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Distinctive chemical and aromatic composition of red wines produced by *Saccharomyces cerevisiae* co-fermentation with indigenous and commercial non-*Saccharomyces* strains

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ABSTRACT

The use of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast species as mixed starters has advantages over pure culture fermentation because of increased wine sensory characteristics. The aim of the present study was to evaluate the divergences of wine compositions fermented by indigenous non-*Saccharomyces* strains (*Torulaspora delbrueckii* TD12 and *Lachancea thermotolerans* LT9) or commercial non-*Saccharomyces* strains (*T. delbrueckii* Prelude and L. *thermotolerans* Concerto) combined with *S. cerevisiae* D254, respectively. Results evidenced that although belong to the same species, the content of chemical and aromatic compounds of red wines produced by indigenous and commercial strains was significantly different after alcoholic fermentation (ALF) and malolactic fermentation (MLF). TD12/D254 was characterized with a higher amount of glycerol, ethyl esters, and volatile acids, whilst Prelude/D254 was distinguished by a higher intensity of isoamyl acetate and a lower production of acetic acid. LT9/D254 increased the intensity of higher alcohols, esters, and β-damascenone compared with Concerto/D254. After MLF, the diversities variation of glycerol and lactic acid were increased, but acetic acid and most volatile compounds were reduced. TD12/D254 obtained better aromatic quality as assessed by calculating the odor activity values (OAVs). Our results highlighted the strain-specificity of non-*Saccharomyces* strains in shaping the aromatic characteristic of wine, and suggested that more attention should be paid to the strain-specific characteristics when selecting non-*Saccharomyces* strains to improve aroma diversity and quality of the wine. In this regard, the indigenous strain is a suitable choice because of better adaptation to fermentation conditions and generating typical sensory characteristics specific to the wine region.

1. Introduction

The fermentation of grape juice into wine is a complex biochemical process, in which yeasts are primarily responsible for the alcoholic fermentation of the juice, including *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts (Jolly, Varela, & Pretorius, 2014). It is widely accepted that a selected and inoculated strain of *S. cerevisiae* will dominate the fermentation process and produce most of the ethanol and a broad range of aroma-active compounds in wine (Comitini et al., 2011). Nowadays, non-*Saccharomyces* yeasts received significant attention because they have some desired enological characteristics that are absent in *S. cerevisiae*, such as producing high levels of aroma compounds (esters, higher alcohols and acids) and producing and secreting several enzymes (esterase, β-glycosidase, lipase and protease, among others), which can interact with odorless grape precursors and improve wine complexity in controlled fermentation manner (Ciani, Comitini, Mannazzu, & Domizio, 2010; Domizio et al., 2011; Escribano et al., 2018; Padilla, Gil, & Manzanares, 2016; van Wyk, Grossmann, Wendland, von Wallbrunn, & Pretorius, 2019). To data, several non-*Saccharomyces* yeasts species, such as *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri* were commercialized and recommended to be co-inoculated with *S. cerevisiae* to improve the specific organoleptic characteristics of wine (Prior, Bauer, & Divol, 2019; Roudil et al., 2020). For example, the

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co-fermentation with *T. delbrueckii* and *S. cerevisiae* can improve major volatile compounds (such as ethyl octanoate, isoamyl acetate, 2-phenylethyl alcohol) and thiols concentration (Benito, 2018; Chen et al., 2018; Puertas, Jiménez, Cantos-Villar, Cantoral, & Rodríguez, 2017). And, the sequential inoculation of *L. thermotolerans* with *S. cerevisiae* can lead to an enhancement of lactic acid, resulting in the increased acidity of the final wines (Gobbi et al., 2013; Santiago, 2018).

China is a rapidly developing country and has a great potential for improved wine production and consumption. Currently, there are over ten major viticultural areas and each has distinct ecological characteristics. Among them, Xinjiang and Ningxia are the main two historic and major wine regions, however, the hot climate in summer and intense sun exposure in both regions results in red wines with high alcohol content (up to 14–15% vol), low acidity, and a lack of fruit notes and elegance in aroma (Duan et al., 2018). To solve the problems of less distinctive aroma and low acidity in wines, inoculating commercial non-*Saccharomyces* strains become increasingly popular in both wine regions, such as commercial *T. delbrueckii* and *L. thermotolerans*. However, because of the usually poor fermentation dynamics of non-*Saccharomyces* yeasts, the application of commercial non-*Saccharomyces* strains is doubted about the improvement of oenological quality and styles of regional wines (Nisiotou et al., 2018; Rainieri & Pretorius, 2000). Moreover, highly strain-dependent variability of the same non-*Saccharomyces* species also leads to the divergences of wine chemical and aroma quality (Binati et al., 2020; Renault et al., 2009; van Breda, Jolly, & van Wyk, 2013), which further increased the troubles of choosing suitable non-*Saccharomyces* strains with the aim of improving the quality of regional wines. To address this problem, inoculating indigenous or locally selected wine yeasts with excellent oenological characteristics is encouraged, owing to these yeasts having high environment adaptability, and more importantly, indigenous yeasts are considered as key factors for the 'terroir' characteristics of regional wine (Li et al., 2020; Raymond, Eder, Reynoso, Lauret, & Rosa, 2017).

In our previous study, two indigenous non-*Saccharomyces* yeasts *T. delbrueckii* CVE-TD12 and *L. thermotolerans* CVE-LT9 with good technological characteristics, including high fermentation speed, high tolerance to SO_2 and sugar, and high activity of β-glucosidase, have been isolated and preliminary evaluated. Hence, in this study, we assess the improvement effects of indigenous strains *T. delbrueckii* TD12 and *L. thermotolerans* LT9 co-fermentation with *Saccharomyces cerevisiae* on red wines in 20 L fermenter using Cabernet Sauvignon grape as the must, respectively. The same design was carried out in commercial non-*Saccharomyces* strains (*T. delbrueckii* Prelude and L. *thermotolerans* Concerto) mixed cultures to identify the similarities and differences with indigenous non-*Saccharomyces* strains. The content of major fermentation products and aromatic compounds of the red wine was determined after alcoholic fermentation (ALF) and malolactic fermentation (MLF), respectively. The results are expected to enrich our understanding of the beneficial effects of local non-*Saccharomyces* strains in improving basic parameters and aroma composition, and shaping regional wine quality.

2. Materials and methods

2.1. Yeast strains

Two indigenous non-*Saccharomyces* strains (*T. delbrueckii* CVE-TD12 and *L. thermotolerans* CVE-LT9) with desirable physiological properties were used in this study. They were identified by means of 26S rDNA-RFLP analysis (Kurtzman & Robnett, 1998) and deposited in the Centre for Viticulture and Enology, China Agricultural University. Two additional commercial non-*Saccharomyces* strains, Prelude™ (*T. delbrueckii*, Chr. Hansen, Denmark) and Concerto™ (*L. thermotolerans*, Chr. Hansen, Denmark) were isolated as a single colony and used in co-fermentation with *S. cerevisiae* D254 (Lalvin, France) for comparison. These strains were stored at −80 °C in the YPD medium with glycerol (20% v/v final concentration).

