS) and *S. pombe* (4.0 ± 0.3 mm for AS3C1, 7.9 ± 0.2 mm for ASB3R and 6.0 ± 0.2 mm for 14AS). No inhibition against *S. cerevisiae*, *S. cerevisiae* F33, and *P. terricola* was found. These data confirm the scientific reports on the antimicrobial action of *M. pulcherrima* against some undesired wild spoilage yeasts [42]. Interestingly, the antimicrobial activity did not have an influence on the growth of *S. cerevisiae* [18], confirming that the selected *M. pulcherrima* strains may be used in controlled multi-starter fermentations with *S. cerevisiae* [9]. The *M. pulcherrima* strains showed different inhibitory activities against the indicator LAB species. In detail, a strong antimicrobial activity was exerted by *M. pulcherrima* ASB3R against *L. brevis*, *P. acidilactici*, and *Lp. plantarum* (inhibition zone diameter was 19.0 ± 0.5 mm, 19.0 ± 0.4 mm, and 18.9 ± 0.3 mm, respectively). A lower inhibition against the same LAB strains was showed by *M. pulcherrima* AS3C1

(13.0 ± 0.3 mm, 11.9 ± 0.4 mm, and 10.1 ± 0.2 mm, respectively). No antimicrobial activity was observed for *M. pulcherrima* 14AS. The results obtained are in line with the few data in the literature confirming the antagonistic activity of *M. pulcherrima* against certain Gram-positive bacteria [14]. The antimicrobial activity is strain-dependent and is probably connected to the intraspecific diversity in terms of the molecular basis responsible for the antagonistic phenotype [43]. The inhibitory activity of *M. pulcherrima* is due to the production of low molecular weight metabolites, among them also pulcherriminic acid [44].

Specifically, *L. brevis* have been frequently isolated from must and wine, and it is a spoiling microorganism due to its ability to produce biogenic amines, ethyl carbamate, volatile phenols, and off-flavours [45,46]. *L. brevis* produces high levels of acetic acid that have a detrimental impact on wine quality, while *Pediococcus* spp. and some *Lp. plantarum* strains cause wine spoilage including off-flavours and excess acetic acid, ropiness, and high level of diacetyl [47,48]. The results obtained show that the use of *M. pulcherrima* in oenology could prevent the development of undesired LAB species, especially in wines in which sulphites are not used or used in low doses. The use of this species could be an alternative to sulphiting during the pre-fermentation maceration of the grapes [21].

3.3. Oenological Properties and Enzymatic Activities

The enzymatic activities and oenological properties of the *M. pulcherrima* strains are reported in Table 4. The cryotolerance test confirmed that all the yeast strains were able to grow at 4 °C. This is an important criterion in the selection of oenological yeast starters, since low-temperature fermentation affects the final sensory quality of the wine by influencing yeast metabolism and reducing metabolite volatilisation [49,50]. The application of the low-temperature treatments of grapes or must before the alcoholic fermentation can be used also for the winemaking of red grapes to improve the colour and aroma profile of wines. The use of yeast strains during the cold pre-fermentation maceration possessing pectinolytic activities can increase the colour intensity and the anthocyanin and polyphenol contents of wines [51]. A negative contribution that could be given by some yeasts to the wine quality is due to their ability to produce volatile sulphur compounds, such as hydrogen sulfide (H_2S), known for its strong "rotten egg" aroma [52]. In particular, H_2S is a metabolite with a low sensory threshold (50–80 µg/L) and it can be produced by yeasts in different amounts during wine fermentation. Our results showed that the tested strains of *M. pulcherrima* produced low (strain ASB3R) or very low (strains AS3C1 and 14AS) amounts of H_2S and can, therefore, be used as starters without a negative impact on the aroma and flavour of the wines. With regard to pulcherrimin, all the yeast strains tested were able to produce this pigment. In this context, it is important to emphasise that pulcherrimin-forming strains are of great interest in oenology, as this compound is involved in the inhibition of undesirable microorganisms [14], thus enabling the use of sulphur dioxide to be reduced [53].

Table 4. Enzymatic activities and oenological properties from *M. pulcherrima* AS3C1, ASB3R, and 14AS strains.

