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ORIGINAL PAPER

Effects of inoculation timing and mixed fermentation with *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* on the aroma and sensory properties of Falanghina wine

Francesca Coppola[1] · Bruno Testa[2] · Rosaria Cozzolino[1] · Julian Karaulli[3] · Gianfranco Pannella[4] · Massimo Di Renzo[2] · Cristina Matarazzo[1,2] · Mariantonietta Succi[2] · Massimo Iorizzo[2]

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Abstract

In recent years, non-*Saccharomyces* yeasts, including *Metschnikowia pulcherrima*, have gained renewed interest in biotechnology applied to the wine industry due to their pro-technological properties. *M. pulcherrima* has been particularly studied for its antimicrobial activity, its potential to produce wines with a low alcohol content, and its enzymatic activities that enhance some sensory characteristics of wines, especially when used in co-culture with *Saccharomyces cerevisiae*. This study evaluated the production of Falanghina wine using *M. pulcherrima* AS3C1 as an initial starter, followed by sequential inoculation with a commercial *S. cerevisiae* after 2 and 4 days. Moreover, a parallel vinification was performed using *S. cerevisiae* as a single starter culture. Volatile Organic Compounds (VOCs) data, obtained through Headspace Solid phase Microextraction couple to Gas Chromatography-Mass Spectrometry (HS-SPME/GC–MS), were analyzed using multivariate statistical methods (PCA and sPLS-DA). The results showed significant differences in the VOC profiles of the wines produced. Notably, the wine obtained through sequential inoculation of *M. pulcherrima* AS3C1 followed by *S. cerevisiae* after 4 days received the highest overall sensory evaluation by the panel test. This wine was distinguished by its elevated levels of ethyl acetate, phenylethyl acetate, benzeneethanol and 2-methylpropanol.

Keywords *Metschnikowia pulcherrima* · Sequential inoculum · *Saccharomyces cerevisiae* · VOCs · Sensory properties

Introduction

Globally wine production relies on commercial starter cultures composed of selected strains from the *Saccharomyces* genus, particularly *Saccharomyces cerevisiae*. These selected strains have enabled winemakers to better control the fermentation process, maximize production standardization, and enhance the organoleptic quality of wines [1].

However, the widespread use of single *Saccharomyces* strains has reduced the microbial biodiversity of fermentation processes, consequently decreasing the complexity of wines [2–4]. The sensory profiles of wines produced using monoculture starter fermentations are markedly different from those of wines produced through spontaneous fermentation [5]. Spontaneous alcoholic fermentation involves a dynamic succession of yeasts from different species present in the grape must, with both non-*Saccharomyces* and *Saccharomyces* yeasts participating in a mixed and sequential manner [6].

Historically, non-*Saccharomyces* yeasts were often associated with the production of undesirable compounds and were therefore regarded as unwanted microorganisms [7, 8]. However, in recent years, their role has been reassessed. Numerous studies have demonstrated that mixed or

✉ Gianfranco Pannella
g.pannella@unicampus.it

1 Italian National Research Council (CNR), Institute of Food Sciences (ISA), 83100 Avellino, Italy

2 Department of Agricultural, Environmental and Food Sciences, University of Molise, 86100 Campobasso, Italy

3 Faculty of Biotechnology and Food, Food and Research Center, Agricultural University of Tirana, 1001 Tirana, Albania

4 Department of Science and Technology for Sustainable Development and One Health, Università Campus Bio-Medico di Roma, 00128 Rome, Italy

sequential fermentations involving both non-*Saccharomyces* and *Saccharomyces* yeasts can result in a greater variety and concentration of volatile compounds, enhancing wine complexity and imparting distinctive sensory characteristics [9–16].

Modern gene sequencing techniques have revealed that certain non-*Saccharomyces* yeasts possess a genetic makeup that encodes for the production of a wider range of extracellular enzymes—such as esterases, β-glycosidases, and lyases—than *Saccharomyces cerevisiae* [17]. As a result, non-*Saccharomyces* yeasts are increasingly regarded as valuable biotechnological resources for improving wine quality [7, 14, 18, 19].

However, the use of non-*Saccharomyces* yeasts, whether in spontaneous fermentations or as pure cultures, comes with risks. These include the possibility of slow or incomplete fermentation, as well as the production of off-flavors or undesirable compounds—such as biogenic amines, acetic acid, ethyl acetate, acetaldehyde, and acetoin—in excessive concentrations [12, 20, 21].

In recent years, to mitigate these challenges and harness the oenological potential of non-*Saccharomyces* yeasts, controlled multi-starter fermentations, have been developed. These fermentations primarily use selected cultures of both *Saccharomyces* and non-*Saccharomyces* yeasts to enhance the chemical composition and aroma profile of wines [15, 16, 22–32].

This approach has driven the scientific community to intensify efforts towards the identification and selection of new yeast strains, as reflected in the growing number of studies focused on single or mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts to enhance wine quality [13, 33].

With the renewed interest in the role of non-*Saccharomyces* yeasts in winemaking and their potential as selected starters in mixed fermentations with *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima* has frequently been proposed as a co-fermentative species. Its wide array of enzymatic activities can interact with odorless grape must precursors, leading to the production of aromatic compounds able to enhance wine aroma [12, 34–44].

Additionally, *M. pulcherrima* has been successfully used in several studies for the production of wines with a reduced alcohol content [45–50]. It has also been explored as a bioprotective alternative to reduce the use of sulfur dioxide in white grape musts and to prevent enzymatic and non-enzymatic browning [51].

Furthermore, due to its specific antimicrobial activity [52–54], *M. pulcherrima* has been proposed as a starter culture in controlled mixed fermentations with *S. cerevisiae* to inhibit undesired microflora in must and wine [55–59].

In a recent online review, several commercial strains of *M. pulcherrima* were identified as being available on the market (Table 1).

To date, there has been limited willingness to address the growing demand from wine producers seeking to enhance or diversify the quality of their products [60]. Recent studies have shown that aerobic conditions and the timing of sequential inoculations, using *M. pulcherrima* in co-culture with *Saccharomyces* yeasts, play a crucial role in defining the compositional characteristics of wine [23, 48, 50].

Building on these findings, this study evaluates the impact of sequential inoculation timing on the chemical and sensory profile of Falanghina wine, using the pre-commercial *M. pulcherrima* AS3C1 in combination with a commercial *S. cerevisiae* strain.

M. pulcherrima AS3C1, isolated from grapes, was chosen for its antimicrobial activity, distinctive enzymatic activities, such as β-glucosidase and β-lyase, and its low production of acetic acid and H_2S [44, 61].

Falanghina (*Vitis vinifera* L.), typical white grape cultivar of the Campania Region (Southern Italy), [62], is characterized by a medium-sized trunk-conical bunch, winged with medium-sized berries with thick and waxy skin, crunchy pulp with a slightly floral flavor, flavor, which gives rise to musts with variable pH and acidity depending on the harvest time [63, 64].

Materials and methods

Starter cultures

M. pulcherrima AS3C1, isolated from grapes and characterized in previous studies [44, 61] and the commercial yeast *S. cerevisiae* ENOFERM T306 (Lallemand, Hunter Valley, NSW, Australia) were used in the present experiment. Both yeasts were pre-cultured in 500 mL shake flasks containing 300 mL of YPD medium at 30 °C with shaking at 150 rpm. After 24 h, the yeast cells were collected by centrifugation (3000 rpm for 5 min) and washed three times with sterile saline before being used as starter cultures. For the subsequent trials, initial cell density was assessed using a Thoma counting chamber (Thermo Fisher Scientific, Waltham, MA, USA).

