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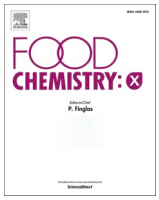

Wine aroma modification by *Hanseniaspora uvarum*: A multiple-step strategy for screening potential mixed starters

Zhong Zhang [a,b], Huiqing Wang [c], Hongchuan Xia [c,d], Lijun Sun [c], Qingchen Zhang [e], Hui Yang [a], Junxiang Zhang [a,b,*]

[a] School of Life Sciences, Ningxia University, Yinchuan, Ningxia 750021, China
[b] College of Enology and Horticulture, Ningxia University, Yinchuan, Ningxia 750021, China
[c] School of Agriculture, Ningxia University, Yinchuan, Ningxia 750021, China
[d] Project Management Department, Yinchuan Wine Industry Development Service Center, Yinchuan, Ningxia 750021, China
[e] College of Pharmacy, University of Florida, Gainesville, FL 32610, United States of America

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ABSTRACT

Hanseniaspora uvarum is a prevalent yeast species in vineyards. However, its application in grape wine fermentation remains limited. This study used culture-dependent and -independent approaches to investigate the dynamics of *H. uvarum* during the spontaneous fermentation of Cabernet Sauvignon grapes. The results revealed that *H. uvarum* constituted 77.49 % of the non-*Saccharomyces* yeast population during fermentation. An indigenous strain, QTX-C10, was isolated from the 148 *H. uvarum* strains using a multistep screening strategy. The 1:1 co-inoculation of QTX-C10 with *Saccharomyces cerevisiae* proved to be an optimal strategy for mixed fermentation, resulting in a 48.54 %–59.55 % increase in ethyl esters in Cabernet Sauvignon wine and a 96.94 %–110.92 % increase in Chardonnay wine. Furthermore, this approach reduced the acetic acid levels by 12.50 %–17.07 % for Cabernet Sauvignon wine and 10.81 %–17.78 % for Chardonnay wine. Additionally, increased ethyl ester content may enhance the tropical fruit flavor of Cabernet Sauvignon wines.

1. Introduction

In the past decade, microorganisms in vineyards and wineries have been proven to be an essential part of wine *terroir*, as they exhibit a nonrandom distribution pattern across different regions, thus contributing to the unique characteristics and distinctiveness of wine (Bokulich et al., 2014, 2016). Recent advances in culture-independent strategies using high-throughput sequencing (HTS) have allowed the identification of numerous grape- and wine-related microorganisms, enhancing our understanding of the "microbial *terroir*" of wine (Gilbert, van der Lelie & Zarraonaindia, 2014; Wei et al., 2022). The entire microbiota plays a role in shaping the characteristics of wine; however, yeasts are particularly crucial. Yeasts dominate alcoholic fermentation (AF), converting sugars to ethanol and carbon dioxide. Additionally, yeasts produce a wide array of volatile organic compounds (VOCs) essential for creating a distinct aroma in wine (Fleet, 2003). More than 100 yeast species have been identified in various wine regions worldwide as they participate in designing microbial *terroir*. They are typically divided into *Saccharomyces* and non-*Saccharomyces* (NS) (Carrau, Boido & Ramey, 2020). Inoculating grape must or juice with selected *Saccharomyces cerevisiae* strains is common in contemporary winemaking. This method is employed to minimize the risk of microbial spoilage, prevent issues like stuck or sluggish fermentations, and ensure a consistent and predictable outcome in the final wine product (Belda, Ruiz, Alastruey-Izquierdo, Navascués, Marquina & Santos, 2016). However, extensive application of commercial *S. cerevisiae* starters can suppress the biodiversity of native yeast taxa, resulting in wine products with a homogeneous profile (Sidari et al., 2021). Therefore, using mixed starter cultures (NS yeast and *S. cerevisiae* inoculated simultaneously or sequentially) to improve the aroma quality of regional wines is a better choice for winemakers.

Because vinification is not performed under sterile conditions, ripe berries naturally harbor approximately 1–5 $\times$ 10^4 yeast cells per mL in their final must (Carrau et al., 2020). Among these, more than 99 % are NS species that predominantly fall within specific genera, such as *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Kluyveromyces*, *Candida*, and *Issatchenkia* (de Filippis, la Storia & Blaiotta, 2017). The beneficial influence of NS yeasts on wine quality primarily stems from their role in modulating aroma. This effect cannot be solely attributed to the conversion of

* Corresponding author at: College of Enology and Horticulture, Ningxia University, No. 489 Helanshan West Road, Yinchuan, Ningxia 750021, China.
E-mail address: zhangjunxiang@126.com (J. Zhang).

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odorless precursors into primary aromatic compounds via the hydrolytic actions of β-glucosidases and β-lyases. NS yeasts also contribute to wine aroma by engaging in esterase, decarboxylase, and dehydrogenase activities, which enhance secondary aromas (Belda et al., 2016; Hu, Jin, Xu & Tao, 2018). However, most NS cells are vulnerable to several stressors, including the addition of sulfur dioxide during grape crushing, high osmolarity, and elevated ethanol levels. These factors can explain the gradual supplantation of NS yeast communities by *S. cerevisiae* during the fermentation process. It is important to note that not all NS yeasts improved the wine quality. Some NS strains can potentially negatively affect wine quality by producing undesirable off-odor compounds. For example, *Hanseniaspora uvarum* was observed as early as the Louis Pasteur era and has been found to dwell in various wine regions worldwide. However, the practical applicability of *H. uvarum* has been constrained by the excessive production of acetic acid and ethyl acetate with oxidative odors (Padilla et al., 2016). As mentioned, the present task of screening potential mixed starters requires consideration of both technological traits (e.g., resistance to stress, growth rate, and fermentative vigor) and qualitative profiles (e.g., enzymatic activities and the yield of VOCs associated with pleasant or undesirable notes) (Sidari et al., 2021).

Ningxia is part of a select group of regions in China that have earned the prestigious "Geographical Indication" status for their wine products. The favorable climate of Ningxia allows the prevalent Cabernet Sauvignon grapes (*Vitis vinifera* L.) to achieve satisfactory ripeness. However, Ningxia wines lack complex and distinct aromas. This may be attributed to the widespread use of commercial *S. cerevisiae* strains. These commercial strains can persist in wineries for extended periods, making it challenging to identify the indigenous *S. cerevisiae* strains unique to the region (Alexandre, 2020; Carrau et al., 2020). Fortunately, adopting foreign commercial NS products is uncommon in Ningxia, creating an opportunity to isolate native NS strains. The primary objective of this study was to conduct a comprehensive examination of the indigenous NS population within Ningxia and to select potential starters for use in future mixed fermentation processes.

2. Materials and methods

2.1. Grapes

The Cabernet Sauvignon and Chardonnay grapes (*Vitis vinifera* L.) used in this study are summarized in Supplementary Table S1, which shows their sugar content, titratable acid, yeast assimilable nitrogen, and pH.

2.2. Spontaneous fermentation and sampling

Cabernet Sauvignon grapes (vintage 2020) from three subregions named Yinchuan (YC, 106°05′ E, 38°56′ N), Yuquanying (YQY, 106°08′ E, 38°26′ N), and Qingtongxia (QTX, 105°88′ E, 38°08′ N) of Ningxia were spontaneously fermented, as previously described by Zhang et al. (2022). Samples were collected at 0, 1, 2, 4, 8, and 12 d (A, B, C, D, E, and F stages) and quenched with liquid nitrogen before being stored at − 80 °C for HTS analysis.

2.3. HTS analysis

Genomic DNA was extracted using the EZNA DNA Kit (Omega Bio-Tek, Norcross, GA, USA). The internal transcribed spacer (ITS) region of the fungi was amplified using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) synthesized by Sangon Biotechnology (Shanghai, China). The polymerase chain reaction (PCR) procedure was performed as described by Wei et al. (2022). Purification, quantification, and sequencing of PCR products were performed following Zhang et al. (2022). Raw reads were quality-filtered, merged, and processed, as stated by Zhang et al. (2022). Raw data are available in the National Center for Biotechnology Information under BioProject PRJNA810594.

