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Enhancing blueberry wine quality and antioxidant capacity through mixed fermentation with *S. cerevisiae* and *O. oeni*

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This study investigates the impact of *O. oeni* on the quality of blueberry wine, employing various analytical methods to determine parameters such as alcohol content, antioxidant capacity, organic acids, amino acids, and volatile components. The results demonstrated that co-inoculation with *O. oeni* significantly decreased the titratable acidity of blueberry wine. Among the organic acids, the malic acid content of ST and SE groups decreased by (26.24 ± 1.24)% and (35.75 ± 4.02)%, respectively. The main consumed amino acids were aspartate, lysine, and arginine after co-inoculation with *O. oeni*. Additionally, co-inoculation with *O. oeni* significantly increased the content of medium-chain fatty acid ethyl esters, especially isoamyl acetate content. The antioxidant capacity of blueberry wine was significantly enhanced by co-inoculation with *O. oeni*, particularly in terms of measurement of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) clearance. The findings of this study provide a solid basis for improving the quality of blueberry wine and promoting the growth of blueberry wine industry.

Blueberry (*Vaccinium spp.*) is a perennial shrub fruit tree belonging to the Ericaceae family[1]. Blueberries are rich in vital nutrients, including vitamins, anthocyanins, phenolic acids, and other bioactive compounds. These nutrients offer significant health benefits such as neuroprotection, cardiovascular support, and immune enhancement[2]. Recognized by the International Blueberry Organization as one of the top five healthiest fruits globally, blueberries in China have a processing rate of 53.85%, surpassing the global average. However, most processed blueberry products on the market have low added value and limited competitiveness, including fruit juice, dried fruits, and jams. Fermenting blueberries into wine can address storage challenges and enhance the product's shelf life and value.

Currently, blueberry wine fermentation mainly uses commercially available brewing yeast strains[3]. The high organic acid content in blueberries often leads to elevated levels of malic and tartaric acids in the resulting wines, contributing to an excessively acidic taste profile. Therefore, it is essential to develop methods to reduce acidity in blueberry wine. The primary approaches for mitigating acidity in fruit wine are chemical, biological, and physical acid reduction[4]. Chemical methods can introduce excessive metal ions, causing wine turbidity[5]. Physical methods reduce organic acid content while adsorbing pigments, negatively affecting sensory quality[5]. Biological methods use lactic acid bacteria to facilitate malo-lactic fermentation (MLF), converting malic acid into lactic acid, thereby reducing acidity with minimal impact on wine quality and stability[5]. MLF also enhances aroma complexity and balances acidity and sweetness[6]. Various lactic acid bacteria conduct MLF with *O. oeni* being the most suitable strain due to its adaptability to low pH (<3.5), high alcohol concentration (>13% v/v), and a glucose/fructose environment in wines. Limited studies have explored lactic acid bacteria for acidity mitigation in fruit wines or juices, with most research focusing on apple juice, cider, and wine rather than blueberry wine. Therefore, investigating the influence of *O. oeni* on blueberry wine quality is crucial.

Many studies suggest that *O. oeni* OI can mitigate the sour taste of fruit during fermentation[7,8]. However, its impact on overall blueberry wine quality remains uncertain. This study employed simultaneous or sequential inoculated fermentation with *S. cerevisiae* FR and *O. oeni* OI to analyze various parameters, including ethanol content, titratable acidity, pH level, soluble solids, reducing sugar concentration, color intensity, anthocyanin content, total phenolic compounds, and flavonoids levels as well as in vitro antioxidant capacity. Additionally, organic acid profiles, amino acids, and volatile components were analyzed to investigate the influence of *O. oeni* on enhancing blueberry wine quality.

Result and discussion

Basic physical and chemical indices

Table 1 demonstrates the ethanol content, soluble solids, reducing sugars, titratable acids, pH values, and malic acids of blueberry wine under different inoculation groups. Compared to the SG group, the ST and SE groups exhibited a slight increase in the pH and a reduction in titratable acid. Titratable acids in the ST and SG groups were significantly lower than those

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Table 1 | Basic physical and chemical indices of blueberry wine

Indexes	Ethonal (%,v/v)	Soluble solid (°Bx)	Titrable acid (g/L)	pH	Reducing sugar (g/L)	Malic acid (mg/mL)
ST	12.05 ± 0.26^{b}	8.03 ± 0.06^{a}	8.33 ± 0.04^{b}	2.80 ± 0.01^{a}	22.62 ± 2.58^{b}	2.51 ± 0.08^{b}
SE	11.75 ± 0.13^{b}	6.97 ± 0.06^{b}	7.08 ± 0.06^{c}	2.81 ± 0.01^{a}	35.64 ± 2.57^{a}	2.18 ± 0.11^{c}
SG	13.63 ± 0.29^{a}	8.10 ± 0.10^{a}	10.25 ± 0.05^{a}	2.65 ± 0.02^{b}	38.43 ± 1.29^{a}	3.40 ± 0.06^{a}

Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR; Different lowercase letters in the same column in the table indicate significant differences ($p < 0.05$).

