Preliminary Analysis of Yeast Communities Associated with the Spontaneous Fermentation of Musalais, a Traditional Alcoholic Beverage of Southern Xinjiang, China

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Submitted for publication: September 2011
Accepted for publication: January 2012

Key words: Musalais, yeast, community

Musalais is a traditional alcoholic beverage made by the Uighur people in southern Xinjiang, China. The initial fermentation juice is obtained by prolonged boiling of local grape juice and grape residues. In the current study, 242 yeast isolates were obtained from 18 samples (grapes, derived starting products, and progressive stages of fermentation), and 20 phenotypes were distinguished, based on colony characteristics on WL nutrient agar. Fifty representative isolates were selected and found to belong to eight genera (based on rRNA gene sequence analysis). Among the non-*Saccharomyces* species present on the grapes and related derived substrates, *Hanseniaspora* spp. was the dominant species. However, nearly all of these species were absent in early fermentation. *Saccharomyces cerevisiae* was not found until the onset of spontaneous fermentation and quickly became the dominant species. The identified yeast community could be used to further develop indigenous yeast strains to serve the traditional technology of Musalais. The production of Musalais, from a starting substrate that has been boiled for 15 hours to kill all, or nearly all, yeast cells, provides fresh insights into the production of ethanol by the fermentation of grape juice.

INTRODUCTION

Musalais is a traditional alcoholic beverage made with local grapes that has been prepared by the Uygur minority people in the Xinjiang province of China using natural fermentation for more than 2 000 years. Musalais is an important element of the tradition and folklore of the Daolang culture and has an important role in the local tourist economy. Local people consuming Musalais have few instances of high blood pressure and hyperlipidemia, which may be related to the wine's reported contribution to the prevention of cardiovascular disease (Stein *et al*., 1999; Folts, 2002; Gorelik *et al*., 2008). In fact, Musalais is rich in quercetin, rutin, catechin, anthocyanin and resveratrol. Tests in mice showed that Musalais could enhance the immune system, lower cholesterol, and improve the body's antioxidant ability (an unpublished report from Xinjiang Medical University, 2003).

Musalais is produced by a traditional process. Local Hetianhong grapes are pressed and the juice is collected. The grape residues are added to water to prevent drying and to extract colorants, and are then gently boiled for more than fifteen hours in an iron pan in a clay oven. After heating, the mixture is filtered and mixed with the collected grape juice and heated as before. The boiling temperature of the grape substrates is about 92°C. The solution is allowed to cool overnight to room temperature to form the Musalais initial fermentation juice, before being transferred into earthenware jugs, sealed, and allowed to ferment for around 45 days. The Musalais production process and how it differs from traditional wine production are shown in Fig. 1.

Musalais is made at oases around the Taklamakan Desert, located in the southern part of Xinjiang province in China. The dry climate and sandy soil are very suitable for grape production. The main production region is A'wati, where there are more than 200 traditional Musalais producers. However, the quality of Musalais can be very unpredictable (Lixia & Xujie, 2008), possibly because of the unknown

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Acknowledgements: The authors would like to thank Prof. Zhang Lili, who provided the initial funding for this study. We are grateful to Jin Chen Yan, chairman of the A'wat Scientific Bureau, and Abudurexiti Gayiti, chairman of the A'wat Musalais Association, for their kind help in collecting and transporting the samples. This research was supported by the National Natural Science Foundation of China (Project No. 31060223) and the Xinjiang Production & Construction Corps Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin (Project No. NCET-06-0917).

microorganisms surviving in the traditional spontaneous fermentation.

Many studies have investigated indigenous yeasts associated with wine production (Fleet, 1993; Constantí *et al*., 1997; Fell *et al*., 2000; Renouf *et al*., 2005; Romancino *et al*., 2008). Although there are studies on the development of the Musalais industry (Lixia *et al*., 2008), the analysis of physicochemical characteristics (Lixia & Xujie, 2008; Yinping *et al*., 2009) and investigations of traditional technology (Lixia *et al*., 2011), there are no published reports on the yeasts associated with Musalais production.

The present study hoped to gain a better understanding of the indigenous yeast community involved in Musalais fermentation.

MATERIALS AND METHODS

Source material

Samples of grapes (G), pressed grape juice (PGJ), juice boiled with grape residues (JBR), initial fermenting juice (IFJ), and fermentations during a single Musalais spontaneous fermentation (Fig. 1) of around 1 000 L volume were collected aseptically in sterile polythene bags (1 kg for G) and bottles (600 mL for each liquid sample). The samples were put into insulated containers to maintain the temperature at collection, and were transferred to the laboratory within two hours. The samples covered all stages of the spontaneous fermentation. Specific collection times are shown in Table 1.

The physicochemical parameters, including temperature (28°C), reducing sugar (211.6 g/L), total acid (7.01 g/L) and pH (3.66) of the starting substrate (IFJ), indicated that the initial conditions of the Musalais fermentation were close to those of grape juice.

Isolation and enumeration of yeast colonies

Bean sprout (BS), potato dextrose agar (PDA) and yeast peptone dextrose agar (YPD) were tested for their suitability for the isolation of a wide range of yeasts. BS medium was prepared from 200 g of bean sprouts that were boiled for 20 min, after which 20 g of agar and 20 g of glucose were added. The solution was made up to 1 000 mL with water and sterilised at 121°C for 20 min at 0.1 Mpa. PDA was prepared by boiling 300 g of unpeeled sliced potatoes in water for 30 minutes, followed by straining of the broth through gauze. The volume was made up to 1 000 mL with water. Twenty grams of dextrose and 20 g of agar powder were added and the medium was sterilised at 121°C for 20 min at 0.1 Mpa. YPD contained 1% yeast extract, 1% peptone, 2% glucose and 2% agar. WLN agar (Amyl Media, Melbourne, VIC, Australia) was used to sort yeast isolates by their colony characteristics.

