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Candida zemplinina Can Reduce Acetic Acid Produced by *Saccharomyces cerevisiae* in Sweet Wine Fermentations

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In this study we investigated the possibility of using *Candida zemplinina*, as a partner of *Saccharomyces cerevisiae*, in mixed fermentations of must with a high sugar content, in order to reduce its acetic acid production. Thirty-five *C. zemplinina* strains, which were isolated from different geographic regions, were molecularly characterized, and their fermentation performances were determined. Five genetically different strains were selected for mixed fermentations with *S. cerevisiae*. Two types of inoculation were carried out: coinoculation and sequential inoculation. A balance between the two species was generally observed for the first 6 days, after which the levels of *C. zemplinina* started to decrease. Relevant differences were observed concerning the consumption of sugars, the ethanol and glycerol content, and acetic acid production, depending on which strain was used and which type of inoculation was performed. Sequential inoculation led to the reduction of about half of the acetic acid content compared to the pure *S. cerevisiae* fermentation, but the ethanol and glycerol amounts were also low. A coinoculation with selected combinations of *S. cerevisiae* and *C. zemplinina* resulted in a decrease of ~0.3 g of acetic acid/liter, while maintaining high ethanol and glycerol levels. This study demonstrates that mixed *S. cerevisiae* and *C. zemplinina* fermentation could be applied in sweet wine fermentation to reduce the production of acetic acid, connected to the *S. cerevisiae* osmotic stress response.

Candida zemplinina is a psycrotolerant and osmotolerant yeast, properties that can be advantageously exploited in sweet wine production, which is characterized by a high sugar concentration and low fermentation temperatures (26). Sipiczki recognized it as a distinct new species and named it *C. zemplinina* in 2003 (25). However, already back in 2002, in the Napa valley, California, Mills et al. isolated a strain of *Candida* sp., named *Candida* species strain EJ1, from *Botrytis cinerea*-infected grapes, with interesting features, such as the ability to deplete fructose from a Chardonnay juice without affecting the glucose concentration (19).

Yeast ecology studies carried out over the last 5 years have highlighted the frequent presence of this species in wine fermentations (3, 16, 17, 20, 28, 29, 31, 33). Moreover, it has been demonstrated that strains of *C. stellata*, deposited in several culture collections and isolated from grapes, belong to the *C. zemplinina* species (10). Altogether, all this evidence points out the need for a better understanding of the role of this yeast during wine transformation.

Sweet wines, such as "Passito wines," "Icewines," "Sauternes," and others, are produced from must obtained from dried grapes and are characterized by a very high sugar concentration. The *Saccharomyces cerevisiae* strains used for these fermentations should be able to promptly respond to osmotic stress and be able to increase their load immediately after inoculation. It has been shown that the response of *S. cerevisiae* to osmotic stress can result in increased acetic acid contents due to the upregulation of genes encoding for aldehyde dehydrogenases. In fact, yeast grown in 40% (wt/vol) sugar juice produced 1.35 g of acetic acid/liter compared to 0.3 g/liter at the lower sugar concentration (22% [wt/vol]) (12). The acetic taste in these wines is in part masked by the high residual sugars after fermentation. However, winemakers would like to reduce its content, which generally penalizes the final sensory quality of wines, becoming a limit to its commercialization, also in view of international legal limits for the acetic acid (11, 14).

Several studies have demonstrated that non-*Saccharomyces* yeasts are able to survive during alcoholic fermentations (9, 19). Such evidence opens the way toward new applications of non-*Saccharomyces* in wine fermentation, which would make the organoleptic profiles of the wines more complex due to enzymatic activities that such yeasts possess (27).

Mixed *Saccharomyces* and non-*Saccharomyces* fermentations have been tested since the 1990s (32), but it is only in recent years that the interest of researchers has been growing, as evidenced by recent reviews published on this subject (5, 13). *Hanseniaspora uvarum*, *Torulaspora delbrueckii*, *Lachancea* (*Kluyveromyces*) *thermotolerans*, and *C. stellata* have been the main species investigated thus far in mixed fermentations with the aim of adding complexity to the wine (4, 6, 9, 15, 32). The mixed fermentation strategy has also been used in high sugar musts in order to reduce the acetic acid content of the final wine. For this purpose, strains of *T. delbrueckii*, which have been described as low acetic acid producers (23), have been combined with *S. cerevisiae*, and a 53% reduction in volatile acidity has been obtained (2).

In this context, one possibility that could be exploited is to combine *S. cerevisiae* with *C. zemplinina* during fermentation. Since the latter yeast is osmotolerant and fructophylic and generally produces low amounts of acetic acid, together with relevant quantities of glycerol from sugar fermentation (18, 29), it might be able to consume sugars at the very beginning of fermentation, in this way alleviating the *S. cerevisiae* osmotic stress, thereby reducing production of acetic acid.

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TABLE 1 Source of isolation of the *C. zemplinina* strains used in this study

Geographical region (country)	Winery	Source	Strain(s)
Abruzzo (Italy)	G	Grape juice	L37
		Cooked must	L191, L35, L491, L23, L34, L364, L344, L36, L365, L477
Friuli Venezia Giulia (Italy)	B	Picolit grapes	BC16, BC20
		Picolit grape juice	BC55, BC60
		Picolit fermentation (3 days)	BC115, BC116
		Picolit fermentation (14 days)	BC224, BC226
	F	Picolit grapes	FC50, FC54
	R	Ramandolo grapes	R1, R5
Trentino Alto Adige (Italy)	To	Nosiola dried grapes	TOHA07
	T	Nosiola dried grapes	TOBLINO02
	Pd	Nosiola dried grapes	PEDRO10
	Pi	Nosiola grapes	PISO02, SANTA01
Veneto (Italy)	M	Amarone grape juice	C2CY2, C1AY2, T1Y3
		Amarone fermentation (7 days)	C2AY9, C2BY10
California (USA)	ND[a]	Botritized grapes	EJ1
Attika (Greece)	ND	Grape juice	D1

[a] ND, not defined.

In the present study, we performed mixed fermentations, combining *C. zemplinina* obtained from grapes and wines of different origin with three strains of *S. cerevisiae* (one commercial and two wine isolates), in order to evaluate the potential effect on the reduction of acetic acid.

MATERIALS AND METHODS

Yeast strains. Thirty-five *C. zemplinina* isolates, mainly from Italy, but also including one isolate from Greece and one from the United States, were used in the present study (Table 1). Three strains of *S. cerevisiae* were also selected for mixed fermentations. Two of these, coded FB40 and ELCF3WC, came from the spontaneous fermentation of sweet wines in northern Italy, from Picolit and Erbaluce, respectively. The third *S. cerevisiae* strain, Lalvin EC1118 (Lallemand, Montreal, Quebec, Canada), a commercial active dried yeast (ADY), was selected because it is widely used by local wineries for the production of sweet wines.

