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Technological application of autochthonous *Meyerozyma guilliermndii* cultures in Chardonnay

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Abstract

Using Chardonnay grape, the fermentation characteristics of sequential inoculation with *M. guilliermondii* and *S. cerevisiae* in the pilot fermentation process of dry white wine were examined. In this study, the physical and chemical indexes, color indexes, volatile aroma compound composition, and sensory indexes of 2 tons of samples at the end of alcoholic fermentation (AF) and malolactic fermentation (MLF) were analyzed. The results showed that the *M. guilliermondii* biomass in the treatment group (inoculated *M. guilliermondii* and *S. cerevisiae sequentially*) was always higher than 10^6 CFU/mL during AF, and the basic physicochemical indexes of samples met the requirements of the national standard GB/T15038-2006 (Wine). Also *M. guilliermondii* NM218 can significantly increase the color saturation of Chardonnay white wine. Regarding aromatic characteristics, the total alcohol, ester, and terpene contents of wine samples after mixed fermentation were higher than those of control group (only inoculated *S. cerevisiae*). Compared with control wine samples (only with *S. cerevisiae*), the treatment group had significantly increased ethyl caprylate, ethyl nonanoate, phenethyl acetate, and ethyl laurate contents, including *n*-heptanol, which can provide Chardonnay dry white wine a richer fruity fragrance. meanwhile, the sensory scores of wine samples were higher in the treatment group. In conclusion, mixed fermentation could boost the aroma quality and sensory pleasure of dry white wine, with the potential for industrial application.

Keywords *Meyerozyma guilliermondii*, Dry white wine, Color, Aroma, Pilot production

Introduction

Nowadays, the winemaking industry almost completely relies on commercial fermentation agent inoculation; although this treatment can guarantee successful alcoholic fermentation (AF), it could lead to the decline in fermentation yeast flora diversity, yeast metabolism of chemical reaction complexity, and inhibit the growth of beneficial microbial metabolism, thereby leading to a monotonous wine style and flavor homogeneity issues. The key microorganisms in wine fermentation are non-*Saccharomyces cerevisiae* (*S. cerevisiae*) strains with excellent characteristics, which can metabolize and produce glycosidase, esterase, protease, glycerol, mannoprotein, and other substances; boost the flavor and taste of wine; and enhance the diversity and typicality of wine [1–2].

Pichia yeast is commonly detected in the natural fermentation process of wine, and it is one of the essential ester-producing yeasts, including phenylethyl acetate and ethyl acetate. Moreover, *Pichia* yeast can potentially increase fragrance [3–5]. *Meyerozyma guilliermondii* (*M.*

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guilliermondii) as a species in the *Pichia* genus, has the characteristics of high-yield glycosidase in *Pichia* strain, tolerance to high glucose and ethanol concentrations, and is categorized as flavor yeast in wine fermentation [6–7]. Aplin et al. [8] showed that Merlot fermentation by sequential inoculation of *M. guilliermondii* (P40D002) and *S. cerevisiae* produced high concentrations of ethyl acetate and other aromatic substances and reduced the ethanol content of wine. Yan et al. [9] also reported that *M. guilliermondii* could produce high concentrations of 2-phenylethanol, enhancing rose and honey's aroma. A high β-glucosidase activity in *M. guilliermondii* GXDK6 increases the terpene concentration by releasing the glycoside conjugate, thereby increasing the fragrance and fruity fragrance intensity [10].

In recent years, to solve the problem of wine homogenization and highlight the regional characteristics, the development and application of local non-*S. cerevisiae* strains has become a research hotspot [1–2]. The *M. guilliermondii* NM218 strain applied in this trial was isolated from naturally fermented grape must in the Hexi Corridor of China, with a high yield of β-glucosidase viability under low acid conditions [11]. In the laboratory-scale microfermentation trial, under the sequential inoculation and fermentation of *S. cerevisiae*, adding the β-glucosidase to the *M. guilliermondii* NM218 strain could increase terpene, C13-norisoprenoid, higher alcohol, ester, and fatty acid concentrations, thereby enriching the aroma complexity of wine [12]. However, its performance on an industrial scale remains unclear. Therefore, to clarify the potential of the *M. guilliermondii* NM218 strain in the industrial production of dry white wine, this study investigated the effects of the *M. guilliermondii* NM218 strain on chemical indexes, color indexes, volatile compounds, and sensory characteristics of Chardonnay dry white wine at a pilot scale and provided data reference for the industrial application of *M. guilliermondii*.

Materials and methods

Yeast agent and medium

The *M. guilliermondii* NM218 strain was screened in Gansu Key Laboratory of Viticulture and Enology (Gansu, China), with 26 S rDNA D1/D2 sequencing, identified as *M. guilliermondii* yeast, and stored in China General Microbiological Culture Collection Center (CGMCC NO: 23155). *M. guilliermondii* NM218 bioactive dry powder was prepared by our team. Commercial wine yeast (*S. cerevisiae* CX9) bioactive dry powder was purchased from LAFFORT (Bordeaux, France).

WL (Wallerstein) nutrient agar medium was purchases from Beijing Auboxing Biotechnology Co., Ltd (Beijing, China).

Raw material

Chardonnay grape, which was harvested in September 2021 at the east foot of Helan Mountain Bona Baifu winery, had a sugar content of 226 g/L (by reducing sugar), a titratable acid of 6.75 g/L (by tartaric acid), and a pH value of 3.57.