In a preliminary experiment, a 5 g G (grape) sample was rinsed with 45 ml of sterilised saline solution (0.9% NaCl), after which the suspension was diluted in a 10-fold series using sterile saline solution. One hundred microlitres of the appropriate dilutions were spread onto YPD, PDA and the BS medium, followed by incubation at 25°C for three days. Colonies with different characteristics were counted and two to seven colonies were isolated. Subsequently, YPD agar was chosen as the most suitable medium for the isolation of yeasts from the different starting materials, as it gave better growth of colonies than BS medium and PDA. Isolates were purified by streaking on YPD and then stored at 4°C.

All liquid samples were diluted in a 10-fold series with sterile saline solution, and 100 µl of the appropriate dilutions were spread onto YPD and WLN agar plates. After incubation at 25°C for three days on YPD and five days on

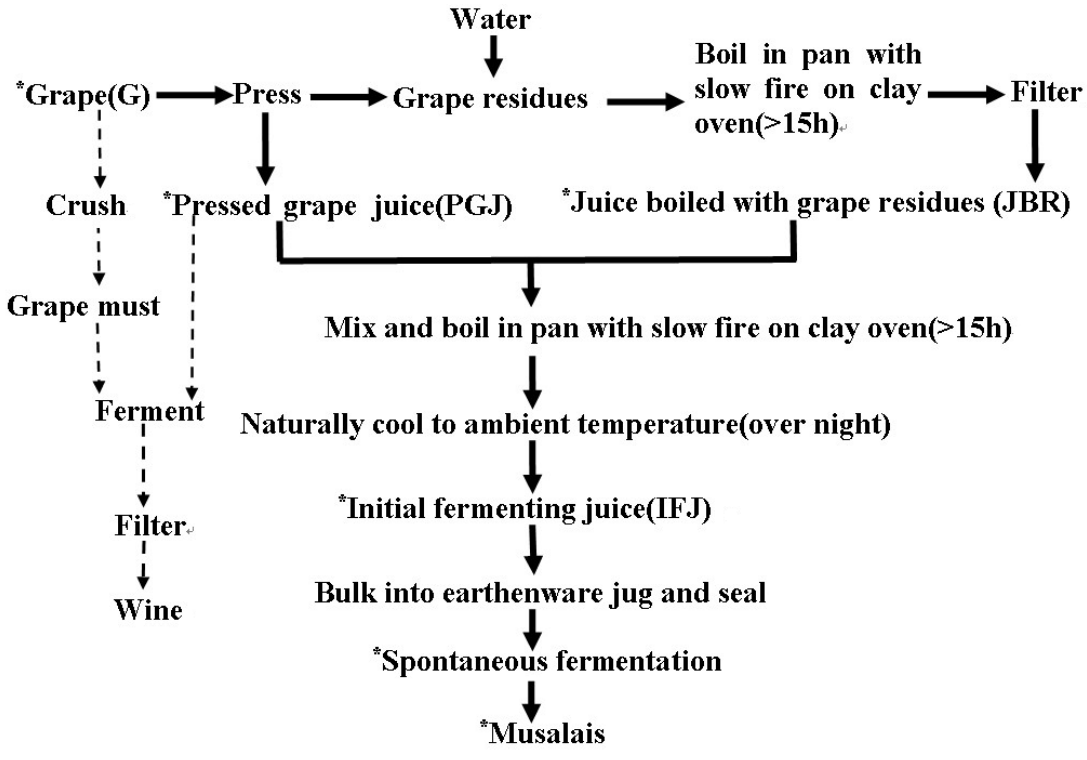

FIGURE 1

The general process of Musalais and wine production. *Samples collected for yeast isolation and enumeration.

TABLE 1
Number of isolates, phenotypes sorted by WLN agar, and representative isolates.

WL-Type	G		PGJ		JBR		IFJ		Fermentations		Total
	Number of isolates	Rep. isolate	Number of isolates	Rep. isolate	Number of isolates	Rep. isolate	Number of isolates	Rep. isolate	Number of isolates	Rep. isolate	Number of Isolates
1 T	0		4		0		0		2	NL1 (4 days)	6
2 T	4		5		3	NL2	2		5 (4, 9, 25 days)		19
3 T	5	NL3, NL71	0		0		0		0		5
4 T	0		0		0		2		121	See Fig. 3	123
5 T	6		6		3	NL50	2		0		17
6 T	7	NL73	7		7	NL52, NL69	0		0		21
7 T	5	NL54, NL66	0		0		0		0		5
8 T	2	NL55	0		2		2	NL59	0		6
9 T	0		0		0		2	NL61	0		2
10 T	5	NL67, NL57	3		2		2		0		12
11 T	5		4		0		2	NL72	0		11
12 T	7	GUOA	4	YzA	4		0		0		15
Total	46	□	33	□	21	□	14	□	128	□	242

WL-type = phenotypes sorted by WLN agar; Rep. isolate = representative isolate

WLN medium, the colonies were counted. Colonies with different characteristics were counted and two to seven colonies were isolated and purified by streaking on YPD isolating medium. The isolates from G were also purified by the same method. Pure isolates were stored at 4°C.

Pure isolates were sorted again according to their colony morphology on WLN agar. If two or more phenotypes originally distinguished could no more be separated, they were considered to belong to the same grouping and their total cell counts were combined. Representative isolates from the different phenotypes were selected for 26S rRNA gene sequence analysis.

