Use of *Candida zemplinina* Sicilian yeast strains to improve red wines quality.

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ABSTRACT

During the 2010-2012 vintages *Candida zemplinina* (Cz) yeast strains isolated from Sicilian grapes, were used in a winery to ferment red musts. Several aliquots (from a single mass of must) were fermented separately. In 2010, aliquots were also fermented with commercial *S. cerevisiae* yeast strains; in addition to that, in 2011 and 2012 aliquots were left to spontaneous fermentation. Different Cz strains were tested and in all cases, we always obtained a fermentation in two phases: one dominated by the *C. zemplinina* yeasts, the other by the *Saccharomyces* yeasts coming from the grapes or the winery.
The wines produced using our Cz strains always had more glycerol (at least 50%) than those made using the commercial *S. cerevisiae* strain or obtained by spontaneous fermentation. Sometimes a lower alcohol level was also obtained. Since glycerol might contribute to the sensory properties of the wines, the use of *C. zemplinina* strains in mixed fermentations, can lead to a sensitive amelioration of red wines.

RIASSUNTO

Durante le vendemmie del 2010-2012 abbiamo usato in cantina ceppi di lievito *Candida zemplinina* (Cz) precedentemente isolati da uve siciliane. In ogni esperimento un'unica massa di mosto di uve rosse è stata suddivisa in serbatoi differenti, per l'inoculo dei diversi ceppi di lievito. Nel 2010 si è anche proceduto alla fermentazione con ceppi commerciali di *S. cerevisiae*; in più nel 2011 e 2012 si è proceduto alla fermentazione spontanea dei mosti. Sono stati testati ceppi Cz differenti, ottenendo sempre una fermentazione in due fasi: la prima dominata da *C. zemplinina* e la seconda dominata da lieviti *Saccharomyces*, provenienti dalle uve e/o dalla cantina. I vini fermentati dai ceppi Cz avevano sempre più glicerolo (circa il 50%) degli altri vini. A volte l'aumento di glicerolo era accompagnato da una diminuzione del grado alcolico. Considerato che il glicerolo può dare un contributo significativo alla qualità del vino, l'impiego di ceppi di *C. zemplinina* per la realizzazione di fermentazioni miste può rappresentare un modo per produrre una tipologia migliorativa di vini rossi.

INTRODUCTION

In the recent years non-*Saccharomyces* yeast species have gained the attention of researchers and winemakers, because they can help obtain wines of higher complexity (reviewed in (Suárez-Lepe and Morata, 2012)). These are used together with *S. cerevisiae* yeast strains to obtain mixed fermentation wines. Among the species considered *Candida zemplinina* is one of the most abundant in musts (Csoma and Sipiczki, 2008); laboratory studies showed that through its utilization wines with increased glycerol levels and decreased alcohol content can be obtained (Ciani and Ferraro, 1996; Di Maio et al., 2012a; Magyar and Tóth, 2011). However very few studies exist demonstrating the transferability of these laboratory studies into a non sterile winery environment.

In addition to that, an interest has developed for products which can relate to specific regions of the world, something that might be obtained also by exploiting the indigenous yeast flora.
We have previously shown the presence of *C. zemplinina* (Cz) yeasts in the Sicilian grapes and musts (Di Maio et al., 2012a). In this work we report on the winery utilization of three different Cz strains during three different vintages (Cz3 in 2010 and 2011; Cz 12 and 26 in 2012). Eventually we developed a mixed fermentation protocol in which a first phase dominated by the Cz yeast strain inoculated, was followed by a second phase driven by indigenous *Saccharomyces* yeasts, resident in the winery or present on the grapes.

MATERIALS AND METHODS

Yeast strains

The *Candida zemplinina* Cz3, Cz12 and Cz26 strains belongs to the non-*Sacharomyces* IRVO collection (Romancino et al., 2008). The NDA21 (Di Maio et al., 2012b) and the AR06 strains are distributed by Biospringer. Cz strains were grown on Malt Agar and kept at 4^{o}C.

