Presence of *Candida zemplinina* in Sicilian Musts and Selection of a Strain for Wine Mixed Fermentations

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The purpose of this work was to investigate the presence of *C. zemplinina* yeasts in Sicilian musts and grapes and to identify strains of oenological interest. We report on the taxonomical reclassification of *Candida* yeast isolates from Sicilian musts and on the selection of one strain of oenological interest (Cz3), based on mixed micro-fermentation experiments in sterile Nero d'Avola musts. Our results show that *Candida zemplinina* is abundant in Sicilian grapes and musts, and that the Cz3 strain is suitable for *Candida zemplinina/Saccharomyces cerevisiae* mixed fermentations. The higher glycerol content and the lower ethanol level stood out as the most promising features of the wines obtained upon sequential inoculation of the Cz3 and (*S. cerevisiae*) NDA21 yeast starters. We therefore have isolated a Sicilian Cz strain endowed with very promising features for the future development of mixed fermentation protocols.

INTRODUCTION

The possibility of increasing wine complexity via mixed fermentation represents an attractive opportunity offered by recent oenological research. This exploits the contribution of some non-*Saccharomyces* yeast species that are present early in spontaneous fermentations. Although attractive, spontaneous fermentations are not easy to control in their progression and outcome. On the other hand, mixed fermentations, where selected non-*Saccharomyces* species are inoculated together with a commercial *Saccharomyces* strain, may be a more manageable way to increase the appeal of certain wines. Non-*Saccharomyces* species can synthesise compounds and enzymes that can positively affect the quality of the wines (e.g. Languet *et al*., 2005). Furthermore, several negative features of non-*Saccharomyces* yeasts are attenuated in mixed fermentations (Du Toit & Pretorius, 2000; Anfang *et al*., 2009).

It has been reported that *Candida zemplinina* and *Hanseniaspora* spp. are the yeasts most frequently found in spontaneous fermentations (Andorrà *et al*., 2010). The higher glycerol content and the relatively low ethanol levels are the most appealing features of the wines made using *Candida* yeasts (Magyar & Tóth, 2011). These have a commercial interest, since glycerol ameliorates taste, while low ethanol levels can attract a wide range of consumers. Among *Candida* species, *C. zemplinina* occurs most frequently in musts (Csoma & Sipiczki, 2008). Compared to *C. stellata*, *C. zemplinina* yeasts have been suggested to be better suited for mixed fermentations, since they could reduce the osmotic stress imposed on *Saccharomyces* cells more efficiently by removing more sugar from the culture medium (Cavagna *et al*., 2008; Rantsiou *et al*., 2008).

For this paper we analysed 59 *Candida zemplinina* isolates from musts of autochthonous cultivars belonging to the non-*Saccharomyces* collection of the Istituto Regionale della Vite e del Vino (IRVV-Palermo, Italy). We present the oenological characterisation of these isolates and we report on the identification of one of these strains (Cz3), which featured the best results in sequential inoculation protocols with a commercial *Saccharomyces* strain.

MATERIALS AND METHODS

Yeasts

The 59 *Candida zemplinina* isolates (Cz) analysed in this work belong to the IRVV non-*Saccharomyces* collection and were previously reported as *C. stellata* isolates (Romancino *et al*., 2008). The *Saccharomyces cerevisiae* (Sc) strain NDA21 (Di Maio *et al*., 2006) belongs to the IRVV collection and is commercialised by Biospringer. The Sc yeast strains L1, L2, L3 and L4 belong to the *Saccharomyces* IRVV collection (Di Maio *et al*., 2012). The commercial Sc strains ICV-k1, EC1118, ICV-D254, RC212, QD145, Uvaferm43 and Ba11 are distributed by Lallemand; Zymaflore F10 is distributed by Laffort. The *S. cerevisiae* L404 strain belongs to the DIPROVAL collection of the University of Bologna,

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Italy. The *H. uvarum* Hu03 and *M. pulcherrima* Mp03 strains belong to the IRVV collection. The *C. stellata* 6714 strain was provided by DBVPG (Department of Applied Biology, Industrial Yeast Collection, University of Perugia, Italy). *C. zemplinina* and *C. stellata* yeasts were grown in malt agar and kept at 4°C. The *Saccharomyces*, *Hanseniaspora* and *Metschnikowia* yeasts were cultured in Sabouraud dextrose agar (Oxoid), with 10 g/L of yeast extract (Oxoid), at 4°C. All cultures were renewed periodically.

