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Evaluation of Yeast Diversity During Wine Fermentations with Direct Inoculation and *pied de cuve* Method at an Industrial Scale

Li, Erhu[†‡], Chuanhe Liu[†], and Yanlin Liu[*]

College of Enology, Northwest A&F University/ Research Centre for Viti-Viniculture of Shaanxi Province, Yangling, Shaanxi, China

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The diversity and composition of yeast populations may greatly impact wine quality. This study investigated the yeast microbiota in two different types of wine fermentations: direct inoculation of a commercial starter versus *pied de cuve* method at an industrial scale. The *pied de cuve* fermentation entailed growth of the commercial inoculum used in the direct inoculation fermentation for further inoculation of additional fermentations. Yeast isolates were collected from different stages of wine fermentation and identified to the species level using Wallersterin Laboratory nutrient (WLN) agar followed by analysis of the 26S rDNA D1/D2 domain. Genetic characteristics of the *Saccharomyces cerevisiae* strains were assessed by a rapid PCR-based method, relying on the amplification of interdelta sequences. A total of 412 yeast colonies were obtained from all fermentations and eight different WL morphotypes were observed. Non-*Saccharomyces* yeast mainly appeared in the grape must and at the early stages of wine fermentation. *S. cerevisiae* was the dominant yeast species using both fermentation techniques. Seven distinguishing interdelta sequence patterns were found among *S. cerevisiae* strains, and the inoculated commercial starter, AWRI 796, dominated all stages in both direct inoculation and *pied de cuve* fermentations. This study revealed that *S. cerevisiae* was the dominant species and an inoculated starter could dominate fermentations with the *pied de cuve* method under controlled conditions.

Keywords: Yeast microbiota, *S. cerevisiae*, interdelta typing, *pied de cuve*

*Corresponding author
Phone: +86-29-87092931; Fax: +86-29-87092931;
E-mail: yanlinliu@nwsuaf.edu.cn

[†]Both authors contributed equally to this work.

[‡]Current address: College of Food Science and Technology, Huazhong Agricultural University, Wuhan, China

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Wine fermentation is a complex microbiological process that involves the growth of a succession of several yeast species. The diversity and evolution of *non-Saccharomyces* and *Saccharomyces cerevisiae* yeasts play important roles in the taste, flavor, bouquet, and even the color of the wine [8]. Numerous studies have investigated yeast species and population dynamics during wine fermentation [14, 16, 17, 21, 36] and suggest using indigenous yeast to ferment wines with enhanced complexity [26]. Furthermore, strains of *S. cerevisiae* have been selected for their enological properties and various commercial starters are available to ensure the success of alcoholic fermentation [23]. Therefore, understanding the diversity of yeast species and the stability of yeast strains with sound enological properties during winemaking is important for better control of alcoholic fermentation.

China is a fast developing country and has a great potential for improved wine production and consumption. Currently, there are over 10 major viticulture regions in China and its wine production reached over 10 million hl in 2010. However, the history of modern winemaking and wine research is relatively short in China. The character, style, and quality of Chinese wines and winemaking technologies have not yet been fully identified, developed, and optimized. For wine fermentations, a number of imported commercial active dry yeast (ADY) strains are available and most wineries use ADY to inoculate wine fermentations. However, some wineries in China apply a traditional yeast starter preparation method called "*pied de cuve*" to induce grape must fermentations. The *pied de cuve* method uses yeast from a successfully running fermentation inoculated with a commercial starter to start a new grape must fermentation. In this study, the re-use of a commercial inoculum was conducted by the *pied de cuve* method. The advantage of this method is that the desirable enological properties of yeast strains from the successful fermentation are transferred to the new fermentations. A comparison study [11] analyzed the chemical parameters and sensory

characteristics of white wines made from the same yeast starter by direct inoculation and the *pied de cuve* method, and found that inoculation methods did not affect the sensory profile and the quality of the final wines significantly. However, it must be pointed out that no studies have been carried out to investigate the stability of yeast strains during these fermentations. It is possible that the yeast strains used in the direct inoculation may not have been the same ones that predominated in the *pied de cuve* fermentations.

