SELECTED YEAST UTILIZATION AND BIODIVERSITY

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Abstract

The use of commercial wine yeast strains as starters has been extensively generalised over the past two decades. Wine yeast strains are annually released in wineries environment and on an annual basis. However, little is known about the fate of these strains in the vineyard. To evaluate the industrial starter yeasts' ability to survive and spread in nature, and become part of the natural microflora of musts, we have devised a large-scale sampling plan over a period of three years in six different vineyards (3 in Portugal and 3 in France). Each vineyard has used the same industrial yeast strain(s) continuously in the last 5 years. A total of 198 grape samples were collected at various distances from the wineries, before and after harvest. Towards the end of the spontaneous fermentations, the composition of the yeast flora was determined by different typing methods (PCR-amplification of ∂-sequences, pulse field electrophoresis, RFLP of mitochondrial DNA, and microsatellite typing). Among 3780 yeast strains identified, 296 isolates had a genetic profile identical to that of commercial yeast strains. For a large majority (94%), these strains were recovered at very close proximity to the winery (10-200m). Commercial strains were mostly found in the post harvest samples, reflecting immediate dissemination. Analysis of population variations from year to year indicated that permanent implantation of commercial strains in the vineyard did not occur, but instead that these strains were subject to natural fluctuations of periodical appearance/disappearance like autochthonous strains. Overall the data show that dissemination of commercial yeast in the vineyard is restricted to short distances and limited periods of times and is largely favoured by the presence of water runoff.

Introduction

Since the beginning of the 1980's, the use of active dried *S. cerevisiae* yeast starters has been extensively generalised. Today, the majority of wine production is based on the use of active dried yeast, which ensures rapid and reliable fermentations, and reduces the risk of sluggish or stuck fermentations and of microbial contaminations. Most commercial wine yeast have been selected in the vineyard for enological traits such as fermentation performance, ethanol tolerance, absence of off-flavors and production of desirable metabolites. These and other technological developments have contributed to improve wine quality, and have enhanced the ability of winemakers to control the fermentation process and achieve specific outcomes.

Commercial yeasts are classically used in winemaking without any special containment and are annually released in large quantities, together with liquid and solid wine-making residues, in the environment around the winery. The behaviour of these yeasts in the ecosystem of the vineyard is totally unknown as is their potential impact on the natural microflora. In particular, it is not known if commercial strains are able to survive in nature and to become members of the vineyard microflora. Only very few data are available that could contribute to the evaluation of the importance of starter yeast's dissemination and permanence in the vineyard (Frezier and Dubourdieu, 1992; Vezinhet *et al.* 1992; Guillamón *et al*., 1996). Recently, a large-scale biogeographical study in South African vineyards was carried out in five areas situated in the Coastal Region vineyards of the Western Cape. Commercial yeasts were recovered in 3 of 13 samples (van der Westhuizen *et al*., 2000a and 2000b).

The present large-scale study, that was carried out in different geographical localizations of France and Portugal, aims to evaluate the industrial starter yeasts' ability to spread and survive in nature. The data will serve as a strong basis to evaluate if inoculated strains may become members of the natural microflora and affect biodiversity, and if they may influence the fermentations of the following years, especially those performed according to traditional practices which rely on spontaneous fermentation. Such data will also serve as strong basis to evaluate potential risks associated with the use of genetically modified (GM) yeasts.

Methodology

The sampling plan included 36 sites in 6 vineyards, 3 located in the South of France (Languedoc) and 3 in the North of Portugal (Região Demarcada dos Vinhos Verdes). The overall duration of these studies is 3 years (2001-2003). The wineries selected used consecutively one or more commercial yeast strains in the last 5 years. The three Portuguese wineries used mainly Zymaflore VL1, a strain originally selected in France, while the three French wineries used predominantly K1M ICV-INRA. A total of 34 commercial wine yeast strains have been used in the six wineries during the three years study.

