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Analysis of yeast populations during alcoholic fermentation: A six year follow-up study

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Summary

Wine yeasts were isolated from fermenting Garnatxa and Xarel.lo musts fermented in a newly built and operated winery between 1995 and 2000. The species of non-*Saccharomyces* yeasts and the *Saccharomyces cerevisiae* strains were identified by ribosomal DNA and mitochondrial DNA RFLP analysis respectively. Non-*Saccharomyces* yeasts, particularly *Hanseniaspora uvarum* and *Candida stellata*, dominated the first stages of fermentation. However *Saccharomyces cerevisiae* was present at the beginning of the fermentation and was the main yeast in the musts in one vintage (1999). In all the cases, *S. cerevisiae* took over the process in the middle and final stages of fermentation. The analysis of the *S. cerevisiae* strains showed that indigenous strains competed with commercial strains inoculated in other fermentation tanks of the cellar. The continuous use of commercial yeasts reduced the diversity and importance of the indigenous *S. cerevisiae* strains.

Key words: wine yeast – *Saccharomyces cerevisiae* – *Hanseniaspora uvarum* – *Candida stellata*

Introduction

The conversion of grape must to wine is a complex biochemical process involving interactions between yeasts, bacteria and other microbial species. Of these microorganisms, yeasts are primarily responsible for alcoholic fermentation. Yeast species with lower fermentative activity than *Saccharomyces cerevisiae*, such as *Hanseniaspora*, *Candida* and *Pichia*, grow during the first period of spontaneous fermentations but then the population size of non-*Saccharomyces* species decreases progressively, leaving the most ethanol-tolerant species of *Saccharomyces cerevisiae* to take over the fermentation (HEARD and FLEET, 1985).

The microbiology of wine and the origin of indigenous wine yeasts have been extensively studied. There are two possible sources of the yeasts responsible for alcoholic fermentation: the vineyard (including the grapes), and the winery (including the winery equipment). Many studies from several countries have described the isolation and identification of yeasts from grape surfaces (FLEET and HEARD, 1993). However, most of these ecological studies are now considered to be erroneous because enrichment methods, rather than direct plating methods, were used for yeast isolation (MARTINI, 1993). Subsequent quantitative studies, excluding any enrichment effect, showed that *Hanseniaspora uvarum* (and its anamorphic form *Kloeckera apiculata*) was the predominant species on the surface of grape berries (50–75% of the total yeast population), and at lesser populations there were species of *Candida*, *Pichia*, *Cryptococcus*, *Rhodotorula*, *Metschnikowia*, *Kluyveromyces* and *Hansenula*. *Saccharomyces cerevisiae*, however, was isolated with extreme difficulty and in low percentage from natural habitats such as vineyard soil or the surface of ripe grapes but could be found colonising the surfaces of the winery equipment (ROSINI, 1982; MARTINI, 1993; FLEET and HEARD, 1993; VAUGHAN-MARTINI and MARTINI, 1995).

Every vintage, the surfaces of winery equipment are exposed to billions of yeast cells, so they are easily colonised and become locations for the development of a resident or winery yeast flora. These *Saccharomyces cerevisiae* strains resident on the winery surfaces are much more abundant than those that might come from the grapes or vineyard and it is very probable that they will predominate during the spontaneous fermentations or play a role in other fermentations inoculated with pure yeast culture (ROSINI, 1984; MARTINI et al., 1996; CONSTANTÍ et al., 1997).

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Nevertheless, MORTIMER and POLSINELLI (1999) demonstrated that damaged grape berries were rich depositories of such microorganisms as *S. cerevisiae*, and the main source of this yeast in natural wine fermentations. But they did not exclude that they may be present on cellar walls and equipments.

The diversity, composition and evolution of yeast flora in grape musts depend on a variety of factors: geographic location, climatic conditions (FLEET et al., 1984; PARISCH and CARROL, 1985), age of the vineyard and grape variety (MARTINI et al., 1980; ROSINI et al., 1982). Many studies have analysed the population dynamics of wild yeast during spontaneous fermentations (SCHÜTZ and GAFNER, 1993; QUEROL et al., 1994; CONSTANTÍ et al., 1998), in different wine-producing cellars (VEZINHET et al., 1992; VERSAVAUD et al., 1995) and different wine regions (LONGO et al., 1991; KHAN et al., 2000; VAN DER WESTHUIZEN et al., 2000; TORIJA et al., 2001), or over several years in the same cellar (VEZINHET et al., 1992; SCHÜTZ and GAFNER, 1994; CONSTANTÍ et al., 1997; SABATÉ et al., 1998).