Mixed fermentations of indigenous and commercial non-*Saccharomyces* strains with *S. cerevisiae* in 20 L fermenter.

Multi-starter fermentations were performed by sequential inoculation using indigenous (TD12 and LT9) or commercial (Prelude and Concerto) non-*Saccharomyces* strains followed by *S. cerevisiae* strain D254 after 2 days. The pure culture of D254 starter was set as the control trials. The inoculum ratio of non-*Saccharomyces* and *Saccharomyces* species was 10:1, and the initial active population of non-*Saccharomyces* and *Saccharomyces* were 1.0×10^7 CFU/mL and 1.0×10^6 CFU/mL pre-cultured in pasteurized Cabernet Sauvignon grape juice, respectively (Renault, Coulon, de Revel, Barbe, & Bely, 2015). Cabernet Sauvignon grapes (18 kg) picked from the Manasi region were added into 20 L stainless steel fermenter after destemming, crushing, and adding 60 mg/L of sulfur dioxide and 30 mg/L pectinase. The basic parameters of the must were measured as follows: pH 3.23, 5.21 g/L of titratable acid, 253 g/L of sugar. Triplicated fermentations were carried out at 20–23 °C with regular punching skins down to improve extraction. After alcoholic fermentation (sugar content was below 4 g/L), grape pomace was separated out of wine carefully. The wines were transferred to 10 L glass fermenter for settle for 2 days and started malolactic fermentation by commercial *Oenococcus oeni* (Viniflora® Oenos, Chr. Hansen) according to the manufacturer's instructions. The samples were taken after alcoholic fermentation and malolactic fermentation, centrifuged, and stored at −20 °C for analysis of glucose, fructose, glycerol, ethanol, acetic acid, non-volatile acids and volatile aroma compounds.

2.2. Analytical techniques

The polymerase chain reaction (PCR) fingerprinting of indigenous strains (TD12 and LT9) and commercial strains (Prelude and Concerto) used in mixed fermentation trials were examined by RAPD (Random Amplified Polymorphic DNA) analysis, using the primer sequences (5′-GCT CGT CGC T-3′) according to the method of Michel et al. (2015) with some modifications. Yeast DNA was isolated using TIANamp Yeast DNA Kit (Tiangen, Beijing, China). Typical PCR was performed with 12.5 μL Master Mix 2 × (TsingKe, China), 5 μL PCR water, 5 μL primer (Sangon Biotech, China) and 2.5 μL template DNA with a total reaction volume of 25 μL. PCR parameters were: (i) 94 °C/4 min; (ii) 30 cycles of 94 °C/45 s, 57 °C/45 s, 72 °C/1 min; and (iii) 72 °C/10 min, and PCR was carried out using a thermal cycler (C1000 TouchTM, Bio-rad, Singapore). Amplicons were detected using 1% agarose gel electrophoresis by PowerPac Basic TM (Bio-rad, Singapore).

Glucose, fructose, glycerol, ethanol, acetic acid, and non-volatile acids of the final wines were determined by HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an HPX-87H Aminex ion-exchange column (300 mm × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) (Liu, Lu, Duan, & Yan, 2016). The mobile phase was 5 mmol sulfuric acid. Glucose, fructose, ethanol, and glycerol were detected with a refractive index detector with the column maintained at 45 °C. Acetic acid, malic acid, lactic acid, and succinic acid were detected with a photodiode array detector at 214 nm with the column maintained at 60 °C. The samples prior to HPLC analysis were filtered through a 0.22 μm membrane filter (Dikma Technologies, Lake Forest, CA, USA).

The aroma compounds of wines after alcoholic and malolactic fermentation were determined by headspace solid-phase micro-extraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC-MS) according to our previous study (Lan et al., 2016; Xu et al., 2015). The identification of the aroma compounds was based on retention indices of reference standards and mass spectra matching in the standard NIST 11 library. For the quantification of these aroma compounds, the relative peak areas of each identified compounds were measured and then compared with the relative peak area of the added internal standard (4-methyl-2-pentanol). Analyses were performed in duplicate. The detailed quantitation information about linear fit, R^2 value, linear range, aroma characteristics and aroma type for the volatile compounds used in this study were showed in Supplementary Table 1.

2.3. Statistical analysis

One-way ANOVA using the Duncan test at significance level $P < 0.05$ was carried out to uncover statistical differences between the wines produced from the different inoculation protocols. Principal component analysis (PCA) was conducted using volatile compounds (OAV>0.1) after alcoholic and malolactic fermentation, respectively. Statistical analyses were performed with the IBM SPSS Statistical Package (version 24.0, IBM Corp, NY, USA).

3. Results and discussion

3.1. PCR fingerprinting of commercial and indigenous non-saccharomyces strains

Inoculation with different wine yeast strains for wine fermentation can influence wine parameters and volatile profiles due to their genetic variations (Casu, Pinu, Fedrizzi, Greenwood, & Villas-Boas, 2016; Erasmus, Cliff, & Vuuren, 2004). To distinguish the indigenous and commercial non-*Saccharomyces* strains at the strain level, the genetic characterizations of TD12 and Prelude, LT9 and Concerto were determined and compared by RAPD analysis, according to Michel et al. (2015). In Fig. 1, there was high length variation between TD12 and Prelude strains, and additional two bands (480 bp and 1050 bp) appeared in TD12 strain. As to L. *thermotolerans* strains, a band of 1500 bp appeared in LT9 strain, which was not observed in Concerto strain. These data indicated that although belonging to the same species, *T. delbrueckii* or *L. thermotolerans* strains in this study had significant differences in genetic characterizations, which is in good agreement with the results of Renault et al. (2009).

3.2. Fermentation progress and basic parameters of wine

The fermentation progress during ALF in the pure or mixed cultures was monitored by must density and presented in Fig. 2. The pure culture of *S. cerevisiae* D254 had the highest fermentation rate and completed ALF within 8 days. In comparison, the duration time of mixed cultures was extended to 12 d. Noticeably, the fermentation activities of *T. delbrueckii* TD12 and *L. thermotolerans* LT9 were all higher than those

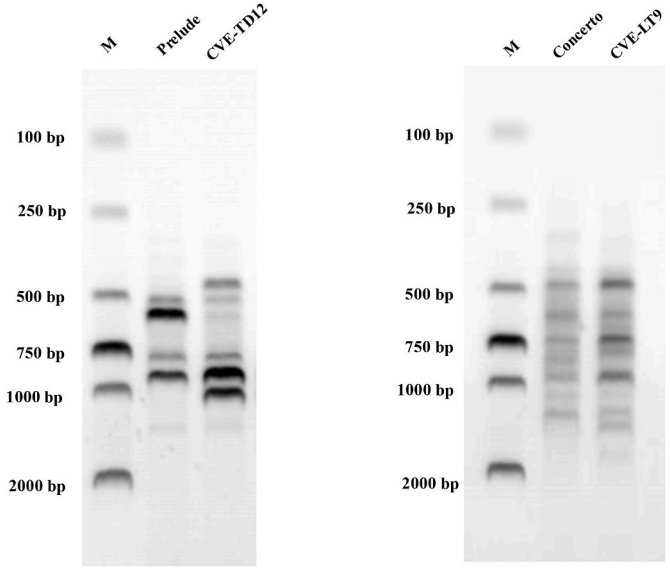

Fig. 1. Electrophoretic patterns of *T. delbrueckii* and *L. thermotolerans* strains with 1% agarose gel resulting from RAPD fingerprint method. *T. delbrueckii* strains include TD12 and commercial strain Prelude; *L. thermotolerans* strains include LT9 and commercial strain Concerto. M: 2000 bp DNA ladder.