All of the isolates were identified through molecular methods, and they are deposited in the culture collections of the Universities of Turin, Teramo, and Verona (Italy). They were routinely cultivated in YPD broth (glucose [20 g/liter], peptone [20 g/liter], and yeast extract [10 g/liter], all from Oxoid, Milan, Italy) for 36 to 48 h at 30°C.

Molecular characterization of the *C. zemplinina* strains. DNA was extracted from the *C. zemplinina* strains by using a mechanical treatment in a bead-beater machine (FastPrep-24; MP Biomedical, Solon, OH) as described elsewhere (7). The DNA was subsequently quantified with a NanoDrop instrument (Celbio, Milan, Italy) and standardized at 100 ng/μl. Molecular characterization of the isolates was carried out using RAPD [random(ly) amplified polymorphic DNA] and SAU-PCR methods, as described by Cocolin et al. (8). All of the *C. zemplinina* strains were subjected to both methods at least twice.

***C. zemplinina* fermentations.** Laboratory fermentations were carried out in a total volume of 100 ml of an Erbaluce dried grape must, containing 210 g of glucose, 193 g of fructose, and 3.5 g of glycerol/liter and 1.8% ethanol. The pH and titratable acidity, expressed as g of tartaric acid/liter, were 3.23 and 8.12, respectively. Before inoculation, the must was pasteurized at 90°C for 90 min, and the absence of live yeast populations was assessed by plating 100 μl on YPD agar, incubated at 30°C for 48 h. Despite the long time necessary for the pasteurization, no caramelization of the sugars occurred. The *C. zemplinina* isolates were preadapted in the same sweet must for 48 h and then inoculated to reach a final concentration of 10^5 to 10^6 cells/ml, which was determined through a microscopic count. Fermentations were carried out at 25°C for 14 days. Samples were collected in triplicate at 0, 1, 2, 6, 8, 12, and 14 days of fermentation and microbiological counts and high-pressure liquid chromatography (HPLC) analysis were carried out. The CFU/ml were determined by plating the serially diluted samples on the differential WLN medium (21) (Oxoid) and by incubating them for 3 to 5 days at 30°C. *S. cerevisiae* forms convex, creamy-white colonies in this medium, whereas *C. zemplinina* forms flat, light to intense green ones, this difference enabling their concurrent enumeration on the same medium (31). The glucose and fructose consumption, as well as the glycerol, ethanol, and acetic acid production, were quantified by means of HPLC (Thermo Electron Corp., Waltham, MA) equipped with a UV detector (UV100), set to 210 nm, and a refractive index detector (RI-150). The analyses were performed isocratically at 0.8 ml/min and 65°C with a cation-exchange column (300 by 7.8 mm [inner diameter];

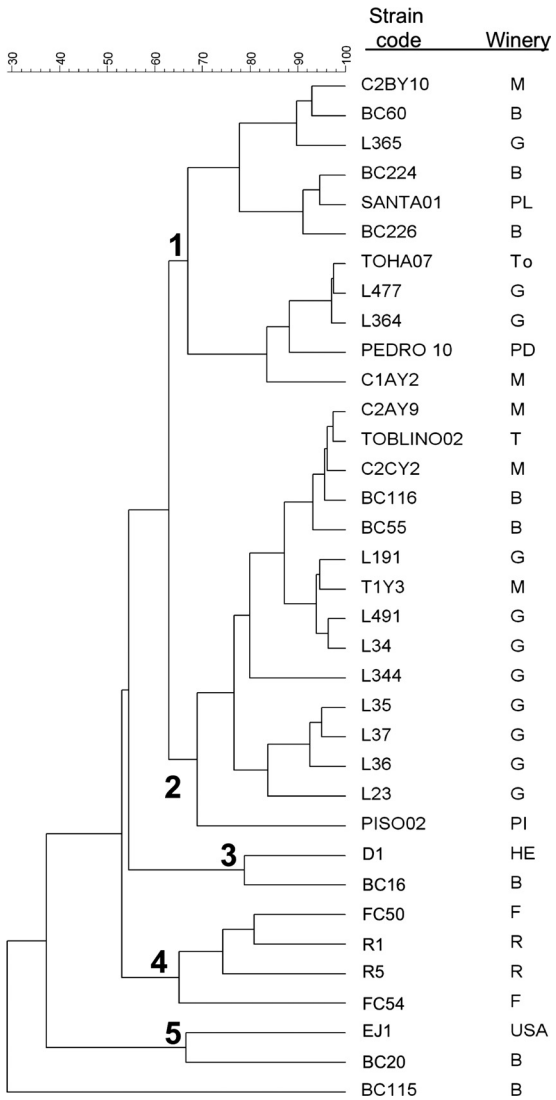

FIG 1 Dendrogram of similarity constructed taking into consideration the RAPD and SAU-PCR profiles of the *C. zemplinina* strains used in the present study. Clusters are indicated by Latin numerals.

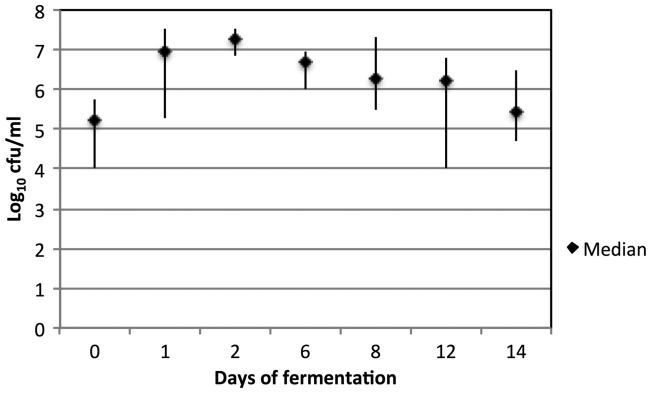

FIG 2 Growth dynamics of *C. zemplinina* during fermentation of the must obtained from dried grapes. The results of the 35 strains are reported as minimums and maximums (indicated by the lines) and median values.

Aminex HPX-87H) and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA), using 0.0026 N H_2SO_4 as the mobile phase (14, 24).

The three *S. cerevisiae* strains were also tested for their fermentation performance in the dried grape must, as described for the *C. zemplinina* strains. All of the fermentation trials were carried out twice.

