Candida zemplinina for Production of Wines with Less Alcohol and More Glycerol

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We developed a new protocol for winery mixed fermentations, using the selected *Candida zemplinina* yeast strain Cz3. The results of a two-year study, in which red musts (Merlot in 2010; Merlot, Nero d'Avola and Frappato in 2011) were inoculated with Cz3, is discussed. These wines were compared with wines obtained by inoculation with commercial *Saccharomyces cerevisiae* yeast strains (NDA21 and AR06 in 2010; NDA21 in 2011), or with those obtained by spontaneous fermentation (only in 2011). The inoculation of Cz3 always resulted in a two-phase fermentation: the first phase was driven by the *C. zemplinina* strain, while the second was dominated by the indigenous *S. cerevisiae* yeasts coming from the grapes and/or the winery. In both years the Cz3 wines contained less alcohol and more glycerol than those made with the commercial yeast strains or those obtained by spontaneous fermentation. Triangle tests showed that a sensorial difference between wines could only be achieved through the utilisation of Cz3.

INTRODUCTION

The standardisation of winery fermentation protocols through the use of selected *S. cerevisiae* yeast strains has represented a very important advancement in winemaking technology. However, over the last decade, the taste of consumers has evolved toward new and more complex products, and a new market sector has developed for wines made under less standardised conditions. This has been possible thanks to the revaluation of the role of non-*Saccharomyces* yeast species (reviewed by Suárez-Lepe & Morata, 2012). The contribution of these yeasts has been appreciated in the context of spontaneous fermentations; however, this kind of fermentation is seldom used in wineries since its outcome is difficult to control (Pretorius, 2000). On the other hand, the possibility of using selected non-*Saccharomyces* yeast strains, together with representatives of the *S. cerevisiae* species in mixed fermentations, has recently gained attention. Several laboratory studies (*e.g.* Ciani *et al*., 2010; Di Maio *et al*., 2012a; Suzzi *et al*., 2012) have shown how the chemical composition of the fermented musts can be affected positively by the intervention of these yeasts. These results have suggested that wines enriched with novel sensory features might be obtained through this practice.

Among non-*Saccharomyces* yeast species, those involved in spontaneous fermentations have received special attention (reviewed by Suárez-Lepe & Morata, 2012). Recently, we have demonstrated the presence of *C. zemplinina* yeasts in Sicilian musts of Catarratto, Nero d'Avola, Muscat and Frappato (Romancino *et al*., 2008; Di Maio *et al*., 2012a). This species is one of the most abundant during the early phases of spontaneous fermentation (Sipiczki, 2003; Csoma & Sipiczki, 2008; Lopandic *et al*., 2008; Urso *et al*., 2008; Andorrà *et al*., 2010). Several reports (Comitini *et al*., 2011; Di Maio *et al*., 2012a) have shown that, in a sterile laboratory setup, *C. zemplinina*/*S. cerevisiae* mixed fermentations could be obtained and that the wines that were produced had more glycerol and less alcohol than the wines obtained from *S. cerevisiae* single-starter fermentations.

In this work we report the results of fermentations conducted in a winery in the course of 2010 and 2011 for

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which the *C. zemplinina* strain Cz3 was used to ferment red musts (Merlot in 2010; Merlot, Nero d'Avola and Frappato in 2011). In a first attempt we inoculated Cz3 together with commercial *S. cerevisiae* yeast strains (NDA21 and AR06). Although we obtained mixed fermentations, these were due to the intervention of *S. cerevisiae* yeasts resident in the winery or coming from the grapes. Therefore, in 2011, we decided to inoculate just the Cz3 strain. Once again mixed fermentations were successful, so we can suggest a protocol for obtaining *C. zemplinina*/*S. cerevisiae* mixed fermentations in a winery environment.

MATERIALS AND METHODS

Yeast strains

The *C. zemplinina* Cz3 yeast strain was described in Di Maio *et al*. (2012a). The NDA21 (Di Maio *et al*., 2006) and the AR06 *S. cerevisiae* yeast strains are distributed by Biospringer (Maisons-Alfort, France).

Wine making

Grapes were delivered to the IRVO winery "G. Dalmasso" in Marsala (Trapani, Italy). Upon arrival, the grapes were de-stemmed and crushed. Musts were supplemented with 100 mg/L potassium metabisulphite. Chemical and microbiological analyses were performed at every step during the processing of the musts.

