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OENOLOGICAL CHARACTERISATION OF INDIGENOUS STRAINS OF *S. CEREVISIAE* ISOLATED IN A BIODYNAMIC WINERY IN THE CORTONA DOC AREA

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ABSTRACT

Enhancement of Italian wines requires a full knowledge of the variables that influence winemaking, and one of the most important of these regards the features of *S. cerevisiae* strains, considering their significance in alcoholic fermentation. With this intention we performed genotypic and technological characterisation of the *S. cerevisiae* population isolated in a biodynamic winery in the Cortona DOC area. The analysis revealed a remarkable variability in terms of *S. cerevisiae* strains, despite the homogeneity of wine features, underlining the high levels of biodiversity characterising biodynamic agriculture. Some strains were found in wines of different vintages, suggesting the presence of an established microbiota in the winery. Oenological tests demonstrated that while some yeasts provided reliable oenological performance, other strains were not able to accomplish prompt and effective alcoholic fermentation, or were characterised by spoilage characteristics, such as excessive production of volatile phenols or acetic acid. In conclusion, indigenous strains of *S. cerevisiae* could be a useful instrument for performing reliable winemaking without altering the native microbiota of each oenological environment. However, characterisation of their oenological suitability, and the application of practices able to drive the evolution of microflora, must be employed to reduce the risk of wine spoilage.

KEY WORDS

Spontaneous fermentation, yeasts, microbial selection, biodiversity, biodynamic, Syrah

FINDINGS

In the last few years there has undoubtedly been growing interest among consumers regarding wines with features more closely linked to their areas of origin. This trend has led winemakers to rethink the approach to alcoholic fermentation, avoiding the use of selected yeast, and entrusting the fermentation process to microflora naturally present in the cellar and on the grapes. This approach might seem a step back in terms of the scientific and technological state of the art of oenology, but given its increasing diffusion and economic significance, it deserves attention and further scientific study. Grapes and cellar equipment are populated by a wide range of yeasts and bacteria that evolve during the production process, according to the environmental conditions and technological

choices (Capozzi et al. 2015). It is common to observe a yeast population of around 10^4 cells/g on ripe bunches and on grape musts, composed mainly of yeasts not belonging to the genus *Saccharomyces* (Barata et al. 2012). It is also widely known that an increase in ethanol content, due to alcoholic fermentation, leads to the selection of microflora with a prevalence of *Saccharomyces cerevisiae*, after accumulation of 5 - 6 % v/v ethanol in fermenting grape must. The use of selected strains of *S. cerevisiae* does not alter the evolution of wine microbiota, but accelerates it, encouraging a faster increase in the alcohol content of grape must (Guzzon et al. 2014, Ciani et al. 2016). However, if "spontaneous" fermentation is well managed from the technological and analytical point of view, it is possible to obtain wines qualitatively comparable with those obtained by inoculating active dry yeasts (Chaves-López et al. 2009). The risk of microbial spoilage in the case of alcoholic fermentation performed without the use of active dry yeast could be associated with incomplete knowledge of the specific features of the native microbiota in each specific oenological environment, and consequently in incorrect technological approaches. The study of biodiversity associated with spontaneous fermentation, and oenological characterisation of identified yeasts, contributes to increasing comprehension of the microbial dynamics of winemaking, preventing the risk of wine spoilage. Many papers have already discussed this topic (Comitini et al. 2017). In this note we report on the study of a *S. cerevisiae* population isolated during the winemaking process in a winery operating in the Cortona DOC area (southern Tuscany, Italy), following a biodynamic approach that excludes the use of active dry yeast, avoiding the risk of contamination of the microbiota native to this scenario.