Instruments and equipment

The instruments used included TU-1080 ultraviolet-visible spectrophotometer (Beijing General Instrument Co., Ltd., Beijing, China), WINESCAN S20 FLEX multi-function wine analyzer (Fox Beijing Science and Trade Co., Ltd., Beijing, China), H2050R high-speed refrigerated centrifuge (Changsha Xiangyi Centrifuge Instrument Co., Ltd., Changsha, China).

The reagents used included sodium chloride (analytical pure, 99%) and absolute ethanol (analytical pure) (Tianjin Guangfu Technology Co., Ltd.), pectinase (food grade) (LAFFORT, France), 2-octanol (chromatographic pure, 99%) (Sigma-Aldrich, Shanghai, Trading Co., Ltd.).

Pilot fermentation trial

After fruit sorting, Chardonnay grape was sent to destemmer-crusher into 2 t stainless steel temperature-control tank. The tank was filled up to approximately 70% of its capacity. Subsequently, 40 mg/L of pectinase (ABF Ingredients company, Hampton, Britain) was added. Potassium metabisulfite (Shouguang Nuomeng Chemical Co., Ltd., Shandong, China) was added in batches to 70 mg/L, the must was stirred well, and macerated at a low temperature (10 °C) for 24 h, and returned to room temperature (20 ± 2 ℃) for yeast inoculation, trial as follows:

The yeast bioactive dry powder was inoculated at 200 mg/L, followed by warm water activation, and expanded incubation in 50 L of grape must for 24 h. The treatment group was initially inoculated with *M. guilliermondii* NM218 and subsequently with *S. cerevisiae* CX9 after 48 h; only *S. cerevisiae* CX9 was inoculated in the control group, with 18–20 °C temperature-control fermentation. A specific gravity (SG) of approximately 1.058 was regarded as the middle stage of AF, approximately 1.015 as the final stage of AF, and 0.993–0.996 as the end of AF. During fermentation, yeast growth was monitored at 12 h, 48 h, middle stage, and final stage after inoculation with strain *M. guilliermondii* NM218. After AF, the wine sample was racked, and 1 g/T of activated lactic bacteria solution was added to initiate malolactic fermentation (MLF), temperature was controlled at 18–20 °C, aged, and stored at 15 °C following MLF. Whereafter, the wine was sampled at the end of AF and MLF, respectively, control group was recorded as CK-AF and CK-MLF, and the treatment group as NM-AF and NM-MLF. Each group's trial was repeated three times.

Analysis of relevant indicators

Specific gravity monitoring

To determine the specific gravity (SG) of the wine, a hydrometer (Guangdong Hongtuo Instrument Technology Co., LTD, Guangdong, China) was used in this study [11].

Yeast growth dynamics monitoring

Wine samples were diluted at appropriate concentrations using plate counting and spread on WL medium. Colonies were counted after 72 h of incubation at 28 °C. *M. guilliermondii* NM218 colonies on the WL medium were characterized by round milky-white dots, whereas *white and light green colonies characterized S. cerevisiae*. The morphological difference of the middle bulge was counted separately.

Determination of common physical and chemical parameters

Ethanol, total acidity, volatile acidity, residual sugar, glycerol, and malic acid in the trial wine samples were measured using a multifunctional wine analyzer (Foss Instrument Co., LTD) [13].

Color parameter determination

CIELab color parameter determination The wine samples to be examined were filtered through a 0.45-μm filter membrane, distilled water as blank, 2-mm light path quartz cuvette, and continuously scanned at 380–780 nm spectral segments at 5-nm intervals using a UV spectrophotometer. According to the SN/T 4675.25–2016 [14], even color space and color difference formula, calculate the color parameters (L*, a*, b*, C*ab, and hab) of CIE 1976 (L * a * b *) for color space when observed at D65 and 10°.

CIELab color parameters were calculated as follows:

$$L^* = 116 \times \ (Y/Y_0)^{1/3} - 16 \tag{1}$$

$$a^* = 500 \times \ [(X/X_0)^{1/3} - (Y/Y_0)^{1/3}] \tag{2}$$

$$b^* = 200 \times \ [(Y/Y_0)^{1/3} - (Z/Z_0))^{1/3}] \tag{3}$$

$$C^*{}_{ab} = [(a^*)^2 + (b^*)^2]^{1/2} \tag{4}$$

$$h^*{}_{ab} = arctan\,(b^*/a^*) \tag{5}$$

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \tag{6}$$

In the abovementioned formulas, X_0 (94.825), Y_0 (100.00), and Z_0 (107.381) are the standard white light three stimulation values, and X, Y, and Z are the sample three stimulation values.

Total phenols 0.5 mL of wine sample was diluted 10 times with 10% ethanol solution (Tianjin Guangfu Technology Co., Ltd, Tianjin, China). Next, take0.25 mL of the diluted wine sample was mixed 0.25 mL of 0.1% HCl–95% ethanol and 4.5 mL of 2% hydrochloric acid solution, and allowed to stand at 25 ℃ temperature for 15 min. Sample absorbance was determined at 280 nm, the 10-mm cuvettes were used. The total phenol was calculated from the standard curve established in the dilution of 10% ethanol gallic acid (Beijing Solaibao Technology Co., LTD., Beijing, China) at each wavelength [15].

Browning index Browning index was performed according to the method by Pati et al. [16] with slight modifications. After filtering the wine sample through a 0.45-μm filter, the absorbance value was measured at 420 nm using the 1-cm cuvettes, and the absorbance value at this wavelength was used as the benchmark for browning index of the wine sample.