Molecular characterisation

The 59 *Candida* isolates (IRVV) were characterised as representatives of the *C. zemplinina* species by amplifying the ITS1-5.8S-ITS2 region and by digesting it with DraI and MboI endonucleases. The *C. stellata* DBVPG 6714 strain was used for reference. All our isolates showed the same digestion pattern, identical to that described for *C. zemplinina* (Csoma & Sipiczki, 2008). Sequencing and analysis of the D1/D2 domain of the 26S rDNA was done at the DBVPG. To distinguish the isolates, the DNA was extracted following the procedure of Querol *et al*. (1992); the DNA was then digested with the HaeIII and HpaII endonucleases (which allow an intra-specific distinction in the closely related *C. stellata* species) and the restriction patterns of the mt-DNA were analysed (Pramateftaki *et al*., 2000).

Oenological characterisation

Acetic acid production was measured according to Caridi *et al*. (2002). The L404 strain and the *H. uvarum* Hu03 strain were used for reference. H_2S production was assessed on BiGGY agar (Nickerson, 1953). L1-L4, EC1118, L404, ICV-D254, QD145, Uvaferm43, NDA21, Zymaflore F10 strains were used for reference.

ß-glucosidase activity was measured as in Strauss *et al*. (2001), with modifications: yeasts were seeded on arbutin with/without 200g/L glucose-fructose (1:1). No-arbutin plates were used for control. The control strains were L404 (negative), and Hu03 and Mp03 (positive).

Killer activity was determined as in Regodón *et al*. (1997), seeding the yeasts over a layer of Ba11 cells (Lallemand). The ICV-k1 and EC1118 strains were used as positive controls; the ICV-D254 and RC212 strains were used as negative controls. The killer activity of the Cz and NDA21 strains versus each other was evaluated by seeding each strain over a layer of the other.

Fermentative vigour was measured according to Caridi *et al*. (2002) in 100 mL of sterile must obtained by diluting concentrated must down to 20°Brix and adjusting the pH to 3.2. Fermentative vigour (weight loss due to CO_2 production; average of two replicates) was measured after two and seven days at 25°C. L404-inoculated, NDA21-inoculated and non-inoculated must were used for reference. After 12 days the musts were filtered and oenochemical analyses were performed.

SO_2 tolerance (Caridi *et al*., 2002) and fermentative power (Zambonelli *et al*., 2000) were determined in the same kind of must. For SO_2 tolerance, musts were supplemented with 200 mg/L potassium metabisulphite. To measure fermentative power, the musts were supplemented with glucose up to 300g/L sugars. Fermentative power was measured every day until the measured weight loss was less than 0.01 g/L. Measurements were performed in duplicate and average values were determined.

Fermentative activity of Cz strains in 250 ml fermentations

Nero d'Avola rosé must sample (100 mg/L potassium metabisulphite; filter sterilised) aliquots were inoculated with each one of the Cz strains or the NDA21 strain alone (pure fermentations (P): $2 \times 10^6 \pm 0.2 \times 10^6$ cfu/mL); or with each of the three Cz strains and the NDA21 strain (co-inoculations (CI): Cz strains, $1.5 \times 10^6 \pm 0.2 \times 10^6$ cfu/mL; NDA21, $0.5 \times 10^6 \pm 0.2 \times 10^6$ cfu/mL); or with each Cz strains and, after five days, with NDA21 (sequential inoculations (Seq): both strains $2 \times 10^6 \pm 0.2 \times 10^6$ cfu/mL; at the time of the NDA21 strain inoculation, the Cz strains were at $61 \times 10^6 \pm 3.5 \times 10^6$ cfu/mL). The yeasts came from monocultures in the same kind of must. Fermentations were conducted at 28°C; every day, microbiological analyses were performed (Cavazza & Poznanski, 1998) and the methods described by Di Maio *et al*. (2011a, 2011b) were utilised to distinguish morphologically between the *Saccharomyces* and *Candida* yeasts. Fermentations took 15 to 23 days. Sc fermentations (pure and mixed) were considered completed when the level of residual glucose was less than 1 g/L (as assessed by Keto-Diabur-Test® 5000, Roche). Since such criterion could not be used for Cz pure fermentations, Cz strains were allowed to ferment until (and beyond) the plateau of glucose consumption.

