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Original article

Application of Bioprotection in Winemaking: Reducing Sulfite Use *with non-Saccharomyces* Yeasts

Mamica Ruci [a,*], Renata Kongoli [b], Massimo Iorizzo [a], Bruno Testa [a], Julian Karaulli [a], Fatbardha Lamce [a], Rozarta Nezaj [a] & Onejda Kycyk [a]

[a] Department of Food and Research Centre, Faculty of Biotchenology and Food, Agricultural University of Tirana, Albania
[b] Department of Agri-Food Technology, Faculty of Biotchenology and Food, Agricultural University of Tirana, Albania
[c] Department of Food Science and Biotechnology, Faculty of Biotchenology and Food, Agricultural University of Tirana, Albania
[d] Department of Agriculture, Environmental and Food Sciences, University of Molise, 86100 Campobasso, Italy

Abstract

In the traditional winemaking practices, the sulfur dioxide (SO_2) has been used for its antimicrobial and antioxidant properties. However, in recent decades, increasing consumer demand for healthier, low-intervention, and additive-free wines has promoted researchers and producers to explore natural alternatives. Among these, *non-Saccharomyces* yeasts have been proposed as a sustainable substitute of SO_2, addressing both: health and consumer concerns. This method consists of the early inoculation of must or grapes with selected yeast strains, such as *Metschnikowia pulcherrima*, which produces antimicrobial metabolites to protect the wine and enrich wine aromatic complexity. This study aimed to evaluate the effectiveness of a selected *M. pulcherrima* strain as a bio protection agent in wine production. Controlled fermentations were conducted in triplicate, comparing conventional sulfiting with bio protected must. The kinetic and microbial populations were monitored during the fermentation process, and the respective wines were analyzed for key quality parameters. The results demonstrated that *M. pulcherrima* effectively limited undesirable microflora and improved fermentation kinetic without compromising wine quality. These findings support the potential of Bioprotectionas a promising strategy for reducing SO_2 in winemaking. This approach, previously unexplored in the Albanian wine industry, offers a pathway for producing organic, natural and sustainable wines, using native cultivars, while contributing to preservation of local biodiversity.

Keywords: Bioprotection, *non-Saccharomyces* Yeasts, Sulfite Reduction, Natural Wine, Biodiversity.

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* **Corresponding author:**

Ruci Mamica is a doctoral candidate in the Department Food and Research Centre at Agricultural University of Tirana in Albania. Her research interests include the Biotechnology and Wine sector She has lived, worked, and studied in Tirana, Albania.
Email: mruci@ubt.edu.al

