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Effects of mixed fermentation on the flavor quality and in vitro antioxidant activity of Zaosu pear-Merlot grape composite alcoholic beverage

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A B S T R A C T

In this study, a mixed fermentation strategy using grape-blended pear juice co-inoculated with *Metschnikowia pulcherrima* 346 and *Saccharomyces cerevisiae* ES488 was used to characterize the modifications of the flavor and antioxidant activity of Zaosu pear-Merlot grape alcoholic beverage. The optimum fermentation parameters identified using a fuzzy mathematical sensory evaluation model were an initial pH of 4.22, a ratio of *M. pulcherrima* 346 and *S. cerevisiae* ES488 inoculated 1.13:1, sequential inoculation time 47.02 h and making temperature 19 °C. The optimal mixed fermentation increased the content of terpenes and ethyl esters in pear-grape beverage by 16.5 % and 11.2 % respectively, enhancing floral and fruity aromas and attaining the highest sensory score. Due to the accumulation of flavonoid, anthocyanin, and phenol, the optimized alcoholic beverage exhibited the highest DPPH (97.6 %) and OH (93.3 %) radical scavenging rate as well as iron ion reducing power (3.25), which is conducive to extending the shelf life of beverages.

1. Introduction

Low-alcohol fruit beverage is favored by consumers due to its low alcohol content, unique flavor profile, and rich nutritional value (Saeed et al., 2019). Zaosu pear (*Pyrus bretschneideri Rehder*) is cultivated in the central and western regions of Gansu province on an area of 18, 667 ha with an annual output of 0.31 million tons. The fruit of this pear is crispy, juicy, and rich in nutrients, phenols, and antioxidant components (Li et al., 2024; Yang, Zhao, Yang, Li, & Zhu, 2022). The production of pear fermented beverage not only enriches consumer choice, but also extends the industrial chain of Zaosu pear, enhancing its economic benefits. However, perry fermented using Zaosu pear usually has drawbacks, such as bland taste and insufficient aroma (Yang et al., 2019; Yin et al., 2020). Therefore, it is necessary to explore new processing technology to improve the aroma and sensory quality of Zaosu pear fermented beverage.

At present, a specific blend of two or more fruit raw materials in an appropriate ratio has been utilized in the fermentation process to enrich the aroma substances in low-alcohol fruit wines and beverages (Raji, Adebayo, & Sanusi, 2022). For example, the addition of pear juice during cider making enhances the contribution of esters such as ethyl 2,4-decadienoate and ethyl caprylate to the aroma profile, and changes the flavor quality of apple cider (Kliks, Kawa-Rygielska, Gasinski, Głowacki, & Szumny, 2020; Kliks, Kawa-Rygielska, Gasinski, Rębas, & Szumny, 2021). Peng (2022) found that a blend of hawthorn and Yali pear juice can effectively make up for the drawbacks of bland taste and aroma of Yali pear alcoholic beverages, providing satisfactory sensory characteristics to beverage sample. Our previous study confirmed that apple- or Merlot grape-Zaosu pear alcoholic beverage exhibits higher levels of ethyl ester, higher alcohols, terpenes, and C_{13}-norisoprenoid compounds when compared to Zaosu pear alcoholic beverage. As a consequence, the composite fruit fermented beverage was featured by distinctive fruity and floral aroma, along with a fuller and more harmonious taste (Yang et al., 2022; Zhao, Yuan, Zhang, Yang, & Zhu, 2022).

In addition to improving the flavor quality of low-alcohol fruit wines and beverages, a specific blend of multiple raw materials can also be used to promote the antioxidant activity, extend the shelf life, and enhance the nutritional value of fruit alcoholic beverage. A recent study has suggested that the antioxidant activity of grape and blueberry compound fermented beverage is higher than that of grape wine (Martín-Gómez, García-Martínez, Varo, Mérida, & Serratosa, 2023). Brezan, Bădărău, and Woinaroschy (2020) also found that the addition of blueberries and black carrots can effectively increase the antioxidant activity and free radical scavenging capacity of cider. This may be attributed to the addition of other fruit materials not only improving the

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level of antioxidant components in the alcoholic beverages, but also enhances the absorption and utilization of these active ingredients by the organisms used in the fermentation process.

Saccharomyces cerevisiae (*S. cerevisiae*) has long been used in the production of traditional fruit wine due to its high fermentation capacity and alcohol productivity (Kanter et al., 2020). However, an increasing number of studies have discovered that non-*Saccharomyces* yeasts make positive contributions to the aroma (Delač Salopek et al., 2022; Yang et al., 2022), color (Minnaar, du Plessis, Jolly, van der Rijst, & du Toit, 2019; Zhang et al., 2024), and flavor (Li et al., 2023; Maicas & Mateo, 2023) of fruit wine and alcoholic fruit juice beverage through the release of extracellular enzymes and metabolites. Moreover, non-*Saccharomyces* yeasts generate significantly less ethanol than *S. cerevisiae* due to their inferior sugar conversion capabilities. Therefore, the mixed fermentation of *S. cerevisiae* and non-*Saccharomyces* yeasts has great potential in low-alcohol fruit beverage production.

Several studies have demonstrated that the remarkable flavor traits of fruit wine or alcoholic fruit juice beverages fermented with the co-inoculum of non-enological and enological yeasts (Delač Salopek et al., 2022; Li et al., 2023). Notably, the effect of paired yeasts on the quality of fruit alcoholic beverage is related to its compatibility with the fermentation broth. Even when using the same yeast strain, the optimum parameters used to manufacture different fruit fermented beverages are not identical (Yuan, Li, Yan, Wu, & Due, 2022). Although some researchers have confirmed that non-*Saccharomyces* yeasts with specific metabolites can effectively enhance the aroma profile of certain fruit wines, investigations focusing on the effects of mixed fermentation on the volatile compounds of pear-grape composite fruit wine are scarce, and there is no specific research dedicated to low-alcohol pear-grape fermented beverages. In addition, although the antioxidant capacity is also an important indicator reflecting the properties of fruit alcoholic beverage, few studies have utilized this criterion to improve the overall quality of the beverages.

In this study, we explore the effect of the co-inoculum comprised of *S. cerevisiae* ES488 and the non-*Saccharomyces* yeast *M. pulcherrima* 346 on the physicochemical indexes, volatile compounds, sensory quality, and antioxidant capacity of a composite low-alcohol fruit beverage from Zaosu pear and Merlot grape. Meanwhile, the optimal parameters for this mixed fermentation process were confirmed using Box-Behnken Design coupled with a fuzzy mathematical sensory evaluation method. Overall, our study will promote the application of the co-inoculation for *S. cerevisiae* and *M. pulcherrima* in the field of low-alcohol fruit beverage fermentation and construct a comprehensive evaluation system for alcoholic beverage.