In 2010, four aliquots of Merlot must (80 litres each from the same initial mass) were prepared for yeast inoculation. One was inoculated with the NDA21 strain, one with the AR06 strain, one with the Cz3 strain and the NDA21 strain ("Cz3 A"), and one with Cz3 strain and the AR06 yeast strain ("Cz3 B").

In 2011, three aliquots of 80 litres for each must (Nero d'Avola, Merlot and Frappato) were prepared. These were either not inoculated (spontaneous fermentations), or inoculated with the NDA21 strain (NDA21 fermentations), or inoculated with the Cz3 strain (Cz3 fermentations).

In 2010 and 2011, Cz3 cells where inoculated at $42 \pm 2 \times 10^6$ CFU/mL; in the single-starter fermentations, commercial *S. cerevisiae* cells (NDA21 and AR06 in 2010; NDA21 in 2011) were inoculated at $11.5 \pm 0.5 \times 10^6$ CFU/mL, following the manufacturer's instructions. This difference in the concentrations of the inoculated starters was based on microscopic observations, which allowed us to calculate that the volume of an *S. cerevisiae* cell is equal to four to five times that of a Cz3 cell (450 ± 150 μm^3 and 150 ± 40 μm^3 respectively; n = 50).

For the inoculation of the Cz3 strain cells, liquid cultures were prepared, which were obtained by pre-multiplication in sterile must (reconstituted from concentrated must; 16 °Brix; pH 3.2; filter sterilised 0.2 μm). Yeast cells were collected, washed with water (to eliminate must traces) and inoculated.

In the mixed fermentations in 2010 (Cz3A and Cz3 B), commercial *S. cerevisiae* cells were inoculated at 500 CFU/mL, one day after the inoculation of the Cz3 cells. This was based on the results of laboratory experiments conducted in 700 mL of sterile must: in these conditions the growth of the *S. cerevisiae* cells would be slow enough to allow the Cz3 cells to proliferate and drive the early phase of the fermentation.

Crushed grapes were fermented at 25°C. Microbiological analyses and temperature controls were performed once every day. Two punching down of the cap were also performed daily. Samples were taken daily for sugar determination. All equipment was carefully sanitised at each step to prevent contamination. Fermentations took 11 days in 2010; and 14, 10 and eight days in 2011 for the Merlot, Nero d'Avola and Frappato musts, respectively.

In 2010, all the wines were inoculated with *Oenococcus oeni* (Viniflora Oenos, Chr Hansen) following the manufacturer's instructions. Samples were taken before and after malolactic fermentation for chemical analyses. At the end of the malolactic fermentation, samples were supplemented with 60 mg/L of potassium metabisulphite. Vinifications were performed between August and September. After racking and the further addition of 60 mg/L of potassium metabisulphite, the wines were bottled in December of the same year. Chemical and microbiological controls were performed at wine bottling.

In 2011, all the wines were inoculated with *Oenococcus oeni* (Viniflora Oenos, Chr Hansen), similarly to what was done in 2010. The Frappato and Merlot wines underwent malolactic fermentation and were supplemented with 60 mg/L of potassium metabisulphite at the end of the fermentation. The Nero d'Avola wines never underwent malolactic fermentation. Wine fining occurred at 20°C, lasting two months for the Frappato and five months for the Merlot and Nero d'Avola wines. After a further transfer and a final addition of 60 mg/L of potassium metabisulphite, the Frappato wines were bottled in December 2011, and the Merlot and Nero d'Avola wines were bottled in March 2012. All the wines were stored under the same conditions (at 16°C in 750 ml bottles).

Microbiological analyses

Samples from the fermenting musts were taken every day, diluted in sterile peptone water (0,1% Bacteriological Peptone, Oxoid) and plated in duplicate on WL nutrient agar (Oxoid), lysine agar (LA, Oxoid) and (only when Cz3 was inoculated) WL differential (WLD). As described in Di Maio *et al*. (2011, 2012c), the use of LA allowed all the non-*Saccharomyces* yeasts to be counted; the use of WL nutrient agar allowed *Saccharomyces* and non-*Saccharomyces* yeasts to be counted and distinguished; and the use of WLD (with 2 mg/L cycloheximide) allowed a selective count of the *C. zemplinina* colonies.