Using active dry yeast in fermentation is becoming one of the most common practices in winemaking because it ensures a reproducible product and reduces the lag phase and the risk of wine spoilage. However, the winemaking community is still widely divided about this practice because of a widespread belief that native yeast strains give a distinctive style and quality to wine (MATEO et al., 1991; FUGELSANG 1996; HEARD, 1999). In fact, the use of active dry yeast reduces the number of different indigenous *Saccharomyces cerevisiae* strains due to the starter imposition, but does not completely prevent them from growing until several days after the inoculation. During this time, wild strains may have an important effect on wine flavour and characteristics (QUEROL et al., 1992a).

In the present study, we analyse the yeast population in a new winery (and therefore with new equipment and no yeast resident flora) for six consecutive years and in two varieties. The aim of the present study was to analyse the cellar yeast ecology in a situation of industrial wine-making. The effect of the common practice of inoculation upon the diversity of indigenous *S. cerevisiae* strains and the development of alcoholic fermentation was also one of the main goals of the present study. The species of the non-*Saccharomyces* yeasts were identified by RFLPs of rDNA (GUILLAMÓN et al., 1998; ESTEVE-ZARZOSO et al., 1999), and *Saccharomyces* sensu stricto were analysed at species and strain level by RFLPs of mtDNA (QUEROL et al., 1992b; GUILLAMÓN et al., 1994).

Materials and Methods

Fermentations

This study was made in a new winery in Tarragona (Spain). The cellar was built in 1994 for the Faculty of Enology, adjacent to its experimental fields with 6.8 ha of cultivated grapevines. All the equipment was brand new the first year of operation and had not been previously used.

Two grape varieties were studied over 6 vintages (1995–2000): Xarel.lo for white fermentations and Garnatxa for red ones. Musts were fermented spontaneously in 100 l steel vats, at controlled temperature for Xarel.lo (18 ºC) and uncontrolled temperatures for Garnatxa (22–28 ºC). In all cases, sulphur dioxide (0.05 g l^{-1}) was added to the musts before the fermentation started. Must samples were taken before sulphitation. Xarel.lo must was clarified by settling before alcoholic fermentation to separate the clear juice from the sediments. Xarel.lo and Garnatxa musts inoculated simultaneously with commercial starters were used as controls in other steel vats.

In all cases fermentation kinetics were monitored by measuring the density of the fermenting must, which has a 99% correlation with reducing sugar content (RIBÉREAU-GAYON et al., 2000)

Sampling

Samples were taken periodically during the fermentation for the isolation and enumeration of yeasts. The samples were plated on YEPD agar in serial decimal dilutions and incubated at 28 ºC for two days. Twenty colonies were randomly taken and isolated from each sample. A selective Lysine medium was used to distinguish between *Saccharomyces* and non-*Saccharomyces* yeasts. *Saccharomyces* species were unable to grow on this medium (ANGELO and SIEBERT, 1987).

mtDNA and rDNA restriction analysis

DNA was extracted and mitochondrial DNA restriction patterns of the strains were determined as previously described by QUEROL et al. (1992b) with the restriction endonuclease *Hin*fI (Boehringer Mannheim, Germany). Restriction fragments were separated on horizontal 0.8% agarose gels (Ecogen, Barcelona, Spain) in TBE buffer (Tris 89 mM; boric acid 89 mM; EDTA 2mM; pH 8) at 5.5 V cm^{-1} and visualized on a UV transilluminator after ethidium bromide staining (5 µg ml^{-1}).

rDNA was amplified and restricted as previously described by GUILLAMÓN et al. (1998). The primers used to amplify the ITS region were ITS1 and ITS4, described by WHITE et al. (1990). The PCR products were digested with 3 restriction endonucleases: *Hin*fI, *Cfo*I and *Hae*III (Boehringer). Restriction fragments were separated on a horizontal 2% multipurpose agarose (Boehringer) gel in TBE buffer and compared with standards (100-bp DNA ladder; Gibco-BRL).