The aim of the present study was to evaluate the diversity of yeast species and *S. cerevisiae* strains during wine fermentations by direct inoculation and *pied de cuve* methods at an industrial scale. To achieve this, yeast colonies from different fermentation stages were sorted into groups according to growth morphology on Wallersterin Laboratory nutrient (WLN) agar, and representative colonies from each WLN biotype were identified by sequencing the 26S rDNA D1/D2 domain[16, 22]. The species identity of all colonies from each *S. cerevisiae* group was confirmed by PCR-RFLP of the 5.8S-ITS rDNA region [7]. In addition, the genetic biodiversity of *S. cerevisiae* strains during fermentations was determine by a molecular method based on analysis of the polymorphisms exhibited by the PCR amplification of the interdelta sequences [15].

Materials and Methods

Industrial Scale Wine Fermentation and Yeast Isolation

Fermentations were carried out at Suntime Winery in Manasi, Xinjiang Province of China in 2009. Cabernet Sauvignon grapes were harvested and destemmed according to standard red winemaking procedures. The grape must had a sugar content of 185 g/l, and the titratable acidity was 8.7 g/l, expressed as tartaric acid. Sulfite was added to a total SO_2 concentration of 30 mg/l. Fermentations with different inoculation methods were performed in two tanks at an industrial scale. Fermentation tanks were of stainless steel (AISI 304 quality) with cooling jackets and a capacity of 110,000 L.

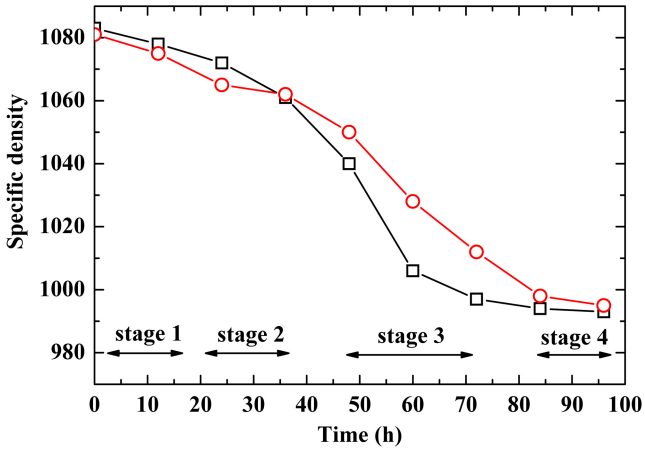

Fig. 1. Time course of specific density of grape must during fermentations with direct inoculation (□) and the *pied de cuve* method (○).
The double arrows indicate sampling stages for yeast population separation and identification.

For direct inoculation, active dry yeast of AWRI 796 (Lallemand Inc., Montreal, Quebec, Canada) was suspended in warm water at 35°C according to the manufacturer's instructions. Then the yeast suspension was added to a fermentation tank containing 88,000 L (80% of the tank capacity) of fresh must. For the *pied de cuve* method, when the specific density of the fermenting must (obtained from the direct inoculation fermentation) had reduced 10–20 g/l, 4,400 L of fermenting must was transferred to the second tank containing about 88,000 L fresh must. Fermentation temperatures were maintained at less than 28°C and musts were pumped over twice a day. Samples were taken at four fermentation stages [stage 1 (at inoculation), stage 2 (early fermentation), stage 3 (mid fermentation), and stage 4 (after fermentation)], which were determined by the range of the specific density of the fermention as shown in Fig. 1. Duplicate aliquots of 0.1 ml from serially diluted samples were plated on YEPD agar (yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l, agar 20 g/l). After incubation at 28°C for 2–3 days, 25–30 colonies from each plate were selected, cultured, and maintained on YEPD agar.