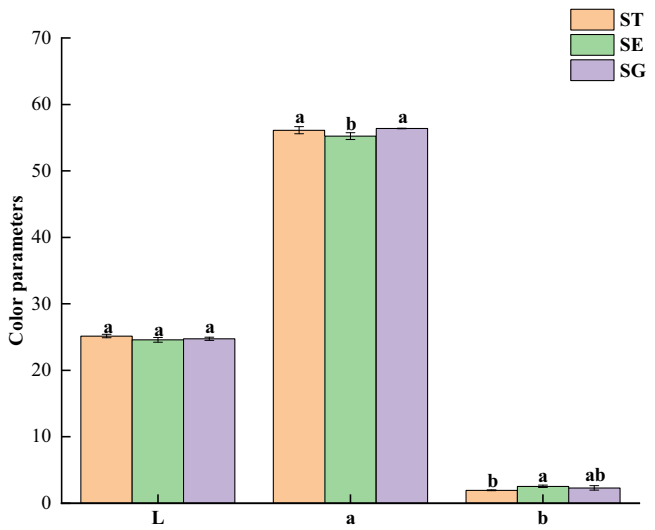

Fig. 1 | The color parameters of blueberry wine. Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR; L (lightness), a (green to red), and b (blue to yellow). Different lowercase letters in the figure indicate significant differences ($p < 0.05$).

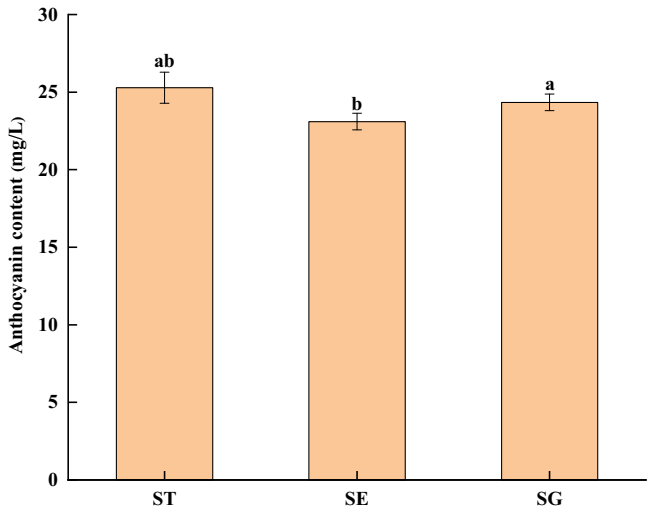

Fig. 2 | The anthocyanin of blueberry wine. Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR; Different lowercase letters in the figure indicate significant differences ($p < 0.05$).

in the SG group; indicating that *O. oeni* OI can effectively reduce the titratable acids through MLF. The titratable acid in the ST group (8.33 ± 0.04 g/L) was significantly higher than in the SE group (7.08 ± 0.06 g/L) ($p < 0.05$). This can be attributed to factors such as the difference in fermentation temperature, the gradual increase of ethanol content, the decrease of dissolved oxygen, the yeast concentration was higher than the bacteria concentration at the beginning of the fermentation, and interactions between strains inhibiting the growth of *O. oeni* OI during fermentation, affecting its acid-reducing ability[9,10]. Blueberry wine fermented by the ST and SE groups had significantly lower ethanol contents (12.05 ± 0.26% v/v and 11.75 ± 0.13% v/v) than the SG group. The addition of *O. oeni* OI likely inhibited the uptake or utilization of amino acids by *S. cerevisiae* FR, there by reducing yeast growthas, as well as the competition for sugars between the two strains, both affecting alcohol fermentation[11]. Reducing sugar in the three different treatment groups ranged from 22.62 ± 2.58 g/L to 38.43 ± 1.29 g/L, meeting the requirement for semi-sweet wine. The ST group's reducing sugar (22.62 ± 2.58 g/L) was significantly lower compared to the SE and SG groups ($p < 0.05$). This study confirmed that MLF can occur in the presence of fermentable sugars, leading to a significant reduction in reducing sugar during simultaneous alcohol fermentation and MLF[9,12–14]. Compared with the SG group, the malic acid content of the ST group and SE group decreased by (26.24 ± 1.24)% and (35.75 ± 4.02)%, respectively.

Color analysis

Chromaticity parameters, including L (lightness), a (green to red), and b (blue to yellow), which can be affected by several factors such as pH, processing and storage temperature, chemical structure, and pigment concentrations, were measured[15]. Figure 1 demonstrates no significant difference in L value between the three groups ($p > 0.05$). However, the a value of the SE group (55.24 ± 0.51) was significantly lower than the other two groups ($p < 0.05$). This result is related to the anthocyanin content and total phenols content in SE group in Figs. 2 and 3. This result aligns with previous studies reporting that acetaldehyde-producing *O. oeni* can restore color loss associated with MLF[16,17]. The b value of the SE group (2.54 ± 0.18) was significantly higher than that of the ST group (1.95 ± 0.05) ($p < 0.05$), possibly due to the pH increase during MLF, shifting anthocyanin towards a blue color[17].

Anthocyanin analysis

Anthocyanins contribute to multiple bioactivities, including anti-oxidation, anti-inflammation, anti-cardiovascular disease, anti-skin damage, and reproductive system protection[18]. As shown in Fig. 2, the anthocyanin content in the SE group (23.10 ± 0.53 mg/L) was significantly lower than in the SG group ($p < 0.05$), possibly due to cell adsorption by *O. oeni* OI and anthocyanin cleavage[19]. *O. oeni* OI can also release SO_2, which binds with anthocyanins, reducing their concentration[20]. Interestingly, there was no significant difference in anthocyanin content between the ST group (25.29 ± 1.00 mg/L) and the SG group (24.34 ± 0.54 mg/L) ($p > 0.05$), likely due to acetaldehyde promoting the synthesis of stable ethylene-linked pigments such as pyranoanthocyanins[21].