The study began with the isolation of yeasts, carried out considering 8 different samples of fermenting grape must (Table 1), after degradation of 90% of initial sugar content. This approach differs from that frequently carried out in works studying the biodiversity of the oenological environment (Settanni et al. 2012). The yeasts were isolated from fermenting grape must having more than 10% ethanol, in order to identify yeast strains with the best ability to drive alcoholic fermentation, and resistance to wine limiting factors. The must was made from Syrah grapes, after gentle crushing, without sulphur dioxide and the addition of selected yeast. Yeasts were counted on WL agar medium (Oxoid, UK), according to OIV standards (OIV, 2016); determination of non-*Saccharomyces* yeast (Agar Lysine, Oxoid), lactic acid bacteria (MRS agar, Oxoid), and acetic bacteria (ACTS agar, Oxoid) was also carried out following the same protocol. The results (Table 1) confirmed the hypothesis on the basis of sampling. The difference in plate counts obtained on WL Agar (mean of $8.0\pm0.6\times10^7$ cfu/mL) and Lysine Agar (mean of $1.8\pm0.9\times10^4$ cfu/mL) revealed a large population of yeasts attributable to the *Saccharomyces* genus, with a negligible presence of other yeast genera of oenological interest. Furthermore, acetic acid bacteria were not detectable ($<5\times10^2$ cfu/mL), while a population of lactic bacteria potentially able to spoil wine (Liu 2002) was present in all samples. The significant contamination, on average $9.3\pm6.8\times10^4$ cfu/mL, of lactic bacteria during alcoholic fermentation was probably favoured by the high pH of the grape must and the absence of SO_2.

100 yeast colonies were isolated and purified on WL agar, on the basis of provenience (vineyard) and morphology. The yeasts isolated were transferred into YM broth (Oxoid), to encourage rapid growth, and after 3 days of incubation at 30 °C, total DNA was extracted and purified using the Insta Gene matrix kit (Bio-Rad). Strain typing was performed through analysis of interdelta sequences (ISA-PCR, Charpentier et al. 2009; Legras and Karst 2003), obtaining discrimination of 11 strains. Their appurtenance to the *S. cerevisiae* species was confirmed by sequencing the D1/D2

region of 26S rDNA using NL1 and NL4 primers (Kurtzman & Robnett 1998). The identities (≥ 97%) of the sequences were verified with a BlastN (Altschul et al. 1997) search against the National Centre for Biotechnology Information (NCBI) non redundant sequence database located at http://www.ncbi.nlm.nih.gov. There was no correlation between the strain and the vineyard lots from which the grapes came, with different strains widespread in the entire set of samples. This result is reasonable considering the small size of the winery, and the need to use the same oenological equipment, with the occurrence of cross-contamination. However, the biodiversity observed in the *S. cerevisiae* species was quite high, despite the uniformity of the features in the isolation source, and the small size of the vineyard (about 8 hectares, located in the same area). These observations agree with the results of previous studies carried out in wineries that operate following a biodynamic process (Morrison-Whittle et al. 2017). Pure strains, named from A1 to A11, were stored in a suitable synthetic medium (YM + 20 % of glycerol) at – 80 °C.

