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ORIGINAL ARTICLE

Changes of diversity and population of yeasts during the fermentations by pure and mixed inoculation of *Saccharomyces cerevisiae* strains

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Abstract Mixed inoculation of *Saccharomyces cerevisiae* strains is used in winemaking for achieving high sensory quality of the wine. However, information on the diversity and population of yeasts during inoculated fermentation is very limited. In this study, we evaluated the effect of mixed inocula with different inoculation timing on the yeast community during fermentations of Cabernet Sauvignon. Grape must was inoculated with pure cultures of *S. cerevisiae* RC212 or *S. cerevisiae* R312, and simultaneous and sequential inoculation of both strains. Wallersterin Laboratory Nutrient (WLN) medium and sequence of the 26S rDNA D1/D2 domain were used to compare the diversity of yeast species. Five species, including *Candida diversa*, *Hanseniaspora opuntiae*, *H. uvarum*, *Issatchenkia orientalis* and *I. terricola*, were identified in the grape must, with *Issatchenkia* sp. being predominant (67.5 %). Three to four species were involved in each fermentation treatment. The fermentations by mixed inocula presented more yeast species than by pure inocula. Interdelta sequence typing was used to identify *S. cerevisiae* strains. Ten genotypes were identified among 322 isolated *S. cerevisiae* strains. Their distribution varied among different stages of fermentations and different inoculation treatments. The inoculated strains were not predominant, while indigenous genotypes I, III, and V showed strong competitiveness during fermentation. In general, this study provided information on the change of population structure and genetic diversity of yeasts in fermentations inoculated with pure and mixed *S. cerevisiae* strains.

Keywords Yeast diversity · Mixed fermentation · 26S rDNA D1/D2 · Interdelta sequence typing

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Introduction

Alcoholic fermentation of grape must mainly involves evolution and activity of various yeast species and strains. It contributes to the complex chemical composition and sensory qualities of the wine. Understanding changes in the diversity and population of yeast flora is important for wine-makers to control the alcoholic fermentation and therefore the wine quality. Nowadays, commercially available strains of *Saccharomyces cerevisiae* have been widely used for winemaking. Consequently, whether they successfully displace native yeasts during wine fermentation or whether they are replaced by native ones become critical factors of determining the complexity and quality of wines. As reported by Vigentini et al. (2009), wine making was not carried out only by the inoculated commercial starter because of the detection of indigenous strains of *S. cerevisiae*. Inoculation of multiple strains of *S. cerevisiae* contributing differently to the final flavor profile of wines has been extensively reported (Romano et al. 2003; Howell et al. 2006; Blanco et al. 2008; Romano et al. 2008). Moreover, Howell et al. (2006) reported that it was impossible to mix monoculture wines to create the same chemical and sensory profile as those fermented by mixed *S. cerevisiae* strains.

New indigenous yeast strains (Swiegers and Pretorius 2005) or mixed *S. cerevisiae* strains (Saberi et al. 2012) could be utilized to influence wine characteristics and complexity to avoid the similar character of wine. Therefore, having more information about the change of yeast diversity and

population during fermentations with different inoculation treatments is helpful for wine-makers to monitor the alcoholic fermentation, and control wine flavor. Recently, the impacts of performing the mixed fermentations using non-*Saccharomyces* species and *S. cerevisiae* on fermentation behavior and chemical profiles of wines have been reported (Ciani et al. 2006; Andorrà et al. 2010; Comitini et al. 2010; Rodríguez et al. 2010). However, there are few reports on the impact of different *S. cerevisiae* strains by different inoculation treatments on the biodiversity and interaction of yeast species and strains during fermentation.