Results and Discussion

This 6-year follow-up study was carried out in a completely new experimental winery, built in 1994, ready for the 1995 vintage. All equipment was brand new and neither the cellar nor the equipment had been previously exposed to musts, fruit juices, wines, or any beverage or fruit. Thus, no resident yeast flora could be considered before the 1995 vintage.

This study had several drawbacks. First, not all the years were good enough for harvest. For example, in 1997, no grapes from the two varieties being studied were harvested because of adverse climatic conditions. Second, no initial estimation of population was made in the first year and the non-*Saccharomyces* population was only determined in Garnatxa must. Finally, it should be pointed out that the yeasts and strains were determined randomly over 20 colonies of each point. Thus, the approach was semiquantitative and this must be taken into account when drawing the final conclusions.

As has been previously reported, non-*Saccharomyces* yeasts are the most usual microorganisms in grape must (Figure 1), probably because they are present in grape skins and vineyards, where *Saccharomyces* yeasts are mostly absent (PARRISH and CARROLL, 1985; MARTINI et al., 1996; CONSTANTÍ et al., 1997). All the species found were identified by RFLP analysis of the rDNA region (GUILLAMÓN et al., 1998; ESTEVE-ZARZOSO et al., 1999). Although most of the non-*Saccharomyces* yeasts were *Candida stellata* or *Hanseniaspora uvarum*, other yeasts were also found, but mainly in one year and in low quantities. This clearly points to a predominance of *H. uvarum* or *C. stellata* in vineyards and grape surfaces, whereas our climatic conditions probably limit the presence of other yeasts, including *Saccharomyces cerevisiae*

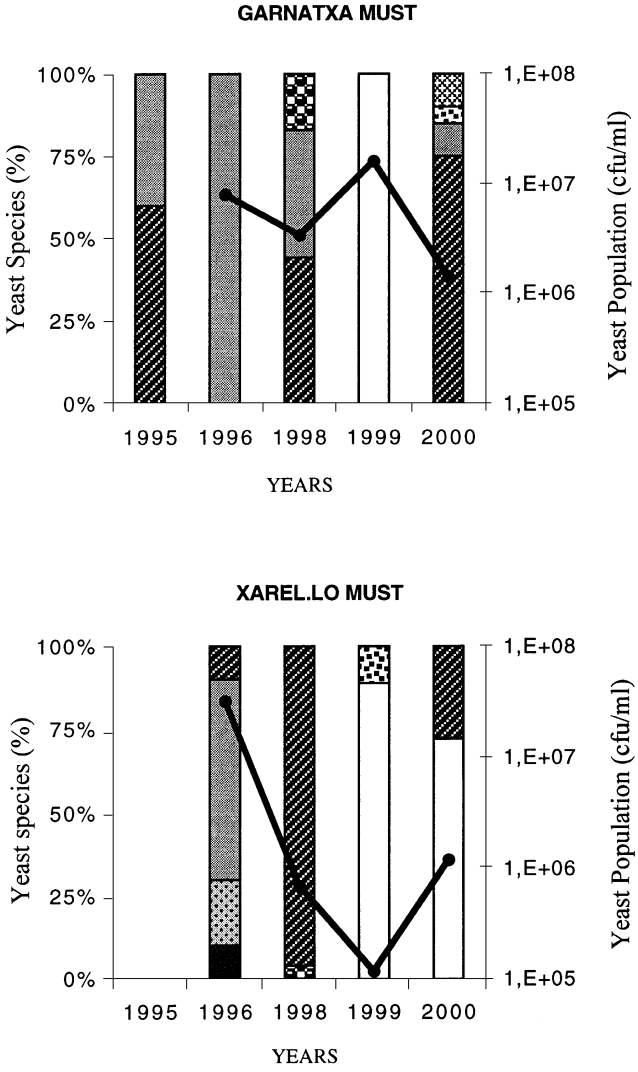

Figure 1. Biodiversity of yeasts species and total population in Garnatxa and Xarel.lo musts in the five years studied. (□*Saccharomyces*, ▨ *Hanseniaspora uvarum*, ▨ *Candida stellata*, ▨ *Candida sorbosa*, ▨ *Debaromyces hansenii*, ▨ *Issatchenkia terricola*, ■ *Metschnikowia pulcherrima*, ▨ *Zygoascus hellenicus*, —●— Yeast Population).