2. Materials and methods

2.1. Materials and reagents

Zaosu pear and 'Merlot' wine grape were produced in Wuwei, Gansu, China. The reducing sugar content (expressed as glucose), pH value, and assimilable nitrogen content in these two fruits were 55.80 g/L, 4.03, and 85.12 mg/L and 196.54 g/L, pH 3.56, and 206.08 mg/L, respectively. *S. cerevisiae* ES488 (ES488) and *M. pulcherrima* 346 (MP346) were purchased from Enartis Inc. (Novara, Italy) and Lallemand Inc. (Quebec, Canada), respectively. Aroma compound standards, including citronellol, geraniol, linalool, isoamyl alcohol, phenylethanol, ethyl acetate, amyl acetate, hexyl acetate, isoamyl acetate, heptyl acetate, ethyl 3-hydroxybutyrate, methyl caproate, ethyl phenylacetate, ethyl heptanate, ethyl caprylic acid, and 2-octanol (internal standard) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

2.2. Composite fruit alcoholic beverage fermentation

2.2.1. Preparation of blended fruit juice

The preparation of fruit juice was carried out using a previously described method (Zhao et al., 2022). Zaosu pears (86 % juice yield) were washed, cored, and cut into pieces and soaked in water containing 10 mg/L vitamin C and 0.1 g/L citric acid for color protection. The chopped fruits were then squeezed with a juicer (DS − 1, Shanghai Precision Instruments Co., Ltd., Shanghai, China) and 60 mg/L SO_2 added into the resulting juice. Approximately 100 mg/L of pectinase (100,000 U/g, Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) was added to the pear juice, which was hydrolyzed in a water bath heated at 40 °C for 3 h and subsequently filtered through 8 layers of gauze. 'Merlot' grapes (74 % juice yield) were manually destemmed, crushed, and placed in a 5-L brown fermentation bottle with the addition of 40 mg/L SO_2 and 100 mg/L pectinase for maceration at 4 °C, and the grape pomace was separated using 4 layers of gauze 24 h later.

Based on our previous study (Zhao et al., 2022), pear and grape juices were blended at a ratio of 50:50 (*v*/v) and the potential alcohol content was controlled at <7 % vol. The initial pH of the composite juice was adjusted using citric acid or calcium carbonate.

2.2.2. Activation of yeast strains

The active dry *S. cerevisiae* ES488 yeast strain was rehydrated using ten times its volume of distilled water at 37 °C for 15 min and activated in an equal volume of pear-grape juice at 25 °C for 15 min. The active dry *M. pulcherrima* 346 powder was rehydrated using ten times its volume of distilled water at 28 °C for 20 min and then incubated in an equal volume of blended fruit juice at 25 °C for 20 min.

2.2.3. Optimization of the alcohol fermentation process parameters

According to our previous study, the effects of the initial pH (3.6, 3.8, 4.0, 4.2, and 4.4), inoculation ratio of *M. pulcherrima* 346 and *S. cerevisiae* ES488 (2:1, 1.5:1, 1:1, 1:1.5, and 1:2, where 1 represents an inoculation amount of 1×10^6 cfu/mL), inoculation time of *S. cerevisiae* ES488 (*M. pulcherrima* 346 was inoculated 0, 24, 48, 72, and 96 h before *S. cerevisiae* ES488), and fermentation temperature (16, 18, 20, 22, and 24 °C) on the quality and fuzzy mathematical sensory scores of pear-grape composite alcoholic beverage were examined at the end of the fermentation process. Based on our single-factor experiment results, a response surface optimization test was conducted using the four factors and fuzzy mathematical sensory scores as independent variables and response values to establish a mathematical model and calculate the optimal fermentation parameters. All treatments were conducted in triplicate.

2.3. Determination of the physicochemical indexes

The level of reducing sugar, titratable acid, volatile acid, and pH were determined with reference to the GB/T 15038–2006 standard. The alcohol content was determined with reference to the GB 5009.225-2016 standard. Determination of the total phenols, total anthocyanins, and flavonoids contents were based on the method of Filipe-Ribeiro, Rodrigues, Nunes, and Cosme (2021). The chromaticity and hue of the alcoholic beverages were measured using a previously described method with slight modification (Uysal, Issa-Issa, Sendra, & Carbonell-Barrachina, 2023). 1 mL of the beverage sample was accurately aspirated and then diluted into a 10-mL volumetric flask using disodium hydrogen phosphate-citric acid buffer at the same pH as the beverage sample. The diluted samples were placed into 1 × 1 cm cuvettes and their absorbance values at 420, 520, and 620 nm measured, respectively. The sum of these three values is the chromaticity of fermented beverage and the ratio of the first two absorbance values is the hue.

2.4. Determination of the volatile aroma compounds

The aroma compounds in the pear-grape fermented beverage were extracted using headspace solid-phase microextraction (HS-SPME) and analyzed using gas chromatography–mass spectrometry (GC–MS) (Thermo Fisher Scientific, Waltham, MA, USA) on a DB-Wax column (60 m × 0.25 mm × 0.25 μm, J & W Scientific, Santa Clara, CA, USA) in accordance with our previously reported method (Zhu, Zhao, Li, Han, & Yang, 2021). Briefly, the beverage sample (8 mL) was mixed with NaCl (2.4 g) and an internal standard solution (2-octanol prepared at a concentration of 81.06 mg/L, 10 μL) in a 15-mL headspace vial, and then incubated at 40 °C for 30 min, followed by volatiles extraction using a 50/30 μm DVB/CAR/PDMS fiber (57329-U, Supelco, Bellefonte, PA, USA) for 30 min. The fiber was desorbed in the GC injector at 250 °C for 5 min, then carried by helium at 1 mL/min. The GC program was set 40 °C for 5 min, increased to 180 °C at 3.5 °C/min, maintained for 15 min, then increased to 220 °C at 6 °C/min, and finally held for 10 min. The MS transfer line and ion source temperatures were set at 180 and 250 °C, respectively. The source was operated in electron ionization mode at 70 eV at a scanning interval of 0.2 s in the *m/z* range from 30 to 350. The separated compounds were identified, based on their chromatographic retention times and the mass spectra obtained for authentic standards available in the NIST and Wiley libraries. They were then quantified using the calibration curves ($R^2 > 0.995$) established for pure standards plotted under the same conditions as the beverage samples. For volatiles without any standards available, their concentrations were semi-quantified using standards with similar chemical structures and functional groups with a similar number of carbon atoms.

2.5. Fuzzy mathematics sensory evaluation

2.5.1. Sensory evaluation

The sensory evaluation of Zaosu pear-grape composite alcoholic beverage was performed by 10 professionally trained personnel (5 males and 5 females, ranging in age from 20 to 35). The entire sensory analysis was guided by sensory ethical standards, and each candidate participated voluntarily with their consent and knowledge obtained. All attributes including appearance, aroma, taste, and typicality were assessed via a mode established using the fuzzy mathematics method. Approximately 30 mL of each sampled beverage was equilibrated at 24 °C for 3 h and randomly placed in ISO wine glasses, then evaluated under white light in isolated booths at 22–24 °C (Yang et al., 2019). The evaluation criteria are shown in Supplementary Table S1. The number of voters for each sample as determined by the panelists is shown in Supplementary Table S2. The Ethics Commission of Gansu Agricultural University gave positive ethical approval of this study.