INTRODUCTION

The winemaking industry has undergone a paradigm shift toward more sustainable and natural production methods, driven by consumer demand for authenticity, transparency and environmental stewardship. Application of Bioprotectionin winemaking has gained increasing interest among researchers due to its potential to reduce or eliminate the use of sulfites, a common preservative with known allergenic potential (Agarbati et al. 2023; Yao et al. 2023). Over the past decades, the exploration of novel Bioprotection strategies has increasingly focused on the use of selected non-*Saccharomyces* yeasts, particularly *Metschnikowia pulcherrima*, as a natural alternative to chemical additives (Testa et al. 2024; Puyo, Simonin, et al. 2020). Traditionally, *Saccharomyces cerevisiae* has dominated alcoholic fermentation due to its high ethanol tolerance and robust fermentative capabilities. However, studies have reported that non-*Saccharomyces* yeasts, can effectively reduce spoilage microorganisms and influence phenolic composition and aroma complexity, highlighting their practical and commercial relevance (Vejarano and Gil-Calderón 2021; Alexandre et al. 2023). Despite these promising advances, the specific mechanisms and efficacy of *M. pulcherrima* as a bioprotective agent remain only partially understood, leaving a notable gap in current knowledge (Puyo,et al. 2023b; Kuchen et al. 2021). While some studies have shown its antimicrobial activity against spoilage yeasts and bacteria, others report variability in its protective capacity depending on strain, fermentation conditions and grape health (Windholtz et al. 2021; Bustamante et al. 2024; Agarbati et al. 2022) Bioprotection in winemaking consists of the early inoculation of selected non-*Saccharomyces* yeasts during fermentation process to inhibit wine spoilage microorganisms through different antagonistic mechanisms, including competition for space, nutrients and oxygen as well as the secretion of antimicrobial molecules (Martín et al. 2024; Puyo, et al. 2023b). The antimicrobial activity of *M. pulcherrima* is primarily attributed to the production of pulcherriminic acid, which is the precursor of the red pulcherrimin pigment. This pigment depletes the iron from the medium, making it unavailable to competing yeasts and thereby contributing to microbial control (Testa et al., 2024; Torres-Díaz et al., 2025; Oro et al., 2014). Additionally, *M. pulcherrima* has been observed to consume oxygen faster than *S. cerevisiae* and other non-*Saccharomyces* species in must, a characteristic that may help limit oxygen availability for other microorganisms while preventing early oxidation of grape must (Windholtz et al. 2023). Furthermore, the studies have shown that *M. pulcherrima* does not inhibit *S. cerevisiae* and can be used in sequential inoculation as a starter culture (Comitini et al. 2011). Nevertheless, its effectiveness as a bioprotectant agent has been reported to vary depending on the initial microbial populations and environmental conditions.

M. pulcherrima offers significant potential in winemaking, not only by protecting must and wine from spoilage microorganisms but also by positively influencing aroma, mouthfeel and color, thereby enhancing the overall structure of the final product (Binati et al. 2023). These attributes are because of

the enzymatic activities of *M. pulcherrima* strain. Enzymes such as β-glucosidase and β-lyase, enhance aroma complexity and phenolic composition, contributing to improved wine's sensory profiles (Martín et al. 2024; Torres-Díaz et al. 2025). Furthermore, sequential inoculation strategies have been shown to optimize fermentation performance and sensory characteristics (Padilla et al. 2017).

This study aimed to explore the effectiveness of a selected *M. pulcherrima* strain as a viable bioprotective agent to support sulfite reduction in the production of *Kallmet* wine produced by Kallmet autochthonous Albanian red grape variety. The research highlights the relevance of Bioprotection as a context-specific innovation for Albania's growing wine industry. The findings underscore the importance of standardized methodologies and optimized Bioprotection protocols to diverse wine types and vintages. Importantly, Bioprotection aligns with the principles of sustainable and organic viticulture particularly significant for Albania as the country looks for strengthening its identity in organic wine production, reducing chemical inputs and promote microbial biodiversity within its unique terroirs.

MATERIALS AND METHODS

Yeast strains and growth condition

The *M. pulcherrima* strain AS3C1 (GenBank accession no. OM038321), used in this study, was isolated from a vineyard and belonging to the culture collection of the Department of Agricultural, Environmental and Food Sciences (Di.A.A.A.), University of Molise, Campobasso, Italy. Its oenological characteristics were extensively studied by Testa et al. (2024).

The commercial strain *S. cerevisiae* Zymaflore F15 *(*Laffort Co., Bordeaux, France) was used as control.The *M. pulcherrima* AS3C1 strain was cultured in YEPD broth (Merck Millipore, Darmstadt, Germany) at 28 °C under aerobic condition for 48 h. After incubation the broth culture was centrifuged at 5000 rpm for 10 min at 4 °C, and the cell pellet was washed three times with sterile physiological solution (0.9% NaCl) prior to used. Inoculation with *M. pulcherrima* AS3C1 was carried out at an initial concentration log 6.8 CFU/mL. The commercial dry yeast Zymaflor F15 was rehydrated according to the manufacturer's instructions and inoculated at a concentration of log 6.8 CFU/mL. Cell density was assessed using a Thoma hemocytometer (Thermo Fisher Scientific, Waltham, MA, USA).

