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Functional Screening of Native *Metschnikowia pulcherrima* Strains for Wine Fermentation and Biocontrol of *Botrytis cinerea* in a Sustainable Production Framework

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Abstract

Metschnikowia pulcherrima is increasingly valued in sustainable vitiviniculture for its dual role as a biocontrol agent and as a contributor to wine quality. However, a coordinated dual-purpose selection strategy has not yet been systematically implemented for this species. This study aimed to identify native strains with combined wine-related traits and biocontrol potential by screening a collection of 179 isolates for key phenotypic traits—β-glucosidase and β-lyase activities, hydrogen sulfide (H_2S) production, and pulcherrimin biosynthesis—and assessing their genotypic diversity. Dereplication yielded 106 unique strains, from which five with the most favorable wine-related traits and distinct genotypic profiles were selected for subsequent evaluation of antagonistic potential. Safety-related traits, including growth at 37 °C, invasive growth, pseudohyphal formation, and proteolytic activity, were assessed to exclude virulence-associated behaviors. Antagonistic activity against *Botrytis cinerea* was evaluated through in vitro dual-culture assays and in vivo grape-berry inoculations, revealing strain- and pathogen-dependent inhibition, with volatile-mediated effects generally exceeding direct-contact interactions. Among the tested strains, NLSFS4 showed strong and consistent biocontrol potential. Microvinification trials further confirmed its oenological relevance, demonstrating the ability to modulate wine aroma composition while preserving fermentation performance. Overall, this study highlights the substantial functional diversity within *M. pulcherrima* and identifies a promising native strain for integrated use in wine fermentation and biological control in sustainable production systems.

Keywords: *Metschnikowia pulcherrima*; dual-role; biocontrol; *Botrytis cinerea*; wine fermentation; sustainable viticulture

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1. Introduction

Metschnikowia pulcherrima, a non-*Saccharomyces* yeast commonly associated with grapes, fruits, flowers, and other plant surfaces, has increasingly attracted interest for its ecological adaptability and biotechnological potential [1–3]. Recent evidence highlights

its dual relevance in vitiviniculture, supporting both the biocontrol of plant pathogens and the improvement of fermentation dynamics and wine quality [4,5].

In biocontrol applications, *M. pulcherrima* displays pronounced antagonistic activity against several phytopathogenic fungi, most notably *Botrytis cinerea*, the causal agent of grey mold and a major source of pre- and post-harvest losses in many crops [6,7]. A key determinant of this antagonism is the biosynthesis of pulcherrimin, a distinctive red pigment formed by extracellular chelation of pulcherriminic acid with ferric ions. The resulting iron-pulcherrimin complex restricts iron availability to competing microorganisms, thereby inhibiting their growth [1,8–11]. Additional mechanisms contributing to antifungal activity include competition for nutrients and colonization sites, secretion of cell wall-degrading enzymes such as chitinases and glucanases, and the potential induction of host defense responses [9,12–15]. Moreover, volatile organic compounds (VOCs) produced during fermentation can exert antimicrobial effects, further enhancing the yeast's biocontrol capacity [16,17]. The efficacy of *M. pulcherrima* in controlling fungal pathogens has been demonstrated in both pre- and post-harvest systems, where it can significantly reduce decay and storage losses [11,18–20]. These findings underscore its potential as a biological control agent (BCA), providing a sustainable alternative to chemical fungicides, whose efficacy is increasingly limited by resistance development and environmental concerns [20–22].

In winemaking, the antimicrobial properties of *M. pulcherrima* support its use as a bio-protective agent capable of limiting the proliferation of spoilage microorganisms during the early stages of fermentation and reducing reliance on sulphite addition. This aligns with consumer demand for low-intervention wines and current trends toward minimizing chemical inputs [23,24]. The commercialization of *M. pulcherrima* bioprotective starters such as Shemer® and Level^2 Initia™ and Level^2 Flavia™ further demonstrates their successful applications at the industrial scale [25,26].

Beyond its protective functions, *M. pulcherrima* exhibits enzymatic versatility and metabolic traits, which can enhance wine composition and sensory quality. Although non-*Saccharomyces* yeasts were historically regarded as spoilage microorganisms, they are now increasingly used in sequential or co-inoculation strategies with *Saccharomyces cerevisiae* to improve wine aroma [27,28]. In this context, *M. pulcherrima* is particularly valued for its β-glucosidase and β-lyase activities, which release aroma-relevant compounds from non-volatile glycosidic precursors present in grape musts. These enzymes contribute to the liberation of varietal thiols and terpenes, thereby enriching aromatic complexity and varietal expression of wines [4,29–34]. *M. pulcherrima* also produces esters and higher alcohols, which modulate aroma [35], and its predominantly respiratory metabolism under aerobic or microaerophilic conditions allows a reduction in ethanol yield—an increasingly desirable trait as grape sugar levels rise due to climate change [1,2,8].

Recent genomic and phenotypic studies have revealed substantial strain-dependent variability in key traits, including enzymatic activity, pulcherrimin production, and safety-associated phenotypes. These differences are largely shaped by the ecological origin and genetic background of individual strains, underscoring the critical importance of targeted strain selection for specific applications in agriculture and oenology [6,36]. Most studies have assessed the oenological properties and biocontrol potential of non-*Saccharomyces* yeasts independently. An integrated selection strategy that simultaneously evaluates wine-related traits, biocontrol mechanisms, and genotypic diversity has not yet been systematically applied to *M. pulcherrima*, limiting the exploitation of its full multifunctional potential.

To address this gap, the present study implemented a comprehensive screening pipeline to pre-select *M. pulcherrima* strains with dual potential for oenological applications and biocontrol. Wine-related traits were used as early-stage selection markers

to efficiently screen a large strain collection, focusing on enzymatic activities linked to aroma precursor release (β-glucosidase and β-lyase) and hydrogen sulphite production as an indicator of off-flavor risk. This approach enabled the identification of genetically distinct candidates suitable for subsequent evaluation of safety attributes, antagonistic activity against *Botrytis cinerea*, and its impact on fermentation performance and wine aroma profiles in microvinification trials.

2. Materials and Methods

2.1. Yeast and Mold Strains and Growth Conditions

In this study, a collection of 179 native *M. pulcherrima* isolates, as listed in Table S1, was examined together with subcultures of two *M. pulcherrima* commercial strains (on the market as Level^2 Flavia and Level^2 Initia, hereinafter referred to as Flavia and Initia) and one *Saccharomyces cerevisiae* strain (EC 1118). All commercial products were provided by Lallemand Inc. (Castel d'Azzano, Italy) and served as reference controls for comparative evaluations. The *S. cerevisiae* strain Zymaflore X5 (hereinafter X5; Laffort, Bordeaux, France) was used as the control in the microvinification trials.

Based on their functional traits, five native *M. pulcherrima* strains were selected for subsequent assessment of biocontrol potential. Two *Botrytis cinerea* strains were used as model pathogens: TOB62, a wild environmental isolate obtained from withered Nosiola grapes (Trentino-Alto Adige/Südtirol, Italy), and BCZG1, a strain from the culture collection maintained at the University of Verona.

Yeasts were reactivated following a standardized protocol across all experimental assays. Frozen cultures stored at −80 °C were first reactivated on WL (Wallerstein Laboratory) Nutrient Agar (Oxoid, Freiburg, Germany), followed by incubation in YPD broth (yeast extract, 1.0%; bacteriological peptone, 2.0%; glucose, 2.0%), under agitation overnight at 27 °C, to reach the early stationary phase. Cells were harvested by centrifugation (3000× *g*, 5 min; centrifuge 5417 R, Eppendorf, Hamburg, Germany), washed twice with sterile physiological solution (0.9% *w*/*v* NaCl), and resuspended to an optical density of 0.6 at 600 nm (OD_{600}), measured using a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), corresponding to approximately 1×10^7 cells/mL.

Botrytis cinerea strains were grown on PDA medium (potato extract, 0.4%; glucose, 2.0%; agar, 1.5%) for 5 days at 27 °C. The resulting mycelium was used for in vitro assays involving simultaneous growth. To obtain conidial suspensions for dual-culture and in vivo assays, PDA plates were incubated at room temperature for 15 days under natural light conditions. Conidia were collected using sterile distilled water supplemented with 0.05% (*v*/*v*) Tween 80, centrifuged (20,800× *g*, 5 min), and resuspended in the same solution. Conidial concentration was determined microscopically using a Burker counting chamber and adjusted to 1×10^6 conidia/mL. All reagents were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Phenotypical Characterization of Oenological Traits

Functional phenotypic traits were assessed using spot assays on Petri dishes containing specific differential media. A 10 mL aliquot of a 48-h YPD culture was deposited onto the agar surface and allowed to dry under a biosafety cabinet. Each yeast strain was tested in triplicate.

- β-Glucosidase Activity

The β-glucosidase activity was determined following the protocol of González Flores et al. (2017) [37]. The assay was performed on Esculin Agar Medium, which is composed of glucose (0.2%), peptone (0.1%), yeast extract (0.1%), esculin (0.3%), and agar (1.5%), adjusted to pH 5.0. After sterilization, 20 mL/L of a sterile filtered 1% (*w*/*v*) ferric ammonium citrate

solution was added. Yeast cultures grown in YPD broth were spotted onto the medium and incubated at 26 °C for 5 days. The formation of a dark halo around the yeast spot indicated enzymatic activity, which was scored as 0 (absent), 50 (moderate), or 100 (high).