2.5.2. Establishment of the fuzzy mathematics sensory evaluation model

The model of our fuzzy mathematics sensory evaluation for composite fruit alcoholic beverages was developed as follows (Yu et al., 2022). Four assessment indices were selected and denoted as factor set: $U = \{U_1, U_2, U_3, U_4\}$ = {appearance, aroma, taste, typicality}, and three grades were selected as the evaluation set: $V = \{V_1, V_2, V_3\}$ = {excellent, good, ordinary}. A 9-point system was used to assign the middle value of each grade score and the evaluation grade $W = \{W_1, W_2, W_3\} = \{8, 5, 2\}$ was obtained. The weights of the four evaluation indicators were analyzed and averaged to obtain the weight set K, $K = \{0.235, 0.265, 0.285, 0.215\}$.

The evaluation results were divided according to the different grades and the votes of each grade were counted. The number of votes at each level was divided by the total number of votes to define fuzzy relation matrix A and the evaluation result $M_i = K \times A_i$ for the i th sample was then obtained. Comprehensive evaluation matrix Y of the beverage sample could be calculated using $Y_i = M_i \times W$.

Taking Group 1 samples in Table 1 as an example:

$$\begin{aligned} M_1 = K \times A_1 &= \left| 0.235 \;\; 0.265 \;\; 0.285 \;\; 0.215 \right| \times \begin{vmatrix} 0.8 & 0.2 & 0.0 \\ 0.8 & 0.2 & 0.0 \\ 0.7 & 0.3 & 0.0 \\ 0.6 & 0.3 & 0.1 \end{vmatrix} \\ &= \left| 0.7285 \quad 0.2500 \quad 0.0215 \right|; \end{aligned}$$

$$Y_1 = M_1 \times W = \left| 0.7285 \quad 0.2500 \quad 0.0215 \right| \times \begin{vmatrix} 8 \\ 5 \\ 2 \end{vmatrix} = 7.12.$$

Table 1
Box–Behnken design and response values of the pear-grape fermented beverages.

Run	A Initial pH	B Inoculationratio	C Inoculation time (h)	D Fermentation temperature (°C)	M Fuzzy mathematics sensory score (Scores)
1	−1(4.00)	−1(1.5:1)	0(48)	0(20)	7.12
2	1(4.40)	−1	0	0	7.41
3	−1	1(1:1.5)	0	0	6.86
4	1	1	0	0	7.27
5	0(4.20)	0(1:1)	0	0	7.71
6	0	0	1(72)	−1(18)	7.32
7	0	0	1	−1	7.38
8	0	0	−1(24)	1(22)	7.57
9	0	0	1	1	7.10
10	0	0	0	0	7.85
11	−1	0	0	−1	7.39
12	1	0	0	−1	7.42
13	−1	0	0	1	6.85
14	1	0	0	−1	7.46
15	0	−1	−1	0	7.38
16	0	0	0	0	7.80
17	0	1	−1	0	7.34
18	0	−1	1	0	7.26
19	0	1	1	0	6.75
20	−1	0	−1	0	6.92
21	1	0	−1	0	7.30
22	−1	0	1	0	6.74
23	1	0	1	0	7.02
24	0	0	0	0	7.86
25	0	−1	0	−1	7.77
26	0	1	0	−1	7.40
27	0	−1	0	1	7.43
28	0	1	0	1	7.52
29	0	0	0	0	7.93

The experiment for each fermentation parameter combination were carried out in triplicate.
All sensory scores are expressed using the average value to eliminate the errors.

2.6. Determination of the antioxidant activity of fruit fermented beverage in vitro

2.6.1. DPPH free radical scavenging ability

2 mL of DPPH (0.20 mmol/L) solution was added to 1 mL of the 100-fold diluted beverage sample and protected from light for 30 min at room temperature. Subsequently, the absorbance was determined at 517 nm with ethanol and 0.15 % Vc solution serving as the blank and positive control, respectively (Yang, Su, Wang, Wang, & Wang, 2021). The formula was as follows:

$$\text{DPPH free radical scavenging rate}(\%) = \frac{1-(\text{A–B})}{\text{C}} \times 100$$

where A represents the absorbance value of the 1-mL beverage sample with 2 mL of DPPH solution, B is the absorbance of 1 mL beverage with 2 mL of ethanol, and C is the absorbance of a mixture comprised of 1 mL ethanol and 2 mL DPPH solution.

2.6.2. OH free radical scavenging ability

2 mL ferric sulfate solution (6 mmol/L) and 2 mL hydrogen peroxide solution (6 mmol/L) were added to 1 mL of the 100-fold diluted beverage sample. After standing in the dark for 10 min, 2 mL of salicylic acid solution (6 mmol/L) was added and kept under dark conditions at room temperature for 30 min. The absorbance was measured at 510 nm using 0.15 % Vc solution as the positive control (Liu, 2019). The formula was as follows:

$$\text{OH free radical scavenging rate}(\%) = \frac{1-(\text{A}-\text{A}_1)}{\text{A}_0} \times 100$$

where A represents the absorbance of the 1 mL beverage sample with 2 mL of iron sulfate, hydrogen peroxide, and salicylic acid, A_1 is the absorbance of 1 mL beverage with 2 mL of iron sulfate, hydrogen peroxide, and water, and A_0 is the absorbance of 1 mL water with 2 mL of iron sulfate, hydrogen peroxide, and salicylic acid.

2.6.3. Iron ion reducing power

Determination of the iron ion reduction force was conducted using a previously described method with some modifications (Cavia, Arlanzón, Busto, Carrillo, & Alonso-Torre, 2023). A 1 mL of the 100-fold diluted beverage sample was mixed with 2.50 mL of 0.20 mol/L phosphate buffer (pH 6.60) and 2.50 mL of 1 % potassium ferricyanide, and incubated in a water bath heated at 50 °C for 20 min. After rapid cooling, 4 mL of 10 % trichloroacetic acid was added and the resulting mixture allowed to stand for 5 min. Exactly 2.50 mL of the reaction solution was added to a 10-mL centrifuge tube and 2.50 mL distilled water and 0.50 mL of 0.10 % ferric chloride were then added to react in the dark for 20 min. The absorbance of the beverage sample was measured at 700 nm, zeroing with distilled water.

2.7. Data analysis

One-way analysis of variance (ANOVA) was completed utilizing SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) and the Duncan test was used for multiple mean comparisons. Data and charts were produced using Microsoft Office 2010. TBtools software was used for heat map analysis; other analyses were performed using Origin 2021.

3. Results and discussion

3.1. Optimization of the mixed fermentation parameters for composite fruit alcoholic beverage

3.1.1. Single factor test

The sensory score and alcohol content of composite fruit alcoholic beverage are affected by the initial pH of fruit juice, fermentation temperature, inoculation ratio, and time-point of the yeast strains (Fig. 1). The fermentation matrix pH may influence the yeast growth and fermentation performance through altering the cell permeability and cell wall structure (Stanzer et al., 2023), thereby affecting the quality of alcoholic beverage. Both the alcohol content and fuzzy mathematics sensory scores first increased and then decrease with an increase in the pH, and the sensory scores reached the maximum value of 7.84 at pH 4.20 (Fig. 1A). A previous study also found that the initial pH not only alters the accumulation of alcohol, but also affects the mouthfeel of the low-alcohol beverage (Johnson et al., 2023). This may be attributed to the fact that a high pH value destroys the juice components and accelerates juice browning, thereby bringing about the imbalance of sugar and acid in fruit fermented beverage as well as its rough texture. Meanwhile, a low pH slows down the growth of the yeast strains, prolongs the fermentation process, and leads to the low alcohol content and sour taste of composite fruit alcoholic beverage.