DNA extraction and PCR

The yeast cells were disrupted by a freeze-boiling method. Yeast cells grown on YPD agar (25°C for two to three days) were re-suspended in 100 µL of lysis solution (100 mM Tris-HCL, 30 mM EDTA, 0.5% SDS), frozen at -85°C for 5 min, and then boiled for 2 min. This procedure was repeated three times. DNA was extracted according to the procedure described by Fengyan *et al*. (2002).

The amplification was performed in a 50 µL mix containing 5 µl 10 × PCR buffer, 3 µL $MgCl_2$ 25 mmol/L, 1 µl dNTPs 10 mmol/L, 1.5 µL DNA polymerase Takara Ex Taq, 1 µL 10 pmoles DNA template, 1 µL 10 pmoles each of primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (synthesised by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. China.), and 37.5 µL double distilled water. Amplification was carried out in an MJ Research PJC-100 thermal cycler (Waltham, MA, USA), as follows: an initial denaturation step of 95°C for 5 min; 36 cycles at a denaturation temperature of 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 20 s and 1 min; and a final extension at 72°C for 8 min. The reactions were maintained at 4°C until electrophoretic analysis. The PCR products were sent to the Shanghai Sangon Biological Engineering Technology & Services Co. Ltd to be purified and sequenced.

Sequence analysis

The sequences were compared with available sequences in GenBank using the web-based nucleotide-nucleotide BLAST search engine hosted by the National Center for Biotechnology Information, Bethesda, USA (http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1997). Typical strains showing identity scores ≥ 99% were collected, as these levels of similarity are within the values considered to indicate conspecificity (Kurtzman & Robnett, 1997, 1998).

Sequences of typical strains with high similarity to isolates from this study were aligned using Clustal X version 1.81 (Thompson *et al*., 1997). For phylogenetic analysis, regions of sequences with poor alignment were removed. Phylogenetic trees were constructed from distance data transformed according to Kimura (1980), using the neighbour-joining method (Saitou & Nei, 1987) with a bootstrap of 1 000 replicates in MEGA 4.

RESULTS

Isolation of yeasts and sorting by characteristics on WLN agar

Based on the colony characteristics of yeasts growing on YPD and WLN agar, 242 isolates were obtained from 18 samples from different stages of Musalais production, including fermentation. They were further sorted into 12 phenotypes by colony characteristics on WLN agar, and 50 representative isolates were selected for identification (Table 1 and Fig. 2).

Identification and phylogeny analysis based on 26S rDNA

The fifty representative yeast isolates were identified by sequencing the D1/D2 domain of the large-subunit (26S) ribosomal DNA. The sequences of the 50 yeast isolates were submitted to GenBank under accession numbers HM191632 to HM191681. The phylogenetic tree was constructed with the 50 isolates and 17 typical strains (Fig. 2).

In the phylogenetic tree (Fig. 2), 32 isolates - NL4, NL5, NL6, NL7, NL8, NL9, NL11, NL12, NL14, NL16, NL17, NL18, NL19, NL20, NL21, NL25, NL26, NL27, NL29, NL30, NL32, NL34, NL38, NL45, NL51, NL60, NL63, NL64, NL74, NL9-9, NL5*, and NL34* - are identified as *Saccharomyces cerevisiae,* with over 99% similarity value with the reference strain of *S. cerevisiae* (U44806). Two of these isolates, NL20 and NL34, showed variance from the other isolates, having substitution rates of 1.7% (nine of 529 nucleotides) and 1.9% (10 of 529 nucleotides) respectively. This is supported by a comparatively low bootstrap value (65%). More variation within the sequences of 26 S rDNA was revealed among the 32 *S. cerevisiae* isolates and was revealed over the range of sampling dates (Fig. 3).

The other 18 representative isolates were identified as non-*Saccharomyces* yeasts, with high matches (≥ 96% bootstrap value), although NL52 and 69, GUOA, NL2, NL61 and NL1 had different branch lengths from their corresponding reference strains. NL50 was identified as *Lachancea thermotolerans*, NL73 as *Hanseniaspora uvarum*, NL52 and 69 as *H. vineae*, NL54 and 66 as *Meyerozyma guilliermondii* (formerly *Pichia guilliermondii)*, NL55 and 59 as *Wikerhamomyces anomalus* (synonym *P. anomala*), GUOA as *Metschnikowia pulcherrima*, YzA as *M. fructicola*, NL72 as *P. manshurica,* NL67 and 57 as *P. membranifaciens*, NL2 as *Candida zemplinina*, NL61 as *Issatchenkia hanoiensis*, and NL1 as *P. kudriavzevii* (synonym *I. orientalis*).

NL71 was identified as *P. kluyveri*, as it was clustered only with *P. kluyveri* reference strains, as shown in Figs 2 and 4. NL3 clustered with a representative strain of *P. kluyveri* (EF116919) and a reference strain of *P. kluyveri var. kluyveri* (EF550251) (Fig. 2). It also clustered with 17 *P. kluyveri* and 10 *P. kluyveri* var. *kluyveri* strains (Fig. 4).

Thus, the 50 representative yeasts isolates were ascribed to eight genera and fifteen species.

Yeast community structure during Musalais production, including fermentation

The 242 isolates obtained were assigned to 12 phenotypes (T) using WLN agar (Table 1). The most common phenotype was 4T with 123 isolates, which was obtained following the onset of fermentation. The second most common phenotype was 6T, with 21 isolates. The representative isolates of the two phenotypes were identified as *S. cerevisiae* and *Hanseniaspora* ssp. respectively (Table 2, Fig. 2, Fig. 3). The distribution and identification of the other 10 yeast phenotypes is also shown in Table 2.