Analysis of total flavonoids and total phenols

The content of phenols and flavonoid compounds significantly impacts the color, aroma, taste, and antioxidant effect of wine[22,23]. As shown in Fig. 3, total flavonoids and phenols decreased with MLF, consistent with previous findings[24]. Total phenols in the ST group (715.93 ± 30.08 μg/mL) and SE group (690.35 ± 24.20 μg/mL) were significantly lower than in the SG group

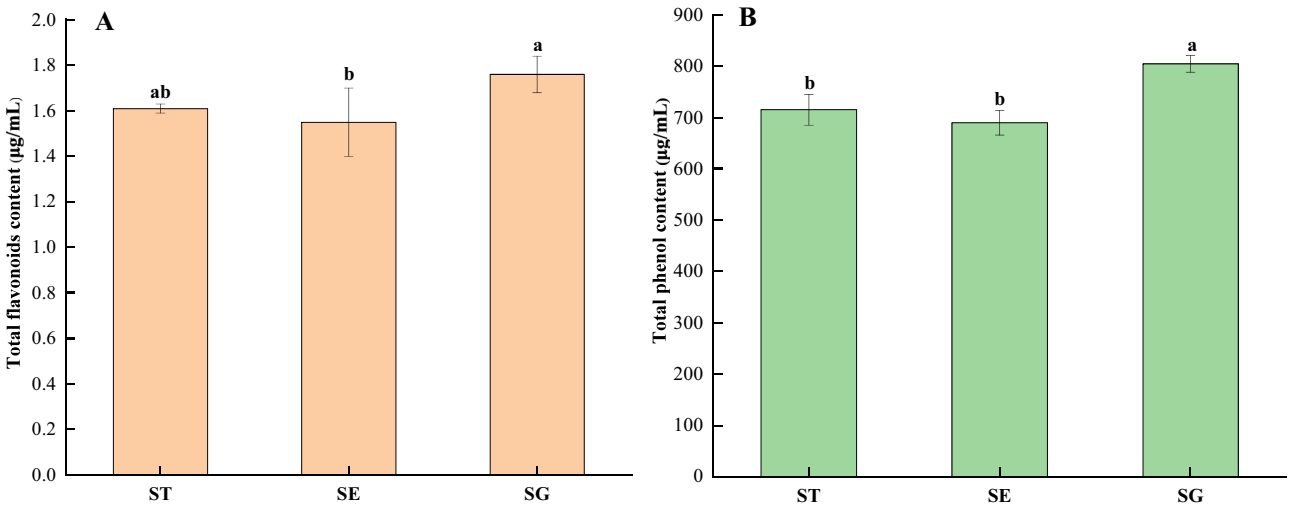

Fig. 3 | The total flavonoids and total phenols content of blueberry wine. The total flavonoids content (**A**) and total phenols content (**B**) of blueberry wine. Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR; Different lowercase letters in the figure indicate significant differences ($p < 0.05$).

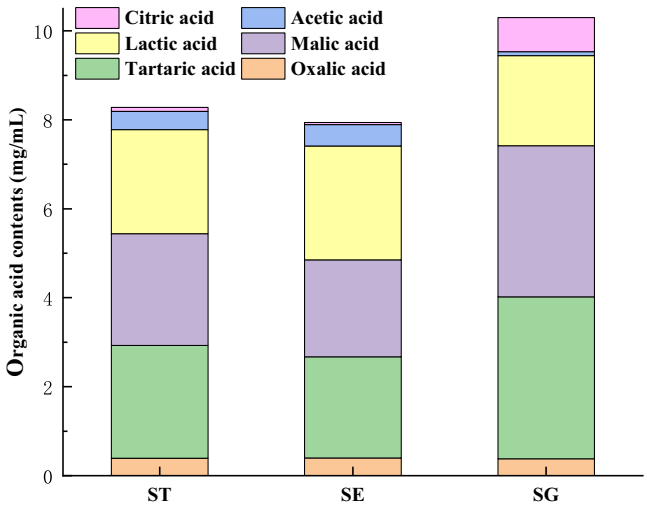

Fig. 4 | The organic acid content of blueberry wine. Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR.

(805.08 ± 18.83 μg/mL) ($p < 0.05$). The decrease in total flavonoids and phenols may be due to changes in ethanol and pH after adding *O. oeni* OI, leading to the precipitation or oxidation of phenolic substances and flavonoids[25]. Additionally, *O. oeni* OI can metabolize phenolic compounds by glucosidase, producing important flavor substances in wine[26].

Analysis of organic acids

Organic acids significantly influence the sensory characteristics and quality of fruit wine[27]. As shown in Fig. 4, malic and tartaric acid contents in the ST and SE groups were significantly lower than in the SG group ($p < 0.05$). The decrease in malic acid was due to *O. oeni* catalyzing the decarboxylation of L-malic acid into L-lactic acid and CO_2[28]. The reduction in tartaric acid was attributed to its precipitation as potassium hydrogen tartrate[29]. The malic acid content in the SE group (2.18 ± 0.08 mg/mL) was significantly lower than in the ST group (2.51 ± 0.08 mg/mL) ($p < 0.05$), possibly due to the two strains were inoculated not at the same concentration, competition for amino acids and nutrients between *S. cerevisiae* and *O. oeni*, and ethanol production by *S. cerevisiae* inhibiting *O. oeni* growth[30,31].