The inoculation ratio of different yeast strains has a significant effect on the quality of fruit fermented beverage (Li et al., 2023; Liu et al., 2023). In particular, lower levels of alcohol are observed in fermentation processes performed with higher inoculation ratios of non-*Saccharomyces* yeasts (Liu, Lou, et al., 2023; Liu, Wan, et al., 2023). However, there was no significant change in the alcohol content of the fruit beverages fermented with different paired yeasts used in this study, which was mainly related to the reducing sugar content that could be converted to alcohol in the blended juice. We obtained the highest sensory score of 7.55 for a 1:1 ratio of *M. pulcherrima* 346 to *S. cerevisiae* ES488 (Fig. 1B). It should be noted that a higher or lower inoculation ratio of *M. pulcherrima/S. cerevisiae* imparted an unpleasant sour or spicy odor to the beverage samples.

Lai et al. (2022) confirmed that low-temperature fermentation can increase the unsaturation of fatty acids in the yeast cell membranes and make yeast strains more ethanol tolerant. In fact, excessively low temperature may inhibit or even interrupt the growth and fermentative activity of yeast strains (Stanzer et al., 2023). We found that temperatures below 16 °C cause a delay in the onset of alcohol fermentation and overall fruit beverage making duration. The highest fuzzy mathematical sensory score (7.86) was obtained for the low-alcohol composite fruit beverage prepared at a fermentation temperature of 20 °C (Fig. 1C), indicating that this temperature is conducive to the growth and metabolism of yeasts in the co-inoculation mode.

During the mixed fermentation process, the full growth of *M. pulcherrima* may positively influence the aroma composition of fruit fermented beverage (Delač Salopek et al., 2022). However, the persistent predominance of non-*Saccharomyces* yeasts will not merely restrain the growth of *S. cerevisiae* but may also result in the accumulation of undesirable flavor substances, which will have a negative impact on the quality of fruit alcoholic beverage. All these findings can explain why the fuzzy mathematical sensory scores of Zaosu pear-grape composite alcoholic beverage initially increase and then decrease with the delay of the *S. cerevisiae* ES488 inoculation time (Fig. 1D). Similarly, Zhang et al. (2024) also confirmed that the sensory quality of kiwi wine fermented using a mixed modality in which non-*Saccharomyces* yeast was inoculated 48 h before *S. cerevisiae* was optimal.

3.1.2. Response surface test

Based on the results of our single factor tests, a response surface design containing 29 experimental combinations was used to optimize the fermentation parameters of Zaosu pear-Merlot grape composite alcoholic beverage (Table 1). A multiple quadratic regression equation for the independent variable and fuzzy math sensory score (Y) was obtained by fitting the results of the Box-Behnken test as follows:

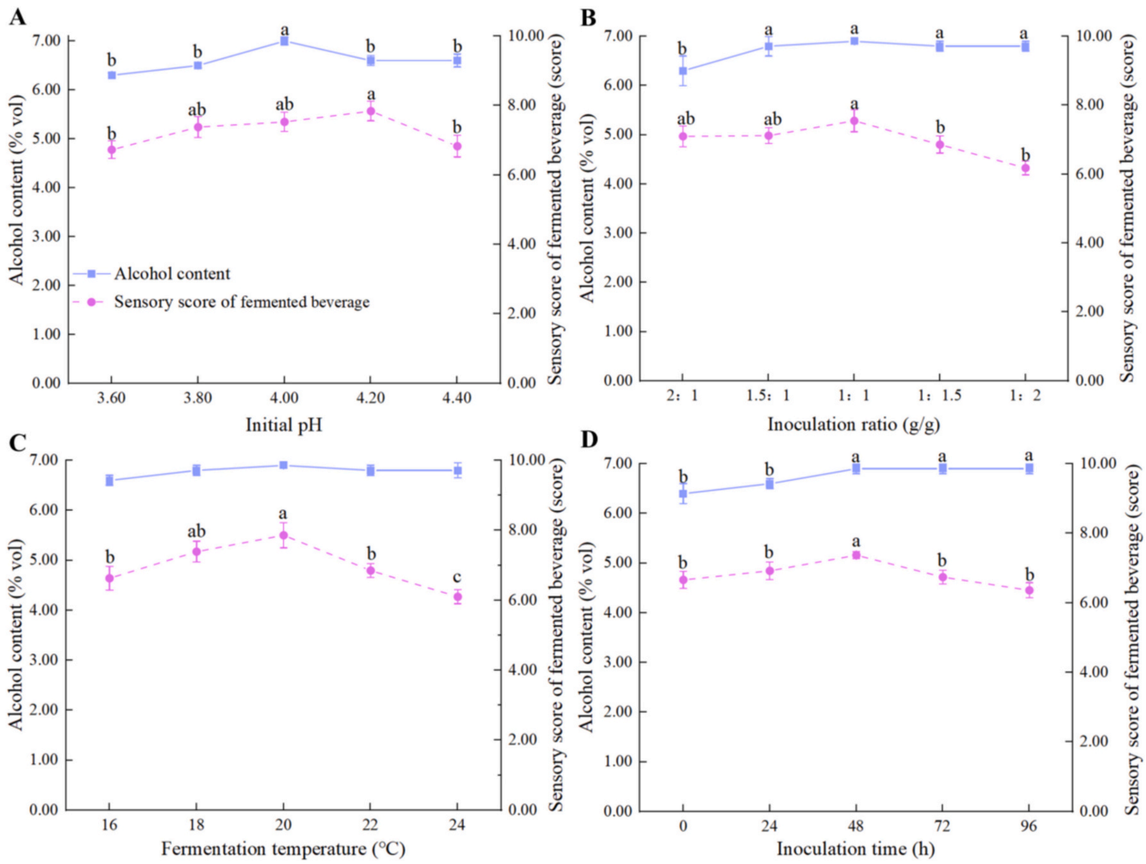

Fig. 1. Effects of mixed fermentation parameters on alcohol content and fuzzy mathematics sensory score of Zaosu pear-Merlot grape alcoholic beverages. A) Initial pH, B) inoculation ratio of *M. pulcherrima* 346 to *Saccharomyces cerevisiae* ES488, C) fermentation temperature, and D) *M. pulcherrima* 346 was inoculated 0, 24, 48, 72 and 96 h before *Saccharomyces cerevisiae* ES488. Error bars represent the standard errors of three replicates. Different letters among fermented beverage samples indicate the significant differences at $P < 0.05$.

$$Y = 7.83 + 0.17A - 0.10B - 0.13C - 0.07D + 0.15AD - 0.12BC - 0.12BD - 0.12CD - 0.44A^2 - 0.22B^2 - 0.41C^2 - 0.09D^2$$

Our ANOVA results (Supplementary Table S3) showed that the effects of the model's primary term coefficients A (initial pH), B (inoculation ratio), C (*S. cerevisiae* ES488 inoculation time), and D (fermentation temperature), as well as their quadratic terms A^2, B^2, C^2, and D^2, on the fuzzy mathematical sensory scores (Y) of the composite fruit beverages were all highly significant ($P < 0.01$). With the except for the interaction terms AC and AB, all of the other interaction terms had significant effects on the response values ($P < 0.05$) (Supplementary Fig. S1). According to the absolute values of the regression coefficients of the model factors, it could be inferred that the factors affecting the sensory quality of Zaosu pear-grape alcoholic beverage are the initial pH, followed by inoculation time, and finally the inoculation ratio and fermentation temperature.