Volatile aroma compounds determination

Referring to the method of Gao et al. [17], the aroma compounds in wine were determined using a headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS, ThermoTRACE-1310 ISQ, Seattle, Washington).

The following were the solid-phase microextraction (SPME) conditions: take 8 mL of the wine sample in a headspace bottle (20 mL) filled with 2.5 g of NaCl, 10 μL of internal standard 2-octanol (1,025.86 μg/L), added magnetic stirring rotor and sealed in 40 ℃ water bath equilibrium for 30 min, followed by headspace extraction under the same equilibrium conditions for 30 min using DVB/CAR/PDMS fibres equipped with a manual holder (50/30 μm, Supelco, Bellefonte, PA, United States). Subsequent desorption in a gas chromatography injector at 240 ℃ for 5 min.

The following were the GC conditions: DB-WAX column (60 m×2.5 mm×0.25 m); heating procedure, 40 °C for 5 min, up to 180 °C at 3.5 °C/min for 15 min; carrier gas (He) flow rate, 1 mL/min; no shunt injection. MS conditions: electronic ionization source; electronic energy, 70 eV; inlet temperature, 240 °C; transmission line temperature, 180 °C; ion source temperature, 250 °C; and mass scan range, approximately 50–350 m/z.

The NIST spectrum database was used to search for qualitative and quantitative analyses, and the relative retention index (RI) was used. RI is calculated according to components' retention time and normal alkane retention time (C6-C22) under the same chromatogram conditions. Compared with NIST and Wiley mass

spectrometry library RI, components with absolute difference less than 50 can be characterized as this compound. The internal standard (2-octanol) was used for semi-quantification.

$$X = \frac{A_1 \times C}{A} \times f \quad (7)$$

Where X is the mass concentration of the aroma substance (μg/L); A_1 is the measured peak area of the aroma substance; f is the correction factor of the internal standard, f = 1; C is the mass concentration of the internal standard(μg/L); and A is the peak area of the internal standard.

Table 1 Criteria for sensory evaluation of wine

Project		Full marks	Code of points
Appear-ance	Color	10	Dry white: golden (0 – 3 points); pale lemon (4 – 6 points); lemon (7 – 9 points)
	Clarity	10	Defect (0 – 3 points); turbidity and precipitation (4 – 6 points); clear and transparent (7 – 9 points)
Nose	Fragrance	10	No fragrance or weak (0 – 3 points); medium but obvious (4 – 6 points); pure and intense fragrance (7 – 9 points)
	Fruity	10	No fruity aroma or weak aroma (0 – 3 points); medium but obvious (4 – 6 points); pure and intense fruity (7 – 9 points)
	Odor	10	No oxidation and sour odor (0 – 3 points); weak odor (0 – 3 points); obvious odor (0 – 3 points)
Palate	Acidity	10	No acidity (0–3 points); low acidity and plain taste (4 – 6 points); moderate acidity and balance in cavity (7–9 points)
	Sweet–sour balance	10	Poor balance between sweet and acid (1 – 3 points); acceptable balance between sweet and acid (4 – 6 points); good balance between sweet and acid, wine coordination (7 – 9 points)
	Aftertaste	10	Short aftertaste (0 – 3 points); medium aftertaste (4 – 6 points); long and good aftertaste (7 – 9 points)
Overall score		10	The wine is light, has poor taste, insufficient aroma, and short aftertaste (0 – 3 points); full bodied, complex, obvious aroma, good taste, and medium aftertaste (4 – 6 points); round and harmonious body, typical, rich, comfortable aroma, long, and good aftertaste (7 – 9 points)

Odor activity value (OAV)

The OAV is the ratio of the content of the aroma substance to the odor threshold. To assess the overall aroma of the wine, the odor descriptors were divided into different aromatic series, and each compound was divided into an aromatic series on the basis of the main odor descriptors. The total intensity of each aromatic series was calculated as the ΣOAV for each compound in the series. The odor series used in this trial was presented referring to the methods described by Roldán et al. [18], which was divided into fruity, sweet, fatty, floral, grass, spicy, earthy, mushroom, and chemical flavors.

Sensory analysis

Sensory testing was performed in a sensory laboratory that adheres to international standards for sensory analysis. The protocol used for data collection complied with national ethical requirements and were implemented in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) on experiments involving humans. All panelists provided written or verbal informed consent, were not coerced to participate, were given full disclosure of the study requirements and risks, and there was no release of participant data without their knowledge.

The sensory evaluation table is made in accordance with national standard GB/T15038-2006 [19]. The sensory laboratory is practiced according to national standard GB/T 13,868 – 2009 [20]. Referring to the methods by Zhao et al. [21], the test wine was assessed by a tasting team of professional tasters (10 females and 10 males) with wine-tasting certificates. Wine samples were evaluated based on ten aspects, including color, clarity, fragrance, fruit, odor, acidity, wine body, aftertaste, sweet and sour balance, and overall score; the 10-point structure value was quantified as the standard. The sensory scoring criteria are shown in Table 1.

Data processing and analysis

The data for physicochemical parameters and volatile aroma compounds were organized and processed using Excel (Version 2016 Microsoft, Washington, United States) and Origin (Version 2021 Origin Lab, Massachusetts, United States). Statistical analysis of data was performed using one-way ANOVA and Duncan's multiple comparisons through SPSS (Version 26.0 IBM, Chicago, United States). The principal component analysis (PCA) was plotted using SIMCA software (SartoriusAG, Gottingen, Germany).