2.4. Culture-dependent analysis

Wine samples from each fermentation stage were diluted in decimal series and streaked on Wallerstein Laboratory nutrient agar (Hope-Biol, Shanghai, China). After being cultured at 28 °C for five days, 20 colonies were randomly isolated from each phase, purified on yeast extract peptone dextrose (YEPD) plates (20 g/L of glucose, 10 g/L of peptones, 5 g/L of yeast extract, and 14 g/L of agar powder), and stored in 50 % (v/v) glycerol at − 80 °C. Three hundred sixty isolates were obtained and identified by sequencing the 26S D1/D2 rRNA gene. DNA extraction and the PCR procedure were conducted as described by Wang and Liu (2013). The PCR products were sequenced by Sangon Biotechnology. BLAST searches [http://www.ncbi.nlm.nih.gov/blast (accessed on 6 October 2021)] were performed to identify strains at the species level when homologies with type strains were greater than 99 %. Nucleotide sequences of identified isolates have been deposited with GenBank under the accession numbers in Supplementary Table S2.

2.5. Gas chromatography–olfactometry/mass spectrometry (GC-O/MS) analysis

The GC-O/MS analysis of the spontaneously fermented wines was performed according to our previous research (Sun, Zhang, Xia, Zhang & Zhang, 2023), using a 7890B GC equipped with a 7000D MS (Agilent Technologies, Santa Clara, CA, USA) and an ODP-4 olfactory detection port (Gerstel, Mülheim, Germany).

2.6. Sniff test

For the primary selection of *H. uvarum* strains, 13 trained judges performed the sniff test. The panelists had more than three years of experience in wine evaluation at Ningxia University (China). The precision and repeatability of the participants were assessed using the PanelCheck software (version 1.4.2, Nofima Mat, Tromsø, Norway). The native *H. uvarum* was inoculated in 100 mL of sterilized Cabernet Sauvignon grape juice (vintage 2021) at 1×10^6 CFU/mL cell density and cultured at 25 °C for 7 d without shaking. A preferred *H. uvarum* strain, Yun-268 (Hu et al., 2016, 2018, 2019; Sun, Hu, Zhang, Zhu & Tao, 2018), was inoculated in the same cell density as the control. The panelists scored the purity, intensity, elegance, and coordination of the aroma of the resulting wine on a scale of 0 to 7.

2.7. Enzyme activity test

In the second selection, an effective semiquantitative method (Hu et al., 2016; Sun et al., 2018) was introduced with modifications to determine the activities of β-D-glucosidase and esterase. The β-D-glucosidase measurement was performed in a final volume of 480 μL containing 350 μL of sterilized YEPD medium (10 g/L of peptone, 20 g/L of glucose, and 5 g/L of yeast extract), 100 μL of 1 mmol/L *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), and 30 μL of yeast culture at 1×10^6 CFU/mL cell density. Esterase activity was measured in 1 mL of reaction mixture consisting of 940 μL of YEPD medium, 10 μL of 100 mmol/L *p*-nitrophenyl octanoate (*p*NPO), and 50 μL of 1×10^6 CFU/mL yeast cells. Tubes without *p*NPG or *p*NPO served as the controls. The mixtures were incubated at 28 °C for 3 d. Strains with β-D-glucosidase or esterase activities hydrolyzed *p*NPG or *p*NPO into *p*-nitrophenol (*p*NP) and formed a yellow color at 400 nm when added with 400 μL of 1 mol/L Na_2CO_3 or 100 μL of 0.5 mol/L NaOH.

2.8. Stress tolerance test

SO_2, glucose, and ethanol tolerances were evaluated for the third

selection procedure. Each isolate was cultured in YEPD medium at 28 °C for 24 h, with SO_2 concentrations of 20, 40, 60, and 80 mg/L; glucose concentrations of 220, 240, 260, and 280 g/L; ethanol concentrations of 2 %, 4 %, 6 %, and 8 % (v/v); and two combined stresses of (1) 80 mg/L of SO_2, 260 g/L of glucose, and 6 % (v/v) ethanol and (2) 80 mg/L of SO_2, 150 g/L of glucose, and 6 % (v/v) ethanol. YEPD medium with 0 mg/L SO_2, 20 g/L glucose, and 0 % (v/v) ethanol served as a control. Cell growth kinetics were monitored at 600 nm at 6 h-intervals. The stress tolerance of each strain was evaluated using the following equations:

$$CV = (Abs600_6 + Abs600_{12} + Abs600_{18} + Abs600_{24}) - (Abs600_0 \times 4), \quad (1)$$

$$SA = CV_{\text{stress}} - CV_{\text{YEPD}}, \quad (2)$$

where for Eq. (1), *CV* is the cumulative variation in absorbance at 600 nm over 24 h, and $Abs600_0$, $Abs600_6$, $Abs600_{12}$, $Abs600_{18}$, and $Abs600_{24}$ are the absorbances at 600 nm at 0, 6, 12, 18, and 24 h, respectively. For Eq. (2), *SA* is the adaptability to the stress of each strain, CV_{stress} is the average *CV* at different gradients of a given stress, and CV_{YEPD} is the *CV* in the YEPD medium.

2.9. Fermentation kinetics of H. uvarum strains

H. uvarum strains obtained from the previous step were inoculated into 40 mL of sterilized Cabernet Sauvignon grape juice (vintage 2021) at 1×10^6 CFU/mL and cultured at 25 °C for 7 d without shaking. The fermentation kinetics were monitored by measuring the weight loss of CO_2. Fermentation rates (FR1 and FR2) (Lee and Park, 2020; Teixeira, Caldeira & Duarte, 2015), fermentation purity (FP) (Teixeira et al., 2015), and aroma production ratio (APR) were evaluated according to the following equations:

$$FR1 = W_{CO2} \times (M_{\text{Glu}}/M_{\text{CO2}}) \times (1/W_{\text{Glu}}) \times 100, \quad (3)$$

$$FR2 = (W_{\text{Loss3}}/6) \times (1/V) \times 100, \quad (4)$$

$$FP = (C_{\text{Aa}}/C_{\text{Et}}) \times 100, \quad (5)$$

$$APR = (M_{\text{VOCs}}/DCW) \times 100, \quad (6)$$

where for Eq. (3), W_{CO2} (g) is the total weight loss of carbon dioxide, M_{Glu} and M_{CO2} are the molecular masses of glucose and carbon dioxide, respectively, and W_{Glu} (g) is the initial glucose weight in grape juice. For Eq. (4), W_{Loss3} (g) corresponds to the weight loss of carbon dioxide during three consecutive days (72 h) with the highest fermentation rate, and *V* (L) is the volume of grape juice. For Eq. (5), C_{Aa} (g/L) and C_{Et} (g/L) are the concentrations of acetic acid and ethanol, respectively, produced during AF. For Eq. (6), M_{VOCs} (g) is the overall weight of VOCs generated in 40 mL of fermentative broth, whereas *DCW* (g) indicates the final dry cell weight of each strain.

2.10. VOC production of H. uvarum strains

Volatile compounds were extracted using the HS-SPME protocol described by Sun et al. (2023). The volatile extract was desorbed at 250 °C for 10 min in the split-less mode. The oven temperature for the DB-Wax column (60 m × 0.25 mm id × 0.25 μm; J&W Scientific, Folsom, CA, USA) was initially held at 50 °C for 1 min, then elevated to 220 °C at 3 °C/min, and maintained for 5 min. The temperature of the transfer line was set at 250 °C. In the MS detector, the scan mode (29–350 *m/z*) and electron ionization source were used, with a source temperature of 230 °C and electron energy of 70 eV. The VOCs were identified using the NIST 17 spectral library and verified using the linear retention indices (LRIs) of C8–C20 alkanes (Sigma-Aldrich, Shanghai, China) on a DB-Wax column. Stock solutions of standards were prepared for quantification (Table S3), and any VOC whose reference standard was not available was quantified according to the calibration curve of one with a similar chemical structure.

