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Dissemination and survival of commercial wine yeast in the vineyard: a large-scale, three years study

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Abstract

The use of commercial wine yeast strains as starters has been extensively generalised over the past two decades. In this study, a large-scale sampling plan was devised over a period of three years in six different vineyards to evaluate the dynamics and survival of industrial yeast strains in the vineyard. A total of 198 grape samples were collected at various distances from the wineries, before and after harvest, and yeast strains isolated after spontaneous fermentation were subsequently identified by molecular methods. 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. Our 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.

1. Introduction

The predominant yeast species used in the production of wine is *Saccharomyces cerevisiae*, universally known as "wine yeast". Under selective conditions of grape must fermentation, yeasts efficiently compete with other microorganisms present in musts, such as moulds and lactic and acetic acid bacteria. A succession of various yeast species – the apiculate yeasts *Hanseniaspora uvarum* (= *Kloeckera apiculata*) and other yeasts of the genera *Metschnikowia*, *Candida* or *Pichia* - is found in the early stages of fermentation [1]. As the concentration in ethanol increases, these species are rapidly outgrown by *S. cerevisiae* and related species, which invariably dominate the later stages of the process.

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 strains available today 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 an improvement in the quality of wine, and have enhanced the ability of winemakers to control the fermentation process and achieve specific outcomes.

As a result of modern winemaking practices and diversification of wine products, there is an increasing quest for specialised wine yeast strains. During the last two decades a considerable knowledge of *S. cerevisiae* genetics and physiology has been generated as well as numerous genetics tools. Recombinant DNA technologies have been successfully applied to wine yeast, generating specialized wine yeast strains which have been engineered for

specific traits, such as improved fermentation performance and process efficiency, wine sensory quality and health benefits for consumers [2-8].

From the perspective of a future possible use of genetically modified wine yeasts, a sound evaluation of the potential environmental impact of genetically modified wine yeast is absolutely required. In this context, industrial yeasts used as fermentation starters are a good study model to evaluate the competition and the influence of inoculated strains on the fermentations of the following years, especially those performed according to traditional practices which rely on spontaneous fermentations. 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.

There is very little available data that could contribute to the evaluation of the importance of starter yeast dissemination and permanence in the vineyard [9-11]. Recently, a large-scale biogeographical study in South African vineyards was carried out over four years. In five areas situated in the Coastal Region vineyards of the Western Cape 13 samples were collected and commercial yeasts were recovered from three samples [12, 13]. These studies have made it necessary to carry out this type of study on a larger scale, with the aim of increasing the statistic significance of the results obtained.

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.

2. Materials and methods

2.1. Sampling plan and wineries selection

Grapes were harvested during three consecutive years (2001-2003) in six vineyards, three of which were located in the south of France and three in the northwest of Portugal, as shown in Figure 1. In France, the wineries were located in the Languedoc-Roussillon Region, around the Mediterranean city of Montpellier, and the vineyards were situated at a distance of between 30 and 80 km. In Portugal, the three wineries were located in the north, centre and south of the Região Demarcada dos Vinhos Verdes, the distance between each being approximately 50 km. In each vineyard, six sampling points were defined according to the predominating wind direction at a distance of between 20 to 1000 m from the winery, as shown in Figure 1.

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. The gap between the pre and post harvest campaigns was 10 days, during which time waste water was released from the wineries. In the consecutive years, samples were always collected from the same area at a radius of 5 m. With the present experimental design, 72 grape samples were collected each year. The wineries selected have used one or more commercial yeast strains consecutively in at least the last five years. Tables 1 and 2 show the commercial yeasts used in each winery during the studied period (2001-2003) and their geographic origin respectively.