Chemical parameters, anthocyanins and colorimetric determinations

Alcohol, reducing sugars, total acidity, colour intensity (the sum of the absorbencies at wavelengths of 420, 520 and 620 nm measured over a 1 cm path length) and hue (or tint: the ratio of the 420 nm and 520 nm absorbencies measured over a 1 cm path length) were determined according to EEC-2676 (1990). Glucose and glucose+fructose, glycerol, acetic acid, malic acid, lactic acid, citric acid and tartaric acid were determined using an Enotech Steroglass apparatus (code SQRQ053586, Steroglass, Italy). Fructose was determined by subtracting glucose from glucose+fructose. Yeast available nitrogen (YAN) was determined according to Gump *et al*. (2002). Total polyphenols (TPFs), total anthocyanins and total flavonoids (in ppm) were determined according to Di Stefano *et al*. (1989). All measurements were performed in duplicate.

RESULTS

Characterisation of the *Candida zemplinina* (Cz) isolates

Several surveys have so far been performed on the yeast populations of Sicilian cultivars, none of which highlighted the presence of *C. zemplinina* (Castelli, 1954; Balloni & Filpi, 1979; Favaloro *et al*., 1984; Pinzauti *et al*., 2004; Romancino *et al*., 2008; Capece *et al*., 2010 and references therein). In this work we report for the first time on the presence of *Candida zemplinina* strains in Sicilian grapes and musts and we provide an oenological characterisation of some representative isolates. We have conducted our analysis by considering 59 *Candida* isolates of the non-*Saccharomyces* IRVV collection. These were initially identified as

representative of *Candida stellata* (Romancino *et al*., 2008), based on microbiological (Cavazza & Poznanski, 1998) and molecular analyses (Esteve-Zarzoso *et al*., 1999; Granchi *et al*., 1999). As new studies showed the prevalence of the *C. zemplinina* species in wine musts (Csoma & Sipiczki, 2008), we reanalysed our isolates and were able to establish that all of them belonged to the *C. zemplinina* species. Sequencing of the D1/D2 domain of one of the isolates (Cz3) further supported this notion (not shown).

We tested our Cz isolates for several oenological characteristics (see Methods). The results of these plate assays highlighted their oenological potential, showing behaviours comparable to those of the reference commercial strains. In our tests, no isolate had an H_2S production level higher than that of the references: 95% of the isolates produced medium-high levels; 5% produced medium levels. As for acetic acid, a greater variability was observed: 8.4% produced very low levels; 22.4% produced low levels; 6.7% produced medium-low levels; 42.3% produced medium-high levels; 6.7% produced high levels and 13.5% produced very high levels. Some β-glucosidase activity was observed (16.2% of the isolates had medium activity; 75% had medium-low activity; 6.7% had no activity), which was completely inhibited at sugar concentrations comparable to those of the musts (200 mg/L). No killer activity was observed, as in Comitini *et al*. (2011).

The mitochondrial DNA of each isolate was analysed by mt-DNA RFLP analysis and the isolates were found to belong to 14 polymorphism groups (Fig. 1). One isolate per group was then chosen to ferment 100 ml sterile white must aliquots and assessed for the 12 oenological parameters shown in Table 1, in comparison with two commercial *Saccharomyces cerevisiae* (Sc) strains (L404 and NDA21). Not-inoculated must was used as a negative control ("Blank"). After two days, all the Cz strains had a fermentation vigour at best equal to 50% of that of the commercial Sc yeast strains: among all strains, the Cz3 was the one with the best SO_2 tolerance value. At seven days, the fermentation vigour and the tolerance to SO_2 of all the Cz strains increased, although they never reached the level of the references. At 30 days, almost all the Cz strains had fermentative power values between 8 and 11 grams of CO_2/100 mL, much lower than the Sc strains: Cz3 had the highest fermentative power among the *Candida* isolates.

Sugar consumption was lower in all the Cz strains compared to the Sc strains. At the end of the fermentation, the Sc strains had utilised almost all the sugars available (about 216 g/L, see "Blank"). In the Cz fermentations, between 50 and 100 g/L of the sugars were left (Cz3 and Cz37 having the lowest amount), in general agreement with Magyar and Tóth (2011). Acetic acid levels of all fermentations (except Cz1) were within the range defined by the two commercial Sc strains. All Cz yeasts were able to reduce malic acid at least to levels comparable to those of the commercial strains (most strains achieving an even greater reduction). Lactic acid levels were minimal and similar in all the fermentations analysed. Citric acid content increased sensitively at the end in all the Cz fermentations (a smaller increase was observed in Sc fermentations). Tartaric acid levels remained close to the blank in all fermentations.