Further microbiological analyses were performed on WL nutrient agar (Oxoid), agar-lysine (Oxoid) and tomato juice agar (Fluka) before bottling (Cavazza & Poznanski, 1998).

Mitochondrial DNA analyses

DNA analyses were performed to assess the identity of the *C. zemplinina* and *S. cerevisiae* yeasts taking part to the fermentations. For the *C. zemplinina* yeasts, must samples were taken on the fourth day (in 2010) or on the third day (in 2011), when the highest concentrations of cells were recorded. For the *S. cerevisiae* yeasts, wine lees samples were taken at the end of the fermentation from the bottom of the tanks, after the first racking.

To verify that Cz3 was the only *C. zemplinina* strain

present in the *Candida* trials, 30 colonies were selected and the mt-DNA RFLP pattern was analysed after digestion with the *HpaII* restriction enzyme (NEB, Ipswitch, MA-USA), according to Di Maio *et al*. (2012a) and Pramateftaki *et al*. (2000). To obtain and analyse the mt-DNA of the *S. cerevisiae* yeasts, the protocol described in Di Maio *et al*. (2012b, based on Ribéreau-Gayon *et al*., 1998) was followed: lees pre-cultures were prepared in YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) supplemented with tetracycline (30 ppm) to prevent bacterial growth. *S. cerevisiae* yeast mt-DNA was extracted and digested with the *HinfI* or *RsaI* endonucleases (NEB).

Chemical parameters and statistical analysis

Before the start of the fermentations, musts were subjected to the analyses listed in Table 1. For the determination of the wines' alcohol content, the OIV official method (OIV, 2006) was followed. Glucose, fructose, glycerol, acetic acid, malic acid, lactic acid, citric acid and tartaric acid concentrations were determined using an Enotech Steroglass apparatus (code SQRQ053586; Steroglass-Italy) by monitoring the changes in absorbance. Yeast-available nitrogen was determined according to Gump *et al*. (2002). A number of additional parameters (volatile compounds, anthocyanins and colorimetric determinations) were measured. These data are available upon request.

Chemical parameters were measured in duplicate in 2010 and in triplicate in 2011; in this latter case a statistical analysis was performed using analysis of variance (ANOVA) and the least significant difference (LSD) test to determine statistically different values at a significance level of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Triangle test

The wines of 2010 and 2011 were subjected to a triangle test in May 2011 and June 2012 respectively. Tests were conducted according to UNI EN ISO 4120 (2008). For the 2010 wines, the panel consisted of 30 trained assessors: 12 males and 18 females, 20 to 24 years old, and for the most part students of the University of Catania. For the 2011 wines, the panel consisted of 16 judges: 13 males and three females, 22 to 48 years old, and for the most part wine experts. Red light filters were used to eliminate colour differences. Samples were kept covered until used; water was provided for mouth rinsing between samples and triads. The presentation order was balanced across judges, and sample presentation was randomised within triads. Evaluations were conducted between 10:00 and 12:00 each day. Tests were done under controlled temperature (22°C to 24°C) and in booths that complied with UNI ISO 8589 (2007). The statistical significance of the results was determined based on tabulated thresholds for triangle tests (UNI EN ISO 4120, 2008): these are reported as the critical numbers of correct responses in Tables 3 and 5 of this paper.

RESULTS AND DISCUSSION

Microbiological, molecular, chemical and sensory aspects of the 2010 fermentations

The purpose of these experiments was to replicate in the winery the results that were previously obtained in the laboratory (Di Maio *et al*., 2012a). To achieve this aim the *C. zemplinina* strain Cz3 was used together with the commercial *S. cerevisiae* strains NDA21 or AR06 to ferment Merlot musts. To understand the contribution of the Cz3 strain to the fermentation outcome, we compared the chemical and sensory properties of the wines we obtained with this strain ("Cz3 fermentations" and "Cz3 wines") with those of the wines made using just the *S. cerevisiae* commercial strains ("*Saccharomyces* fermentations" and "*Saccharomyces* wines").