In each vineyard, six sampling points were defined according to the local conditions (size and orientation of the vineyard, predominating wind direction). The distance between winery and the sampling sites varied between 20 to 1000 m. In order to evaluate the remanence over years of commercial yeast, a first sampling campaign was performed before the winery started wine production with the use of commercial yeast strains (pre-harvest samples). In a second post-harvest sampling campaign, the grapes were collected, after the onset of wine production, in order to evaluate the immediate commercial yeast dissemination from the winery. With the present experimental design, 72 grape samples were collected each year. From each sampling point, approximately 2 kg of grapes were asseptically collected, and the extracted grape juice was fermented in small volumes (200 – 500 ml), with mechanical agitation at 20ºC. Daily weight determinations allowed the monitoring of the fermentation progress. The yeast flora was analysed when the must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content. Must samples were diluted and spread on plates with YPD medium (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v), and after 2 days of incubation 30 randomly selected colonies were collected from each spontaneous fermentation. The *Saccharomyces* strains were first selected on a selective medium with L-Lysin as sole nitrogen source. The *Saccharomyces* not able to growth on L-lysin medium were subjected to molecular identification based on mitochondrial DNA restriction profiles [Querol *et al*, 1992], microsatellite analysis using six loci (ScAAT1-ScAAT6) [Perez *et al*, 2001], karyotype pattern using pulsed field gel electrophoresis (PFGE) [Blondin and Vezinhet, 1988] and interdelta sequence amplification patterns (Ness *et al.*, 1993; Legras and Karst, 2003]. Before starting the study, we evaluated the discriminatory power of different typing methods on a total of 23 commercial yeast strains used in the wineries of the two countries. Among the 23 commercial yeast strains analysed, 22 different

patterns were obtained using karyotyping analysis and 21 using the three other methods (Schuller *et al*, 2004). Due to the verified similarity of the discriminatory power of these methods any of them can be used for our study and the results obtained will be comparable.

Results

A total of 198 samples were collected during three consecutive campaigns (2001-2003), 108 of which were taken in France and 90 in Portugal (Table 1)

Table 1: Distribution of global data by country and year

	2001		2002		2003		Total
	France	Portugal	France	Portugal	France	Portugal	
Samples	36	36	36	18	36	36	198
Spontaneous fermentations	24	19	33	12	15	23	126
Isolates	720	570	990	360	450	690	3780
Saccharomyces strains	406	570	120	360	209	690	2355

Of the 198 samples, 126 musts (64%) produced spontaneous fermentations, 20% and 44% in must from pre-harvest and post-harvest campaigns respectively. The percentages of spontaneous fermentations were similar in both countries, 66% in France and 60% in Portugal. A total of 3780 colonies were isolated, of which 2355 were identified as *Saccharomyces* strains.

Table 2: Commercial yeast strains recovered in each vineyard over the 3 years

Vineyards	**A**	**B**	**C**	**D**	**E**	**F**	**Total**
Spontaneous fermentations	19	24	29	16	23	15	126
Spontaneous fermentations with ≥ 1 commercial yeast strains	0	2	1	11	9	2	25
Isolates	570	720	870	480	690	450	3780
Commercial yeasts strains	0	15*	1	206	54+18*	2	296
% Commercial yeast / nb of isolates	0	2	0.1	43	10	0.5	7.8

* strains originated from the same area

Molecular characterisation of the 2355 *Saccharomyces* isolates led to the identification of 296 strains with a genetic profile similar to that of commercial yeasts (Table 2). These strains represent 7.8% of the fermentative yeast community, the majority of which (5.8%) were recovered in post-harvest campaigns. It should be noted that since fermentation is used as an enrichment tool for *Saccharomyces* strains, the present results do not allow conclusions about the number of strains occurring on the surface of the grape, which is in fact very low. Instead, the number of fermentations with at least one commercial yeast strain gives a better picture of the situation as it occurs in vineyards; commercial yeast strains were recovered in 12% of samples.

The global data reflect very different situations. In four vineyards where the sampling sites were placed at a greater distance from the winery, i.e. vineyard F in Portugal and the three French vineyards (A, B, C), the occurrence of commercial yeast was very low, representing between 0% and 2% of the fermentative community, and these strains were isolated from only five samples (Table 2). In France the genetic profile of 16 clones out of 735 *Saccharomyces* isolates (2%) was identical to that of commercial yeasts. These strains correspond to 0.8% of the yeast strains isolated after fermentation. With only one exception, these strains (15 isolates) had an identical profile to that of the autochthonous strain ICV D254 and were found in the same site (winery B), in pre-harvest samples taken in 2001. This fact could indicate previous dissemination, but it cannot be confirmed since the commercial yeast strain ICV D254 was initially isolated from the same region of the South of France where the study was carried out. One colony was isolated in 2003 in winery C, which had the same profile as K1M ICV-INRA, used in the three French wineries for the last 5-15 years. It is noteworthy that this yeast, which has been used extensively for a considerable length of time, has never been found in the vineyard, except in this case. In the Portuguese winery F, only two isolates with the same profile as the extensively used commercial yeast, Zymaflore VL1, in use for five years, were found. The results were very different in the Portuguese wineries D and E, for which a high number of commercial strains was isolated representing 43 and 10% of the fermentative yeast community respectively.