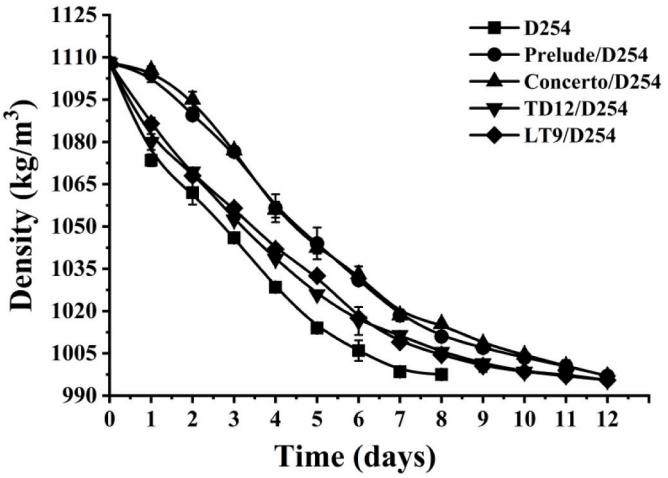

Fig. 2. The fermentation progress of trials inoculated by different strains.

of commercial strains, confirming that indigenous strains can well adapt to grape must composition and environmental conditions of the regional winery (Nisiotou et al., 2018).

Table 1 showed the basic compositions in wines after ALF and MLF. All treatments successfully completed fermentation with total reducing sugar content below 4 g/L. The production of glycerol was 6.95–9.05 g/L after ALF and 8.69–9.73 g/L after MLF, in which TD12/D254 had a significantly increased level. Minor differences of treatments were observed in the case of ethanol concentration after ALF ($P = 0.128$), but the significant diversities appeared after MLF ($P < 0.001$), and the highest values always produced in D254 single fermentation. Acetic acid is a negative fermentation by-product and provides vinegar character to wine with the level above 0.8 g/L (Benito, 2018). TD12/D254 and Prelude/D254 that involved *T. delbrueckii* yeasts had a significantly lower production of acetic acid (0.21 g/L), which was in consistent with the previous finding that *T. delbrueckii* generated a lower level of acetic acid during winemaking (Canonico, Comitini, & Ciani, 2017; Chen et al., 2018; Liu, Laaksonen, Kortesniemi, Kalpio, & Yang, 2018). However, the level of acetic acid after MLF was a range from 0.51 g/L to 0.60 g/L, and no significant differences ($P = 0.751$) were observed in all treatments. Lactic acid usually is perceived as sour and spicy (Vilela, 2019). The obviously higher concentration of lactic acid was produced in wines of Concerto/D254 (1.49 g/L) and LT9/D254 (1.18 g/L), confirmed that *L. thermotolerans* can increase the lactic acid level and improve the acidity of wine (Balikci, Tanguler, Jolly, & Erten, 2016; Porter, Divol, & Setati, 2019). The content of lactic acid further increased after MLF, ranging from 3.02 g/L (D254) to 4.58 g/L (Concerto/D254). It should be noted that although having less amount after ALF, lactic acid in *T. delbruecki/S. cerevisiae* (4.25–4.26 g/L) became comparable with *L. thermotolerans/S. cerevisiae* (4.23–4.58 g/L) after MLF. This implied that the interaction between *T. delbrueckii* strain and lactic acid bacteria might favor lactic acid formation, or specific intermediates produced by *T. delbrueckii* strain could be transformed to lactic acid. The detailed mechanism needs to be further investigated.

3.3. Aromatic quality of wines fermented by different yeasts after ALF and MLF

HS-SPME-GC-MS was applied to detect the volatile compounds of red wines inoculated with different *T. delbrueckii* and *L. thermotolerans* strains in this study. Thirty-one aroma compounds in wines after ALF and MLF were identified and quantified (Table 2 and Table 3). The compounds with odor activity value (OAV) greater than one were highlighted, and the compounds of OAV > 0.1 were underlined.

The higher alcohols were the largest group of aroma compounds in this study, the concentration of 300–400 mg/L in wine is acceptable, and

Table 1
The contents of principal products of the wines fermented by different strains after alcoholic fermentation (ALF) and malolactic fermentation (MLF); F value and P value of main products were analyzed by one-way ANOVA.

	Compounds	D254	Prelude/D254	Concerto/D254	TD12/D254	LT9/D254	F value	P value
ALF	Glucose (g/L)	0.28 ± 0.05b	0.15 ± 0.01a	0.16 ± 0.04a	0.26 ± 0.06b	0.28 ± 0.05b	7.863	0.001
	Fructose (g/L)	2.04 ± 0.24a	2.04 ± 0.53a	2.31 ± 0.48ab	2.72 ± 0.08b	2.71 ± 0.2b	3.748	0.026
	Glycerol (g/L)	8.12 ± 0.32b	7.01 ± 0.76a	6.95 ± 0.22a	9.05 ± 0.2c	8.1 ± 0.2b	19.252	<0.001
	Ethanol (%v/v)	14.45 ± 1.31b	13.62 ± 0.08ab	13.36 ± 0.4a	14.23 ± 0.36ab	13.53 ± 0.29ab	2.127	0.128
	Malic acid (g/L)	2.35 ± 0.46ab	2.45 ± 0.38ab	2.16 ± 0.14a	3.26 ± 0.12c	2.76 ± 0.08b	9.260	0.001
	Lactic acid (g/L)	0.66 ± 0.26b	0.38 ± 0.2a	1.49 ± 0.12d	0.49 ± 0.13ab	1.18 ± 0.06b	32.177	<0.001
	Acetic acid (g/L)	0.54 ± 0.08c	0.21 ± 0.03a	0.24 ± 0.05a	0.21 ± 0.02a	0.37 ± 0.06b	30.512	<0.001
MLF	Fructose (g/L)	1.02 ± 0.59a	0.5 ± 0.01a	0.3 ± 0.06a	0.43 ± 0.12a	0.57 ± 0.02a	2.041	0.227
	Glycerol (g/L)	8.69 ± 0.16b	9.01 ± 0.14b	7.95 ± 0.05a	9.73 ± 0.13c	9.53 ± 0.26c	37.707	0.001
	Ethanol (%v/v)	15.32 ± 0.16c	15.42 ± 0.04c	13.95 ± 0.06a	14.94 ± 0.13b	15.26 ± 0.03c	75.275	<0.001
	Lactic acid (g/L)	3.02 ± 0.12a	4.26 ± 0.01b	4.58 ± 0.06b	4.25 ± 0.18b	4.23 ± 0.23b	35.248	0.001
	Acetic acid (g/L)	0.57 ± 0.1a	0.51 ± 0.06a	0.51 ± 0.01a	0.56 ± 0.08a	0.6 ± 0.11a	0.481	0.751

Values are given as mean ± standard deviation of three biological replicates and two HPLC detection runs. (a, b, c, d, e) represents significantly different statistical groups ($p < 0.05$) using the data after alcoholic fermentation and malolactic fermentation, respectively.