Citric acid contents in the ST and SE groups were 0.09 ± 0.02 mg/mL and 0.05 ± 0.01 mg/mL, respectively, significantly lower than in the SG group (0.77 ± 0.03 mg/mL) ($p < 0.05$). *O. oeni* OI metabolizes citric acid to produce acetic acid and aroma compounds like diacetyl, ethoin, and 2,3-butanediol[19,29]. High acetic acid content can mask fruit wine aromas, reduce flavor coordination, and result in a harsh, bitter taste. However, all three groups were within the normal range (<0.7 mg/mL)[29].

Analysis of amino acids

Amino acid contents in different groups are shown in Fig. 5A. Based on the polarity of the R group, amino acids are categorized as acidic, alkaline, neutral polar, and neutral non-polar[32]. Acidic amino acids, such as aspartic acid, decreased with MLF, potentially due to conversion into diacetyl, acetoin, and 1,3-butanediol by aminotransferase[32]. Glutamic acid in the ST group (1.50 ± 0.18 mg/mL) was significantly lower than in the SE and SG groups ($p < 0.05$). The alkaline amino acids (histidine, arginine, and lysine) were significantly lower in the ST and SE groups than in the SG group ($p < 0.05$). The reduction in arginine content suggests that *O. oeni* can catabolizes arginine through the arginine deiminase pathway (ADI) to protect against acidic environment[33]. Neutral polar amino acids (glycine, serine, and threonine) and the neutral non-polar amino acid (alanine) were also significantly lower in the ST and SE groups than in the SG group ($p < 0.05$). These amino acids may provide precursors for free amino acids[34].

Analysis of volatile components

A total of 69 volatile compounds were identified in the blueberry wine. The SG and ST groups each detected 44 volatile components, while the SE group detected 37 (Fig. 6A). Twenty-one compounds were common to all groups. MLF with *O. oeni* OI led to the formation of 25 new volatiles: 12 in the ST group, 10 in the SE group, and 3 shared by both ST and SE groups. These volatiles were categorized into alcohols (11), esters (26), aldoketones (5), hydrocarbons (20), acids (2), phenols (1), and others (4) (Fig. 6B).

As shown in Fig. 6B, there was no significant difference in the alcohol content between the three groups, except for ethanol ($p > 0.05$). This indicates that *O. oeni* OI doesn't promote the production of higher alcohols, which can impart unpleasant aromas[35]. Isoamyl alcohol and phenethyl alcohol were the main alcohol volatile flavor compounds in all three groups. Phenyl ethanol has a soft, pleasant, and long-lasting aroma of rose, violet, jasmine, and spice[8]. There were no significant differences in content among

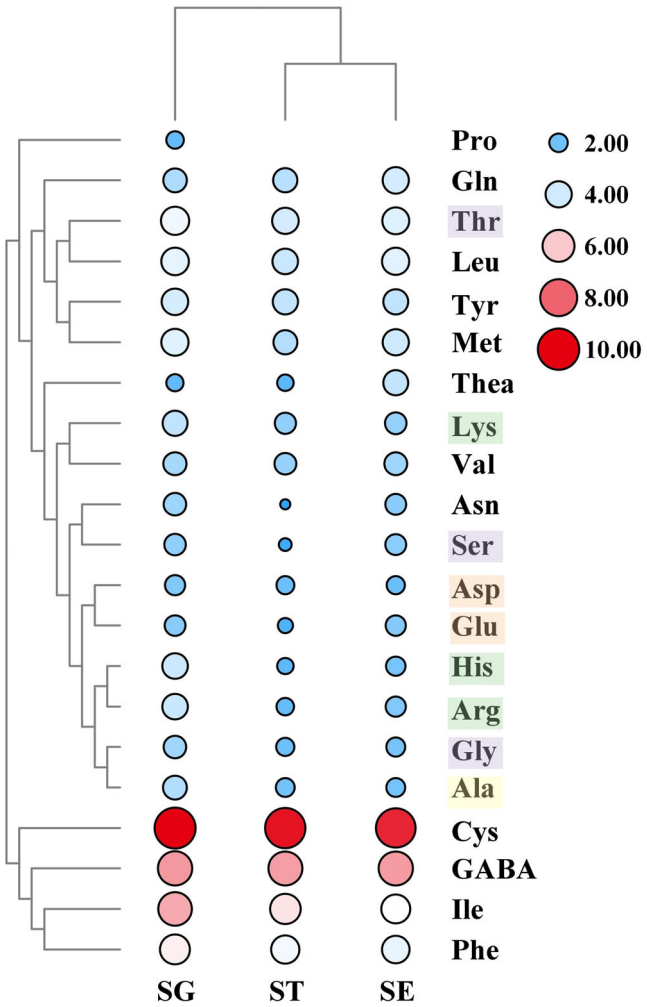

Fig. 5 | The amino acids of blueberry wine. The circle bar denoted the relative content of each individual amino acids. The redder (the bigger) of the circle, the higher content of the amino acids, and the bluer (the smaller) of the circle, the lower content of the amino acids. In the color panels for the amino acids classification, orange, green, purple and yellow indicated acidic, alkaline, neutral polar and neutral non-polar, respectively. Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR.

the three groups, indicating consistent characteristic flavors of the blueberry wine. However, the SE group produced 2-methyl-1-butanol (11.24 ± 4.76 mg/L), absent in the other groups, contributing to the aroma of the wine[36].