	Yeast Strains		
	ASB3R	**AS3C1**	**14AS**
Polygalacturonase *	+	+	+
β-glucosidase *	+	+	+
β-lyase *	+	+	+
Protease *	+	+	+
H_2S **	3	2	2
Pulcherrimin *	+	+	+
Cryotolerance *	+	+	+

* Enzymatic activities (Polygalacturonase, β-glucosidase, β-lyase, and protease), cryotolerance and pulcherrimin production, and qualitative test (+ positive; − negative). ** H_2S production, semi-quantitative test: (1) white colour—no production; (2) light brown; (3) brown; (4) dark brown; and (5) dark brown/black.

However, our results showed that the inhibitory phenomena by *M. pulcherrima* cannot be linked to pulcherrimin alone, as the 14AS strain, which produces this pigment, was not able to inhibit the indicator bacteria used in the antimicrobial assay (Table 3). This aspect, therefore, merits further investigation in order to investigate the causes that determined the antibacterial activity of the AS3C1 and ASB3R strains.

Several studies have reported that some yeast species can produce killer toxins that show inhibitory effects not only against other yeasts and moulds, but also against various bacterial species [3].

Recently, the production of a killer toxin by a strain of *M. pulcherrima* TB26, active against Gram-negative and Gram-positive bacteria, has been demonstrated [54–56].

The co-inoculum and sequential inoculum of non-*Saccharomyces* yeasts and *S. cerevisiae* in winemaking can improve the sensorial characteristics of wines because non-*Saccharomyces* yeasts, like *M. pulcherrima*, are rich in enzymes which can release aroma compounds [8]. Our qualitative enzymatic tests showed that all the strains of *M. pulcherrima* possessed protease, β-glucosidase, β-lyase, and polygalacturonase activity. Proteases are enzymes responsible for the hydrolysis of proteins present in musts and wines. Proteins are responsible for the appearance of sediments or floccules that can produce turbidity affecting the stability of wines before or after bottling [57]. Yeasts with proteolytic activity have the capacity to hydrolyse proteins to small peptides and amino acids that can be easily consumed as a source of nitrogen. *S. cerevisiae*, the principal wine yeast, is not recognised as a significant producer of extracellular proteases, unlike some non-*Saccharomyces* yeast species, such as *M. pulcherrima*, which instead possess proteolytic activity. In our study, all the strains of *M. pulcherrima* showed protease activity. Therefore, the use of these yeasts, especially in white and rosé winemaking, can avoid the turbidity of wines and also reduce the use of bentonite or other chemicals in clarification processes [58]. *M. pulcherrima* have attracted attention for use in winemaking also thanks to the high β-glucosidase activity, the ability to decrease volatile acidity, and to increase the production of esters, terpenols, medium-chain fatty acids, higher alcohols, and glycerol [59]. In our study, all the yeast strains possessed β-glucosidase activity. Mixed fermentation combining *M. pulcherrima* with *S. cerevisiae* results in changes in the formation of aromas, with an increase in the final concentration of higher alcohols and variations in the production of ethyl esters and acetate esters [60,61]. Several non-*Saccharomyces* yeasts have been shown to release significant concentrations of volatile thiols. β-lyase is an enzymatic activity that allows the release of aromatic thiols. *M. pulcherrima* strains tested possessed this activity. The thiols 3-mercaptohexan-1-ol (3-MH) and 4-mercapto-4-methylpentan2-one (4-MMP) are known to be derived from cysteine and glutathione conjugate precursors, which break down during fermentation due to β-lyase activity of the yeast with the release of the free thiol. The selection of wine yeasts able to produce volatile thiols constitute an important goal for the wine industry and can contribute to improve wines organoleptic quality [62]. Regarding polygalacturonase activity, all the *M. pulcherrima* strains tested were positive to this activity. *Aureobasidium pullulans*, *M. pulcherrima*, and *Metschnikowia fructicola* are reported in the literature for their polygalacturonase activity, which can affect filterability and turbidity and increase the colour intensity and the anthocyanin and polyphenol contents of wines when fermented in combination with *S. cerevisiae* [63]. A recent study has shown that *M. pulcherrima* possesses a polygalacturonase activity very similar to a highly productive strain of *A. pullulans* [35]. The selection of pectinolytic yeast strains for their use as inoculum in wine fermentations could represent a useful tool to produce higher quality wines without the addition of expensive commercial enzyme preparations [63].