Characterization of Yeast Species

Identification of yeast isolates at the species level was carried out on Wallerstein Laboratory nutrient (WLN) medium and sequence analysis of the 26S rDNA D1/D2 domain. Isolates were restreaked on WLN agar [22], and after 5 days of incubation at 28°C, colonies were sorted into groups according to morphology and color on WLN agar. For groups with less than three colonies, all isolates were subjected to the 26S rDNA D1/D2 domain sequences analysis. For groups with colony numbers over three, 2–6 representative isolates from each group were selected for the 26S rDNA D1/D2 domain sequences analysis.

DNA Extraction

Yeast cells were grown in 3 ml of YEPD cultures. Total DNA was isolated using a MasterPure Yeast Purification kit (Epicentre, Madison, WI, USA), according to the manufacturer's instructions. DNA was quantified with a ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). DNA was stored at 0°C for subsequent use.

26S rDNA D1/D2 Domain Sequencing

The DNA fragment of 26S rDNA D1/D2 domain sequences was amplified with primers NL1 and NL4 [13, 15], with thermocycler parameters as follows: 95°C for 5 min; followed by 36 cycles at 94°C for 1 min, 52°C for 1 min, 72°C for 1 min and 20 s, and 72°C for 8 min. PCR products were purified and sequenced by Beijing Sunbiotech Co., Ltd (Sunbiotech, Beijing, China). Sequences were analyzed using the BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast).

Characterization of *S. cerevisiae* Strains

Colonies from the *S. cerevisiae* group were confirmed by PCR-RFLP of the 5.8S-ITS rDNA region [7]. This procedure included amplification with primers ITS1 and ITS4 and subsequently digestion with *Hae*III, *Hpa*II, and *Scr*FI restriction enzymes. Restriction profiles were analyzed by comparison with the fragments in the database (http://www.yeast-id.com/).

Further identification of *S. cerevisiae* at the strain level was performed by the PCR amplification of the interdelta sequences. DNA extraction was as described above. PCR amplifications were carried out in 25 μl reaction volumes containing PCR buffer (10 mmol/l Tris, pH 8.4, 50 mmol/l KCl), 30–100 ng yeast DNA, 200 μmol/l dNTPs, 2.5 mmol/l $MgCl_2$, 0.5 μmol/l of each oligonucleotide primer δ12 (5'-TCAACAATGGAATCCCAAC-3') and δ21 (5'-CATCTT AACACCGTATATGA-3'), and 2.0 U *Taq* polymerase. Amplification reactions were performed in a Peltier Thermal Cycler (PTC-200, MJ Research) as previously described by *et al*. [20] and Legras and Karst [15]. DNA fragments were separated on a 1.5% (w/v) agarose gel or 1.5% agarose at 100 V for 3 h in 0.5× TBE buffer, visualized, and photographed.

Wine Characterization and Sensory Evaluation

Wine analyses included titratable acidity, pH, ethanol content, residual sugars, volatile acid, and total and free SO_2 and were determined according to the National Standard of the People's Republic of China (GB15038-2005). Sensory profiles were evaluated by a panel of 7 wine experts. The panelists were all experienced winemakers. A total intensity scale of 20 points was used to evaluate each wine. Three attributes were selected: appearance (maximum 3 points), aroma (maximum 7 points), and oral perception (maximum 10 points). The judges were also requested to describe the global impression of each wine. Evaluations were conducted at 20–22°C according to standardized procedures.