Prior to the onset of fermentation, the yeast species present on the grapes (G) and in the grape juice (PGJ) were all non-*Saccharomyces* species. The yeasts present in the juice that had been boiled with the residues following the pressing of the grapes (JBR) were also all of non-*Saccharomyces* species. The first presence of *S. cerevisiae* came from the combined fractions (IFJ) following the overnight cooling after the 15 h of gentle boiling. In the G, PGJ, JBR and IFJ samples, 12, nine, eight and seven yeast species were detected respectively. The total yeast concentration in the four samples was $1.59 \pm 0.38 \times 10^5$ colony-forming units (CFU) per millilitre in G, $3.81 \pm 0.18 \times 10^7$ CFU/mL in PGJ, $9.25 \pm 0.97 \times 10^4$ CFU/mL in JBR, and 45.27 ± 27.86 CFU/mL in IFJ. During fermentation, the non-*Saccharomyces* yeasts almost completely disappeared and *S. cerevisiae* became the predominant population.

S. cerevisiae was detected in only trace amounts in the initial fermenting juice, but had increased to $3.21 \pm 0.92 \times 10^6$ CFU/mL after four days. The levels detected remained at the 10^7 to 10^8 CFU/mL range until day 44 of the fermentation, when the level detected was $3.63 \pm 0.05 \times 10^6$ CFU/mL. The levels decreased over the next 46 days, to $6.00 \pm 0.52 \times 10^4$ CFU/mL at day 62 and $1.78 \pm 0.52 \times 10^3$ CFU/mL at day 90.

Among the non-*Saccharomyces* species, the *L. thermotolerans* populations ranged from high in the non-boiled substrates to low in the two substrates that had been boiled: G ($1.18 \pm 0.64 \times 10^4$ CFU/mL), PGJ ($3.12 \pm 1.7 \times 10^4$ CFU/mL), JBR ($5.02 \pm 0.67 \times 10^2$ CFU/mL), and IFJ (10.5 ± 0.7 CFU/mL). *C. zemplinina* was present in the four starting substrates and was also present on days 4, 9, and 25. The *M. pulcherrima* and *M. fructicola* population was high on grapes ($3.81 \pm 0.49 \times 10^4$ CFU/mL), and survived in PGJ and JBR with populations of $1.8 \pm 0.14 \times 10^2$ and $5.00 \pm 1.41 \times 10^2$ CFU/mL respectively. *Meyerizyma guilliermondii* had a similar concentration ($4.0 \pm 1 \times 10^4$ CFU/mL) on grapes as the combined population of *Metschnikowia* ssp., but was not identified in PGJ and JBR. *W. anomalus* was present in the G, JBR and IFJ (with just 20 ± 1.0 cell counts), but not in PGJ. *I. hanoiensis* was only present in the initial fermentation sample (cell counts not determined).

P. kudriavzevii was isolated from only PGJ and the fermenting sample at day 4, with $2.01 \pm 0.5 \times 10^2$ and $1.12 \pm 0.78 \times 10^4$ CFU/mL respectively. Other *Pichia* species were distributed in G, PGJ and JBR, but were not detected once fermentation had commenced. All of these *Pichia* species were associated with grapes, the largest population being that of *P. manshurica*, with $1.06 \pm 0.68 \times 10^4$ CFU/mL. *P. membranifaciens* was present in all four substrates, with a maximum cell number of 1 to 2×10^2 CFU /mL being present in G and PGJ. *P. kluyver*i and *P. kluyver*i *var. kluyver*i were detected only on grapes.

H. vineae and *H. uvarum* were present in G, PGJ and JBR, constituting 99.8% and 87.8% percent of the total yeast population in the PGJ and JBR samples respectively. Both species were not detected in the initial fermentation sample.