Physiological tests were carried out in order to evaluate the oenological performance of the 11 *S. cerevisiae* strains, and the possible presence of spoilage characteristics, such as poor fermentative activity, excessive production of acetic acid, sulphur dioxide or volatile phenols (Guzzon et al. 2014). The 3 grape musts considered had sugar content between 220 and the 240 g/L, pH between 3.24 and 3.81, YAN over 164 and 118 mg/L, and they were supplemented with 500 mg/L of *p*-cumaric acid to stimulate vinyl phenol production. Cellular growth was monitored with plate counts; the evolution of alcoholic fermentation was followed by measuring the weight loss of samples due to CO_2 production. The main chemical parameters of the wines obtained were determined with FT-IR spectroscopy using a Wine Scan (Foss) apparatus and a Crison titrator to monitor sulphur dioxide. Volatile phenols were quantified using HPLC, equipped with a colorimetric array detector as proposed by Larcher et al. (2007). From the kinetic point of view, we chose to focus our attention on 3 moments: the lag phase (2 days after yeast inoculum), the end of the exponential phase (5 days after yeast inoculum), and complete fermentation (arbitrarily established at 10 days after yeast inoculum); the progress of alcoholic fermentation was expressed as a % of the theoretical total weight loss (Figure 1A). The initial yeast inoculum was set at 10^5 cfu/mL, ensuring the prompt start of alcoholic fermentation in all tests, with a mean weight loss of 7.9±1.3% after 2 days (Data separated for grape must in table 1). After 5 days, the first differences were already highlighted. The mean weight loss was 59.1±6.5%, but 4 strains - A2, A3, A8 and A11 - showed progress in alcoholic fermentation below the mean for the population. A similar trend was observed after 10 days' fermentation for 3 strains that showed performance below the mean (96.0±3.8%). The measurement of residual sugars in the wine obtained (Table 1) showed that strains A4, A7 and A9 achieved alcoholic fermentation in the 3 tests, residing in wines with less than 5 g/L of sugars. As expected, most of the problems in achieving alcoholic fermentation were observed at the highest potential ethanol content (grape must 3), with 3 strains - A8, A10 and A11 - that were unable to completely degrade sugars in the full set of tests. This observation is particularly significant because the problem of incomplete consumption of sugars, with the consequential possibility of developing spoilage microorganisms such as *Brettanomyces* or lactic acid bacteria (Chatonnet et al. 1995, Loureiro and Malfeito-Ferreira 2003), has been more frequent in recent years, due to climate change and the resulting higher sugar content of grapes. The potential value of 15 % of alcohol established in grape must sample 3 is not unusual in the Mediterranean oenological area. It is therefore important to underline that native yeast strains, developing in the presence of specific environmental factors, are not always suitable for guaranteeing efficient alcoholic fermentation. Spontaneous fermentation must be adequately monitored with microbiological assays designed to provide rapid and reliable

information about the physiological state of the yeast population (Guzzon and Larcher 2015, OIV, 2016). On the other hand, it was possible to isolate at least 3 strains of *S. cerevisiae* endowed with good fermentative activity and high resistance to ethanol from the complex microbial population present in fermenting grape must samples. The three spoilage characteristics taken into consideration, the production of acetic acid, sulphur dioxide and volatile phenols, showed different trends (Table 1). The accumulation of acetic acid (mean of 0.3±0.1 g/L) was generally low, considering the high sugar content of grape must samples, which induces osmotic stress and accumulation of acetic acid (Bely et al. 2003, Teixeira et al. 2011). The production of 222±98 mg/L of volatile phenols, corresponding to a conversion rate of 44.5±19.5%, was comparable to that of some *S. cerevisiae* strains used as fermentation starters (Guzzon et al., 2014), and acceptable for the production of red wines (Rojas et al 2012). The accumulation of sulphur dioxide appeared to be more closely linked to the initial sugar content than to the features of each *S. cerevisiae* strain (Table 1). However, strains A1 and A2 produced the least over the full set of tests, while strains A9 and A10 accumulated the highest amount of sulphur dioxide in all wines. The level of SO_2 reached at the end of fermentation of grape must 3 (46±3 mg/L) is potentially able to lead to stuck malolactic fermentation; this aspect deserves particular consideration, because the combination of high ethanol, pH and sulphur dioxide content in wines could stimulate the development of spoilage lactic bacteria such as *Pediococcus* spp. (Bartowsky 2009).

Isolated strains were employed in the subsequent vintage to drive alcoholic fermentation, to avoid an excessive lag-phase which could cause uncontrolled proliferation of alterative microorganisms (Renouf 2015). The strains were inoculated by preparing a pied de cuve before the harvest, having a volume of 1% of the overall mass of grapes harvested. At the end of alcoholic fermentation, yeasts belonging to the *S. cerevisiae* species were isolated and characterised at strain level, according to the previously described experiments. The objective of this second set of microbiological assays was to verify the capacity of isolated strains of *S. cerevisiae* to remain active in the microbial population of the winery. Figure 1B shows the electrophoretic pattern of *S. cerevisiae* strains identified at the end of alcoholic fermentation. The presence of 8 different biotypes confirmed the wide biodiversity observed in this oenological scenario. 4 strains, of those inoculated, were identified in lanes 1, 2, 3 and 4 in Figure 1B. Some new *S. cerevisiae* strains (lanes 4, 6, 8 and 10 in Figure 1B) involved in the fermentative process were also found.