Laboratory scale fermentation trials

The Kallmet red grapes (*Vitis vinifera*.cv), an autochthonous Albanian variety grown in the Koplik, Malsi and Madhe area, in North Albania, were used for the laboratory scale fermentation trials. The experiments were carried out at Research Food Center, Faculty of Biotechnology and Food, Agricultural University of Tirana. The harvested grapes were destemmed, crushed and divided into three trials (M1, M2, M3). Fermentations were carried out in triplicate in 10 L glass bottles filled with grape must and skins. The resulting grape must, showed the following chemical composition: pH 3.60, sugar 23 °Brix, total acidity 6.50 g/L, and YAN (yeast assimilable nitrogen) 182 mg/L. The first (M1) and

second (M2) trials were inoculated at pre-fermentative stage (at 16 °C) with *M. pulcherrima AS3C1* strain to assess potential biocontrol activity over 48 and 72 h, followed by the sequential inoculation with the commercial *S. Cerevisiae* F15 to complete the fermentation process. In the third (M3) trial, the must was treated with 50 mg/L potassium metabisulphite and inoculated only with commercial yeast *S. cerevisiae* F15 serving as the control test. The inoculation was performed with log 6.8 CFU/mL of *M. pulcherrima* strain and the same concentration for the *S. cerevisiae*. Alcoholic fermentations were conducted at temperature 22 ± 2 °C and monitored by measuring sugar consumption and by assessing ethanol production.

Monitoring of yeast population

The population of both wild and inoculated yeasts was monitored during the fermentation by viable cell counting on WL nutrient agar medium *(*Merck KGaA, Darmstadt, Germany*)* supplemented with 100 mg/L chloramphenicol to inhibit bacterial growth. Plates were incubated aerobically at 28 °C for four days and colony morphology and color diversity were assessed to differentiate *S. cerevisiae*, *M. pulcherrima*, and other yeast species (Pallmann et al., 2001). Yeast identification was further confirmed by molecular analysis through sequencing of the D1/D2 domain of the 26S rDNA gene (Testa et al., 2024).

Chemical analyses of wines

At the end of alcoholic fermentation, the chemical parameters of the wines were determined, including pH, total acidity (g/L as tartaric acid), volatile acidity (g/L as acetic acid), alcohol content % (v/v), free and total SO_2 (mg/L), reducing sugar (g/L) following OIV methods (ET and VIN, 2011). Glycerol (g/L) and acetaldehydes (mg/L) were determined using enzymatic kits (Steroglass, Perugia, Italy), according to the manufacturer's instructions.

Statistical analysis

The SPSS software was used for performed the statistical analysis of the data obtained from the experiments. Analysis of variance (ANOVA) followed by Duncan's multiple range test was used to assess significant differences among the results. Data from three replicates for each fermentation trial (n=3), are presented as mean ± standard deviation (SD) and differences were considered statistically significant at P-values ≤ 0.05.

RESULTS and DISCUSSION

Fermentation Kinetics

The *M. pulcherrima* AS3C1 strain previously characterized for its antimicrobial properties (Testa et al. 2024) was investigated for the first time in an Albanian grape variety as a potential alternative to

sulfite for protecting must and wine from spoilage microorganisms. The growth dynamics of the selected strain *M. pulcherrima* AS3C1 during the fermentation process are shown in Figure 1