Mixed fermentations. Three *S. cerevisiae* strains and five strains of *C. zemplinina* were selected and used for mixed fermentations in a total volume of 100 ml of the same must described above. Two different approaches were used: (i) inoculation of both yeasts at the same time (coinoculation) and (ii) the addition of *S. cerevisiae* 48 h after *C. zemplinina* inoculation (sequential inoculation). All of the strains were preadapted in the same sweet must and always added to a final concentration of 10^5 to 10^6 cells/ml. The fermentations lasted 14 and 16 days for the coinoculation and sequential inoculation, respectively. Both the plate counts and the HPLC analysis of these experiments were performed as described above. Samplings were carried out in triplicate at 0, 1, 6, 8, 12, and 14 days of fermentation. In the case of sequential inoculation, samples were also collected 48 and 24 h prior to inoculation of *S. cerevisiae* (−2 and −1 days of fermentation). All of the fermentation trials were carried out twice.

Statistical analysis. Gels containing the RAPD and SAU-PCR profiles of the *C. zemplinina* isolates were normalized, using the 1-kb molecular ladder (Sigma, Milan, Italy), loaded into each gel, and subjected to cluster analysis using BioNumerics software (Applied Maths, Kortrijk, Belgium). The Pearson product moment correlation coefficient was used to calculate the similarities in profile patterns, and dendrograms were obtained by means of the unweighted pair group method using arithmetic averages (UPGMA) clustering algorithm.

Statistical analyses of the chemical composition of the wines were performed using the SPSS statistical software package (version 17.0; SPSS, Inc., Chicago, IL). The Tukey test for $P < 0.05$ was used in order to establish any statistical differences by one-way analysis of variance (ANOVA). A multifactorial ANOVA test was used to explore the effect of the three tested factors (i.e., the strain of *S. cerevisiae*, the strain of *C. zemplinina*, and the type of inoculum) and to verify the existence of any interaction between them.

RESULTS

Molecular characterization of the *C. zemplinina* strains. The dendrogram, which combines the results of the RAPD and SAU-PCR molecular characterization of the *C. zemplinina* strains included in the present study, is shown in Fig. 1. As can be observed, 26 of 35 strains clustered in two groups (1 and 2), while the remaining 9 strains formed three minor clusters, each including 2 to 4 strains, and one single-strain cluster. If the composition of the clusters is analyzed, it can be observed that no correlation could be found with the source of isolation; strains from different Italian regions, from Greece, and from the United States in fact clustered together. Only cluster 4 was composed of four strains from the same geographic region in the northeast of Italy, and the majority of the strains from winery G, in the Abruzzo region (Central Italy), were grouped in cluster 2.

Dynamics and analytical results of the fermentations inoculated with pure cultures of *C. zemplinina* and *S. cerevisiae*. The growth kinetics of the *C. zemplinina* strains, when inoculated in pure culture in must obtained from dried grapes, are summarized in Fig. 2, where the counts of all of the tested strains are taken into

TABLE 2 HPLC analyses of wines obtained from pure cultures of *C. zemplinina* and *S. cerevisiae*[a]

Strain	Residual reducing sugars (g/liter)	Consumption		Production			Ethanol/consumed sugar yield (g/g)
		Glucose (g/liter)[b]	Fructose (g/liter)	Glycerol (g/liter)	Acetic acid (g/liter)	Ethanol (% vol)	
All *C. zemplinina* strains							
Avg	297.49	23.76	82.33	5.99	0.31	5.07	0.373
Minimum	229.75	ND	29.22	1.84	0.19	1.91	0.154
Maximum	360.60	67.82	107.34	10.10	0.79	8.44	0.504
All *S. cerevisiae* strains							
FB40	179.47	158.32	65.21	10.89	1.54	13.70	0.484
EC1118	269.97	89.56	43.47	10.33	0.95	7.83	0.464
ELCF3WC	181.15	157.37	64.48	12.34	1.29	13.54	0.482
Selected *C. zemplinina* strains							
PEDRO10	328.31	ND	74.69	6.16	0.33	4.71	0.498
L37	300.45	16.07	86.48	5.60	0.21	4.67	0.359
BC60	305.16	22.03	75.81	3.82	0.20	2.01	0.162
EJ1	234.13	67.82	101.05	7.73	0.39	6.88	0.321
T1Y3	271.87	33.77	97.36	6.01	0.19	5.48	0.330

[a] Results are shown as minimum, maximum and average amounts for the 35 strains of *C. zemplinina.* Moreover, the analytical results for the three *S. cerevisiae* and the five *C. zemplinina* selected in this study are presented as averages of two fermentation trials. The glucose and fructose contents of the sweet must used in the fermentations were 210 and 193 g/liter, respectively.

[b] ND, not detectable.

consideration, and the results are shown as the minimum, the maximum, and the median growth. As can be seen, *C. zemplinina* was able to grow, reaching a count of ~10^7 CFU/ml in the first 2 days of fermentation, and then started to decrease from day 6 onwards. At the end of the monitored period, the strains still presented good vitality, with counts ranging from 10^5 to 10^6 CFU/ml.

The results obtained from the HPLC analyses of the wines, after 14 days of fermentation, using either strains of *C. zemplinina* or *S. cerevisiae* in pure culture, are shown in Table 2. The *C. zemplinina* strains were characterized by a higher consumption of fructose than glucose, and some strains showed a total fructophylic character, having left the content of glucose originally found in the must before inoculation untouched (Table 2). The *C. zemplinina* strains generally produced relevant quantities of glycerol and low amounts of acetic acid and ethanol, although it should be underscored that some isolates were able to produce up to an 8.0% volume of ethanol. A completely different picture emerged from the chemical analysis of the wines obtained from the *S. cerevisiae* fermentation. Strains FB40 and ELCF3WC performed rather well, consuming more than half of the sugars present in the must and producing more than an 13.5% volume ethanol (Table 2). However, both strains showed a very high production of acetic acid: 1.54 and 1.29 g/liter for FB40 and ELCF3WC, respectively. A different behavior was observed for the EC1118 commercial strain, which showed a limited consumption of sugars (~130 g/liter), which was correlated to a very low ethanol and acetic acid content. All of the *S. cerevisiae* strains were able to produce a relevant amount of glycerol (Table 2).

Five *C. zemplinina* strains were selected, on the basis of the fermentation performances and their genetic diversity, from the 35 tested. The details regarding sugar consumption, yield, and glycerol, acetic acid, and ethanol production are reported in Table 2. These strains were chosen because of the low acetic acid, low ethanol, and relevant glycerol content of the final wines and the preferred consumption of fructose with respect to glucose. The *C. zemplinina* EJ1strain did not comply with the above-mentioned criteria; however, it was selected due to its origin (from a different continent) and limited genetic similarity with the other *C. zemplinina* isolates, as shown in Fig. 1.