(GUILLAMÓN et al., 1996; CONSTANTÍ et al., 1997, 1998). However, the fact that these other species were present in certain years pointed toward a consistent presence of other non-*Saccharomyces* species, which in a qualitative study would probably show up consistently. During the initial stages of fermentations, some authors have also isolated other minor species, belonging to the genera *Metschnikowia*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Kluyveromyces* and *Hansenula* (FLEET et al., 1984; LONGO et al., 1991; SCHÜTZ and GAFNER, 1993; TORIJA et al., 2001).

The microbiota of grapes mainly depends on grape variety, climatic conditions and viticultural practices (PRETORIUS, 2000). As mentioned above, *H. uvarum* and *C. stellata* were the predominant non-*Saccharomyces* species in the musts obtained from both grape varieties. The proportion of these species varied in the years studied but the predominace of both varieties did not change, i.e. *H. uvarum* was the major non-Saccharomyces species in both varieties in the 1996 vintage and *C. stellata* was predominant in 2000. Therefore, according to our results, the grape variety does not have a great influence on yeast microbiota. As far as climatic conditions are concerned, temperature and rainfalls were very similar in the years studied. The total annual rainfall was about 400 mm and 100 mm during the summer. The year when the grapes were not suitable for vinification (1997) the precipitation recorded was 700 mm. Therefore, the differences in the proportion of *C. stellata* and *H. uvarum* in the period studied could not be explained by these parameters. A more exhaustive study of other climatic and cultural conditions should be made if their influence on the development of these species is to be understood.

It has been widely reported that *H. uvarum* is present in the initial phases of the fermentation of several musts. This might be important in the taste and flavour of wine, and could produce considerable amounts of volatile acids, acetate and glycerol (BENDA, 1982; LAFON-LAFOURCADE, 1983; SCHÜTZ and GAFNER, 1993). *C. stellata* was the other strain that was most found in the first stages of fermentation (FLEET et al., 1984). If it continues to develop in the latter stages, a film may be formed on the surface of wines, which can change their chemical composition and flavour (SPONHOLZ, 1993). Anyway, neither *H. uvarum* nor *C. stellata* lasted too long in the fermentations in any of the years tested and consequently the final product was affected little or not at all. Vinifications in red and white wines differ in temperature and the fact that skins are in contact with the fermenting musts, but these factors only slightly affected the permanence of these species (Table 1). Other non-*Saccharomyces* strains were present in these conditions. The 1999 vintage was surprising because most of the colonies collected from both musts were identified as *Saccharomyces cerevisiae*. These results are certainly difficult to reconcile with previous observations (SCHÜTZ and GAFNER, 1993; MARTINI et al., 1996; GUILLAMÓN et al., 1998; TORIJA et al., 2001) about the unfrequent presence of *S. cerevisiae* in grape musts. The principal characteristic of the vintage was the exceptional sanity of the grapes, which implies a very low

Table 1. Percentage and permanence of yeast species during spontaneous fermentations of Garnatxa and Xarel.lo in the five years studied. (EF: end of fermentation).

		Day of fermentation	*Sac. cerevisiae*	non-*Saccharomyces*		
				C. stellata	*H. uvarum*	Others
1995	Garnatxa	0		60	40	
		3	5	50	45	
		5	90		10	
		EF	100			
1996	Garnatxa	0			100	
		1		30	70	
		3		50	45	5
		6	72	6	21	
		EF	100			
	Xarel.lo	0		10	60	30
		1		100		
		3	100			
		5	100			
		EF	100			
1998	Garnatxa	0		44	39	17
		1		79	21	
		2	14	72	10	4
		5	100			
		EF	100			
	Xarel.lo	0		96		4
		1		42	4	54
		2	100			
		5	100			
		EF	100			
1999	Garnatxa	0	100			
		2	100			
		5	100			
		EF	100			
	Xarel.lo	0	90			10
		2	100			
		5	100			
		EF	100			
2000	Garnatxa	0		75	10	15
		2	76		12	12
		5	100			
		EF	100			
	Xarel.lo	0	72	28		
		2	70			30
		5	100			
		EF	100			

population of microorganisms such as yeasts, and lactic and acetic acid bacteria on the grape surface. As a possibility, the low yeast population in the grape and the high yeast contamination in the wine environment after several years of operating may explain why *S. cerevisiae* strains were detected in the musts.