2.2. Sample collection and yeast isolation

From each sampling point, approximately 2 kg of grapes were collected aseptically and placed directly into sterile plastic bags, which were transported to the laboratory in cool bags. At the laboratory, grapes were crushed by hand in the plastic bags, which were opened and 180 ml of juice was poured into 250 ml sterile fermentors. The fermentors were placed in a temperature-controlled room at 20°C with mechanical agitation. 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^{-1}, corresponding to the consumption of about 2/3 of the sugar content. Must samples were diluted and spread on plates with YEPD medium (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v, agar 2% w/v), and after 2 days of incubation 30 randomly selected colonies were collected from each spontaneous fermentation.

2.3. Selection of Saccharomyces

To rapidly discriminate between *Saccharomyces* and non-*Saccharomyces* yeast, every isolate was evaluated according to its ability to grow in L-lysine [14]. All isolates that were not able to grow on the YNB medium with L-lysine as the sole nitrogen source but grew on the control medium YNB with ammonium sulphate were considered as *Saccharomyces* and selected for molecular identification.

2.4. Molecular identification methods

DNA was extracted from yeast cells cultivated in 1 ml YEPD medium (36 h, 28ºC, 160 rpm) as previously described [15] with a modified cell lysis procedure, using 25 U of Zymolase (SIGMA). Cell lysis was dependent on the strain and lasted between 20 minutes and 1 hour (37°C).

Mitochondrial DNA restriction profiles were established as previously described [16]. Digestions (*Hinf*I) were performed overnight at 37ºC in a final volume of 20 µl [17].

Microsatellite analysis was performed using six loci (ScAAT1-ScAAT6) previously described by Pérez *et al*. [18] that were amplified (Bio-Rad iCycler thermal cycler) in two multiplex reactions. The samples were denatured and separated by capillary electrophoresis in an ABI Prism 310 DNA sequencer (Applied Biosystems) and analysed using Genescan software. The complete method was described by Schuller *et al*. [17].

Chromosomal profiles were established by pulsed field gel electrophoresis (PFGE) using yeast chromosomal DNA prepared in plugs and the TAFE (transverse alternating field electrophoresis) system (Geneline, Beckman) as previously described [19]. The gels were run for 6 h at 250 V with 35 s pulse time followed by 20 h at 275 V with 55 s pulsed time, at a constant temperature (14°C).

3. Results and discussion

3.1. Sampling sites and isolation of Saccharomyces

A large sampling plan was followed: a total of 198 samples were collected during three consecutive campaigns (2001-2003), 108 of which were taken in France and 90 in Portugal. It is to be noted, as can be observed in Figure 1, that due to geographical constraints, the samples in Portugal were collected much closer to the winery than in France. In the French wineries (A, B and C), the sample sites were located at a distance of between 100 and 1000 m from the winery, whereas in the Portuguese wineries (D, E and F) half of the sampling sites were located at a distance of less than 70 m from the winery and none was located further than 400 m.

Table 3 shows the global data in each country broken down into years. 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 from these fermentations (2160 and 1620 in France and Portugal respectively).

Discrimination between *Saccharomyces* and *non-Saccharomyces* isolated in Languedoc was performed using a selective medium with L-lysine as the sole nitrogen source [14]. According to this method only 2 species of the genus *Saccharomyces* (i.e. *S. kluyveri* and *S. unisporus*), which do not occur in enological environments, are capable of growing with L-lysine. From this we concluded that yeasts isolated after fermentation, which can utilize L-lysine, do not belong to the genus *Saccharomyces*. To confirm this hypothesis isolates from the fastest fermentations that grew in L-lysine medium were identified by PCR-RFLP analysis of the rDNA ITS region [20]. The results confirmed that they were non-*Saccharomyces* yeast strains, belonging mainly to the genus *Kloeckera* (data not shown). All isolates not able to grow on the L-lysine medium were therefore selected for molecular identification. In Portugal, all isolates were assigned to different groups according to their

mtDNA RFLP pattern. One representative strain from each group was randomly withdrawn, and its ability to grow on L-lysine was tested. Based on these methods, 2355 *Saccharomyces* strains were selected from the 3780 isolates collected during the three years.