***S. cerevisiae* and *C. zemplinina* mixed fermentations.** The microbial dynamics of the mixed fermentations are shown in Fig. 3. Two different strategies were tested, a coinoculation of the two species and a sequential inoculation, with a delay of 48 h in the addition of *S. cerevisiae* with respect to *C. zemplinina*. The data are presented separately for the three *S. cerevisiae* strains, and the results of the counts (CFU/ml) are expressed as means ± the standard deviations. The *C. zemplinina* trends are the results of the five fermentations conducted in duplicate with the selected strains. The counts of *S. cerevisiae* FB40 when mixed separately with the 5 *C. zemplinina* strains are reported in Fig. 3A. In this case in either the coinoculated or the sequential inoculation, the *C. zemplinina* strains were not able to compete with *S. cerevisiae*, although a balance of the two species can be observed in the first 6 days. After this point, the *C. zemplinina* counts started to decrease and reached 10^3 to 10^4 CFU/ml after 14 days, compared to a population of *S. cerevisiae* that remained stable at 10^6 to 10^7 CFU/ml throughout the whole period. Very similar behavior was observed for *S. cerevisiae* ELCF3WC (Fig. 3B). Again, in this case, the *C. zemplinina* strains could not compete and started to decrease in numbers

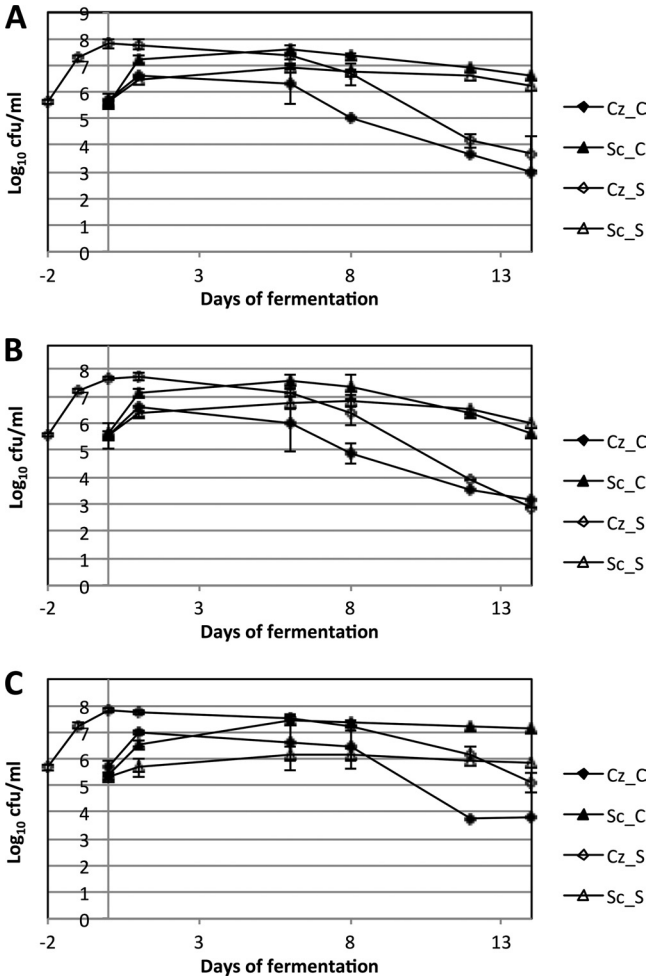

FIG 3 Growth dynamics of the *S. cerevisiae* strains (FB40 [A], ELCF3WC [B], and EC1118 [C]) coinoculated (solid symbols) or sequentially inoculated (empty symbols) with *C. zemplinina*, as determined on the WLN medium. The mean CFU/ml values ± the standard deviations of five fermentations (each with a different *C. zemplinina* strain) are shown for each *S. cerevisiae*, whereas all of the data were combined and are presented as the mean CFU/ml ± the standard deviations for the five selected strains of *C. zemplinina*. Abbreviations: Cz_C, *C. zemplinina* in coinoculation; Sc_C, *S. cerevisiae* in coinoculation; Cz_S, *C. zemplinina* in sequential inoculations; Sc_S, *S. cerevisiae* in sequential inoculations.

after 6 days. In order to exclude possible killer toxin activity of *S. cerevisiae* FB40 and ELCF3WC toward the *C. zemplinina* strains, tests were carried out as described by Pérez et al. (22) and resulted in no inhibition in any of the cases (data not shown). A completely different picture emerged for the EC1118 commercial strain, which was only able to dominate the fermentation in the case of the coinoculation (Fig. 3C). It is interesting that in the case of the sequential inoculation, the *C. zemplinina* strains were able to dominate EC1118 until day 12, and this was most probably due to the scarce capability of growth that this *S. cerevisiae* showed when inoculated after *C. zemplinina*.

The chemical composition of the wines obtained from the fermentations carried out by coinoculation and sequential inoculation are presented in Tables 3 and 4, respectively. In the coinoculated fermentations, the three different *S. cerevisiae* strain-*C. zemplinina* combinations resulted in a significantly different con-

TABLE 3 Chemical composition of wines obtained from coinoculated fermentations of *S. cerevisiae* and *C. zemplinina* strains