Although the differences in glycerol between the Cz and the Sc fermentations were small, it should be noted that these levels were obtained with a smaller sugar consumption in the Cz fermentations compared to the Sc ones. These results would give us hope that an even higher glycerol level could be obtained in mixed Cz-Sc fermentations, improving wines in an important positive aspect. Of all the strains, Cz3, Cz12 and Cz26 were those producing on average slightly higher amounts of glycerol. No Cz strain showed killer activity towards the Sc strain NDA21 in the tests performed on plates (not shown).

Based on these results, three Cz strains were selected for mixed fermentations trials with the Sc strain NDA21: Cz3, Cz12 and Cz26 (Table 1).

Mixed fermentations in 250 ml of must

In what follows we present the results of single fermentation experiments conducted in 250 ml of Nero d'Avola must (rosé). Further experiments were also performed, which confirmed the results reported here (Di Maio *et al*., in preparation).

In all the P fermentations, growth of the NDA21 and the Cz yeast strains reached a plateau before the end of the second day (close to 100 x 10^6 cfu/mL) and maintained it for the next 10 or five days respectively (not shown).

In all CI fermentations, the growth of NDA21 quickly overwhelmed that of the Cz strains, which declined sharply. This agrees with what was reported by Comitini *et al*. (2011). The NDA21 growth plateau (close to 100 x 10^6 cfu/mL) was reached in two to three days and was maintained until the 11th to 12th day. Even after that, the concentration of the NDA21 strain was maintained at conspicuous levels (between 5 and 10 x 10^6 cfu/mL). An example of this trend is shown in Fig. 2A.

In the Seq inoculations, the growth of the Cz strains inoculated at day 0 reached a plateau (100 x 10^6 cfu/mL) after one to two days. Considerable growth levels were observed even on the 8th or 9th day (three to four days after inoculation of the NDA21 strain). Growth of the NDA21 strain reached a plateau four to five days after its inoculation, and remained stable throughout. This is illustrated in Fig. 2B and indicates that the presence of the Cz yeast strains did not interfere with the growth of NDA21.

The contribution of the Cz strains to the variation of the chemical parameters of the must can be appreciated by looking at the results in Table 2. In the CI fermentations, the contribution of the Cz strains was minimal (compared to what was obtained by the inoculation of NDA21 alone: F250-1 P- NDA21). This is consistent with the result in Fig. 2A.

On the other hand, the contribution of the Cz strains became quite evident in the Seq fermentations. All fermentations where the NDA21 strain was present had a markedly reduced level of sugars left, while the amount of sugars left in the P-Cz fermentations (F250-2, F250-3 and F250-4) was considerable; therefore the presence of the Cz strains did not affect the ability of the NDA21 strain to metabolise sugars in mixed fermentations. Acetic acid increased in all the fermentations. The Cz strains maintained lower levels in the P fermentation; however, these increased