RESULTS

Fermentation Kinetics and Yeast Counts

Fermentations with different inoculation methods were carried out in two separate tanks. Tank 1 was inoculated directly with commercial starters and tank 2 was inoculated by the *pied de cuve* method. Sugar degradation kinetics, measured as the reduction of specific density, were similar (Fig. 1) in both fermentations. Both fermentations reached a sugar content of <4 g/l within 100 h. The sugar consumption rate of the *pied de cuve* fermentation was faster than the direct inoculation at stage 1 and stage 2, but slower at stage 3 and stage 4 (Fig. 1). Yeast cell numbers throughout the fermentations were determined by spread plating (Table 1). The *pied de cuve* method had higher viable cell numbers than the direct inoculation at stage 1, stage 2, and stage 3, but lower numbers at stage 4 of the fermentation.

Diversity of Yeast Species During Fermentation

A total of 412 yeast isolates were collected from different stages of the two fermentations. Based on the colony color and morphology on WLN agar, eight different morphotypes were observed (Supplementary Fig. 1). The highest frequencies were types A and C, with 68.0% and 27.2%, respectively (Table 2). The presence of other WLN biotypes was relatively low with colony frequencies ranging from 0.24% to 2.7% (Table 2). Yeasts of type A were found to dominate all fermentation stages except stage 1 of the direct inoculation. The colony frequency of type A was higher at stage 1 in the *pied de cuve* fermentation than the direct inoculation (Table 2). Type C colonies mainly appeared in the grape must and at the beginning of both fermentations. Two colonies of type C and one of type D were isolated at stage 4 of the direct inoculation. Other WLN biotypes were only observed in grape musts (Table 2).

All colonies of groups D, E, F, G, and H were subjected to the 26S rDNA D1/D2 domain sequences analysis and were identified as species of *Candida sorbosivorans*, *Metschnikowia aff. fructicola*, *Metschnikowia pulcherrima*, *Pichia anomala*, and *Candida zemplinina*, respectively (Table 3). Two to six representative colonies from WLN biotypes A, B, and C were selected for 26S rDNA D1/D2 domain sequencing. The D1/D2 fragment size of sequenced strains and their identity with related yeasts are also shown in Table 3. Representatives from group A were identified as *Saccharomyces cerevisiae*, group B as *Hanseniaspora uvarum*, and group C as species of *Candida diversa*, *Hanseniaspora clermontiae*, and *Hanseniaspora uvarum* (Table 3).

Interdelta Sequence Typing of *S. cerevisiae* Strains

All colonies of WLN biotype A were confirmed as *S. cerevisiae* by PCR-RFLP of the 5.8S-ITS rDNA region. A total of 241 *S. cerevisiae* isolates were obtained and subsequently differentiated at the strain level by a rapid PCR-based protocol relying on the amplification of interdelta sequences. Fig. 2 is an example of the electrophoretic profiles of 17 of the strains analyzed. Frequencies of *S. cerevisiae* strains with different interdelta sequence patterns during fermentation by direct inoculation and the *pied de cuve* method are shown in Table 4. Seven distinct patterns were found: I to VII. Pattern I was from the

Table 1. Number of yeast cells at different fermentation stages with different types of inoculation methods.

Type of inoculation	Number of yeast cells (×10^6 CFU/ml)				
	Grape must	Stage 1	Stage 2	Stage 3	Stage 4
Direct seed	0.32 ± 0.08	2 ± 0.35	37 ± 1.48	2,300 ± 46	70 ± 1.21
Pied de cuve	0.43 ± 0.11	4.5 ± 0.48	105 ± 2.51	2,500 ± 38	39 ± 0.59

Stage 1, after inoculation; stage 2, at the beginning of fermentation; stage 3, in the middle of fermentation; and stage 4, the end of fermentation.

Table 2. WLN biotypes and yeast species during fermentations with different types of inoculation methods.