Therefore, the aim of this study was to obtain information on the differences in the dynamics of yeast populations during mixed inoculation as well as pure culture fermentations of the Cabernet Sauvignon must and their effect on sensory profile. Commercial *S. cerevisiae* RC212 and native Xinjiang (China) *S. cerevisiae* R312, with characteristics of reduced acidity, were used to perform the fermentations. *S. cerevisiae* R312 was isolated from spontaneous fermentation of Big Thompson Seedless grapes. The characterization of yeast species was evaluated according to colony morphologies on WLN agar and sequence analyses of the 26S rDNA D1/D2 domain. Subsequently, *S. cerevisiae* strains were discriminated by interdelta sequence typing using modified primers (Legras and Karst 2003). The sensory profile of different wines was evaluated according to the method described by Li et al. (2012). In general, the results of this research are important to understand the distribution of different yeast species during mixed inoculation of *S. cerevisiae* strains, and very useful for wine-makers in monitoring and controlling mixed fermentation processes.

Materials and methods

Wine fermentations and yeast isolation

The grapes for the Cabernet Sauvignon in this study was harvested in Jingyang County, China, in 2009. Ripe and physically undamaged grape berries were crushed and put into ten 20-l stainless glass fermenters. Each of them contained 16 l of grape must with 40 mg l^{-1} SO_2. The grape must with 166.7 g l^{-1} reducing sugars, pH 2.84, total acid 8.5 g l^{-1}, expressed as tartaric acid, was chaptalized to an alcohol content of 12 % (v/v) by adding sucrose at 18 h after crushing (Fig. 1). Five types of fermentations with different yeast inocula and inoculation times were conducted in duplicate as single inoculated fermentations F1 and F2, inoculated with 10^6 cells ml^{-1} of pure cultures of Lalvin RC212 (Lallemand) and R312 (isolated from Xinjiang, China, and made into active dry yeast in our previous study) after crushing (t=0), respectively; mixed inoculated fermentations F3, inoculated with both 10^6 cells ml^{-1} of RC212 and 10^6 cells ml^{-1} of R312 at the same time (t=0); sequential inoculated fermentations F4, where 10^6 cells ml^{-1} of RC212 were inoculated at 48 h (t=48) following 10^6 cells ml^{-1} of R312 (t=0); and F5, where 10^6 cells ml^{-1} of RC212 were inoculated at 0 h (t=0) followed by 10^6 cells ml^{-1} of R312 (t=48). Active dry yeasts RC212 or R312 were suspended in clean water at 40 °C for 30 min according to the manufacturer's instructions. Fermentations were carried out in the sterile bio-hood (BCN 1360B, Hadonglian Company, Harbin, China) at 24 °C to 26 °C and monitored by measuring specific gravity. Fermentations were considered to be finished when the level of reducing sugars was below 4 g l^{-1}.

Four fermentative stages, including stage 0 (grape must after crushing), stage 1 (specific gravity of 1.079–1.081), stage 2 (specific gravity of 1.049–1.051), and stage 3 (specific gravity of 0.999–1.001), were sampled to isolate the yeast strains. Time of sugar addition and sample taking are shown in Fig. 1. Yeast growth was analyzed by enumeration of viable cells using the classical plate count method. Aliquots of 0.1 ml from serially diluted samples were spread on WLN agar (Pallmann et al. 2001) in triplicate, supplemented with 100 mg l^{-1} chloramphenicol (Wang and Liu 2013) to inhibit bacterial growth. Colonies were counted, recorded and sorted into different phenotypes after incubation at 28 °C for 5 days. For groups with colony numbers less than three colonies, all isolates were selected for the 26S rDNA D1/D2 domain sequences. For groups with over three colonies, two to six representative isolates from each group were subjected to 26S rDNA D1/D2 domain sequences. Fifteen colonies from each of the appropriately diluted samples were randomly selected, purified, and preserved at -80 °C after addition of glycerol at 20 % (v/v) final concentration. The yielded 322 *S. cerevisiae* strains were subjected to interdelta sequence typing.

DNA extraction from pure cultures

DNA isolation was carried out from pure cultures as described by Li et al. (2011).

Sequence analysis of the 26S rDNA D1/D2 domain

PCR amplification of the 26S rDNA was performed on extracted DNA samples with primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett 1998). The PCR procedure was performed as described by Wang and Liu (2013). Aliquots (5 μl) of PCR products were routinely checked by 1.0 % agarose gel electrophoresis at 100V for about 1 h. The products producing positive results were sent to Beijing Sunbiotech Co. Ltd. for purification and sequencing. Blast searches of the sequences were performed at the National Centre for Biotechnology Information (NCBI) GenBank data library (http://www.ncbi.nlm.nih.gov/blast).