The concentration and types of esters increased significantly with MLF. The ST group produced seven new esters compared to the SG group. Isoamyl acetate, ethyl n-caproate, ethyl caprylate, phenyl ethyl acetate, and ethyl 9-decenoate were significantly higher in the ST and SE groups than in the SG group ($p < 0.05$), with isoamyl acetate in the ST group was increasing nearly 6-fold. These esters contribute fruity aromas to the wine[35]. One study of Negroamaro wine similarly found that the co-inoculation of *S. cerevisiae* and *O. oeni* increased ester contents[37]. The formation of ethyl fatty acid esters and acetate esters is likely due to esterase and acyltransferase from *O. oeni*. However, ethyl formate was absent in the ST and SE groups, indicating potential decomposition of transformation by *O. oeni* OI.

In aldoketones, only the ST group detected acetaldehyde (1.54 ± 0.59 mg/L), possibly contributing to the minor reduction of anthocyanins in this group. Acetaldehyde can be rapidly metabolized by *O. oeni*, but the rate of acetaldehyde degradation by *O. oeni* is affected by malic acid concentration, which may account for the detection of acetaldehyde only in the ST group[38]. The SE group had the highest variety of hydrocarbons, with 8 unique to it. Acetic acid, a by-product of MLF, increases significantly with MLF; with caprylic acid only detected in the SG and ST groups. The phenolic compound 2, 4-di-tert-butylphenol was present in all groups, potentially related to phenolic acid degradation[39]. Sulfur dioxide, converted by the added potassium metabisulfite, was detected in the ST and SE groups.

Multivariate statistical analysis of volatile aroma compounds

The influence of different groups on the volatile aroma compounds of blueberry wine was evaluated by principal component analysis (PCA). As revealed in Fig. 7A, the contributions of variance for PC1 and PC2 were 48.6% and 35.9%, respectively, with a cumulative variance contribution of 84.5%. It could also be seen that the three groups were located in different quadrants, indicating that there was a great deal of variation in the composition and content of the volatile aroma compounds.

To further recognize the volatile aroma compounds that result in the flavor differences of blueberry wines, the supervised PLS-DA method was used to analyze them. The PLS-DA score plot and VIP value plot were shown in Fig. 7B, C. In the score plot, PC1 and PC2 explain 47.1% and 36.1% of the variance, respectively, explaining 83.2% of the total variance. The sample points were scattered between groups, while samples within groups were independently clustered. Compared with

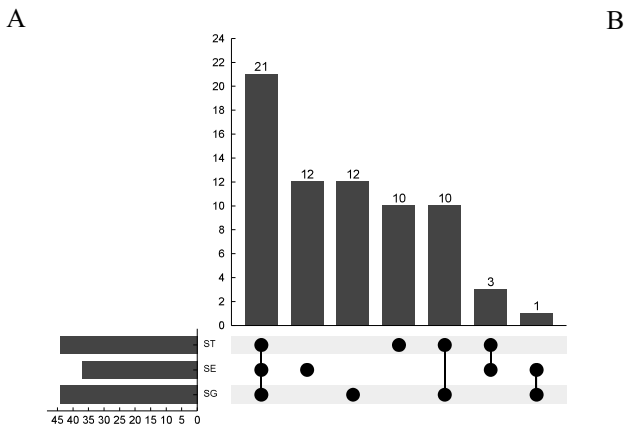

Fig. 6 | Aroma profile of different fermentation trials. A The distribution of volatile components. The bar chart at the bottom-left represents the number of volatile components included in each blueberry wine. The bar chart on the right represents the numbers of common volatile compounds in the wine samples. The solid black dot at the bottom-right shows the wine samples contained in the group. **B** Individual aroma compounds. Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR.