Strain(s)		Avg ± SD[a]					
C. zemplinina	*S. cerevisiae*	Reducing sugar (g/liter)	Glucose/fructose (−)	Ethanol (% vol)	Ethanol/consumed sugar yield (g/g)	Glycerol (g/liter)	Acetic acid (g/liter)
EJ1	FB40	$240 \pm 2^{b,\gamma}$	$0.69 \pm 0.01^{a,\alpha}$	$11.2 \pm 0.1^{a,\alpha}$	$0.454 \pm 0.009^{a,\alpha}$	$13.2 \pm 0.1^{a,\alpha}$	$1.21 \pm 0.01^{b,\beta}$
	EC1118	$255 \pm 5^{c,\beta}$	$0.87 \pm 0.07^{b,\alpha}$	$10.6 \pm 0.4^{a,\alpha}$	$0.467 \pm 0.028^{a,\alpha}$	$14.0 \pm 0.2^{b,\alpha}$	$1.12 \pm 0.07^{b,\beta}$
	ELCF3WC	$223 \pm 4^{a,\delta}$	$0.70 \pm 0.05^{a,\alpha}$	$12.6 \pm 0.3^{b,\alpha}$	$0.471 \pm 0.023^{a,\alpha}$	$15.2 \pm 0.1^{c,\alpha}$	$0.92 \pm 0.05^{a,\alpha}$
	Sig[1]	***	**	***	NS	***	***
T1Y3	FB40	$228 \pm 3^{b,\beta}$	$0.66 \pm 0.04^{a,\alpha}$	$12.3 \pm 0.2^{b,\beta}$	$0.471 \pm 0.018^{a,\alpha}$	$14.2 \pm 0.1^{a,\delta}$	$1.50 \pm 0.04^{b,\gamma}$
	EC1118	$235 \pm 4^{c,\alpha}$	$0.91 \pm 0.05^{b,\alpha}$	$11.6 \pm 0.3^{a,\beta}$	$0.462 \pm 0.025^{a,\alpha}$	$14.7 \pm 0.1^{b,\beta}$	$0.96 \pm 0.05^{a,\alpha}$
	ELCF3WC	$183 \pm 3^{a,\alpha}$	$0.60 \pm 0.04^{a,\alpha}$	$14.9 \pm 0.2^{c,\gamma}$	$0.468 \pm 0.015^{a,\alpha}$	$15.2 \pm 0.1^{c,\alpha}$	$1.02 \pm 0.04^{a,\beta}$
	Sig[1]	***	***	***	NS	***	***
BC60	FB40	$206 \pm 3^{a,\alpha}$	$0.61 \pm 0.05^{a,\alpha}$	$13.4 \pm 0.3^{b,\gamma}$	$0.465 \pm 0.019^{a,\alpha}$	$13.8 \pm 0.1^{a,\gamma}$	$1.12 \pm 0.05^{a,\alpha\beta}$
	EC1118	$248 \pm 5^{b,\beta}$	$0.83 \pm 0.07^{b,\alpha}$	$11.1 \pm 0.4^{a,\alpha\beta}$	$0.462 \pm 0.037^{a,\alpha}$	$14.5 \pm 0.2^{b,\beta}$	$1.55 \pm 0.07^{b,\gamma}$
	ELCF3WC	$197 \pm 4^{a,\beta}$	$0.65 \pm 0.06^{a,\alpha}$	$13.6 \pm 0.4^{b,\beta}$	$0.454 \pm 0.023^{a,\alpha}$	$15.1 \pm 0.2^{c,\alpha}$	$1.22 \pm 0.06^{a,\gamma}$
	Sig[1]	***	*	***	NS	***	***
L37	FB40	$222 \pm 2^{b,\beta}$	$0.70 \pm 0.03^{a,\alpha}$	$12.3 \pm 0.1^{b,\beta}$	$0.460 \pm 0.011^{a,\alpha}$	$13.5 \pm 0.1^{a,\beta}$	$1.04 \pm 0.03^{a,\alpha}$
	EC1118	$249 \pm 3^{c,\beta}$	$0.91 \pm 0.05^{b,\alpha}$	$10.8 \pm 0.3^{a,\alpha\beta}$	$0.463 \pm 0.025^{a,\alpha}$	$14.5 \pm 0.1^{b,\beta}$	$1.62 \pm 0.05^{b,\gamma}$
	ELCF3WC	$189 \pm 1^{a,\alpha}$	$0.63 \pm 0.02^{a,\alpha}$	$14.7 \pm 0.1^{c,\gamma}$	$0.474 \pm 0.007^{a,\alpha}$	$15.3 \pm 0.2^{c,\alpha\beta}$	$1.02 \pm 0.02^{a,\beta}$
	Sig[1]	***	***	***	NS	***	***
PEDRO10	FB40	$225 \pm 3^{b,\beta}$	$0.68 \pm 0.04^{a,\alpha}$	$12.0 \pm 0.2^{b,\beta}$	$0.453 \pm 0.017^{a,\alpha}$	$13.4 \pm 0.1^{a,\beta}$	$1.13 \pm 0.04^{a,\alpha\beta}$
	EC1118	$238 \pm 3^{c,\alpha}$	$0.82 \pm 0.04^{b,\alpha}$	$11.4 \pm 0.2^{a,\beta}$	$0.459 \pm 0.020^{a,\alpha}$	$14.6 \pm 0.1^{b,\beta}$	$1.22 \pm 0.04^{ab,\beta}$
	ELCF3WC	$209 \pm 2^{a,\gamma}$	$0.65 \pm 0.05^{a,\alpha}$	$13.8 \pm 0.1^{c,\beta}$	$0.488 \pm 0.011^{a,\alpha}$	$15.7 \pm 0.1^{c,\beta}$	$1.27 \pm 0.05^{b,\gamma}$
	Sig[1]	***	**	***	NS	***	*
Sig[2]		***, **, ***	NS, NS, NS	***, *, ***	NS, NS, NS	***, **, ***	***, ***, ***

[a] The ethanol content of the initial must was 1.8%. All data are expressed as averages ($n = 2$). Different superscript Roman letters within the same column indicate significant differences (Sig[1]) among the different *S. cerevisiae* strains inoculated with the same strain of *C. zemplinina* (Tukey's test; $P < 0.05$). Different superscript Greek letters within the same column indicate significant differences (Sig[2]) for different *C. zemplinina* strains inoculated with the same strain of *S. cerevisiae* (Tukey's test; $P < 0.05$). *, **, ***, and NS indicate significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$ and no significant difference, respectively.

sumption of sugars. As can be seen, the EC1118 strain combination always performed poorly, leaving high quantities of sugars at day 14, regardless of what *C. zemplinina* was used. On the contrary, the ELCF3WC combination always showed good fermentation properties, and this resulted in high ethanol content and low residual sugars. The glucose/fructose (G/F) ratio was similar for strains FB40 and ELCF3WC, while it was significantly higher for all of the wines produced with EC1118. The acetic acid content was influenced to a great extent by the *S. cerevisiae* strain that was used. It is interesting that *S. cerevisiae* EC1118, which showed the worst performance, also produced the highest amount of acetic acid unless it was inoculated with *C. zemplinina* T1Y3. This observation could be considered in conflict with its behavior in pure culture; however, in such a condition, the strain consumed very little sugar and produced limited amounts of ethanol and acetic acid. On the contrary, wines fermented with the ELCF3WC strain always contained higher amounts of alcohol (>12.6%vol.) and glycerol (>15 g/liter), whereas strain FB40 always produced low quantities of glycerol. However, it did not always produce low quantities of alcohol.

In the case of the sequential inoculation (Table 4), *S. cerevisiae* EC1118 was again the worst performer, producing wines with high residual sugars and with a G/F ratio of between 1.85 and 2.13. When this inoculation approach was used, it is important to note that the acetic acid content was not connected to the *S. cerevisiae* strain but was influenced by the *S. cerevisiae*-*C. zemplinina* strain combination used in the fermentation process. *C. zemplinina* EJ1 was associated with wines with a low ethanol content and a generally high acetic acid content, and similar results were also obtained for the BC60 strain. The combination *C. zemplinina* PEDRO10 and *S. cerevisiae* ELCF3WC produced wines with less acetic acid (<0.40 g/liter) without affecting the ethanol (11.4% [volume]) or glycerol (12 g/liter) contents.