3.2. Geographic distribution of recovered commercial yeast strains

The global composition of the yeast population isolated after fermentation from the 6 wineries over the 3 years studied, in pre- and post-harvest campaigns, is shown in Figure 2. Table 4 shows the distribution and frequency of commercial yeasts in each vineyard.

Identification of *Saccharomyces* strains was performed by different molecular typing methods depending on the specific resources of each laboratory. An example of genetic profiles, both of natural isolates and commercial yeast strains from France and Portugal is shown in Figure 3. Chromosomal pattern analysis of 735 *Saccharomyces* isolates from France (wineries A, B and C) was performed, and compared with that of the 19 commercial yeasts used in the 3 wineries. In Portugal, all 1620 isolates were analysed by mtDNA RFLP (*Hinf*I), and their patterns compared to those of a strain collection including all strains used by the three wineries. At least one representative isolate of each group of strains showing identical mtDNA RFLP patterns to commercial strains was further confirmed by microsatellite analysis. In order to evaluate the discriminatory power of these three methods, we have previously performed a survey of the genetic polymorphisms generated by distinct methods on a total of 23 commercial yeast strains used in the wineries of the two countries [17]. The results showed that the discriminatory power of microsatellite typing using these six different loci and that of mtDNA RFLP patterns generated by the enzyme *Hinf*I was the same and similar to that of karyotype analysis. Among the 23 commercial yeast strains analysed, 21

different patterns were obtained using the first two methods and 22 using the last. 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.

The analysis of genetic profiles of 2355 out of 3780 *Saccharomyces* isolates resulted in the identification of 296 commercial yeasts, representing 7.8% of the fermentative yeast community (Table 4), the majority of which (5.8%) were recovered in post-harvest campaigns (Fig. 2). It should be noted that in this study, fermentation is used as an enrichment tool for *Saccharomyces* strains. Therefore, 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, but reflect only those strains that could possibly have some enological use. 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 (Tables 3 and 4).

These global data reflect very different situations. In the 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 4). In France the genetic profile of 16 clones out of 735 *Saccharomyces* isolates (2%) was identical to that of commercial yeasts (Fig. 2). 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 (Fig. 2). 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 (Table 2) where the study was carried out. No commercial yeasts were found

from winery A and one colony, isolated in 2003 in winery C (site 3), 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. The same situation occurs in the Portuguese winery F, where only two isolates with the same profile as the extensively used commercial yeast, Zymaflore VL1, in use for five years, were found. Since strain ICV D254 was initially isolated in the region from which it was recovered, dissemination in these four vineyards was proofed only by the presence of three isolates (0.1% of fermentative flora), one of K1M ICV-INRA and two of Zymaflore VL1. It could be considered that their presence is due to immediate dissemination, probably mediated by insects or another occasional dissemination vector. It is, in any case, evident that the presence of the most widely used commercial yeast for the last 5-10 years in French (i.e. K1M ICV-INRA) and in Portuguese wineries (i.e. Zymaflora VL1) was incidental and that it does not ever dominate the microflora of any of these four vineyards. These results, in accordance with those obtained previously in South African vineyards [12, 13], indicate a very poor level of dissemination/implantation of commercial yeast in the vineyard ecosystem. The results were very different in the Portuguese wineries D and E, for which a high number of commercial strains was isolated from 20 spontaneous fermentations, representing 43 and 10% of the fermentative yeast community respectively. Indeed, the large majority (94%) of the commercial strains isolated within the six vineyards were recovered from these two vineyards only, and 70% solely from vineyard D. It can be observed from Figures 1 and 2 that the majority of the commercial strains in these 2 vineyards were recovered from sites closest to the winery, namely sites 4, 5 and 6 in vineyard D and sites 1, 2 and 6 in vineyard E. The major difference between these two vineyards and the four others is that the sample sites in the first two were placed in close proximity to the winery (Fig. 1). In addition, the presence of water runoff in these sites