WLN type	Colony number/frequency (%)										Yeast species[b]
	Total[a]	Direct inoculation					*Pied de cuve*				
		Must	Stage1	Stage2	Stage3	Stage4	Must	Stage1	Stage2	Stage3	Stage4	
A	280/68.0	-	13/30.2	39/95.1	41/100.0	36/92.3	1/2.4	35/68.6	35/97.2	40/100.0	40/100.0	*Saccharomyces cerevisiae*
B	11/2.7	6/15.0	2/4.7	-	-	-	3/7.3	-	-	-	-	*Hanseniaspora uvarum*
C	112/27.2	29/72.5	28/65.1	2/4.9	-	2/5.1	34/82.9	16/31.4	1/2.8	-	-	*Candida diversa, Hanseniaspora clermontiae, Hanseniaspora uvarum*
D	4/1.0	2/5.0	-	-	-	1/2.6	1/2.4	-	-	-	-	*Candida sorbosivorans*
E	1/0.24	1/2.5	-	-	-	-	-	-	-	-	-	*Metschnikowia aff. fructicola*
F	1/0.24	1/2.5	-	-	-	-	1/2.4	-	-	-	-	*Metschnikowia pulcherrima*
G	1/0.24	1/2.5	-	-	-	-	-	-	-	-	-	*Pichia anomala*
H	2/0.48	-	-	-	-	-	1/2.4	-	-	-	-	*Candida zemplinina*

[a]Frequency was the total of five stages during must fermentations with two types of inoculation methods.
[b]Yeast species were identified by sequencing the D1/D2 domain of the 26S rDNA gene of representative colonies from each WLN biotype.

Table 3. 26S rDNA D1/D2 fragment size of the sequenced strains and the identity with related yeast.

WL type	Strain	Size (bp)	Related members of the family yeast	Type strain	Identity (%)	GeneBank Accession No.
A	CEC Y518	610	*Saccharomyces cerevisiae* GU565213	DX6-2	100	JN083824
A	CEC Y431	610	*Saccharomyces cerevisiae* AY048154	NRRL Y-12632^T	99.7	JN083825
B	CEC F15	596	*Hanseniaspora uvarum* AM160628	HA 1670	100	JN083810
B	CEC F12	610	*Hanseniaspora uvarum* AF257273	KCTC 7834	100	JN083811
B	CEC F30	600	*Hanseniaspora uvarum* EU809448	SY2SS−2	100	JN083812
C	CEC Y111	571	*Candida diversa* EF550213	NRRL Y-5713^T	99.8	JN083818
C	CEC Y123	601	*Hanseniaspora clermontiae* AJ512452	CBS 8821^T	99.4	JN083819
C	CEC Y106	595	*Hanseniaspora uvarum* EU807899	F0501-1	100	JN083820
C	CEC F7	599	*Hanseniaspora uvarum* EU809448	SY2S-2	100	JN083821
C	CEC F1	600	*Hanseniaspora uvarum* AM397849	YS 82	100	JN083822
C	CEC F20	598	*Hanseniaspora uvarum* EU268636	N229	100	JN083823
D	CEC C209	479	*Candida sorbosivorans* AJ277846	NCYCD2974	100	JN083807
D	CEC Y621	477	*Candida sorbosivorans* AJ783433	ESAB20	100	JN083808
D	CEC C219	483	*Candida sorbosivorans* AJ783433	ESAB20	100	JN083809
E	CEC F2	538	*Metschnikowia aff. fructicola* AJ786395	HA 1628	100	JN083815
F	CEC F28	528	*Metschnikowia pulcherrima* EF564392	GSGRX 2	98.6	JN083816
G	CEC C251	601	*Pichia anomala* AF330114	CBS 113^T	100	JN083817
H	CEC F9	529	*Candida zemplinina* EF452197	CBS 6100^T	100	JN083813
H	CEC F18	511	*Candida zemplinina* EF452195	CBS 2779^T	99.6	JN083814

T = type strain; NRRL: Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.