If the chemical parameters determined for the wines obtained with the two different inoculation approaches are analyzed together, significant differences emerge in their compositions, especially for the G/F ratio, glycerol, and acetic acid (Table 5). The influence of the strains (both *S. cerevisiae* and *C. zemplinina*), their interaction, as well as the type of inoculation used, always resulted in significant differences ($P < 0.001$) (data not shown). The wines obtained with sequential inoculation resulted to have higher residual sugars, with an increased G/F ratio, thereby potentially influencing their final organoleptic properties. In these cases, the fermentation products, such as ethanol and glycerol, decreased. However, the quantity of acetic acid produced was between 0.60 and 0.75 g/liter and was half that of the fermentations conducted inoculating only the *S. cerevisiae* strains or both species at the same time.

DISCUSSION

In the last couple of years, a number of studies that have focused on the potential application of *C. zemplinina* in wine fermentations have been published (1, 18, 29, 30), mainly due to its ethanol

TABLE 4 Chemical composition of wines obtained from sequential inoculated fermentations of *S. cerevisiae* and *C. zemplinina* strains

Strain(s)		Avg ± SD[a]					
C. zemplinina	*S. cerevisiae*	Reducing Sugar (g/liter)	Glucose/fructose (−)	Ethanol (% vol)	Ethanol/consumed sugar yield (g/g)	Glycerol (g/liter)	Acetic acid (g/liter)
EJ1	FB40	$242 \pm 2^{b,\alpha}$	$2.06 \pm 0.03^{b,\gamma}$	$11.3 \pm 0.2^{b,\beta,\gamma}$	$0.465 \pm 0.016^{a,\beta}$	$14.6 \pm 0.1^{c,\delta}$	$1.03 \pm 0.03^{b,\gamma}$
	EC1118	$288 \pm 3^{c,\gamma}$	$2.04 \pm 0.04^{b,\beta}$	$8.9 \pm 0.2^{a,\beta\gamma}$	$0.466 \pm 0.030^{a,\beta}$	$12.1 \pm 0.1^{a,\beta}$	$0.70 \pm 0.04^{a,\beta}$
	ELCF3WC	$228 \pm 3^{a,\beta\gamma}$	$1.56 \pm 0.04^{a,\alpha}$	$11.4 \pm 0.3^{b,\beta,\gamma}$	$0.432 \pm 0.019^{a,\alpha}$	$13.5 \pm 0.1^{b,\beta}$	$0.75 \pm 0.04^{a,\gamma}$
Sig[1]		***	***	***	NS	***	***
T1Y3	FB40	$245 \pm 5^{b,\alpha}$	$1.54 \pm 0.07^{a,\alpha}$	$10.9 \pm 0.4^{b,\beta}$	$0.453 \pm 0.036^{b,\beta}$	$12.9 \pm 0.2^{b,\beta}$	$0.58 \pm 0.07^{a,\alpha}$
	EC1118	$262 \pm 2^{c,\alpha}$	$2.13 \pm 0.03^{b,\beta}$	$8.4 \pm 0.1^{a,\alpha\beta}$	$0.373 \pm 0.013^{a,\alpha}$	$11.4 \pm 0.1^{a,\alpha}$	$0.50 \pm 0.03^{a,\alpha}$
	ELCF3WC	$236 \pm 3^{a,\gamma}$	$1.56 \pm 0.04^{a,\alpha}$	$11.4 \pm 0.2^{b,\beta}$	$0.454 \pm 0.019^{b,\alpha}$	$13.9 \pm 0.1^{c,\beta,\gamma}$	$0.56 \pm 0.04^{a,\beta}$
Sig[1]		***	***	***	**	***	NS
BC60	FB40	$247 \pm 4^{a,\alpha}$	$1.64 \pm 0.05^{a,\alpha}$	$9.1 \pm 0.3^{b,\alpha}$	$0.370 \pm 0.023^{a,\alpha}$	$12.1 \pm 0.1^{b,\alpha}$	$0.74 \pm 0.05^{b,\beta}$
	EC1118	$271 \pm 5^{b,\alpha\beta}$	$1.85 \pm 0.07^{b,\alpha}$	$7.7 \pm 0.4^{a,\alpha}$	$0.355 \pm 0.035^{a,\alpha}$	$11.2 \pm 0.2^{a,\alpha}$	$0.61 \pm 0.07^{a,\alpha,\beta}$
	ELCF3WC	$250 \pm 4^{a,\delta}$	$1.51 \pm 0.05^{a,\alpha}$	$10.4 \pm 0.3^{c,\alpha}$	$0.444 \pm 0.026^{b,\alpha}$	$14.0 \pm 0.1^{c,\gamma}$	$1.01 \pm 0.05^{c,\delta}$
Sig[1]		***	***	***	**	***	*
L37	FB40	$247 \pm 3^{b,\alpha}$	$1.64 \pm 0.04^{a,\alpha}$	$9.6 \pm 0.2^{b,\alpha}$	$0.391 \pm 0.018^{a,\alpha}$	$12.2 \pm 0.1^{b,\alpha}$	$0.59 \pm 0.04^{ab,\alpha}$
	EC1118	$264 \pm 4^{c,\alpha}$	$2.04 \pm 0.06^{b,\beta}$	$8.5 \pm 0.3^{a,\alpha,\beta}$	$0.378 \pm 0.029^{a,\alpha}$	$11.5 \pm 0.2^{a,\alpha}$	$0.50 \pm 0.06^{a,\alpha}$
	ELCF3WC	$214 \pm 5^{a,\alpha}$	$1.52 \pm 0.07^{a,\alpha}$	$11.9 \pm 0.4^{c,\beta}$	$0.422 \pm 0.029^{a,\alpha}$	$13.7 \pm 0.2^{c,\beta,\gamma}$	$0.67 \pm 0.07^{b,\beta,\gamma}$
Sig[1]		***	***	***	NS	***	***
PEDRO10	FB40	$242 \pm 3^{b,\alpha}$	$1.79 \pm 0.05^{a,\beta}$	$11.9 \pm 0.3^{b,\gamma}$	$0.475 \pm 0.023^{b,\beta}$	$14.1 \pm 0.1^{b,\gamma}$	$0.56 \pm 0.05^{b,\alpha}$
	EC1118	$278 \pm 4^{c,\beta}$	$2.09 \pm 0.06^{b,\beta}$	$9.4 \pm 0.4^{a,\gamma}$	$0.462 \pm 0.020^{b,\beta}$	$12.3 \pm 0.2^{a,\beta}$	$0.51 \pm 0.06^{b,\alpha}$
	ELCF3WC	$226 \pm 5^{a,\beta}$	$1.88 \pm 0.05^{a,\beta}$	$11.4 \pm 0.4^{b,\beta}$	$0.417 \pm 0.031^{a,\alpha}$	$12.0 \pm 0.2^{a,\alpha}$	$0.37 \pm 0.05^{a,\alpha}$
Sig[1]		***	**	***	*	***	*
Sig[2]		NS, ***, ***	***, **, ***	***, **, **	***, **, NS	***, ***, ***	***, **, ***