Wine making

Vinifications were performed between August and September of 2010, 2011 and 2012. Four aliquots of Merlot grapes (80 liters each) were used in 2010; three aliquots of 80 liters each, per each cultivar (Merlot, Nero d'Avola and Frappato) were used in 2011; four aliquots (80 liters each) of Merlot grapes were used in 2012.
Grapes were delivered to the IRVO "G. Dalmasso" winery in Marsala (TP-Italy), de-stemmed and crushed. Musts were supplemented with 100 mgl^{-1} potassium metabisulfite and microbiological and chemical analyses were performed.
In 2010 aliquots were inoculated with NDA21, AR06, Cz3+NDA21 and Cz3+AR06. In the mixed fermentations Cz3 was inoculated the first day and the *S. cerevisiae* yeast cells were inoculated 1 day after.
In 2011 aliquots were inoculated with the NDA21 yeast strain cells, or with Cz3 yeast strain cells, or were left to spontaneous fermentation. In 2012 aliquots were inoculated with the NDA21 yeast strain cells, or with Cz12 yeast strain cells, or with the Cz26 yeast strain cells, or were left to spontaneous fermentation.
Cz3 liquid cultures were prepared for inoculation, by pre-multiplication in white must (reconstituted from concentrated must; 16 Brix; pH 3.2). Cells were collected, washed and inoculated at approximately $40 \cdot 10^{6}$ cfu ml^{-1}.
The NDA21 and AR06 yeast strains were inoculated as active dry yeast (following manufacturer's instructions) at a final concentration of 30g/hL (single starter fermentations) or 500 cfu ml^{-1} (only in the 2010 mixed fermentations).
Cz12 was inoculated at approx $40x10^{6}$ CFU /mL; Cz 26 at approx $40x10^{6}$ cfu/mL.
Crushed grapes were fermented at 25°C. Three punching down of the cap per day were performed, carefully cleaning the equipment between samples. Daily microbiological analyses were performed as well as controls to assess the amount of sugar and the temperature

Single and mixed fermentations took 11 days in 2010; 14, 10 and 8 days in 2011 (Merlot, Nero d'Avola and Frappato respectively); 11 days in 2012. During the fermentations and at the end wine lees samples were taken and criopreserved for downstream molecular analyses.
After racking, all wines were inoculated with *Oenococcus oeni* (Viniflora Oenos, Chr Hansen) following manufacturer instructions. Samples were taken before and after malolactic fermentation for downstream chemical and microbiological analyses. At the end of malolactic fermentation, samples were supplemented with 60 mg l^{-1} of potassium metabisulfite. After further addition of 60 mg l^{-1} of potassium metabisulfite, wines were bottled in December of the same year. Chemical and microbiological controls were also performed at wine bottling.

Microbiological analyses

Everyday, fermenting must samples were diluted in sterile peptone water (0,1% Bacteriological Peptone, Oxoid) and plated in duplicate on WL Nutrient Agar (Oxoid), Lysine Agar (Sigma) and (only for mixed fermentations) WL Differential (Cavazza & Poznanski, 1998; Di Maio et al, 2011).
Further microbiological analyses were performed on WL Nutrient Agar, Agar-Lysine and Tomato Juice Agar (Fluka) before bottling (Cavazza & Poznanski, 1998).

Molecular analyses

Mt-DNA RFLP analyses were performed on *Saccharomyces* yeasts in wine lees samples collected after the first tracking; mt-DNA RFLP analyses were performed on must samples from the mixed fermentations taken at 4th day, when *Candida* proliferation was at plateau. Mt-RFLP analyses were performed following the protocols of (Querol et al., 1992; Ribéreau-Gayon et al., 1998). The first protocol was used for all *Candida-Saccharomyces* mixed fermentation samples (25 and 50 colonies were analyzed respectively); the second for the single fermentations with just the commercial *Saccharomyces* strains. In this latter case the lees pre-cultures were prepared in YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose) supplemented with tetracycline (30 ppm), to prevent bacterial growth. *Saccharomyces* and *Candida* DNA was digested with the *RsaI* and *HpaII* endonucleases (NEB) respectively (Pramateftaki et al., 2000). Restriction fragments were separated by electrophoresis on 0.7% TBE Agarose gels. Gels were stained at the end of the run and photographed using a Gel Doc 2000 (Biorad) apparatus provided with the Quantity One (Biorad) analysis software.