The data presented in Fig. 1 show how the microbial populations present in the musts evolved over time. In both the *Saccharomyces* fermentations (a and b), the *S. cerevisiae* yeasts took control of the entire process and the growth of non-*Saccharomyces* yeasts was kept at low levels. In both the Cz3 fermentations (c and d), a first phase dominated by the *C. zemplinina* yeasts was followed by a second one with a robust proliferation of *S. cerevisiae* yeasts. The growth of other non-*Saccharomyces* yeasts was kept at low levels, comparable to those seen in the *Saccharomyces* fermentations (Fig. 1). The contribution of *C. zemplinina* yeasts to the progress of the Cz3 fermentations was also clearly indicated by the higher fructose consumption, in agreement with the fructophilic character of this species (Magyar & Tóth, 2011). In all the fermentations, residual sugar levels were consistent with the definition of "dry wines" (Commission Regulation of the European Union (EC) No 753, 2002).

Mitochondrial DNA analyses showed that each of the *Saccharomyces* fermentations was driven by the commercial yeast strain that was inoculated. In the Cz3 fermentations, the first phase was always controlled by the Cz3 yeast strain: 100% of the *C. zemplinina* colonies we recovered from the musts on the fourth day of fermentation had a mt-RFLP pattern identical to that of the Cz3 strain. On the other hand, the mt-DNA RFLP pattern of the *S. cerevisiae* yeasts recovered from the lees was different from that of the commercial strains (data not shown). Therefore the second phase of the Cz3 fermentation was not dominated by the inoculated *S. cerevisiae* starter yeasts. We concluded that this phase was instead driven by indigenous *S. cerevisiae* yeasts resident in

TABLE 1
Enological parameters for 2010 Merlot and 2011 Merlot, Nero d'Avola (NdA) and Frappato musts, before inoculation with yeast.

Parameter	Merlot 2010	Merlot 2011	NdA 2011	Frappato 2011
Glucose+ fructose (g/L)	244.06 (0.00)	269.95 (11.62)	228.23 (3.10)	241.95 (5.10)
pH	3.37 (0.02)	3.38 (0.01)	3.28 (0.00)	3.46 (0.07)
Total Acidity (g/L)	5.60 (0.06)	6.50 (0.06)	8.90 (0.03)	7.60 (0.03)
Yeast available nitrogen (mg/L)	195 (0.00)	215 (0.18)	300(10.50)	211 (2.70)

the winery, in agreement with Ciani *et al*. (2004), or originally present on the grapes. Several chemical parameters were determined to understand the difference between the Cz3 wines and those obtained with the commercial *S. cerevisiae* strains. Compared to the *Saccharomyces* wines, Cz3 wines contained about half a degree less alcohol and up to 50% more glycerol (see Table 2). This was in good agreement with the laboratory results obtained by Di Maio *et al*. (2012a). Also, the acetic acid levels in the Cz3 wines were higher than in the *Saccharomyces* wines, but always below the prescribed limit for red wines (Commission Regulation (EC) No 606, 2009).

Finally, to evaluate the sensory aspects of our wines we subjected them to triangle tests. As can be seen in Table 3, no statistically significant differences were found between the Cz3 wines; the same result was found for the *Saccharomyces* wines; however, the Cz3 wines were found to be different from the *Saccharomyces* wines in a statistically significant way.

Microbiological, molecular, chemical and sensory aspects of the 2011 fermentations

The results of the experiments performed in 2010 indicated that, although we could not control the fermentation process in all its aspects, we were still able to obtain mixed fermentations in which the Cz3 strain would co-operate together with *S. cerevisiae* yeasts, giving rise to wines with specific chemical and sensory properties. We considered this an interesting outcome and wanted to see if we could obtain mixed fermentations in 2011 by inoculating wine musts with the Cz3 strain alone. At this time we performed three tests, using different red musts: Merlot, Nero d'Avola and Frappato. The results obtained with the Cz3 strain ("Cz3 wines") were compared with those obtained by letting musts ferment spontaneously ("spontaneous wines"), or by fermenting them with *S. cerevisiae* strain NDA21 ("NDA21 wines").