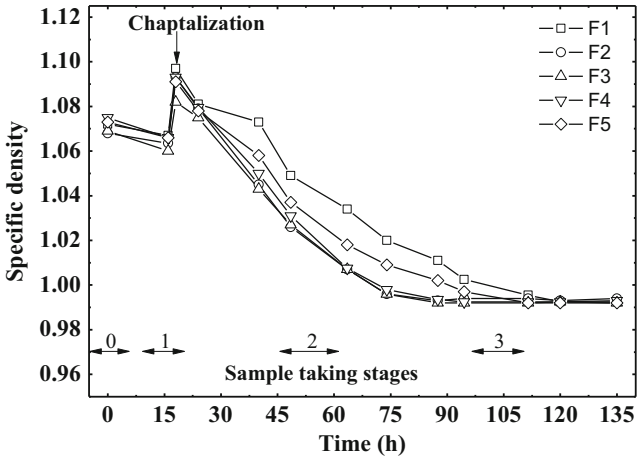

Fig. 1 Time courses of specific density of wines made with different types of inoculated S. *cerevisiae*. F1 and F2, inoculated with pure cultures of RC212 (LALVIN) and R312 (isolated from Xinjiang, China, and made into active dry yeasts in our previous study) after crushing (t=0); F3, inoculated with both RC212 and R312 at the same time (t=0); F4, where RC212 was inoculated 48 h (t=48) later than R312 (t=0); F5, where RC212 was inoculated 48 h (t=0) earlier than R312 (t=48). The double *arrows* indicate sampling stages for yeast population separation and identification

5.8S-ITS-RFLP analysis

Colonies from the *S. cerevisiae* group were confirmed by profiles of PCR-RFLP targeting the 5.8S-ITS rDNA region. The PCR amplification was performed with primers ITS1 and ITS4 as described by White et al. (1990) and the following restriction digestions were carried out using *Hae*III and *Hinf*I. Digestions were performed according to instructions of the supplier TaKaRa Biotechnology (Dalian) Co. Ltd. The restriction fragments were separated on 3 % agarose gel stained with ethidium bromide and then photographed. Type strain *S. cerevisiae* (CGMCC 2.1882=CBS 1171^T) was purchased from the China General Microbiological Culture Collection Center.

Interdelta sequence typing

Interdelta sequence typing was used for *S. cerevisiae* strain typing. PCR was carried out using delta12 primer (5'-TCAA CAATGGAATCCCAAC-3') and delta21 primer (5'-CATC TTAACACCGTATATGA-3') (Legras and Karst 2003). The amplification reaction was performed under the following conditions: a 25 μl reaction mix prepared with 2.5 μl of 10× PCR buffer (*Taq* buffer with KCl); 2.5 μl of 25 mmol $l^{-1}MgCl_2$; 2 μl of 2.5 mmol l^{-1} dNTPs; 1.25 μl of each primer; 0.4 μl of 5U μl^{-1} Taq DNA polymerase (Fermentas), 1 μl of template DNA and 14.1 μl of ultrapure water. The mixture was subjected to an initial denaturation cycle of 4 min at 95 °C, followed by 35 cycles consisting of 30s at 95 °C, 30 s at 46 °C, and 90s at 72 °C, and a final extension step of 10 min at 72 °C. The PCR products were separated by electrophoresis and then visualized under a UV transilluminator. The Interdelta sequence types of RC212 and R312 were used as references to compare the genetic profiles of all obtained *S. cerevisiae*.

Chemical analysis and sensory evaluation

When fermentation was finished, yeast lees were allowed to settle for 7 days. Then the wines were racked in 10-l bottles at room temperature for 3 months. Conclusively, the wines were bottled (750 ml) and stored at 15–20 °C for up to 6 months before chemical and sensory evaluations were performed. Residual sugars, ethanol content, total and volatile acids and were determined according to the National Standards of the People's Republic of China (GB15038-2005). Sensory test data were analyzed using Friedman test (Li et al. 2012) with the least significant difference at 5 % level. Twenty trained judges were asked to evaluate the samples. For each of the judges, the samples were ranked first to last—from 1 to 5—for the five wine samples, respectively. Result of each wine (Ri) was the sum of the ranks given by the judges. The wines with smaller sum indicated better quality. Sensory tasting was conducted at 20–22 °C according to standardized procedures.