- β-Lyase Activity

β-lyase activity was assessed using the YCB-SMC medium developed by Belda et al. (2016) [38], composed of S-methyl-L-cysteine (0.1%), pyridoxal-5-phosphate (0.01%), Yeast Carbon Base (Sigma-Aldrich, Darmstadt, Merck, 1.2%), and agar (2%) and adjusted to pH 3.5. The 48-h YPD cultures were spotted onto the medium, and the plates were incubated at 20 °C for 48–72 h. Colony growth was considered indicative of β-lyase activity. To minimize false positives, all strains were subjected to two consecutive passages on the YCB-SMC medium. β-lyase activity was semi-quantitatively scored as 0 (no growth, no activity), 50 (moderate growth, moderate activity), or 100 (abundant growth, high activity).

- Hydrogen Sulphite (H_2S) Production

H_2S production was assessed using Bismuth Sulfite Glucose Glycine Yeast (BIGGY; Sigma-Aldrich, Merck) agar, following the protocol described by Iorizzo et al. (2021) [39]. Each *M. pulcherrima* strain was streaked onto BIGGY agar and incubated at 15 °C for 5 days. H_2S-negative strains formed white colonies, whereas H_2S-producing strains exhibited brown to dark brown coloration. A chromatic scale (0, 1, 2.5, 5) was used to classify the intensity of colony coloration.

The patterns of β-glucosidase and β-lyase activities, as well as H_2S production, were then combined to distinguish the phenotypic profile distribution among the 179 isolates.

2.3. Pulcherrimin Production

Pulcherrimin production was evaluated on YG agar plates composed of yeast extract (0.5%), glucose (2.0%), and agar (2.0%) and supplemented with 0.05% (w/v) $FeCl_3$, following Pawlikowska et al. (2020) and Troiano et al. (2022) [40,41]. After incubation at 27 °C for 72 h, colonies were assessed for pulcherrimin production using a color-based scale from 1 to 5, reflecting pigmentation intensity.

2.4. Genotypic Clustering and Strain Definition

Total genomic DNA was extracted from 2-mL of yeast cultures grown in YPD medium using the Wizard Genomic DNA Purification Kit (Promega, Milano, Italy), according to the manufacturer's instructions. DNA yield and purity were assessed with a NanoDrop ND1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). A combined fingerprinting approach was applied, integrating the banding patterns from Rep-PCR using the $(GTG)_5$ primer with those from RAPD-PCR with the M13 random primer [41].

PCR reactions were performed in a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles of 95 °C for 1 min, 40 °C for 1 min, and 72 °C for 8 min; followed by a final elongation at 72 °C for 16 min.

Amplification products were separated on 1.5% (w/v) agarose gels in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 0.4 mM EDTA), stained with Atlas Clearsight (Bioatlas, Tartu, Estonia), and run at 110 V for 2 h. Each gel included an O'Gene Ruler DNA ladder (Thermo Scientific) for normalization. Visualization and image capture were performed under UV light using a UVITEC Gel Documentation System (Cleaver Scientific, Rugby, England).

Fingerprint profiles were analyzed with TotalLab CLIQS 1D Pro (v1.0) for band detection, normalization, and clustering. Similarity among profiles was calculated using Pearson's correlation coefficient, and dendrograms were generated using the un-

weighted pair group method with arithmetical average (UPGMA). A similarity cut-off of 0.20—empirically defined from the clustering distance of a reference profile included on all gels—was used to identify genotypically identical isolates. This internal reference also enabled consistent normalization and dereplication across gels.

Genetic clusters identified through fingerprinting served as the primary criterion for strain assignment. Phenotypic traits were subsequently used to resolve isolates within each cluster. Isolates were considered the same strain only if they shared both identical genotypic fingerprints and phenotypic profiles. In contrast, genotypically identical isolates exhibiting divergent phenotypes were classified as distinct strains.

By integrating enzymatic screening with genotypic clustering, five isolates (FIANO12, FM15, MONL18, G12, and NLSFS4) displaying suitable wine-related traits and clear genetic distinctiveness were selected for subsequent pathogenicity and biocontrol assays.

2.5. Tests of Pathogenicity

Growth at 37 °C, invasive growth, formation of pseudohyphae, and proteolytic activity were assessed by spot inoculation on specific media, as described by de Llanos et al. (2006) [42]. From a 48-h YPD culture, 10 µL droplets were deposited onto the agar surface and allowed to dry under a biosafety cabinet. Ten spots were applied per plate, and all isolates were tested in quadruplicate. *Saccharomyces cerevisiae* EC 1118 was used as a control.

Growth at human body temperature was evaluated on YPD agar after 3 days of incubation at 37 °C and compared to control plates incubated at 28 °C. Invasive growth was assessed on the same YPD plates after 10 days of incubation at room temperature by washing off surface colonies with deionized water to evaluate agar penetration.

Pseudohyphae formation was evaluated using Synthetic Low Ammonium Dextrose (SLAD) medium, which contained $(NH_4)_2SO_4$ (6.61 mg/L), Yeast Nitrogen Base (YNB; Sigma-Aldrich, Merck) without amino acids (6.7 g/L), dextrose (20 g/L), and agar (20 g/L). The plates were incubated at 30 °C for 10 days. Results were classified based on colony morphology, as negative (plain margin), intermediate (irregular margin), or positive (visible pseudohyphae).

Proteolytic activity was determined using a medium containing malt extract (20 g/L), $MgSO_4$ (0.2 g/L), K_2HPO_4 (2.5 g/L), NaCl (5 g/L), yeast extract (1 g/L), dextrose (20 g/L), and agar (20 g/L). After sterilization, bovine serum albumin (BSA; Sigma-Aldrich, Merck) was added at a concentration of 2.5 g/L. Plates were observed after 4 days of incubation at 37 °C; a clear halo around the colonies indicated proteolytic activity.

2.6. In Vitro Antagonistic Bioassays

The antimicrobial activity of *M. pulcherrima* strains against *B. cinerea* TOB62 and BCZG1 was evaluated using the dual culture method on PDA agar plates, following the procedures described by Parafati et al. (2015) [43] and Lemos Junior et al. (2016) [44], with the modifications detailed below.

- Contemporary Growth on Agar Plates

In this assay, each yeast and mould strain was simultaneously inoculated on the same PDA agar plate. A 10 µL aliquot of yeast cell suspension, prepared as previously described, was streaked orthogonally across the center of the Petri dish. Subsequently, two 6 mm diameter plugs were excised from the actively growing edge of *B. cinerea* mycelium and placed on opposite sides of the plate, each 3 cm from the yeast streak (corresponding to a 6 cm distance between plugs) and 1.5 cm from the plate's edge. Plates were incubated at 25 °C for 5 days. Each treatment was performed in triplicate. Control plates were prepared by inoculating only the *B. cinerea* strain. Radial growth inhibition was calculated using the following formula: $I(\%) = [(C - T)/C] \times 100$, where $I(\%)$ represents the percentage

inhibition of radial mycelial growth, *C* is the radial growth of *B. cinerea* on the control plate, and *T* is the radial growth of *B. cinerea* on the plate containing the yeast strain [43].

- Opposite Growth on Agar Plates

This assay required two PDA plates for each yeast/*B. cinerea* pair. A 100 µL aliquot of yeast suspension was spread evenly on one PDA plate. On the second plate, a 10 µL drop of *B. cinerea* conidial suspension, prepared as previously described, was inoculated at the center of the plate. The two plates were then positioned face-to-face, sealed together at the edges with Parafilm®, and incubated at 25 °C for 5 days. Controls consisted of *B. cinerea*-inoculated plates paired with uninoculated PDA plates. The percentage of inhibition was calculated using the same formula as above [43].

2.7. In Vivo Antagonistic Bioassay

The antagonistic activity of selected *M. pulcherrima* strains against *B. cinerea* TOB62 and BCZG1 was evaluated on wounded white table grapes, following the protocol described by Nadai et al. (2018) [29], with minor modifications. Briefly, healthy, undamaged grape berries were cleaned and disinfected by immersion in a 0.1% (*v*/*v*) sodium hypochlorite solution for 2 min, followed by rinsing with sterile distilled water. After air-drying at room temperature, each berry was wounded at the equatorial region using a sterile needle. Each wound was inoculated with 10 µL of a conidial suspension of *B. cinerea* (1×10^6 conidia/mL). Two hours after conidial inoculation, 10 µL of yeast cell suspension (1×10^7 cells/mL) were applied to the same wound. Control berries were treated with 10 µL of sterile physiological solution. The experiment was repeated twice, each time in duplicate. For each *M. pulcherrima*/*B. cinerea* pair, a total of 80 berries was used. The berries were incubated in plastic boxes at 25 °C for 5 days, with a moistened paper towel placed inside each box to maintain high humidity. Treatment efficacy was evaluated using the same inhibition formula reported above.

2.8. Microvinification Trials and Analysis of VOCs

Metschnikowia pulcherrima NLSFS4 was evaluated in microvinification trials according to a previously described protocol [26]. Fresh untreated white grape must was distributed into sterile 500 mL fermentation bottles fitted with airlocks to allow CO_2 release. Fermentations were performed in triplicate at 16 °C. Sequential inoculations consisted of *M. pulcherrima* followed 48 h later by *S. cerevisiae* Zymaflore X5 (Laffort, Bordeaux, France), while a single-strain *S. cerevisiae* fermentation was used as the control. All yeast cultures were inoculated at approximately 1×10^6 cells/mL.

Volatile organic compounds (VOCs) were quantified by gas chromatography–mass spectrometry (GC–MS) following the method previously described by Bertazzoli et al. (2025) [26].

2.9. Statistical Analysis

All experiments were performed with at least three biological replicates, and data are reported as mean values ± standard deviation (SD). Statistical analyses were conducted using ExcelStat software (version 2021).