During the course of all fermentations we monitored the

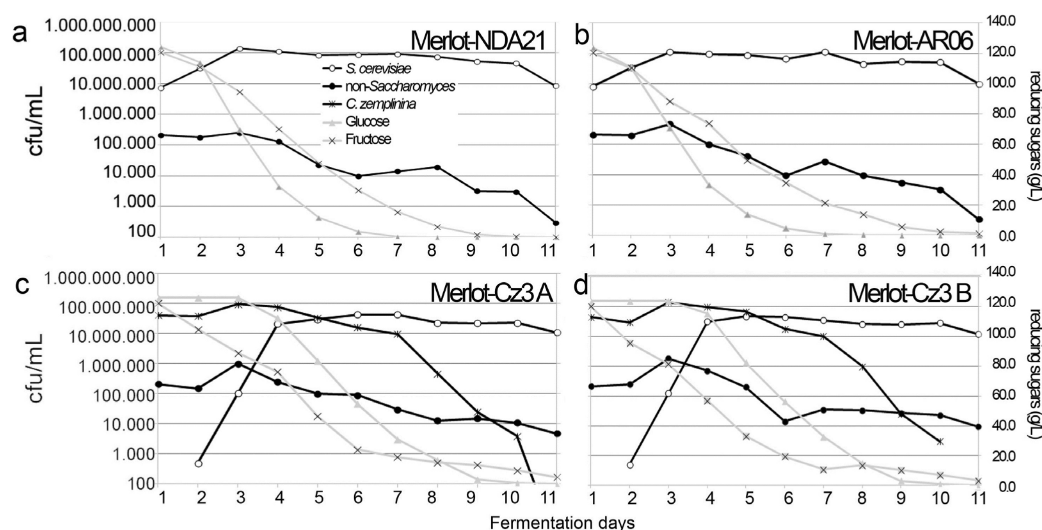

FIGURE 1
Growth curves of *Saccharomyces*, non-*Saccharomyces* and *Candida* yeasts in 2010 Merlot fermentations. Musts were inoculated with NDA21 (a), AR06 (b), Cz3 and NDA21 (c) or Cz3 and AR06 (d). Symbols are explained in the legend. The concentration of glucose and fructose is shown by the light grey curves (values are reported on the secondary axis).

TABLE 2
Chemical parameters of the 2010 Merlot wines.

	NDA21	AR06	Cz3 A	Cz3 B
Alcohol (%)	14.73 (0.03)	14.82 (0.00)	14.24 (0.03)	14.23 (0.04)
Glycerol (g/L)	8.10 (0.00)	8.20 (0.00)	12.00 (0.00)	12.50 (0.00)
pH	3.64 (0.01)	3.59 (0.00)	3.59 (0.00)	3.50 (0.00)
Titratable acidity (g/L)	5.80 (0.14)	6.20 (0.14)	5.85 (0.07)	5.90 (0.14)
Tartaric Acid (g/L)	2.77 (0.24)	3.00 (0.25)	2.87 (0.23)	2.84 (0.19)
Malic Acid (g/L)	1.02 (0.03)	1.08 (0.02)	1.05 (0.00)	1.14 (0.01)
Lactic Acid (g/L)	0.09 (0.09)	0.05 (0.03)	0.01 (0.00)	0.01 (0.01)
Citric Acid (g/L)	0.41 (0.00)	0.42 (0.00)	0.57 (0.01)	0.61 (0.02)
Acetic Acid (g/L)	0.42 (0.01)	0.20 (0.01)	0.61(0.01)	0.62 (0.01)
Glucose (g/L)	0.00 (0.00)	0.01 (0.00)	0.01 (0.00)	0.00 (0.00)
Glucose+Fructose (g/L)	0.15 (0.00)	0.15 (0.00)	0.14 (0.00)	0.12 (0.00)

Values are averages of two measurements, standard deviations are indicated in parenthesis.

growth of the yeast species in the musts, as well as the glucose and fructose consumption (Fig. 2). As can be seen in Fig. 2a, d and g, when musts were left to ferment spontaneously, the first few days were characterised by the growth of non-*Saccharomyces* yeasts. Soon, however (within two to three days), *S. cerevisiae* yeasts began proliferating in the musts. As the ratio between these yeasts and the non-*Saccharomyces* yeasts increased, a decline in the growth curve of the non-*Saccharomyces* yeasts followed in all musts.