Table 2
The contents of thirty-one volatile compounds of the wines produced by different strains after alcoholic fermentation; F value and P value of aroma compounds were analyzed by one-way ANOVA.

Compounds (μg/L)	D254	Prelude/D254	Concerto/D254	TD12/D254	LT9/D254	F value	P value
1-Hexanol	**3883.59 ± 147.13b**	**3245.57 ± 89.87a**	**3265.97 ± 81.53a**	**4733.83 ± 115.83c**	**4542.29 ± 239.82c**	67.538	<0.001
Isobutyl alcohol	32239.58 ± 1334.1b	24835.61 ± 3345.94a	21269.32 ± 1111.61a	28913.34 ± 1004.04b	24089.12 ± 2271.68a	13.698	<0.001
Isoamyl alcohol	**167743.02 ± 11572.48b**	**151842.72 ± 10191.81ab**	**137589.33 ± 8198.61a**	**197874.98 ± 12585.69c**	**164345.06 ± 2475.89b**	16.028	<0.001
2,3-Butanediol	111215.2 ± 2242.45b	105875.3 ± 1285.78a	114950 ± 2148.92b	139531.1 ± 2563.87c	130310.2 ± 1896.39c	5919287	<0.001
1-Octanol	13.62 ± 0.93c	10.03 ± 1.41b	7.22 ± 0.31a	22.42 ± 1.21e	20.18 ± 1.51d	94.268	<0.001
1-Decanol	3.35 ± 0.03a	3.34 ± 0.1a	3.37 ± 0.07a	4.08 ± 0.22b	4.37 ± 0.16c	41.043	<0.001
Benzyl alcohol	16109.36 ± 2149.44b	15493.54 ± 727.35b	9257.79 ± 1130.95a	15595.42 ± 583.54b	10512.17 ± 1710.27a	16.263	<0.001
2-Phenylethyl alcohol	**53202.98 ± 7725.3a**	**71088.75 ± 3911.39c**	**56548.31 ± 7924.3ab**	**84707.01 ± 6389.57d**	**66573.21 ± 6464.95bc**	10.692	0.001
Total of higher alcohols	384410.7 ± 18665.87b	372394.87 ± 17414.07ab	342891.31 ± 18018.44a	471382.19 ± 19896.22c	400396.6 ± 8012.18b	24.017	<0.001
Ethyl butanoate	**571.72 ± 50.3b**	**713.84 ± 21.15c**	**424.44 ± 13.64a**	**1127.7 ± 45.61d**	**756.03 ± 41.07c**	149.829	<0.001
Ethyl hexanoate	**1460.15 ± 122.94b**	**1343.7 ± 27.05b**	**1040.55 ± 53.68a**	**2512.5 ± 59.23d**	**1764.69 ± 42.62c**	195.674	<0.001
Ethyl heptanoate	0.74 ± 0.04a	0.92 ± 0.02bc	0.81 ± 0.02ab	1.32 ± 0.14d	0.95 ± 0.06c	29.501	<0.001
Ethyl octanoate	**2537.79 ± 179.09c**	**1468.4 ± 64.76b**	**1155.54 ± 47.06a**	**4297.72 ± 222.74d**	**2645.28 ± 140.76c**	211.812	<0.001
Ethyl nonanoate	1.26 ± 0.07a	1.25 ± 0.08a	1.17 ± 0.03a	1.51 ± 0.03c	1.38 ± 0.04b	19.368	<0.001
Ethyl decanoate	**553.82 ± 35.87b**	**379.15 ± 20.11a**	**347.83 ± 19.41a**	**994.85 ± 14.44d**	**664.39 ± 47.23c**	227.867	<0.001
Ethyl phenylacetate	–	–	0.18 ± 0.13a	0.61 ± 0.11b	0.64 ± 0.09b	42.062	<0.001
Ethyl dodecanoate	36.99 ± 2.25b	31.69 ± 1.39a	28.58 ± 1.79a	85.49 ± 4.27d	54.92 ± 2.55c	237.914	<0.001
Isoamyl acetate	**853.42 ± 115.31a**	**1314.52 ± 108.18b**	**837.56 ± 28.82a**	**1241.55 ± 76.6b**	**952.59 ± 131.04a**	15.303	<0.001
Hexyl acetate	8.7 ± 1.64a	15.09 ± 2.01b	7.08 ± 0.73a	19.86 ± 2.41c	15.18 ± 3.2b	17.667	<0.001
2-Phenylethyl acetate	13.42 ± 2.35a	23.3 ± 3.61c	18.48 ± 0.96b	19.71 ± 1.82bc	18.73 ± 1.77b	7.25	0.005
Ethyl acetate	**155274.99 ± 4844.3d**	**110221.65 ± 8925.97b**	**81197.93 ± 3068.01a**	**163305.87 ± 6451.87d**	**124867.59 ± 10648.21c**	63.09	<0.001
Methyl octanoate	6.18 ± 0.42d	3.59 ± 0.14b	2.84 ± 0.08a	8.57 ± 0.37e	5.49 ± 0.12c	219.607	<0.001
Isoamyl octanoate	5.59 ± 0.32c	4.15 ± 0.13b	3.53 ± 0.18a	11.45 ± 0.25e	6.14 ± 0.49d	323.077	<0.001
Isopentyl hexanoate	5.76 ± 0.37c	5.15 ± 0.13b	4.06 ± 0.1a	11.07 ± 0.5e	6.57 ± 0.25d	232.516	<0.001
Diethyl succinate	223.58 ± 29.04b	153.42 ± 7.24a	134.77 ± 12.16a	525.21 ± 15.23d	330.28 ± 34.85c	154.746	<0.001
Total of esters	161554.09 ± 5338.84d	115679.81 ± 9104.07b	85205.36 ± 3158.86a	174164.99 ± 6658.03d	132090.84 ± 10974.99c	66.736	<0.001
Hexanoic acid	257.65 ± 25.86cd	157.49 ± 83.4ab	85.2 ± 36.87a	308.71 ± 15.01d	191.76 ± 23.48bc	11.603	0.001
Octanoic acid	**534.77 ± 58.38b**	472.5 ± 40.94ab	388.34 ± 22.04a	**743.82 ± 78.28c**	**526.04 ± 76.17b**	14.804	<0.001
Decanoic acid	82.06 ± 2.65ab	91.2 ± 3.21b	73.91 ± 6.7a	120.72 ± 13.85d	96.7 ± 6.83c	15.83	<0.001
Total of volatile acids	874.48 ± 85.4b	721.18 ± 120.93ab	547.46 ± 65.52a	1173.26 ± 106.45c	814.5 ± 104.79b	16.357	<0.001
Citronellol	3.84 ± 0.40a	3.66 ± 0.41a	4.02 ± 0.48a	3.68 ± 0.16a	3.48 ± 0.45a	0.789	0.558
Linalool	10.83 ± 0.18a	11.27 ± 0.12b	11.28 ± 0.21b	13.32 ± 0.08c	14.79 ± 0.11d	862.701	<0.001
β-Damascenone	**4.56 ± 0.12a**	**5.24 ± 0.08b**	**5.68 ± 0.05c**	**6.38 ± 0.10e**	**6.01 ± 0.12d**	186.165	<0.001
Geraniol	11.89 ± 0.14a	14.91 ± 0.05b	14.99 ± 0.15b	17.91 ± 0.01c	18.44 ± 0.05d	3478.07	<0.001