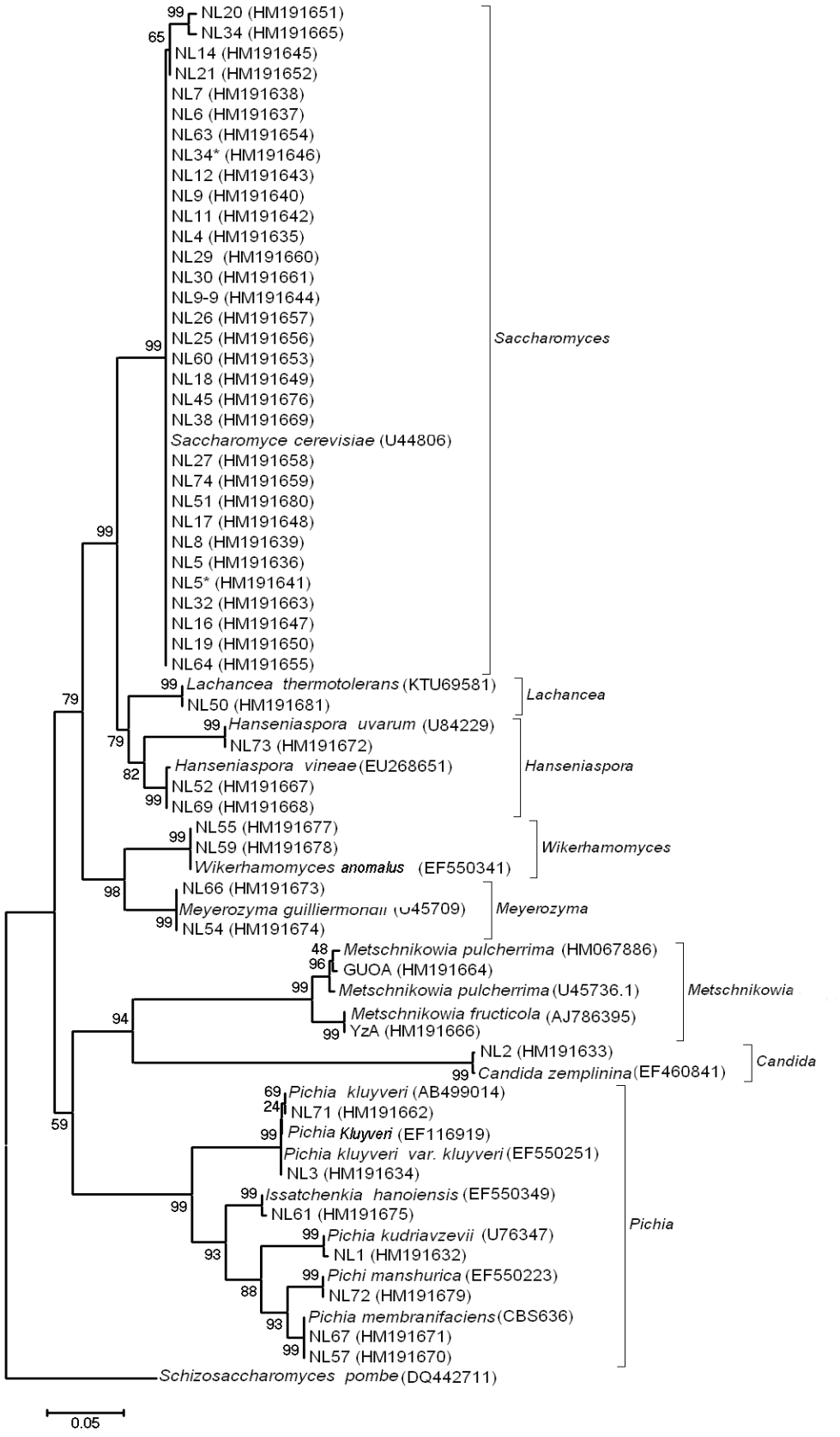

FIGURE 2

A phylogenetic tree constructed by the neighbour-joining method based on the 26S rRNA D1/D2 domain sequence alignment, showing the relationships of the yeast isolates studied. Bootstrap percentages over 50% are shown. *Schizosaccharomyces pombe* was used as the outgroup. The numbers at the nodes indicate the level of bootstrap support based on 1 000 replicates. Branch lengths were proportional to the scale given in substitutions per nucleotide.