Based on our model analysis, the optimal parameters obtained for low-alcohol pear-grape beverage fermented with the mixed culture were as follows: initial pH of 4.22, access to *S. cerevisiae* ES488 47.02 h after *M. pulcherrima* 346 inoculation, an *M. pulcherrima* 346 to *S. cerevisiae* ES488 inoculation ratio of 1.13:1, and fermentation temperature of 19.05 °C. In order to verify the true accuracy of this result, three parallel validation tests were conducted under the optimized fermentation conditions. As expected, the ultimate fuzzy mathematical sensory score was 7.85, which was in good accordance with the predicted value of 7.87, demonstrating that the model was reliable and can predict the relationship between the mixed fermentation parameters and sensory quality of low-alcohol Zaosu pear-grape beverage.

3.2. Quality analysis of fruit alcoholic beverage

3.2.1. Physicochemical indexes

The reducing sugar contents of pure Zaosu pear fermented beverage and non-optimized and optimized pear-grape composite fermented beverage were < 4 g/L, and there were significant differences among the three beverage samples ($P < 0.05$) (Table 2). Due to the low level of initial sugar in pear juice, the alcohol content of pure pear beverage was significantly lower than that of the composite fruit beverage with or without optimization ($P < 0.05$). Whether the mixed fermentation parameters were optimized or not, the content of titratable acidity and volatile acidity in the composite fruit alcoholic beverage were always significantly higher than those in pure pear beverage ($P < 0.05$). In particular, the highest level of titratable acidity (5.38 g/L) was detected in the pear-grape alcoholic beverage with optimization, and correspondingly, the pH was the lowest (3.68). A similar result has been observed in the fermentation of cactus pear and *Lantana camara* composite alcoholic beverage (Tsegay, 2020). This may be related to production of acetic acid by yeast cells in response to hypertonic stress

Table 2
Oenological parameters in fermented beverages.

Parameters	Non-optimized pear-grape fermented beverage	Optimized pear-grape fermented beverage	Pear fermented beverage
Reducing sugars (glucose, g/L)	3.98 ± 0.03a	3.61 ± 0.02b	3.07 ± 0.02c
Ethanol (% vol)	6.67 ± 0.09b	6.87 ± 0.09a	2.60 ± 0.00c
pH	3.71 ± 0.01b	3.68 ± 0.00c	3.78 ± 0.00a
Titratable acidity (tartaric acid, g/L)	5.19 ± 0.09b	5.38 ± 0.09a	4.69 ± 0.00c
Volatile acidity (acetic acid, g/L)	0.46 ± 0.00a	0.46 ± 0.00a	0.23 ± 0.01b
Color density	3.36 ± 0.02a	3.36 ± 0.02a	2.94 ± 0.00b
Color tonality	0.70 ± 0.01b	0.69 ± 0.00b	1.12 ± 0.01a
Total phenols (mg/L)	1130 ± 40b	1187 ± 37a	820.4 ± 8.2c
Total anthocyanins (mg/L)	132.1 ± 1.2a	136.1 ± 4.1a	0.71 ± 0.43b
Flavonoids (mg/L)	140.4 ± 25.3a	138.3 ± 14.4a	39.88 ± 0.63b
Color characterization			

All values are reported as the mean (± SD) of three experiments.
The values with different letters in a row are statistically different according to Tukey's test ($P < 0.05$).

during the alcohol fermentation process at high initial sugar concentrations (Chidi, Bauer, & Rossouw, 2018).

Color is an important parameter for identifying the age, type, and component of low-alcohol fruit beverage. The chroma-hue of the pear-grape beverages was significantly different from that of pure pear beverage, but there was no significant difference between the composite fruit alcoholic beverage produced with and without optimization, which was consistent with our color characterization results (Table 2). When compared with pure pear beverage, the grape-blended pear beverage samples contain more anthocyanins and therefore, have a higher color density. The contents of total anthocyanins and flavonoids in the samples were only significantly different between pear fermented beverage and pear-grape alcoholic beverage ($P < 0.05$). As for the total phenols, their contents ranged from 1130 to 820 mg/L, and significant differences were observed among the three samples ($P < 0.05$), indicating that both the raw materials and fermentation parameters affect the accumulation of total phenols in low-alcohol fruit beverages.