[a] The ethanol content of the initial must was 1.8%. All data are expressed as averages ($n = 2$). Different superscript Roman letters within the same column indicate significant differences (Sig[1]) among the different *S. cerevisiae* strains inoculated with the same strain of *C. zemplinina* (Tukey's test; $P < 0.05$). Different superscript Greek letters within the same column indicate significant differences (Sig[2]) for different *C. zemplinina* strains inoculated with the same strain of *S. cerevisiae* (Tukey's test; $P < 0.05$). *, **, ***, and NS in indicate significance at $P < 0.05$, $P < 0.01$, $P < 0.001$, and no significant differences, respectively.

and low temperature tolerance, osmotic resistance and fructophylic character.

In the present study, we have specifically investigated the possibility of using *C. zemplinina* in sweet wine fermentations, sequentially or coinoculated with *S. cerevisiae*. All of the fermentations were carried out in natural must obtained from dried grapes in order to mimic the real conditions encountered during the production of sweet wines and avoid the use of laboratory media that can give a totally different picture in terms of yeast fermentative behavior.

A set of 35 isolates of *C. zemplinina* was first molecularly characterized, and the results obtained underlined a relative genetic homogeneity within the strains tested. There were no differences, in terms of clustering, on the basis of the geographic distribution, and most of the strains formed two large clusters. When these strains were tested in fermentation trials of must obtained from dried grapes, their fructophylic character was confirmed, as previously described (25, 26). Moreover, their ability to produce relevant quantities of glycerol and low amounts of acetic acid was also confirmed, in agreement with other studies (18, 29). Interestingly, the behavior of *S. cerevisiae* was different between the commercial strain and the wild isolates from sweet wine fermentations. As shown in Table 2, the EC1118 commercial strain performed worse than the two wine isolated strains, since a high level of residual sugar, associated with lower ethanol production, was detected at the end of the monitoring period. A notable production of acetic acid was concomitantly observed, most likely due to the osmotic stress provoked by the high concentration of sugars, responsible for the upregulation of the genes encoding for aldehyde dehydrogenases (12). It should be underlined that differences in the vitality counts were observed for the three *S. cerevisiae* strains when inoculated as pure culture in the must utilized here. Although the wild strains were able to promptly increase in cell counts, reaching 10^8 CFU/ml at day 1, EC1118 never reached a load of 10^7 CFU/ml and started to decrease after 6 days of fermentation (data not shown). This evidence allows us to speculate that the wild strains may adapt well to an environment, similar to the one from where they were isolated (i.e., musts with a high sugar concentration), and underlines the need for better performing strains than the commercial one used in the present study.

Five strains of *C. zemplinina* were selected, on the basis of their genetic characteristics and fermentation performances, for the mixed fermentation experiments. As shown in Fig. 1, the BC60 and PEDRO10 strains were located in cluster 1 (although in two different subclusters), while the T1Y3 and L37 strains grouped in cluster 2 (but again in two different subclusters). The last strain selected, EJ1, was genetically far from the first four described. Considering the chemical parameters of the wines obtained with these strains (Table 2), it can be observed that they all resulted to be homogeneous, apart from *C. zemplinina* EJ1, which again showed relevant differences with respect of the other strains selected.

TABLE 5 Statistical differences for the chemical composition of wines obtained from coinoculated (data from Table 3) and sequentially inoculated (data from Table 4) fermentations of *S. cerevisiae* and *C. zemplinina* strains

Strain(s)		Significance[a]					
C. zemplinina	*S. cerevisiae*	Reducing sugar (g/liter)	Glucose/fructose (−)	Ethanol (% vol)	Ethanol/consumed sugar yield (g/g)	Glycerol (g/liter)	Acetic acid (g/liter)
EJ1	FB40	NS	***	NS	NS	***	**
	EC1118	**	***	**	NS	***	***
	ELCF3WC	NS	***	**	NS	**	*
T1Y3	FB40	*	***	**	NS	**	***
	EC1118	***	***	***	**	***	***
	ELCF3WC	***	***	***	NS	***	***
BC60	FB40	***	***	***	**	***	***
	EC1118	**	***	***	*	***	***
	ELCF3WC	***	***	***	NS	**	*
L37	FB40	***	***	***	**	***	***
	EC1118	**	***	**	*	***	***
	ELCF3WC	**	***	***	**	***	**
PEDRO10	FB40	**	***	NS	NS	**	***
	EC1118	***	***	**	NS	***	***
	ELCF3WC	**	***	**	*	***	***

[a] *, **, ***, and NS indicate significance at $P < 0.05$, $P < 0.01$, $P < 0.001$, and no significant differences, respectively.

Remarkably, all of the selected *C. zemplinina* showed comparable growth kinetics, regardless of which *S. cerevisiae* strain was used. This aspect is highlighted by the contained standard deviations reported in Fig. 3. In other words, the five selected *C. zemplinina* showed similar growth curves when coupled with *S. cerevisiae*, both in the coinoculation and in the sequential inoculation. The main differences were observed in the trends of *S. cerevisiae* EC1118, which was not able to dominate the fermentation in the case of sequential inoculation. It is interesting that the counts reached by this strain in mixed fermentations were higher than in pure culture, underscoring that *C. zemplinina* strains could facilitate the growth of *S. cerevisiae* EC1118.

Although no variations were observed, in terms of growth, in the mixed fermentation experiments for each strain of *S. cerevisiae*, relevant differences were detected for the chemical composition of the wines. Again, in this case, *S. cerevisiae* EC1118 showed poor fermentation power, whereas *S. cerevisiae* ELCF3WC was able to produce more ethanol and glycerol and less acetic acid. However, as described above, *C. zemplinina* influenced the production of acetic acid by *S. cerevisiae*. More specifically, a coinoculation of *C. zemplinina* T1Y3 or L37 with *S. cerevisiae* ELCF3WC resulted in wines with a high ethanol content (>14.5% [by volume]) and acetic acid of ~1 g/liter (21% acetic acid reduction, compared to the pure ELCF3WC fermentation).