indicates that dissemination is probably largely favoured by liquid effluents. In vineyard D, due to the ground inclination, water runoff flowing from the winery to the vine may contribute to the frequent occurrence of commercial strains in these sites. It is also noteworthy that site 1 of winery E, where the highest number of VL1 strains was recovered, is located close to a rill that transports runoff water from the winery, emphasizing the importance of water as a vehicle for yeast strain dissemination. Furthermore, the dumping site of macerated grape skins is adjacent to site 1, constituting a fermenting sugary substrate harbouring large amounts of yeast that are distributed throughout the vineyard.

An overview of the dissemination of commercial strains in relation to their distance from the winery is shown in Figure 4. Nine 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. With the exception of the autochthonous ICV D254 strain collected in French winery B, commercial yeasts in pre-harvest campaigns were only collected in sites very close to winery D (10-50 m) and the strain found in the greatest quantity (87%) was Zymaflore F15. In the post-harvest samples, strain VL1 represented 49% of commercial strains recovered after harvest. This strain was derived from sites close to the area where macerated grape skin was deposited or water runoff occured, and never further than 10-20 m from the winery (Figs 1 and 2). A lower percentage of other predominant strains Zymaflore F10 and F15, the formerly used minority strains Uvaferm BDX and ICV D254, and the autochthonous strain Lalvin QA23, were found at sites closer to the winery (10-50 m). Zymaflore F15, F10 and ICV D254 were also found at about 100 m from the winery. The occurrence of several isolates found at 200 m (site 1, winery D) can be attributed to the presence of a small building for storage of harvest transport equipment. Two samples taken in France at a distance of 400

and 1000 m contained yeasts with an identical karyotype to that of indigenous strain ICV D254. In very rare cases, dissemination to sites located further from the winery (i.e. 2 isolates at 400 m and 1 at 1000 m from a total of 3780 strains) was revealed and may be attributable to other factors, such as insects or wind.

3.3. Dissemination of commercial strains as a function of their utilisation

As shown in Table 5, 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. In most instances, the strains with a profile similar to a commercial strain were recovered from a vineyard in which the same commercial yeast was used, except for ICV D254 and Uvaferm BDX, which were collected in vineyard D and not used during the study. However, these strains were used previously (1998-2000) in the same vineyard. The other exception was strain Lalvin QA23, which was used in vineyard A and collected only from vineyard E. Since this strain was initially isolated in this Portuguese region, the most likely explanation is that the strain isolated in vineyard E is not the result of dissemination but that is a member of the indigenous yeast community.

The industrial yeasts most commonly used in the wineries were usually collected in great abundance in the vineyard. However, this was not always the case, because 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. In Portuguese wineries, Zymaflore VL1 was predominantly and continuously used for more than 10 years, followed by Zymaflore F10 and VL3 (Table 1). The strains VL1 and F10 were frequently recovered, but this could be due to the fact that the sites where they had been collected were located in close proximity to the winery (i.e. vineyards D or E, Fig. 2). The

strain Zymaflore F15, although frequently collected in the same vineyard D, was used to a lesser extent. Over the period of the study, Zymaflore VL3 was also widely used in Portuguese wineries, and the genetic profile of only one isolate was identical to this commercial yeast. As a whole these data indicate that there is no strict correlation between the utilisation level and the frequency of dissemination.

3.4. Evolution of fermentative yeast community

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 5. From a total of 296 commercial yeasts recovered during this period in the six vineyards, 76% were found in 2001, in pre- and post-harvest samples collected in vineyard D and post-harvest samples collected in vineyard E. In the following two years commercial yeasts were detected only in certain post-harvest but not in pre-harvest samples. As can be observed in Figure 5, five different commercial yeast strains were found in the pre-harvest campaign of winery D in 2001, namely 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.