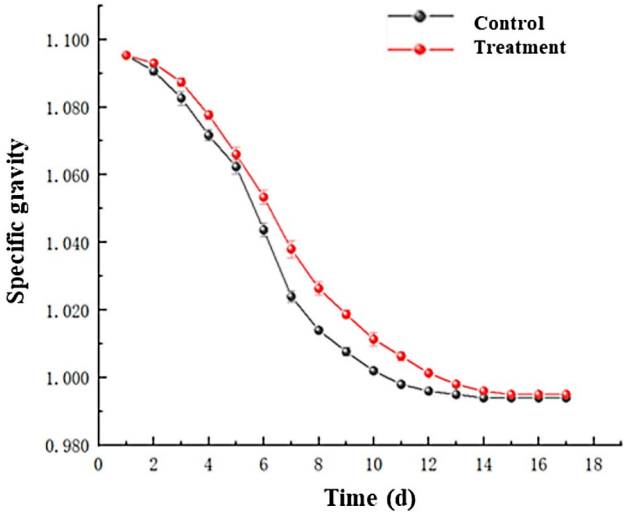

Fig. 1 Changes in specific gravity during alcoholic fermentation. Note: The data presented in the figure are expressed as averages. The vertical lines within the figure represent the error bars

Table 2 Yeast biomass during different fermentation periods under the two inoculation modes

Fermentation period	Control group	Treatment group	
	S. cerevisiae (×10^7 CFU/mL)	M.guilliermondii (×10^7CFU/mL)	S. cerevisiae (×10^7CFU/mL)
12 h (1 day) after NM218 inoculation	2.13 ± 0.06^b	0.17 ± 0^c	5.20 ± 0.05^a
48 h after NM218 inoculation (2 days)	6.77 ± 0.55^b	1.64 ± 0.16^c	10.00 ± 0.66^a
Middle stage (6 days)	3.23 ± 0.06^b	0.04 ± 0.01^c	8.73 ± 0.21^a
Final stage (14 days)	0.08 ± 0.01^b	0^c	0.10 ± 0.01a

Note: Different lowercase letters indicate significant differences between data ($P < 0.05$), and the same lowercase letters indicate insignificant differences between data ($P > 0.05$, ANOVA–Tukey HSD test, $\alpha = 0.05$, same as below). Statistical difference corresponded to the same row

Results

SG change during AF

The SG was monitored under a controlled fermentation temperature of approximately 18–20 °C and the results are shown in Fig. 1. The SG changes in the Chardonnay-treated group showed less reduction and slower fermentation rate than those in the control group. In the control group, the SG sharply decreased from 2 to 8 days, indicating that the fermentation rate was high at this stage; on day 8, the SG decrease slowed down, indicating that the fermentation rate began to decelerate until the end of AF on day 17. The treated group fermented faster at 3–9 days and subsequently slowed down, extending by 2–3 days compared with the control group.

Analysis of yeast growth status during fermentation

The number of living yeasts during AF is presented in Table 2. In the Chardonnay-treated group, *S. cerevisiae* and *M. guilliermondii* biomass tended to increase first and subsequently decrease. *M. guilliermondii* biomass reached its maximum value of 1.64×10^7 CFU/mL at 48 h following inoculation, to the middle stage of fermentation, owing to the rapid growth of *S. cerevisiae* and the increase in alcohol content, the number of *M. guilliermondii* (10^5 CFU/mL) was significantly inhibited and no *M. guilliermondii* was detected at the end of fermentation, whereas the number of *S. cerevisiae* reached a maximum of 1×10^8 CFU/mL at the 48 h after NM218 inoculation (2 days), the number of viable yeasts at the end of fermentation was approximately 10^6 CFU/mL. In the control group, the *S. cerevisiae* participated in the whole AF procedure, the number of living yeasts initially increased, subsequently decreased, and maintained at the end of fermentation at 10^5 CFU/mL.

Analysis of common physical and chemical indicators

The basic physical and chemical indexes of the samples after the end of AF and MLF are presented in Table 3. All samples met the requirements of the national standard GB/T15038-2006 [19]. The residual sugar content of both groups was less than 4 g/L, and the malolactic acid content was less than0.2 g/L, indicating that the wine samples were completely fermented. The ethanol content ranged from 12.17 to 12.76% (v/v), and the treated group had a significantly lower ethanol content than the control group, indicating that *M. guilliermondii* participation in fermentation can reduce the alcohol content. The volatile acid content of the wine samples in the treatment group was significantly higher at the end of AF than of the control group during the same period; and the volatile acid content in the treatment group and control group samples was less than0.66 g/L, indicating no abnormal fermentation process. There was no significant difference in total acid content between the treated group and the control group at the end of AF and MLF, but after MLF, the total acid content of all wine samples was significantly reduced, indicating that M. guilliermondi is not a non-saccharomycete that can reduce total acid.The glycerol content of the wine samples was significantly different ($P < 0.05$), and the glycerol content of the treated group was significantly higher at the end of AF and MLF than that of the control group ($P < 0.05$), indicating that *M. guilliermondii* can increase glycerol content as a non-*S. cerevisiae* yeast.