Chemical parameters

For the determination of wines' alcohol content, the OIV official method (OIV, 2006) was followed. Glucose, fructose, glycerol, acetic acid, malic acid, lactic acid, citric acid, tartaric acid concentrations were determined using a Enotech Steroglass apparatus (code SQRQ053586; Steroglass-Italy), by monitoring the changes in absorbance, induced by chemical (tartaric acid) or enzymatic reactions (for all the other parameters).
Chemical parameters were measured in duplicate in 2010 and in triplicate in 2011; in duplicate in 2012.

RESULTS AND DISCUSSION

Microbiological and molecular aspects of the 2010, 2011 and 2012 fermentations.

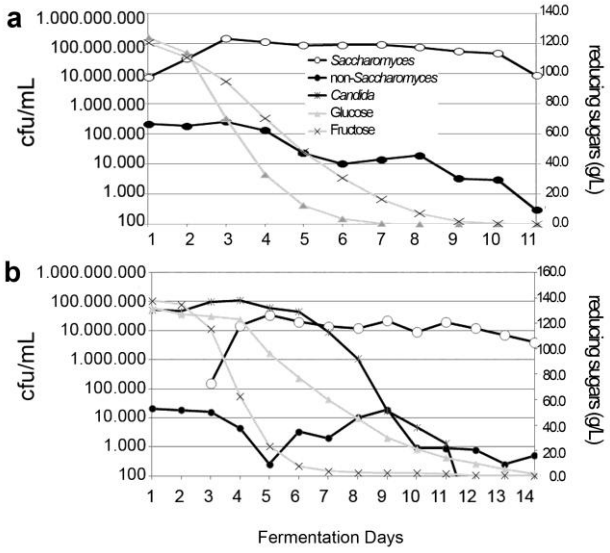
During the course of each fermentation the evolution of the microbial populations present in the musts was monitored by way of plate assays and by molecular analyses. Although our inoculation protocols and experimental design differed from one year to the other, we

observed the same general behavior. In brief, *S. cerevisiae* single starter fermentations were performed and the wines obtained were compared with those obtained by mixed fermentation. These were always dominated by the yeast strains inoculated (NDA21 or AR06 in 2010; NDA21 in 2011 and 2012). The concentration of non-*Saccharomyces* yeasts was always kept a low levels. This is exemplified in fig 1a where the result of one NDA21 fermentation (2010, Merlot must) is shown. The RFLP analysis of the Mt-DNA confirmed the identity of the inoculated yeast strain.

In 2010 we tried to obtain mixed fermentations by inoculating Cz3 cells and (after one day) either NDA21 or AR06 cells. While the first phase of the fermentation was dominated by Cz3, the second phase was driven by *Saccharomyces* yeasts different from those we inoculated. We concluded that these were *Saccharomyces* yeasts resident of the winery or present on the grapes. Since mixed fermentations were however obtained, in 2011 we inoculated musts of three different cultivars (Merlot, Nero d'Avola and Frappato) with just Cz3 cells. To provide an additional control, aliquots from the same initial masses were also left to spontaneous fermentation. In all the Cz3 fermentations, after the initial proliferation of the *C. zemplinina* cells inoculated, a proliferation of *Saccharomyces* yeast cells was observed. Once again the growth of other-non *Saccharomyces* yeast species was kept at low levels (comparable to those seen in the NDA21 fermentations).

Molecular analyses confirmed that the Cz3 cells were the only *Candida* yeasts proliferating in the musts. These results are exemplified in fig 1b, where the evolution of the microbial population in a Merlot fermentation conducted in 2011 is shown.

Figure 1



a. Growth curves of *Saccharomyces* and non-*Saccharomyces* yeasts in a 2010 Merlot fermentation.
b. Growth curves of *Saccharomyces*, non-*Saccharomyces* and *Candida* yeasts in a 2011 Merlot fermentation.

Symbols are explained in the legend. The amount of glucose and fructose are shown by the light grey curves (values are reported on the secondary axis). This allows to appreciate the contribution of the fructophilic *C. zemplinina* yeasts to the fermentation.

In 2012 similar results were obtained. Single fermentations were always driven by the inoculated yeast strains, while in mixed fermentations a first phase driven by the Cz yeast strain inoculated was followed by a second phase dominated by indigenous *Saccharomyces* yeast strains. These results are not illustrated here, for brevity.

Chemical aspects of the 2010, 2011 and 2012 fermentations.