Normality of data distribution was assessed using the Shapiro–Wilk test. When both normality and homogeneity of variance assumptions were satisfied, differences among treatments were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. When parametric assumptions were not met, the Kruskal–Wallis test was applied, with the Conover–Iman procedure used for pairwise comparisons.

To determine whether isolates were randomly distributed across phenotypic profiles, a chi-square (χ^2) goodness-of-fit test was performed by comparing observed and expected frequencies under a uniform distribution. Standardized residuals were examined to identify profiles contributing disproportionately to the χ^2 statistic, with values >+2 or <−2 indicating strong over- or under-representation.

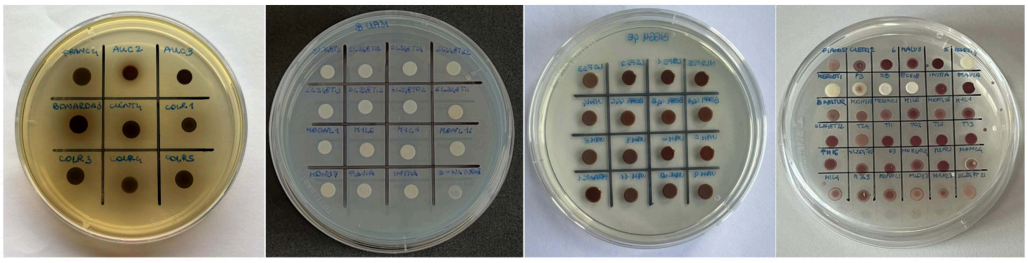
For in vitro and in vivo antagonistic assays, inhibition percentages were compared across yeast strains and *B. cinerea* isolates using the appropriate parametric or non-parametric tests as described above. A significance threshold of $p < 0.05$ was applied to all analyses.

3. Results and Discussion

3.1. Oenologically Relevant Phenotypic Traits

Strains intended for vineyard application may persist during fermentation and influence the wine's sensory profile; therefore, an initial pre-screening of some functional phenotypic traits was performed to exclude isolates with potentially undesirable aroma-related characteristics and retain only those exhibiting a favorable oenological background. This approach ensured that subsequent evaluations of biocontrol and bioprotection potential were conducted exclusively on strains suitable for dual-purpose use.

A total of 179 isolates were screened for three traits relevant to winemaking: β-glucosidase and β-lyase activities and H_2S production. The results of this phenotypic screening are reported in Figures 1 and S1 and Table S2.



(**A**) (**B**) (**C**) (**D**)

Figure 1. Representative examples of wine-relevant traits and pulcherrimin production in *Metschnikowia pulcherrima* isolates. Reference control strains are included. (**A**) β-glucosidase activity on Esculin Agar Medium, incubated at 26 °C for 5 days. (**B**) β-lyase activity on YCB-SMC medium (20 °C for 48–72 h). (**C**) H_2S production on Bismuth Sulfite Glucose Glycine Yeast medium (15 °C for 5 days). (**D**) Pulcherrimin production on YG agar plates supplemented with 0.05% (*w*/*v*) $FeCl_3$, (27 °C for 72 h).

β-glucosidase activity was detected in 67.04% of isolates, while β-lyase activity was detected in 98.88% of isolates. This indicates that β-lyase is nearly ubiquitous within the collection, whereas β-glucosidase is more variable—a pattern consistent with previous studies reporting strain-dependent β-glucosidase activity in *M. pulcherrima* [31]. Conversely, the high prevalence of β-lyase activity aligns with recent findings describing this function as a characteristic and strong feature of the species [45]. Together, these enzymatic profiles reinforce the potential of *M. pulcherrima* to contribute to varietal thiol release and aromatic enhancement during fermentation.

Regarding H_2S production, colony staining intensity on the BIGGY medium revealed that 4.47% of isolates were classified as low producers (score 1), 13.40% as medium producers (score 2.5), and 81.56% as high producers (score 5). Only two isolates showed no detectable H_2S formation, highlighting the predominance of elevated sulfite reductase activity within the collection. This variability is consistent with reports showing that H_2S production in non-*Saccharomyces* yeasts is strongly strain-dependent and influenced by ex-

perimental and nutritional conditions, rather than being a fixed species-level trait [1,31,35]. The rarity of H_2S-negative strains reflects the physiological requirement for basal activity of the sulfate reduction pathway, as complete suppression would impair the biosynthesis of essential sulfur amino acid [46]. Overall, these results indicate that H_2S production is a highly variable and discriminating trait within *M. pulcherrima* populations, representing an important criterion for selecting strains suitable for dual applications in viticulture and fermentation.

The 179 isolates were grouped into ten phenotypic profiles based on their combined β-glucosidase, β-lyase, and H_2S production patterns (Table S3). Approximately 77.75% of isolates clustered within profiles D, G, and I. These profiles were distinguished by specific combinations of oenologically relevant traits: profile D was characterized by the absence of β-glucosidase activity, the presence of β-lyase activity, and moderate H_2S production; profiles G and I exhibited moderate to high β-glucosidase activity, β-lyase activity, and moderate H_2S production.

The distribution of isolates across profiles was significantly non-random ($\chi^2 = 605.02$, $p < 0.05$). Profiles I, D, and G were markedly over-represented, with profile I comprising 63 isolates (standardized residual of +18.07), and profiles D and G each comprising 38 isolates (residuals of +9.71). In contrast, most other profiles exhibited negative residuals (-2 to -2.66), indicating frequencies lower than expected under a random distribution model.

These findings highlight the predominance of specific phenotypic profiles within the yeast collection. The strong overrepresentation of profiles I, D, and G suggests potential selective advantages or environmental factors that favor these trait combinations. Importantly, the identification of these dominant profiles provides a robust framework for prioritizing isolates with desirable oenological characteristics, supporting strategic strain selection for dual applications in fermentation and biocontrol.

3.2. Pulcherrimin Production

The screening also included evaluating pulcherrimin production on iron-supplemented media, the characteristic reddish pigment of *M. pulcherrima* that reflects its ability to chelate iron and inhibit competing microorganisms [47,48].

Overall, the isolates displayed a broad and heterogeneous distribution of pulcherrimin synthesis levels (Table S2). None received a score of 0, confirming that pigment production was widespread. Low production (score 1) was observed in 29 isolates (16.2%), whereas the majority belonged to intermediate to high categories: 38 isolates (21.23%) scored 2, 37 (20.67%) scored 3, 38 (21.23%) scored 4, and 37 (20.67%) reached the maximum score of 5. The near-uniform distribution across scores 2–5 highlights the substantial metabolic variability within the collection.

High-producing isolates (scores 4–5) were predominantly recovered from withered grape must, mirroring the pattern observed in isolates with desirable oenological traits.

This heterogeneity highlights the considerable metabolic diversity within the *M. pulcherrima* population, likely reflecting strain-specific traits shaped by environmental factors or the ecological origin of the isolates. In this context, recent taxonomic revisions, including the reclassification of *Metschnikowia citriensis* (lower producer of pulcherrimin) as *M. pulcherrima*, have effectively expanded the recognized genetic and phenotypic diversity of the species, which should be considered when interpreting variability in pulcherrimin production [49]. Although previous studies have reported spontaneous or induced loss-of-function mutations yielding pulcherrimin-negative phenotypes [3,49–51], the absence of score-0 isolates in this study indicates that such variants were rare or absent in the sampled environments.

The strong association between high pulcherrimin output and isolates from withered grapes must suggests that stressful or nutrient-limited conditions may select for strains with enhanced iron-chelating capacity, providing a competitive advantage. This trait is particularly relevant for biocontrol applications, where iron sequestration is a key mechanism by which phytopathogens are inhibited [50]. Conversely, the presence of low-producing phenotypes highlights the importance of careful strain selection, as reduced pigment synthesis may limit antagonistic performance despite favorable oenological or enzymatic characteristics [50].

3.3. Genotypic Clustering and Strain Definition

The UPGMA dendrogram obtained from the combined Rep-PCR ($(GTG)_5$) and RAPD-PCR (M13) fingerprint profiles (Figure S2) provides an overview of the genetic relationships among the 179 *M. pulcherrima* isolates. Prior to dereplication, genotypic clustering was broadly consistent with isolation source, geographical origin, and sampling year. In particular, isolates from the same origin-defined series (Table S1) are frequently grouped into the same clusters, indicating a pronounced local genetic structure within the *M. pulcherrima* population. This pattern suggests limited dispersal and possible adaptation to specific vineyard or must-associated niches, as previously proposed for non-*Saccharomyces* yeasts [31]. Conversely, several clusters contained isolates originating from different sources, geographical locations, or sampling years. The presence of these mixed clusters suggests that certain genotypes may exhibit broader ecological plasticity, enabling persistence across diverse environments and sampling campaigns. For instance, isolates from the FIANO and M0ML series consistently clustered together despite being sampled across different vintages, whereas mixed clusters such as MALV–MOML–COLR indicate broader ecological distribution of specific genotypes. The isolates included in this study had not been previously genotyped, and the applied fingerprinting approach enabled a detailed assessment of intraspecific genetic diversity within this native collection.

Although genotypic clustering generally mirrored isolation history, marked phenotypic heterogeneity was observed among genetically related isolates (Table S3, Figure S3). This finding confirms that genotypic similarity alone does not reliably predict functional behavior, and underscores the importance of integrating molecular typing with phenotypic screening when selecting strains for applied purposes. These results support the rationale for adopting an integrative dereplication strategy that combines genotypic and phenotypic information with metadata on isolation source, geographical origin, and sampling year to achieve a more realistic representation of *M. pulcherrima* biodiversity. Although strain-level dereplication is traditionally based exclusively on genetic criteria using one or more fingerprinting techniques [52–56], our findings indicate that reliance on genotyping alone—particularly when a single fingerprinting method is applied—may substantially underestimate the functional diversity within a strain collection.