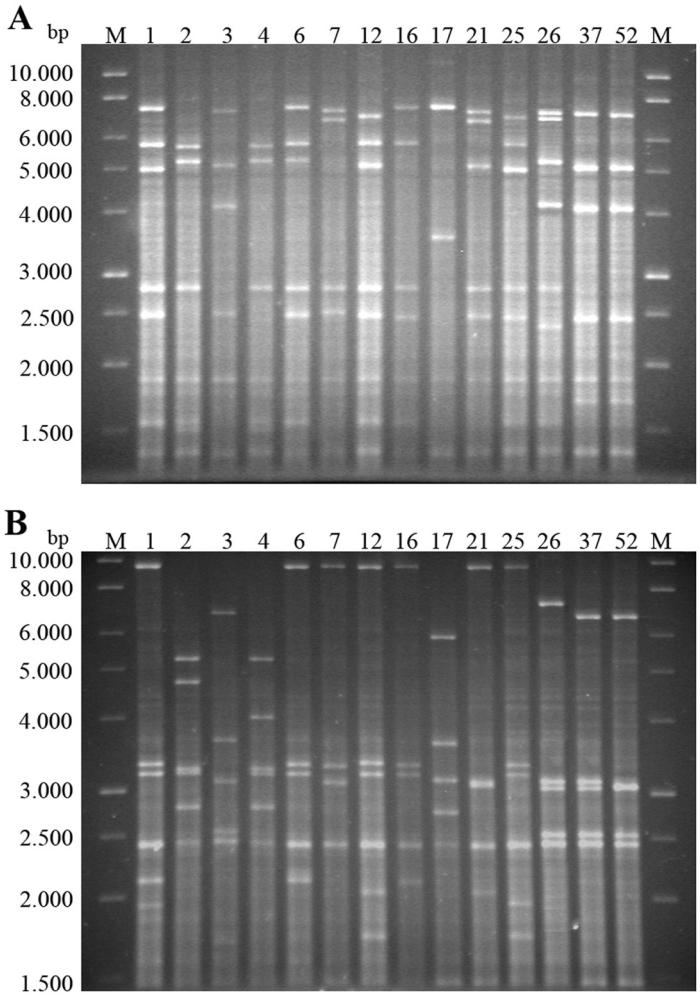

FIGURE 1
Mt-RFLP pattern analyses: one Cz representative isolate (indicated with its numeral) per each polymorphism group (strain) is shown. M: Molecular marker, A: *Hpa*II pattern, B: *Hae*III pattern

in all the mixed fermentations. Note that the acetic acid values found here relate to the high surface/volume ratio favouring oxidation: fermentations conducted in larger volumes produced lower acetic acid levels (Di Maio *et al*., in preparation).

The Cz strains favoured the synthesis of citric acid: this was evident in the P fermentations; and higher levels of citric acid were found in the Seq fermentations compared to the CI fermentations. YAN levels (in the NDA21 P and the Seq fermentations) indicated that the amount of nitrogen was enough to sustain the growth of the yeast cells. Total polyphenols (TPFs) were lower in all wines compared to the must. However, Cz (P and Seq) wines maintained a higher TPF content than NDA21 and CI wines; the Cz3 wines had the highest TPF content. Anthocyanins decreased in all fermentations. The NDA21 wines had the lowest amount of these compounds, while the presence of the Cz strains (in the P or Seq fermentations) helped reduce this loss. The Cz3/NDA21 Seq fermentation showed the highest level of anthocyanins among the mixed fermentation wines.

The fructophilic character of the Cz isolates was assessed by monitoring the level of glucose and fructose consumption. In those fermentations in which the contribution of the *Candida* yeasts could be assessed (the Cz (P) and the Cz (Seq) fermentations before the inoculation of NDA21), the preference for fructose over glucose appeared evident: in all (P) fermentations, fructose consumption was six to 16 times higher than glucose consumption; in the (Seq) fermentations, the former was two to three times higher than the latter.

Interestingly, the contribution of the Cz yeasts became most evident (in the Seq-fermentations) in the observed increases in glycerol content and decreases in ethanol levels. Following our sequential inoculation protocols, glycerol levels comparable to (or even higher than) those of the Cz (P) fermentations were obtained. Compared to the NDA21 P fermentation, these increases were on average 3 g/L.

DISCUSSION

We have reported for the first time on the presence of *C. zemplinina* strains in Sicilian grapes and musts. Furthermore, we have presented an oenological characterisation of these strains and we have shown that at least one of them is of potential interest for the development of mixed fermentation protocols with *S. cerevisiae* strains.

As we reclassified all the *Candida* isolates of the Sicilian must collection (IRVV non-*Saccharomyces* collection) as *C. zemplinina* representatives, our results speak for the ecological prevalence of this species in Sicily and are in

TABLE 1

Oenological parameters measured in 100 mL fermentations for the 14 *C. zemplinina* isolates indicated (belonging to 14 strains). Average values from two fermentation experiments are indicated (with standard errors in parenthesis).