Results

Yeast counts and morphology on WLN plates

In this study, the viable yeast counts were determined using WLN agar. On WLN agar plates, five different yeast morphotypes were observed altogether. WLN agar and sequencing of D1/D2 region of 26S rDNA were used to identify the isolated yeasts. Table 1 shows 26S rDNA D1/D2 fragment size of the sequenced strains and identity with related yeasts. Six different species in four genera were identified to be *S. cerevisiae* (type A), *Hanseniaspora opuntiae* (type B), *H. uvarum* (type B), *Issatchenkia terricola* (type C), *I. orientalis* (type D) and *Candida diversa* (type E).

The inoculated fermentations showed different trends of viable yeast numbers (Table 2). The fresh grape must exhibited a total minimal yeast count on WLN medium of 0.02×10^7 CFU ml^{-1}. Both single inoculated fermentations F1 and F2 exhibited maximum numbers of 110×10^7 and 335×10^7 CFU ml^{-1}, respectively, at stage 2, before decreasing to 20×10^7 and 19.5×10^7 CFU ml^{-1}, respectively, at stage 3. In mixed inoculated fermentations F3, the number of viable cells experienced a declining trend and decreased from 295×10^7 CFU ml^{-1} at stage 1 to 13×10^7 CFU ml^{-1} at stage 3. In contrast, the yeast numbers at stage 3 in sequential inoculated fermentations F4 and F5

Table 1 26S rDNA D1/D2 fragment size of the sequenced strains and their identity with the reference species

WLN type	Strain	Fragment sequenced (bp)	Related members of the family yeasts	Type strain [a]	Identity (%)	GenBank accession No.
A	CEC 23w66	622	*Saccharomyces cerevisiae* AY048154.1	NRRLY-12632	100	JX110693
A	CEC 23w61	609	*Saccharomyces cerevisiae* AY048154.1	NRRLY-12632	100	JX110697
A	CEC 23w65	600	*Saccharomyces cerevisiae* AY048154.1	NRRLY-12632	99.8	JX110688
B	CEC 13w76	618	*Hanseniaspora uvarum* DQ377648.1	VTTC-04561	100	JX110690
B	CEC 23w69	600	*Hanseniaspora opuntiae* AJ512451.1	CBS 8820	100	JX110695
B	CEC 23w64	601	*Hanseniaspora opuntiae* AJ512453.1	CBS 8733	100	JX110694
B	CEC 01w25	600	*Hanseniaspora opuntiae* AJ512453.1	CBS 8733	100	JX110698
B	CEC 23w68	621	*Hanseniaspora opuntiae* AJ512453.1	CBS 8733	100	JX110699
B	CEC 01w23	617	*Hanseniaspora opuntiae* DQ872866.1	MH502	100	JX110700
C	CEC 01w22	602	*Issatchenkia terricola* EF550233.1	NRRLYB-4310	99.7	JX110687
C	CEC 23w62	599	*Issatchenkia terricola* EF550233.1	NRRLYB-4310	99.8	JX110692
C	CEC 13w73	599	*Issatchenkia terricola* EF550233.1	NRRLYB-4310	99.8	JX110689
D	CEC 01y37	609	*Issatchenkia orientalis* EF550222.1	NRRLY-5396	99.8	JX110701
D	CEC 01w29	612	*Issatchenkia orientalis* EF550222.1	NRRLY-5396	99.8	JX110702
E	CEC 13w74	577	*Candida diversa* EF550213.1	NRRLY-5713	99.8	JX110691

[a] CBS Centraalbureau voor Schimmelcultures, Delft/Baarn, The Netherlands; NRRL Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA

increased to 330×10^7 and 460×10^7 CFU ml^{-1}, respectively, from the beginning of the fermentations. Overall, in all fermentations, the changes of the number of *S. cerevisiae* were correlated to those of the total yeast number.