Measurement of color indicators

The results of the CIE Lab color parameters of wine samples are shown in Table 3. The L* values of the wine samples in the treatment group were almost as high as

Table 3 Common physical, chemical and color-related indicators of samples after the end of AF and MLF

Sample name	CK-AF	NM-AF	CK-MLF	NM-MLF
Common physical and chemical indicators of Chardonnay samples				
Alcohol level (% vol)	12.51 ± 0.02^{b}	12.17 ± 0.02^{d}	12.76 ± 0.02^{a}	12.33 ± 0.03^{c}
Total acidity (g/L)	5.87 ± 0.01^{a}	5.76 ± 0.02^{b}	5.28 ± 0.02^{c}	5.25 ± 0.02^{c}
Volatile acidity (g/L)	0.34 ± 0.01^{c}	0.65 ± 0.02^{a}	0.43 ± 0.02^{b}	0.66 ± 0.02^{a}
Residual sugar (g/L)	1.57 ± 0.02^{c}	1.89 ± 0.01^{a}	1.22 ± 0.02^{d}	1.73 ± 0.02^{b}
pH	3.46 ± 0.02^{c}	3.57 ± 0.02^{b}	3.66 ± 0.02^{a}	3.68 ± 0.02^{a}
Glycerol (g/L)	7.57 ± 0.02^{c}	8.06 ± 0.02^{b}	7.43 ± 0.03^{d}	8.53 ± 0.03^{a}
Malic acid (g/L)	2.45 ± 0.01^{a}	2.13 ± 0.02^{b}	0.08 ± 0.01^{c}	0.08 ± 0.01^{c}
Color-related indicators of Chardonnay samples				
L^*	98.88 ± 0.22^{a}	98.69 ± 0.29^{a}	97.05 ± 0.44^{b}	96.80 ± 0.20^{b}
a^*	-2.20 ± 0.09^{a}	-2.33 ± 0.05^{a}	-1.84 ± 0.06^{c}	-2.02 ± 0.12^{b}
b^*	8.98 ± 0.33^{c}	9.92 ± 0.16^{b}	9.97 ± 0.32^{b}	11.35 ± 0.34^{a}
C^*_{ab}	9.24 ± 0.34^{c}	10.19 ± 0.17^{b}	10.14 ± 0.3^{b}	11.53 ± 0.33^{a}
h_{ab}	-76.24 ± 0.37^{a}	-76.81 ± 0.07^{a}	-79.56 ± 0.68^{b}	-79.96 ± 0.74^{b}
ΔE^*_{ab}	0^{d}	1.06 ± 0.19^{c}	2.11 ± 0.23^{b}	2.97 ± 0.29^{a}
Total phenol (mg/L)	243.07 ± 6.49^{b}	261.80 ± 11.24^{a}	224.34 ± 6.49^{c}	239.33 ± 11.24^{bc}
Browning index	0.026 ± 0^{b}	0.027 ± 0^{b}	0.033 ± 0^{a}	0.037 ± 0^{a}

Note: Different lowercase letters indicate significant differences between treatment and control groups for the same physicochemical index at the end of AF and MLF ($P < 0.05$); the same letter indicates non-significant differences ($P > 0.05$).The wine was sampled at the end of AF and MLF, control group was recorded as CK-AF and CK-MLF, and the treatment group as NM-AF and NM-MLF

those in the control group and better wine gloss. L* at the end of AF and MLF showed a non-significant difference in values ($P > 0.05$), indicating that the participating fermentation of the NM218 strain had no significant effect on the brightness of wine color. a* represents the wine sample red and green degrees. The a* values of the two Chardonnay sample groups were < 0 and between − 2.33 and − 1.84, indicating a large green content in wine color at the same periods. b* represents the yellow and blue degrees of the wine sample. The b* value of the test wine sample was between 8.98 and 11.35, indicating that the yellow component in wine color was larger, which was also consistent with the color characteristics of white wine [22]. At the end of AF, the b* value of the treatment group increased to 10.47% compared with the control; at the end of MLF, the b*value of the control increased to 13.71%, indicating that the *M. guilliermondii* NM218 strain significantly increased the proportion of wine yellow component during AF ($P < 0.05$), making the value C^*_{ab} (color saturation) of the treatment group also increase accordingly. However, as shown in Table 3, at the end of AF and MLF, the ΔE*ab values of each group are < 3; therefore, the color difference between the wine samples was not significant ($P > 0.05$), indicating that the participating fermentation of the *M. guilliermondii* NM218 strain had no significant effect on the total color difference of white wine. Studies showed that the total phenolic content was significantly correlated with the antioxidant activity in white wine [23]. As shown in Table 3, the total phenolic content of the samples is between 224.34 and 261.80 mg/L. At the end of MLF, the total phenolic content of the treated group increased by 6.68% more than the control. Absorbance values at 420 nm were used as a useful indicator to assess the extent of browning due to non-enzymatic oxidation [16]. According to the browning index of the samples, no significant difference was observed between the browning indexes of the wine samples at the same period, which is consistent with the abovementioned CIELab parameters.

Analysis of the aroma compounds

Different volatile aroma compounds in wine samples were analyzed using GC-MS. Overall, 68 volatile aroma compounds were detected in Chardonnay wine samples, including 16 alcohols, 38 esters, 5 terpenes, 3 acids, 6 aldols, and other categories. The types and contents of the volatile compounds of various wine samples were different.

Higher alcohols are one of the secondary products of yeast amino acid metabolism and are the main aroma substances in wine. However, when the content exceeds 400 mg/L, it gives wine a pungent smell [24]. The results are shown in Table S1. Overall, 16 alcohols were detected in the two samples, with a total concentration of 5,999.03 μg/L (NM-AF) and 7,601.25 μg/L (NM-MLF). The total concentration of alcohol in each sample was lower than 300 mg/L, which can increase the complexity of the wine aroma, providing the wine a pleasant floral and fruity fragrance. In wine samples, higher alcohols of NM-AF decreased by 10.30%, those of NM-MLF increased by 19.52%, and o-pentyl alcohol (greater than58.68%) and 2-phenylethanol (greater than25.97%) dominated the total alcohols, which brought wine banana, honey, and lilac aromas; the OAV value

of 2-phenylethanol was greater than 1. 2-phenylethanol content of treatment group samples increased by 17.76% and 7.98% at the end of AF and MLF, respectively. Notably, euheptanol had an oil odor that was detected only in the treated group; however, its content was well below the threshold.