M. pulcherrima AS3C1 during the fermentation process are shown in Figure 1.

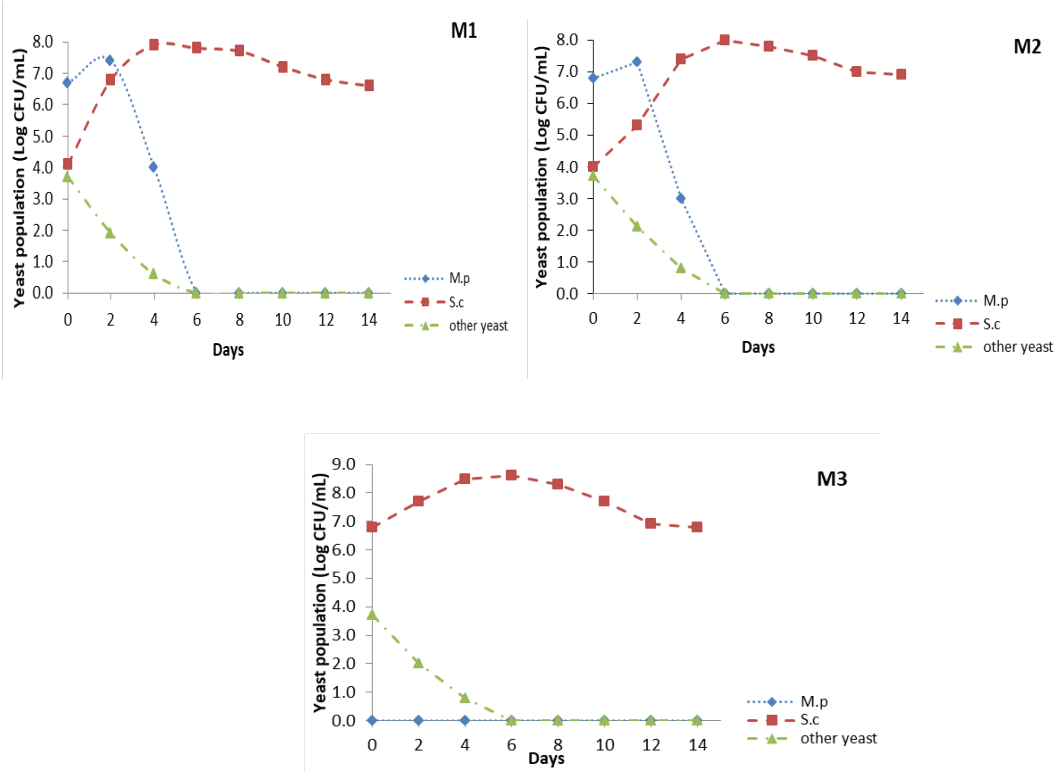

Figure 1. Dynamics of yeast population (log CFU/mL) during fermentation. **M1:** *M. pulcherrima AS3C1* inoculated at the start followed *by S. cerevisiae* F15 after 48 h; **M2:** *M. pulcherrima AS3C1* inoculated at the start, followed by *S. cerevisiae* F15 after 72 h**; M3:***S.cerevisiae* F15 inoculated at the start of fermentation**.**

In the first trial (M1) was inoculated *M. pulcherrima* AS3C1 at the pre-fermentative stage at a concentration of log 6.7 CFU/mL. After two days, the yeast population increased to log 7.4 CFU/mL followed by a gradual decline until day six, when *M. pulcherrima* yeast became undetectable. The populations of endogenous non-*Saccharomyces* yeasts in the must were initially present at log 3.7 CFU/mL, rapidly declining to log 1.9 CFU/mL after two days and becoming undetectable by day six. *S. cerevisiae* F15, was inoculated 48 h into fermentation at a concentration of log 6.8 CFU/mL, after which it increased to dominate the population, reaching a maximum by day four and remaining stable until the end of fermentation process. In the second trial (M2), *M. pulcherrima* AS3C1 was inoculated at concentration of log 6.8 CFU/mL. After two days, the population increased and began to decline by day four, becoming undetectable by day six. *S. cerevisiae* F15 was inoculated 72 h into fermentation at concentration of log 6.8 CFU/mL; its cell density reached the concentration of log 8.0 CFU/mL by day six followed by a slight decrease to log 7.0 CFU/mL, which remained stable until the end of alcoholic