Values are given as mean ± standard deviation. Data with different letters (a, b, c, d, e) within each column are different according to Duncan tests (0.05%). Aroma compounds of OVA>1 were highlighted; aroma compounds of OVA>0.1 were underlined.

below 300 mg/L gives a pleasant character (Rapp & Versini, 1995). In this work, the total concentrations of higher alcohols are ranged in 355.39 mg/L (Concerto/D254) to 453.53 mg/L (TD12/D254) after ALF and 474.30 mg/L (Concerto/D254) to 538.77 mg/L (Prelude/D254) after MLF, respectively. Isoamyl alcohol (fatty and chemical notes), 2, 3-butanediol (fruity and sweet notes), 2-phenylethyl alcohol (flowery and sweet notes), isobutyl alcohol (green and chemical notes), and 1-hexanol (herbaceous note) are the main higher alcohols of the red wines. D254 single fermentation produced a higher concentration of isobutyl alcohol than those of the mixed culture trials. TD12/D254 sequential fermentation had a positive effect on 1-hexanol, isoamyl alcohol, 2,3-butanediol and 2-phenylethyl alcohol formation, with 121.89%, 117.96%, 125.46% and 159.21% increment compared to D254 single fermentation, respectively. In particular, the trials involving

Table 3
The contents of thirty-one volatile compounds of the wines produced by the mixed cultures after malolactic fermentation; F value and P value of aroma compounds were analyzed by one-way ANOVA.

Compounds (μg/L)	D254	Prelude/D254	Concerto/D254	TD12/D254	LT9/D254	F value	P value
1-Hexanol	**4071.95 ± 344.94bc**	**3268.34 ± 203.70a**	**3827.52 ± 31.62b**	**4485.96 ± 116.95d**	**4323.28 ± 31.86cd**	19.388	<0.001
Isobutyl alcohol	41352.42 ± 2686.09c	51129.19 ± 3765.92d	32259.97 ± 194.30a	39367.64 ± 1755.84bc	36492.60 ± 2466.33ab	24.292	<0.001
Isoamyl alcohol	**215681.1 ± 20256.07a**	**224554.79 ± 11140.43a**	**203915.6 ± 511.34a**	**210011.04 ± 3871.44a**	**206868.99 ± 8664.11a**	1.6	0.249
2,3-Butanediol	104542.02 ± 1245.21b	100014.30 ± 2145.28a	109945.05 ± 2298.32c	125553.45 ± 2893.12d	130001.22 ± 1986.35e	8008.999	<0.001
1-Octanol	14.77 ± 2.52b	5.3 ± 1.31a	14.64 ± 0.93b	20.41 ± 1.16c	18.13 ± 1.01c	44.171	<0.001
1-Decanol	3.8 ± 0.1b	3.32 ± 0.08a	4.75 ± 0.18d	4.11 ± 0.06c	4.14 ± 0.09c	67.348	<0.001
Benzyl alcohol	40871.41 ± 3637.23c	33727.83 ± 3265.55bc	22673.89 ± 4445.67a	26598.83 ± 6211.6ab	28167.54 ± 5945.72ab	6.373	0.008
2-Phenylethyl alcohol	**103589.34 ± 4713.43a**	**126069.97 ± 14256.83a**	**101657.46 ± 17052.45a**	**91652.91 ± 19692.79a**	**111974.02 ± 24849.71a**	1.637	0.240
Total of higher alcohols	510126.98 ± 30308.99ab	538773.04 ± 25552.72b	474298.88 ± 22083.78a	497694.35 ± 25459.16ab	517849.92 ± 36531.14ab	2.125	0.152
Ethyl butanoate	**643.7 ± 68.39b**	**757.71 ± 74.72c**	**495.29 ± 3.11a**	**1022.21 ± 53.91d**	**806.55 ± 22.5c**	42.044	<0.001
Ethyl hexanoate	**1444.11 ± 161.29bc**	**1075.65 ± 146.87a**	**991.95 ± 6.07a**	**1580.09 ± 133.1c**	**1326.24 ± 37.76b**	13.693	<0.001
Ethyl heptanoate	0.94 ± 0.08a	0.89 ± 0.31a	1.01 ± 0.01a	0.85 ± 0.08a	0.87 ± 0.03a	0.524	0.720
Ethyl octanoate	**4390.88 ± 448.18b**	**2562.01 ± 340.14a**	**2446.16 ± 51.11a**	**4435.78 ± 363.54b**	**4173.12 ± 31.72b**	33.668	<0.001
Ethyl nonanoate	2.42 ± 0.12a	3.16 ± 0.38b	4 ± 0.06c	2.17 ± 0.19a	3.66 ± 0.07c	43.862	<0.001
Ethyl decanoate	**1662.56 ± 136.24b**	**1160.95 ± 137.09a**	**1189.34 ± 42.95a**	**1655.69 ± 93.14b**	**1627.76 ± 13.46b**	21.082	<0.001
Ethyl phenylacetate	0.5 ± 0.25a	0.35 ± 0.22a	1.39 ± 0.23b	1.07 ± 0.28b	1.38 ± 0.18b	13.403	0.001
Ethyl dodecanoate	118 ± 8.27c	97.35 ± 12.57ab	84.57 ± 5.89a	106.08 ± 1.98bc	96.2 ± 3.72ab	8.33	0.003
Isoamyl acetate	**664.18 ± 77.64a**	**996.02 ± 119.32c**	**756.6 ± 6.32ab**	**874.53 ± 61.8bc**	**918.66 ± 25.19c**	10.537	0.001
Hexyl acetate	11.29 ± 1.42b	10.12 ± 1.61b	6.21 ± 0.15a	14.48 ± 1.54c	16.36 ± 0.3c	32.908	<0.001
2-Phenylethyl acetate	13.71 ± 0.86a	24.22 ± 2.64c	20.23 ± 1.43b	19.02 ± 1.49b	23.43 ± 0.28c	21.828	<0.001
Ethyl acetate	**219574.04 ± 5602.09c**	**177375.74 ± 15974.58b**	**125863.43 ± 438.77a**	**194228.16 ± 10945.22b**	**183153.69 ± 4380.36b**	41.54	<0.001
Methyl octanoate	9.4±1c	4.43 ± 0.75a	5.16 ± 0.06a	7.64 ± 0.71b	7.15 ± 0.2b	28.257	<0.001
Isoamyl octanoate	14.08 ± 1.02bc	12.29 ± 1.65ab	11.87 ± 0.48a	14.35 ± 0.86c	12.72 ± 0.26abc	3.792	0.040
Isopentyl hexanoate	10.28 ± 0.89c	8.6 ± 1.05ab	7.51 ± 0.1a	10.46 ± 0.79c	9.23 ± 0.13bc	8.809	0.003
Diethyl succinate	773.95 ± 42.88c	445.75 ± 33.14b	284.59 ± 42.72a	446.88 ± 61.72b	474.35 ± 64.27b	37.422	<0.001
Total of esters	229334.02 ± 6535.27d	184535.24 ± 16834.02b	132169.29 ± 437.31a	204419.46 ± 11564.55c	192651.38 ± 4533.79bc	40.005	<0.001
Hexanoic acid	**479.19 ± 50.9b**	355.85 ± 5.91ab	282.35 ± 37.41a	342.6 ± 141.88ab	360.6 ± 76.74ab	2.555	0.104
Octanoic acid	**1224.61 ± 132.13b**	**683.86 ± 21.64a**	**796.08 ± 103.74a**	**861.05 ± 255.07a**	**814.7 ± 204.02a**	4.676	0.022
Decanoic acid	151.3 ± 28.13b	111.07 ± 7.25a	141.23 ± 5.65ab	118.91 ± 19.04ab	115.66 ± 18.98a	2.907	0.078
Total of volatile acids	1855.1 ± 198.10b	1150.78 ± 20.27a	1219.66 ± 146.18a	1322.56 ± 411.77a	1290.96 ± 299.47a	3.682	0.043
Citronellol	5.23 ± 1.14c	2.70 ± 0.32a	4.23 ± 0.58bc	3.17 ± 0.31ab	3.25 ± 0.39ab	7.752	0.004
Linalool	11.51 ± 0.04a	11.54 ± 0.10a	11.48 ± 0.21a	13.62 ± 0.14b	15.3 ± 0.12b	8.892	0.002
β-Damascenone	**2.5 ± 0.21a**	**2.28 ± 0.08a**	**2.38 ± 0.13a**	**3.25 ± 0.09b**	**2.49 ± 0.03a**	29.143	<0.001
Geraniol	11.00 ± 0.14a	14.21 ± 0.11b	14.86 ± 0.05b	17 ± 0.01c	18.74 ± 0.01c	25.875	<0.001