Winemaking process

Fermentation trials were performed using Falanghina grapes from the 2020 vintage. The vines used for this study are planted in rows and cultivated on calcareous clay soil in San Lorenzello (Benevento province; 41,25,385° N, 14,52,881° E; 200 m a.s.l.) by Double Guyot (DG) farmed system,

Table 1 Oenological Properties of Commercial *Metschnikowia pulcherrima* Strains Available on the Market

Commercial Product	Productor	Declared oenological properties	References of the oenological properties
LEVEL^2 FLAVIA™	Lallemand (Canada	Antimicrobial Activity and Increased Aromatic Complexity of Wine	https://www.lallemandwine.com/en/canada/products/wine-yeasts/level2-flavia
LEVEL^2 GUARDIA™	Lallemand (Italy)	Antimicrobial activity	https://www.lallemandwine.com/it/italy/prodotti/wine-yeasts/level2-guardia
LEVEL^2 INITIA™	Lallemand (Canada)	Antimicrobial and Antioxidant activities	https://www.lallemandwine.com/en/canada/products/wine-yeasts/level2-initia/
Oenoferm® MProtect	Erbslöeh (Germany)	Antimicrobial activity and prevention of off-flavours in wines	https://erbsloeh.com/produkt/oenoferm-mprotect/
AWRI Obsession	AB Biotek (United Kingdom)	Increase the colour and the aromatic complexity of red wines	https://www.abbiotek.com/perch/resources/next-generation-awri-obsession-product-information-may-2019-web.pdf
LEVULIA® Pulcherrima	AEB Group (Italy)	Increase the aromatic complexity of wines	https://www.aeb-group.com/media/catalogo-unico/levulia_pulcherrima-2888/docs/it/LEVULIA%20PULCHERRIMA_TDS_IT_0140917_OENOLIA_Italy.pdf
Primaflora® VB BIO	AEB Group (Italy)	Antimicrobial Activity and Increased Aromatic Complexity of Wine	https://www.aeb-group.com/it/primaflora-vb-bio-17601
Excellence® B-Nature	Lamothe-Abiet (France)	Antimicrobial Activity and Increased Aromatic Complexity of Wine	https://lamothe-abiet.com/it/lieviti/excellence-b-nature/
EnartisFerm Q MCK	Enartis (Italy)	Antimicrobial and Antioxidant activities	https://www.enartis.com/wp-content/uploads/2024/09/Enartis_Flyer_EnartisFermQMCK_USA-1.pdf

planted 2.40 × 1.40 m. The grapes were harvested in the first week of October.

Following the harvest, the grapes were transferred to stainless-steel containers, treated with 0.01 g/kg of pectolytic enzyme (pectinase, Lallzyme Ex), and subjected to the crushing-destemming process.

The must obtained was subjected to the following pre-fermentation treatments:

- Sulphiting with 50 mg/L of potassium metabisulfite;
- Soft pressing;
- Static decantation at 8 °C for 24 h after the addition of 10 mg/L of pectolytic enzyme;
- Pumping over and the addition of 100 mg/L of polyvinylpolypyrrolidone (PVPP).

The resulting grape must was then characterized according to the official methods established by the European Commission (EEC). All analyses were conducted in triplicate. The basic parameters were as follows: 216.35 ± 3.4 g/L total sugar, pH 3.34 ± 0.0, 6.37 ± 0.82 g/L total titratable acids, 2.7 ± 0.2 g/L malic acid, and 90.24 ± 2.1 mg/L Yeast Assimilable Nitrogen (YAN).

Before the fermentation phase, 50 mg/L of potassium metabisulfite was added to the grape must, followed by diammonium phosphate to achieve a YAN of 200 mg/L. For the sequential fermentations, *M. pulcherrima* AS3C1 was inoculated immediately into the must, while *S. cerevisiae* T306 was added after 2 (Batch A) and 4 days (Batch B). As a control, *S. cerevisiae* T306 was used as the single starter culture (Batch C). The starter yeasts were inoculated to achieve an initial cell density of approximately 10^7 CFU/mL. The fermentations were conducted in duplicate at 18 ± 1 °C in stainless steel tanks (100 L capacity) containing 80 L of grape must.

For the clarification phase, at the end of fermentation (residual sugar < 2 g/L), the wines were treated with bentonite (400 mg/L), PVPP (100 mg/L), plant-based protein fining agents (300 mg/L), and potassium metabisulfite (100 mg/L). After 10 days, the wines were filtered using a 0.45 µm membrane cartridge, supplemented with potassium metabisulfite to achieve about 30 mg/L of free SO_2, bottled in 750 mL dark brown glass bottles sealed with crown caps, and stored at 5 °C for 2 months prior to analysis.

All processing aids used in winemaking were supplied by Enartis (Enartis S.r.l., San Martino Trecate, Italy).

Fermentation kinetics

The fermentations were monitored by evaluating yeast viability and the decrease in sugar content. Yeast viable cell counts were determined using WL nutrient agar (Oxoid, Hampshire, UK). After incubation at 25 °C for 72 h, colony color and topography were used as parameters to differentiate *S. cerevisiae*, *M. pulcherrima*, and other yeasts [44]. The taxonomic identification of the yeast species was confirmed by sequencing the ITS 1 and 4 regions. The ITS1-5.8S rRNA-ITS2 region was amplified by PCR (Polymerase Chain Reaction) using the primer pair ITS1 (5′-TCCGTAG GTGAACCTCGCG-3′) and ITS4 (5′-TCCTCCGCTTTAT TGATATGC-3′) [65].

Oenological parameters

Alcohol content, pH, total acidity, and reducing sugars were determined according to the methods of the International Organization of Vine and Wine [66]. Acetaldehyde, L-malic acid, L-lactic acid, glycerol, and catechins were measured using enzymatic kits provided by Boehringer Mannheim (Boehringer Mannheim Biochemicals, Mannheim, Germany).

Total YAN was determined using the K-PANOPA and K-LARGE kits from Megazyme (Megazyme, Wicklow, Ireland), following the manufacturer's instructions. The browning index (BI) was evaluated by measuring the absorbance at 420 nm [67].

All chemicals used were of analytical grade (≥99%) and were purchased from Sigma-Aldrich (Merck Life Science, Milan, Italy).

Analysis of volatile organic compounds

Volatile organic compounds (VOCs) in wine samples were detected using headspace solid-phase microextraction (HS-SPME) coupled with Gas Chromatography/Mass Spectrometry (GC/MS).

For sample preparation, 5 mL of wine was placed in a 20 mL headspace vial, and 2 g of NaCl was added. To ensure sample reproducibility, 5 µL of 3-octanol (Merck Life Science, Milan, Italy), in a hydro-alcoholic solution (1/1, v/v) at 100 µg/L, was used as the internal standard (IS). The vial was sealed and homogenized with a vortex shaker. VOCs were extracted following the procedure described by [68] with minor modifications. Briefly, the extraction and injection were carried out automatically by an autosampler device (MPS 2, Gerstel, Mülheim, Germany). The fiber (DVB/CAR/PDMS; 50/30 mm, 2 cm) was exposed to the headspace for 30 min at 40 °C, after which it was automatically transferred to the GC injector for VOC desorption at 240 °C for 10 min.

VOCs were separated using an Agilent 7890A gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) coupled to a 5975A mass spectrometer (5975A, Agilent Technologies, Santa Clara, CA, USA), equipped with a capillary HP-Innowax column (30 m × 0.25 mm × 0.5 µm). The oven temperature was initially held at 40 °C for 1 min, then gradually increased: first to 60 °C at a rate of 2 °C/min, then to 150 °C at 3 °C/min, followed by a third increase to 200 °C at 10 °C/min, and finally to 240 °C at 25 °C/min, maintaining this temperature for 7 min. Helium, with a flow rate of 1.5 mL/min, was used as the carrier gas. The ion source and quadrupole temperatures were set to 230 °C and 150 °C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV, in splitless mode, and in the m/z range of 30–300.

VOCs were identified or tentatively identified by comparing mass spectra with existing libraries (Nist05/Wiley07), linear retention indices (LRI), and retention times of available commercial standards, analyzed under the same experimental conditions.

Semi-quantitative data for individual volatile components (Relative Peak Area, RPA%) were calculated relative to the peak area of 3-octanol (IS). Areas of the identified VOCs were obtained from the total ion chromatogram (TIC). Wine samples were analyzed in triplicate using a randomized sequence, with blanks also included.

Sensory analysis

To evaluate the sensory characteristics of the wines, a panel of 6 professional tasters, between the age of 30–50 and with a sex-ratio of 1:1, belonging to the National Organization of Wine Tasters (ONAV, Italy) was conducted, following the procedures outlined by [69]. Several sensory attributes were assessed using a score range from 0 (absence of perception) to 9 (maximum perception). The sensory attributes and their corresponding descriptors were as follows: visual (color intensity), olfactory (apple, fresh fruit, vegetal, hazelnut, vanilla, spicy), taste (sweetness, acidity, bitterness, tastiness), tactile (astringency), retro-nasal olfaction (apple, vegetal, fruity), and overall rating. Two independent replicates of each wine batch were sensory analyzed.