Yeast Strain	Fermentative Vigour[1] (2 days)	SO_2 Tolerance[1] (2 days)	Fermentative Vigour[1] (7 days)	SO_2 Tolerance[1] (7 days)	Fermentative Power[1] (30 day)	Reducing Sugars[2] (g/L)	Acetic Acid[2] (g/L)	Malic Acid[2] (g/L)	Lactic Acid[2] (g/L)	Citric Acid[2] (g/L)	Tartaric Acid[2] (g/L)	Glycerol (g/L)[2]
Cz1	1.68 (0.16)	1.10 (0.06)	6.30 (0.82)	5.74 (0.42)	8.76 (2.76)	84.17 (9.67)	0.27 (0.04)	1.20 (0.07)	0.02 (0.00)	1.33 (0.17)	1.22 (0.02)	8.14 (0.88)
Cz2	1.72 (0.12)	0.94 (0.06)	7.14 (0.34)	5.82 (0.30)	10.32 (0.16)	67.26 (4.26)	0.41 (0.02)	1.06 (0.06)	0.05 (0.00)	1.30 (0.15)	1.19 (0.00)	9.43 (0.17)
Cz3	1.90 (0.14)	1.28 (0.04)	7.52 (0.52)	6.16 (0.52)	11.72 (0.04)	59.88 (3.72)	0.46 (0.00)	1.12 (0.02)	0.05 (0.04)	1.25 (0.05)	1.23 (0.02)	10.21 (0.25)
Cz4	1.46 (0.10)	0.86 (0.06)	6.64 (0.48)	4.52 (0.24)	9.40 (0.32)	72.12 (2.64)	0.51 (0.00)	1.10 (0.02)	0.07 (0.05)	1.23 (0.07)	1.25 (0.01)	9.45 (0.00)
Cz6	1.78 (0.08)	1.10 (0.02)	7.02 (0.62)	5.38 (0.18)	10.26 (0.02)	73.08 (5.16)	0.41 (0.01)	1.07 (0.09)	0.01 (0.00)	1.25 (0.15)	1.28 (0.01)	9.35 (0.45)
Cz7	1.62 (0.06)	1.12 (0.04)	6.90 (0.06)	6.04 (0.08)	10.46 (0.30)	85.62 (13.50)	0.42 (0.00)	1.11 (0.09)	0.05 (0.00)	1.25 (0.05)	1.26 (0.03)	9.23 (0.15)
Cz12	2.00 (0.08)	0.92 (0.12)	7.94 (0.06)	5.56 (0.72)	11.24 (1.08)	63.06 (5.70)	0.50 (0.04)	1.04 (0.01)	0.06 (0.00)	1.00 (0.05)	1.26 (0.04)	10.12 (0.11)
Cz16	1.68 (0.08)	0.82 (0.26)	7.08 (0.12)	6.08 (0.24)	10.36 (0.32)	70.68 (0.60)	0.48 (0.03)	1.12 (0.07)	0.07 (0.01)	1.33 (0.05)	1.26 (0.02)	9.62 (0.14)
Cz17	1.80 (0.04)	1.12 (0.00)	7.32 (0.04)	6.08 (0.12)	10.22 (0.06)	71.88 (1.44)	0.43 (0.01)	1.18 (0.00)	0.08 (0.00)	1.20 (0.02)	1.26 (0.02)	9.48 (0.14)
Cz21	2.04 (0.04)	0.88 (0.08)	7.84 (0.08)	5.22 (0.78)	10.70 (0.26)	67.98 (0.84)	0.52 (0.00)	1.20 (0.02)	0.07 (0.00)	1.15 (0.02)	1.26 (0.01)	10.04 (0.23)
Cz25	1.53 (0.10)	1.08 (0.06)	6.81 (0.30)	5.80 (0.33)	11.14 (0.40)	92.90 (5.40)	0.43 (0.00)	1.27 (0.01)	0.04 (0.00)	1.25 (0.02)	1.23 (0.00)	9.79 (0.15)
Cz26	2.02 (0.14)	1.00 (0.04)	7.90 (0.18)	4.90 (0.38)	9.54 (0.46)	70.14 (1.02)	0.52 (0.00)	1.21 (0.02)	0.07 (0.00)	1.25 (0.10)	1.27 (0.00)	10.46 (0.10)
Cz37	1.82 (0.22)	1.12 (0.08)	7.12 (0.36)	5.24 (0.44)	10.38 (0.02)	54.00 (14.16)	0.44 (0.03)	1.13 (0.02)	0.03 (0.00)	1.08 (0.07)	1.33 (0.00)	9.52 (0.35)
Cz52	1.72 (0.16)	1.00 (0.04)	7.28 (0.56)	4.72 (0.56)	11.04 (0.32)	64.56 (9.12)	0.39 (0.00)	1.12 (0.16)	0.04 (0.00)	1.25 (0.10)	1.33 (0.05)	9.79 (0.60)
L404	4.46 (0.26)	4.70 (0.02)	10.16 (0.84)	10.70 (0.14)	17.02 (0.18)	0.70 (10.1)	0.37 (0.01)	1.27 (0.14)	0.09 (0.00)	0.60 (0.05)	1.37 (0.00)	9.19 (0.20)
NDA21	4.08 (0.12)	3.54 (0.14)	10.46 (0.10)	9.86 (0.14)	19.54 (0.38)	1.48 (0.6)	0.66 (0.03)	1.23 (0.07)	0.06 (0.01)	0.45 (0.02)	1.35 (0.02)	8.82 (0.14)
Blank	0.00 (0.00)	0.02 (0.02)	0.00 (0.00)	0.02 (0.02)	0.12 (0.04)	216.45 (0.42)	0.07 (0.02)	1.54 (0.00)	0.06 (0.04)	0.35 (0.05)	1.20 (0.01)	0.83 (0.30)