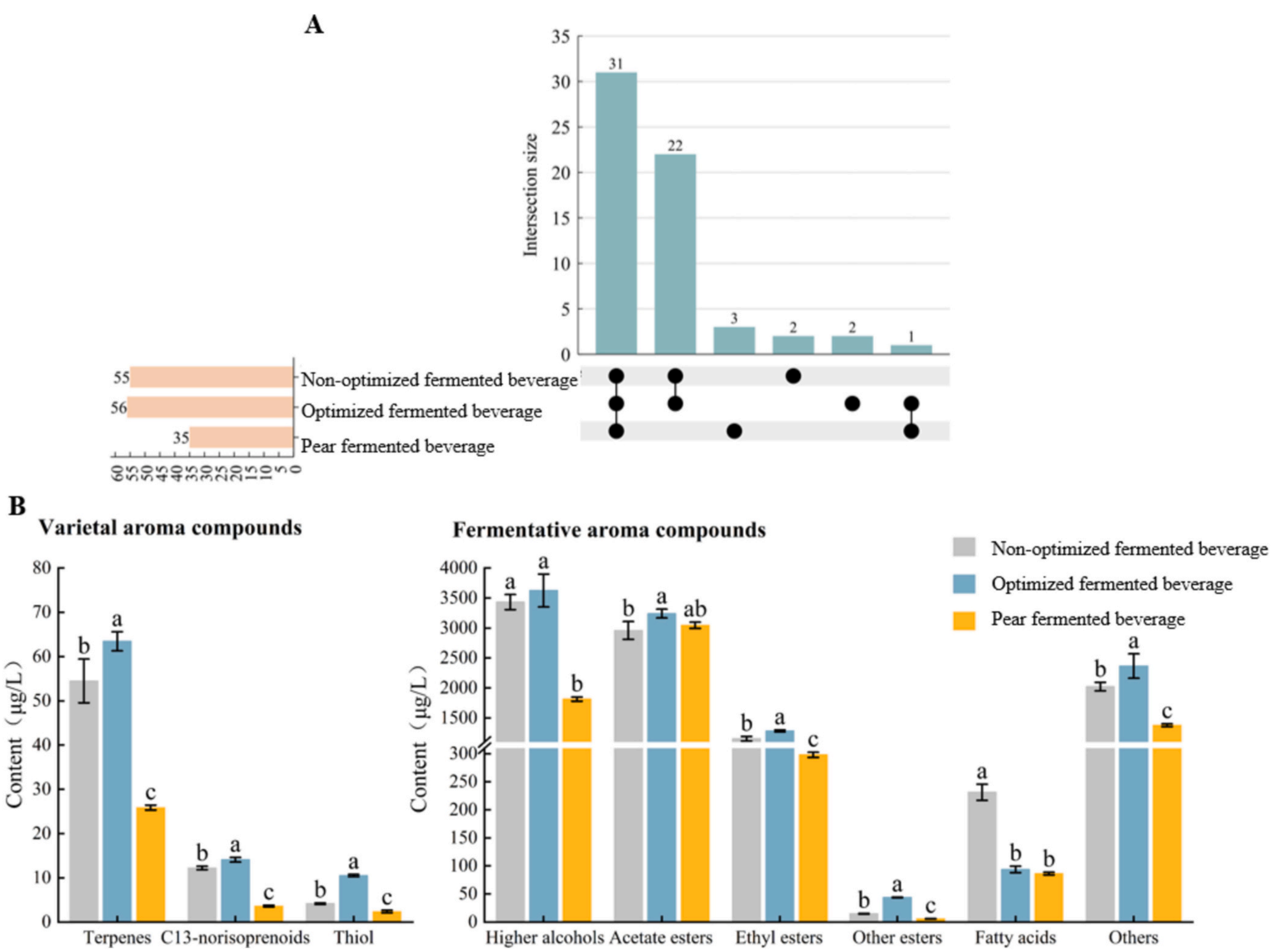

Fig. 2. Distribution and content of aroma compounds in fermented beverages. A) Distributions of aroma components in different fermented beverages, B) Content of aroma components in different fermented beverages. Error bars represent the standard errors of three replicates. Different letters among fermented beverage samples indicate the significant differences at $P < 0.05$.

3.2.2. Volatile aroma compounds

A total of 61 volatile aroma compounds were detected in the fruit beverage samples (Supplementary Table S4). Among them, there were 31 volatile compounds in all of the samples and only 22 volatiles were present in the composite fruit fermented beverage. The types of aroma compounds in pure pear beverage (35) were much lower than those in the non-optimized (55) and optimized (56) pear-grape beverage samples (Fig. 2A). Both the addition of grape juice and optimization of the mixed fermentation process can obviously enrich the aroma components in Zaosu pear fermented beverage, such as isobutyric acid, ethyl linoleate, lauryl alcohol, and decanoate, which were only found in the composite fruit alcoholic beverages prepared with or without optimization. In addition, the total content of volatile compounds in the pear-grape beverages increased by at least 48.4 % when compared to Zaosu pear beverage. This may be explained that the addition of grape juice increased the level of assimilable nitrogen in the substrate, thus improving yeast growth and fermentation performance which then promoted the accumulation of secondary metabolites such as aroma compounds (Lytra, Miot-Sertier, Moine, Coulon, & Barbe, 2020).

The varietal aroma compounds of the samples were mainly composed of 5 terpenes, 1 C_{13}-norisoprenoid, and 1 thiol, with terpenes accounting for >50 % of the varietal aroma content. It should be noted that no methoxypyrazines were detected even in the pear-grape alcoholic beverages. Several studies have suggested that the content of this compound decrease rapidly as the grape ripens (Gao et al., 2019; Wimalasiri et al., 2023). Therefore, we speculated that the disappearance of the methoxypyrazines may be associated with the maturity of the Merlot grapes. In addition to the types, the total contents of varietal aroma compounds in the composite fruit fermented beverages were significantly higher than that in pure pear fermented beverage ($P < 0.05$), especially in the optimized pear-grape beverage, which was ~2.8-fold higher than that observed in pear beverage (Fig. 2B).

54 fermentative aroma compounds were identified in the beverage samples, including 13 higher alcohols, 8 acetates esters, 16 ethyl esters, 6 other esters, 4 fatty acids, and 7 other substances such as phenylethyl, carbonyl compounds, and volatile phenols. The mixed inoculation of *M. pulcherrima* 346 and *S. cerevisiae* ES488 promotes the production of fermentative aroma compounds due to the accumulation of isopentanol, ethyl acetate, isoamyl acetate, ethyl octanoate, and phenethyl alcohol, and optimization of the fermentation parameters further increased the content of these volatile compounds (Fig. 2).

Higher alcohols are formed by the decarboxylation of α-keto acid, an intermediate product of glycolysis or amino acid metabolism (Liu, Lou, et al., 2023; Liu, Wan, et al., 2023; Zhang et al., 2022), which bring a richer and more intense flavor to fermented beverage. 1-Octene-3-ol, n-octanol, and n-hexanol with strong floral aroma were the main higher alcohols (OAV > 0.1) in all of the samples and their high levels were found in pear-grape alcoholic beverage. Due to the increased content of isoamyl alcohol after the addition of grape juice (Supplementary Table S4), the total amount of higher alcohols in the optimized and non-optimized composite beverages (3626 and 3433 μg/L, respectively) were much higher than that in pear beverage (1815 μg/L) (Fig. 2B). Given that isoamyl alcohol is produced via the leucine-Ehrlich pathway, we hypothesized that the addition of grape juice might increase the content of leucine and thereby accelerate the accumulation of isoamyl alcohol in the composite fermented beverage. Higher alcohols can be used as precursors to synthesize acetate during the fruit wine fermentation process (Delač Salopek et al., 2022). It is possible that the pear-grape alcoholic beverages are characterized by the high content of aromatic esters.

Esters are an important group of aroma compounds and some short-chain esters may impart the desirable fruitiness to fermented beverages (Liu, Lou, et al., 2023; Liu, Wan, et al., 2023). We identified 30 acetates and ethyl esters in the different beverage samples among which ethyl acetate was the most abundant compound. The optimized pear-grape composite alcoholic beverage exhibits the highest level (3433 μg/L) of ethyl acetate, isoamyl acetate, isobutyl acetate, octyl acetate, heptyl acetate, and phenyl ethyl acetate (Supplementary Table S4). Although the contents of most of these acetates in the composite fruit beverages were below their threshold, they may enhance the pear and tropical fruit flavors of the samples via a synergistic effect (Yang et al., 2022). Ethyl esters are responsible for a pleasant cheesy and fruity flavor when they are present at high concentrations (Delač Salopek et al., 2022). The content of ethyl esters in the composite fruit beverages was 2.9 times higher than in the pure pear beverage, and the optimized co-inoculation strategy of *M. pulcherrima* and *S. cerevisiae* further increased the content