Finally, the qualitative screening for biogenic amines showed that the three *M. pulcherrima* strains were not capable of producing histamine, tyramine, phenylethylamine, and putrescine. Biogenic amines are non-volatile low-molecular-weight nitrogenous organic bases derived through the decarboxylation of the precursor amino acids during microbial fermentation. The enzymes responsible, amino acid decarboxylases, are widely distributed in yeasts and LAB species [64,65]. A high concentration of biogenic amines can cause

undesirable physiological effects in humans [66], so the lack of biogenic amines production is considered a positive feature for yeast strains to be used as starters in winemaking. The combined results of the tests described above allowed the selection of the *M. pulcherrima* strain for use in the fermentation tests. In particular, *M. pulcherrima* 14AS was excluded as it showed the lowest fermentation vigour, sulphite tolerance, and ability to inhibit LAB strains used as indicators in the antimicrobial test. *M. pulcherrima* ASB3R and AS3C1 exhibited very similar behaviour, but the latter strain was characterised by a higher antimicrobial activity against *H. guilliermondii*, one of the main grape-associated species with a significant impact on wine quality [25,67], and lower H_2S production. In light of these results, *M. pulcherrima* AS3C1 was selected for the winemaking trials.

3.4. Fermentative Kinetics

The yeast population dynamics during the alcoholic fermentation are shown in Table 5. The use of WL medium allowed to differentiate *S. cerevisiae* and *M. pulcherrima* as shown in Figure 2.

Table 5. Evolution of *S. cerevisiae*, *M. pulcherrima* and other yeasts during fermentation expressed as log CFU/mL: test AG-1, inoculation with *S. cerevisiae* F33; test AG-2, *M. pulcherrima* AS3C1 and *S. cerevisiae* F33 in sequential inoculum; and test AG-3, spontaneous fermentation. All values are expressed as mean ± standard deviation (n = 3).

		Fermentation Time (Days)				
Test	**Yeasts**	**0**	**2**	**4**	**6**	**10**
AG-1	*S. cerevisiae*	6.66 ± 0.12 ^{b}	7.86 ± 0.16 ^{a}	7.95 ± 0.17 ^{a}	8.09 ± 0.25 ^{a}	7.96 ± 0.11 ^{a}
	Other yeasts	4.63 ± 0.26 ^{a}	3.40 ± 0.23 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
	M. pulcherrima	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
AG-2	*S. cerevisiae*	4.02 ± 0.13 ^{c}	6.94 ± 0.05 ^{b}	8.17 ± 0.27 ^{a}	8.22 ± 0.29 ^{a}	8.00 ± 0.16 ^{a}
	Other yeasts	4.79 ± 0.10 ^{a}	4.65 ± 0.15 ^{a}	2.79 ± 0.12 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.0 ^{c}
	M. pulcherrima	6.70 ± 0.08 ^{b}	7.03 ± 0.10 ^{a}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}	0.00 ± 0.0 ^{c}
AG-3	*Saccharomyces*	2.91 ± 0.11 ^{d}	4.34 ± 0.18 ^{c}	6.55 ± 0.14 ^{b}	7.71 ± 0.15 ^{a}	7.67 ± 0.09 ^{a}
	Other yeasts	4.95 ± 0.12 ^{a}	4.77 ± 0.20 ^{a}	3.80 ± 0.23 ^{b}	2.53 ± 0.34 ^{c}	0.00 ± 0.00 ^{d}
	M. pulcherrima	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}

Different superscript letters in each row indicate significant differences ($p < 0.05$).

Figure 2. Colonies morphology of *S. cerevisiae* (creamy white) and *M. pulcherrima* (light blue) using WL agar medium.

In test AG-1, the alcoholic fermentation started with the inoculum of *S. cerevisiae* F33 at a concentration of 6.00 log CFU/mL. *S. cerevisiae* maintained throughout the fermentation a cellular concentration around 7.00–8.00 log CFU/mL, with 7.96 log CFU/mL registered after 10 days. The other yeasts were initially present at a concentration of 4.63 log CFU/mL and then rapidly decreased to no longer detectable values after 4 days of fermentation. No colonies of *M. pulcherrima* were detected in this test. In the test AG-2, in which the

strain AS3C1 was initially inoculated at a concentration of 6.00 log CFU/mL, the colonies attributable to the species *M. pulcherrima* increased to a cellular density of 7.03 log CFU/mL after two days, but in the following days, they were no longer detectable. Inoculation with *S. cerevisiae* F33 (6.00 log CFU/mL) was carried out after 48 h, and the concentration of the cells attributable to this species reached approximately 8.00 log CFU/mL after 4 days. This concentration was maintained until 10 days. The other yeasts were found at 4.79 log CFU/mL at the beginning and then decreased rapidly until they were no longer detectable after 6 days. In the test AG-3, that is, spontaneous fermentation, *Saccharomyces* yeasts were initially present at 2.91 log CFU/mL, but from the 2nd day of fermentation onwards, they increased and stabilised at around 7.00 Log CFU/mL until the end of the fermentation. The other yeasts count was 4.95 Log CFU/mL at the beginning of fermentation, and from day 4, it decreased to 3.80 log CFU/mL and were no longer detectable after 10 days. *M. pulcherrima* was never found in this test.