This study has demonstrated that the fermentation of musts, characterized by a high sugar content, with *S. cerevisiae* and *C. zemplinina* mixtures, may contribute to control the acetic acid production by *S. cerevisiae*. The data presented here support the use of this non-*Saccharomyces* species, but at the same time the specific *S. cerevisiae*-*C. zemplinina* combination is important. As shown, the possibility to reduce the acetic acid content is closely connected to the strain combination and the type of inoculation performed. Moreover, other chemical parameters, such as higher alcohols and acetaldehyde should be monitored, since it has been pointed out that *C. zemplinina* can contribute to a great extent to their increase or decrease, respectively (1, 18). Since all of the data presented here were obtained from pasteurized must, more investigations are necessary to assess the ability of *C. zemplinina* to compete with the natural microbiota of grape musts and to confirm that the mechanism responsible for the acetic acid reduction is due to *S. cerevisiae* osmotic stress relief.

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REFERENCES

1. **Andorrà I, et al.** 2010. Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. Eur. Food Res. Technol. **231**: 215–224.
2. **Bely M, Stoeckle P, Masneuf-Pomarède I, Dubourdieu D.** 2008. Impact of mixed *Torulaspora delbrueckii*-*Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. **122**:312–320.
3. **Brezna B, et al.** 2010. Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. Antonie Van Leeuwenhoek **98**:519–529.
4. **Ciani M, Beco L, Comitini F.** 2006. Fermentation behavior and metabolic interactions of multistarter wine yeast fermentations. Int. J. Food Microbiol. **108**:239–245.
5. **Ciani M, Comitini F, Mannazzu I, Domizio P.** 2010. Controlled mixed culture fermentation: a new prospective on the use of non-*Saccharomyces* yeasts in wine making. FEMS Yeast Res. **10**:123–133.
6. **Ciani M, Ferraro L.** 1998. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. J. Appl. Microbiol. **85**:247–254.
7. **Cocolin L, Bisson LF, Mills DA.** 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiol. Lett. **189**:81–87.
8. **Cocolin L, Pepe V, Comitini F, Comi G, Ciani M.** 2004. Enological and genetic traits of *Saccharomyces cerevisiae* isolated from former and modern wineries. FEMS Yeast Res. **5**:237–245.

9. **Comitini F, et al.** 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol. **28**:873–882.
10. **Csoma H, Sipiczki M.** 2008. Taxonomic reclassification of *Candida stellata* strains reveals frequent occurrence of *Candida zemplinina* in wine fermentations. FEMS Yeast Res. **8**:328–336.
11. **Erasmus DJ, Cliff M, van Vuuren HJJ.** 2004. Impact of yeast strain on the production of acetic acid, glycerol, and the sensory attributes of Icewines. Am. J. Enol. Vitic. **55**:371–378.
12. **Erasmus DJ, van der Merwe GK, van Vuurem HJJ.** 2003. Genome-wide expression analyses: metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. FEMS Yeast Res. **3**:375–399.
13. **Fleet G.** 2008. Wine yeast for the future. FEMS Yeast Res. **8**:979–995.
14. **Giordano M, Rolle L, Zeppa G, Gerbi V.** 2009. Chemical and volatile composition of three Italian sweet white Passito wines. J. Int. Sci. Vigne Vin. **43**:159–170.
15. **Herraiz T, Reglero G, Herraiz M, Martin-Alvarez PJ, Cabezudo MD.** 1990. The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. Am. J. Enol. Vitic. **41**:313–318.
16. **Li SS, et al.** 2010. Yeast species associated with wine grapes in China. Int. J. Food Microbiol. **138**:85–90.
17. **Magyar I, Bene ZS.** 2006. Morphological and taxonomic study of mycobiota of noble rotten grapes in the Tokay wine district. Acta Alimentaria **35**:237–246.
18. **Magyar I, Toth T.** 2011. Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum*, and *Saccharomyces cerevisiae*. Food Microbiol. **28**:94–100.
19. **Mills DA, Johannsen EA, Cocolin L.** 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. Appl. Environ. Microbiol. **68**: 4884–4893.
20. **Nisiotou A, Nychas GJE.** 2007. Yeast populations residing on healthy or *Botrytis*-infected grapes from a vineyard in Attica, Greece. Appl. Environ. Microbiol. **73**:2765–2768.
21. **Pallmann CL, et al.** 2001. Use of WL medium to profile native flora fermentations. Am. J. Enol. Vitic. **52**:198–203.
22. **Pérez F, Ramírez M, Regodón JA.** 2001. Influence of killer strains of *Saccharomyces cerevisiae* on wine fermentation. Antonie Van Leeuwenhoek **79**:393–399.
23. **Renault P, et al.** 2009. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: potential applications in the wine industry. Int. J. Food Microbiol. **134**:201–210.
24. **Schneider A, Gerbi V, Redoglia M.** 1987. A rapid HPLC method for separation and determination of major organic acids in grape musts and wines. Am. J. Enol. Vitic. **38**:151–155.
25. **Sipiczki M.** 2003. *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. Int. J. Syst. Evol. Microbiol. **53**:2079–2083.
26. **Sipiczki M.** 2004. Species identification and comparative molecular and physiological analysis of *Candida zemplinina* and *Candida stellata*. J. Basic Microbiol. **44**:471–479.
27. **Strauss MLA, Jolly NP, Lambrechts MG, van Rensburg P.** 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J. Appl. Microbiol. **91**:182–190.
28. **Tofalo R, et al.** 2009. Molecular identification and osmotolerant profile of wine yeasts that ferment a high sugar grape must. Int. J. Food Microbiol. **130**:179–187.
29. **Tofalo R, et al.** 2012. Diversity of *Candida zemplinina* strains from grapes and Italian wines. Food Microbiol. **29**:18–26.
30. **Tofalo R, et al.** 2011. Influence of organic viticulture on non-*Saccharomyces* wine yeast populations. Ann. Microbiol. **61**:57–66.
31. **Urso R, et al.** 2008. Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. FEMS Yeast Res. **8**:1053–1062.
32. **Zironi R, Romano P, Suzzi G, Battistuta F, Comi G.** 1993. Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guillermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. Biotechnol. Lett. **15**:235–238.
33. **Zott K, Miot-Sertier C, Claisse O, Lonvaud-Funel A, Masneuf-Pomarede I.** 2008. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. Int. J. Food Microbiol. **125**:197–203.