An overview of the dissemination of commercial strains in relation to their distance from the winery is shown in Figure 2. Ninety four percent of commercial strains were found in a radius of around 10-200 m from the winery and a large majority (78%) was recovered in sites at very close proximity (10-50 m) to the wineries (vineyards D and E). A major

proportion (73%) was collected in post-harvest campaigns indicating immediate dissemination.

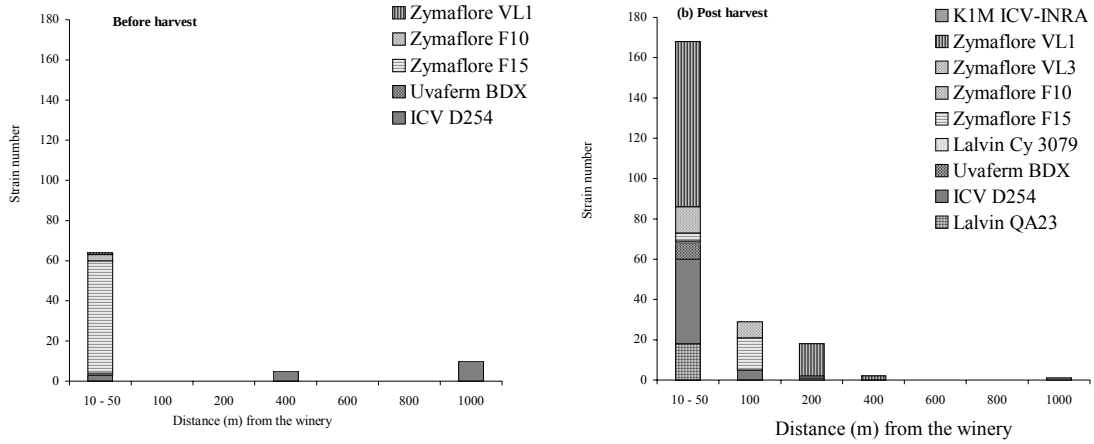


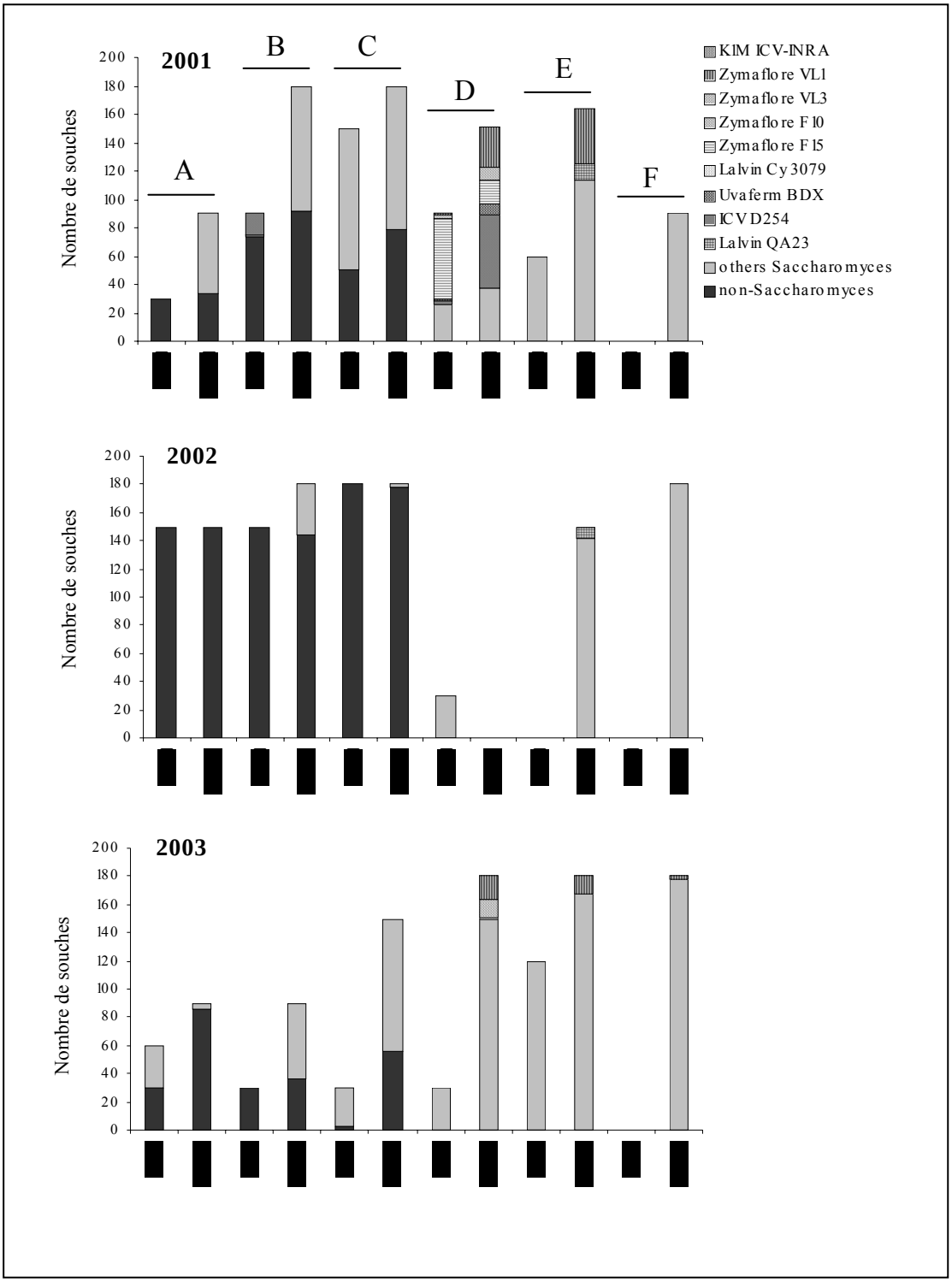
Fig. 1. Overall (three years) distribution of commercial yeast strains according to the distance from the wineries in pre-harvest (a) and in post-harvest (b) campaigns.