The non-*Saccharomyces* dynamics are in agreement with the data reported in the literature, indicating their dominance in the first 3–4 days of fermentations up to an ethanol concentration of about 4–7% (*v*/*v*) [26]. Several non-*Saccharomyces* yeasts are incapable of completing alcoholic fermentation. To reduce the risk of a stuck fermentation, *S. cerevisiae* is usually added as a co-inoculum or sequentially after 24–72 h inoculation of non-*Saccharomyces* yeasts [68]. In this study, alcoholic fermentation was completed in all the trials (Figure 3), and *Saccharomyces* yeasts were dominant in test AG-1 and in test AG-3; they became the most abundant yeasts between 2 and 4 days until the end of fermentation. In test AG-2, *M. pulcherrima* AS3C1 maintained a good cell viability for 48 h. This is in accordance with previous studies showing that in the early stages of alcoholic fermentation, non-*Saccharomyces* yeasts, such as *M. pulcherrima*, are present in the grape must [26], where they are capable to exert a moderate fermentation. Moreover, these yeasts have interesting enzymatic activities involving aromatic and colour precursors. The inoculation of *S. cerevisiae* after 48 h in the AG-2 test and its prevalence after 2–4 days in the AG-3 test reduced the persistence of *M. pulcherrima*, as well as that of other yeasts. In fact, *S. cerevisiae* has an antagonistic effect on the other yeast species due to its high rate of sugar consumption and ethanol production together with a high tolerance to alcohol [69]. The fermentation process was monitored assessing the ethanol evolution during the alcoholic fermentation (Figure 3). As stated above, alcoholic fermentation was completed in all the trials, but the evolution of alcohol production varied within the three tests. In test AG-1, inoculated with *S. cerevisiae* F33, the ethanol content increased exponentially between day 1 and 7, and it reached 12.30% already at day 7 of fermentation. In tests AG-2 and AG-3, the ethanol content after 24 h of fermentation was 0.27% and 0.24%, respectively, significantly different if compared to the value registered in test AG-1 (0.63%). From the 3rd day onward, there has been a gradual increase until the end of alcoholic fermentation. At the end of the fermentation, the alcohol content in the wines obtained from tests AG-1 and AG-2 was 13.53% and 13.16%, respectively, significantly different from that of the wine obtained from AG-3 (12.63%). Overall, these results confirm, as abundantly reported in the literature, the predominance of non-*Saccharomyces* yeasts with low fermentation vigour [25,67,70] at the beginning of spontaneous fermentation. Among them, different species belonging to *Hanseniaspora*, *Candida*, *Pichia*, *Hansenula*, *Metschnikowia*, and *Kluyveromyces* can be detected in different fermentation stages depending on the production area, vineyard age, grape variety, and practical winemaking process [42]. As fermentation continues and ethanol becomes increasingly concentrated, *S. cerevisiae* strains take over the process until the end of alcoholic fermentation [18]. To facilitate the control of fermentation conditions and reduce the risk of stuck fermentation, spoilage, and unpredictable changes in wine flavour, commercial active dry *S. cerevisiae* strains are commonly used in current winemaking [71], recently complemented by indigenous grape yeasts recognised for their importance in the final quality of wines.