The results obtained in this work confirm that there are strains of *S. cerevisiae* of promising technological value in the spontaneous microflora characterising the winemaking process in a biodynamic winery, capable of guaranteeing efficient biotransformation and potentially high quality wines. However, it is crucial to apply a technological approach suitable for stimulating these strains within a complex microbiota that also contains spoilage microorganisms such as lactic bacteria or yeast with poor fermentative activity. In this way, strains of high oenological suitability are able to drive alcoholic fermentation without eliminating the biodiversity characteristic of each harvest

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Table 1 First section. Main features of the wines used to isolate *S. cerevisiae* strains. Second section. Results of oenological tests performed with *S. cerevisiae* strains.(Mean+-SD n:11). Grape must 1: sugars 220 g/L, pH: 3.24, YAN 164 mg/L; Grape must 2: sugars 230 g/L, pH 3.58, YAN 137 mg/L; Grape must 3: sugars 250 g/L, pH 3.81, YAN 118 mg/L. *Sugar value was calculated considering strains that accomplishers alcoholic fermentation, with a residue of sugars below the 5 g/L.

First section. Main features of the wines used to sample S. cerevisiae strains

Number of vat	Date of sampling	Density of must	Yeast	non-*Saccharomyces* yeast	Lactic acid bacteria
	(days from start of AF)		*(ufc/mL)*		
1	26/09 (+6)	995	8.20E+07	2.10E+04	3.80E+04
3	4/10 (+5)	999	8.70E+07	3.80E+04	2.60E+04
4	27/09 (+8)	996	8.90E+07	1.30E+04	1.20E+05
6	05/10 (+8)	997	8.20E+07	1.20E+04	8.90E+04
8	30/09 (+7)	993	7.40E+07	2.20E+04	1.60E+05
11	30/09 (+7)	993	7.80E+07	1.60E+04	2.10E+05
13	03/10 (+11)	992	7.40E+07	1.10E+04	8.80E+04
15	29/09 (+9)	992	7.60E+07	1.40E+04	1.30E+04

Second section. Results of oenological tests performed with the 11 S. cerevisiae strains

Grape must	Advancement of fermentation after 2/5/10 days			Ethanol	Sugars*	Volatile acidity	Malic acid	Tot. SO_2	Volatile phenols	Stuck of AF
	%			*(% vol)*	*(g/L)*			*(mg/L)*		*Strain*
M1	8.5±0.7	62.4±2.6	96.8±3.6	13.0±0.7	3.89±1.39	0.24±0.06	2.80±0.01	35±6	202±103	1, 8
M2	8.7±0.8	53.9±6.0	94.7±4.4	13.2±0.7	3.68±1.34	0.30±0.10	2.61±0.13	42±8	228±104	5,6,8,10,11
M3	6.6±0.9	56.5±8.6	94.9±5.7	14,2±0.7	3.04±1.1	0,38±0.15	2,31±0.12	46±3	236±91	2,3,5,6,8,10,11

Figure 1. A) Sugar consumption (expressed as %) of a population 11 *S. cerevisiae* strains in tests performed in 3 different grape musts with increasingly harsh conditions. M1: sugars 220 g/L, pH: 3.24, YAN 164 mg/L; M2: sugars 230 g/L, pH 3.58, YAN 137 mg/L; M3: sugars 250 g/L, pH 3.81, YAN 118 mg/L. B) Electrophoresis patterns generated by the ISA-PCR products of *S. cerevisiae* isolates at the end of fermentation performed during the 2012 harvest. We observed the presence of some strains isolated and characterised through this work (lanes 1, 2, 3 and 4) and new indigenous strains (lanes 4, 6, 8 and 10).