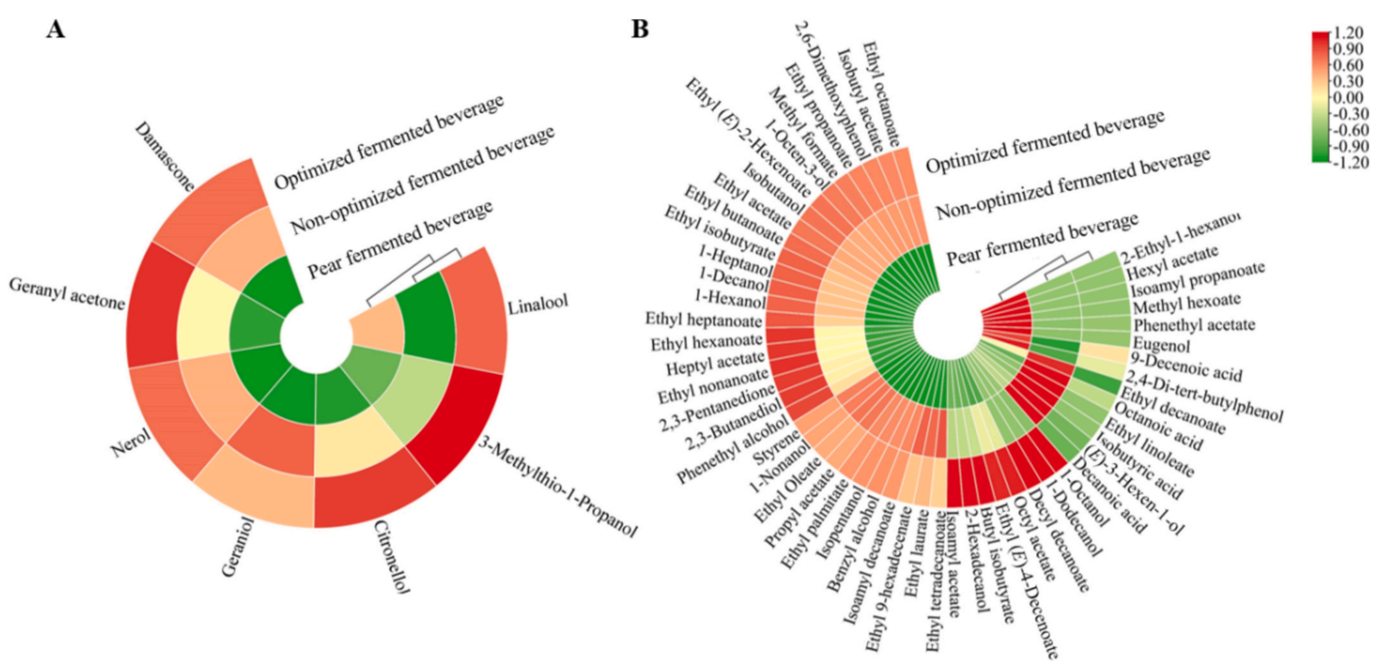

Fig. 3. Hierarchical clustering and heatmap visualization of volatile aroma compounds in fermented beverages. A) Varietal aroma compounds, B) Fermentative aroma compounds.

of these esters by at least 11.2 % (Fig. 3B), which was in accordance with the findings of several researchers (Fejzullahu, Kiss, Kun-Farkas, & Kun, 2021).

Fatty acids, which are the products resulting from the cleavage of higher fatty acids or the oxidation of alcohols and aldehydes, can enhance the aromatic complexity and balance of fermented beverages (Zhang et al., 2022). Such compounds introduce a buttery and cheesy flavor to wine at a low concentration, while they produce rotten and sour flavor at a high concentration (Liu, Lou, et al., 2023; Liu, Wan, et al., 2023; Tarko & Duda, 2024). In this study, caprylic acid, n-decanoic acid, 9-decenoic acid, and isobutyric acid were identified in the fermented beverages. Of these, the content of caprylic acid and n-decanoic acid contributed >70 % of the total fatty acids amount (Supplementary Table S4). When compared with the non-optimized pear-grape alcoholic beverage, the optimized composite fruit beverage exhibited the lower level of fatty acids (Fig. 2B), which prevented the key floral and fruity flavor from being obscured by high concentrations of fatty acids and facilitated the formation of a more complex fermented beverage.

Phenylethyl compounds tend to exhibit a rosy and nutty flavor, but their high thresholds limit the contribution of such substances to the aroma profile of fermented beverages. Phenylethanol was the most prevalent phenylethyl compound, ranging from 1327 to 1875 μg/mL, and the highest level was found in the composite fruit beverage fermented with the mixed culture (Supplementary Table S4). Similarly, Kanter et al. (2020) have reported that a mixed fermentation process involving non-*saccharomyces* yeasts tends to positively influence the formation of phenylethanol and they pointed out that the extracellular enzyme of non-*saccharomyces* yeasts may be a critical variable in the regulation of phenylethanol accumulation. Volatile phenols, which typically possess a vanilla flavor and low threshold, are generally regarded as the cause of off-flavors in fermented beverage (Tarko & Duda, 2024). However, they can enhance the fermentative aroma of wine samples when combined with other oxides (Delač Salopek et al., 2022). The content of volatile phenols in the optimized pear-grape alcoholic beverage was at least 7 % higher than that in the other samples (Supplementary Table S4).

3.2.3. Cluster analysis of the aroma compounds

According to the accumulation of varietal (Fig. 3A) and fermentative aroma (Fig. 3B), pure Zaosu pear beverage was clustered into one category and pear-grape composite beverage into another category, which indicated that there are significant differences in the aroma profile of these two fruit fermented beverages. With the exception of linalool, the contents of the varietal aroma compounds in the pear-grape beverages were higher than those in pure pear beverages, especially in the composite fruit beverages prepared with optimization (Fig. 3A). The addition of grape juice increases the content of varietal aroma compounds in pear fermented beverage, while non-*Saccharomyces* yeasts may promote the release of varietal aroma compounds mainly through the secretion of glycosidase (Maicas & Mateo, 2023). The volatile aroma compounds were more abundant in the composite fruit beverages when compared to pure pear beverage. In contrast, the non-optimized pear-grape alcoholic beverage was characterized by high levels of ethyl decanoate, trans-3-hexen−1-ol, and fatty acid, while the optimized composite fruit alcoholic beverage accumulated rich varietal aroma compounds, higher alcohols, and esters (Fig. 3B).

3.2.4. Principal component analysis of aroma compounds

A total of 20 volatile compounds with OAV > 0.1 (Supplementary Table S4) were chosen to perform principal component analysis (PCA) in order to visualize the discrimination of fermented beverage samples on the first two principal components (PC) (Fig. 4). PC1 and PC2 accounted for 73.1 and 22.0 % of the total variation, respectively. The pure pear and composite fruit fermented beverages could be easily separated, which was consistent with our hierarchical clustering heat map results. To be specific, pure pear beverage was distributed in the negative part of PC1 and related to phenylethyl acetate, ethyl decanoate and linalool, which enhance the floral, fruity, and sweet attributes. On the positive part of PC1, most of the volatile compounds covering esters and terpenes were grouped to the pear-grape alcoholic beverages. The composite fruit beverages prepared with and without optimization were clearly separated on PC2 and the optimized pear-grape beverages showed strong correlations with citronellol, isoamyl acetate, phenylethanol, 1-octanol, and some esters (Fig. 4).