Statistical analysis

The yeast viable cell counts and main chemical parameters were analyzed using ANOVA with the General Linear Model (GLM) tool in SPSS (IBM SPSS Statistics 21). All data are expressed as the mean ± standard deviation (± SD)

Table 2 Evolution of populations of *S. cerevisiae*, *M. pulcherrima*, and other yeasts during fermentation, expressed as log CFU/mL

		Fermentation time (days)				
Test	Yeasts	0	2	4	6	10
Batch A	*S. cerevisiae*	2.02 ± 0.13^{c}	6.94 ± 0.05^{b}	7.67 ± 0.27^{a}	7.22 ± 0.29^{a}	6.80 ± 0.16^{b}
	Other yeasts	4.79 ± 0.10^{a}	4.65 ± 0.15^{a}	n.d.	n.d.	n.d.
	M. pulcherrima	7.10 ± 0.08^{a}	7.23 ± 0.10^{a}	n.d.	n.d.	n.d.
Batch B	*S. cerevisiae*	2.11 ± 0.11^{d}	2.34 ± 0.18^{d}	7.25 ± 0.14^{b}	7.71 ± 0.15^{a}	6.67 ± 0.09^{c}
	Other yeasts	4.95 ± 0.12^{a}	4.77 ± 0.20^{a}	2.80 ± 0.23^{b}	n.d.	n.d.
	M. pulcherrima	7.24 ± 0.10^{a}	7.40 ± 0.11^{a}	2.00 ± 0.07^{b}	n.d.	n.d.
Batch C	*S. cerevisiae*	7.26 ± 0.12^{b}	7.86 ± 0.16^{a}	7.95 ± 0.17^{a}	7.09 ± 0.25^{b}	5.96 ± 0.11^{c}
	Other yeasts	4.63 ± 0.26^{a}	3.40 ± 0.23^{b}	n.d.	n.d.	n.d.
	M. pulcherrima	n.d.	n.d.	n.d.	n.d.	n.d.

Batch A: Initial inoculation of *M. pulcherrima* AS3C1, followed by sequential inoculation of *S. cerevisiae* T306 after 2 days. Batch B: Initial inoculation of *M. pulcherrima* AS3C1, followed by sequential inoculation of *S. cerevisiae* T306 after 4 days. Batch C: Inoculation of *S. cerevisiae* T306 as a single starter

n.d. not detected

Different letters in the same row indicate significant differences. All values are expressed as the mean ± standard deviation of two biological replicates

of two biological replicates. Statistical significance was set at $p \leq 0.05$.

Data analysis and graph processing for the sensory characteristics of the wines and VOCs (Supplementary Material, Table S1) were performed in R (Version 4.2.3) using RStudio software (Version 2022.07.0).

For sensory analysis, the Kruskal–Wallis test was used to assess significant differences ($p < 0.05$) between groups, and the Dunn's post-hoc test was applied for multiple comparisons. The rstatix R package (Version 0.7.2) was used for these analyses. The median value of each dataset was compared between batches.

To explore differences in VOCs content among the wines, two statistical methods based on a multivariate approach were applied: unsupervised principal component analysis (PCA) and supervised Sparse Partial Least Squares Discriminant Analysis (sPLS-DA). The FactoMineR package [70] was used for PCA, and the MixOmics package [71] was used for sPLS-DA.

Results and Discussion

Evolution of yeast populations and sugar consumption

The viability of yeasts in pure and sequential fermentations is reported in Table 2.

Sequential inoculation allowed the persistence of the *M. pulcherrima* population for 2 (Batch A) and 4 days (Batch B). After these time points, the viability of *M. pulcherrima* began to decline, eventually becoming undetectable after day 4 in Batch A and after day 6 in Batch B. In Batch C, *M. pulcherrima* was never detected.

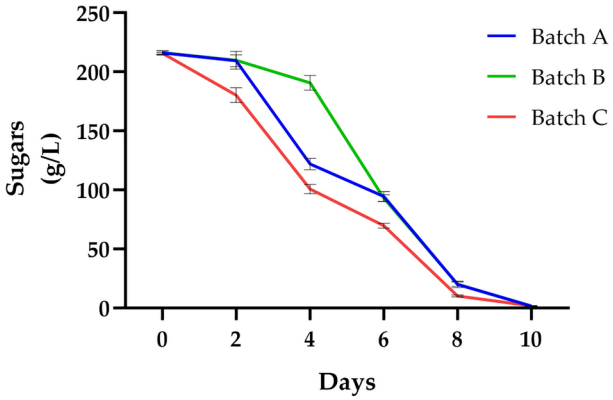

Fig. 1 Evolution of sugar content (g/L) during fermentation. Batch A: Initial inoculation of *M. pulcherrima* AS3C1, followed by sequential inoculation of *S. cerevisiae* T306 after 2 days. Batch B: Initial inoculation of *M. pulcherrima* AS3C1, followed by sequential inoculation of *S. cerevisiae* T306 after 4 days. Batch C: Inoculation of *S. cerevisiae* T306 as a single starter

Previous studies using *M. pulcherrima* and *S. cerevisiae* in sequential fermentations of white wine reported similar population dynamics, with a significant decrease in *M. pulcherrima* following the sequential inoculation of *S. cerevisiae* [58, 72, 73].

Regarding sugar content, all batches followed a similar trend, although with varying consumption rates (Fig. 1).

In Batch C, where *S. cerevisiae* was used as a single starter, sugars decreased more rapidly in the first few days due to the greater fermentative vigor of this species compared to *M. pulcherrima*.

In Batch A and Batch B, sugars decreased slightly until the inoculation of *S. cerevisiae* T306. After the sequential inoculation of *S. cerevisiae*, the rate of sugar consumption increased, and all sugars were completely fermented within 10 days, similar to Batch C.

Comparable trends in sugar consumption rate have also been observed in previous studies [58], and other authors noted similar kinetics in weight loss, which have been correlated with sugar consumption and fermentation metabolism [41].

Therefore, our results show that *M. pulcherrima* AS3C1 did not negatively interfere with the fermentation kinetics of *S. cerevisiae* T306 during sequential fermentation. These findings confirm earlier studies suggesting that *M. pulcherrima*, despite its multiple antimicrobial activities, does not adversely affect the viability and metabolic activity of *S. cerevisiae* [50, 55, 74].

In other studies, simultaneous mixed inoculations of *S. cerevisiae* and *M. pulcherrima* did not allow sufficient development of *M. pulcherrima* [47], limiting its influence on fermentation. In contrast, the sequential inoculum allows *M. pulcherrima* to persist in the grape must, begin alcoholic fermentation slowly, and express its unique metabolic activities.

Main wine parameters

The values of the main physicochemical parameters of the wines are presented in Table 3. *S. cerevisiae* T306, both in co-culture and as a single starter, successfully completed alcoholic fermentation. The wines obtained from the three different batches showed no significant differences in the final alcohol content, with values ranging from approximately 12.2% to 12.3% vol. As indicated by the fermentation kinetics previously discussed, these results further confirm that the antimicrobial activity of *M. pulcherrima* does not negatively affect the growth or fermentative capacity of *S. cerevisiae* [40, 43, 55, 65].

Table 3 Oenological parameters of Falanghina wines

Physical–chemical parameters	Batch A	Batch B	Batch C
Acetaldehyde (mg/L)	20.90 ± 0.45^{a}	16.61 ± 1.80^{b}	24.96 ± 0.53^{a}
Acetic acid (mg/L)	45.00 ± 3.3^{b}	40.0 ± 2.40^{b}	280.02 ± 15^{a}
Alcohol (%vol.)	12.24 ± 0.2^{a}	12.27 ± 0.10^{a}	12.34 ± 0.20^{a}
Browning index (DO 420 nm)	0.12 ± 0.04^{a}	0.10 ± 0.05^{a}	0.13 ± 0.01^{a}
Catechins (mg/L)	9.00 ± 0.11^{b}	7.50 ± 0.20^{c}	10.50 ± 0.22^{a}
glycerol (g/L)	6.51 ± 0.2^{a}	6.50 ± 0.25^{a}	5.24 ± 0.30^{b}
L-lactic acid(g/L)	n.d.	n.d.	n.d.
L-malic acid (g/L)	2.54 ± 0.30^{a}	2.60 ± 0.22^{a}	2.53 ± 0.10^{a}
pH	3.51 ± 0.12^{a}	3.50 ± 0.14^{a}	3.54 ± 0.15^{a}
Residual sugars (g/L)	0.55 ± 0.10^{a}	0.40 ± 0.06^{b}	0.35 ± 0.03^{b}
*Total acidity (g/L)	5.97 ± 0.12^{a}	6.16 ± 0.15^{a}	6.20 ± 0.11^{a}

Batch A: Initial inoculation of *M. pulcherrima* AS3C1, followed by sequential inoculation of *S. cerevisiae* T306 after 2 days. Batch B: Initial inoculation of *M. pulcherrima* AS3C1, followed by sequential inoculation of *S. cerevisiae* T306 after 4 days. Batch C: Inoculation of *S. cerevisiae* T306 as a single starter.

n.d. not detected

Different letters in the same row indicate significant differences ($p < 0.05$). All values are expressed as the mean ± standard deviation of two biological replicates

Glycerol is the most abundant by-product of alcoholic fermentation. This triol is a non-volatile compound that does not directly contribute to wine aroma, but significantly enhances wine quality by providing sweetness, fullness, and smoothness [75]. The amount of glycerol typically produced by *S. cerevisiae* in wine ranges from 2 to 11 g/L [76].