The different presence and endurance of non-*Saccharomyces* species during fermentation may also be due to similar reasons (Table 1). In the first 3–4 vintages, non-*Saccharomyces* species lasted as long as 2 to 6 days in competition with *Saccharomyces* strains. However, after four vintages, there were fewer non-*Saccharomyces* yeast present during fermentation and *Saccharomyces* dominated the very early stages of fermentation. There are several explanations for this. First of all, as the winery was newly built in 1994, the winery itself and the equipment was initially 'clean' and without 'resident' microbiota. After several vintages and fermentations, the equipment, walls and the rest of the cellar became suitable for the development of microbiota adapted to the cellar environment (MARTINI et al., 1996). Thus, a certain number of different *Saccharomyces* strains were always available to initiate and lead the fermentation. Secondly, after several years of running the experimental cellar, many different strains of commercial *Saccharomyces* starters had been tested in the cellar. These strains were 'selected yeasts' and well adapted to the winemaking process (BOULTON et al., 1996). So, the presence of specially selected strains

Table 2. Percentages of indigenous (MF) and commercial (CI) *Saccharomyces* strains in spontaneous fermentations of Garnatxa and Xarel.lo, from 1995 to 2000.

Year of 1st appearance	Indigenous str.	Garnatxa					Xarel.lo				
		1995	1996	1998	1999	2000	1995	1996	1998	1999	2000
1995	MF02	23	0	0	0	0	15	30	0	0	0
	MF05	3	0	0	0	0	6	2	0	0	0
	MF09	0	0	0	0	0	10	0	0	0	0
	MF10	8	0	0	0	0	2	0	0	4	0
	MF14	3	0	0	0	0	2	0	0	0	9
	Others*	50 (9)	0	0	0	0	18 (5)	0	0	0	0
1996	MF19		2	4	0	0		0	0	0	0
	MF20		41	3	0	0		62	0	0	0
	MF25		0	0	0	0		2	0	10	5
	Others*		57 (2)	0	0	0		4 (2)	0	0	0
1999	MF26					21				1	32
	MF27				16	11				71	44
	MF32				4	1				0	0
	MF37				4	4				1	0
	MF38				14	8				0	0
	Others*				24 (1)	0				9 (5)	0
Years of usage	Comercial str.										
95,98,00	Cl 1	13	0	30	0	0	47	0	0	0	0
98,99,00	Cl 2			62	0	0			88	0	0
98	Cl 3			0	0	0			12	0	0
99	Cl 4				16	4				1	0
98,00	Cl 5			0	22	54			0	1	10

*Others means *Saccharomyces cerevisiae* strains that did not showed up in others vintages. In parenthesis the number of strains.

would favour the rapid imposition of *Saccharomyces* over non-*Saccharomyces* species. Finally, neither of the two varieties are the first to be harvested and fermented in the cellar every year; so the cellar environment is already enriched with fermenting *Saccharomyces* strains. 'Cross-contamination' between adjacent vats by means of insects or winery equipment (pumps, must conducts, etc) is very frequent (Rosini, 1984; Constantí et al., 1997; Ribéreau-Gayon et al., 2000), particularly on an industrial scale, and the volumes and machinery of this experimental cellar are similar to those of an industrial, commercial cellar. The presence of natural and commercial *Saccharomyces* strains contaminating the winery probably makes it easier for them to become dominant during fermentation.

Table 2 shows the percentages of indigenous (MF) and commercial (CI) *Saccharomyces* strains throughout spontaneous fermentation. Each year between 80 and 100 colonies were isolated at different stages of the fermentation and analysed. The mtDNA restriction profiles of the colonies analysed during the 6-year period showed that 35 different 'indigenous' (or non-inoculated) *Saccharomyces cerevisiae* strains were involved in the fermentations at some time (Table 2). Although some strains showed higher percentages in the fermentation of one grape variety (i.e. MF27, Table 2), most were isolated in the same year and/or different years from fermenting musts of both varieties. Thus, the predominance of a specific strain was more influenced by the vintage than the grape variety or wine making process. Interestingly, new strains were detected in only three years, two of which were the initial ones when few resident strains were present (Table 2). The other year was 1999 when few non-*Saccharomyces* species were detected in the must of both varieties. The absence of non-*Saccharomyces* yeasts may have enabled a considerable number of *Saccharomyces* strains to be detected. Indigenous yeasts, however, were detected in most of the vintages. In fact, indigenous yeasts detected in 1995, 1996 and 1999 were also detected during the last year of fermentation. These strains had probably colonised the winery environment, and the different conditions of each vintage (a different selective pressure on the *S. cerevisiae* population) determined whether each specific strain would develop to a greater or lesser extent.