Values are given as mean ± standard deviation. Data with different letters (a, b, c, d, e) within each column are different according to Duncan tests (0.05%). Aroma compounds of OVA>1 were highlighted; aroma compounds of OVA>0.1 were underlined.

T. delbrueckii yeasts produced significantly higher level of 2-phenylethyl alcohol than D254 single fermentation, with 1.59-fold of TD12/D254 and 1.34-fold of Prelude/D254, which was in agreement with the previous data (Canonico et al., 2017; Chen & Liu, 2016). It should be noticed that the significantly increased levels of higher alcohols (OAV>0.1) were produced by indigenous *T. delbrueckii* and *L. thermotolerans* strains compared to commercial non-*Saccharomyces* strains, respectively, especially in TD12/D254 wine, 30.31% and 19.16% increment of isoamyl alcohol and 2-phenylethanol were observed compared to Prelude/D254 wine, which may be due to the different genetic characterizations between indigenous and commercial non-*Saccharomyces* strains. After MLF, the highest amount of 1-hexanol was obtained in TD12/D254, however, more isobutyl alcohol, isoamyl alcohol, benzyl alcohol and 2-phenylethyl alcohol were produced in the wine of Prelude/D254. The diversities of higher alcohols (expected for isobutyl alcohol) analyzed by F values of one-way ANOVA in all treatments were decreased when compared to ALF. Those results confirmed that lactic acid bacteria could result in greater modification of high alcohols in wines experienced the mixed starters in ALF (Zhang, Luan, Duan, & Yan, 2018).

A total of sixteen esters were detected in this study, principally comprising ethyl acetate and ethyl octanoate. The total concentrations of esters are ranged in 85.21 mg/L (Concerto/D254) to 174.16 mg/L (TD12/D254) after ALF and 132.17 mg/L (Concerto/D254) to 229.33 mg/L (D254) after MLF, respectively. It is interesting to note that the ester profiles amongst treatments are significantly different ($p < 0.05$). TD12/D254 was characterized by higher intensities of ethyl esters (OAV>0.1), including ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate, contributing to the wine with desirable and fruity sensory properties, including banana, strawberry and green apple (Cai et al., 2014). Prelude/D254 was distinguished by the greater abundance of isoamyl acetate, responsible for the banana note. Our results are in agreement with the prior literature that co-fermentation of *T. delbrueckii* and *S. cerevisiae* can produce a high concentration of esters (Renault et al., 2015), however, this property is largely strain-specificity (Loira et al., 2014). The significant divergences of esters also appeared in *L. thermotolerans/S. cerevisiae* trials, and LT9/D254 produced a higher amount of most esters than Concerto/D254. After MLF, TD12/D254 wine still contained the highest level of ethyl butanoate, ethyl hexanoate and ethyl octanoate, and Prelude/D254 wine had the maximum concentration of isoamyl acetate. Meanwhile, both TD12/D254 and LT9/D254 sequential fermentation favored the production of ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate, especially ethyl octanoate; their levels were increased by 73.14% and 70.60% compared to those produced by Prelude/D254 and Concerto/D254, respectively. The different responses were found in isoamyl

acetate, in which Prelude/D254 produced a 13.89% higher level than TD12/D254. The similar tendency of esters between the experimental groups after ALF and MLF indicated that non-*Saccharomyces* strains could significantly influence the content of esters compounds in final wines. Lactic acid bacteria decreased the diversities of the most ester compounds in all treatments after MLF, mainly including ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate.

Volatile fatty acids are formed by yeasts during fatty acid metabolism, which contributes rancid, pungent, fruity, or cheesy odors to wine when above their thresholds (Swiegers & Pretorius, 2005). Three volatile fatty acids (hexanoic acid, octanoic acid and decanoic acid) were detected in this work, in which TD12/D254 produced the maximum levels of those fatty acids, followed by D254 and LT9/D254. The contents of fatty acids were increased by MLF, and D254 single fermentation produced the highest increment. Contrary to ALF, no significant differences appeared in hexanoic acid and decanoic acid after MLF, suggesting that lactic acid bacteria might have an important impact on fatty acids metabolism in final wines.

Four compounds belonging to the terpene group were identified in this study, i.e., citronellol, linalool, geraniol and β-damascenone. The content of linalool (sweet and floral note), geraniol (floral note), and β-damascenone (sweet and floral note) was enhanced by the mixed fermentation when compared to D254 single fermentation. Among them, TD12/D254 and LT9/D254 generated more terpenes than commercial non-*Saccharomyces* trials. After MLF, β-damascenone level was significantly decreased, especially in the wine of LT9/D254 (only half of the value of alcoholic fermentation). TD12/D254 had the highest intensity of β-damascenone, and LT9/D254 resulted in a larger yield of linalool and geraniol in final wines, whose intensities were 33.28% and 26.11% higher than those in Concerto/D254, respectively.