In our study, the glycerol content was significantly higher in Batch A (6.51 g/L) and Batch B (6.50 g/L) compared to Batch C (5.2 g/L). Other studies have reported increased glycerol concentrations in mixed cultures involving *M. pulcherrima* and *S. cerevisiae* [23, 77–80].

However, complex interactions and substantial differences in the metabolic activity of *S. cerevisiae* have been observed when cultured alone versus in co-culture with non-*Saccharomyces* yeasts [74–81].

In a study by Sadoudi et al. [73] the increase in glycerol concentration in the presence of *M. pulcherrima* was attributed to the overexpression of the GPD1 gene in *S. cerevisiae*. This gene encodes glycerol-3-phosphate dehydrogenase, which is involved in the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate, an intermediate in glycerol formation. Additionally, [82] emphasized that *M. pulcherrima* can respire the dissolved oxygen supplied to the system, leading to a significant repression of the respiration pathway in *S. cerevisiae*. The depletion of available oxygen causes the modulation of glycerol-pyruvic fermentation, shifting *S. cerevisiae*'s metabolism toward a bypass of pyruvate dehydrogenase (PDH), resulting in an increase in glycerol production.

The acetic acid content differed in the wines obtained from Batch A (45 mg/L) and Batch B (40 mg/L) compared to Batch C (280 mg/L), confirming that *M. pulcherrima* produces low amounts of acetic acid [39, 43, 76, 77]. Other studies have also reported no significant differences in the acetic acid content between wines obtained from mixed fermentation of *M. pulcherrima* and *S. cerevisiae*, and those obtained from using *S. cerevisiae* as a single starter [36, 77, 83].

The Browning Index Absorbance (BIA) at 420 nm (A420), which is characteristic of the yellow absorption spectrum, measures the intensity of the yellow color of white wine and provides an indication of the browning index. This parameter allows monitoring of both the intensity and chromatic evolution of white wines over time. In general, except for specific wines or grape varieties, straw-colored wines typically have A420 values between 0.10 and 0.15 [84, 85].

The BIA values we measured showed no significant differences between wines, ranging from 0.10 (Batch B) to 0.13 (Batch C).

Flavanols, particularly catechins present in the skins of grapes, are among the first compounds to oxidize and significantly impact the color evolution and the browning reactions of white wines. Enzymatic browning in wine is a significant issue in oenology, caused by polyphenol oxidase enzymes, such as tyrosinase and laccase, which can oxidize phenolic compounds like hydroxycinnamic acids and catechins in the presence of oxygen. This oxidation forms o-quinones, which polymerize and interact with other compounds to create brown pigmented melanins, responsible for the enzymatic browning observed in white wines [86–89].

Catechins, widespread in the plant world [90], are the most common polyphenols in white wines (10–40 mg/L), are responsible for the bitterness, astringency, and color of the wine [91, 92]. In our vinifications, the catechin values ranged from 7.5 mg/L (Batch B) to 10.5 mg/L (Batch C), in agreement with the concentrations found in white wines [93].

Non-enzymatic oxidation is a primary factor influencing wine quality during bottling or aging [85]. This process occurs when polyphenols, oxygen, and transition metals are present simultaneously [94]. The presence of oxygen significantly impacts wine by generating hydrogen peroxide (H_2O_2) and quinones (formed by oxidizing polyphenols in the presence of iron). These compounds can further oxidize other substrates or form color compounds, thus affecting the color and aroma profiles of the wine [95, 96].

M. pulcherrima appears to have a substantial capacity for oxygen consumption [49, 97]. A reduction in oxygen in the grape must can inhibit the development of other microorganisms [98, 99] and significantly reduce both enzymatic and non-enzymatic oxidation processes that could occur in white grape must [51].

Regarding malic acid, our results showed that the use of *M. pulcherrima* AS3C1 as an initial starter did not affect the concentration of this compound initially observed in the grape must (2.7 g/L). Previous studies have found that some *M. pulcherrima* strains can partially metabolize malic acid in both single and sequential fermentation [26, 100]. However, Varela [23] reported that *M. pulcherrima* does not interfere with the production of malic acid by *Saccharomyces uvarum* in sequential inoculation.

Finally, regarding acetaldehyde levels in the final wines, Batch C showed a higher concentration of this undesirable compound, with a final concentration of about 25 mg/L, which remains below the sensory threshold of 100–125 mg/L [101]. In comparison, Batch A and Batch B had 20.09 mg/L and 16.6 mg/L of acetaldehyde, respectively. Therefore, a longer exposure of *M. pulcherrima* AS3C1 (4 days) in Batch B resulted in a lower concentration of acetaldehyde in the wine.

Acetaldehyde is primarily produced through the partial oxidation of ethanol by the enzyme alcohol dehydrogenase and negatively affects the flavor of wine [102]. Its significant reduction in sequential fermentations involving *M. pulcherrima* has been observed in other studies [58, 77]. Other papers report slight differences in acetaldehyde levels with no statistical significance [26, 76].

Acetaldehyde concentrations in wines typically range from 5 to 100 mg/L [101]. At low concentrations, it can enhance the fruity aroma and intensify the "green apple" and nutty notes. However, concentrations above 100 mg/L are considered a defect, as they can produce undesirable "rotten apple" flavors [101].

Volatile compounds

The raw data of our experimental results are presented in Supplementary Material, Table S1. A total of 41 aroma compounds were identified, belonging to various chemical classes, including 18 esters, 10 alcohols, 8 acids, and 5 terpenes. Many of these volatile compounds are commonly found in wines and are derived both from the grapes and from the yeast metabolism during fermentation [103, 104].

Esters

Esters are widely recognized as some of the most important contributors to the flavor and aroma of wine, imparting fruity and floral notes [105]. Aroma-active esters are formed in yeast cells during fermentation and can diffuse through the plasma membrane into the fermenting grape must. Chemically, esters are formed by a condensation reaction between acetyl/acyl-CoA and an alcohol [106].

Acetate esters are synthesized by the condensation of acetate (acetyl-CoA) with ethanol or high alcohols derived from amino acids through the Ehrlich pathway. In contrast, ethyl esters are produced via an enzyme-mediated esterification of ethanol with fatty acids (acyl-CoA). The amount of ester formation depends on the concentration of substrates and the activity of the enzymes involved in their synthesis and hydrolysis [1, 96, 107, 108].

Our results show that, while the quantities of esters varied across the wines from the three different batches, the most abundant acetate esters were: ethyl acetate, amyl acetate, hexyl acetate, and phenylethyl acetate. The predominant ethyl esters were: ethyl hexanoate, ethyl octanoate, and ethyl-9-decenoate. Based on the available literature, only amyl acetate, ethyl hexanoate, ethyl octanoate, and phenylethyl acetate exceeded their individual thresholds of perception in all three batches, making them particularly important in imparting floral and fruity notes to Falanghina

wines. Ethyl decanoate exceeded the threshold values only in wines from Batch B and Batch C.

The odor thresholds (µg/L) and odor descriptors of the detected volatile compounds are provided in Supplementary Material, Table S2 [109–115].

It seems well-established that the presence of different esters often has a synergistic effect, influencing the overall flavor profile even at concentrations well below their individual threshold values [116–118].

Regarding the ester concentrations, various studies have reported different results on the effect of *M. pulcherrima* on the ester production. Ruiz et al. [72], in their study on Verdejo wine production, found slightly lower ester concentrations after sequential inoculation of *M. pulcherrima* followed by *S. cerevisiae* after 15 g/L of sugar consumption, with the exception of 2-phenylethyl acetate. The highest levels of ethyl butanoate, ethyl hexanoate, and ethyl octanoate were associated with *S. cerevisiae*.