In agreement with previous studies [57,58], integrating phenotypic traits into the dereplication process proved essential to avoid excluding isolates with distinct functional profiles despite close genetic relatedness. Indeed, if dereplication had been performed solely on the basis of genotypic clustering, the number of retained strains would have been markedly reduced, leading to the loss of phenotypically divergent isolates and, consequently, narrowing the spectrum of potentially relevant traits for both enological and biocontrol applications. The lack of a consistent correspondence between genotypic clustering and phenotypic behavior further highlights the complexity of functional differentiation within *M. pulcherrima* populations. Similar observations were reported by Barbosa et al. (2018) [31], who found no significant association between genotypic and phenotypic profiles in *M. pulcherrima* isolates from the Douro wine region using $(GTG)_5$ and M13 fingerprinting.

After dereplication, 106 non-redundant strains were retained, each representing a distinct genotype–phenotype profile (Figure 2). Based on combined genotypic distinctiveness and favorable wine-related traits, five strains (FIANO12, FM15, MONL18, G12, and NLSFS4) were selected for downstream safety evaluation and antagonism assays.

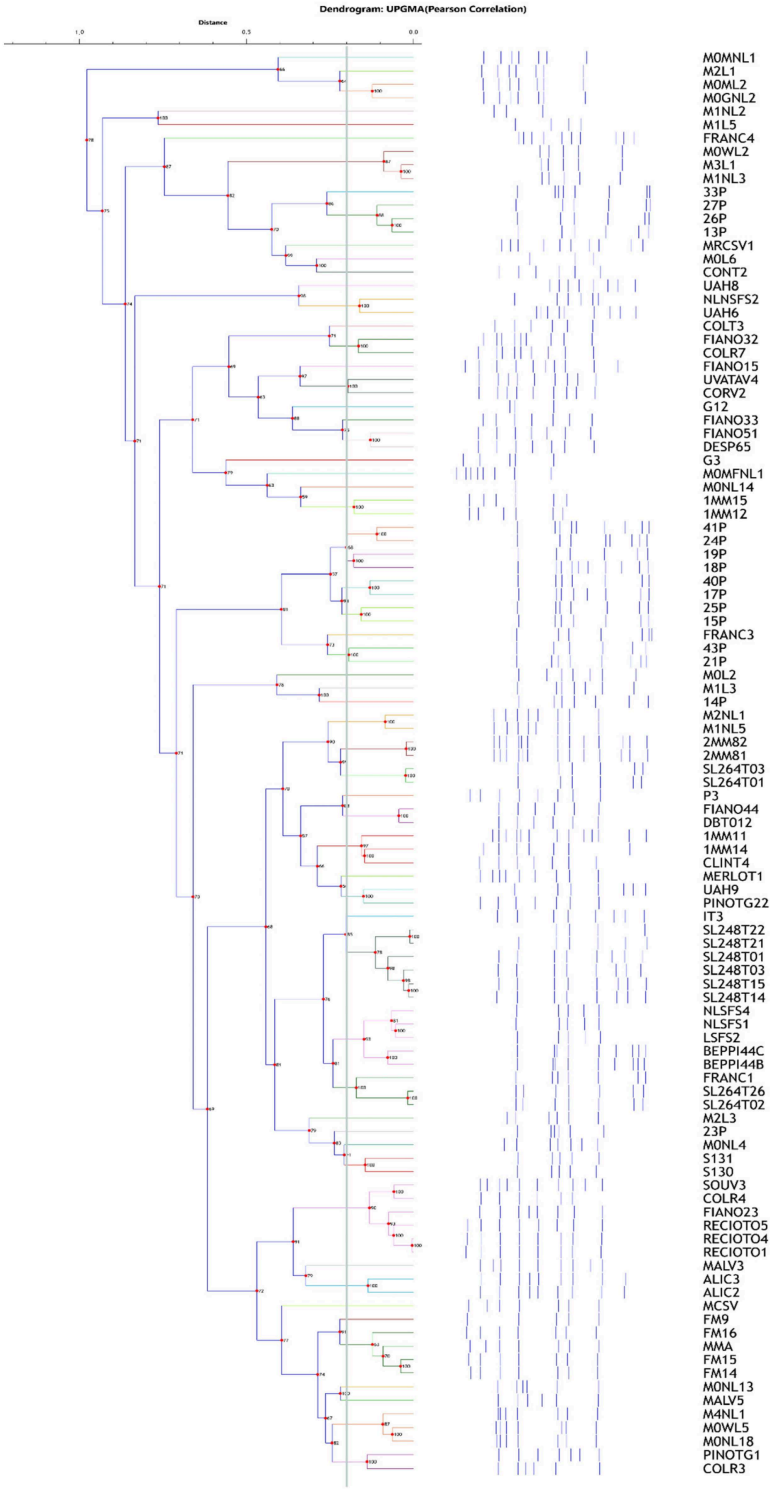


Figure 2. UPGMA dendrogram based on combined Rep-PCR ($(GTG)_5$) and RAPD-PCR (M13) fingerprint profiles of 106 *Metschnikowia pulcherrima* strains. Similarity among profiles was calculated using Pearson's correlation coefficient, and clustering was performed with the unweighted pair group method with arithmetic mean (UPGMA). Genetic clusters were defined using a similarity cut-off of 0.20.

3.4. Tests of Pathogenicity

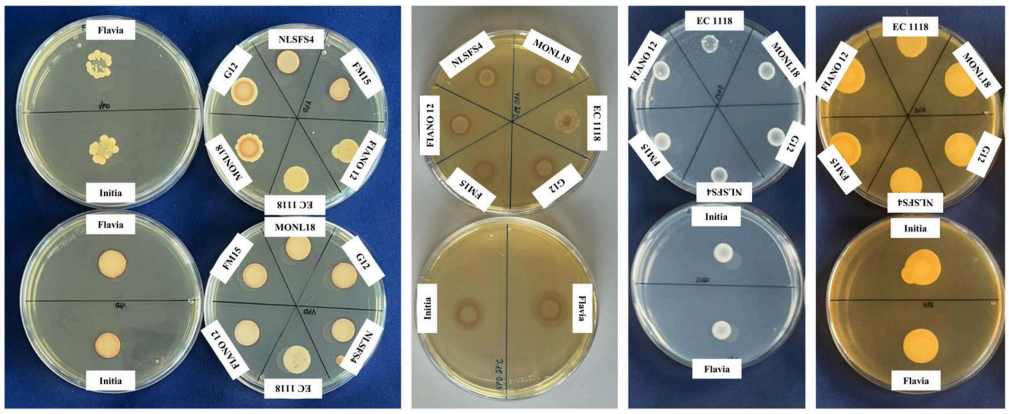
Before evaluating their suitability as biocontrol agents, the five selected *M. pulcherrima* strains were assessed for safety-associated traits, including growth at 37 °C, invasive growth, pseudohyphal formation, and proteolytic activity. These traits are commonly associated with opportunistic pathogenicity in yeasts [42,59].

Table 1, Figures 3 and S2 summarize the results on the native *M. pulcherrima* strains and the commercial yeasts used as controls.

Table 1. Pathogenicity tests on the yeast strains.

Strain	Characteristics			
	Growth at 37 °C [a]	Invasive Growth [b]	Pseudohyphae Formation [c]	Proteolytic Activity [d]
S. cerevisiae				
EC 1118	+	+	-	-
M. pulcherrima				
FIANO12	+	-	-	-
FM15	+	-	-	-
MONL18	+	-	-	-
G12	+	+/-	-	-
NLSFS4	+	+/-	-	-
Flavia	+	-	-	-
Initia	+	-	-	-

[a] growth at 37 °C after 3 days of incubation; [b] penetration on YPD medium; [c] formation of pseudohyphae on SLAD medium; [d] proteolytic activity on BSA medium.



(**A**) (**B**) (**C**) (**D**)

Figure 3. Representative pathogenicity assays on the yeast strains. (**A**) Growth at the human body temperature (37 ± 4 °C) on YPD agar incubated at 37 °C. Plates incubated at 28 °C are shown at the bottom as the control. (**B**) Invasive growth on YPD agar (25 °C for 10 days). (**C**) Pseudohyphae formation on SLAD medium (30 °C for 10 days). (**D**) Proteolytic activity on bovine serum albumin (BSA) medium (37 °C for 4 days).

All strains grew both at 28 °C and at 37 °C, indicating broad environmental adaptability. Interestingly, the commercial control strains Flavia and Initia exhibited non-confluent colony growth at 37 °C, potentially reflecting physiological stress or selection for thermotolerant subpopulations. Similar behavior has been reported in other wine yeasts at elevated temperatures (37 °C and 39 °C) [42].

The invasive growth assay revealed variability among strains. Flavia and Initia remained on the surface of the medium, with a visible halo likely due to pulcherrimin production rather than agar penetration. MONL18, FIANO12, and FM15 also showed su-

perficial growth, whereas G12 and NLSFS4 exhibited partial invasive growth. As expected, *S. cerevisiae* EC 1118 displayed a positive invasive phenotype, consistent with previous reports [42]. Notably, invasive growth in non-pathogenic yeasts may reflect a flexible developmental strategy for environmental adaptation rather than a pathogenic trait [44].

The SLAD medium, a minimal nutrient medium lacking amino acids and containing a low concentration of ammonium sulfate, was used to evaluate the morphological response of the yeast strains under nitrogen-limited conditions. While this morphology is often linked to virulence in pathogens like *Candida albicans*, it also serves as an adaptive mechanism for nutrient foraging and spatial exploration [60,61]. None of the *M. pulcherrima* strains tested formed pseudohyphae, and all displayed smooth colony margins. The reference strain *S. cerevisiae* EC 1118 showed irregular colony edges but no pseudohyphal growth.