The commercial yeasts collected in each site were nevertheless different. The highest number of Zymaflore VL1 isolates was obtained from grapes collected after harvest at site 4, whereas in samples collected before harvest, VL1 and F10 occurred rarely. This contrasts to the abundance of the strain F15 in the pre-harvest campaign in 2001 sites 4 and 6 (Fig. 2). These data may suggest a better ability of strain Zymaflore F15 to remain in the vineyard, although

no isolate of strain F15 was found in 2002 and only one in 2003 (Figs 2 and 4). In this winery no samples were collected in the post-harvest campaign of 2002, and a lower quantity of commercial yeasts was found in 2003. In addition, the presence of one isolate of Zymaflore VL3, not present in 2001, was detected. In the post-harvest campaign of 2001, two commercial yeast strains, Zymaflore VL1 and the autochthonous yeast Lalvin QA23, were isolated in winery E. This last strain was the only commercial yeast found in the same winery in 2002, but it was not present in 2003. Contrarily, Zymaflore VL1 was not found in this winery in 2002, but was present in 2003, although in lower proportions. The situation observed in Portuguese winery F, as described previously, was similar to that in French wineries. No commercial yeasts were detected in 2001 and 2002, and only two isolates of Zymaflora VL1 were found in 2003. In winery B, autochthonous strain ICV D254 was found in the pre-harvest campaign in 2001, but did not occur in the following years. Only one isolate of K1M ICV-INRA was found in 2003 in winery C. 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 has been 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.

In 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. A significant number of non-*Saccharomyces*strains was also found in the spontaneous fermentations, from the French samples but not from the

Portugese grape musts (Table 3). Climatic factors and differences in phytosanitary treatment may be the reason for these discrepancies. In future studies, the occurrence of non *Saccharomyces* during fermentation could be reduced by adding SO_2 to the grape musts prior to fermentation. 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 [22, and unpublished data]. This reflects great differences in the samples regarding the presence of *Saccharomyces* With regard to the initial biodiversity, these data also show that the number of colonies analysed per sample was appropriate. For future studies, increasing the initial amount of grapes collected may increase the number of spontaneous fermentations and therefore of *S. cerevisiae* strains isolated.

Data obtained in the present study show that 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. This situation was observed during the habitual functioning of a winery, where commercial strains are used without any containment. Avoiding grape-skin deposition and canalisation of water-runoff are low-cost measures, which are able to reduce significantly the population sizes number of commercial yeast strains around the winery.

Given that they are used in large quantities, commercial strains tend to out-compete autochthonous strains inside the winery [21]. 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. Moreover, vine-associated autochthonous *Saccharomyces* biodiversity is not affected by long-term use of commercial yeasts [22]. Considering commercial yeast strains as an appropriate model system for genetically modified yeast

strains, our data can contribute to the in-depth environmental risk assessment concerning the use of such strains in the wine industry.

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References

[1] Fleet, G. H. and Heard, G. M. (1993) Yeast-Growth during fermentation. In: Wine microbiology and biotechnology (Fleet, G.H., Ed.), pp. 27-54. Harwood Academic Publishers. Singapore.

[2] Dequin, S., Salmon, J.M., Nguyen, H.V. and B. Blondin. (2003) Wine yeast's. In: Yeasts in Food, beneficial and detrimental aspects (Boekhout, T., Robert, V. Eds.), pp. 389-412. B. Berhr's Verlag GmbH and Co Hamburg, Germany.

[3] Butzke, C. E. and Bisson, L. F. (1996) Genetic engineering of yeast for wine production. Agro Food Industry Hi-Tech. 7, 26-30.

[4] Dequin, S. (2001) The potential of genetic engineering for improving brewing, wine-making and baking yeasts. Appl. Microbiol. Biotechnol. 56, 577-588.