Benito et al. [78], using sterile grape must inoculated with *M. pulcherrima* Flavia® followed by *S. cerevisiae* EC1118 after 24 h, reported an increase in ethyl octanoate compared to fermentation with *S. cerevisiae* as a single starter.

On the other hand, Zhang et al. [119], in a study on Vidal Blanc wort, found that sequential inoculation with *M. pulcherrima* MP20 followed by *S. cerevisiae* SC45, after 2 or 4 days, led to a decrease in ethyl ester production compared to monoculture *S. cerevisiae*. However, co-inoculating *M. pulcherrima* MP20 with *S. cerevisiae* SC45 significantly improved the production of higher alcohols, including isobutanol, isopentanol, and 2-phenylethanol.

Canonico et al. [58], using Verdicchio grape juice, demonstrated that sequential inoculation with *M. pulcherrima* DiSVA 269 followed by *S. cerevisiae* DiSVA 708 after 48 h induced a significant increase in ethyl butyrate, ethyl hexanoate, and isoamyl acetate.

In a more recent study, [39] found that inoculating *S. cerevisiae* SRS1 48 h after *M. pulcherrima* GS80 in Pecorino grape must resulted in an increase in ethyl decanoate, ethyl octanoate, isoamyl acetate, and phenylethyl acetate.

Varela et al. [47] compared wines produced by *S. cerevisiae* AWRI1631 as a single starter and found that inoculating *S. cerevisiae* AWRI1631 after 50% sugar consumption by *M. pulcherrima* AWRI1149 led to wines with significantly higher concentrations of ethyl acetate, 2-methylpropyl acetate, and ethyl butanoate, along with lower concentrations of ethyl 2-methylpropanoate, 3-methylbutyl acetate, hexyl acetate, and ethyl decanoate.

These studies, together with the results from our own research, highlighted that the inoculation times and the specific strains of *M. pulcherrima* used are critical factors, due to their unique enzymatic properties, in determining the qualitative and quantitative differences in the volatile compounds released [44, 120].

Higher alcohols

Higher alcohols are produced by yeasts during fermentation, either from grape amino acids via the Ehrlich pathway or directly from sugars [121]. The α-keto acids, which are derived from sugars via pyruvate or from transamination/deamination of amino acids, are decarboxylated into the corresponding aldehyde which is then reduced to the corresponding alcohol by alcohol dehydrogenase [122].

These volatile compounds significantly influence the sensory profile of wine and are also important precursors for ester formation [1, 122]. Except for 2-phenylethanol (with a ‘rose-like’ odor), which is derived from phenylalanine, an excess of higher alcohols (>400 mg/L) could negatively affect wine quality [123].

In our study, the most abundant higher alcohols across all batches were benzeneethanol (phenylethyl alcohol), 3-methylbutanol (isoamyl alcohol), and 2-methylpropanol (isobutanol). However, the detected concentrations of these volatile compounds were well below their perception threshold values (Supplementary Material, Table S2). The presence and concentration of these alcohols in wine are influenced by various factors, including the yeast strain used during fermentation, grapevine cultivation methods, aeration, fermentation temperature, and the amino acid composition of the must [1].

Some studies suggest that when higher alcohols are present in mixtures, there can be a change in the overall perceived aromatic intensity compared to the intensity of each individual component, due to perceptual interactions or synergistic effects [123–125].

Several studies have reported a decrease in the total amount of higher alcohols in wines fermented with *M. pulcherrima* in sequential fermentations. Notably, Ruiz et al. [72] observed lower concentrations of all higher alcohols, except for 2-phenylethanol, in sequential fermentations of Verdejo grape must involving *M. pulcherrima* NS-EM-34.

Perpetuini et al. [39] found that Pecorino wine, produced by sequential inoculation of *M. pulcherrima* GS80 followed by *S. cerevisiae* SRS1, reduced isoamyl alcohol content and increased phenethyl alcohol content. Conversely, Valera et al. [47], in his study with *S. cerevisiae* AWRI1631 inoculated after 50% sugar consumption by *M. pulcherrima* AWRI1149, reported significantly higher concentrations of 2-methylpropanol in sequential fermentation [47].

Other studies have highlighted increased concentrations of higher alcohols in co-fermentations with *M. pulcherrima* [39, 50, 65]. The different results observed in these studies are attributed to the complex interactions between yeasts

used in sequential inoculations, which influence both the availability of amino acids and sugars in the initial must and during fermentation [126, 127].

Fatty Acids

Yeasts are the primary producers of fatty acids, which serve as the initial substrates for the final formation of ethyl esters [107]. Some fatty acids in wine originate from the grape skins, but the majority are biosynthesized from acetyl-CoA during the early stages of alcoholic fermentation, as they are released by the yeasts. However, they can also be found in low concentrations in the original composition of the must before fermentation. The formation of volatile fatty acids during fermentation is relatively low in quantity and depends on the yeast species, strains, and fermentation conditions [128, 129].

Volatile fatty acids can enhance the complexity of wine at sub-sensory threshold levels, but they may negatively affect the aroma of wine when their concentrations exceed the thresholds, imparting undesirable notes of wine, cheese, fat, or rancidity [104, 130].

In our vinifications, seven fatty acids were identified, including butanoic, hexanoic, octanoic, nonanoic, decanoic, 9-decenoic, and dodecanoic. Except for the butanoic acid that only in the Batch A reached levels over the threshold values, in all the other batches the concentrations of the fatty acids were below the perception threshold values (Supplementary Material, Table S2). Although C6–C10 fatty acids (hexanoic, octanoic, nonanoic, and decanoic) are typically associated with negative odors, they are crucial for the aromatic equilibrium in wine, as they counteract the hydrolysis of the corresponding esters [131].

Terpenes

Previous studies have confirmed that terpenes, particularly monoterpenoids, are important compounds that contribute to the rich flavors and varietal characteristics of wines [130, 132]. Most of these monoterpenoids exist in their flavorless glycoside-bound form and are produced or released upon hydrolysis (acidolysis and enzymolysis) during the wine fermentation process, imparting the floral and fruity notes to wine [63, 133].

The extracellular hydrolytic enzymes produced by yeast strains include β-D-glucosidase, α-L-rhamnosidases, α-L-arabinofuranosidases, and β-D-xylosidases. Some studies have shown that β-D-glucosidase is one of the most important hydrolases, enhancing the aroma quality of wines [133–135].

In our study, the terpenes detected in the three batches included: limonene, linalool, β-citronellol, and nerolidol. Additionally, geranyl acetate, a derivative of the acetate ester of geraniol, was detected as a monoterpenoid. Quantitatively, only linalool exceeded the threshold value (15 µg/L) in all three batches. However, some studies suggest that synergistic or additive effects among aroma compounds, which result in lowered perception thresholds, make difficult the prediction of the sensory consequences [117, 136, 137].

Our results showed a higher total amount of terpenes in Batch A (65.6 ± 12.4 µg/L) and Batch B (63.6 ± 11.1 µg/L), where *M. pulcherrima* AS3C1 was used as the initial starter, compared to Batch C (38.2 ± 7.2 µg/L). These results are consistent with previous studies that demonstrated an increase in total terpene concentration at the end of sequential fermentations involving *M. pulcherrima* strains, compared to a single fermentation with *S. cerevisiae*. This increase is expected to be caused by the enzymatic activity (α-arabinofuranosidase and β-glucosidase) of this non-*Saccharomyces* yeast [39–41, 65, 138]. In this regard, *M. pulcherrima* AS3C1 has also been selected and used in previous studies for its enzymatic activities, including β-glucosidase [44].

Indeed, terpenes, which predominantly originate from grapes, are released and modulated by microbial activity, although de novo synthesis has been reported for both *S. cerevisiae* and non-*Saccharomyces* yeasts [7].

Multivariate statistical analysis

The relationships between the volatile compounds and the wines obtained from the three different batches were explored through multivariate statistical analysis using Principal Component Analysis (PCA) and Sparse Partial Least-Squares Discriminant Analysis (sPLS-DA).

The results from PCA showed that the first two principal components (Dim 1 and Dim 2) accounted for 65.4% of the total variance, with approximately 39% attributed to the first component (Fig. 2A). Specifically, Dim 1 differentiated Batch C from Batches A and B, while Dim 2 separated Batch A from replicate 2 of Batch B (B2a, B2b, B2c), but not from replicate 1 of Batch B (B1a, B1b, B1c). The main contributions to the two principal components were influenced by samples from Batch C1 (~47% on Dim 1) and from Batch B2 (~63% on Dim 2) (Fig. 2B, Supplementary Material, Table S3).