fermentation. The initially endogenous non-Saccharomyces yeast population in M2 was log 3.7 CFU/ml, decreasing to log 2.1 CFU/mL by day three and disappearing by day six. In the third trial (M3, control), the must was inoculated only with commercial yeast *S. cerevisiae* F15 at log 6.8 CFU/ mL. The yeast population gradually increased, reaching at log 8.5 CFU/ mL by day four, then slightly decreased to log 7.7 CFU/ mL after day eight and remained stable until the end of fermentation. Other non-*Saccharomyces* yeasts, initially present in the must at concentration of log 3.7 CFU/mL rapidly decreased to 10^1CFU/ mL by day three and became undetectable by day six.

Alcoholic fermentation was completed within 15 days in all three trials, as shown in Figure 2. Sugar consumption in the first days of M1 and M2 were slow until the inoculation of *S. cerevisiae* F15, after which sugar consumption increased markedly from day four onwards. In contrast, M3 exhibited faster sugar consumption because *S. cerevisiae* F15 was inoculated at the start of fermentation. Therefore, in the control trial M3, the ethanol production was more rapid compared with the other two trials.

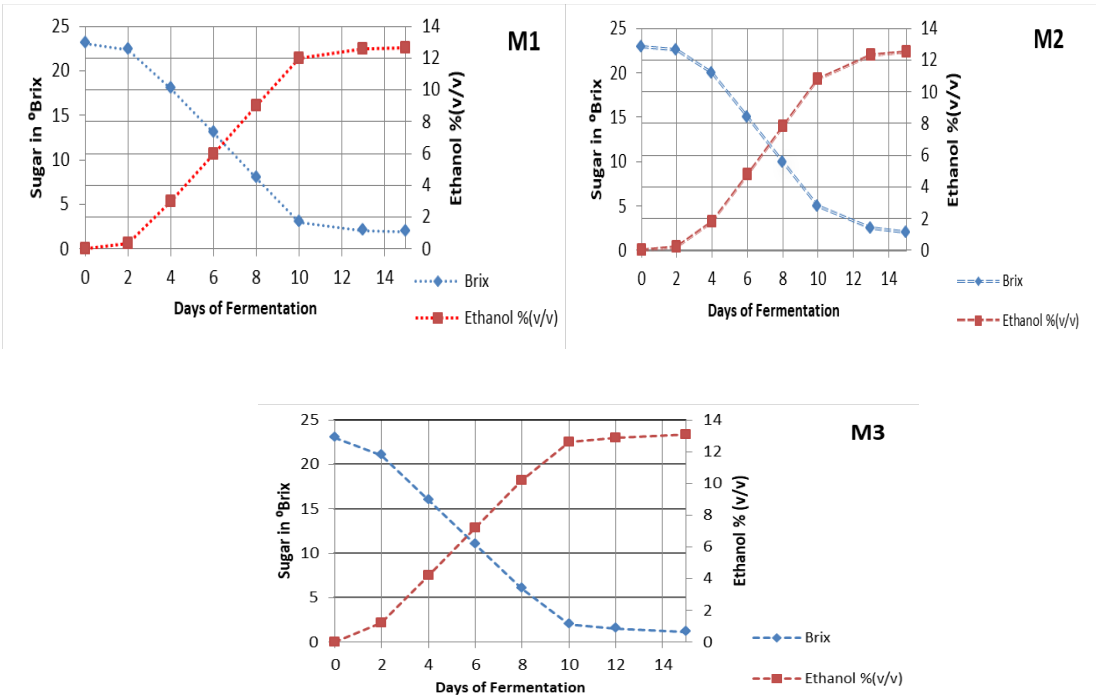

Figure 2. Sugar consumption (º Brix) and ethanol production % (v/v) during the alcoholic fermentation in the three trials. M1 (*M.pulcherrima AS3C1* + *S.cerevisiae* F15 after 48 h), M2 (*M.pulcherrima AS3C1* + *S.cerevisiae* F15 after 72 h), M3 (*S.cerevisiae* F15).