2.11. Mixed fermentation using sterilized grape juice

Mixed fermentations were performed at 25 °C in 1-L glass flasks containing 800 mL of sterilized Cabernet Sauvignon grape juice (vintage 2021). Pure-culture fermentation of the commercialized Ningxia native *S. cerevisiae* strain CECA was used as a control (Sc). In sequentially inoculated fermentation, *H. uvarum* QTX-C10 was inoculated 48 h before CECA in two proportions: 1:1 (Se1) or 10:1 (Se10). In simultaneously inoculated fermentation, QTX-C10 and CECA were added at a 1:1 (Si1) or 10:1 (Si10) ratio. The total yeast cells in each treatment were controlled at 2.0×10^6 CFU/mL. The fermentation process was monitored by assessing the weight loss of CO_2. Fluctuations in the yeast cells were tracked by streaking the diluted fermentation broth onto Wallerstein Laboratory plates. The production of VOCs was measured as described in Section 2.10.

2.12. Mixed fermentation using fresh grape juice

Fresh grape juice was obtained by destemming, crushing, and pressing Cabernet Sauvignon grapes harvested from Yinchuan and Qingtongxia in 2022. The grape juice was then subjected to simultaneous inoculation with equal cells of *H. uvarum* QTX-C10 and *S. cerevisiae* CECA and fermented at 25 °C. Grape juice inoculated with CECA alone served as the control. To further examine the effects of QTX-C10, Chardonnay grapes from the Yinchuan and Qingtongxia subregions were subjected to the same processing steps in 2022. The concentration of VOCs was determined as described in Section 2.10.

2.13. Data analysis

One-way analysis of variance was conducted using the "Agricola" package of R software (version 4.2.0; R Foundation for Statistical Computing, Vienna, Austria). Circos plots depicting the distribution of fungi in different samples were created online (http://circos.ca/). The neighbor-joining method was used to perform the phylogenetic analyses of *H. uvarum* strains via MEGA (version 11.0.13, Bethesda, MD, USA). Heatmaps and line charts were generated using GraphPad Prism 9 (San Diego, CA, USA). The spider diagrams depicting the aroma profile of the wine were made using OriginPro 2023 (Northampton, MA, USA). Network analysis was performed using the OmicStudio tools (https://www.omicstudio.cn/tool).

3. Results

3.1. Dynamics of Hanseniaspora during spontaneous fermentation

We obtained 2,410,415 high-quality fungal ITS sequences using the Illumina MiSeq PE300 platform. The clean tags were classified into 486 OTUs belonging to 214 genera, 117 families, 52 orders, 23 classes, and five phyla. A total of 125, 120, and 189 genera were identified in Yinchuan (YC), Yuquanying (YQY), and Qingtongxia (QTX), respectively (Fig. S1a). The three subregions shared 89 genera; 90.07 % were *Saccharomyces*, *Cladosporium*, *Hanseniaspora*, and *Alternaria* (Fig. S1b). Additionally, all fermentation phases exhibited the presence of *Cladosporium*, *Hanseniaspora*, and *Alternaria*, which accounted for 83.52 % of the 16 ubiquitous genera throughout fermentation (Fig. S1c and S1d).

Among the four prominent genera, *Hanseniaspora* is the NS yeast. The Circos plot shows that the relative abundance of *Hanseniaspora* differed among the three subregions and was higher in the YQY samples (Fig. 1a). The fermentation process also revealed considerable changes in *Hanseniaspora*, which was enriched at stage C (day 2) (Fig. 1b). The relative abundance of *Hanseniaspora* at stage A (day 0) of all subregion fermentations, measured by the culture-independent method, was extremely low (less than 0.04 %) compared to those of *Cladosporium* and

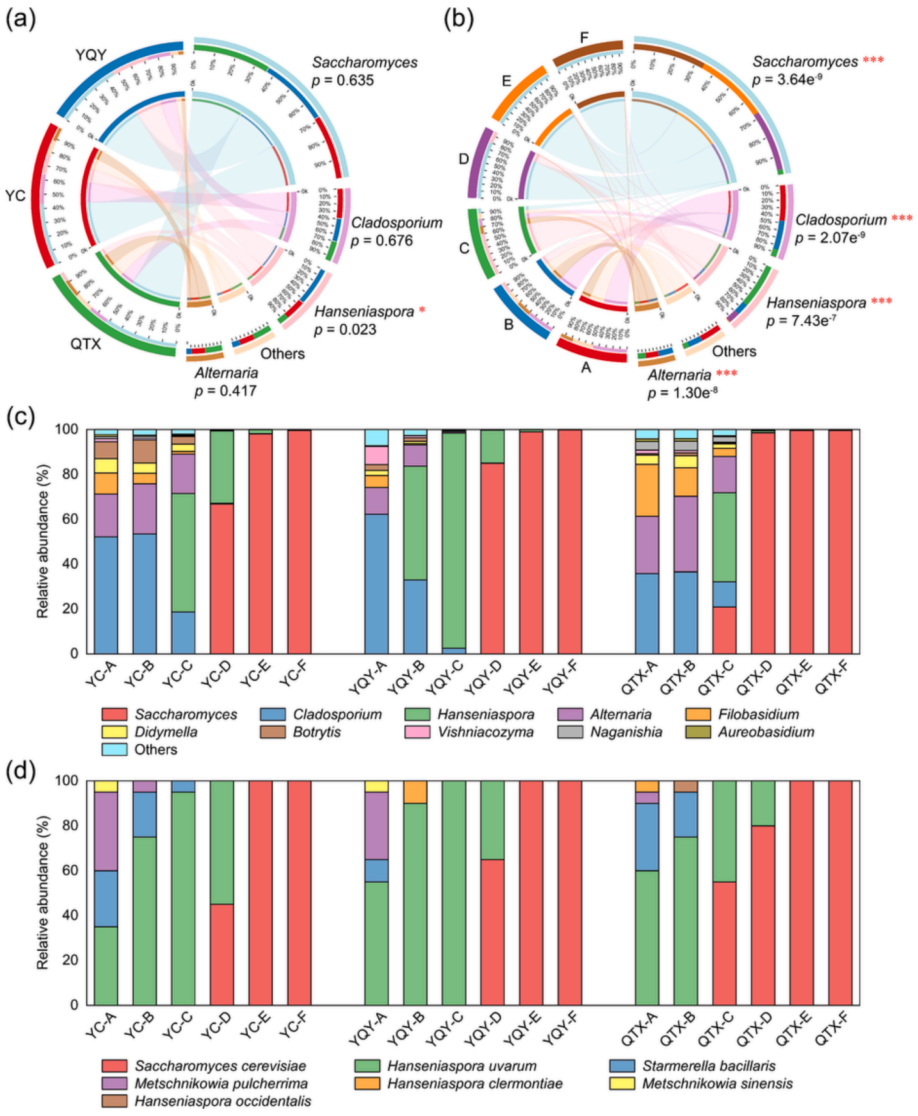

Fig. 1. Changes in the relative abundance of fungi during spontaneous fermentation. Four main fungal genera are distributed in three subregions (a) and six fermentation stages (b). The dynamics of fungi detected by the culture-independent approach (c). The dynamics of yeasts detected by the culture-dependent method (d). Three subregions are Yinchuan (YC), Yuquanying (YQY), and Qingtongxia (QTX). The six stages are A (day 0), B (day 1), C (day 2), D (day 4), E (day 8), and F (day 12). The statistical significance analysis is based on the Kruskal-Wallis *H* test: $*p < 0.05$, $***p < 0.001$.