It was observed that the first component contrasted five volatile compounds from the others, specifically one acid (A1, Acetic acid), one alcohol (Al4, 1-Hexanol), and three esters (E3, Ethyl butyrate; E6, Hexyl acetate; E14, Ethyl decanoate). The second component contrasted 22 variables (7 acids, 8 esters, 4 alcohols, and 3 terpenes) positively correlated with Dim 2, from the remaining 19 variables (1 acid,

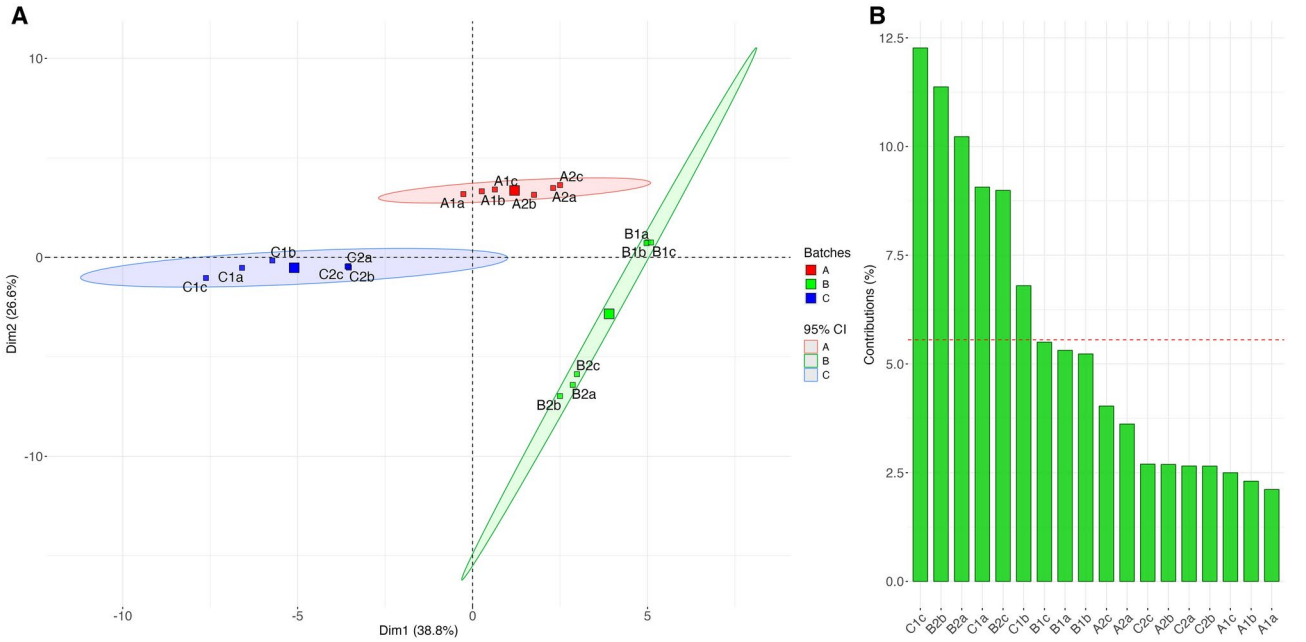

Fig. 2 Principal Component Analysis (PCA) showing: **A** the projection of wine samples along the first principal component (Dim 1) and the second principal component (Dim 2). **B** The percentage contribution of each wine sample to the first two principal components. The capital letters represent the Batches, the numbers indicate the biological replicate, and the lowercase letters represent the technical replicate. Ellipses indicate the 95% confidence interval (95% CI), with the large square at the center of the ellipse representing the mean value. The dashed red line in panel B indicates the expected average contribution of the batches

10 esters, 6 alcohols, and 2 terpenes) that were negatively correlated with Dim 2. Additionally, out of the 41 volatile compounds detected in the wines, 25 (5 acids, 9 esters, 8 alcohols, and 3 terpenes) were primarily responsible for the variation observed (Fig. 3A-B, Supplementary Material, Table S4).

Subsequently, the sPLS-DA method was used for discriminant analysis to identify the VOCs responsible for the differences between the wines obtained in the three batches. The results showed that the batches were well separated along the two main components (Comp1 and Comp2), accounting for a total variance of 58% (Fig. 4A). Component 1 separates Batch B from Batches A and C, while Component 2 separates Batch A from the other two batches. The samples from Batches B1 and B2 were more tightly clustered compared to the PCA results (Fig. 2). Of the 41 volatile compounds detected (Supplementary Material, Table S1), sPLS-DA revealed that 10 of them (Al2, Al9, E18, A1, E14, T3, E3, A3, A4, E1) contributed to the differences between the batches. Specifically, the compounds Al2, Al9, E18, and E1 were positively correlated with Component 1, while A1 was negatively correlated with this component (Fig. 4B). The compounds T3, A3, and A4 were positively correlated with Component 2, whereas the esters E14 and E3 were negatively correlated with Component 2 (Fig. 4C).

Moreover, as clearly reported in the Fig. 5, the wine belonging to the Batch A was associated to geranyl acetate (T3), hexanoic acids (A3) and octanoic acid (A4), which showed higher values compared to those detected in batches B and C (Table 4).

The wine from Batch B was characterized by two alcohols (benzeneethanol, Al9, and 2-methyl propanol, Al2) and two esters (ethyl acetate, E1, and phenylethyl acetate, E18). The wine samples from Batch C showed the highest concentrations of acetic acid (A1) and a high level of ethyl butyrate (E3) and ethyl decanoate (E14) (Fig. 5, Table 4). However, the last two compounds were also present in high amount in Batch B, although they were not distinctive in this case.

It is evident that the different sequential inoculation times led to distinct volatile profiles in the wines from Batches A and B. In detail, when *M. pulcherrima* AS3C1 was added to the wine on the 4th day of fermentation (Batch B), a significant higher concentrations of ethyl acetate, phenylethyl acetate, benzeneethanol, and 2-methyl propanol was observed compared to the wines in which *M. pulcherrima* AS3C1 was added on the 2nd day (Batch A).

The higher amounts of these compounds in batch B are probably due to a longer persistence of *M. pulcherrima* AS3C1, which was able to express its metabolism more widely prior to inoculation with *S. cerevisiae.*

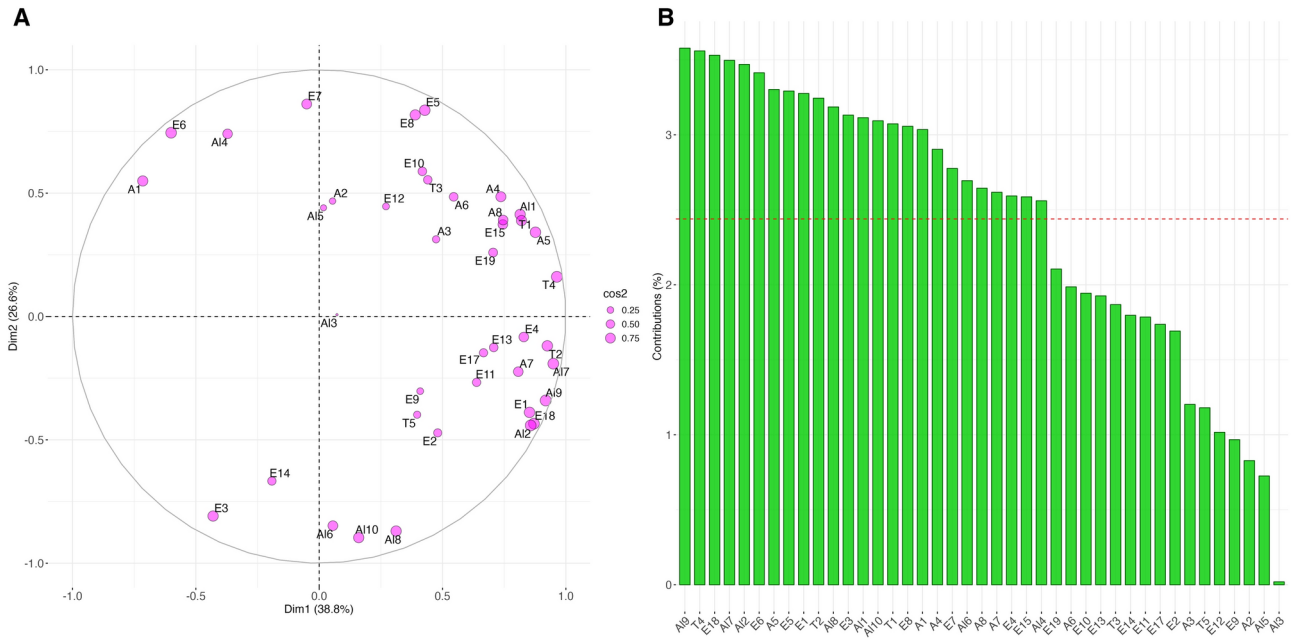

Fig. 3 Principal Component Analysis (PCA) showing: **A** the projection of wine volatile compounds along the first principal component (Dim 1) and the second principal component (Dim 2). **B** The percentage contribution of each wine volatile compound to the first two principal components. The size of the square cosines (cos^2) in panel A indicates the importance of each component for the corresponding volatile compound. The dashed red line in panel B represents the expected average contribution of the volatile compounds