None of the tested strains, including *S. cerevisiae* EC1118, exhibited proteolytic activity on BSA medium. Protease production can contribute to tissue invasion in pathogenic yeasts [62], and its absence supports the favorable safety of the strains.

Collectively, these findings demonstrate that the *M. pulcherrima* strains lack key morphological traits typically associated with fungal pathogenicity. This aligns with recent genomic and phenotypic analysis, which show that *M. pulcherrima* strains do not exhibit known virulence determinants and possess a favorable safety profile [36]. Although the species is not currently included in the European Food Safety Authority (EFSA) Qualified Presumption of Safety (QPS) list [63], the accumulating safety data support its potential future inclusion.

3.5. In Vitro Antagonistic Bioassays Against B. cinerea

In vitro assays on PDA plates were conducted to provide preliminary evidence of the biocontrol potential of the *M. pulcherrima* strains and to explore possible mechanisms of antagonism. Two different experimental setups were used. In the first experiment, the yeast and *B. cinerea* were inoculated simultaneously on the same plate, allowing for direct interactions, including competition for nutrients and space, hyperparasitism, and the production of antimicrobial metabolites. In the second experiment, yeast and pathogen were grown on opposite sides of sealed, divided plates without physical contact, allowing for the assessment of the inhibitory effect of yeast-emitted VOCs [64].

The simultaneous growth assay revealed clear strain-dependent differences in antagonistic ability (Figure 4A).

One-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) showed that inhibition of *B. cinerea* TOB62 was higher for NLSFS4 (~56%), whereas FIANO12, FM15, G12, Flavia, and Initia displayed similar inhibition levels (around 48–52%) and did not differ significantly from NLSFS4. These strains were all significantly more inhibitory than MONL18 and EC 1118.

Against the BCZG1 strain, inhibition values were generally lower. Flavia and EC 1118 displayed moderate inhibition (~40%), while FIANO12, MONL18, and NLSFS4 showed slightly lower values (~34–36%). Initia exhibited no inhibition in this assay.

Altogether, these results demonstrate that antagonism mediated through direct contact is strongly strain-specific and highly dependent on the fungal genotype [65]. The stronger inhibitory response observed against TOB62 suggests that this strain is more susceptible to resource-based competition and/or diffusible metabolites.

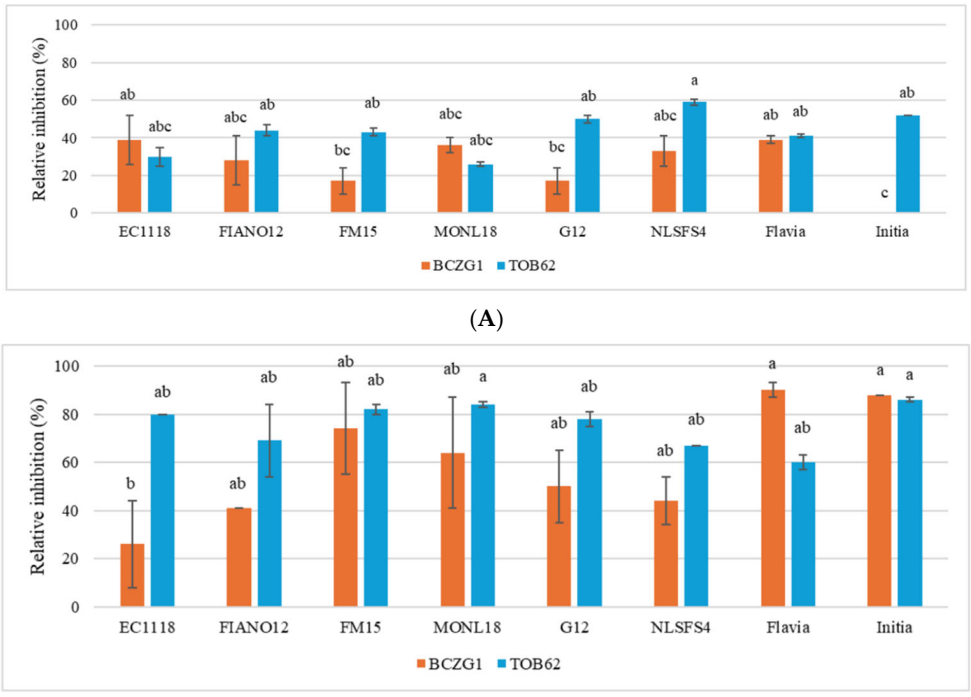


Figure 4. In vitro antagonistic activity of *Metschnikowia pulcherrima* strains against *Botrytis cinerea* BCZG1 and TOB62 strains under two conditions: direct contact and volatile emission. Reference control strains are also included. (**A**) Percentage inhibition of radial mycelial growth in co-culture assays with yeasts (direct contact). (**B**) Percentage inhibition observed in sealed plate assays assessing the effect of volatile organic compounds (VOCs) emitted by the yeast strains. Error bars represent the standard error of the mean (SEM). Data were analyzed using one-way ANOVA. Different letters in a column indicate significantly different percentage inhibition ($p < 0.05$).

In similar assays conducted by Lemos et al. [44], *Starmerella bacillaris* strains isolated from withered Raboso Piave grapes showed inhibition percentages of *B. cinerea* BM05.10 ranging from 12% to 33%. These values are lower than those obtained in the present study, indicating that the yeast strains tested here exhibited a higher in vitro biocontrol efficacy against *B. cinerea*.

When yeasts and pathogens were grown on opposite sides of divided plates, ANOVA revealed significant differences among combinations, and VOC-mediated inhibition was generally higher than in the direct-contact assay (Figure 4B).

For BCZG1, Flavia and Initia were the most effective (90% and 88% inhibition, respectively), almost completely suppressing fungal growth. FM15 showed inhibition levels statistically overlapping with those of the top performers, whereas MONL18, G12, NLSFS4, FIANO12, and EC 1118 showed significantly lower inhibition.

Against TOB62, the strongest inhibitory activity was observed for Initia (87%) and MONL18 (84%). FM15, EC 1118, and G12 also showed strong inhibition (78–80%), whereas FIANO12 and NLSFS4 presented moderate activity (67–69%). Interestingly, Flavia—despite being among the top performers against BCZG1—showed a lower inhibition level (60%) against TOB62.

These results confirm that VOC production is a major contributor to antagonism for many of the tested *M. pulcherrima* strains and that the magnitude of inhibition is both strain and pathogen-dependent [66].

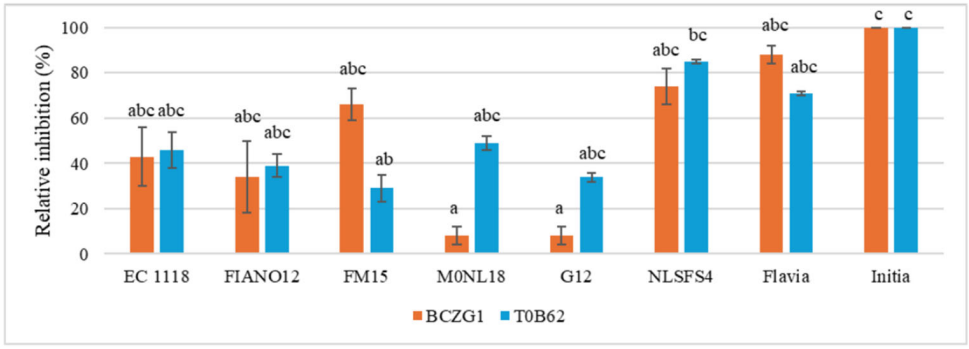
The higher VOC-mediated inhibition compared to direct contact is consistent with earlier work [44], although the inhibition values obtained here (60–90%) exceed those

previously reported (44–79%). Overall, VOC production appears to represent a potent antagonistic mechanism under the tested conditions.

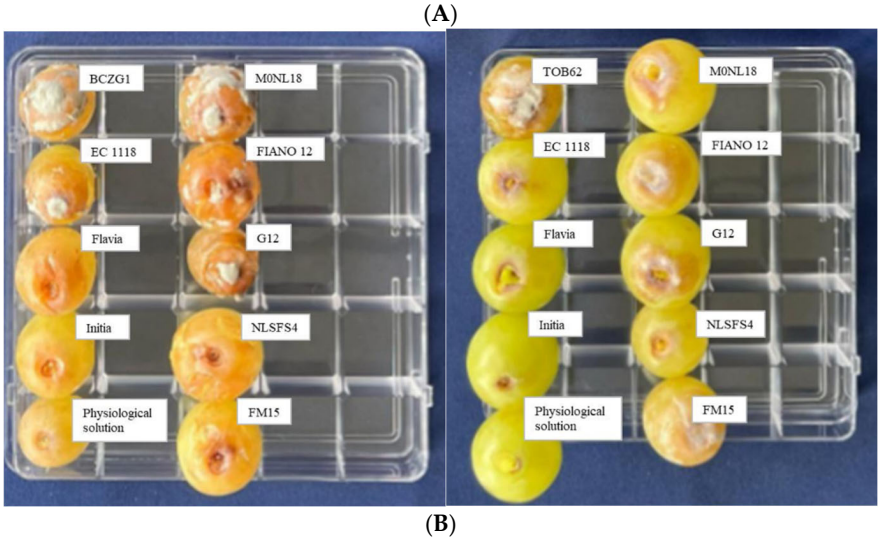
Collectively, the in vitro assays revealed that the inhibition of *B. cinerea* by *M. pulcherrima* relies on multiple mechanisms, including both direct interactions and airborne metabolites, and is strongly influenced by the genotypes of both the yeast and the pathogen. VOC-mediated inhibition was generally more pronounced than direct-contact inhibition, suggesting that airborne metabolites may substantially contribute to biocontrol activity. Based on consistently high inhibition across assays—particularly in VOC-mediated conditions—Initia, FM15, and MONL18 emerge as the most promising strains for further validation [67], while the performance of other strains was more markedly dependent on the target *B. cinerea* strain. Nonetheless, the same set of strains was tested in vivo for antagonistic bioassays.