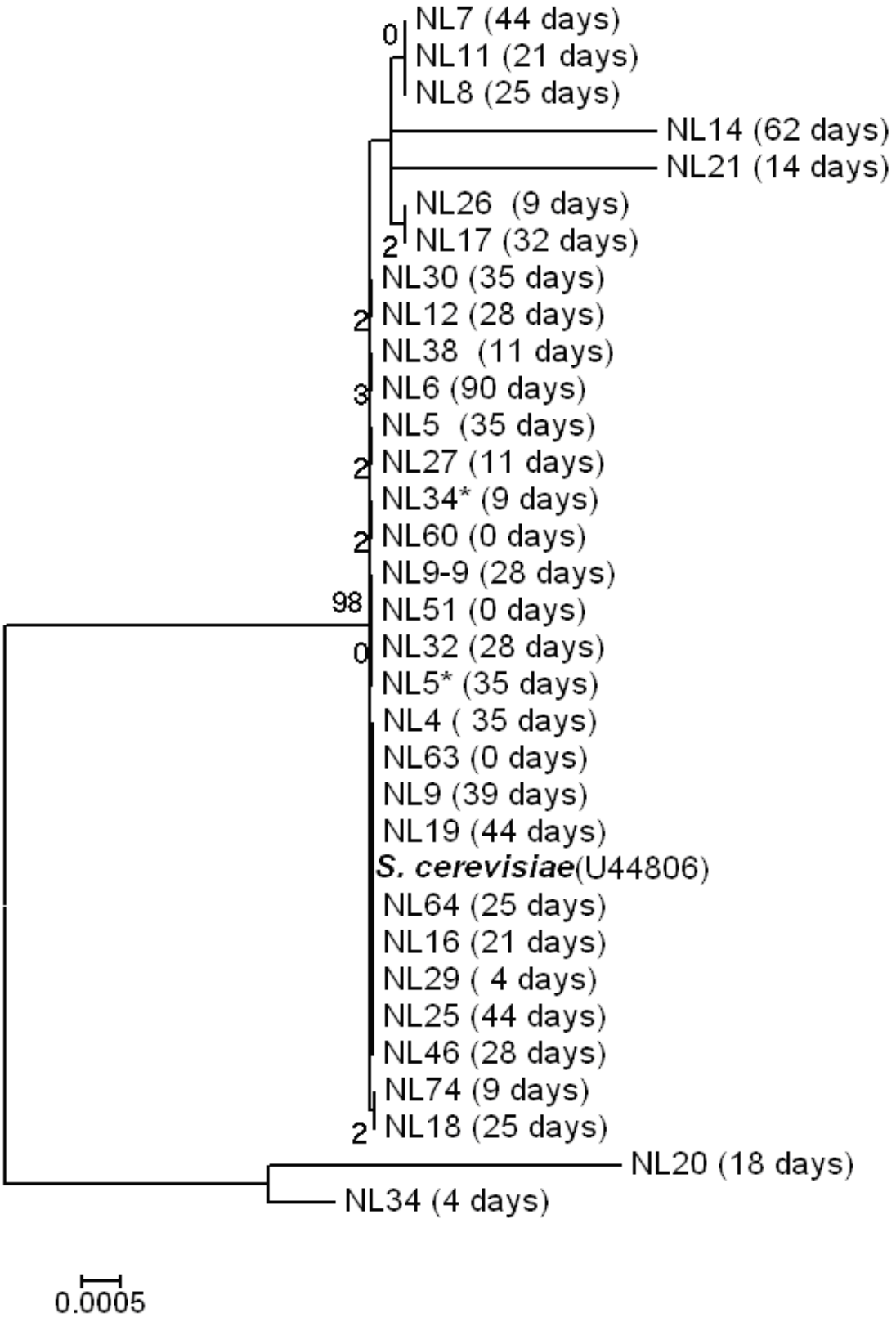

FIGURE 3

The 32 representative isolates of *S. cerevisiae* studied and a typical strain (U44806) were used to construct a phylogenetic tree by the neighbour-joining method based on the 26S rRNA D1/D2 domain sequence alignment, to show the diversity at the strain level. The numbers at the nodes indicate the level of bootstrap support based on 1 000 replicates. Branch lengths are proportional to the scale given in substitutions per nucleotide.

TABLE 2
Population of yeasts distributed in the different samples (CFU/mL)

WL-Type	Species	G	PGJ	JBR	0 day (IFJ)	4 days	9 days	11 days	14 days	18 days
1 T	*P. kudriavzevii*	0	$2.01 \pm 0.5 \times 10^2$	0	0	$1.12 \pm 0.78 \times 10^4$	0	0	0	0
2 T	*C. zemplinina*	$5.03 \pm 0.42 \times 10^2$	$4.02 \pm 0.13 \times 10^4$	$1.03 \pm 0.48 \times 10^4$	12 ± 2.82	$1.01 \pm 0.23 \times 10^4$	$2.24 \pm 0.08 \times 10^4$	0	0	0
3 T	*P. kluyveri**	$3.00 \pm 0.70 \times 10^2$	0	0	0		0	0		0
4 T	*S. cerevisiae*	0	0	0	22 ± 1.73	$3.21 \pm 0.92 \times 10^6$	$1.51 \pm 0.53 \times 10^8$	$2.77 \pm 0.15 \times 10^7$	$4.9 \pm 0.38 \times 1^07$	$4.76 \pm 4.62 \times 10^8$
5 T	*L. thermotolerans*	$1.18 \pm 0.64 \times 10^4$	$3.12 \pm 1.7 \times 10^4$	$5.02 \pm 0.67 \times 10^2$	10.5 ± 0.7	0	0	0	0	0
6 T	*Hanseniaspora* ssp.	$5.74 \pm 0.96 \times 10^4$	$3.73 \pm 0.18 \times 10^7$	$7.96 \pm 3.41 \times 10^4$	0	0	0	0	0	0
7 T	*Meyerozyma guilliermond*ii	$4.0 \pm 1 \times 10^4$	0	0	0	0	0	0	0	0
8 T	*W. anomalus*	Pre.	0	Pre	20 ± 1.0	0	0	0	0	0
9 T	*I. hanoiensis*	0	0	0	Pre.	0	0	0	.	0
10 T	*P. membranifaciens*	$1.1 \pm 0.88 \times 10^2$	$1.80 \pm 0.70 \times 10^2$	24 ± 5	Pre.	*0*	*0*	*0*	*0*	
11 T	*P. manshurica*	$1.06 \pm 0.68 \times 10^4$	$1.81 \pm 0.09 \times 10^2$	0	25 ± 8.66	0	0	0	0	0
12 T	*Metschnikowia* ssp.	$3.81 \pm 0.49 \times 10^4$	$1.8 \pm 0.14 \times 10^2$	$5.00 \pm 1.41 \times 10^2$	0	0	0	0	0	0
	Total	$1.59 \pm 0.38 \times 10^5$	$3.81 \pm 0.18 \times 10^7$	$9.25 \pm 0.97 \times 10^4$	45.27 ± 27.86	$6.44 \pm 1.99 \times 10^6$	$2.53 \pm 0.55 \times 10^8$	$2.77 \pm 0.15 \times 10^7$	$4.9 \pm 0.38 \times 10^7$	$4.76 \pm 4.62 \times 10^8$

TABLE 2 COLUMS CONTINUED

WL-Type	Species	21 days	25 days	28 days	32 days	35 days	39 days	44 days	62 days	90 days
1 T	*P. kudriavzevii*	0	0	0	0	0	0	0	0	0
2 T	*C. zemplinina*	0	$5.21 \pm 0.45 \times 10^4$	0	0	0	0	0	0	0
3 T	*P. kluyveri**	0	0	0	0	0	0	0	0	0
4 T	*S. cerevisiae*	$3.79 \pm 1.75 \times 10^7$	$5.90 \pm 0.23 \times 10^8$	$1.20 \pm 0.22 \times 10^8$	$4.9 \pm 0.32 \times 10^8$	$5.20 \pm 0.47 \times 10^7$	$2.07 \pm 0.25 \times 10^7$	$3.63 \pm 0.05 \times 10^6$	$6.00 \pm 0.52 \times 10^4$	$1.78 \pm 0.52 \times 10^3$
5 T	*L. thermotolerans*	0	0	0		0	0	0	0	0
6 T	*Hanseniaspora* ssp.	0	0	0	0	0	0	0	0	0
7 T	*Meyerozyma guilliermondii*	0	0	0	0	0	0	0	0	0
8 T	*W. anomalus*	0	0	0	0	0	0	0	0	0
9 T	*I. hanoiensis*	0	0	0	0	0	0	0	0	0
10 T	*P. membranifaciens*	0	0	0	0	0	0	0	0	0
11 T	*P. manshurica*	0	0	0	0	0	0	0	0	0
12 T	*Metschnikowia* ssp.	0	0	0	0	0	0	0	0	0
	Total	$3.79 \pm 1.75 \times 10^7$	$6.18 \pm 2.56 \times 10^8$	$1.20 \pm 0.22 \times 10^8$	$4.9 \pm 0.32 \times 10^8$	$5.20 \pm 0.47 \times 10^7$	$2.07 \pm 0.25 \times 10^7$	$3.63 \pm 0.05 \times 10^6$	$6.00 \pm 0.52 \times 10^4$	$1.78 \pm 0.52 \times 10^3$