[5] Henschke, P. A. (1997) Wine yeast. In: Yeast sugar metabolism(Zimmermann, F. K. and Entian, K. D., Eds.), pp. 527-560. Technomic Publishing Co. Lancaster, Pensylvania.

[6] Pretorius, I. S. (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16, 675-729.

[7] Pretorius, I. S. and Bauer, F. F. (2002) Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. 20, 426-432.

[8] Querol, A and Ramon, D. (1996) The application of molecular techniques in wine microbiology. Trends Food Sci. Technol. 7, 73-78.

[9] Frezier, V. and Dubourdieu, D. (1992) Ecology of yeast strain *Saccharomyces cerevisiae* during spontaneous fermentation in a Bordeaux winery. Am. J. Enol. Vitic. 43, 375-380.

[10] Guillamón, J.M., Bairro, E. and Querol, A. (1996) Characterization of wine yeast strains of the *Saccharomyces* genus on the basis of molecular markers; relationships between genetic distance and geographic or ecological origin. System. Appl. Microbiol. 19, 122-132.

[11] Vezinhet F., Hallet, J. N., Valade, M. and Poulard, A. (1992) Ecological survey of wine yeast strains by molecular methods of identification. Am. J. Enol. Vitic. 43, 83-86.

[12] Van der Westhuizen, T.J. Augustyn, O.P.H. and Pretorius, I.S. (2000a) Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the Coastal Regions of the Western Cape in South Africa. S. Afr. J. Enol. Vitic. 21, 3-9.

[13] Van der Westhuizen, T.J. Augustyn, O.P.H., Khan, W. and Pretorius, I.S. (2000) Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards of the Western Cape in South Africa. S. Afr. J. Enol. Vitic. 21, 10-16.

[14] Barnett J. A., Payne, R. W. and Yarrow, D. (1990) Yeasts: Characteristics and identification. 2nd Ed. Cambridge University Press, Cambridge, UK.

[15] López, V., Querol, A., Ramón, D. and Fernández-Espinar, M.T. (2001) A simplified procedure to analyse mitochondrial DNA from industrial yeasts. Int. J. Food Microbiol. 68, 75-81.

[16] Querol, A., Barrio, E. and Ramon, D. (1992) A comparative study of different methods of yeast strain characterization. Syst. Appl. Microbiol. 15, 439-446.

[17] Schuller, D., Valero, E., Dequin, S. and Casal, M. (2004) Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiol. Lett. 231, 19-26.

[18] Pérez, M.A., Gallego, F.J., Martinez, I. and Hidalgo, P. (2001) Detection, distribution and selection of microsatellites (SSRs) in the genome of the yeast *Saccharomyces cerevisiae* as molecular markers. Lett. Appl. Microbiol. 33, 461-466.

[19] Blondin, B. and Vezinhet, F. (1988) Identification de souches de levures oenologiques par leurs caryotypes obtenus en électrophorèse en champs pulsées. Rev. Fr. Oenol. 28, 7-11.

[20] Granchi, L, Bosco, M. Messini, A, and Vicenzini, M. (1999) Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. J. Appl. Microbiol. 87, 949-956.

[21] Beltran, G., Torija, M. J., Novo, M., Ferrer, N., Poblet, M., Guillamon, J. M., Rozes, N. and Mas, A. (2002) Analysis of yeast populations during alcoholic fermentation: A six year follow-up study. Syst. Appl. Microbiol. 25, 287-293.

[22] Schuller, D., Alves, H., Dequin, S. and Casal, Margarida. (2005) Ecological survey of Saccharomyces strains from vineyards in the Vinho Verde Region of Portugal. FEMS Microbiol. Ecol. 51, 167-177.

Figure Legends

Fig. 1. Geographic localization of the vineyards belonging to the Languedoc (A, B, C) and Vinho Verde (D, E, F) wine regions with an indication of the sampling sites in each of the six vineyards. In each site, 2 samples (pre- and post-harvest campaign) were collected. Factors that may influence the dissemination of the yeasts are indicated in the figure.