Alternaria (Fig. 1c). As soon as AF was initiated, *Hanseniaspora* began to increase until it peaked at stage C when it was replaced by *Saccharomyces* (Fig. 1c).

Regarding the culture-dependent investigation, seven species were found by sequencing the 26S D1/D2 rRNA gene of 360 yeast strains isolated from spontaneous fermentation (Table S4). Among the 191 NS isolates, 77.49 % (148 isolates) were *H. uvarum*. *H. uvarum* dominated the yeast niche during the early stages of fermentation (0–2 d in YC and YQY and 0–1 d in QTX), coexisted with *S. cerevisiae* at stage D (day 4), and was suppressed by *S. cerevisiae* in stages E–F (8–12 d) (Fig. 1d).

3.2. Screening for indigenous strains of *H. uvarum*

According to culture-independent and culture-dependent analyses, *H. uvarum* was extensively prevalent during the early stages of spontaneous fermentation in Ningxia Cabernet Sauvignon wine. This may contribute to the formation of wine aroma. Consequently, we employed a multistep screening strategy to evaluate 148 indigenous strains of *H. uvarum* (Table S5; Fig. 2).

In the sniff test, 13 skilled judges evaluated the aroma of wines fermented using 148 autochthonous strains. Nine judges with reliable accuracy and repeatability were selected using the PanelCheck software (Fig. S2). The average scores of the four aroma traits (purity, intensity, elegance, and coordination) were used to indicate the overall olfactory quality of the wine (Table S5). Only fermented wine strains with an olfactory quality value at or greater than the first quartile of all 148 scores qualified, and 113 local *H. uvarum* strains were obtained (Fig. 2). In the enzyme activity test, 84 of the 113 indigenous *H. uvarum* strains exhibited β-D-glucosidase activity higher than or equal to the first quartile in the ranking list, and 63 of the 84 strains exhibited higher esterase activity (Table S5; Fig. 2). Based on these screening criteria, the following strains were obtained: 47 strains of *H. uvarum* in the SO_2 resistance test, 35 in the osmotic endurance test, and 26 in the ethanol tolerance test. These strains were then subjected to multiple stress treatments. Finally, 13 local *H. uvarum* strains were identified.

3.3. VOCs produced by indigenous strains of *H. uvarum*

The fermentation curves of the 13 *H. uvarum* strains and Yun-268 are shown in Fig. S3, which was further evaluated using several indices, as

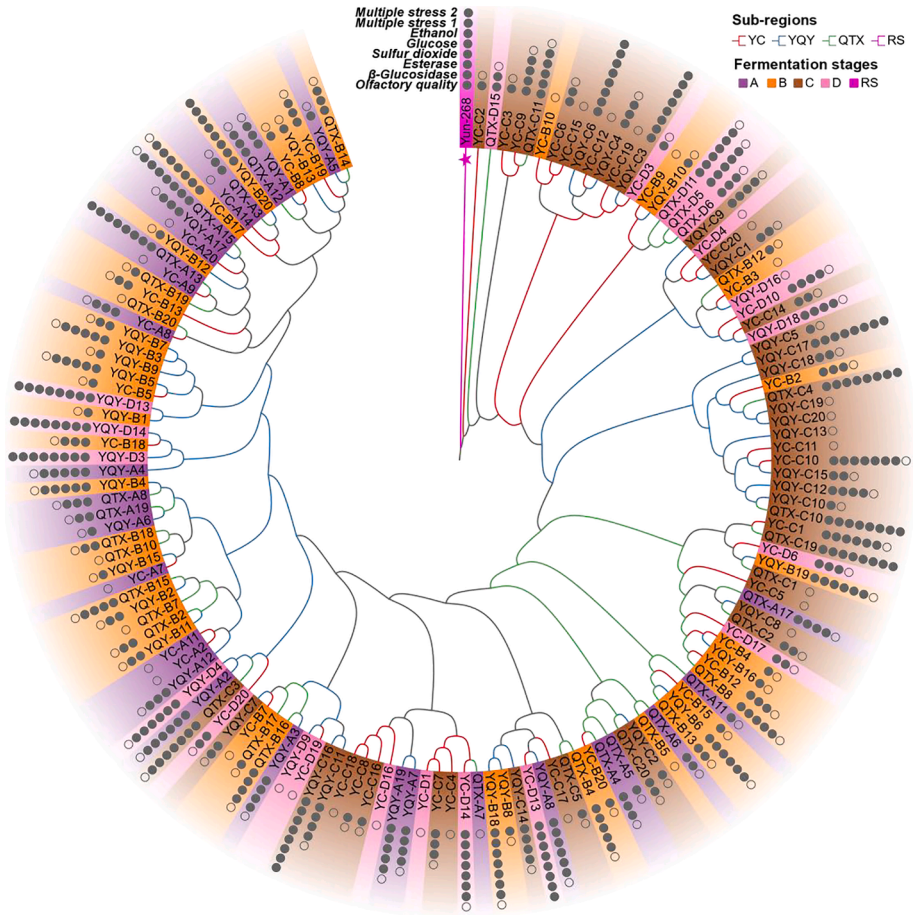

Fig. 2. Screening for indigenous *Hanseniaspora uvarum* strains. The colored branch corresponds to each subregion in the legend. The colored leaf corresponds to each fermentation stage in the legend. Three subregions are Yinchuan (YC), Yuquanying (YQY), and Qingtongxia (QTX). Fermentation stages are A (day 0), B (day 1), C (day 2), and D (day 4). RS is the reference strain Yun-268. The solid dot outside represents that the strain is retained in each round of screening, whereas the hollow dot represents a strain that is eliminated.

shown in Fig. 3a. The strains labeled YQY-C16, YQY-C17, and QTX-C10 produced ethanol at concentrations higher than 4.0 % (v/v) [4.16 % (v/v), 4.38 % (v/v), and 4.36 % (v/v)]. Compared with Yun-268, QTX-C10 fermented wine much more quickly (higher FR2) and yielded VOCs efficiently (higher APR). Furthermore, the FP of QTX-C10 was comparable with that of Yun-268.

The VOCs generated by *H. uvarum* strains are presented in Supplementary Table S6. The strains labeled YC-D13, YQY-B20, YQY-C3, YQY-C6, YQY-C16, and YQY-D13 produced total amounts of VOCs comparable to those produced by Yun-268 (Fig. 3b). However, a significant portion consisted of ethyl acetate and volatile phenols, which are undesirable for wine quality (Hu et al., 2018). YQY-C17, YQY-D14, QTX-A13, and QTX-C19 produced fewer undesirable VOCs than Yun-268; however, they were not recommended because the total VOCs that they produced were significantly lower than that of Yun-268. Some VOCs, including ethyl esters, terpenes, and aromatic alcohols, contribute to fruity and floral odors and are desirable in grape wine (Carpena et al., 2020). A negligible disparity existed in the amounts of desired VOCs generated by the 13 indigenous *H. uvarum* strains and Yun-268. Ten substances, referred to as key VOCs, were crucial for the olfactory perception of wine because they had posterior intensity values of no less than 3.0 (Table S7). Unfortunately, YC-D13, YQY-B20, YQY-C3, and YQY-C16 could not produce enough "key VOCs" (Fig. 3b). As a result, the strain QTX-C10 was retained because of a balanced performance on fermentation kinetic and VOC production.