In all the NDA21 fermentations (Fig. 2 b, e, h) it took two to three days for the growth curve of the starter to reach a plateau. In both the Merlot and Nero d'Avola musts, the growth of non-*Saccharomyces* yeasts was kept at levels that were always much lower than the initial ones and than those seen in the spontaneous fermentation.

Inoculation of the Cz3 strain (Fig. 2 c, f, i) helped control the growth of non-*Saccharomyces* yeasts; this was kept at lower levels (compared to the initial ones) in the Merlot and Nero d'Avola musts for most of the fermentation, while higher levels were observed in the Frappato must (probably due to the initially higher concentration of these yeasts in the must). In this case, however, the *C. zemplinina*/non-*Saccharomyces* ratio was higher than 40 in the first five days. The onset of the proliferation of the indigenous *S. cerevisiae* yeasts helped reduce and maintain the growth of the non-*Saccharomyces* yeasts at low levels in all fermentations. Similarly to what was observed in 2010, the contribution of the *C. zemplinina* yeasts to the progress of the fermentations was consistent with the different levels of glucose and fructose consumption. Once again, residual sugar levels were consistent with the definition of "dry wines" (Commission Regulation of the European Union (EC) No 753, 2002).

Molecular monitoring by mtDNA-RFLP analysis, performed on the DNA of the yeast colonies grown from the musts, showed that, when inoculated, Cz3 was the only *C. zemplinina* yeast proliferating in the musts. Analyses

TABLE 3
Triangle test on 2010 Merlot wines.

Yeast pairs	Number of correct answers received (out of 30)	Critical number of correct answers [a]	Significant difference (α risk = 0.05)
NDA21 versus AR06	12	21	No
NDA21 versus Cz3 A	24	21	Yes
NDA21 versus Cz3 B	22	21	Yes
AR06 versus Cz3 A	22	21	Yes
AR06 versus Cz3 B	21	21	Yes
Cz3 A versus Cz3 B	11	21	No