3.6. In Vivo Antagonistic Bioassays Against B. cinerea

In vivo assays on wounded grapes aimed at validating antagonistic performance under conditions closer to natural infection environments. Figure 5A,B show the in vivo biocontrol efficacy of the *M. pulcherrima* strains against *B. cinerea* inoculated on wounded grape berries.



(**A**)



(**B**)

Figure 5. In vivo antagonistic activity of *Metschnikowia pulcherrima* strains against *Botrytis cinerea* BCZG1 and TOB62 strains inoculated on wounded grape berries for a 5-day incubation period at 25 °C. Reference control strains are also included. (**A**) Error bars indicate the standard error of the mean (SEM). Data were analyzed using the Kruskal–Wallis test, followed by Conover-Iman's test. Different letters in a column indicate significantly different percentage inhibition ($p < 0.05$). (**B**) Representative in vivo applications on the grape.

All strains reduced *B. cinerea* growth on grape berries, although the magnitude of inhibition differed significantly among treatments (Kruskal–Wallis, $p < 0.05$), as confirmed by the post hoc test. The inhibition percentages are reported in Figure 5A.

Among the tested strains, Initia showed the highest antagonistic activity, completely inhibiting the growth of both *B. cinerea* strains (BCZG1 and TOB62) with 100% inhibition. This indicates a maximal biocontrol effect comparable to that of the healthy control berries treated with physiological solution.

Flavia also displayed strong biocontrol potential, with inhibition rates of 88% against BCZG1 and 71% against TOB62; NLSFS4 showed high inhibition levels (50–85%), while FM15, EC 1118, and FIANO12 showed moderate inhibition (34–66%). In contrast, MONL18 and G12 exhibited the lowest antifungal activity (8–49%).

These results are in line with previous findings that high in vitro antagonistic activity does not necessarily translate into proportional efficacy in vivo [68] and are consistent with earlier studies on wild grape-associated yeasts [43,66]. Comparable inhibition ranges (39–85%) were reported for *Starmerella bacillaris* on artificially infected grapes [44]. The high in vivo performance of Initia and Flavia, as well as NLSFS4, observed here further supports their potential as effective biocontrol agents.

Overall, these findings emphasize that the efficacy of yeast-based biocontrol is strain-dependent and influenced by factors such as environmental conditions, fruit surface characteristics, and pathogen variability [29]. The variable inhibition observed among strains—particularly FM15 and G12—underscores the importance of selecting strains based on both the target fungal isolate and the ecological context. Taken together, the present results confirm *M. pulcherrima* as a promising reservoir for selecting biocontrol-useful strains against *B. cinerea*, with possible applications in both pre- and post-harvest management strategies.

3.7. Microvinification Trials and Analysis of VOCs

Both sequential inoculations and the *S. cerevisiae* X5 control were completed within 19 days, indicating that the presence of *M. pulcherrima* NLSFS4 did not negatively affect fermentation performance or the ability of *S. cerevisiae* to complete sugar consumption. This demonstrates the technological compatibility of the NLSFS4 strain with sequential inoculation strategies and confirms the absence of inhibitory interactions toward the fermentative yeast, as previously observed for selected non-*Saccharomyces* yeasts [26,64].

Sequential fermentation resulted in a modified volatile profile compared with the single-strain *S. cerevisiae* control. In particular, total ester concentration was lower in the sequential treatment (37.7 vs. 51.3 mg/L), whereas higher alcohols were increased (167.6 vs. 122.2 mg/L). At the individual compound level, variations in specific esters suggested a shift in aroma balance rather than a uniform reduction, as shown in Supplementary Table S4. In particular, ethyl acetate concentrations were lower under sequential fermentation, which may be favorable as excessive levels can mask varietal characteristics [69]. Conversely, the average concentrations of several medium-chain ethyl esters (e.g., ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate) were higher in the sequential treatment, suggesting an enhanced -fruity fermentative contribution. Notably, 2-phenethyl acetate showed similar values between treatments, indicating that floral notes were preserved and potentially enhanced. Overall, the results suggest that sequential fermentation with NLSFS4 modulated the balance of volatile esters, highlighting its potential as a tool for tailoring the wine aroma profile.

4. Conclusions

This study provides the most comprehensive characterization to date of a large *M. pulcherrima* collection, revealing pronounced intraspecific variability and substantial functional diversity relevant to wine fermentation and biocontrol. By integrating phenotypic screening with genotypic clustering, we propose a robust and scalable pre-selection framework for identifying candidate strains with dual potential, making it suitable for further validation in applied oenological and agronomic contexts. The combined assessment of wine-related enzymatic traits, hydrogen sulphite production, pulcherrimin biosynthesis, and safety-associated phenotypes allowed the selection of five genetically distinct native strains for targeted biocontrol evaluation. In vitro and in vivo assays confirmed strain- and pathogen-dependent antagonistic activity against *B. cinerea*, with volatile-mediated interactions emerging as a significant mechanism of inhibition. Microvinification trials further confirm the oenological relevance of the selected strain NLSFS4, demonstrating its ability to modulate aroma composition while maintaining fermentation efficiency. Although full-scale fermentation trials and comprehensive aroma profiling remain essential to confirm industrial suitability, the present study establishes a solid foundation for rational strain selection and highlights *M. pulcherrima* as a valuable resource for developing sustainable, low-input strategies in viticulture and winemaking.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12030139/s1, Table S1. Collection of 179 native *Metschnikowia pulcherrima* isolates with associated provenance metadata. Table S2. Wine-relevant traits and pulcherrimin production in *Metschnikowia pulcherrima* isolates. Reference control strains are also included. β-glucosidase activity was scored as 0 (absent), 50 (moderate), or 100 (high). β-lyase activity was semi-quantitatively scored as 0 (no growth, no activity), 50 (moderate growth, moderate activity), or 100 (abundant growth, high activity); H_2S-producing strains exhibited brown to dark brown coloration, with a chromatic scale (0, 1, 2.5, 5) used to classify the intensity of colony coloration; Pulcherrimin production was evaluated using a color-based scale ranging from 1 to 5, reflecting the intensity of pigmentation. The phenotypic profiles were obtained by combining the results of the wine-related traits. Table S3. Distribution of the *Metschnikowia pulcherrima* isolates within the different phenotypic profiles. Table S4. Concentrations of individual volatile compounds (esters and higher alcohols) detected in wines obtained by sequential inoculation (*Metschnikowia pulcherrima* NLSFS4 followed by *Saccharomyces cerevisiae* Zymaflore X5) and by pure *S. cerevisiae* Zymaflore X5 fermentation. Values are expressed as μg/L and reported as mean ± standard deviation of three independent fermentations. Volatile compounds were quantified by GC–MS as described in the Materials and Methods section. Figure S1. Complete phenotypic screening of wine-relevant traits and pulcherrimin production in *Metschnikowia pulcherrima* isolates. Reference control strains are included. (A) β-glucosidase activity on Esculin Agar Medium, incubated at 26 °C for 5 days. (B) β-lyase activity on YCB-SMC medium (20 °C for 48–72 h). (C) H_2S production on Bismuth Sulfite Glucose Glycine Yeast medium (15 °C for 5 days). (D) Pulcherrimin production on YG agar plates supplemented with 0.05% (*w*/*v*) $FeCl_3$, (27 °C for 72 h). Figure S2. UPGMA dendrogram based on combined Rep-PCR ($(GTG)_5$) and RAPD-PCR (M13) fingerprint profiles of the 179 *Metschnikowia pulcherrima* isolates. Similarity among profiles was calculated using the Pearson's correlation coefficient, and clustering was performed with the unweighted pair group method with arithmetic mean (UPGMA). Genetic clusters were defined using a similarity cut-off of 0.20. Figure S3. Complete set of pathogenicity-related assays for *Metschnikowia pulcherrima* strains, including experimental replicates. (A) YPD agar incubated at 37 °C and 28 °C (control). (B) Invasive growth on YPD agar (25 °C for 10 days). (C) Pseudohyphae formation on SLAD medium (30 °C for 10 days). (D) Proteolytic activity on bovine serum albumin (BSA) medium (37 °C for 4 days).