Yeast species diversity during fermentations

The presence of yeast populations during the fermentations was evaluated by yeast count on WLN agar (Table 2). The predominant yeast flora in the crushed must was *Issatchenkia* (67.5 %), followed by *Hanseniaspora* and *C. diversa* (20 % and 12.5 %, respectively). However, *S. cerevisiae* was not found in the fresh must sample. During the fermentation process, *S. cerevisiae* became the most abundant species at stages 2 and 3, with a decreasing number of non-*Saccharomyces* species. More specifically, *S. cerevisiae* dominated in the whole fermentation process, except at stage 1 in F1 and F5 treatments, where *Hanseniaspora* was the most abundant genus (61.54 and 58.06 %, respectively). In general, *Hanseniaspora* was the second most abundant genus at stages 1 and 2 of all the fermentations, while only a small proportion of the population was presented in F1 (7.50 %), F4 (1.52 %) and F5 (1.09 %) at stage 3. *Issatchenkia*, the third largest population, participated at stages 1 and 2 of the fermentations. However, they were not identified at stage 3 in all the fermentations. *C. diversa* did not appear in the fermentations by pure cultures inoculation, but in the fermentation by mixed inocula F3 at stage 2, F4 at stage 1 and F5 at stage 1, with clone frequencies of 3.23 %, 1.92 % and 6.45 %, respectively.

Genetic diversity and distribution of *S. cerevisiae* during inoculated fermentations

In this study, information on *S. cerevisiae* strain diversity and changes of the strain level during inoculated fermentation was obtained by the interdelta sequence typing. The generated banding patterns of the 322 strains had ten different genetic profiles called I to X (Fig. 2). The inoculated yeast used in this study were named genotypes IV (R312) and VI (RC212). The ratio of each genotype among 322 strains is shown in Table 3. Indigenous genotypes I, III, and V were the three main isolates that showed strong competitiveness during the alcoholic fermentation, with clone frequencies of 20.81 %, 15.22 % and 13.35 %, respectively. Meanwhile, the proportions of the inoculated active dry yeasts RC212 and R312 were 24.84 % and 14.91 %, respectively.

In all five treatments, the fermentation process was completed by more than two genotypes of *S. cerevisiae* strains. The genetic profiles of these strains and the distribution of the 322 strains are shown in Table 3. The number of different *S. cerevisiae* strains detected for each stage varied in all the fermentations. Two different genotypes were identified throughout F1 and F2. This indicated a lower biodiversity in F1 and F2, which was in contrast with the results found in the fermentations of mixed and sequential inocula (seven genotypes in F3 and three genotypes in both F4 and F5, respectively). In F1 and F2, the fermentation process was mainly finished by the inoculated strains and another different type of the *S. cerevisiae* strain (genotypes VII and III, respectively). In contrast, of the 96 colonies from F3 which both RC212 and

Table 2 The total yeast counts and frequency of yeast species on WLN agar. F1 and F2, inoculated with pure cultures of RC212 (LALVIN) and R312 (isolated from Xinjiang, China, and made into active dry yeasts in our previous study) after crushing (t=0); F3, inoculated with both RC212 and R312 at the same time (t=0); F4, where RC212 was inoculated 48 h (t=48) later than R312 (t=0); F5, where RC212 was inoculated 48 h (t=0) earlier than R312 (t=48)

Yeast	Number of yeast cells (× 10^7 CFU ml^{-1}) [a] /Colony frequency (%) [b]							
	Stage 0	F1			F2			F3
		Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	Stage 1
S. cerevisiae	—	0.95±4.60/29.23	75±4.43/68.18	18.5±9.36/92.50	4.3±7.27/65.15	180±8.39/53.73	19.5±14.53/100	220±5.44/74.58
Hanseniaspora sp.	0.004±4.58/20	2±8.53/61.54	35±4.86/31.82	1.5±6.78/7.50	1.7±5.80/25.76	145±9.53/43.28	—	55±11.61/18.64
Issatchenkia sp.	0.0135±5.57/67.5	0.3±4.29/9.23	—	—	0.6±2.53/9.09	10±5.59/2.99	—	20±8.25/6.78
C. diversa	0.0025±7.21/12.5	—	—	—	—	—	—	—
Total [c]	0.02±7.55	3.25±14.24	110±8.02	20±11.68	6.6±9.12	335±15.07	19.5±14.53	295±8.97

