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

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ABSTRACT

The valorization of Italian's wines passes through a complete knowledge of variables that influence the winemaking and, among them, the features of *S. cerevisiae* strains is one of the most important considering their relevance in the alcoholic fermentation. With this intention we performed a genotypic and technological characterization of S. cerevisiae population isolated in a biodynamic winery of the Cortona DOC area. Analyses revealed a remarkable variability in terms of strains of *S. cerevisiae*, despite the homogeneity of features of wines, underling the high levels of biodiversity that characterizes the biodynamic agriculture. Some strains were found in wines of different vintages suggesting the presence of a microbiota established in the winery. Oenological tests demonstrated that, alongside yeasts with reliable oenological performances, some strains are not able to accomplish a prompt and effective alcoholic fermentation, or are characterized by spoilage characters, such as excessive production of volatile phenols or acetic acid. In conclusion, indigenous strains of *S. cerevisiae* could be a useful instrument to perform reliable winemaking without altering the native microbiota of each oenological environment. However, a characterization of their oenological aptitude, and the application of practices able to drive the evolution of microflora, must be employed to reduce risk of wine spoilage.

KEY WORDS

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

FINDINGS

In the last years is indubitable the growing interest of consumers about wines having features more closely linked to the territories of origin. This trend leading winemakers to rethink the approach to the alcoholic fermentation, avoiding the use of selected yeast, and entrusting the fermentation process to the microflora naturally present in the cellar and on the grapes. This approach might seem a step backwards from the scientific and technological state of the art of oenology but, given its increasing diffusion and economic relevance, it deserves attention and scientific deepening. Grapes and cellar equipment are populated by a wide range of yeasts and bacteria that evolves during the production process, according to the environmental conditions and the technological choices (Capozzi et al 2015). It is common to

observe, on ripe bunches and on grape musts, a population of yeasts in the order of 10^4 cells/g, composed mainly of yeasts not belonging to the genera *Saccharomyces* (Barata et al. 2012). It is also widely known that the increase of ethanol content, due to alcoholic fermentation, leads the selection of microflora with the prevalence, after the accumulation of 5 - 6 % v/v of ethanol in the fermenting grape must, of *Saccharomyces cerevisiae*. The use of selected strains of *S. cerevisiae* does no alter this process of evolution of wine microbiota, but accelerates it, favoring a faster increase in the alcohol content of grape must (Guzzon et al. 2014, Ciani et al., 2016). However, if the "spontaneous" fermentations are 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 to an incomplete knowledge about the peculiar features of native microbiota of each specific oenological environment, and in consequent wrong technological approaches. The study of biodiversity associated with spontaneous fermentations, and the oenological characterization of identified yeasts, contributes to increase the comprehension of microbial dynamics of winemaking, preventing risk of wine spoilage. Many papers have been already discussed about this topic (Comitini et al. 2017). In this note we report about the study of a population of *S. cerevisiae* isolated, trough the winemaking process, in a winery that operates in the area of Cortona DOC (south of Tuscany, Italy) following a biodynamic approach that excludes the use of active dry yeast, avoiding risk of contamination of microbiota native of this scenario.

The study starts from the isolation of yeasts, carried out considering 8 different samples of fermenting grape must (Table 1), after the degradation of 90% of initial sugars content. This approach differs to date frequently carried out in works that studied the biodiversity of oenological environment (Settanni et al. 2012). Yeast were isolated from fermenting grape must having more than 10% of ethanol, in order to identify yeast strains having the highest ability to drive alcoholic fermentation, and resistance to wine limiting factors. Must was made from Syrah grapes, after gentile crushing, without sulphur dioxide and selected yeast addiction. Yeast were counted onto WL agar medium (Oxoid, UK), according to the 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. Results (Table 1) confirmed the hypothesis on the base of sampling. The difference of plate counts obtained onto WL Agar (meanly $8.0\pm0.6\times10^7$ cfu/mL) and Lysine Agar (meanly $1.8\pm0.9\times10^4$ cfu/mL) revealed a large population of yeasts, attributable to the *Saccharomyces* genus, with a negligible presence of other genus of yeast having oenological interest. Also, acetic acid bacteria were not detectable ($<5\times10^2$ cfu/mL), while a population of lactic bacteria, potentially able to spoil wine (Liu 2002), is present in all samples. The relevant contamination, meanly $9.3\pm6.8\times10^4$ cfu/mL, of lactic bacteria already during alcoholic fermentation is probably favoured by the high pH of grape must and the absence of SO_2.