3.4. Multivariate analysis

To visualize the differences of aroma composition produced by different inoculations, PCAs (principal component analysis) were applied using nineteen main aromatic compounds (OAV>0.1) after ALF (Fig. 3A) and MLF (Fig. 3B). The first and second accounted for 68.1% (PC1) and17.9% (PC2) of the total variation after alcoholic fermentation, and 37.6% (PC1) and 28.0% (PC2) after malolactic fermentation, respectively. For ALF, the pure fermentation of D254 was separated by PC2 from the mixed fermentation trials. The wines inoculated of commercial non-*Saccharomyces* strains located in the negative part of PC1, and the wines of TD12/D254 and LT9/D254 were loaded in the positive part of PC1. The main separated compounds between TD12/D254 and Prelude/D254 was most esters (ethyl butanoate, ethyl octanoate, ethyl hexanoate and ethyl decanoate) and volatile fatty acids (octanoic acid and decanoic acid), whereas in LT9/D254 and Concerto/D254 wines was isoamyl acetate, which further confirmed that the volatiles characteristics of wine inoculated with indigenous and commercial non-*Saccharomyces* strains were distinct. After MLF, the wine composition was further varied. The wine of D254 pure culture was in the first quadrant, characterized with benzyl alcohol, isoamyl alcohol, hexanoic acid, octanoic acid and decanoic acid. The differences of wine components inoculated with indigenous and commercial non-*Saccharomyces* strains were clear as well, and principal aroma compounds were 2-phenylethyl alcohol, isobutyl alcohol, isoamyl acetate and ethyl butanoate.

3.5. Wine odor profile

An aromatic series of wines could be defined as a group of aroma compounds with similar odor descriptors (Duan et al., 2015; Liu et al., 2016). In this study, six aromatic series were established by the combination of OAVs of a group of active aroma compounds (OAV>0.1) with similar aroma type to better understand the influence of different non-*Saccharomyces* strains on wine odor profile, including fruity, floral, sweet, herbaceous, rancid, and solvent (Fig. 4, Supplementary Table 1). A fruity attribute was prominent in six aromatic series, followed by floral, sweet, herbaceous, solvent, and rancid series. The sequential fermentation trials after ALF had improvement effects on fruity, floral and sweet attributes compared to D254 single fermentation excepted for Prelude/D254 and Concerto/D254 on the fruity aroma. Among them, the wine from TD12/D254 sequential fermentation obtained the highest values of fruity, sweet and floral aroma series due to the increased contents of 2-phenylethyl alcohol, ethyl esters, β-damascenone and fatty acids. There were no considerable discrepancies in the herbaceous, rancid, and solvent aroma series among different treatments, which are due to the low concentration or high odor threshold of related aroma compounds. After MLF, the divergences of aroma series among different wines were decreased, and TD12/D254 still scored the high aromatic quality of fruity, sweet and floral. These results indicated that TD12/D254 can intensify the pleasant aroma attributes of wine, and confirmed that indigenous strains with excellent enological characteristics have more potential to improve the aroma profiles of regional wines compared to extraneous strains (Nisiotou et al., 2018).

In recent years, co-inoculating non-*Saccharomyces* strains with *S. cerevisiae* received increasing attention in the winemaking industry because it can produce wine products with distinctive and diversified aroma expected by consumers. To data, there are more than twenty-six non-*Saccharomyces* strains have been commercialized (Roudil et al., 2020). However, how to choose suitable non-*Saccharomyces* strains paired with *S. cerevisiae* becomes a difficult issue for winemakers when aims to produce wine products with distinctive and regional characteristics. In previous studies, inoculating autochthonous yeast is strongly recommended (Calabretti et al., 2012). The results of the present study well supported this conclusion. The autochthonous TD12 combined with D254 generated distinct aromatic characteristics (high concentrations of ethyl esters, higher alcohols, linalool and β-damascenone) in comparison with the pair of commercial strain *T. delbrueckii* and D254, which was featured by high production of acetate esters (especially isoamyl acetate). Both LT9 and Concerto strains co-fermented with D254 could generate a high amount of lactic acid compared to pure fermentation, but LT9/D254 had obviously higher aroma intensity than the wine produced by Concerto/D254. The lower fermentation rates of Prelude/D254 and Concerto/D254 relative to TD12/D254 and LT9/D254, respectively, verified the previous reports that the commercial strains cannot well adapt to the micro-conditions of the wine region and grape must composition (Rainieri & Pretorius, 2000), which could partly explain the differences of wines produced by different non-*Saccharomyces* mixed inoculations. In addition, the diverse genetic characteristics of indigenous and commercial non-*Saccharomyces* yeasts, although belonging to the same species, are also responsible for the high divergences of wine compositions. Actually, there is a reported study have revealed a wide phenotypic variability existing in *T. delbrueckii* strain, such as volatile acidity (0.01–0.57 g/L), glycerol (3.30–6.03 g/L), higher alcohols (56–133 mg/L), esters (3.15–3.45 mg/L) and volatile fatty acids (1.51–3.16 mg/L) (van Breda et al., 2013). Our data well demonstrate these results from the viewing of the mixed inoculated cultures. To explain the different enological and aromatic properties of the same species strains, it is necessary to compare the transcriptional and metabolic profiles under single fermentation and mixed fermentation conditions.

After MLF, the chemical and aromatic compositions of wines produced by different mixed fermentations were further modified. For example, the production of glycerol, acetic acid, lactic acid, higher alcohols, ethyl esters, and volatile fatty acids was enhanced, while acetate esters and β-damascenone were attenuated compared to after ALF. Meanwhile, the diversity of most aroma compounds between different treatments were decreased after MLF, which attenuated the improvement effects of mixed fermentation compared to the single fermentation of *S. cerevisiae*. These results indicated that the differences of nutrition status in wines after ALF and (or) the interaction of yeast and lactic acid bacteria could further influence the formations of primary and