Yeast	Number of yeast cells (× 10^7 CFU ml^{-1}) [a] /Colony frequency (%) [b]							
	F3		F4			F5		
	Stage 2	Stage3	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
S. cerevisiae	11.5±7.77/74.19	13±7.72/100	23±16.17/88.46	16.5±11.17/78.57	325±13.84.02/98.48	0.45±11.15/29.03	90±12.35/54.55	455±10.37/98.91
Hanseniaspora sp.	3±9.84/19.35	—	2±12.10/7.69	4±6.93/19.05	5±4.34/1.52	0.9±16.42/58.06	60±11.24/36.36	5±0.84/1.09
Issatchenkia sp.	0.5±2.28/3.23	—	0.5±3.52/1.92	0.5±5.06/2.38	—	0.1±6.81/6.45	15±4.56/9.09	—
C. diversa	0.5±4.15/3.23	—	0.5±4.98/1.92	—	—	0.1±3.85/6.45	—	—
Total [c]	15.5±8.99	13±7.72	26±15.03	21±14.46	330±11.64	1.55±11.21	165±17.44	460±11.08

—: undetected

[a] The data are express as the mean value plus or minus the standard deviation

[b] Percentage calculated from each stage

[c] Total yeast populations of each stage during the fermentations

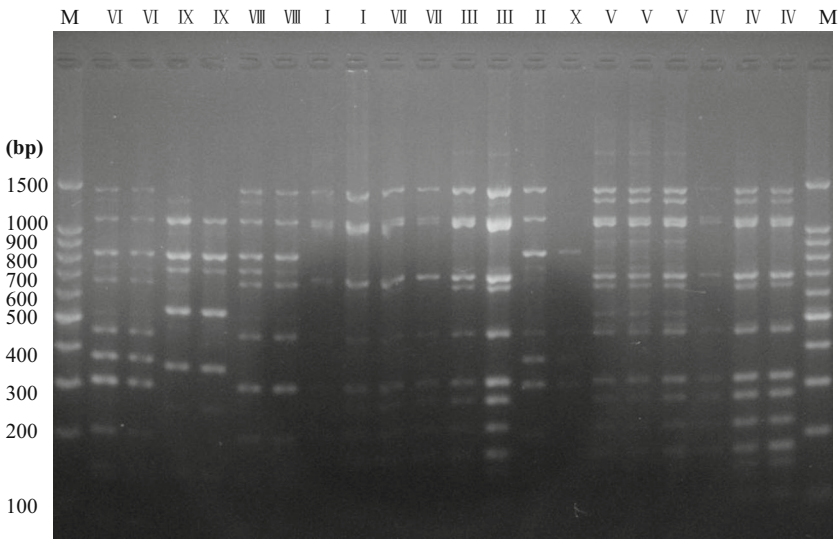

Fig. 2 The electrophoretic profiles of interdelta sequences obtained from 322 S. *cerevisiae* strains studied. The Lane M is 100 bp DNA Ladder. Roman numerals represent different genotypes

R312 were inoculated at the same time, 43 colonies presented indigenous strains genotype V, while active dry yeasts (genotype IV and genotype VI) were rarely identified (six colonies in total). In F4, with RC212 inoculated 48 h after R312 inoculation, genotype III represented at a level of 18.06 % at stage 1, while the number of genotype I increased to a level of 38.89 % at stage 3 among the 72 isolates. Surprisingly, only five isolates (namely genotype IV, R312) of the inoculated strains were detected in 72 colonies. Among the 44 colonies studied in F5, the genotype VI (RC212) increased from 4.54 % at stage 1 to 65.91 % at stage 3, with four colonies of genotype VII and two colonies of genotype IX.