Chemical analysis of the wines

The results of the chemical parameters of the wines at the end of alcoholic fermentation are reported in Table 1. Wines from trials M1 (*M. pulcherrima* AS3C1 + *S. cerevisiae* F15 after 48 h) and

M2 (*M. pulcherrima* AS3C1 + *S. cerevisiae* F15 after 72 h**),** showed lower ethanol content (12.6% v/v and 12.5% v/v, respectively) compared with the control trial M3, which was inoculated with *S. cerevisiae* F15 (12.9 % v/v). No significant differences were observed among the trials for pH or total acidity. However, volatile acidity was significantly higher in the control trial M3 (0.5 g/L acetic acid) compared with the bio protected wines M1 and M2, (0.28 g/L; 0.30 g/L). Glycerol content was highest in M1 (5.05g/L), follow by M2 (4.25 g/L) and lowest in M3 (3.03 g/L), Significant differences were also observed in acetaldehyde levels: the control trial M3 showed the highest concentration (11.2 mg/L), followed by M2 (6.61 mg/L) and M1 (6.02 mg/L). Reducing sugar levels were lower in trial M3 compared with M1 and M2. For L-malic acid the lowest concentration was found in M2 (1.8 g/L), followed by M3 (1.94 g/L) and M1 (2.2 g/L).

Table 1. Chemical parameters of the wines at the end of alcoholic fermentation in trial **M1** (*M. pulcherrima AS3C1 + S. cerevisiae* F15 after 48h), **M2** (*M. pulcherrima AS3C1 + S. cerevisiae* F15 after 72h**), M3 (***S. cerevisiae* F15**).**

Chemical Parameters	M1	M2	M3
pH	3.74 ± 0.010^{a}	3.73 ± 0.017^{a}	3.70 ± 0.021^{a}
Total acidity (g/L)	6.31 ± 0.015^{b}	6.39 ± 0.006^{a}	6.24 ± 0.051^{c}
Volatile acidity (g/L)	0.27 ± 0.006^{c}	0.31 ± 0.015^{b}	0.51 ± 0.015^{a}
Alcohol (% v/v)	12.6 ± 0.012^{b}	12.4 ± 0.061^{c}	12.9 ± 0.006^{a}
Total SO_2 (mg/L)	24.2 ± 0.064^{c}	38.2 ± 0.153^{b}	48.5 ± 0.487^{a}
Free SO_2 (mg/L)	10.2 ± 0.075^{c}	17.8 ± 0.155^{b}	21.7 ± 0.050^{a}
L-Malic acid (g/L)	2.20 ± 0.010^{a}	1.80 ± 0.010^{c}	1.94 ± 0.010^{b}
Glycerol (g/L)	5.05 ± 0.010^{a}	4.25 ± 0.006^{b}	3.03 ± 0.006^{c}
Reducing sugar (g/L)	2.01 ± 0.010^{b}	2.33 ± 0.015^{a}	1.88 ± 0.010^{c}
Acetaldehyde (mg/L)	6.02 ± 0.010^{c}	6.61 ± 0.051^{b}	11.20 ± 0.015^{a}

The values are expressed as mean ± standard deviation (n = 3). Different superscript letters in each row indicate significant differences ($p < 0.05$).