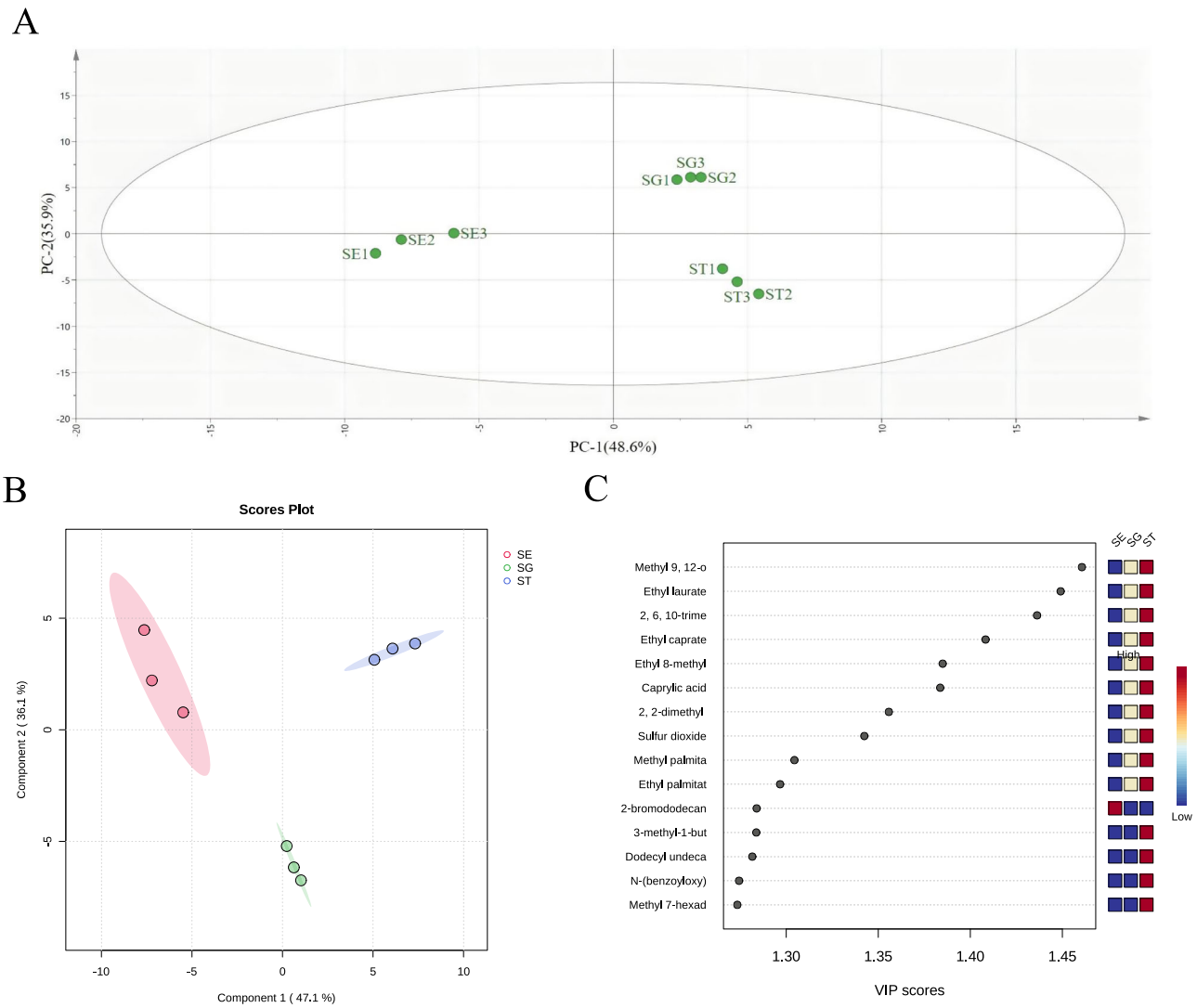

Fig. 7 | Multivariate statistical analysis of volatile aroma compounds. Multivariate statistical analysis of volatile aroma compounds (**A**: PCA analysis of volatile aroma compounds; **B**: The scores plot of PLS-DA; **C**: VIP value plot).

Table 2 | Antioxidant capacity of blueberry wine

Antioxidant capacity	ABTS (%)	DPPH (%)	Hydroxyl radical (%)
ST	86.67 ± 0.95^{b}	95.76 ± 5.54^{a}	47.62 ± 3.83^{a}
SE	92.61 ± 1.18^{a}	71.23 ± 3.71^{b}	16.39 ± 2.30^{b}
SG	83.25 ± 0.48^{c}	50.40 ± 4.86^{c}	15.45 ± 2.87^{b}

Note: ST: *S. cerevisiae* FR and *O. oeni* OI were inoculated simultaneously; SE: *S. cerevisiae* FR and *O. oeni* OI were sequentially inoculated; SG: Inoculated with *S. cerevisiae* FR; Different lowercase letters in the same column in the table indicate significant differences ($p < 0.05$).

the PCA, the PLS-DA more clearly showed intra-group consistency. On the basis of the VIP score, 15 compounds with VIP scores greater than 1 were recognized as potential markers for differentiating between samples[40]. Among them, methyl 9, 12-octadecadienoate, ethyl laurate, 2,6, 10-trimethylpentadecane, ethyl caprate, ethyl 8-methyl-8-pelanoate, caprylic acid, 2, 2-dimethyl ethylene oxide, sulfur dioxide, methyl palmitate, ethyl palmitate, 3-methyl-1-butanol formate, dodecyl isoamyl ester, n-(benzoyloxy) cyclohexylamine and methyl 7-hexadecenoate had strong correlations with the ST group. 2-bromododecane showed a strong correlation with the SE group. These results illustrated that blueberry wines fermented by different groups presented different aroma profiles, thus enriching the complexity of blueberry wine.

Analysis of antioxidant capacity

DPPH, ABTS, and hydroxyl radical scavenging rates are shown in Table 2. The ABTS clearance rate of the SE group was significantly higher than both the SG and ST groups ($p < 0.05$). Conversely, the DPPH clearance rate of the SE group was significantly lower than the ST group ($p < 0.05$), but still higher than the SG group. The hydroxyl radical scavenging rate in the SE group was significantly lower than in the ST group ($p < 0.05$). Typically, higher antioxidant activity in fermented foods is attributed to increased total phenolics and flavonoids[41]. However, our findings showed increased antioxidant capacity without a corresponding increase in total phenolics and flavonoids. Instead, there was a reduction in total phenols with MLF, suggesting the formation of new phenolic compounds from glycoconjugate precursors or degradation[42]. This enhances the bioavailability of health-promoting phenolics in blueberry wine with *O. oeni* OI. Similar results were reported in one study, which found higher bioactive compounds after fermentation in sorghum ting despite a decrease in total flavonoid, tannin, and phenolic content[43].

Materials and Methods

Blueberry wine fermentation

Rabbit eye blueberry (*V. ashei* Reade), 'Emerald' cultivars (Reducing sugar: 26.6 g/kg; Soluble solid:12 °Bx) from Yubei, Chongqing, was commercially harvested in August 2023. After harvesting, the raw material was immediately transported to our laboratory and stored at -20 °C until use. Immature

and damaged blueberries were discarded and the rest were homogenized using a food grinder (PB-110, Suzhou Shangyi Electric Technology Co. Ltd). Potassium pyrosulfite (0.374 g/kg, Aladdin, Shanghai, China) and pectinase (0.2 g/kg, Aladdin, Shanghai) was added to the mixture, which was incubated in a water bath at 50 °C for 1 h. After cooling, the sugar content was adjusted to 25% soluble solid with sucrose.