[1]g CO_2/100 mL

[2]Measured at 12 days

TABLE 2

Oenological parameters measured in the pre-fermentation must ("Blank"; pH 3.11) and in 250 mL fermentations. F1–F4, pure fermentations (P). F5–F10, mixed fermentations. F5–F7, co-inoculated fermentations (CI). F8–F10, sequential (five days delayed) fermentations (Seq). Starter yeasts are indicated for each fermentation. TPF, total polyphenols. YAN, yeast available nitrogen.

Sample	Reducing Sugars (g/L)	Alcohol %	Glycerol (g/L)	Total Acidity (g/L)	Acetic Acid (g/L)	Malic Acid (g/L)	Lactic Acid (g/L)	Citric Acid (g/L)	TPF (mg/L)	Anthocyanins (mg/L)	Colour Intensity 620+520+420 nm	Hue 420/520 nm	YAN (mg/L)
Blank	187	-	-	5.80	0.05	1.15	0.00	0.21	250	12.20	1.670	1.170	221
F250-1 P- NDA21	0.20	13.27	5.30	6.90	0.78	0.93	0.02	0.36	144.0	2.82	0.268	1.110	69
F250-2 P-Cz3	67.76	7.25	7.97	6.80	0.28	1.12	0.02	0.95	192.0	4.13	0.751	1.230	132
F250-3 P-Cz12	53.10	9.28	7.39	6.80	0.56	0.86	0.02	0.58	175.0	4.22	0.749	1.270	126
F250-4 P-Cz26	64.00	8.61	7.22	6.60	0.55	0.91	0.02	0.57	174.0	3.84	0.622	1.250	212
F250-5 CI-Cz3/NDA21	1.44	13.17	5.83	6.90	0.93	0.93	0.03	0.38	151.0	3.16	0.356	1.130	81

TABLE 2 CONTINUED

Sample	Reducing Sugars (g/L)	Alcohol %	Glycerol (g/L)	Total Acidity (g/L)	Acetic Acid (g/L)	Malic Acid (g/L)	Lactic Acid (g/L)	Citric Acid (g/L)	TPF (mg/L)	Anthocyanins (mg/L)	Colour Intensity 620+520+420 nm	Hue 420/520 nm	YAN (mg/L)
F250-6 CI-Cz12/NDA21	0.27	13.26	5.98	6.60	0.86	0.93	0.02	0.38	145.0	2.95	0.325	1.120	69
F250-7 CI-Cz26/NDA21	0.28	13.45	5.80	6.80	0.90	0.98	0.02	0.38	146.0	3.16	0.370	1.120	84
F250-8 Seq-Cz3/NDA21	0.92	12.95	8.59	7.10	0.76	0.96	0.02	0.49	172.0	3.50	0.750	1.210	64
F250-9 Seq-Cz12/NDA21	1.15	13.18	7.94	6.80	0.85	0.96	0.02	0.51	166.0	3.38	0.607	1.230	75
F250-10 Seq-Cz26/NDA21	0.72	13.03	7.86	6.80	0.91	0.97	0.00	0.48	162.0	3.16	0.635	1.230	73