Chemical properties and sensory evaluation

The chemical composition and sensory evaluation of wines derived from Cabernet Sauvignon using different types of inoculations are summarized in Table 4. Sugar level decreased to a final content under 4 g l^{-1}, while the final ethanol concentrations reached about 12 %. There were significant differences in the content of total acidity, except for the differences between F2 and F4. Sensory analysis proved that the quality of wines were different by the fermentation treatment. With the lowest total acidity of 7.19 g l^{-1} and Ri value of 27, the wine fermented from F5 was well accepted by the judges with the best sensory quality.

Discussion

In the present study, WLN agar and sequence analyses of the 26S rDNA D1/D2 domain were used to profile the yeast communities present during fermentations by pure and mixed inoculation. The changes of species diversity and population during fermentations of five different treatments of inoculated *S. cerevisiae* strains were observed. In our study, *Issatchenkia* sp. and *Hanseniaspora* sp. were dominated in the grape must. The findings were similar to the previous studies (Zott et al. 2008; Clavijo et al. 2010). As the fermentation continued, *Hanseniaspora* and *Issatchenkia* became the second and the third genera at stages 2 and 3. It showed that they had similar ethanol tolerances to *S. cerevisiae* to survive in the alcohol fermentation process (Xufre et al. 2006; Li et al. 2011).

Although the biodiversity of non-*Saccharomyces* yeasts were inhibited by the use of the starter culture (Henick-kling et al. 1998; Ganga and Martínez 2004), in this study, a noteworthy difference in the yeast community during the inoculated fermentations was the population change of non-*Saccharomyces*. The percentage of genus *Issatchenkia* (67.5 %) was clearly higher than that of *Hanseniaspora* (20 %) in the grape must, whereas *Hanseniaspora* was mostly present in the process of the inoculated fermentations. The impact of inocula and inoculation timing on the development and diversity of yeast species during the fermentations of Cabernet Sauvignon was significant. *C. diversa* was not isolated during the monoculture fermentation compared to the fermentations of the mixed *S. cerevisiae* strains, regardless of simultaneous or sequential inoculations. In addition, the presence of *C. diversa* at stage 2 in simultaneous inoculations *vs*. In sequential inoculations, stage 1was changed by the inoculation treatment. A recent study of microbiota found that *C. diversa* was linked with sour rotten grape (Barata et al. 2012). However, in this study, its presence was not found at the end stage of the fermentation.

The presence and diversity of *S. cerevisiae* strains during wine fermentation are complex and unpredictable. Many factors, such as climatic conditions, specific factors (age and size) associated with vineyards and fermentation processes (Guerra et al. 2001; Valero et al. 2007), and grape variety (Schuller

Table 3 Frequency of each genotype found among the 322 *S. cerevisiae* strains using interdelta sequence typing

Genotype	Frequency of each genotype														
	Total number [a]	F1[b]			F2[b]			F3			F4			F5		
		Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
I	67 (20.81)	—	—	—	—	—	—	9	10	4	8	8	28	—	—	—
II	6 (1.86)	—	—	—	—	—	—	—	2	2	—	—	2	—	—	—
III	49 (15.22)	—	—	—	6	9	11	—	—	2	13	—	8	—	—	—
IV (R312)	48 (14.91)	na	na	na	11	6	25	1	—	—	5	—	—	—	—	—
V	43 (13.35)	—	—	—	—	—	—	14	14	15	—	—	—	—	—	—
VI (RC212)	80 (24.84)	2	6	29	na	na	na	1	2	2	—	—	—	2	7	29
VII	16 (4.97)	—	—	—	—	—	—	4	8	4	—	—	—	—	—	—
VII	9 (2.80)	2	—	3	—	—	—	—	—	—	—	—	—	—	—	4
IX	2 (0.62)	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—
X	2 (0.62)	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—

—: undetected

[a] The number in bracket indicates the percentage of each type calculated from the 322 strains

[b] na: not applicable

Table 4 Chemical parameters and sensory levels of wines made with different types of inoculated *S. cerevisiae*