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References

1. Morata, A.; Loira, I.; Escott, C.; Del Fresno, J.M.; Bañuelos, M.A.; Suárez-Lepe, J.A. Applications of *Metschnikowia pulcherrima* in wine biotechnology. *Fermentation* **2019**, *5*, 63. [CrossRef]
2. Tatay-Núñez, J.; Albi-Puig, J.; Garrigós, V.; Orejas-Suárez, M.; Matallana, E.; Aranda, A. Isolation of local strains of the yeast *Metschnikowia* for biocontrol and lipid production purposes. *World J. Microbiol. Biotechnol.* **2024**, *40*, 88. [CrossRef]
3. Sipiczki, M. *Metschnikowia pulcherrima* and related pulcherrimin-producing yeasts: Fuzzy species boundaries and complex antimicrobial antagonism. *Microorganisms* **2020**, *8*, 1029. [CrossRef]
4. Canonico, L.; Agarbati, A.; Galli, E.; Comitini, F.; Ciani, M. *Metschnikowia pulcherrima* as biocontrol agent and wine aroma enhancer in combination with a native *Saccharomyces cerevisiae*. *LWT* **2023**, *181*, 114758. [CrossRef]
5. Binati, R.L.; Maule, M.; Luzzini, G.; Martelli, F.; Felis, G.E.; Ugliano, M.; Torriani, S. From bioprotective effects to diversification of wine aroma: Expanding the knowledge on *Metschnikowia pulcherrima* oenological potential. *Food Res. Int.* **2023**, *174*, 113550. [CrossRef]
6. Liu, Y.; Yan, H.; Yao, J.; Yan, Y.; Zhou, J.; Shi, W. Bioprospection of *Metschnikowia pulcherrima* as biocontrol agents against gray mold on grapes with their potential modes of action. *Int. J. Food Microbiol.* **2025**, *442*, 111383. [CrossRef] [PubMed]
7. Urazova, M.; Satenova, A.; Askarova, M.; Tuyakova, A.; Abilkhadirov, A.; Shaikhin, S. Biocontrol activity of *Metschnikowia pulcherrima* strains isolated from local varieties of apples in Kazakhstan. *Int. J. Agric. Biol.* **2025**, *27*, 6. [CrossRef]
8. Puyo, M.; Simonin, S.; Bach, B.; Klein, G.; Alexandre, H.; Tourdot-Maréchal, R. Bio-protection in oenology by *Metschnikowia pulcherrima*: From field results to scientific inquiry. *Front. Microbiol.* **2023**, *14*, 1252973. [CrossRef] [PubMed]
9. Sipiczki, M.; Czentye, K.; Kállai, Z. High intragenomic, intergenomic, and phenotypic diversity in pulcherrimin-producing *Metschnikowia* yeasts indicates a special mode of genome evolution. *Sci. Rep.* **2024**, *14*, 10521. [CrossRef] [PubMed]
10. Sipiczki, M.; Czentye, K. Reversible stochastic epigenetic-like silencing of the production of pulcherriminic acid in the antimicrobial antagonist *Metschnikowia pulcherrima*. *Sci. Rep.* **2024**, *14*, 29677. [CrossRef]
11. Saravanakumar, D.; Ciavorella, A.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples through iron depletion. *Postharvest Biol. Technol.* **2008**, *49*, 121–128. [CrossRef]
12. Ribes, S.; Fuentes, A.; Talens, P.; Barat, J.M. Prevention of fungal spoilage in food products using natural compounds: A review. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 2002–2016. [CrossRef]
13. Spadaro, D.; Droby, S. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. *Trends Food Sci. Technol.* **2016**, *47*, 39–49. [CrossRef]
14. Saravanakumar, D.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. Detection of enzymatic activity and partial sequence of a chitinase gene in *Metschnikowia pulcherrima* strain MACH1 used as post-harvest biocontrol agent. *Eur. J. Plant Pathol.* **2009**, *123*, 183–193. [CrossRef]
15. Droby, S.; Wisniewski, M.; Macarisin, D.; Wilson, C. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biol. Technol.* **2009**, *52*, 137–145. [CrossRef]

16. Wei, J.; Wang, S.; Zhang, Y.; Yuan, Y.; Yue, T. Characterization and screening of non-*Saccharomyces* yeasts used to produce fragrant cider. *LWT* **2019**, *107*, 191–198. [CrossRef]
17. Oro, L.; Feliziani, E.; Ciani, M.; Romanazzi, G.; Comitini, F. Volatile organic compounds from *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* inhibit growth of decay causing fungi and control postharvest diseases of strawberries. *Int. J. Food Microbiol.* **2018**, *265*, 18–22. [CrossRef] [PubMed]
18. Piano, S.; Neyrotti, V.; Migheli, Q.; Gullino, M.L. Biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple. *Postharvest Biol. Technol.* **1997**, *11*, 131–140. [CrossRef]
19. Tuyakova, A.K.; Urazova, M.S.; Satenova, A.M.; Shaikhin, S.M. The prospect of using strains of *Metschnikowia pulcherrima* to combat pathogens of postharvest spoilage of fruits. Bulletin of the L. N. Gumilyov Eurasian National University. *Biosci. Ser.* **2022**, *140*, 76–82. [CrossRef]
20. Liu, W.; Liu, C.; Zeren, D.; Wang, S.; Tan, Z.; Hang, F.; Liang, X.; Xie, C.; Li, K. Biocontrol ability and possible mechanism of *Metschnikowia pulcherrima* against major diseases of postharvest citrus fruit and its biopreservative application. *Int. J. Food Microbiol.* **2025**, *438*, 111230. [CrossRef]
21. Hahn, M. The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. *J. Biol. Chem.* **2014**, *7*, 133–141. [CrossRef]
22. Nicolopoulou-Stamati, P.; Maipas, S.; Kotampasi, C.; Stamatis, P.; Hens, L. Chemical pesticides and human health: The urgent need for a new concept in agriculture. *Front. Public Health* **2016**, *4*, e00148. [CrossRef]
23. Bene, Z.; Hegyi, Á.I.; Weninger, H.; Váczy, K.Z. *Metschnikowia pulcherrima* as a tool for sulphite reduction and enhanced volatile retention in noble rot wine fermentation. *Fermentation* **2025**, *11*, 491. [CrossRef]
24. Torres Díaz, L.L.; Sáenz de Urturi, I.; Iribarren, M.; Murillo Peña, R.; Marín San Román, S.; González Lázaro, M.; Pérez Álvarez, E.P.; Garde-Cerdán, T. Evaluation of the potential of *Metschnikowia pulcherrima* to reduce SO_2 in winemaking: Impact on wine phenolic compounds and their bottle evolution. *Eur. Food Res. Technol.* **2025**, *251*, 705–718. [CrossRef]
25. Piombo, E.; Sela, N.; Wisniewski, M.; Hoffmann, M.; Gullino, M.L.; Allard, M.W.; Levin, E.; Spadaro, D.; Droby, S. Genome sequence, assembly and characterization of two *Metschnikowia fructicola* strains used as biocontrol agents of postharvest diseases. *Front. Microbiol.* **2018**, *9*, 593. [CrossRef] [PubMed]
26. Bertazzoli, G.; Pelizza, E.; Luzzini, G.; Felis, G.E.; Ugliano, M.; Torriani, S. Influence of fermentation temperature and *Metschnikowia pulcherrima/Saccharomyces cerevisiae* multi-starter cultures on the volatile compounds of Lugana wine. *Foods* **2025**, *14*, 3538. [CrossRef]
27. Padilla, B.; Gil, J.V.; Manzanares, P. Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front. Microbiol.* **2016**, *7*, 411. [CrossRef] [PubMed]
28. Benito, Á.; Calderón, F.; Benito, S. The influence of non-*Saccharomyces* species on wine fermentation quality parameters. *Fermentation* **2019**, *5*, 54. [CrossRef]
29. Nadai, C.; Lemos Junior, W.J.F.; Favaron, F.; Giacomini, A.; Corich, V. Biocontrol activity of *Starmerella bacillaris* yeast against blue mold disease on apple fruit and its effect on cider fermentation. *PLoS ONE* **2018**, *13*, e0204350. [CrossRef]
30. Lorenzini, M.; Simonato, B.; Slaghenaufi, D.; Ugliano, M.; Zapparoli, G. Assessment of yeasts for apple juice fermentation and production of cider volatile compounds. *LWT* **2019**, *99*, 224–230. [CrossRef]
31. Barbosa, C.; Lage, P.; Esteves, M.; Chambel, L.; Mendes-Faia, A.; Mendes-Ferreira, A. Molecular and phenotypic characterization of *Metschnikowia pulcherrima* strains from Douro wine region. *Fermentation* **2018**, *4*, 8. [CrossRef]
32. Vicente, J.; Ruiz, J.; Belda, I.; Benito-Vázquez, I.; Marquina, D.; Calderón, F.; Santos, A.; Benito, S. The genus *Metschnikowia* in enology. *Microorganisms* **2020**, *8*, 1038. [CrossRef]
33. Ruiz, J.; Belda, I.; Beisert, B.; Navascués, E.; Marquina, D.; Calderón, F.; Rauhut, D.; Santos, A.; Benito, S. Analytical impact of *Metschnikowia pulcherrima* in the volatile profile of Verdejo white wines. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 8501–8509. [CrossRef]
34. Muhl, J.R.; Pilkington, L.I.; Fedrizzi, B.; Deed, R.C. Insights into the relative contribution of four precursors to 3-sulfanylhexan-1-ol and 3-sulfanylhexylacetate biogenesis during fermentation. *Food Chem.* **2024**, *449*, 139193. [CrossRef]
35. Coppola, F.; Testa, B.; Cozzolino, R.; Karaulli, J.; Pannella, G.; Di Renzo, M.; Matarazzo, C.; Succi, M.; Iorizzo, M. Effects of inoculation timing and mixed fermentation with *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* on the aroma and sensory properties of Falanghina wine. *Eur. Food Res. Technol.* **2025**, *251*, 1699–1717. [CrossRef]
36. Larini, I.; Ferrara, M.; Troiano, E.; Gatto, V.; Mulè, G.; Vitulo, N.; Capozzi, V.; Salvetti, E.; Felis, G.E.; Torriani, S. Unlocking the potential of *Metschnikowia pulcherrima*: A dive into the genomic and safety characterization of four plant-associated strains. *Appl. Microbiol. Biotechnol.* **2025**, *109*, 128. [CrossRef]
37. González Flores, M.; Rodríguez, M.E.; Oteiza, J.M.; Barbagelata, R.J.; Lopes, C.A. Physiological characterization of *Saccharomyces uvarum* and *Saccharomyces eubayanus* from Patagonia and their potential for cidermaking. *Int. J. Food Microbiol.* **2017**, *249*, 9–17. [CrossRef] [PubMed]