3.4. Application of QTX-C10 in mixed fermentation using sterilized grape juice

QTX-C10 and commercial *S. cerevisiae* strain CECA were inoculated into sterile grape juice in different proportions and orders. Fermentation curves for each treatment are shown in Fig. 4a. The fermentation dynamics of simultaneous inoculation with QTX-C10 and CECA (Si1 and Si10) were similar to those of Sc. However, the fermentation dynamics of sequential inoculation (Se1 and Se10) were slower. In all treatments, CECA peaked on the fourth day after inoculation, followed by a decrease (Fig. 4b). However, peak and final cell counts of CECA in the mixed fermentations (Se1, Se10, Si1, and Si10) were slightly lower than those in pure fermentation (Sc). In the mixed fermentation, the cell count of QTX-C10 peaked on the second day after inoculation, followed by a rapid decline (Fig. 4c). With simultaneous inoculation, the inhibitory effect of CECA on QTX-C10 was more pronounced than with sequential inoculation, and the inhibition was more evident in Si1 than in Si10.

The wine samples obtained from mixed fermentation exhibited a lower acetic acid content (Table S8) and improved FP (Fig. 4d) compared to Sc. Except for Se10, all mixed inoculations could ferment grape juice into dry wine (residual sugar < 4.0 g/L). Si1 exhibited the highest VOC production efficiency.

Fifty-three VOCs were detected using HS-SPME-GC–MS. Based on similarities in their chemical structures, these VOCs were classified into 16 distinct categories (Table S9; Fig. 4e). Simultaneous inoculation, particularly with Si1, had a more pronounced effect on wine VOCs than sequential inoculation. Compared to Sc, sequential inoculation (Se1 and

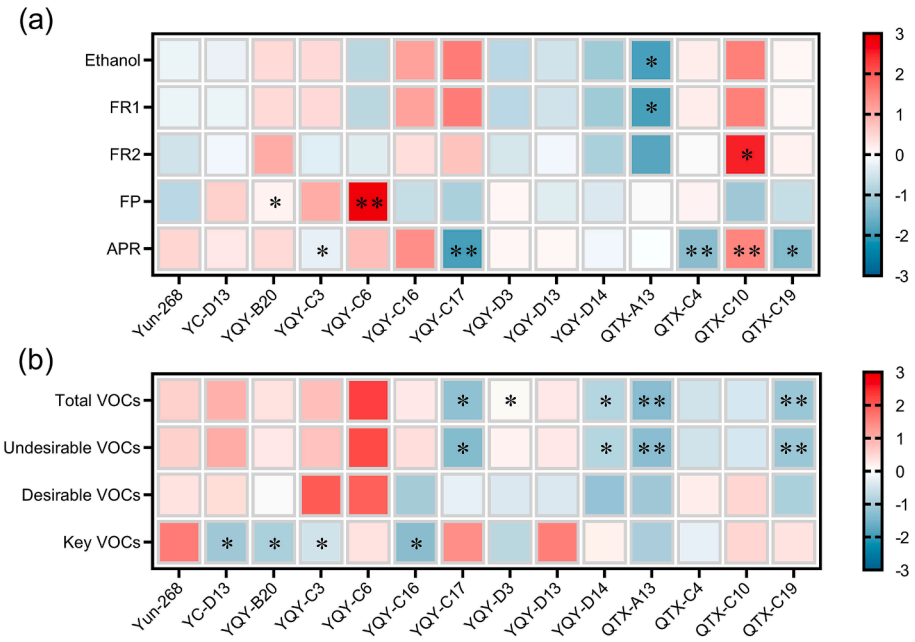

Fig. 3. Fermentation performances of indigenous *Hanseniaspora uvarum* strains. The heatmap of fermentation kinetics (a) and VOC production (b). Asterisks in a red or a blue block indicate a significantly higher or lower value than the reference strain Yun-268 (Duncan's test, $*p < 0.05$, $**p < 0.01$). FR1, fermentation rate I, calculated by Eq. (3) (higher value, higher FR1); FR2, fermentation rate II, calculated by Eq. (4) (higher value, higher FR2); FP, fermentation purity, calculated by Eq. (5) (lower value, better FP); APR, aroma production ratio, calculated by Eq. (6) (higher value, higher APR). Desirable VOCs, ethyl esters, terpenes, and aromatic alcohols. Undesirable VOCs, ethyl acetate, and volatile phenols. Key VOCs, volatiles with posterior intensity values no less than 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Se10) led to reduced levels of C6 alcohols and fatty acids in the wine. In the simultaneous inoculations (Si1 and Si10), significant reductions were observed in C6 alcohols, acetate esters, and undesirable VOCs, whereas ethyl and isoamyl esters increased significantly. The decrease in acetate esters and undesirable VOCs may be associated with the reduction in ethyl acetate (Table S9). Furthermore, in Si1, the contents of straight-chain fatty alcohols and furan derivatives decreased, whereas those of fatty acids, aromatic alkenes, total VOCs, and key VOCs increased (Fig. 4e).

3.5. Application of QTX-C10 in mixed fermentation using fresh grape juice

Considering the effectiveness of Si1 treatment, we applied this inoculation approach to the fermentation of fresh Cabernet Sauvignon and Chardonnay grape juice. The influence of the co-inoculation of QTX-C10 and CECA on the VOCs in wine varied depending on the subregion and variety (Fig. 5a). For Cabernet Sauvignon, mixed fermentation had a more pronounced effect on VOCs in the YC subregion than in QTX, whereas the opposite was observed for Chardonnay. Consistent with all treatments, mixed fermentation increased the content of ethyl esters by 59.55 % in YC and 48.54 % in QTX for Cabernet Sauvignon wines and 96.94 % in YC and 110.92 % in QTX for Chardonnay wines (Table S10). Similar to mixed fermentation with sterile grape juice, co-inoculation of QTX-C10 and CECA strains reduced acetic acid levels in Cabernet Sauvignon wines by 12.50 % in YC and 17.07 % in QTX and Chardonnay wines by 10.81 % in YC and 17.78 % in QTX (Table S11).

The panelists evaluated 12 aroma descriptors of the wine samples using the quantitative descriptive analysis described by Zhang et al. (2022) (Fig. S4). In comparison to inoculation with CECA alone, co-inoculation with QTX-C10 and CECA enhanced the flavors of tropical fruits, nuts, and jams in Cabernet Sauvignon wines from the YC subregion (Fig. 5b) and the tropical fruit and drupe flavors in Cabernet Sauvignon wines from the QTX subregion (Fig. 5c). However, the impact of mixed fermentation on the aromatic characteristics of Chardonnay wines in both the YC and QTX subregions was not significant (Fig. 5d and 5e).

3.6. Correlation between VOCs and aroma descriptors

Network analysis was utilized to explore the contribution of VOCs to the intensity of aroma descriptors based on the Pearson correlation coefficient ($|\rho| \geq 0.5$). In general, a more significantly connected module was observed in the Cabernet Sauvignon wines ($p < 0.05$) (Fig. 6a and 6b) than in the Chardonnay wines ($p < 0.1$) (Fig. 6c and 6d). The primary aroma characteristics of the Cabernet Sauvignon wines included tropical fruits, jams, nuts, spices, green grass, and dried fruits (Fig. 6a and 6b). The tropical fruit flavor was significantly and positively correlated with ethyl esters and branched-chain fatty alcohols. At the same time, the jam odor is notably positively correlated with acetate esters and methyl esters. Despite the minor impact of mixed fermentation on Chardonnay wine aromas in the QTX subregion, tropical fruits in Chardonnay wine displayed positive connectivity with ethyl esters and branched-chain fatty alcohols. However, this was not statistically significant (Fig. 6d).