For the fermentation process, *S. cerevisiae* FR (2.3×10^{10} CFU/g, 0.25 g, Yantai Diboshi Brewing Machine Co. Ltd) and *O. oeni* OI (2.28×10^{9} CFU/g, 2.5 mg, Yantai Diboshi Brewing Machine Co. Ltd) were inoculated into 250 mL of blueberry juice and fermented at 19 °C for 13 days (The temperature is the optimal temperature for *O. oeni* OI strains to work obtained from our previous studies), constituting the ST group. In the SE group, *S. cerevisiae* FR was inoculated for 8 days at 24 °C (The temperature is the optimal temperature for *S. cerevisiae* FR strains to work obtained from our previous studies), followed by co-inoculation with *O. oeni* OI for 5 additional days at 19 °C. As a control, only *S. cerevisiae* FR was inoculated for 13 days at 24 °C, forming the SG group. The resulting fermented blueberry wine was stored at 4 °C until analysis. All fermentations were conducted in triplicate.

Basic physical and chemical indexes

Following the analytical methods prescribed by the International Organization of Vine and Wine[44], the pH value, ethanol content, and titratable acidity in wines were analyzed. The soluble solid content was determined using a hand-held refractometer (WZS-32, Shanghai Yili Electrophysical Optical Instrument Co, Ltd). All samples were analyzed in three replicates.

Reducing sugar content was determined using the anthrone colorimetric method with a slight modification[45]. The absorbance was measured at 620 nm using a double-beam ultraviolet–visible (UV–vis) spectrophotometer (TU-1950, Shanghai Jinghua Scientific and Technical Instrument Co, Ltd). All samples were analyzed in three replicates.

Color parameters

The color of blueberry wine was measured using a colorimeter (WR-10, Weifu Photoelectricity Technology Co, Ltd) in transmission mode under light avoidance. L, a, and b values were recorded to represent the brightness, redness-greenness, and yellowness-blueness characteristics of the samples. All samples were analyzed in three replicates.

Anthocyanin content

The total anthocyanin content was determined by the pH differential method[46]. Blueberry wine was diluted 10-fold with 0.2 mol/L potassium chloride buffer at pH 1.0 and 1.0 mol/L acetate buffer at pH 4.5, followed by incubation at 4 °C for 2 h. Absorbance was recorded at 510 nm and 700 nm at pH 1 and pH 4.5 using a double beam UV–vis spectrophotometer, calibrated with distilled water as a blank. All samples were analyzed in three replicates.

Total flavonoid content

Total flavonoid content was determined using the aluminum chloride method with slight modifications[47]. The blueberry wine was diluted 2-fold with 80% ethanol-water solution, followed by sonication for 30 min using an ultrasonic washer (SB-5200 DTD, Ningbo Xinzhi Biotechnology Co. Ltd). Samples were filtered, and 1 mL of the diluted sample was mixed with 5 mL of 30% ethanol and 1 mL of 5% sodium nitrite (Chongqing Taixin Chemical Co. Ltd,) solution, followed by a reaction at room temperature for 6 min. The reaction system was then transferred into a solution containing 4 mL of 1 mol/L sodium hydroxide (Chongqing Taixin Chemical Co, Ltd) and diluted to 25 mL using 30% ethanol. Absorbance at 510 nm was measured, calibrating with rutin standard solution. All samples were analyzed in three replicates.

Total phenol content

Total phenol content was measured using the Folin–Ciocalteu method[39]. The blueberry wine (1 mL) was diluted 10-fold with anhydrous ethanol, followed by ultrasound treatment for 10 min and filtration. Treated sample (1 mL) was mixed with distilled water (6 mL) and 1.0 mol/L Folin-Phenol reagent (1 mL). After incubation for 6 min, sodium carbonate solution (10.6%, 4 mL) was added and incubated for 60 min, then diluted to 25 mL with distilled water. Absorbance at 760 nm was measured, calibrating with a gallic acid standard solution. All samples were analyzed in three replicates.

Organic acids

Organic acid content was determined using the method described by Yang et al.[48]. Blueberry wine was centrifuged at 6000 g at 4 °C for 10 min. The treated sample was diluted 1.5-fold with 0.01 mol/L sodium dihydrogen phosphate (pH 2.8, Chongqing Taixin Chemical Co. Ltd) and filtered using a 0.22 μm organic phase needle filter. The processed sample was injected into the high-performance liquid chromatography (HPLC) (LC-20A, Shimadzu Co. Ltd, Japan) with a C18 column (250 mm × 4.6 mm, particle size: 5 μm, Agilent, USA). The mobile phase consisted of sodium dihydrogen phosphate (pH 2.8): methanol = 97:3 (v/v). A flow rate of 1 mL/min was maintained, and samples (20 μL) were injected into the column. Organic acid concentrations were determined by measuring their UV absorbance at 210 nm. All samples were analyzed in three replicates.