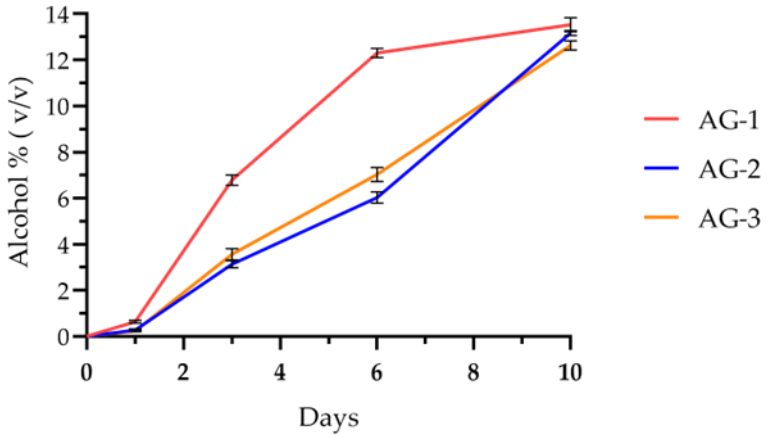

Figure 3. Ethanol evolution (% *v*/*v*) during fermentation in test AG-1 (inoculation with *S. cerevisiae* F33), test AG-2 (*M. pulcherrima* AS3C1 and *S. cerevisiae* F33, sequential inoculum), and test AG-3 (spontaneous fermentation).

3.5. Main Chemical Parameters of Wines

The results of the main chemical parameters analysis of the wines at the end of alcoholic fermentation are shown in Table 6. The alcoholic fermentation in all the tests was considered finished when there was not any longer variation in the alcohol content—Figure 3 (10 days). The values of reducing sugar detected in the final wines obtained from tests AG-1, AG-2, and AG-3 were 0.65 g/L, 1.06 g/L, and 1.72 g/L, respectively. Contemporarily to the decrease in sugars during the alcoholic fermentation, there was a gradual increase in the alcohol content and the value find into the final wines in tests AG-1, AG-2, and AG-3 were 13.53% (*v*/*v*), 13.16% (*v*/*v*), and 12.63% (*v*/*v*), respectively. The wine obtained from test AG-3 showed the lowest alcohol content and the highest concentration in residual sugars, significantly different from the values found in the wines obtained from the other tests. This is due to the fact that non-*Saccharomyces* yeasts, predominant in the early stage of spontaneous fermentation, are quickly overwhelmed by the indigenous *Saccharomyces*. The latter become predominant and can grow and produce enough alcohol to inhibit the other indigenous yeasts and dominate the main alcohol fermentation. If non-*Saccharomyces* yeasts become dominant instead of Saccharomyces species, they potentially produce undesirable aromatic defaults such as acetic acid and ethyl acetate, and they may cause stuck or sluggish fermentations [72]. The volatile acidity values found in the wines obtained from the AG-1, AG-2, and AG-3 tests were 0.15 g/L, 0.30 g/L, and 0.76 g/L, respectively, in accordance with the non-*Saccharomyces* counts, including *M. pulcherrima* in test AG-2. In general, non-*Saccharomyces* yeasts play a significant role in producing aroma compounds, such as esters, higher alcohols, acids, and monoterpenes, but some species found in spontaneous fermentations can produced high amounts of acetic acid, with a significant increase in the volatile acidity of wines [73]. Significant differences were found in the concentration of phenolic compounds into the wines. The highest amounts of anthocyanins and catechins were found in the wine obtained from AG-2 test, which were 334.42 mg/L and 243.92 mg/L, respectively. In tests AG-1 and AG-3, 302.25 mg/L and 313.89 mg/L of anthocyanins and 185.66 mg/L and 230.00 mg/L of catechins were detected. This result could be attributable to the polygalacturonase activity of *M. pulcherrima* AS3C1 inoculated in test AG-2 during the grape maceration phase. This activity probably may also have contributed to increasing the colour intensity (CI) of the resulting wine (16.84 versus 12.93 and 13.74 registered in tests AG-1 and AG-3, respectively). Moreover, together with polygalacturonase, the other enzymatic activities (β-glucosidase, β-lyase, and protease) of *M. pulcherrima* AS3C1 could contribute to improving the technological process and impacting the sensory characteristics of wine by releasing aromatic precursors, increasing the intensity and colour stability of red wines and facilitating the clarification process [74]. The glycerol concentration in the wines obtained from tests AG-1 and AG-2 were 6.10 g/L

and 5.84 g/L, respectively, which did not differ significantly. The highest glycerol content was detected in the wine obtain from test AG-3, which was 7.65 g/L.

Table 6. Chemical parameters of the wines. Test AG-1, inoculation with *S. cerevisiae* F33; test AG-2, *M. pulcherrima* AS3C1 and *S. cerevisiae* F33 in sequential inoculum; and test AG-3, spontaneous fermentation. All the values are expressed as mean ± standard deviation (n = 3).