Esters are mainly produced in the fermentation and aging process of wine, which can provide the wine a pleasant fruity fragrance [24]. Overall, 38 esters were detected in the two wine samples; however, their types and contents differed significantly. The total concentration of esters was 59,777.05 μg/L (CK-MLF) and 74,186.52 μg/L (NM-AF); a total of 15 ester aroma compounds had OAV values of > 0.1. Their large aroma substances were consistent with Chardonnay fruit, and most of the esters showed floral and fruity fragrances. Ethylic acid, ethyl acid, ethyl acetate, and ethyl lauric acid contents in NM-AF and NM-MLF were higher than those in the control group in the same period. The content of total esters was not significant at the end of AF; however, it increased by 11.75% after MLF, which further strengthened the aroma of pineapple, pear, and coconut in the wine samples.

Fatty acids are the product of the fatty acid metabolism of yeast. The nutty and cheese flavor can increase the complexity of wine aroma; however, excessive concentration can produce "rot," "fatty," and "sweaty" smell [25]. Caproic acid, octanoic acid, and decanoic acid were detected from the wine samples, among which the OAV value of the octanoic content was > 0.1, and the octanoic content increased to different degrees after MLF but did not exceed the threshold.

Terpenes mainly show the aroma characteristics of floral and citrus smell. Five kinds of terpenes including linalool and citronella alcohol were detected in the wine samples. Among the samples, linalool, the citronellol, geranylacetone, and total terpenes in NM-MLF increased by 100%, 17.4%, 27.4%, and, 15.7% compared with CK-MLF, respectively. Four ketoaldehyde and two other compounds were also detected in the wine samples, and the total mass concentration of the wine was 437.31–485.28 μg/L.

As shown in Table S1, floral and fruity fragrances have the highest OAV values and are the main aroma categories. At the end of MLF, floral and fruity fragrances of NM-MLF (61.90 and 593.12, respectively) were significantly higher than CK-MLF (54.16 and 544.61, respectively).

Principal component analysis (PCA) of the main aroma compounds

The OAV value can be used as an indicator for evaluating the contribution of a single aroma component to the overall aroma of the wine [26]. PCA could indicate the correlation and dispersion between single aroma components and different treated wine samples [27].

As shown in Fig. 2, PC1 and PC2 accounted for 66.3% and 26.5% of the overall variance, respectively, indicating that the PCA model clustered well and reflected 92.8% of the aroma in Chardonnay dry white wine samples. CK-AF wine samples were distributed in the third quadrant and

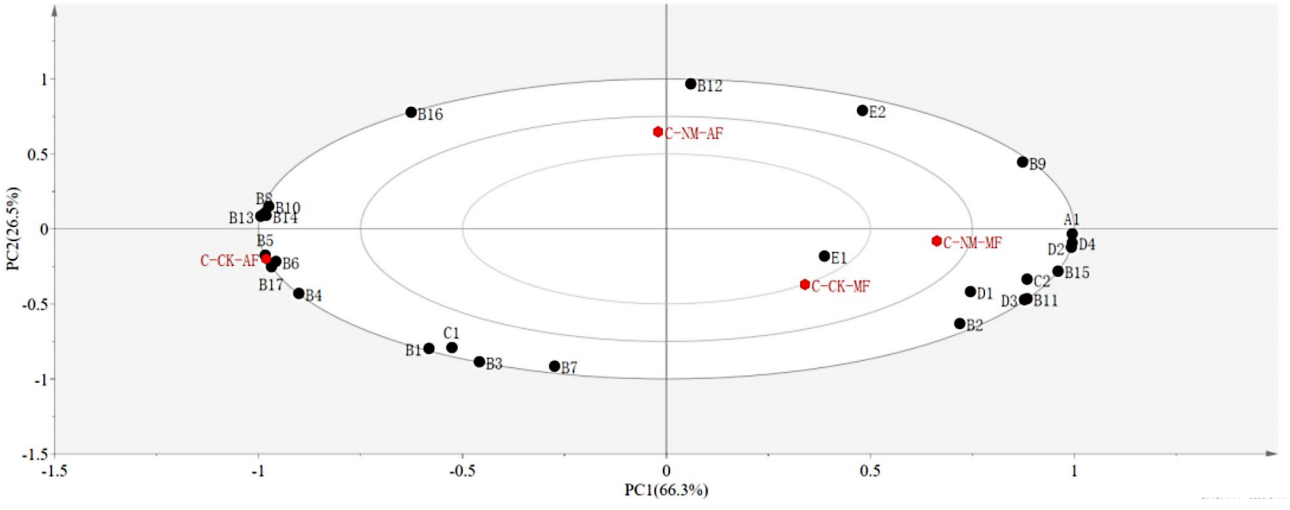

Fig. 2 Factor load value and liquor sample distribution of Chardonnay wine OAV > 0.1 aroma compounds. A1 denotes 2-phenethanol, B1 denotes ethyl acetate, B2 denotes ethyl 2-methylpropionate, B3 denotes ethyl butyrate, B4 denotes isoamyl acetate, B5 denotes hexyl acetate, B6 denotes ethyl hexanoate, B7 denotes trans-2-ethyl hexanoate, B8 denotes methyl octanoate, B9 denotes ethyl caprylate, B10 denotes isoamyl hexanoate, B12 denotes ethyl decanoate, B13 denotes isoamyl caprylate, B14 denotes 3-ethyl methylbutyrate, B15 denotes phenethyl acetate, B16 denotes ethyl laurate, B17 denotes ethyl palmitate, C1 denotes hexanoic acid, C2 denotes octanoic acid, D1 denotes linalool, D2 denotes citronellol, D3 denotes Damascus, D4 denotes geranyl acetone, E1 denotes decanal, and E2 denotes nonanal