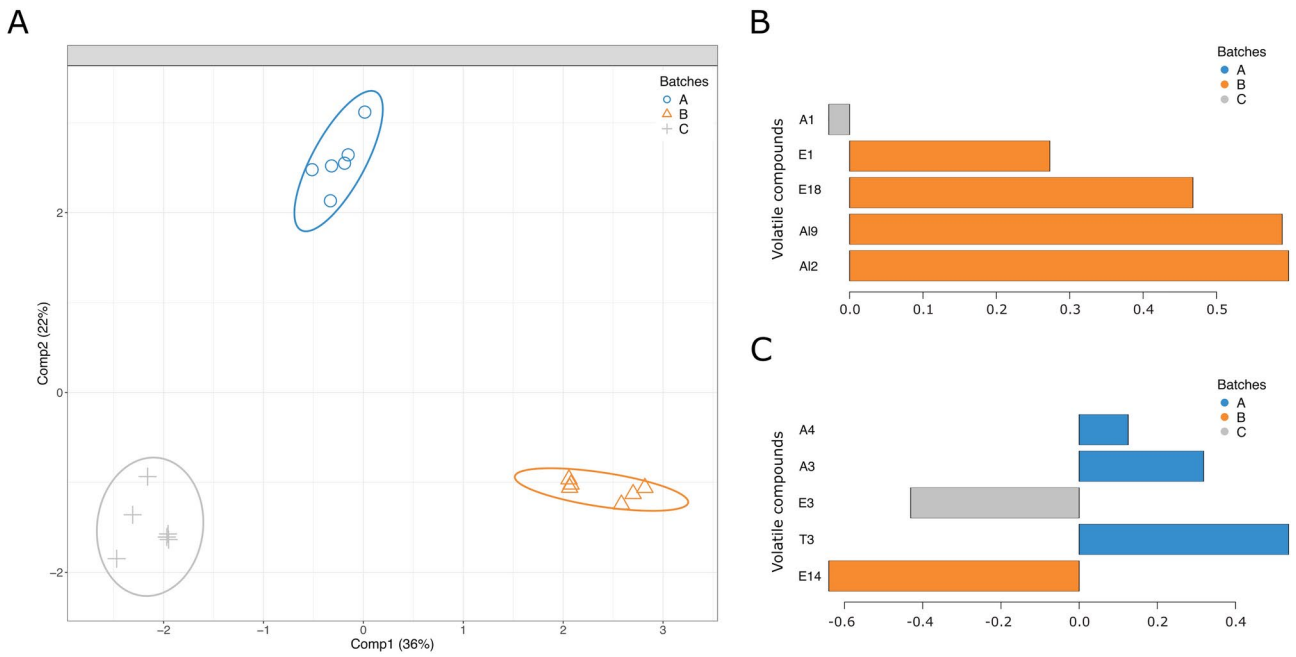

Fig. 4 Plots showing **A** the sparse partial least-squares discriminant analysis (sPLS-DA) of volatile compounds in different batches at the end of the winemaking process. The ellipse represents the 95% confidence level. **B** Volatile compounds driving differences along Component 1. **C** Volatile compounds driving differences along Component 2. The length of the bars indicates the loading coefficient, ranked by importance from bottom to top. The color of the bars indicates the group in which each volatile compound has the highest median abundance

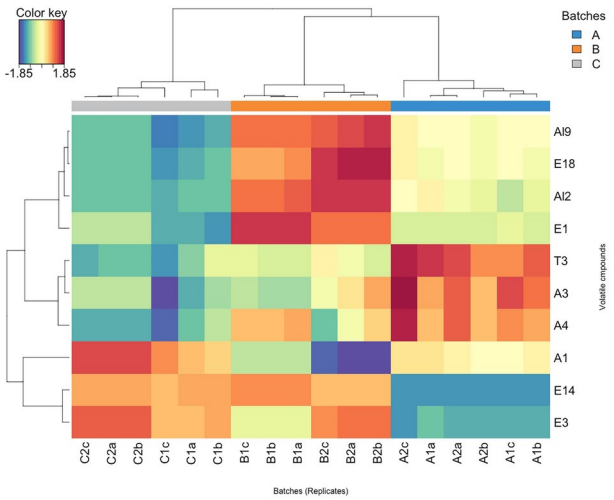

Fig. 5 Clustered Image Map (CIM) of the 10 volatile organic compounds (VOCs) selected by sPLS-DA from the 41 volatile compounds dataset across both principal components

Sensorial analysis

The results of the sensory analysis are presented in Figs. 6 and 7. Regarding the olfactory descriptors (Fig. 6A), a significant difference was observed among the three batches only for the spicy descriptor. Specifically, the wine from Batch A showed a very low score compared to Batches B and C, where the score values were 4 and 4.5, respectively. No significant differences ($p > 0.05$) were found for the other descriptors (apple, fresh fruit, hazelnut, vanilla, and vegetal).

For the taste descriptors (Fig. 6B), the median values were similar ($p > 0.05$) between groups, except for the "bitterness" and "acidity" descriptors, where significantly lower values were observed for Batch C compared to Batch A. Additional differences were found between the batches for the fruity and apple descriptors of retro-nasal olfaction (Fig. 6C). Specifically, the score for the apple attribute was higher in Batch A compared to Batches B and C. In contrast, the fruity descriptor received a higher score in Batch B compared to Batch C, with no significant differences observed between Batches A and C.

These results could be explained by the significant amounts of ethyl acetate, phenylethyl acetate, benzeneethanol, and 2-methyl propanol detected in Batch B, as these compounds are often associated with fruity and floral aromas (Supplementary Material, Table S2).

Regarding the color intensity descriptor (Fig. 7A), the wine from batch B showed a lower score (median value of 6.0) compared to the wines from batches A and C. In contrast, the wines from batches A and B received higher overall acceptance.

Conclusions

Although numerous studies have proposed the use of *M. pulcherrima* in co-culture with *S. cerevisiae* in winemaking, it is clear that further techno-scientific investigations are necessary both to better understand the interactions between the strains used and to optimize the inoculation times. The complexity of the biochemical reactions during fermentation is influenced by the enzymatic activities of the yeasts and the interactions among the compounds they produce, which ultimately determine the volatile content and the sensory profiles of the wine. Our results demonstrated that different inoculation times contributed to the distinctive characterization of the wines produced. Therefore, while the use of multiple starter cultures represents an interesting biotechnological innovation, further studies are needed to optimize application protocols to achieve more predictable and reproducible results.