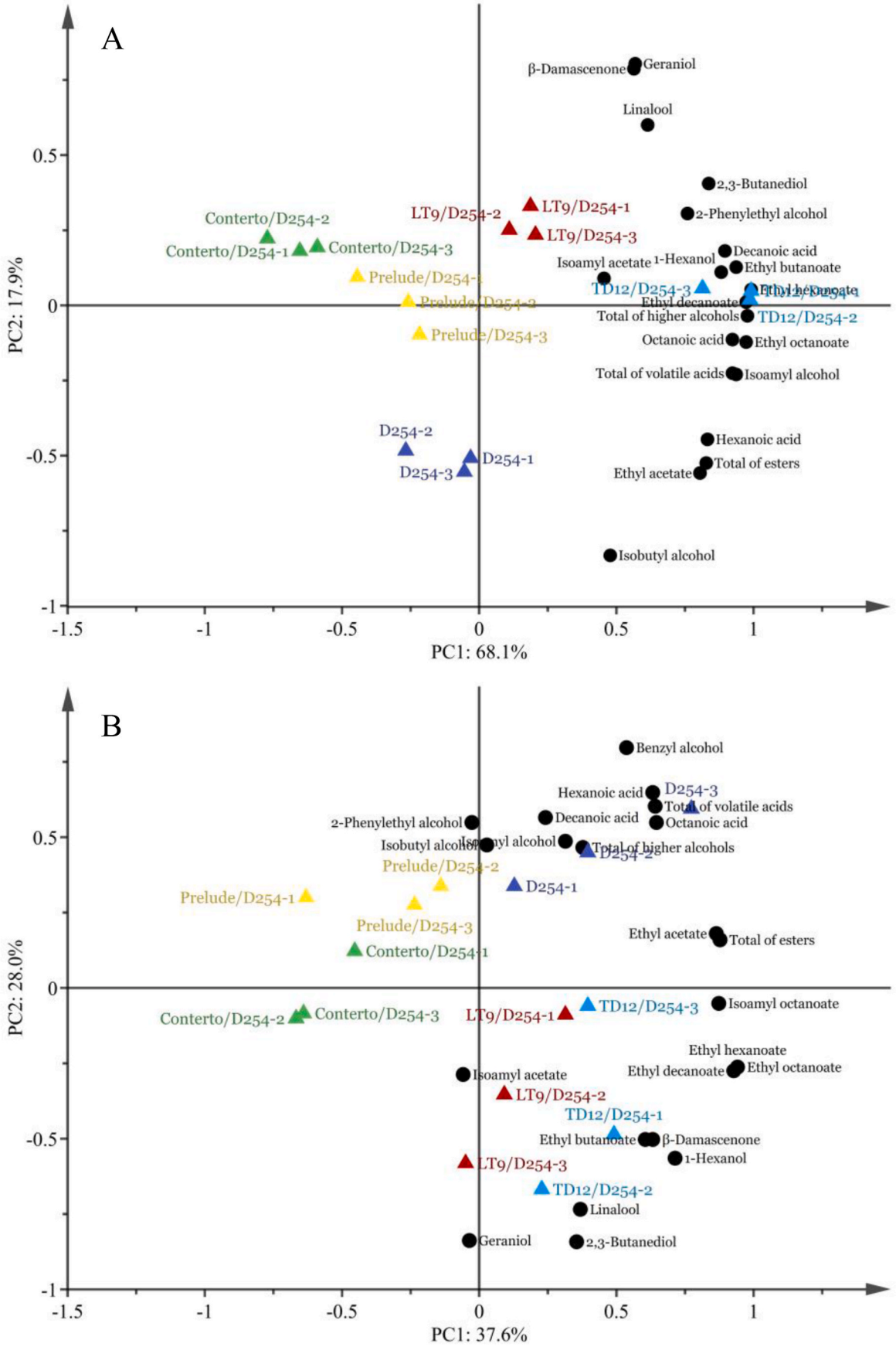

Fig. 3. PCA biplots performed on aromatic compounds (OAV>0.1) produced by different strains after alcoholic fermentation (A) and malolactic fermentation (B), respectively.

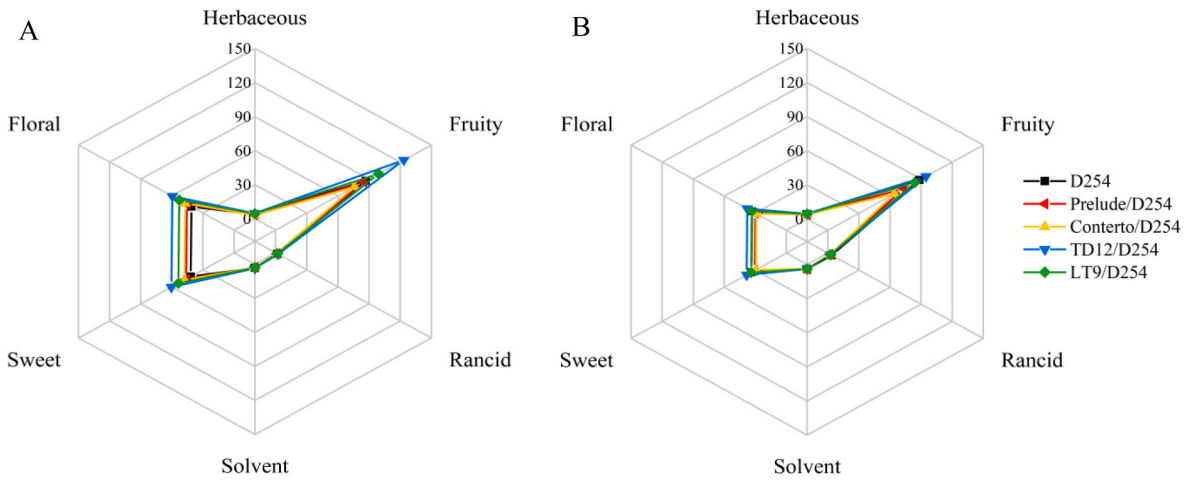

Fig. 4. Odor profile of wines produced by different strains after alcoholic fermentation (A) and malolactic fermentation (B) based on OAVs, respectively.

secondary compounds in final wines, and also suggested that the winemakers should take more considerations when choosing a suitable inoculated combination of yeasts and lactic acid bacteria to improve the quality of regional wines.

4. Conclusions

In this work, the enological traits of indigenous strains (*T. delbrueckii* TD12 and *L. thermotolerans* LT9) and commercial strains (*T. delbrueckii* Prelude and L. *thermotolerans* Concerto) in mixed fermentation with *S. cerevisiae* D254 were evaluated and compared in 20 L fermenter, respectively. The compositions of wines produced by indigenous and commercial strains were significantly different although belonging to the same species. TD12/D254 was characteristic with high contents of ethyl esters, higher alcohol, linalool, β-damascenone, and lactic acid, while Prelude/D254 was featured with the highest concentration of acetate esters (mainly isoamyl acetate) and a low amount of acetic acid. LT9/D254 produced more ethyl esters, acetate esters and comparable level of lactic acid relative to Concerto/D254. The divergences of aromatic properties in wines were reduced after MLF, and TD12/D254 still scored high aromatic quality. Our results highlighted the importance of non-*Saccharomyces* strains in shaping the aromatic quality of wine in mixed fermentation, and suggested that their enological properties are largely strain-specificity, which usually lead to distinctive basic parameters and aroma profiles of the final wines. Thus, more attention should be paid to the strain level when selecting non-*Saccharomyces* strains in multi-starters wine fermentation. In this regard, the indigenous non-*Saccharomyces* strains are highly recommended because they not only improve aroma quality and complexity due to better adapt to fermentation conditions, but also impart the wines with typical sensory characteristics specific to each wine area. Due to the red wine quality is determined not only by chemical and aromatic composition, but also by the appearance, structure and taste involving polyphenol substances, more extensive research and large-scale co-fermentation experiments need to be conducted to further confirm the beneficial properties of the both indigenous strains in red wine fermentation, and this is underway in our lab.

Declaration of competing interest

The authors confirm that there are no conflicts of interest in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2021.100925.

Author statement

Designed the experiments: C-QD, V-IP and G-LY. Conducted the experiments: B-QZ and G-LY. Analyzed the experimental data: B-QZ, V-IP and G-LY. Wrote the paper: B-QZ and G-LY.

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