38. Belda, I.; Ruiz, J.; Navascués, E.; Marquina, D.; Santos, A. Improvement of aromatic thiol release through the selection of yeasts with increased β-lyase activity. *Int. J. Food Microbiol.* **2016**, *225*, 1–8. [CrossRef] [PubMed]
39. Iorizzo, M.; Letizia, F.; Albanese, G.; Coppola, F.; Gambuti, A.; Testa, B.; Aversano, R.; Forino, M.; Coppola, R. Potential for lager beer production from *Saccharomyces cerevisiae* strains isolated from the vineyard environment. *Processes* **2021**, *9*, 1628. [CrossRef]
40. Pawlikowska, E.; Kolesińska, B.; Nowacka, M.; Kregiel, D. A New approach to producing high yields of pulcherrimin from *Metschnikowia* yeasts. *Fermentation* **2020**, *6*, 114. [CrossRef]
41. Troiano, E. Biodiversity of *Metschnikowia pulcherrima* as a Resource for Innovation in Fermented Beverages. Ph.D. Thesis, University of Verona, Verona, Italy, 2022. Available online: https://tesidottorato.depositolegale.it/handle/20.500.14242/181247 (accessed on 19 January 2026).
42. de Llanos, R.; Fernández-Espinar, M.T.; Querol, A. A comparison of clinical and food *Saccharomyces cerevisiae* isolates on the basis of potential virulence factors. *Antonie Van Leeuwenhoek* **2006**, *90*, 221–231. [CrossRef]
43. Parafati, L.; Vitale, A.; Restuccia, C.; Cirvilleri, G. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. *Food Microbiol.* **2015**, *47*, 85–92. [CrossRef]
44. Lemos Junior, W.J.F.; Bovo, B.; Nadai, C.; Crosato, G.; Carlot, M.; Favaro, F.; Giacomini, A.; Corich, V. Biocontrol ability and action mechanism of *Starmerella bacillaris* (Synonym *Candida zemplinina*) isolated from wine musts against gray mold disease agent *Botrytis cinerea* on grape and their effects on alcoholic fermentation. *Front. Microbiol.* **2016**, *7*, 1249, Erratum in: *Front Microbiol.* **2016**, *7*, 1499. [CrossRef]
45. Testa, B.; Coppola, F.; Iorizzo, M.; Di Renzo, M.; Coppola, R.; Succi, M. Preliminary characterisation of *Metschnikowia pulcherrima* to be used as a starter culture in red winemaking. *Beverages* **2024**, *10*, 88. [CrossRef]
46. Kumar, G.R.; Ramakrishnan, V.; Bisson, L.F. Survey of hydrogen sulfide production in wine strains of *Saccharomyces cerevisiae*. *Am. J. Enol. Vitic.* **2010**, *61*, 365–371. [CrossRef]
47. Nardi, T. Microbial resources as a tool for enhancing sustainability in winemaking. *Microorganisms* **2020**, *8*, 507. [CrossRef]
48. Wang, S.; Tan, Z.; Wang, C.; Liu, W.; Hang, F.; He, X.; Ye, D.; Li, L.; Sun, J. Iron competition as an important mechanism of pulcherrimin-producing *Metschnikowia* sp. strains for controlling postharvest fungal decays on citrus fruit. *Foods* **2023**, *12*, 4249. [CrossRef] [PubMed]
49. Sipiczki, M. Taxonomic revision of the pulcherrima clade of *Metschnikowia* (Fungi): Merger of species. *Taxonomy* **2022**, *2*, 107–123. [CrossRef]
50. Gore-Lloyd, D.; Sumann, I.; Brachmann, A.; Schneeberger, K.; Ortiz-Merino, R.; Moreno, M. Snf2 controls pulcherriminic acid biosynthesis and antifungal activity of the biocontrol yeast *Metschnikowia pulcherrima*. *Mol. Microbiol.* **2019**, *112*, 317–332. [CrossRef]
51. Sipiczki, M. When barcoding fails: Genome chimerization (admixing) and reticulation obscure phylogenetic and taxonomic relationships. *Mol. Ecol. Resour.* **2022**, *22*, 1762–1785. [CrossRef]
52. Caputo, L.; Quintieri, L.; Baruzzi, F.; Borcakli, M.; Morea, M. Molecular and phenotypic characterization of *Pichia fermentans* strains found among Boza yeasts. *Food Res. Int.* **2012**, *48*, 755–762. [CrossRef]
53. Meier-Dörnberg, T.; Kory, O.I.; Jacob, F.; Michel, M.; Hutzler, M. *Saccharomyces cerevisiae* variety *diastaticus*: Friend or foe? Spoilage potential and brewing ability of different *Saccharomyces cerevisiae* var. *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization. *FEMS Yeast Res.* **2018**, *18*, foy023. [CrossRef] [PubMed]
54. Garofalo, C.; Berbegal, C.; Grieco, F.; Tufariello, M.; Spano, G.; Capozzi, V. Selection of indigenous yeast strains for the production of sparkling wines from native Apulian grape varieties. *Int. J. Food Microbiol.* **2018**, *285*, 7–17. [CrossRef]
55. Tufariello, M.; Maiorano, G.; Rampino, P.; Spano, G.; Grieco, F.; Perrotta, C.; Capozzi, V. Selection of an autochthonous yeast starter culture for industrial production of Primitivo "Gioia del Colle" PDO/DOC in Apulia (Southern Italy). *LWT—Food Sci. Technol.* **2019**, *99*, 188–196. [CrossRef]
56. Mateus, D.; Sousa, S.; Coimbra, C.S.; Rogerson, F.; Simões, J. Identification and characterization of non-*Saccharomyces* species isolated from Port wine spontaneous fermentations. *Foods* **2020**, *9*, 120. [CrossRef] [PubMed]
57. Pfliegler, W.P.; Horváth, E.; Kállai, Z.; Sipiczki, M. Diversity of *Candida zemplinina* isolates inferred from RAPD, micro/minisatellite and physiological analysis. *Microbiol. Res.* **2014**, *169*, 402–410. [CrossRef]
58. Pfliegler, W.P.; Sipiczki, M. Does fingerprinting truly represent the diversity of wine yeasts? A case study with interdelta genotyping of *Saccharomyces cerevisiae* strains. *Lett. Appl. Microbiol.* **2016**, *63*, 406–411. [CrossRef]
59. Sundh, I.; Melin, P. Safety and regulation of yeasts used for biocontrol or biopreservation in the food or feed chain. *Antonie Van Leeuwenhoek* **2011**, *99*, 113–119. [CrossRef]
60. Gognies, S.; Belarbi, A. Endopolygalacturonase of *Saccharomyces cerevisiae*: Involvement in pseudohyphae development of haploids and in pathogenicity on *Vitis vinifera*. *Plant Sci.* **2002**, *163*, 759–769. [CrossRef]
61. Sudbery, P.; Gow, N.; Berman, J. The distinct morphogenic states of *Candida albicans*. *Trends Microbiol.* **2004**, *12*, 317–324. [CrossRef]
62. Kumamoto, C.A.; Vinces, M.D. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell. Microbiol.* **2005**, *7*, 1546–1554. [CrossRef]

63. Koutsoumanis, K.; Allende, A.; Álvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; Hilbert, F.; Lindqvist, R.; Nauta, M.; et al. Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 9: Suitability of taxonomic units notified to EFSA until September 2018. *EFSA J.* **2019**, *17*, 5555. [CrossRef]
64. Binati, R.L.; Lemos Junior, W.J.F.; Luzzini, G.; Slaghenaufi, D.; Ugliano, M.; Torriani, S. Contribution of non-*Saccharomyces* yeasts to wine volatile and sensory diversity: A study on *Lachancea thermotolerans*, *Metschnikowia* spp. and *Starmerella bacillaris* strains isolated in Italy. *Int. J. Food Microbiol.* **2020**, *318*, 108470. [CrossRef] [PubMed]
65. Fernandez-San Millan, A.; Gamir, J.; Larraya, L.; Farran, I.; Veramendi, J. Towards understanding of fungal biocontrol mechanisms of different yeasts antagonistic to *Botrytis cinerea* through exometabolomic analysis. *Biol. Control* **2022**, *170*, 105033. [CrossRef]
66. Cordero-Bueso, G.; Mangieri, N.; Maghradze, D.; Foschino, R.; Valdetara, F.; Cantoral, J.M.; Vigentini, I. Wild grape-associated yeasts as promising biocontrol agents against *Vitis vinifera* fungal pathogens. *Front. Microbiol.* **2017**, *8*, 2025. [CrossRef]
67. Huang, R.; Che, H.J.; Zhang, J.; Yang, L.; Jiang, D.H.; Li, G.Q. Evaluation of *Sporidiobolus pararoseus* strain YCXT3 as biocontrol agent of *Botrytis cinerea* on post-harvest strawberry fruits. *Biol. Control* **2012**, *62*, 53–63. [CrossRef]
68. Dal Bello, G.; Mónaco, C.; Rollán, M.C.; Lampugnani, G.; Arteta, N.; Abramoff, C.; Ronco, L.; Stocco, M. Biocontrol of postharvest grey mould on tomato by yeasts. *J. Phytopathol.* **2008**, *156*, 257–263. [CrossRef]
69. Carpena, M.; Fraga-Corral, M.; Otero, P.; Nogueira, R.A.; Garcia-Oliveira, P.; Prieto, M.A.; Simal-Gandara, J. Secondary aroma: Influence of wine microorganisms in their aroma profile. *Foods* **2021**, *10*, 51. [CrossRef]

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