"Pre." means species is present in the corresponding sample without enumeration. *Metschnikowia spp.* include *M. pulcherrima* and *M. fructicola*, cell numbers of which were counted together instead of separately because of their almost similar colony characteristics when growing on YPD agar; similarly for *Hanseniaspora* spp. (*H. uvarum* and *H. vineae*); "*" means including *P. kluyveri var. kluyveri*.
WL-type = phenotypes sorted by WLN agar.

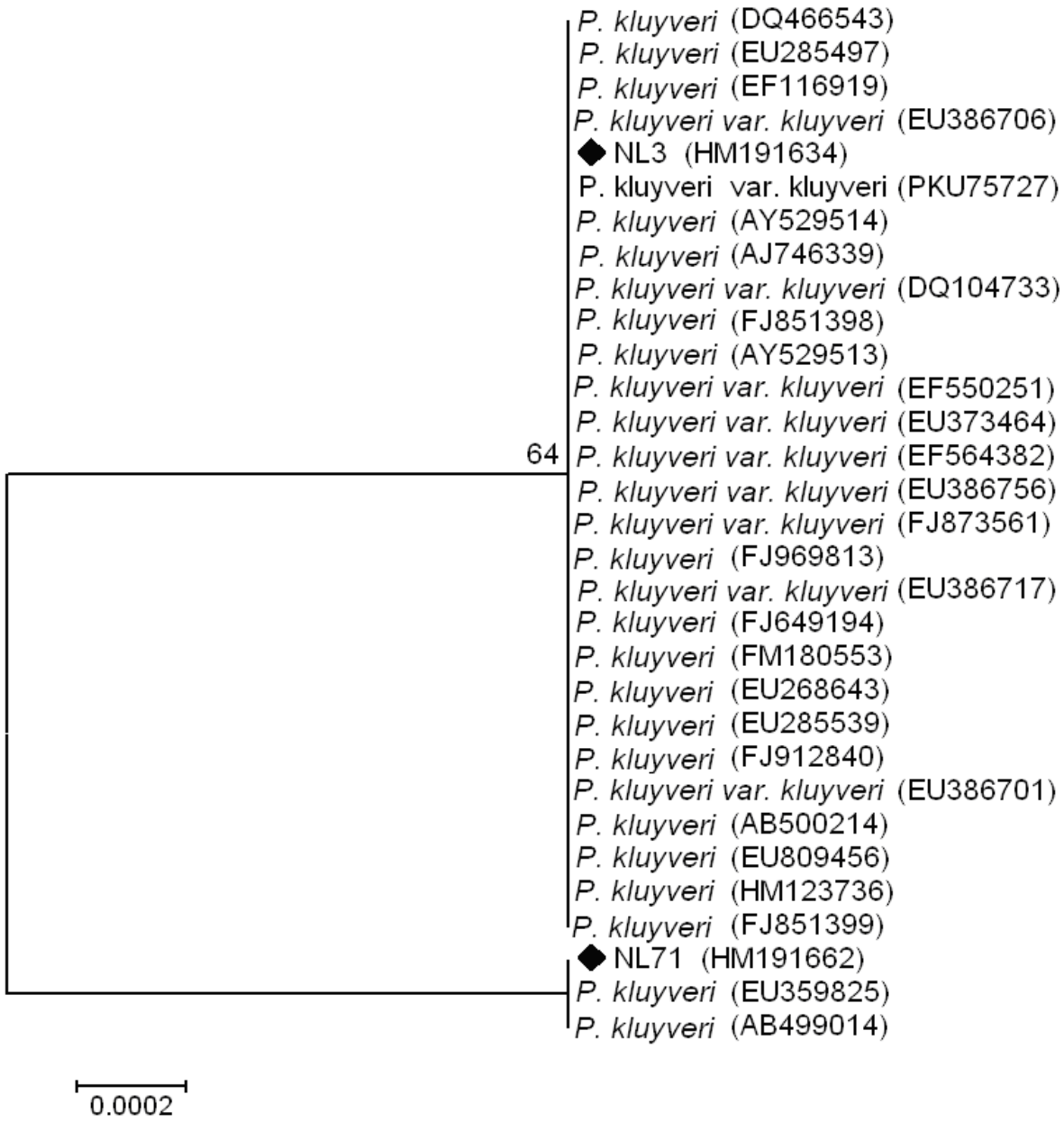

FIGURE 4

A phylogenetic tree constructed by the neighbour-joining method based on the 26S rRNA D1/D2 domain sequence alignment showing the relations between *P. kluyveri* and *P. kluyveri* var. *kluyveri* (including NL3 and NL71 strains). The numbers at the nodes indicate the level of bootstrap support based on 1 000 replicates. Branch lengths are proportional to the scale given in substitutions per nucleotide.

H. vineae and *H. uvarum* populations were also high on grapes, being present in the same 10-fold dilution (10^4) as *L. thermotolerans, Meyerizyma guilliermondii*, *Metschnikowia* ssp. and *P. manshurica.*

DISCUSSION

Yeast communities in the production of Musalais were very diverse at the species and even *S. cerevisiae* strain level, although they were all within the common wine yeast genera (Fleet & Heard, 1993; Kurtzman, 2003). Not surprisingly, there was a large difference in yeast distribution between the un-boiled and boiled substrates. Almost all of the identified non-*Saccharomyces* yeasts were present in the pre-fermentation substrates, with *Hanseniaspora* spp. as the dominant species. However, these non-Saccharomyces yeasts were absent or at low concentrations during fermentation. This was undoubtedly because they did not survive the 15 h of light boiling in the production of the starting substrate.