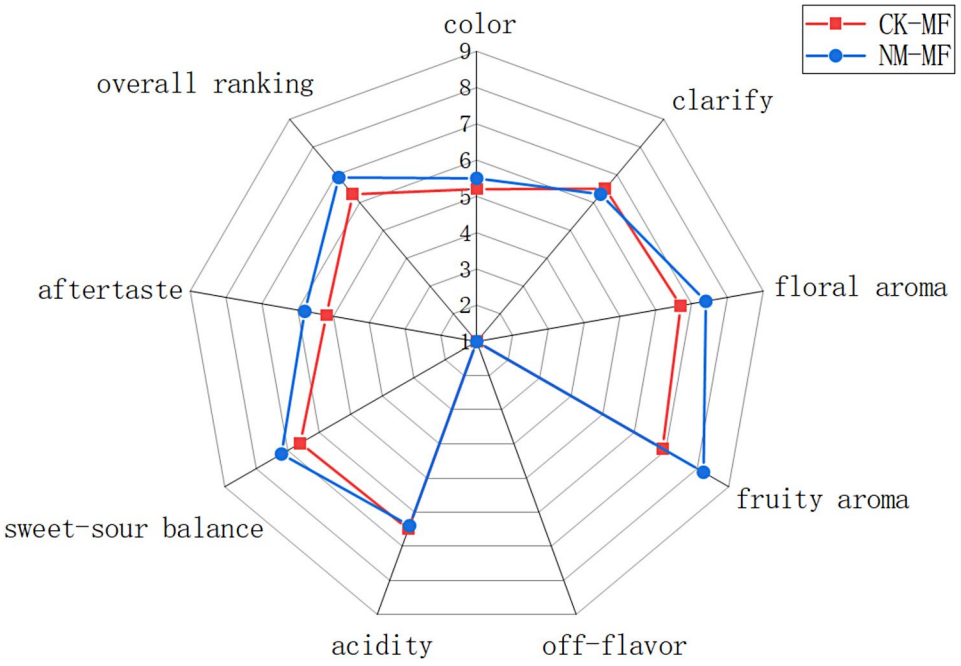

Fig. 3 Sensory analysis radar diagram

strongly associated with ethyl hexanate, ethyl palmitate, isoamyl acetate, and phenyl acetate. NM-AF wine samples were distributed in the second quadrant, which had a strong association with ethyl decanoate, ethyl laurate, and nonaldehyde; however, nonaldehyde had a green flavor, which may have an unpleasant flavor influence on wine. Therefore, further sensory analysis was needed. CK-MLF had a similar aroma composition to NM-MLF in wine samples, distributed in the fourth quadrant, and strongly associated with aroma substances, including beta-damascenone, citronellol, geranylacetone, and phenethyl alcohol. In summary, *M. guilliermondii* NM218 and *S. cerevisiae* mixed fermentation changed the aroma characteristics of purebred fermentation.

Sensory analysis

The sensory evaluation was used to judge the effect of the test strains on the wine quality. In Chardonnay wine (Fig. 3), regarding the appearance, the treatment group NM-MLF and control group CK-MLF were light yellow but had no obvious distinction; regarding nose and palate, the treatment group NM-MLF, which had better sweet–sour balance and longer lasting taste, showed more pleasant fruity characteristics than the control group CK-MLF; meanwhile, the treatment group NM-MLF obtained higher overall score with well-balanced wine body and strong aroma.

Discussion

The application of mixed fermentation of indigenous non-*S. cerevisiae* and *S. cerevisiae* to wine production is a hotspot of research and application in the winemaking industry. In this study, the sequential inoculation of *M. guilliermondii* NM218 and *S. cerevisiae* in Chardonnay must, *M. guilliermondii* biomass could reach 10^7 CFU/mL after 48 h. At the middle stage of fermentation, *M. guilliermondii* biomass could reach approximately 10^5–10^6 CFU/mL, indicating that the *M. guilliermondii* NM218 strain has good tolerance in wine environment, showing good colonization ability and potential for industrial application. Inoculation of non-cerevisiae during sequential inoculation did not inhibit the fermentation performance of cerevisiae during the alcoholic fermentation of Chardonnay, and non-cerevisiae increased the content of flavor substances in Chardonnay. Furthermore, the cellular activity of the *M. guilliermondii* NM218 could not be detected at the later stages of sequential fermentation inoculation, which could be caused by microbial interactions and changes in ethanol concentration [28].