Chemical Parameters	Test AG-1	Test AG-2	Test AG-3
pH	3.32 ± 0.07 ^{a}	3.35 ± 0.06 ^{a}	3.38 ±0.06 ^{a}
Alcohol % (v/v)	13.53 ± 0.30 ^{a}	13.16 ± 0.11 ^{a}	12.63 ± 0.20 ^{b}
Volatile acidity (g/L)	0.15 ± 0.03 ^{c}	0.30 ± 0.04 ^{b}	0.76 ± 0.05 ^{a}
Total acidity (g/L)	8.62 ± 0.13 ^{a}	7.84 ± 0.17 ^{b}	7.13 ± 0.11 ^{c}
D-lactic acid (g/L)	0.77 ± 0.07 ^{a}	0.45 ± 0.07 ^{b}	0.39 ± 0.04 ^{b}
DL-malic acid (g/L)	1.47 ± 0.10 ^{a}	1.32 ± 0.07 ^{a}	1.12 ± 0.09 ^{b}
L-lactic acid (g/L)	0.07 ± 0.01 ^{a}	0.05 ± 0.01 ^{b}	0.09 ± 0.01 ^{a}
Anthocyanins (mg/L)	302.25 ± 9.08 ^{b}	334.42 ± 8.51 ^{a}	313.89 ± 6.32 ^{b}
Reducing sugar (g/L)	0.65 ± 0.10 ^{c}	1.06 ± 0.12 ^{b}	1.72 ± 0.08 ^{a}
Glycerol (g/L)	6.10 ± 0.11 ^{b}	5.84 ± 0.16 ^{b}	7.65 ± 0.22 ^{a}
Colour intensity (CI)	12.93 ± 0.33 ^{b}	16.84 ± 1.52 ^{a}	13.74 ± 0.24 ^{b}
Tonality (T)	0.56 ± 0.05 ^{a}	0.51 ± 0.03 ^{a}	0.48 ± 0.09 ^{a}
Catechins (mg/L)	185.66 ± 9.77 ^{b}	243.92 ± 12.72 ^{a}	230.00 ± 7.93 ^{a}
Acetaldehyde (mg/L)	27.60 ± 2.62 ^{a}	24.73 ± 1.55 ^{a}	10.80 ± 0.80 ^{b}

Different superscript letters in each row indicate significant differences ($p < 0.05$).

Glycerol contributes to smoothness, sweetness, and complexity in wines, but the grape variety and winemaking will determine the extent to which glycerol impacts on these properties. Increased glycerol production is usually linked to increased acetic acid production, which can affect negatively the wine quality [75]. Spontaneously fermented wines have higher glycerol levels, indicating a possible contribution by non-*Saccharomyces* yeasts. There are some factors that can have an impact on the formation of glycerol by non-*Saccharomyces* yeasts, in particular the concentration of nitrogen and sulphites [76]. The higher acetaldehyde values were found in wines from test AG-1 and AG-2 (27.60 mg/L and 24.73 mg/L, respectively), significantly different if compared with the value detected in the wine from test AG-3 (10.80 mg/L). Large amounts of this compound are released by yeasts during alcoholic fermentation as a by-product. Generally, non-*Saccharomyces* yeast species are the lower producers of this compound respect to *S. cerevisiae*. In all the tests, the acetaldehyde values found were under the sensory threshold of 100–125 mg/L [77]. Non-*Saccharomyces* yeasts have been considered for years as contaminating microorganisms in winemaking due to the production of undesirable compounds. This aspect has been changing in recent years due to the growing interest in certain species, like *M. pulcherrima*, which can positively impact the wine organoleptic characteristics thanks to some of their peculiar enzymatic activities [78].

4. Conclusions

The use of non-*Saccharomyces* strains in mixed cultures with *S. cerevisiae* could represent an oenological strategy to differentiate and improve wines. Our results highlighted that *M. pulcherrima* AS3C1 exert an antimicrobial action against some unwanted bacteria and yeasts frequently present in grape must and potentially causing the alteration of wines. Therefore, it could be used for the control of the indigenous microflora of musts, promoting less use of sulphur dioxide. In addition, *M. pulcherrima* AS3C1 possesses some specific enzymatic activities (polygalacturonase, β-glucosidase, β-lyase, and protease) that could facilitate the extraction of aromas and phenolic compounds contained in the grape skin and improve the final clarification process of wines. These peculiarities positively suggest the possible use of *M. pulcherrima* AS3C1 as a starter in red winemaking processes. Further studies are underway to assess the impact of this yeast strain on wine volatile and non-volatile composition included the polyphenols composition and colour stability of red wines.

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