Fermentation	Type of inoculation [a]	Residual sugars (g l^{-1})	Alcohol content (%, v/v)	Total acidity (g l^{-1}) [b]	Volatile acidity (g l^{-1})	Sensory evaluation	
						Ri	Mean value
F1	RC212 (*t*=0)	2.13±0.01c	12.6±0.01	8.41±0.04	0.54±0.00c	58	2.90a
F2	R312 (*t*=0)	1.53±0.014d	12.1±0.01b	8.29±0.03	0.52±0.01	86	4.30b
F3	RC212+R312 (*t*=0)	1.35±0.00a	11.9±0.01a	8.12±0.01b	0.50±0.01	88	4.40b
F4	R312 (*t*=0)+RC212 (*t*=48)	1.35±0.01	11.9±0.02a	8.29±0.04c	0.51±0.00b	41	2.05c
F5	RC212 (*t*=0)+R312 (*t*=48)	1.41±0.01	12.4±0.01	7.19±0.01	0.49±0.00a	27	1.35d

[a] t : inoculation time

[b] Expressed as tartaric acid

The same letters in a column indicate no significant difference at 95 % confidence level

et al. 2012), could influence the genetic diversity of *S. cerevisiae* strains. In this study, the different types and timing of inoculation may also influence this aspect. The diversity of *S. cerevisiae* strains was higher in the F3 compared to other fermentations. It may be contributed by the greater genetic variation or competitiveness of indigenous *S. cerevisiae* strains in F3. Egli et al. (1998) reported that the diversity of *Saccharomyces* yeast strains with the use of starter cultures decreased compared to the non-inoculated one. In this study, the number of indigenous *S. cerevisiae* increased in the mixed fermentations (Table 3). It may result from the intense competition between RC212 and R312. Barrajón et al. (2009) indicated that the ADY implantation could fail by strong competition between wild yeasts and starter cultures when winemaking practices are apparently correct. The results of this study showed that the inoculation timing also affected the successful implantation of the inoculated yeasts. Specifically, the yeast R312 inoculated at the first was found to have only five colonies in F4. Also, RC212 was not successfully implanted after its inoculation, with a pretty high appearance of genotype I all through the fermentation. In contrast, RC212 was successful in its implantation in F5 after the early inoculation.

In this study, the interdelta sequence typing was used to differentiate *S. cerevisiae* strains. Similar to other studies, the use of active dry yeasts did not prevent the growth of indigenous *S. cerevisiae* strains (Barrajón et al. 2009). In F1 and F2, the active dry yeasts were responsible for the whole fermentation. However, there was an exception where the active dry yeasts did not conduct and complete the fermentations. In F3, type V, an indigenous yeast strain was dominant all throughout the fermentation process, due to the strong competition between RC212 and R312 within the same inoculation time. It was reported that a competition of two *S. cerevisiae* strains also occurred during the fermentation with dried yeast product Oenoprox68-72 consisting of strains L2868 and L2827 (Schütz and Gafner 1993). Although inoculated with R312, it was found with a low occurrence among the colonies isolated from the fermentations with mixed inocula. It can be concluded that the capability of adapting to the fermentation conditions of R312 was weaker than RC212 or other indigenous yeast strains. Therefore, it is necessary to monitor the implantation of inoculated *S. cerevisiae* strains during wine fermentation. Moreover, it is worth noting that the majority of strains from local areas could be selected and used as active dry yeasts if they could provide good enological properties of the wine (Sabate et al. 1998).

The wines fermented from F4 and F5 showed better quality and significant differences from other wines. On the other hand, the reduced acidity strain R312 may still play an important role under the circumstance of strong competition, although its population was lower during the fermentation process. Therefore, the role and characters of R312 is unknown and further study is needed in the future.

Conclusion

From the results of this study, it can be concluded that mixed inocula with different inoculation timing results in different diversity and populations of yeast species and *S. cerevisiae* strains. Even in the inoculation of active dry yeasts, there were different types of wild yeast species and strains participating in the fermentation process. The change of diversity and population by the inoculation with mixed cultures of *S. cerevisiae* strains could significantly impact wine flavor and characteristics during alcoholic fermentation.

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