100 colonies of yeast were isolated and purified onto WL agar, on the basis of provenience (vineyard parcel) and morphology. The yeast isolated were transferred in 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). The strain typing was performed by the analysis of interdelta sequences (ISA-PCR, Charpentier et al. 2009; Legras and Karst 2003), obtaining the discrimination of 11 strains. Their appurtenance to the *S. cerevisiae* specie was confirmed by sequencing

of 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 parcels of origin of grapes, which 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 enological equipment with occurrence of cross contamination. However, the observed biodiversity in the *S. cerevisiae* specie is quite high, despite the uniformity of the features of the source of isolation, and the small size of the vineyard (about 8 hectares, located in the same area). These observations agreed with results of previous experiences 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 performances of the 11 strains of *S. cerevisiae,* and the possible presence of spoilage characters, such as poor fermentative activity, excessive production of acetic acid, sulphur dioxide or volatile phenols (Guzzon et al. 2014). The 3 grape musts considered have a sugar content between the 220 and the 240 g/L, pH in the interval between 3.24 and 3.81, YAN beyond 164 and 118 mg/L, and they were supplemented by 500 mg/L of *p*-cumaric acid to stimulate the vinyl phenols production. Cellular growth was monitored by plate count; the evolution of alcoholic fermentation was followed by measure of the weight loss of samples due to the CO_2 production. The main chemical parameters of obtained wines were determined by FT-IR spectroscopy using a Wine Scan (Foss) apparatus and by a Crison titrator, for the monitoring of sulphur dioxide. Volatiles phenols was quantified by HPLC equipped with a colorimetric array detector as proposed by Larcher et al. (2007). From the kinetic point of view, we chosen to focus our attention on 3 moments: the lag phase (24 h after yeast inoculum), the end of exponential phase (5 days after yeast inoculum), and the complete fermentation (arbitrarily established at 10 days after yeast inoculum); the advancements of alcoholic fermentation was expressed as % of the theoretical total weight loss (Figure 1A). The initial yeast's inoculum was settled to 10^5 cfu/mL ensuring a prompt start of alcoholic fermentation in all tests with a mean 7.9±1.3% of weight loss after 2 days (Table 1). Instead, already after 5 days, the first differences were highlighted. The mean weight loss was the 59.1±6.5% but 4 strains - A2, A3, A8, and A11 - showed an advancement of alcoholic fermentation below from the mean of population. Similar trend was observed after 10 days of fermentation with 3 strains that showed performances below the mean (96.0±3.8%). The measurement of sugars residual in the obtained wine (Table 1) indicated that the strains A4, A7, and A9 accomplish the alcoholic fermentation in the 3 tests, residing in wines less than 5 g/L of sugars. As expected, the majority of problems in the accomplishment of alcoholic fermentation were observed at the highest potential ethanol content (grape must 3), with 3 strains - A8, A10 and A11 - that were not able to degrade completely sugars in the entire set of tests. This observation are particularly relevant because the problems of the incomplete consumption of sugars, with consequent possibility of development o spoilage microorganisms such as *Brettanomyces* or lactic acid bacteria (Chatonnet et al. 1995, Loureiro and Malfeito-Ferreira 2003), it is more frequent in recent years, due to climate changes and the consequent higher sugar content of grapes. The value of 15 alcoholic potential, established in the grape must sample n° 3, is not unusual in the Mediterranean oenological area. Therefore, it is important to underline that native yeast strains, developed in presence of specific environmental factors, are not always suitable to guarantee an efficient alcoholic fermentation. Spontaneous fermentation must be adequately monitored with