As mentioned above, the cellar that was studied operates like an industrial winery and most of the fermentations of these and other grape varieties were inoculated with different commercial strains. These commercial strains were also detected in the spontaneous fermentations (Table 2). On the basis of our data, cross contamination between spontaneous and inoculated fermentations by insects, pumps, etc. (Phaff and Knapp, 1956; Lachance, 1994; Mortimer and Polsinelli, 1999) must be considered to be a possibility. So, the strain observed in 1995 (CI1) was the one inoculated this year in other fermentations. This cross contamination between vats

could be facilitated by the fact that these varieties were harvested when the winery was in operation at least one month before, fermentating other varieties.

The commercial strains clearly competed with the indigenous strains throughout the spontaneous fermentation. These commercial strains were selected on the basis of their enological capability and were present in high percentages during wine fermentation. For instance, two commercial strains represented 92% and 100% of the colonies isolated from the 1998 fermentations for Garnatxa and Xarel.lo, respectively. However, high percentages of some indigenous strains were also isolated during the fermentation (i.e. MF27 in Xarel.lo fermentations). Further studies should be carried out into the enological properties of these major indigenous strains because they may be local strains that could be used as future fermentation starters.

Recently, TORIJA et al. (2001) showed that a high degree of the variability of *Saccharomyces* strains (measured as the percentage of different strains found out of the colonies analysed) in two wine regions was due to the fact that no commercial starters had previously been inoculated. The variability of the strains in the present study is much lower than that reported by TORIJA et al. (2001) and similar to other studies carried out in previously inoculated cellars (QUEROL et al., 1994; SCHÜTZ and GAFNER, 1994; NADAL et al., 1996). Moreover, with some exceptions, there are fewer strains actively involved in fermentation after several years of operating than during the first vintage. Therefore, our results support the hypothesis that active dry yeasts reduce the variability of strains that appear in spontaneous fermentations.

Conclusions

H. uvarum and *C. stellata* were the predominant non-*Saccharomyces* species in the fermentations studied. This latter species seems to be specially important in this and other neighboring wine regions that have previously been studied (TORIJA et al., 2001) compared to other areas where *H. uvarum* is clearly the majority species in must and during the first stages of fermentation. The importance of both species alternates in the different vintages but was not influenced by the grape variety. *S. cerevisiae* was isolated in high percentages in 1999 (and Xarel.lo fermentation in 2000). The exceptional sanity of the grapes and the increase in resident *S. cerevisiae* strains in the cellar could explain the high population of this species in the first stages of fermentation. Anyway, in all cases, *S. cerevisiae* strains took over the fermentations, which proceeded faster.

The analysis of the *S. cerevisiae* strains showed that indigenous strains were present in several stages of the spontaneous fermentations. These strains competed with commercial strains inoculated in other fermentations of the cellar, which contaminated the vats. It should be pointed out that the continuous use of commercial and selected yeasts reduced the diversity and importance of the indigenous strains.

Despite the large number of studies about wine and vineyard yeast ecology, the origin of *S. cerevisiae* is quite controversial (PRETORIUS, 2000). Some authors claim that the primary source of this yeast is the vineyard (TÖRÖK et al., 1996; MORTIMER and POLSINELLY, 1999). Others postulated direct association with artificial, man-made environments such as wineries and fermentation plants, and that a natural origin for *S. cerevisiae* should be excluded (MARTINI, 1993; VAUGHAN-MARTINI and MARTINI, 1995). Unfortunately, we have not tried to isolate *S. cerevisiae* strains directly from grape berries but the study of yeast population in a newly established winery is an ideal situation to clarify this controversial question. A natural origin of the indigenous strains isolated could be proposed but a repeated appearance of these strains in different years pointed out to a colonisation of the winery environment with yeasts that go through generations and generations at each vintage. This hypothesis is supported by the analysis of the inoculated, commercial strains. The presence of these strains, even when not inoculated in the same vintage, competing with "natural" strains, in the spontaneous fermentations prove the establishment of these strains as residents of the winery surfaces. Lastly, the early isolation of *S. cerevisiae* strains in high percentages in the fermentations of the latter studied years could be understood as an increase of the yeast contamination in the cellar, which make easier the predominance in the fermentative process.

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