[a] Number of correct answers required for statistical significance

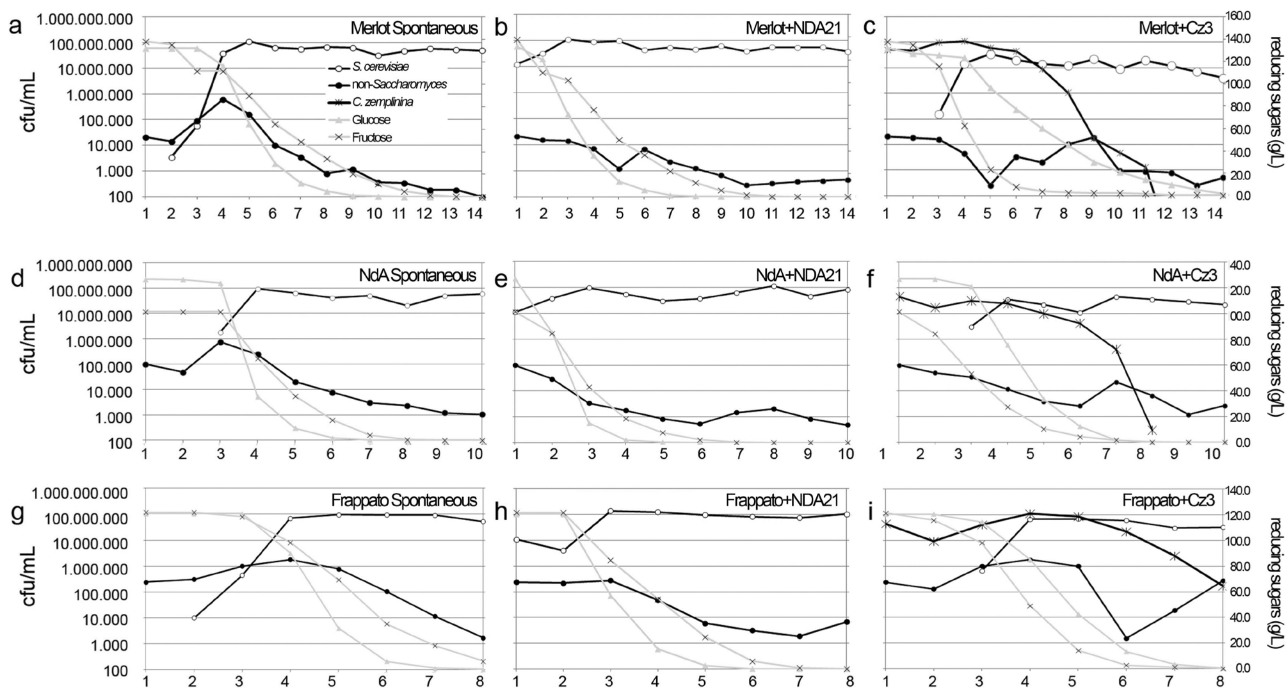

FIGURE 2
Growth curves of *Saccharomyces*, non-*Saccharomyces* and Cz3 yeasts in 2011 fermentations. Merlot (a, b, c), Nero d'Avola (d, e, f) and Frappato (g, h, i) musts, were allowed to ferment spontaneously (a, d, g); or were inoculated with the NDA21 (b, e, h); or with the Cz3 (c, f, i) yeast strains. Symbols are explained in the legend. The amount of glucose and fructose is shown by the light grey curves (values are reported on the secondary axis).

TABLE 4
Chemical parameters measured at bottling in 2011 Merlot, Nero d'Avola and Frappato wines.

Wine	Merlot 2011				Nero d'Avola 2011				Frappato 2011			
Starters	Spont	NDA21	Cz3	P	Spont	NDA21	Cz3	P	Spont	NDA21	Cz3	P
Chemical Parameter												
Alcohol % (v/v)	15.34 b	15.40 b	14.90 a	***	14.69 b	14.61 b	14.31 a	***	13.93 b	13.82 b	13.43 a	***
Glucose (g/L)	0.21 ab	0.27 b	0.20 a	***	0.44 b	0.49 b	0.34 a	***	0.17	0.16	0.14	n.s.
Glucose+Fructose (g/L)	0.35	0.43	0.32	n.s.	0.69	0.74	0.56	n.s.	0.27	0.28	0.27	n.s.
Glycerol (g/L)	6.83 a	6.63 a	11.70 b	***	7.23 a	7.20 a	11.63 b	***	8.13 a	7.80 a	12.46 b	***
pH	3.69 a	3.75 b	3.65 a	***	3.21	3.20	3.21	n.s.	3.80	3.80	3.79	n.s.
Titratable Acidity (g/L)	4.67 a	4.80 a	5.39 b	***	7.66 a	7.79 a	8.14 b	***	4.82 a	5.10 b	4.89 a	**
Acetic Acid (g/L)	0.65 a	0.63 a	0.95 b	***	0.56 a	0.51 a	0.70 b	***	0.56 b	0.37 a	0.76 c	***
Tartaric Acid (g/L)	1.51 a	1.47 a	1.67 b	***	3.54	3.41	3.39	n.s.	1.57	1.61	1.58	n.s.
Malic Acid (g/L)	0.03 a	0.10 ab	0.25 b	***	0.64	0.66	0.67	n.s.	0.01 a	0.03 a	0.05 b	**
Lactic Acid (g/L)	0.63 b	0.62 ab	0.48 a	***	0.01	0.01	0.01	n.s.	1.64 a	1.75 a	1.80 b	**
Citric Acid (g/L)	0.03 a	0.04 a	0.12 b	***	0.25 b	0.26 b	0.23 a	**	0.02 a	0.33 b	0.04 a	**

Values are averages of three measurements. P-values were determined by analysis of variance (least significant difference test); n =3; Different letters (a–c) denote statistically significant differences within a single row at * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$; ns, not significant.

TABLE 5
Triangle test on 2011 wines.

Merlot wines: yeast pairs	Number of correct answers received (out of 30)	Critical number of correct answers [a]	Significant difference (α risk = 0.05)
Spontaneous versus NDA21	7	9	No
Spontaneous versus Cz3	9	9	Yes
NDA21 versus Cz3	10	9	Yes
Frappato wines: yeast pairs	**Number of correct answers received (out of 16)**	**Critical number of correct answers [a]**	**Significant difference (α risk = 0.05)**
Spontaneous versus NDA21	6	9	No
Spontaneous versus Cz3	9	9	Yes
NDA21 versus Cz3	8	9	No
Nero d'Avola wines: yeast pairs	**Number of correct answers received (out of 16)**	**Critical number of correct answers [a]**	**Significant difference (α risk = 0.05)**
Spontaneous versus NDA21	6	9	No
Spontaneous versus Cz3	6	9	No
NDA21 versus Cz3	8	9	No

[a] Number of correct answers required for statistical significance.

performed on the lees collected at racking showed that each NDA21 fermentation was driven by this yeast strain (data not shown).