4. Discussion

4.1. Dynamics of H. uvarum during spontaneous fermentation

In this study, we used high-throughput sequencing (HTS) to investigate the fungal dynamics occurring during the spontaneous fermentation of Cabernet Sauvignon grapes in three subregions of Ningxia, China. Our research revealed that filamentous fungi, specifically *Cladosporium* and *Alternaria*, were the predominant fungi in the initial grape must. As the fermentation progressed, the yeast genus *Hanseniaspora* became dominant in the early stages, ultimately allowing *Saccharomyces* to complete the alcoholic fermentation (AF) process. It is worth noting that despite the initial stage of mold growth during fermentation, their negative influence on wine quality was limited, as yeasts quickly took

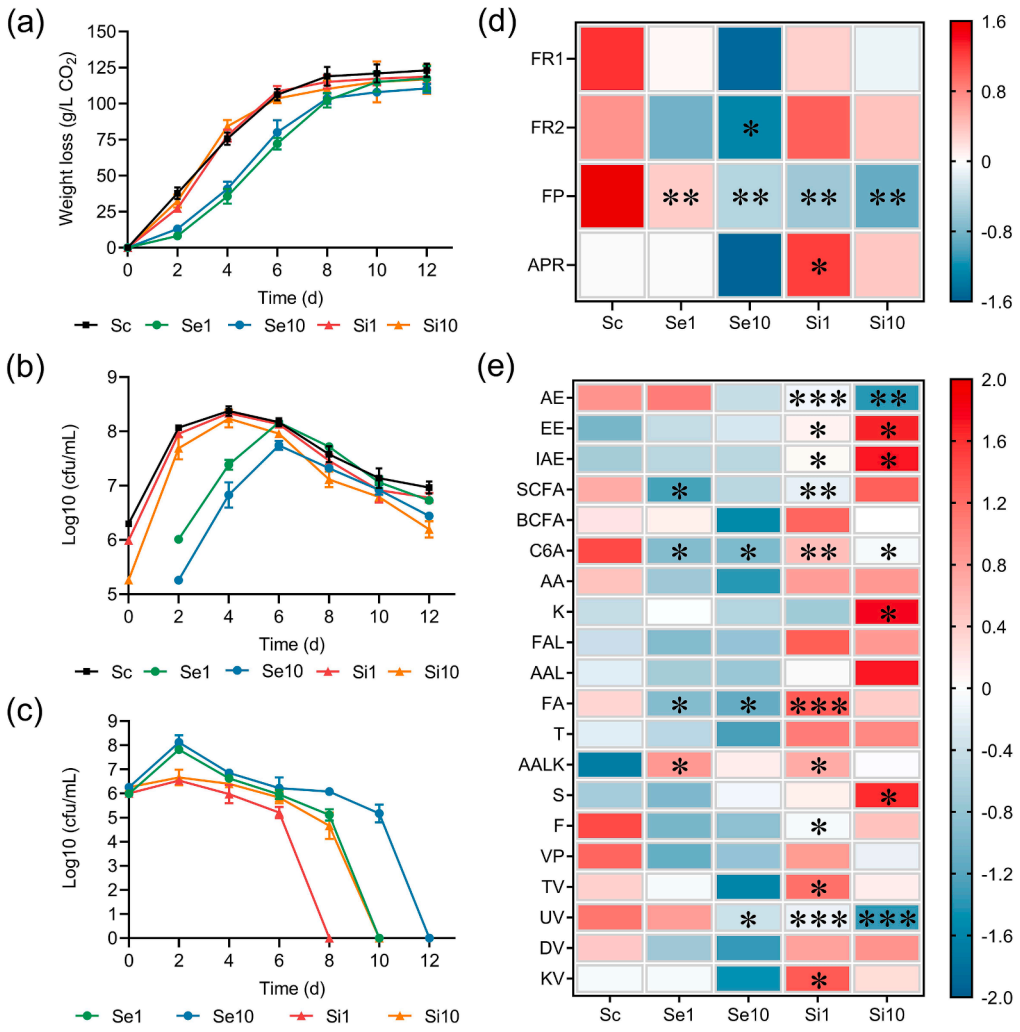

Fig. 4. Mixed fermentation using sterilized grape juice. Weight loss of carbon dioxide during different fermentations (a). Dynamics of *Saccharomyces cerevisiae* during different fermentations (b). Dynamics of *Hanseniaspora uvarum* during different fermentations (c). Fermentation kinetics of different fermentations (d). VOC production of different fermentations (e). Asterisks in a red or a blue block indicate a significantly higher or lower value than the control group labeled Sc (Duncan's test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Sc, pure fermentation of *S. cerevisiae* CECA. Se1 or Se10, *H. uvarum* QTX-C10 is inoculated 48 h before CECA in the ratio of 1:1 or 10:1. Si1 or Si10, QTX-C10 is inoculated simultaneously with CECA in the ratio of 1:1 or 10:1. AE, acetate esters; EE, ethyl esters; IAE, isoamyl esters; SCFA, straight-chain fatty alcohols; BCFA, branched-chain fatty alcohols; C6A, C6 alcohols; AA, aromatic alcohols; K, ketones; FAL, fatty aldehydes; AAL, aromatic aldehydes; FA, fatty acids; T, terpenes; AALK, aromatic alkenes; S, sulfides; F, furan derivatives; VP, volatile phenols; TV, total VOCs; UV, undesirable VOCs; DV, desirable VOCs; KV, key VOCs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

over after initiating AF (Toit and Pretorius, 2000; Wang, García-Fernández, Mas & Esteve-Zarzoso, 2015a). Our findings highlight *Hanseniaspora* as the primary NS yeast that replaced the initial mold. The *Hanseniaspora* genus, which includes species like *H. uvarum*, *H. vineae*, and *H. guilliermondii*, is commonly observed in the early phases of wine fermentation (Carpena et al., 2020; de Filippis et al., 2017; Padilla et al., 2016).

The culture-dependent approach used in our study confirmed that *H. uvarum* was the dominant NS species during both the initial and middle phases of spontaneous fermentation of Cabernet Sauvignon grapes in Ningxia. Similar findings have been reported in various viticultural regions, including Priorat in Spain (Padilla et al., 2016; Wang et al., 2015a), Campania and Marche in Italy (di Maro, Ercolini & Coppola, 2007; Milanović, Comitini & Ciani, 2013), and Attica in Greece (Nisiotou, Spiropoulos & Nychas, 2007). However, our attempts to isolate *H. uvarum* from the fermentation broth after the fourth day were unsuccessful (Table S4), even though HTS analysis indicated a minimal quantity of *Hanseniaspora* spp. in the later fermentation stages (8–12 d) (Fig. 1c). The absence of *H. uvarum* in the later stages may be caused by the inhibitory effects of increasing ethanol levels and the dominance of *S. cerevisiae* in the subsequent AF stages, as previously documented (Fleet, 2003; Schütz & Gafner, 1993). Furthermore, our culture-dependent strategy revealed that *H. uvarum*, rather than *Metschnikowia* spp. or *Saccharomyces* spp., is the predominant yeast species during the initial must stage. This finding aligns with research by Wang, Wu, and Qiu (2019), who also observed discrepancies between culture-dependent and culture-independent approaches when studying yeast diversity during the spontaneous fermentation of *Vitis davidii* Föex grapes. As mentioned above, we advocate the concurrent use of traditional culture protocols alongside next-generation sequencing technology when exploring the microbial diversity of wine, as it can provide a more comprehensive and accurate understanding of the fermentation process.