109 microbiological assay devoted to furnish rapid and reliable information about the physiological state of yeast
110 population (Guzzon and Larcher 2015, OIV, 2016). On the other hand, from the complex microbial population present
111 in fermenting grape must samples was possible to isolate at least 3 strains of *S. cerevisiae* that endowed good
112 fermentative activity and high resistance to ethanol. The three spoilage characters taken under consideration, the
113 production of acetic acid, sulphur dioxide and volatile phenols showed different trends (Table 1). The accumulation of
114 acetic acid (mean 0.3±0.1 g/L) was generally low, considering the high sugar content of grape must samples, which
115 induces osmotic stress and accumulation of acetic acid (Bely et al. 2003, Teixeira et al. 2011). The production of
116 222±98 mg/L of volatile phenols, corresponding to a conversion rate of 44.5±19.5% is comparable to that of some *S.*
117 *cerevisiae* strains used as fermentation starter (Guzzon et al., 2014), and acceptable for the production of red wines
118 (Rojas et al 2012). The accumulation of sulphur dioxide appears more linked at the initial sugars content, that that at
119 the features of each strain of *S. cerevisiae* (Table 1). However, strain A1 and A2 resulted the less producer in the entire
120 set of tests, while strains A9 and A10 accumulated the highest amount of sulphur dioxide in all wines. The level of SO_2
121 reached at the end of fermentation the grape must 3 (46±3 mg/L) are potentially able to stuck malolactic
122 fermentation; this aspect deserve particular consideration because the combination of high ethanol, pH and sulfur
123 dioxide content in wines could stimulate the development of spoilage lactic bacteria such as *Pediococcus* spp.
124 (Bartowsky 2009).

125 Isolated strains were employed in the successive vintage to drive alcoholic fermentation, to avoid excessive lag-phase
126 which could cause an uncontrolled proliferation of alterative microorganisms (Renouf 2015). The strains was
127 inoculated preparing, prior the harvest, a pied de cuve having a volume of 1% of the whole mass of grapes harvested.
128 At the end of alcoholic fermentation yeast belonging to the specie *S. cerevisiae* was isolated and characterized at
129 strain level, according the previous described experiments. The objective of this second set of microbiological assays
130 was the verification of the capacity of isolated strain of *S. cerevisiae* to remain active in the microbial population of
131 winery. Figure 1B exhibited the electrophoretic pattern of *S.cerevisiae* strains identified at the end of alcoholic
132 fermentation. The presence of 8 different biotypes confirmed the large biodiversity, observed in this oenological
133 scenario. 4 strains, among the inoculated, were identified in the lanes 1, 2, 3 and 4 of figure 1B. Were also found some
134 new *S. cerevisiae* strains (Lane 4, 6, 8 and 10 of figure 1B) involved in the fermentative process.

135 The results obtained in this work confirm that in the spontaneous microflora that characterizes the winemaking
136 process of biodynamic winery, there are strains of *S. cerevisiae* having a promising technological value, capable of
137 guaranteeing efficient biotransformation and, in perspective, high quality wines. However, it is crucial to apply any
138 technological approach suitable to stimulate these strains, within a complex microbiota that contains also spoilage
139 microorganisms such as lactic bacteria or yeast having poor fermentative activity. In this way strains having a good
140 oenological aptitude are in condition to drive alcoholic fermentation without eliminating the biodiversity
141 characteristic of each harvest

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Table 1 First section. Main features of wines source of isolation *of S. cerevisiae* strains. Second section. Results of oenological test performed by the strains of *S. cerevisiae*. *Test that showed a stuck of fermentation (10 days of incubation, sugars ≥ 5 g/L). 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.