There were, however, non-*Saccharomyces* yeasts present in the two boiled JBR and IFJ substrates. Their presence may be the result of contamination while these two substrates were being slowly cooled. The filtering of the JBR though gauze was a likely entry point for contaminating yeasts. IFJ was not filtered, so airborne and vessel-borne cells were possible means of contamination. Further evidence for the potential of contamination prior to the onset of fermentation comes from the presence of a mycelial mat that developed within the first four days of fermentation.

S. cerevisiae was only found in the boiled substrates, implying that it must have originated from inside the building where the Musalais was prepared. The means of entry of this yeast species include air, the implements used, and the clay fermentation vessels. Evidence for an airborne inoculum was found by the detection of *S. cerevisiae* in the air of the boiling and fermentation room examined in our study (data not shown). *S. cerevisiae* entry from the winery instead of the vineyard during wine fermentation has also been reported (Davenport, 1976; Martini & Martini, 1990; Longo *et al*., 1991; Constantí *et al*., 1997). Future studies should include testing for their yeast flora of materials such as earthenware jars and buildings used during the production of Musalais.

S. cerevisiae very rapidly became the dominant yeast species during the fermentation of Musalais, possibly because of the boiling of the grape substrates. The boiled starting substrate could provide *S. cerevisiae* with a competitive advantage compared with grape juice or grape must. Boiling might have killed all or most of the yeast and bacteria associated with the grapes, weakening or preventing the competition for nutrients from other microorganisms, and would have increased the concentration of assimilable nitrogen. In addition, boiling would have oxidised phenolic compounds, including tannins, and may have weakened or removed their antimicrobial properties that are associated with wine fermentation (Papadopoulou *et al*., 2005; Rodríguez Vaquero *et al*., 2007). In addition, there is no addition of sulphur dioxide (SO_2) in the processing of Musalais, as is used in traditional wine production to inhibit undesirable microbial growth. This addition of SO_2 to wine is also likely to inhibit *S. cerevisiae* (Henick-Kling *et al*., 1998). It is also possible that local strains of *S. cerevisiae* have a competitive advantage during Musalais production, for example, an ability to tolerate temperatures up to 37°C at the vigorous fermentation stage of Musalais production.

The dominance of *S. cerevisiae*, at over 10^6 CFU/mL, may have prevented the detection of non-*Saccharomyces* yeasts that might have been present, although at much lower population densities. This was indicated by the failure to persist of the mycelial mat that developed during the first four days of spontaneous fermentation. The concentration of bacilli also remained low (within 100 CFU/mL) during spontaneous fermentation (Lixia *et al*., 2010). In addition, the key physicochemical parameters are similar to unprocessed grape juice and are indicative of a favourable substrate for *S. cerevisiae* growth. Other factors, for example an increasing concentration of ethanol, high osmotic pressure, and the presence of possible inhibitory substances, also contribute to the strong selective conditions prevailing during the spontaneous fermentation of Musalais. These might explain how the traditional process reliably and successfully completes the spontaneous fermentation of Musalais in practice. This contrasts with wine, where problems with spontaneous fermentation (referred to as stuck or sluggish fermentation) are not uncommon, and where non-*Saccharomyces* yeasts are the dominant colonisers of the starter grape juice before the dominance of *S. cerevisiae* occurs as the ethanol content increases (Fleet & Heard, 1993; Linda, 1999; Amato *et al*., 2006).

C. zemplinina populations fluctuated in a puzzling way during the fermentation. A possible explanation for this is that the inoculum entered the fermentation vessel when it was opened for sampling. Another possible explanation for its absence is the abovementioned strong selective conditions during the fermentation of Musalais.

The results indicate that the bulk of the microbes present in the post-boiling samples occur as a result of contamination from the materials used or from the facility. Further experiments involving multiple fermentations from the same facility or fermentations from other facilities would aid our understanding of this aspect, as would samples taken from the vessels, materials and the fabric of the buildings.

Our results provide an initial indication of the yeast communities associated with the spontaneous fermentation of Musalais. A more comprehensive analysis, involving a wider choice of culture media (e.g. for non-fermentative yeasts), quantitative RT-PCR, restriction fragment length polymorphisms (RFLPs) and denaturing gradient gel electrophoresis (DGGE) directly from the samples instead of cultures, would further identify and quantify the yeast populations present during the various stages of Musalais production. The application of such techniques should also clarify the confusing findings involving *P. kluyveri* and *P. kluyveri var. kluyveri* strains from NCBI, which could not be differentiated based on the 26S rRNA sequences analysis.

CONCLUSIONS

A yeast community associated with Musalais production has been revealed for the first time. Seven non-*Saccharomyces* species, including *Hanseniaspora* spp. as the dominant species, were present on the grapes and related derived substrates; however, nearly all of them were absent in the early fermentation. *S. cerevisiae* was not found until the onset of the spontaneous fermentation stage and quickly became the dominant species. This obviously differs from wine production, during which ethanol-tolerant *S. cerevisiae* gradually dominates fermentation after non-*Saccharomyces* yeasts initiate fermentation. Diverse *S. cerevisiae* strains were isolated from different fermentation stages. An analysis of the effects of the boiling process involved in the production of Musalais has provided fresh insights into the fermentation of grape juice to produce ethanol.

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