Amino acids

The amino acid content was determined by using the method described by Tian et al.[49]. Briefly, 10 mL of blueberry wine was heated at 60 °C for 30 min and was diluted to 100 mL with distilled water. The diluted sample was filtered via a 0.22-μm organic-phase needle filter. The quantification of amino acids was conducted by using high-performance liquid chromatography (HPLC) with C18 column (250 × 4.6 mm, 5 μm, Agilent, USA) and detector: ultraviolet detector–liquid phase. The chromatographic conditions were as follows: mobile phase A consisted of 0.1 mol/L sodium acetate solution-acetonitrile (97:3, V/V) (acetic acid adjusted pH was 6.5), mobile phase B consisted of acetonitrile: water (4:1, V/V), the flow rate of 1 mL/min, gradient elution: 0–11 min, 0–1.5% A, 100–98.5% B; 11–21.7 min, 1.5%–7.6% A, 98.5–92.4% B; 21.7–23.9 min, 7.6–11% A, 92.4–89% B; 23.9–39 min, 11–30% A, 89–70% B; 39–42 min, 30–70% A, 70–30% B; 42–45 min, 70–100%A, 30–0% B; 45–52 min, 100% A, 0% B; 52–55 min, 100–0% A, 0–100% B; 55–70 min, 0% A, 100% B. The detection wavelength was 254 nm, the column temperature was 36 °C, and the sample size was 20 μL. All samples were analyzed in three replicates.

Volatile components

Volatile compounds were analyzed using headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS)[50]. Blueberry wine (5 mL) with 1 g sodium chloride (Chongqing Taixin Chemical Co. Ltd) and 20 μL 2-octanol (500 μg/L, Chongqing Taixin Chemical Co. Ltd) was prepared in a 20 mL vial. The sample was agitated at 376 g for 10 min at 45 °C, and a manual solid-phase microextraction injector (75 um CAR/PDMS headspace solid-phase microextraction fiber head, Supelco, USA) was inserted into the vial headspace to absorb volatiles at 45 °C for 40 min. The injector was inserted into the gas chromatography(GC) injector for 3 min at 230 °C.

Volatile compounds were analyzed using a gas chromatography-mass spectrometry instrument (GCMS-2010, Shimadzu, Japan) with a DB-5MS capillary column (60 m × 0.25 mm, 0.25 μm thickness). Helium was the carrier gas, with a flow rate of 1.0 mL/min. The injection temperature was 230°C in splitless mode. The oven temperature program was: 35 °C for 3 min, increased to 150 °C at 6 °C/min and held for 1 min, then increased to 230 °C at 12 °C/min and held for 3 min. MS conditions included ion source and interface temperatures of 230 °C, full scan mode from *m/z* 20–550, and an ionization voltage of 70 eV.

Volatile compounds were identified by matching the mass spectrum and the retention indices (RI) with reference standards in the National Institute of Standards and Technology (NIST) 11 MS database. RI values were calculated using the Automated Mass Spectral Deconvolution and Identification System (AMDIS). All samples were analyzed in three replicates.

2,2′-Azinobis-(3-Ethylbenzthiazoline-6-Sulphonate) (ABTS) cationic free Radical clearance

ABTS cationic free radical clearance was determined using a method described by Liu et al.[51]. The ABTS cationic free radical dilution solution was prepared by reacting 7 mmol/L ABTS solution with 140 mmol/L potassium persulfate solution (both obtained from Chongqing Taixin Chemical Co, Ltd, China) in a 1:1 (v/v) ratio, incubated for 24 h at room temperature in the dark. The mixture was then diluted with absolute ethanol to an absorbance value of 0.70 ± 0.02 at 734 nm. Blueberry wine (0.5 mL) was added to 4.5 mL ABTS solution, and absorbance was measured after 6 min. All samples were analyzed in three replicates.

1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical Clearance

DPPH free radical scavenging rate was determined according to the method proposed by Shopska et al.[52]. Blueberry wine (2 mL) was mixed with 2 mL of a DPPH solution (0.2 mmol/L, Chongqing Taixin Chemical Co. Ltd). After 20 min of incubation, absorbance at 517 nm was measured, Calibrating with absolute ethanol. All samples were analyzed in three replicates.

Hydroxyl radical scavenging ability

Hydroxyl radical scavenging ability was determined using the method described by Ma et al.[53]. Ferrous sulfate (6 mmol/L, 2 mL)) was mixed with a salicylic acid solution (6 mmol/L, 2 mL, both obtained from Chongqing Taixin Chemical Co. Ltd), followed by the addition of 2 mL of blueberry wine. The reaction system was then supplemented with 2 mL of hydrogen peroxide solution (6 mmol/L, Chongqing Taixin Chemical Co. Ltd). After incubation at 37 °C for 1 h, absorbance at 517 nm was measured. All samples were analyzed in three replicates.

Statistical Analysis

Standard deviation (SD) represented the errors in triplicate studies for each treatment group. One-way analysis of variance (ANOVA) using SPSS 25.0 software identified significant differences among groups. Duncan's test ($p < 0.05$) discerned significant differences between treatments, with different letters indicating statistically significant differences. Data visualization was done using Origin 2021, MetaboAnalyst 5.0, TBtools, Simca, ChiPlot (https://www.chiplot.online). Values were reported as mean ± SD with a minimum of three replicates per group.

Data availability

All data are available from the corresponding author upon request.

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Author contributions

Liu and Zhang wrote the main manuscript text and processed all the data; Lian, Xie and Gao prepared figures 5-7 and tables; Song and Suo offered suggestions and references for the manuscript; Zhang conducted a full-text analysis and review and made revisions and corrections. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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