Table 4 Absolute quantities of the volatile organic compounds (VOCs) responsible for the diversity observed across the three batches

Compounds	ID	Batch A1	Batch A2	Batch B1	Batch B2	Batch C1	Batch C2
Ethyl acetate	E1	754.5±17.9	750.0±8.3	1332.0±7.1	1158.4±8.3	590.1±10.1	721.2±6.0
Ethyl butyrate	E3	35.7±0.9	34.3±2.1	52.1±0.1	96.8±1.9	80.8±2.8	104.3±0.8
Ethyl decanoate	E14	5.2±0.1	6.1±0.1	4179.6±10.6	1876.2±135.8	2639.1±166.0	2897.9±0.5
Phenylethyl acetate	E18	624.7±48.8	701.3±59.7	1247.8±13.5	2203.0±74.3	276.9±23.4	296.4±1.2
2-Methyl propanol	Al2	143.4±39.6	155.1±12.3	406.0±1.4	516.2±2.6	74.7±3.2	75.4±1.0
Benzeneethanol	Al9	942.9±1.5	989.5±83.9	1917.9±5.0	2424.7±261.6	392.4±36.6	460.3±0.1
Acetic acid	A1	40.9±3.5	40.8±3.4	24.9±0.1	12.6±0.7	54.3±6.5	85.0±0.1
Hexanoic acid	A3	188.9±9.4	196.2±9.9	133.3±1.2	164.2±16.3	119.9±12.4	136.7±1.3
Octanoic acid	A4	1173.7±28.3	1267.4±127.1	1140.3±11.5	977.3±143.1	814.2±83.0	800.6±0.0
Geranyl acetate	T3	9.5±1.0	9.7±1.1	5.4±0.1	5.9±0.5	4.7±0.9	4.2±0.1

Data are presented as the mean±standard deviation of three technical replicates for each biological replicate

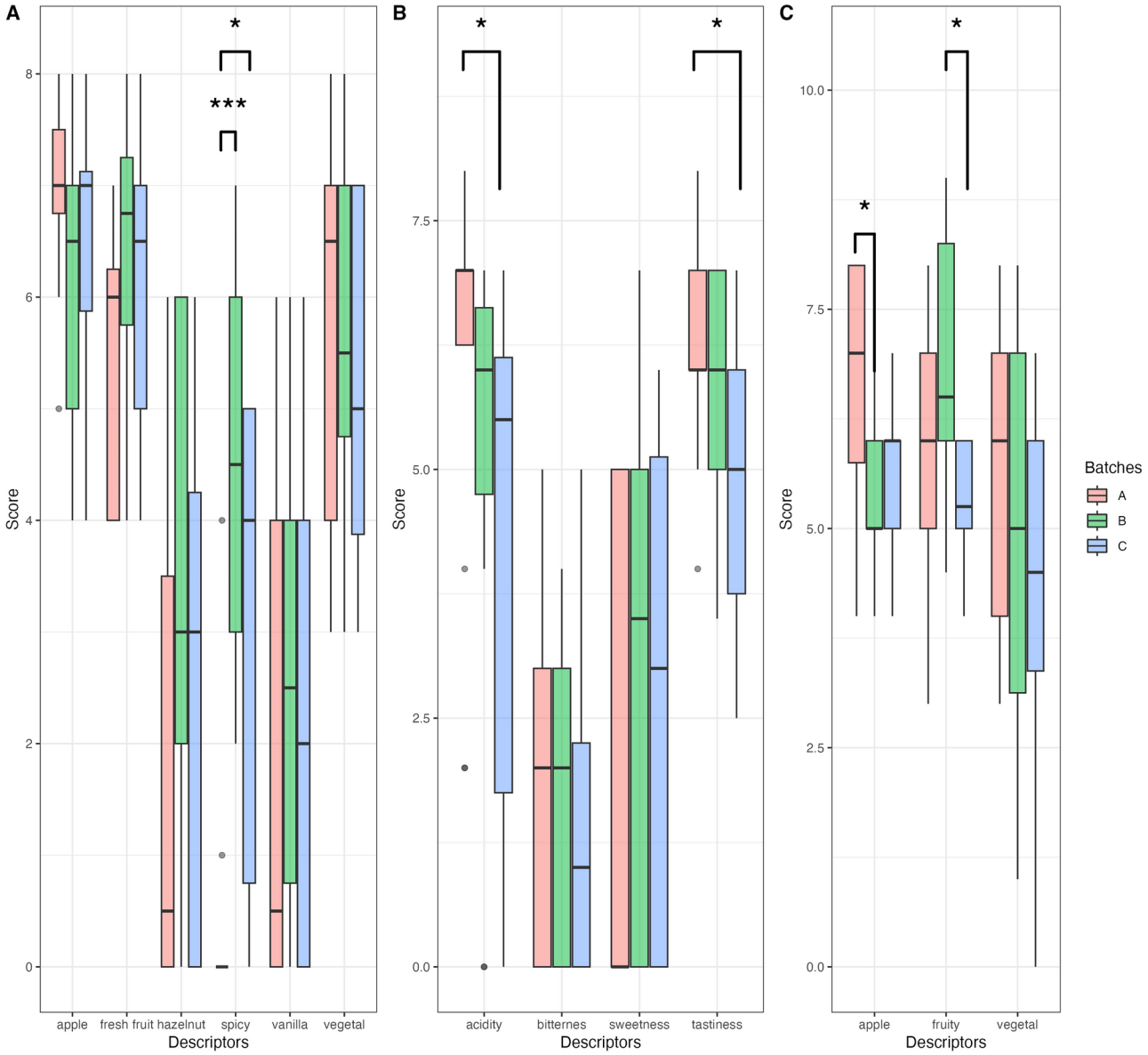

Fig. 6 Boxplot showing the scores of the sensory analysis for the three wine batches regarding **A** olfactory attributes, **B** taste attributes, and **C** retro-nasal attributes. The Kruskal–Wallis test was used to assess significant differences between batches, with the Dunn test used as a post-hoc analysis. Asterisks indicate the following levels of statistical significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

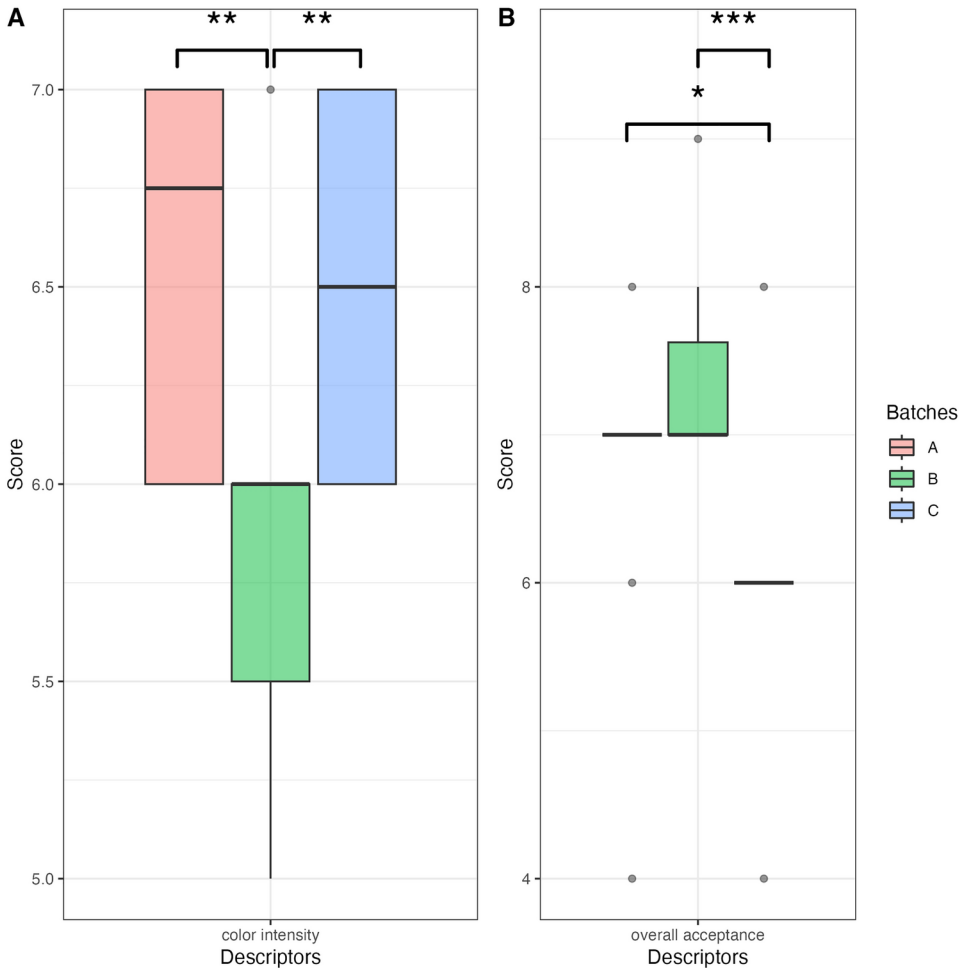

Fig. 7 Boxplot showing the scores from the sensory analysis of the three wine batches regarding **A** color intensity and **B** overall acceptance attributes. The Kruskal–Wallis test was used to calculate significant differences between batches, with the Dunn test applied as the post-hoc test. Asterisk symbols indicate the following levels of statistical significance: *$p<0.05$; **$p<0.01$; ***$p<0.001$

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Author contributions The study was conceptualized and designed by I.M. and S.M. Material preparation, data collection and analysis were performed by C.F., T.B., C.R., K.Y., D.R.M., M.C., and P.G.. The first draft of the manuscript was written by C.F., I.M., S.M., and P.G.. All authors commented on and edited earlier versions of the manuscript and approved the final manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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