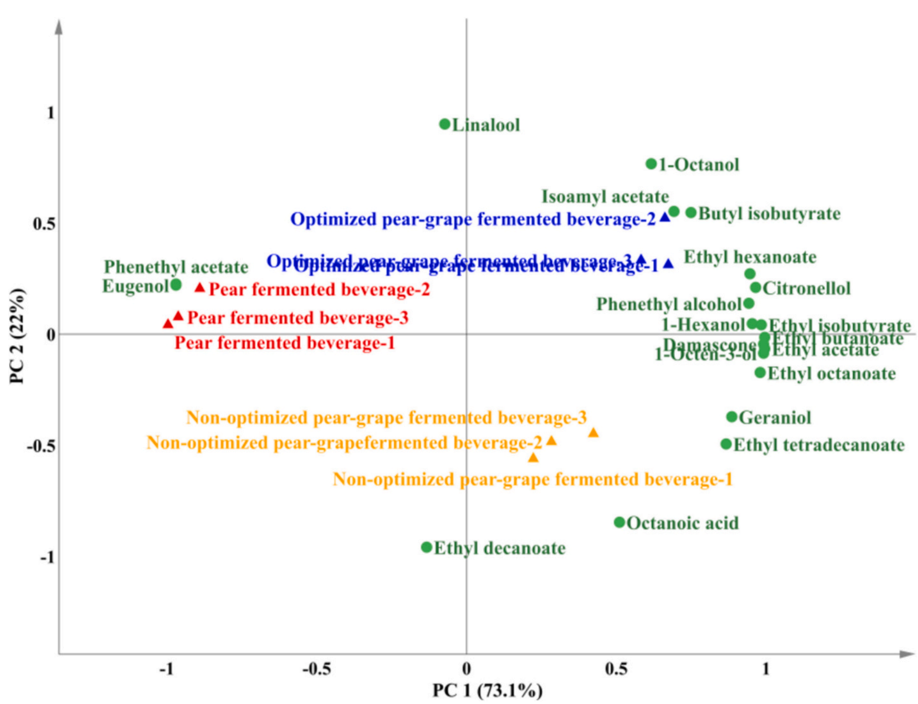

Fig. 4. Bioplot of principal component analysis (PCA) for volatile aroma compounds (OAV > 0.1) in fermented beverages.

Table 3
The vote on the sensory evaluation of fermented beverages.

Run	Color and clarity			Aroma			Taste			Typicality		
	Excellent	Good	Ordinary	Excellent	Good	Ordinary	Excellent	Good	Ordinary	Excellent	Good	Ordinary
1	0	2	8	0	8	2	3	7	0	0	10	0
2	8	2	0	8	2	0	8	1	1	9	1	0
3	9	1	0	8	1	1	8	2	0	9	1	0
Weight	0.235			0.265			0.285			0.215		

3.3. Sensory evaluation of the beverage samples

The low-alcohol pure pear and composite fruit beverages without and with optimization were numbered as 1, 2 and 3, respectively. Based on the sensory scores of the fermented beverages (Table 3), the fuzzy mathematical sensory scores of the pure pear beverage and non-optimized and optimized composite fruit beverages were calculated to be 4.54, 7.20, and 7.46, respectively. The optimized composite fruit alcoholic beverage exhibited the highest scores for color and clarity, which may be associated to the anthocyanins, flavonoids, proteins, and alcohol contents in the samples. In addition, the optimized composite fruit beverage had higher taste scores, which were possibly due to the appropriate ratio of sugar to organic acids in the mixed fermentation process. Regardless of whether the fermentation parameters were optimized or not, the aroma and typicality of the composite fruit alcoholic beverages were significantly superior to those of pure pear alcoholic beverage. These results suggest that both the addition of grape juice and the mixed fermentation strategy may effectively improve the quality of low-alcohol Zaosu pear beverage.

3.4. Antioxidant activity of the fermented beverage

The DPPH and OH free radical scavenging rates and iron ion reducing power of the three beverage samples were significantly different ($P < 0.05$) and the antioxidant activities of the composite fruit beverages were all significantly higher than those of the pure pear beverage ($P < 0.05$) (Table 4). It has been reported that flavonoids, polyphenols, and other substances may affect the free radical scavenging activity of alcoholic beverage (Yang et al., 2021; Yuan et al., 2022). In this study, the addition of grape juice increased the release and dissolution of antioxidants, such as total polyphenols and anthocyanins, which improves the antioxidant activity of composite fruit fermented beverage. The optimized composite fruit beverage exhibited the highest DPPH and OH radical scavenging rate as well as iron ion reducing power, which were 12.64, 10.04, and 41.18 % higher than those of the pure pear fermented beverage, respectively. This may be associated with the high level of flavonoids, anthocyanins, and phenols in the optimized pear-grape alcoholic beverage (Fig. 2). Such functional molecules may generate antioxidant activity through colliding with free radicals and transferring delocalized electrons from non-volatile compounds to free radical conjugation systems (Wang et al., 2023). Meanwhile, the extracellular metabolites (proteins and possibly some alcohols, aldehydes, and ketones) produced by non-*Saccharomyces* yeasts in the early stage of the fermentation process can affect the activity of *S. cerevisiae*, which is conducive to the production and accumulation of antioxidants and enhance the scavenging ability of free radicals.

Table 4
Antioxidant index of fermented beverages.

Parameters	Non-optimized pear-grape fermented beverage	Optimized pear-grape fermented beverage	Pear fermented beverage
DPPH clearance rate (%)	93.23 ± 0.41b	97.64 ± 0.21a	82.77 ± 0.66c
OH free radical clearance (%)	90.00 ± 0.55b	93.30 ± 0.85a	81.79 ± 0.98c
Iron ion reducing power	3.02 ± 0.00b	3.25 ± 0.00a	2.14 ± 0.00c

All values are reported as the mean (± SD) of three experiments.
The values with different letters in a row are statistically different according to Tukey's test ($P < 0.05$).

4. Conclusions

In this study, the mixed fermentation of *M. pulcherrima* 346 and *S. cerevisiae* ES488 as well as the addition of grape juice were used to improve the sensory quality of Zaosu pear alcoholic beverage. Using a fuzzy mathematical sensory evaluation model based on single factor and response surface experiments, the optimum fermentation parameters of pear-grape composite alcoholic beverage were identified as follows: Initial pH of 4.22, access to *S. cerevisiae* ES488 47.02 h after *M. pulcherrima* 346 inoculation, an *M. pulcherrima* 346 to *S. cerevisiae* ES488 inoculation ratio of 1.13:1, and fermentation temperature of 19 °C. The optimized composite fruit beverage showed a significant improvement in varietal and fermentative aroma as well as taste and color. Due to the accumulation of flavonoids, anthocyanins, and phenols, the antioxidant activity of the optimized composite fruit alcoholic beverage was also much higher than that of the pure pear fermented beverage, which is conducive to prevent browning and extending the shelf life of the product. In conclusion, our optimized mixed fermentation strategy will help to improve the flavor quality and nutritional value of low-alcohol Zaosu pear-Merlot grape beverage. This study provides clear implications for exploiting the use of a mixed culture fermentation process in composite fruit alcoholic beverage, further work will focus on the regulatory mechanisms of microbial interaction on flavor quality.

CRediT authorship contribution statement

Binyan Xu: Writing – review & editing, Writing – original draft. **Xueshan Yang:** Supervision, Project administration, Investigation, Funding acquisition. **Jie Zhao:** Validation, Methodology, Formal analysis. **Baihan Yu:** Software, Resources, Methodology. **Jiaxin Li:** Software, Resources, Methodology. **Xia Zhu:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102128.

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