First section. Main features of wine source of sampling of S. cerevisiae strains

Number of vat	Date of sampling	Density of must	Yeast	non-*Saccharomyces* yeast	Lactic acid bacteria
	(days from starts 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 test performed by the 11 strains of S. cerevisiae

S. cerevisiae strain (Grape must)	% of fermentation after 2/5/10 days	Ethanol	Sugars	Volatile acidity	Malic acid	Tot. SO_2	Volatile phenols
		(% vol)		*(g/L)*			*(mg/L)*
A1(1)	8.3/60.8/100.0	13.3	4.0	0.22	2.73	42	128
A1 (2)*	9.9/58.4/92.8	12.9	18.7	0.29	2.68	51	136
A1 (3)	6.9/56/9/98.7	14.8	8.3	0.31	2.22	47	221
A2 (1)	7.7/59.5/98.0	13.0	3.8	0.26	2.94	30	86
A2 (2)	8.6/59.7/96.7	13.3	2.5	0.31	2.66	30	98
A2 (3)*	7.0/53.2/92.5	13.7	28.0	0.36	2.25	47	112
A3 (1)	9.6/60.6/99.0	13.1	2.5	0.16	2.75	23	220
A3 (2)	8.6/58.3/99.3	14.0	3.0	0.14	2.62	36	225
A3 (3)*	6.2/52.9/97.2	14.5	8.1	0.17	2.34	33	326
A4 (1)	8.2/65.0/97.0	12.9	2.6	0.26	2.92	36	150
A4 (2)	7.7/62.3/98.3	13.6	2.7	0.22	2.82	51	165

A4 (3)	6.5/69.2/99.2	14.8	2.5	0.31	2.57	43	189
A5 (1)	9.4/66.2/98.4	13.0	4.1	0.23	2.72	38	321
A5 (2) *	8.9/63.1/96.6	13.5	5.8	0.28	2.36	39	366
A5 (3) *	5.9/57.6/98.3	14.4	6.0	0.31	2.31	38	384
A6 (1)	9.8/66.0/98.1	14.7	3.7	0.20	2.69	34	385
A6 (2) *	9.8/61.0/95.1	12.9	31.2	0.42	2.58	36	396
A6 (3) *	5.9/57.6/87.3	13.1	34.2	0.68	2.36	47	321
A7 (1)	7.8/60.8/98.9	13.1	4.5	0.17	2.76	33	120
A7 (2)	9.1/58.3/98.5	13.9	4.9	0.17	2.39	38	221
A7 (3)	7.3/62.9/99.8	14.9	4.6	0.20	2.10	44	186
A8 (1) *	8.5/60.4/87.8	11.7	> 35	0.33	2.75	nd	325
A8 (2) *	6.9/42.3/89.3	12.3	> 35	0.38	2.64	nd	336
A8 (3) *	5.5/36.8/90.8	13.6	> 35	0.46	2.28	nd	322
A9 (1)	8.5/60.4/98.0	13.0	2.6	0.34	2.86	41	86
A9 (2)	8.8/63.5/99.6	13.7	3.2	0.32	2.67	45	85
A9 (3)	8.0/58.9/98.7	14.8	3.1	0.47	2.31	45	112
A10 (1) *	8.3/61.2/92.6	12.3	7.0	0.26	2.82	41	225
A10 (2) *	8.8/60.1/93.8	12.9	8.2	0.42	2.64	49	263
A10 (3) *	7.7/61.5/92.5	13.8	7.4	0.48	2.21	52	228
A11 (1)	8.3/65.5/96.7	12.7	> 35	0.21	2.89	nd	185
A11 (2) *	8.6/61.2/85.7	11.83	> 35	0.38	2.65	nd	221
A11 (3) *	5.2/48.9/88.3	13.2	> 35	0.45	2.44	nd	196

Figure 1. A) Box plot of sugars consumption (expressed as %) of a population 11 *S. cerevisiae* strains in test performed in 3 different grape must with increasing harsh conditions. B) electrophoresis patterns generated from the ISA-PCR products of *S. cerevisiae* isolates at the end of fermentation performed during the harvest 2012. We observed the presence of some strains isolated and characterized through this work (lane 1, 2, 3 and 4) and new indigenous strains (lane 4, 6, 8 and 10).