Some important chemical were consistently affected in the Cz fermentations, from one year to the other and among the different cultivars. In all the Cz3 fermentations the amount of glycerol was always increased (up to 50% with Cz3 and up to 50-60% more with Cz12 and Cz 26)**.** In 2010 and 2011 this was also accompanied by a decrease in the alcohol content (up to half a degree less; some decrease was also observed in 2012 when Cz12 was inoculated). Residual sugar levels were always reduced and were consistent with the definition of "dry wines" (Commission Regulation of the European Union (EC) No 753, 2002). Acetic acid levels were sometimes higher in the Cz3 wines compared to those made by the single *S. cerevisiae* starters, however they would never exceeded the levels prescribed for red wines (Commission Regulation (EC) No 606, 2009). Some of the results obtained are shown in table 1**.**

Table 1

Year	Cultivar	Starters	Alcohol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)
2010	MERLOT	NDA21	14.73	8.10	0.42
		AR06	14.82	8.20	0.20
		CZ3+NDA21	14.24	12.00	0.61
		Cz3+AR06	14.23	12.50	0.62
2011	MERLOT	Spontaneous	15.34	6.83	0.65
		NDA21	15.40	6.63	0.63
		CZ3+Sacch	14.90	11.70	0.95
	NERO D'AVOLA	Spontaneous	14.69	7.23	0.56
		NDA21	14.61	7.20	0.51
		CZ3+Sacch	14.31	11.63	0.70
	Frappato	Spontaneous	13.93	8.13	0.56
		NDA21	13.82	7.80	0.37
		CZ3+Sacch	13.43	12.46	0.76
2012	MERLOT	Spontaneous	13.46	7.25	0.38
		NDA21	13.24	7.35	0.33
		Cz12+Sacc	13.17	11.05	0.72
		Cz26+ Sacc	13.02	12.03	0.66

These results show that trough the utilization of the *C. zemplinina* yeast strains we selected, wines with a markedly higher glycerol content were obtained. This is an important result since it shows that the technological potential of our Cz strains is transferable into a winery environment.

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BIBLIOGRAPHY

Ciani, M., Ferraro, L., 1996. Enhanced Glycerol Content in Wines Made with Immobilized *Candida stellata* Cells. Appl Environ Microbiol 62, 128–132.

Commission Regulation (EC) No 606, 2009. Laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions.

Commission Regulation of the European Union (EC) No 753, 2002. Laying down certain rules for applying Council Regulation (EC) No 1493/1999 as regards the description, designation, presentation and protection of certain wine sector products.

Csoma, H., Sipiczki, M., 2008. Taxonomic reclassification of *Candida stellata* strains reveals frequent occurrence of *Candida zemplinina* in wine fermentation. FEMS Yeast Res 8, 328–336.

Di Maio, S., Genna, G., Gandolfo, V., Amore, G., Ciaccio, M., Oliva, D., 2012a. Presence of *Candida zemplinina* in Sicilian Musts and Selection of a Strain for Wine Mixed Fermentations. S. Afr. J. Enol. Vitic. 33, 80–87.

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G., Genna, G., Verzera, A., Scacco, A., Amore, G., Oliva, D., 2012b. Biodiversity of Indigenous *Saccharomyces* Populations from Old Wineries of South-Eastern Sicily (Italy): Preservation and Economic Potential. PLoS ONE 7, e30428.

Magyar, I., Tóth, 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.

Pramateftaki, P.V., Lanaridis, P., Typas, M.A., 2000. Molecular identification of wine yeast at species or strain level: a case study with strains from two vine-growing areas of Greece. Journal of Applied Microbiology 89, 236–248.

Querol, A., Barrio, E., Huerta, T., Ramòn, D., 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. Applied and Environmental Microbiology 58, 2948–2953.

Ribéreau-Gayon, P., Dubourdieu, D., Doneche, B., Lonvaud, A., 1998. Traité d'oenologie. 1. Microbiologie du vin. Vinifications. Dunod, Paris.

Romancino, D.P., Di Maio, S., Muriella, R., Oliva, D., 2008. Analysis of non-*Saccharomyces* yeast populations isolated from grape musts from Sicily (Italy). Journal of Applied Microbiology 105, 2248–54.

Suárez-Lepe, J.A., Morata, A., 2012. New trends in yeast selection for winemaking. Trends in Food Science & Technology 23, 39–50.