4.2. Selection of *H. uvarum* with multistep screening process

In recent years, researchers have noted a growing trend in identifying NS species with favorable fermentation characteristics through the characterization of spontaneous fermentations, moving beyond the common practice of isolating these strains from grape skins (Franco,

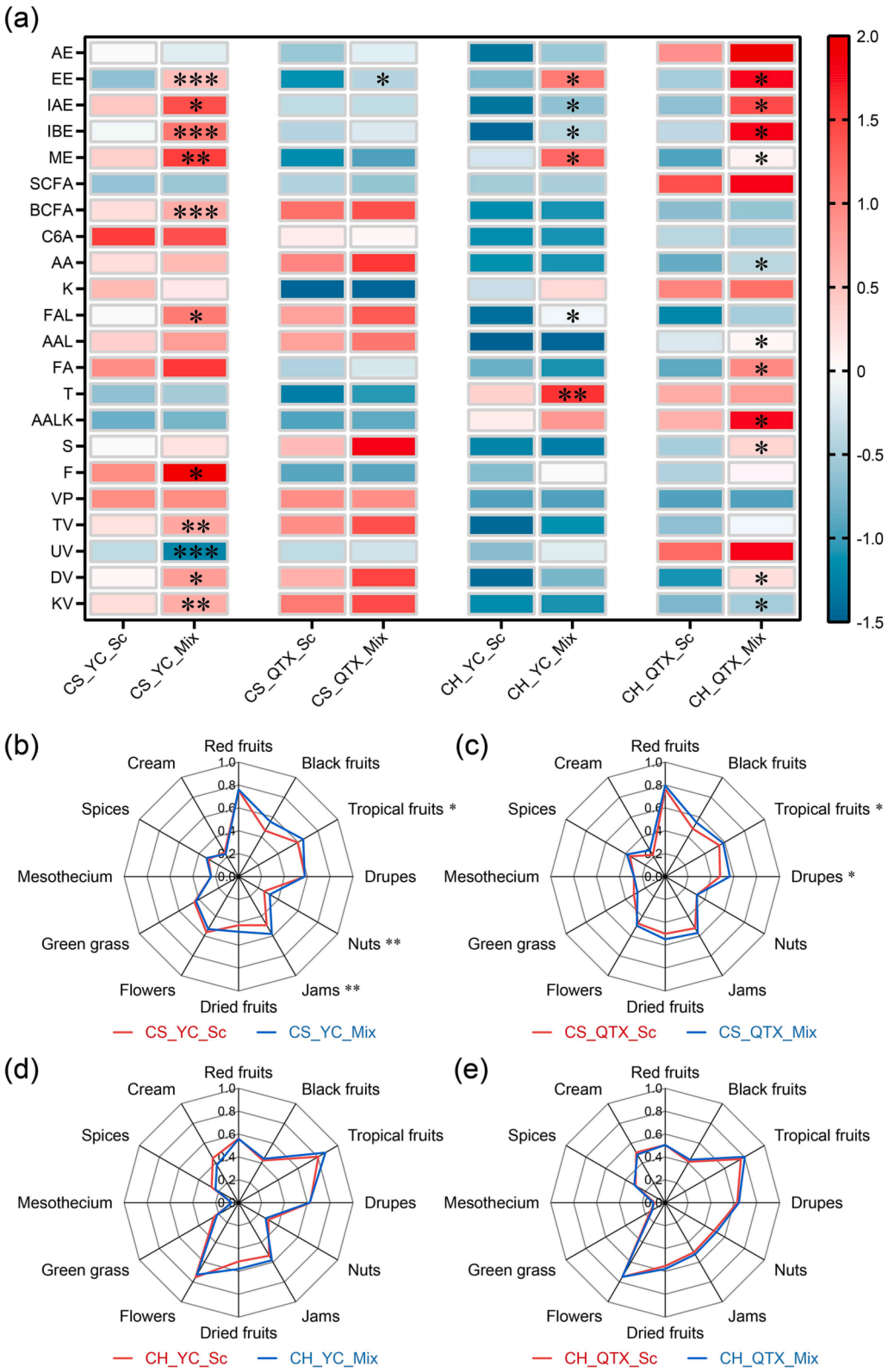

Fig. 5. Mixed fermentation using fresh grape juice. The effects of mixed fermentation on the production of VOCs (a). The effects of mixed fermentation on the aroma profiles of Cabernet Sauvignon wine from Yinchuan (b), Cabernet Sauvignon wine from Qingtongxia (c), Chardonnay wine from Yinchuan (d), and Chardonnay wine from Qingtongxia (e). In (a), the VOCs marked with asterisks represent their concentrations significantly higher or lower in Mix compared to Sc (Duncan's test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). In (b – e), the aroma descriptors marked with asterisks represent their intensity significantly higher or lower in Mix compared to Sc (Duncan's test, $*p < 0.05$, $**p < 0.01$). Sc, inoculation of *Saccharomyces cerevisiae* CECA for fermentation. Mix, simultaneous inoculation of CECA and *Hanseniaspora uvarum* QTX-C10 in equal cells for fermentation. CS, Cabernet Sauvignon. CH, Chardonnay. YC, Yinchuan subregion. QTX, Qingtongxia subregion. AE, acetate esters; EE, ethyl esters; IAE, isoamyl esters; IBE, isobutyl esters; ME, methyl esters; SCFA, straight-chain fatty alcohols; BCFA, branched-chain fatty alcohols; C6A, C6 alcohols; AA, aromatic alcohols; K, ketones; FAL, fatty aldehydes; AAL, aromatic aldehydes; FA, fatty acids; T, terpenes; AALK, aromatic alkenes; S, sulfides; F, furan derivatives; VP, volatile phenols; TV, total VOCs; UV, undesirable VOCs; DV, desirable VOCs; KV, key VOCs.