A number of chemical parameters were measured at bottling (Table 4). The Cz3 wines contained more glycerol (up to 50% more) and less alcohol than the other wines, consistent with what was observed in 2010. They also had more acetic acid than the spontaneous or NDA21 wines (levels were again within the limits for red wines). For these three parameters, the ANOVA revealed significant variations and the three Cz3 wines were consistently different from the NDA21 and spontaneous wines. These results were also in agreement with those obtained in the laboratory (Di Maio *et al*., 2012a). We therefore could conclude that the results were due to the activity of the Cz3 strain.

The results of the triangle tests performed on the 2011 Merlot wines (Table 5) confirmed what was found in 2010, and a statistically significant difference between the Cz3 wine on one hand, and the *Saccharomyces* and spontaneous wines on the other, could be detected by the judges. For the Nero d'Avola, no significant difference could be found between the Cz3 wine and the other wines. On the other hand, a difference between the Cz3 and the spontaneous wine could be detected in the case of the Frappato. This suggests that, although specific chemical changes were consistently produced upon Cz3 inoculation in three different musts, in some cases their sensorial impact could be reduced by the varietal features of the wines that were produced. Nonetheless, every time a difference was noted between the wines, this always coincided with the utilisation of the Cz3

yeast strain.

A number of studies have been devoted to the discovery of non-*Saccharomyces* yeast species, which until now have been utilised in the laboratory to obtain mixed fermentation wines with less alcohol. This process was often accompanied by an increase in glycerol production (*e.g.* Ciani *et al*., 2010; Milanovic *et al*., 2012). Our results suggest the possibility of obtaining such products in a winery environment through the utilisation of a new fermentation protocol. This protocol also allowed effective control over the contamination of unwanted non-*Saccharomyces* species in a non-sterile environment, and at a time when low alcohol levels could have allowed their proliferation.

The contribution of Cz3 was important for accumulating glycerol and maintaining lower ethanol levels, consistently with what observed in our laboratory experiments (Di Maio *et al*., 2012a). This shows that the technological potential of Cz3 can be exploited in a winery environment. The increase in glycerol content of the wines (about 50%) is an important result, since it affects a chemical whose concentration remains stable over time (Scanesl *et al*., 1998) and which might contribute to the sensory properties of the wines, although probably in subtle and wine-dependent ways (Gawel *et al*., 2007). The production of higher levels of acetic acid was never noted during the tasting sessions (data not shown), and the concentration of this chemical was always within the allowed limits for red wines (Commission Regulation (EC) No 606, 2009).

The fructophilic character displayed by *C. zemplinina* yeasts (and by the Cz3 strain in particular) is another important result, since the use of yeast strains endowed with the ability to consume fructose has been indicated as a possible solution (or at least a possible help) for overcoming stuck fermentations (*e.g.* Messias *et al*., 2008). Therefore, our Cz3 strain possesses a number of features that make it an interesting strain for technological applications.

CONCLUSIONS

We have presented a novel fermentation protocol in which the inoculation of just one *C. zemplinina* (Cz3) yeast strain allowed us to obtain *C. zemplinina*/*S. cerevisiae* fermentations in the winery. The wines we obtained had lower ethanol concentrations and markedly higher glycerol levels than those made using the commercial *S. cerevisiae* strain alone.

The fermentation protocol we have set up might represent an interesting compromise between a controlled fermentation and a spontaneous one, with the co-operation of indigenous *S. cerevisiae* yeasts. This might be a way of meeting the general interest in spontaneous fermentations (*e.g.* Capece *et al*., 2012) by providing a way to control (at least to some extent) their outcome. Natural ways of lowering the alcohol in wine might finally be interesting in view of the demonstrated effect of global climate change on the early ripening and enhanced sugar accumulation of grapes (*e.g.* Webb *et al*., 2012).

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