Non-*Saccharomyces* yeasts have received increasing attention among researchers and wine producers due to their biotechnological potential. Over the last decade, several non-*Saccharomyces* species have been investigated as biological protectants in the context of sulfite reduction in winemaking (Yao et al. 2023; Windholtz et al. 2021). The application of non-*Saccharomyces* yeasts as a starter cultures, either in the mixed fermentation or in sequential inoculation with *S. cerevisiae*, has been demonstrated to be an effective strategy for reducing sulfite without compromising wine quality parameters (Mateo and Maicas 2016; Simonin et al. 2020). In regard to bio protection, non-*Saccharomyces* yeasts are added in the early stage of grapes or must in order to colonize the environment, exploiting their competitive advantage through various mechanisms and thereby limiting the growth and survival of other undesirable microorganisms (Puyo et al., 2023a; Agarbati et al. 2022). During the initial days of alcoholic fermentation, when ethanol concentrations are still low, *non-Saccharomyces* yeasts can thrive and showcase their metabolic potential, especially regarding

antimicrobial and enzymatic activities. Studies have shown that the timing of inoculation and the inoculation rate are key factors that impact on the chemical characteristics of wine produced without sulfites (Windholtz et al. 2021).

The findings from this study confirm the dynamic and transient role of *M. pulcherrima* AS3C1 during alcoholic fermentation and highlight its potential as a bioprotective agent against the undesirable microorganisms in winemaking. These results align with recent studies, particularly in the ability of *M. pulcherrima* strains to suppress *non-Saccharomyces* populations through antimicrobial mechanisms without hindering the fermentative capacity of *S. cerevisiae* (Puyo et al. 2023a). In trials M1 and M2 the cell density of *M. pulcherrima* rapidly increased after inoculation, reaching log 7.3 CFU/mL within two days. However, consistent with its known sensitivity to ethanol and nutrient competition, the population declined sharply and became undetectable by day six. These dynamics are characteristic of *M. pulcherrima*, which tends to dominate and express its antimicrobic activities during early stages of fermentation, before ethanol level rise (Canonico et al. 2023)

Notably, the presence of *M. pulcherrima* coincided with a significant suppression of indigenous non-*Saccharomyces* yeasts, supporting its bio productive role. This effect is attributed to the production of pulcherrimin, an iron-chelating pigment with antimicrobial properties, which limits the growth of competing microorganisms (Aragno et al. 2024; Testa et al. 2024). Such antagonistic behavior highlights the potential of *M. pulcherrima* as a natural alternative to sulfite use in sustainable wine production.

After the inoculation of *S. cerevisiae* F15 (at 48 h in M1 and 72h in M2), an increase in fermentative yeast population was observed, with stable dominance maintained throughout the fermentation. This observation aligns with other studies showing *M. pulcherrima* does not negatively interfere with the fermentation kinetics of *S. cerevisiae* during the co-inoculated fermentation (Binati et al. 2023b; Coppola et al. 2025).

Fermentation was completed within 15 days across all trials that confirm the suitability of *M. pulcherrima* for sequential inoculation strategies. Beyond microbial stability, this Bioprotection approach can enhance sustainability by reducing the sulfite reliance and potentially enriching the sensory profiles of wines (Canonico et al. 2023; Denat et al. 2021).

The chemical analysis at the end of fermentation revealed that the use of *M. pulcherrima AS3C1* in sequential inoculation with *S. cerevisiae* F15 influenced several key wine parameters compared to the control fermentation. Ethanol content was slightly lower in wines from trials M1 (12.6% v/v) and M2 (12.5% v/v) than in the control M3 (12.9% v/v). Such reductions have been reported previously when non-*Saccharomyces* yeasts are active during the early stages of fermentation, as they may divert carbon flux towards other metabolites such as glycerol and organic acids rather than ethanol (Canonico et al. 2019). Other studies have emphasized that the extent of ethanol reduction depends on yeast strains,

timing, and inoculation protocol (Coppola et al. 2025; Hranilovic et al. 2020). This suggests the use of *M. pulcherrima* may offer a partial ethanol reduction strategy, aligning with current winemaking trends toward lower-alcohol wines (Aplin and Edwards 2021).