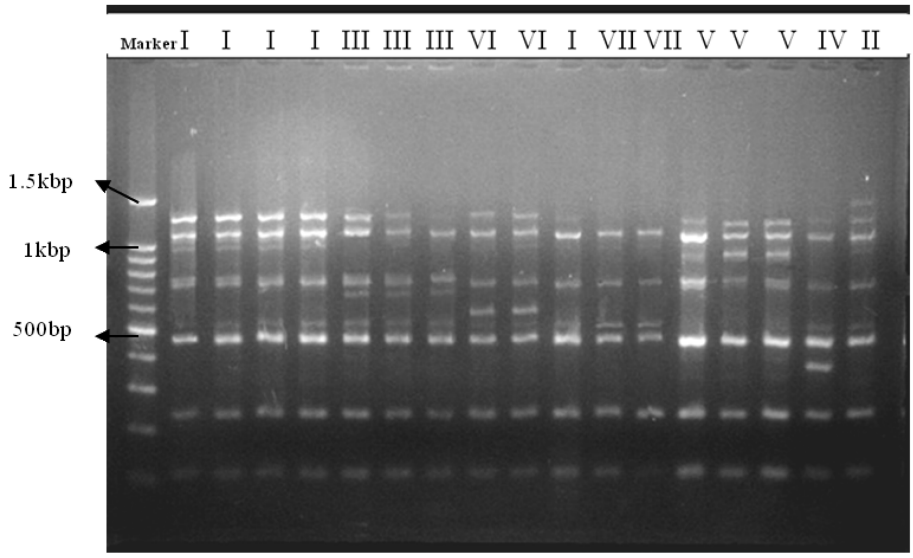

Fig. 2. Electrophoretic profiles of interdelta sequence patterns obtained from the *Saccharomyces cerevisiae* strains isolated during fermentations by direct inoculation and the *pied de cuve* method.

inoculated ADY strain AWRI 796. Other patterns were indigenous *S. cerevisiae* strains isolated from the fermentations.

Six genotypic patterns were identified in fermentation with direct inoculation and five patterns were observed in the *pied de cuve* fermentation. The commercial starter AWRI 796 (pattern I) dominated at all stages in both the direct inoculation and the *pied de cuve* fermentations. Yeast strains of patterns II and IV were only found in the direct inoculation fermentation at the beginning (BF) and in the middle (MF) of fermentation, respectively. Pattern VI was only detected in the *pied de cuve* in the middle (MF) and at the end of fermentation (EF). The other three patterns (III, V, and VII) appeared in both fermentations and persisted in the presence of the commercial starter (Table 4).

Table 4. Frequency (%) of *S. cerevisiae* strains with different interdelta sequence patterns during fermentations by direct inoculation and the *pied de cuve* method.

Polymorphic patterns	Direct inoculation			*Pied de cuve*		
	BF	MF	EF	BF	MF	EF
I (AWRI 796)	91.4	85.7	72.4	96.8	69.0	77.5
II	2.9	–	–	–	–	–
III	2.9	6.5	17.2	–	–	5
IV	–	1.3	–	–	–	–
V	2.9	2.6	10.3	–	10.3	7.5
VI	–	–	–	3.2	6.9	–
VII	-	3.9	-	-	13.8	10

BF: beginning of the fermentation; MF: middle of the fermentation; EF: end of the fermentation.

Chemical Parameters and Sensory Characterization

The analytical parameters and sensory scores of the Cabernet Sauvignon wines from the direct inoculation and *pied de cuve* method are listed in Table 5. The chemical parameters of both wines did not vary significantly and the sensory scores were the same (Table 5). All judges described both wines as having a deep ruby red color, as fruity and floral, and well balanced. The *pied de cuve* method did not affect the sensory profile and the quality of wines in this study.