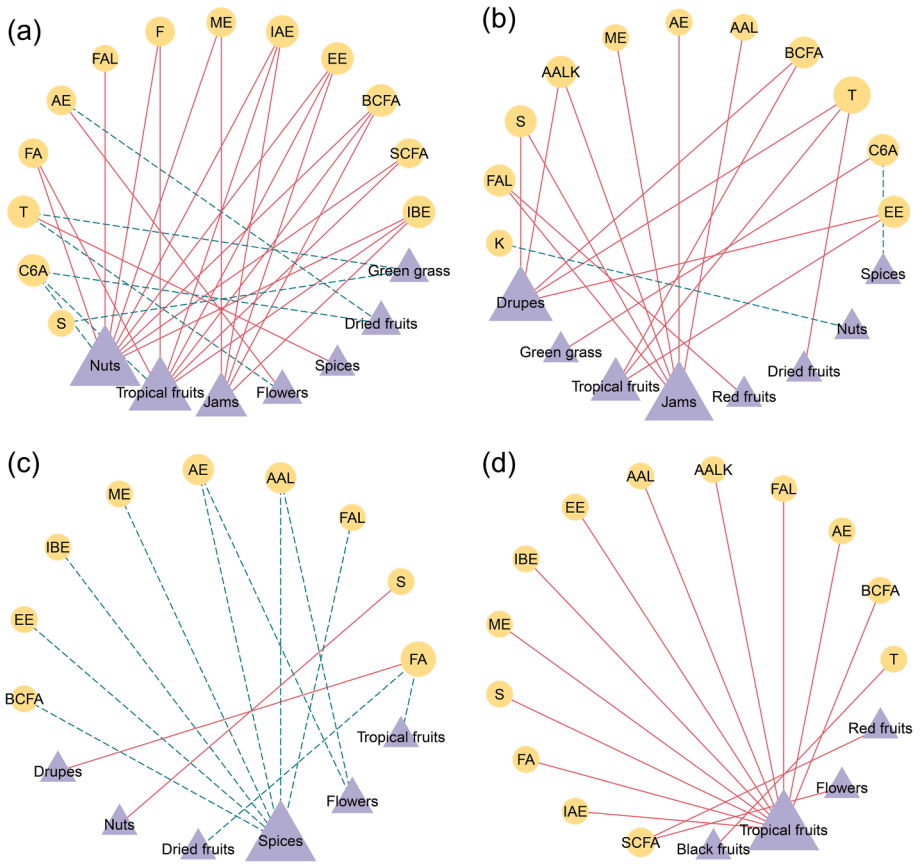

Fig. 6. Correlation between VOCs and aroma descriptors. Network analysis reflected the co-occurrence relationship between VOC categories and aroma characteristics for Cabernet Sauvignon wine from Yinchuan (a), Cabernet Sauvignon wine from Qingtongxia (b), Chardonnay wine from Yinchuan (c), and Chardonnay wine from Qingtongxia (d). Orange circle nodes represent VOC categories. Purple triangle nodes represent aroma characteristics. Direct connections between nodes in (a) and (b) indicate strong correlations (Pearson correlation coefficient, $|\rho| \geq 0.5$, $p < 0.05$). Direct connections between nodes in (c) and (d) indicate positive or negative correlations (Pearson correlation coefficient, $|\rho| \geq 0.5$, $p < 0.1$). The color of the edges represents a positive correlation (pink) or negative correlation (blue). The size of the nodes represents the degree of centrality. AE, acetate esters; EE, ethyl esters; IAE, isoamyl esters; IBE, isobutyl esters; ME, methyl esters; SCFA, straight-chain fatty alcohols; BCFA, branched-chain fatty alcohols; C6A, C6 alcohols; K, ketones; FAL, fatty aldehydes; AAL, aromatic aldehydes; FA, fatty acids; T, terpenes; AALK, aromatic alkenes; S, sulfides; F, furan derivatives. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Benavides, Valencia, Ramírez & Urtubia, 2021). In this study, we isolated 148 native *H. uvarum* strains from Cabernet Sauvignon grape musts and spontaneous fermentation broths from Ningxia. We opted for sensory rather than VOCs in the initial screening phase. This choice was made because, in some instances, volatile components may not fully represent the overall aroma quality of wine, and a sensory evaluation by a trained panel provides immediate and comprehensive results (Alexandre, 2020). Additionally, we adopted a conservative approach, discarding only 25 % of the experimental strains in each round of screening to minimize the possibility of false negatives. It is important to note that the reference *H. uvarum* strain Yun-268 is well-documented for its high β-D-glucosidase and esterase activities (Hu et al., 2016, 2018, 2019; Sun et al., 2018). These enzymatic properties are advantageous for producing terpenes and esters during AF and enhancing the floral and fruity notes in wine. Therefore, in our secondary selection process, we developed a semiquantitative colorimetric method to assess the β-D-glucosidase and esterase activities of each strain. Our findings indicated that most of the tested autochthonous *H. uvarum* strains exhibited similar enzyme activity levels to Yun-268 (Table S5).

Traditionally, grapes are treated with SO_2 after destemming and crushing to protect them from spoilage caused by microorganisms and the detrimental effects of oxygen exposure. In our third screening, we observed that most *H. uvarum* strains exhibited sensitivity to SO_2, with significant inhibition occurring when the SO_2 content exceeded 60 mg/L (data not shown). Fortunately, the established practice in Ningxia involves adding an empirical dose of SO_2 during crushing, typically ranging from 30 to 40 mg/L, depending on the grape quality. *S. cerevisiae* cells often face considerable osmotic stress when dealing with high sugar concentrations (>250 g/L) in grape juice, a condition known as very high gravity (VHG) (Auesukaree, 2017). Remarkably, the activity of *H. uvarum* cells was still facilitated in VHG environments (Table S5), even when exposed to high concentrations of SO_2 and ethanol (e.g., under multiple stress conditions). This resilience of *H. uvarum* to high osmotic stress aligns with findings from Elhalis, Cox, Frank, and Zhao (2021), who proposed that *H. uvarum* has a natural resistance to such stressors. Similarly, Wang, Mas, and Esteve-Zarzoso (2015b) suggested that low sugar concentrations could impact the cultivability of *H. uvarum*.

4.3. QTX-C10 was used in mixed fermentations

We investigated the effects of different proportions and sequences of *H. uvarum* QTX-C10 and *S. cerevisiae* CECA on grape wine fermentation. Our findings revealed that the fermentation rates observed with simultaneous inoculations (Si1 and Si10) were comparable to those with pure *S. cerevisiae* inoculation (Sc). Both simultaneous inoculations were faster by approximately 2 d compared to sequential inoculations (Se1 and Se10) (Fig. 4a). This trend aligns with the observations made by Xia, Zhang, Sun, Zhang, and Zhang (2023). The inhibitory effect of QTX-C10 on CECA was more pronounced in sequentially inoculated fermentations

than in simultaneous fermentations (Fig. 4b). This could be attributed to the prior inoculation of QTX-C10, which might lead to the consumption of some yeast assimilable nitrogen in the grape must, thereby suppressing the subsequent growth of CECA (Bordet, Joran, Klein, Roullier-Gall & Alexandre, 2020). This phenomenon was also evident in the case of the high proportion of simultaneous co-inoculation fermentation (Si10). Conversely, the inhibitory effect of CECA on QTX-C10 was notable (Fig. 4c), particularly during the low-ratio simultaneous co-inoculation fermentation (Si1). This inhibition may be linked to the production of killer toxins by *S. cerevisiae*, including glucanases, protein toxoids, and antimicrobial peptides (Bordet et al., 2020). Furthermore, the increase in ethanol during fermentation may be the main death-inducing factor for QTX-C10 because the ethanol tolerance of *H. uvarum* typically falls within the range of 4–7 % (v/v) (Moreira, Mendes, de Pinho, Hogg & Vasconcelos, 2008).

Various studies have suggested that when simultaneously co-inoculated with *S. cerevisiae*, *H. uvarum* can enhance the production of VOCs such as isobutyl acetate, isoamyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, 2,3-butanediol, and carbonyl compounds (Hu et al., 2018, 2019; Tristezza, Tufariello, Capozzi, Spano, Mita & Grieco, 2016). For sequential inoculation, wine exhibits increased levels of ethyl acetate, isobutyl acetate, isoamyl acetate, 2-phenylethyl acetate, and volatile phenols, as well as reduced concentrations of higher alcohols and fatty acids (Bordet et al., 2020; Hall, Zhou, Qian & Osborne, 2017; Hu et al., 2018; Mestre et al., 2019). *H. uvarum* strain QTX-C10, when co-inoculated with *S. cerevisiae* CECA in sterilized Cabernet Sauvignon grape juice, not only increased the content of ethyl and isoamyl esters but also reduced the levels of acetic acid and ethyl acetate in the final wine. Subsequent experiments conducted with fresh Cabernet Sauvignon and Chardonnay grape juices showed variations compared to the sterilized grape juice experiment, but a consistent trend was observed in the elevation of ethyl esters and reduction of acetic acid among the different treatments (Fig. 5a; Table S11).

5. Conclusions

A comparison between culture-dependent and -independent techniques revealed that the core NS yeast in the spontaneous fermentation of Cabernet Sauvignon grapes in Ningxia (China) belonged to the genus *Hanseniaspora*, identified as *H. uvarum*. *H. uvarum* cells survived in the fermentation broth during the first 4 d and dominated the yeast niche on the second day. Indigenous *H. uvarum* strains are capable of producing β-D-glucosidase and esterase. Most were sensitive to sulfur dioxide and ethanol, but their growth was facilitated in the VHG environment. The performance of *H. uvarum* strain QTX-C10 was confirmed by its ability to produce VOCs and efficiently ferment wine. QTX-C10 was used for mixed fermentation and exhibited the potential to increase the ethyl ester content in wine while reducing the level of acetic acid. More studies are encouraged to introduce QTX-C10 to pilot-scale mixed fermentation of grapes from different regions, varieties, and vintages.

6. Ethical statement

The participants engaged in the sensory analysis are all healthy adults who voluntarily participated in the sensory evaluation. They possess the right to withdraw their assessments at any time, and their personal information must not be disclosed without their knowledge. The wine samples used are harmless, with participants only permitted to engage in olfactory assessment, while consumption is strictly prohibited.

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CRediT authorship contribution statement

Zhong Zhang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. **Huiqing Wang:** Data curation, Software, Validation, Visualization. **Hongchuan Xia:** Formal analysis, Investigation, Methodology. **Lijun Sun:** Investigation, Software, Validation. **Qingchen Zhang:** Software, Writing – review & editing. **Hui Yang:** Methodology, Resources, Writing – review & editing. **Junxiang Zhang:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100930.

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