No significant differences were observed in pH and total acidity between trials, indicating that *M. pulcherrima* activity under these conditions did not change the major acid balance of the wine. However, volatile acidity was significantly higher in the control trial M3, whereas M1, and M2, where *M. pulcherrima* acted as a bioprotectant, maintained lower levels. This finding is consistent with earlier observations that *M. pulcherrima* may suppress acetic acid bacteria and spoilage yeasts responsible for volatile acidity production (Windholtz et al. 2023).

Glycerol is a by-product of yeasts during the alcoholic fermentation and contributes to enhancing the mouthfeel and overall body of wine (Aragno et al. 2024). In this study, higher glycerol production was observed in M1 and M2 trials compared to the control trial M3, which was fermented only with *S. cerevisiae* yeast. This result is consistent with previous findings showing that *non-Saccharomyces* yeasts, such as *M. pulcherrima* can increase glycerol content without producing undesirable compounds such as acetic acid (Coppola et al. 2025; Ivit et al. 2020). The higher glycerol in M1 compared to M2 may reflect the longer co-presence of *M. pulcherrima* before *S. cerevisiae* inoculation in the 48h sequential inoculation strategy.

Acetaldehyde concentrations were significantly lower in trials M1 (6.02 mg/L) and M2 (6.62 mg/L), where *M. pulcherrima* was used during the early stages of fermentation, compared with the control trial M3 (11.2 mg/L), which relied solely on *S. cerevisiae*. These results are aligned with other studies indicating that *M. pulcherrima* may contribute to acetaldehyde reduction, either through enzymatic activity or by moderating the kinetics of early fermentation. Lower acetaldehyde levels are favorable, as they can reduce oxidative aromas and improve wine freshness (Binati et al. 2023a; Denat et al. 2021).

Reducing sugar concentrations were lowest in the M3 trial, reflecting the earlier and uninterrupted activity of *S. cerevisiae*. In contrast, trials M1 and M2, due to the delayed inoculation of *S. cerevisiae*, resulted in slightly higher residual sugar levels, although these remained within the limits for dry wines.

L-malic acid concentrations did not show any significant difference between the trials. Thus, the findings of this study highlight the selected *M. pulcherrima* AS3C1 as a viable bioprotective alternative to sulfite addition, capable of suppressing spoilage microorganisms while contributing positively to chemical composition of the wine. Such an approach represents a promising strategy for reducing chemical inputs, protecting biodiversity and enhancing the quality of wine.

CONCLUSION

This study provides the first data on the application of *Methschnikowuia pulcherrima AS3C1* for Bioprotectionin Albanian winemaking, using a local grape variety to produce sulfite-free wine. *M. pulcherrima AS3C1* strain, previously characterized for its antimicrobial properties, can be effectively applied as a bioprotectant agent in winemaking. Sequential inoculation with *S. cerevisiae* F15 allowed *M. pulcherrima* to dominate the early fermentation stages, suppress indigenous spoilage yeasts, and support the completion of alcoholic fermentation without the use of chemical preservatives. This approach resulted in wines free from chemical preservatives, with slightly reduced ethanol content, lower volatile acidity, increased glycerol, and reduced acetaldehyde concentrations compared to the control fermentation. Although preliminary, these findings highlight that *M. pulcherrima* may be a promising biotechnological strategy and a sustainable innovation for the Albanian wine sector. Future studies should focus on sensory profiling and the long-term wine stability to fully assess its oenological potential.

Author Contributions

In this study, the contribution of the authors was: Experiment and laboratory analysis were carried out at the Agricultural University of Tirana under the supervision of Professor Renata Kongoli and the assistance of Professor Iorizzo and Dr. Bruno Testa from University of Molise. All the authors from Agricultural University of Tirana contributed equally to the development of the research idea, data analysis, writing and proofreading stages. The first author contributed to all stages.

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Responsible Artificial Intelligence Statement

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Ethics Approval

In all processes of this study, the principles of Pen Academic Publishing Research Ethics Policy were followed.

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