DISCUSSION

The use of active dry yeast of the genus *Saccharomyces* in winemaking is a common practice at wineries because of the consistent fermentation and wine quality [9]. Although traditionally a *pied de cuve* method starts with a spontaneous alcoholic fermentation, wine makers apply this technique to transfer successful fermentations harboring commercial starters to fresh must in another tank. The new fermentation starts quickly and wine quality is not influenced significantly [11]. However, if progressively reduced fermentation rates were observed, frequent monitoring for contamination by undesirable yeasts would be necessary to control the wine quality. In addition, periodic assessment of the genetic characteristics of the yeast population is essential. The current study investigated the diversity of yeast species during two types of fermentation and revealed that *S. cerevisiae* was the dominant yeast throughout the fermentation by the *pied de cuve* method. Assessment of the stability of *S. cerevisiae* strains by interdelta sequence

Table 5. Chemical parameters and sensory level of wines made with different inoculation methods.

Wine type	Acidity (g/l)	pH	Alcohol (%v/v)	Residual sugar (g/l)	Volatile acid (g/l)	Free/total SO_2 (mg/l)	Sensory level[a]
Direct inoculation	7.4 ± 0.35	3.64 ± 0.08	11.4 ± 0.11	2.7 ± 0.46	0.23 ± 0.08	0 ± 0.0/2 ± 0.45	B3
Pied de cuve	6.8 ± 0.26	3.69 ± 0.13	11.2 ± 0.07	2.8 ± 0.54	0.34 ± 0.11	0 ± 0.0/5.6 ± 0.62	B3

[a]B3 level is 14.0–14.9 points in the 20-point evaluation scale.

typing demonstrated that the inoculated commercial starter also dominated all stages of fermentation. Three other interdelta sequence patterns of *S. cerevisiae* strains were found in all fermentations and may significantly contribute to the wine characteristics.

In cool climate regions, especially North China, grapes may be harvested at a relatively low temperature (around 10°C) that is maintained at the beginning of fermentation. Using the *pied de cuve* method, fermentation could start rapidly and the total fermentation duration could be reduced. The viable yeast cell at the end of the *pied de cuve* fermentation was low in this study and was probably due to the inability to produce sterols during anaerobic growth by the mother yeast strains [33]. Hence, regular aeration and fermentation control are crucial when applying the *pied de cuve* method.

S. cerevisiae strains differ significantly in their fermentation performance and their contribution to the final bouquet and quality of wine. Although it is difficult to distinguish and identify *S. cerevisiae* at the strain level using classic biochemical methods, numerous molecular methods have been developed that provide new approaches to yeast strain differentiation. These methods include randomly amplified polymorphic DNA (RAPD) [1, 5, 25, 35] mitochondrial DNA (mtDNA) restriction analysis [12, 18, 24, 32], application of microsatellites [10, 14, 15, 28, 30], and PCR amplification of the mitochondrial gene *COX1* [19]. Among these techniques, interdelta sequence typing has been proven to be a convenient method to monitor the diversity of *S. cerevisiae* strains because it is rapid, reproducible, and sensitive [29]. In addition this method does not require sophisticated equipment or highly skilled human resources and could be widely applied in industrial wine fermentations [34]. Using this method, the presence and evolution of *S. cerevisiae* strains were shown to be different during fermentations with the two inoculation methods in this study. However, the intraspecific diversity detected in the analyzed fermentations in the present study is low compared with other inoculated fermentations around the world [3, 4]. This may be related to the introduction of industrialized practices in winemaking in the region where the work was conducted. Santamaria *et al*. [27] reported that an old winery had higher *S. cerevisiae* diversity than a new winery and this situation is in agreement with our study, in which a low number of *S. cerevisiae* interdelta sequence patterns have been detected. The winery involved in our work is relatively new (established in 2000), and only a small number of commercial starters have been used to inoculate wine fermentations. The results are not always comparable with previous studies where the molecular markers applied have been different [2, 6, 31]. It must also be pointed out that analysis of interdelta sequences can be used only to discriminate *Saccharomyces* strains. The diversity of non-*Saccharomyces* strains and the interaction between non-*Saccharomyces* and *S. cerevisiae* should be investigated by other methods in a future study.

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