Sensory evaluation can directly reveal the quality of wine. The score of sensory evaluation of wine samples with sequential inoculation was significantly higher than that of pure fermentation. Liu Yu et al. reported that the use of H. varum BF345 strain and S. Cerevisiae in sequential inoculation fermentation could improve the sensory characteristics of wine. This is also consistent with the results obtained for volatile aroma compounds, which may be due to the increased variety and content

of volatile aroma compounds by mixed fermentation [29]. β-Glucosidase produced by *S. cerevisiae* can hydrolyze glycoside aroma substances and generate monoterpene alcohol (linalool, geranylacetone, and nerol), higher alcohol (2-phenylethanol), and norisoprenoid (damascenone) compounds to improve the aromatic properties of wine [30]. In particular, esters are essential for the aromatic characteristics of most alcoholic beverages and are the main odorant for sensing fruity and floral fragrances [24]. This study observed that the sequential inoculation of *M. guilliermondii* NM218 and *S. cerevisiae* effectively increased ethyl acetate and acetate ester contents in fruit-based wine and improved the aroma complexity and richness. Silva et al. [7] reported that *M. guilliermondii* (*Pichia guilliermondii*) can secrete β-glucosidase that is tolerant to ethanol and glucose, which can be used as a candidate strain for the preparation of aromatic wines. Of note, in this study, ethyl caprylate and ethyl caprate were the characteristic aroma substances of the treated group, which may be metabolic production of the *M. guilliermondii* NM218, whereas the specific mechanism requires further investigation. In this study, *M. guilliermondii* NM218 and *S. cerevisiae* significantly increased the concentration of 2-phenylethanol in Chardonnay wine ($P<0.05$), and its OAV value was >1, which could provide wine its rose, honey, and other flavors, and optimize the overall sensory quality of wine. These study results were consistent with Yan et al.'s [9]. Although the expected increase in 2-phenylethanol content was not correlated with β-glucosidase activity, this increase could be explained by the general metabolism of the yeast strains [31]. Sáez et al. [32] reported that enhanced volatile phenols in wine fermented with *Saccharomyces cerevisiae* and spoiled with *Pichia guilliermondii* (*M. guilliermondii*) and *Dekkera bruxellensis*. 34 volatile compounds were detected in the sequenced inoculation of *M. guilliermondii* Pg1 and *S cerevisiae* FX10 in the fermented plum fruit wine [33]. At present, there are few studies on the fermentation of other alcoholic beverages by *M. guilliermondii*. In addition, it is interesting that *M. guilliermondii* AF01 can degrade aflatoxin B1 in peanut meal through solid fermentation [34], which indicated that *M. guilliermondii* AF01 had the potential to be used in degrading aflatoxin.

Volatile phenols including 4-ethylphenol and 4-ethyl guaiacol are significant components of wine aroma. Meanwhile, *M. guilliermondii* could produce 4-ethylphenol and other substances as well as exhibit smoky smell under low concentrations; however, when its total concentration is higher than 650 μg/L, the flavor of the wine would be destroyed, even exhibiting sweaty and fishy smell [35–37]. In this study, 4-ethyl guaiacol was detected in the treated group, with a much lower content than 650 μg/L. The reason for the presence of 4-ethyl guaiacol was unclear, and its content should be tracked and evaluated during the aging period.

Conclusions

M. guilliermondii showed good colonization ability before mid-AF. The physicochemical indexes met the national standard GB/T15038-2006 Wine [19]. Regarding the color of Chardonnay dry white wine, the b* value, C^*_{ab} value (color saturation), and total phenol content of the treatment group (inoculated *M. guilliermondii* and *S. cerevisiae* sequentially) were significantly increased ($P<0.05$). This may be due to the fact that non-saccharomyces cerevisiae produced some polyphenols during the alcoholic fermentation process, which improved the color of the wine sample and inhibited Browning. Compared with the control samples (only inoculated *S. cerevisiae*), no significant differences were noted in the other color parameters. Regarding the aroma composition, comparing the Chardonnay-treated group alcohol samples with the control alcohol samples during the same period, the contents of ester substances ($P<0.05$). After MLF, the total alcohol, ester, and terpene contents were increased, respectively. In the treatment group, ethyl decanoate and ocoate were significantly higher than the control wine samples and produced three kinds of unique aroma substances. Whether this result is caused by the high β-glucosidase production of this strain needs further investigation. The interaction between M. guilliermondii NM218 and commercial saccharomyces cerevisiae during the alcoholic fermentation of Chardonnay wine needs further study. To sum up, the sequential inoculation of *M. guilliermondii* NM218 with *S. cerevisiae* enriched Chardonnay's fruity and floral fragrances. The wine samples of the treatment group showed light and refreshing fragrance, typical aroma, comfortable taste, and long-lasting aftertaste.

Abbreviations

M. guilliermndii	*Meyerozyma guilliermndii*
S. cerevisiae	*Saccharomyces cerevisiae*
AF	Alcoholic fermentation
MLF	Malolactic fermentation
SG	Specific gravity
HS-SPME	Headspace solid-phase microextraction
GC-MS	Gas Chromatography-mass Spectrometry
RI	Retention index
OAV	Odor activity value
PCA	Principal component analysis

Supplementary Information

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Supplementary Material 1

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Author contributions
L. M.: writing—original draft preparation, data curation, writing—review and editing; W. Z.: methodology, formal analysis, conceptu-alization; X.F. Z.: writing–review & editing; S. P.: supervision, funding acquisition; M. L.: validation, writing–original draft, visualization; J. W.: project administration, resources, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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

Declarations

Institutional review board statement
Ethical review and approval were waived for this study due to the protocol used for data collection complied with the national ethical requirements and were implemented in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) on experiments involving humans.

Informed consent
All panelists provided with informed consent and no coercion to participate, full disclosure of study requirements and risks, written or verbal consent of participants, no release of participant data without their knowledge.

Competing interests
The authors declare no competing interests.

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