The evolution of the total yeast community isolated after fermentation in the different wineries of France and Portugal during the three years studied is shown in Figure 2. For a large part, commercial strains were found in post harvest samples, indicating immediate dissemination (also shown Figure 1). The 296 strains collected had an identical genetic profile to only 9 commercial yeast strains from a total of 34 strains used in the six wineries. Although the industrial yeasts most commonly used in the wineries were usually collected in great abundance in the vineyard, no strict correlation between the utilisation level and the frequency of dissemination was evidenced. In example, the strain K1M ICV-INRA was the most widely used in the three French wineries and only one isolate out of 2160 isolates collection in France had an identical genetic pattern to this strain.

As a whole, the evolution of the fermentative yeast communities over the three years studied showed that the same strains were not found in the same sites from one year to another. This indicates that if some of these strains are able to remain in the ecosystem, as suggested by the presence of commercial yeasts in pre-harvest samples taken in 2001 in Portugal, they are not capable of dominating the natural yeast community of the vineyard. For exemple, five different commercial yeast strains were found in the pre-harvest campaign of winery D in 2001, namely the the predominantly used strains VL1, F10 and F15 and in much smaller quantities, the strains Uvaferm BDX and ICV D254, used from 1998-2000, thus showing their

survival in the vineyard from one year to another. However, given that the two later strains appeared in 2001 only, their permanence is limited.

Fig. 2. Evolution of the total fermentative yeast communities from each of the wineries (A, B, C, D, E, F) during the three years in pre- and post-harvest campaigns.



Conclusion

This systematic study has provided new insights in the impact of commercial yeasts on the communities of fermentative yeasts that inhabit surrounding vineyards. The methodology used, based on analysis of yeast community after spontaneous fermentation, permitted the isolation of a very large number of *Saccharomyces* wine yeasts, which are poorly found on the grapes. It is important to mention that among the 30 colonies analyzed per fermentation, the number of different genetic profiles varied from 1 to 21, with an average of about 5 different *Saccharomyces* biotypes per sample (Schuller *et al*, 2005; unpublished data), indicating that the number of colonies analysed per sample was high enough to show the initial biodiversity.

Based on these data, we conclude that the dissemination of commercial yeasts in the vineyard is restricted to short distances and limited periods of time. More than 90% of commercial yeasts were found at a radius between 10 and 200 m from the winery and did not become implanted in the ecosystem in a systematic way. Dispersal of commercial strains seems to be mainly mediated by water runoff and occurs also from macerated grape skin at dumping sites. Given that they are used in large quantities, commercial strains tend to out-compete autochthonous strains inside the winery (Beltran et al, 2002). In contrast, they do not seem to settle in the vineyard. Rather, they show natural fluctuations of periodical appearance and disappearance just like autochthonous strains do. Considering commercial yeast strains as an appropriate model system for genetically modified yeast strains, our data also contribute to the in-depth environmental risk assessment concerning the use of such strains in the wine industry.

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