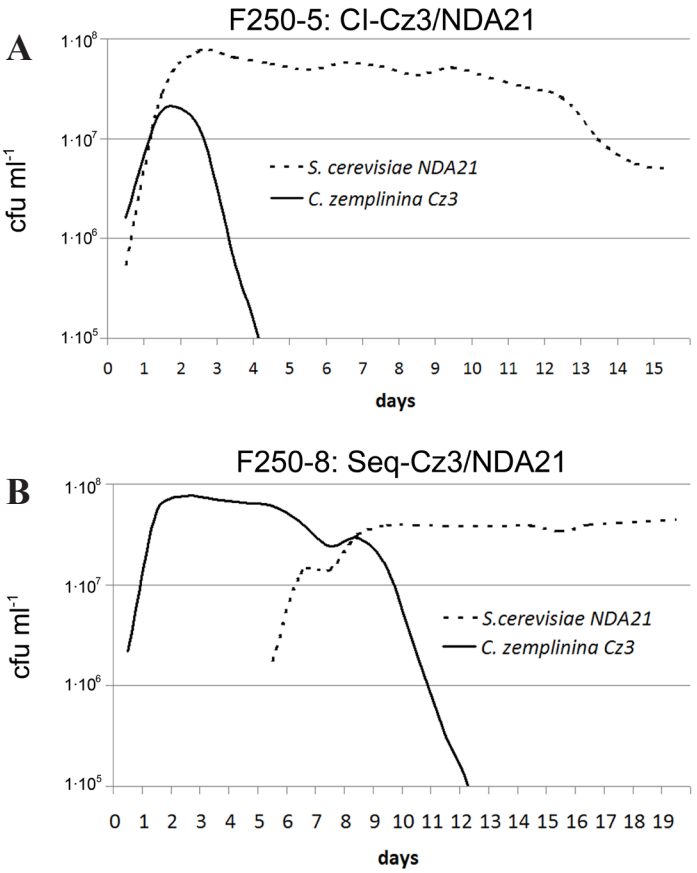

FIGURE 2
A: Growth curve for the mixed co-inoculated fermentation F250-5 (Cz3 and NDA21),
B: Growth curve for the mixed sequential fermentation F250-8 (Cz3 and NDA21).

agreement with those authors (e.g. Csoma & Sipiczki, 2008) arguing that *C. zemplinina* (not *C. stellata*) is the most abundant *Candida* species on grapes and musts.

We showed that care must be taken to ensure an appropriate proliferation of both yeast starters (Cz and Sc) in a mixed fermentation. In fact, in our CI experiments, the fermentation process was carried out by the Sc strain with essentially no contribution from the Cz strains. This is in agreement with the previous results of Comitini *et al*. (2011), which showed (in CI inoculations) two distinct fermentation phases (one carried out by *Candida,* the other by *Saccharomyces*) with a Cz:Sc inoculation ratio of 10 000:1. Therefore, an early, robust proliferation of *Saccharomyces* hinders proliferation of *Candida*.

On the other hand, it could be interesting to investigate how the early proliferation of *Candida* affects the proliferation of *Saccharomyces*. The levels of citric acid found at the end of all Cz (P and Seq) fermentations were higher than those found in the NDA21-dominated fermentations, and it has been reported that citric acid can favour *Saccharomyces* growth (Bardi, 2005). However, other authors have shown that, depending on pH, citric acid might hinder *Saccharomyces cerevisiae* growth (Nielsen & Arneborg, 2007). Further studies in which the parameters of the mixed fermentation are carefully taken into account

might illuminate this issue and reveal interesting details on the yeast population dynamics occurring within the must niche.

The mixed fermentation protocol we have presented here obtains a very similar result to that already obtained by Comitini *et al*. (2011): an early *Candida* proliferation phase is followed by a later *Saccharomyces* one. By comparing the results of P, CI and Seq inoculations, we were able to determine the contribution made by our *Candida* strains. The decrease in ethanol content we obtained was comparable to that obtained by Comitini *et al*. (2011). However, in the Seq inoculation we had an increase in glycerol content about twice that reported by these authors. Therefore the *Candida zemplinina* strains we selected might be better glycerol producers, better suited for utilisation in mixed fermentation. Careful comparative studies will be needed to assess such a proposition, given the number of differences existing between these two studies (musts, starter strains utilised, inoculation protocols).

Finally, although both protocols (our Seq protocol and the 1:10000 CI inoculation of Comitini *et al*., 2011) obtain a similar proliferation result in sterile musts, things might work differently in a winery (where these results eventually must be applied). Several reports have shown the presence of "resident" yeasts in wineries (Ciani *et al*., 2004; Santamaría *et al*., 2005; Mercado *et al*., 2007); our preliminary results show that these hinder the proliferation of the yeast starters if these latter are inoculated at low concentrations (such as those proposed by Comitini *et al*. (2011). On the other hand, our Seq inoculation protocol allows a high concentration of both starters, possibly helping to overcome this problem. We are currently testing this proposition in the winery.

In conclusion, we have presented the oenological characterisation of the *Candida zemplinina* yeast strain Cz3, isolated in Sicily. The analysis of its technological and fermentation properties have shown that this is a promising oenological starter for mixed fermentation protocols with a potential for industrial applications.

CONCLUSIONS

We have shown the presence of *Candida zemplinina* yeasts in Sicilian grapes and musts. All *Candida* isolates of the non-*Saccharomyces* yeasts in the IRVV collection were representatives of the *C. zemplinina* specie. Furthermore, we have selected a *C. zemplinina* strain endowed with very promising features for future industrial applications.

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