Fig. 2. Global composition of the yeast communities isolated from each site at the six wineries during the pre- and post-harvest sampling campaigns over the three years. The motifs show the presence of commercial yeasts, light grey indicates other *Saccharomyces* strains and dark grey the non-*Saccharomyces* strains. Nf: no fermentation; Nc: not collected.

Fig. 3. Examples of molecular fingerprinting of commercial yeast and natural isolates (indicated by numbers). (a) Chromosomal profiles of commercial yeast and natural isolates from France. Profiles 4, 5 and 6 are identical to ICV-D254. (b) mtDNA RFLP profiles of commercial yeast and natural isolates from Portugal. Profiles 245, 13, 5, 105, 157, 12 and 32 are identical to Zymaflore VL3, F10, F15, Lalvin QA23, ICV-D254, Zymaflore VL1 and Uvaferm BDX respectively.

Fig. 4. 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.

Fig. 5. 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.

Table 1

Commercial yeast strains used in each winery during 2001-2003. All strains are *Saccharomyces cerevisiae* strains.

Year	Winery A	Winery B	Winery C	Winery D	Winery E	Winery F
2001	**K1M ICV-INRA*** **ICV D254** **Enolevure K34** **Lalvin QA23** **ICV D47**	**K1M ICV-INRA** **ICV D254** ICV D80 Uvaline BL Lalvin BM45 Maurivin AWRI2	**K1M ICV-INRA** **Zymaflore VL3** **Maurivin PDM** ICV D254 ICV D47 Uvaline arôme Vitilevure-Chardonnay Anchor VIN 13	**ZymafloreVL1** **ZymafloreVL3** **Zymaflore F10** Zymaflore F15 Uvaferm L2056 Lalvin CY 3079 Uvaferm ALB Uvaferm 228	**Zymaflore VL1** Lalvin EC 1118	**Zymaflore VL1**
2002	**K1M ICV-INRA** **ICV D254** **Lalvin QA23**	**K1M ICV-INRA** ICV D80 Uvaline BL Lalvin BM45 Maurivin AWRI2 Uvaline CVR	**K1M ICV-INRA** **Maurivin PDM** ICV D47 Anchor VIN 13 Zymaflore VL3 Anchor NT 116 Vitilevure-Sauvignon	**ZymafloreVL1** **ZymafloreVL3** **Zymaflore F10** Zymaflore F15 Uvaferm ALB Uvaferm 228 Uvaferm CS2	**Zymaflore VL1** Lalvin EC 1118 Levuline BRG Fermichamp	**Zymaflore VL1** Lalvin EC 1118
2003	**K1M ICV-INRA** ICV D254 Enolevure K34	**K1M ICV-INRA** Uvaline BL Lalvin BM45 Anchor NT 45 Anchor NT 50 ICV D80 Uvaline CVR Enolevure K34 Maurivin PDM	**K1M ICV-INRA** **Zymaflore VL3** **Maurivin PDM** Vitilevure-Chardonnay Vitilevure-Sauvignon	**ZymafloreVL1** **ZymafloreVL3** **Zymaflore F10** Zymaflore F15 Zymaflore VL2 Uvaferm ALB	**Zymaflore VL1** Fermafine Fermafruit IOC 18-2007 Lalvin CY 3079	**Zymaflore VL1** Lalvin CY 3079

*The strains shown in bold were used for at least the last 5 years prior to the study.

Table 2

Geographic origin of commercial yeast strains used in the wineries studied

Strains	Origin
Anchor NT 45	South Africa
Anchor NT 50	South Africa
Anchor NT 116	South Africa
Anchor VIN 13	Stellenbosch, South Africa
Enolevure K34	Valencia, Espagne
Fermafine	Not Known
Fermafruit	Not Known
Fermichamp	Alsace, France
ICV D 47	Rhône, France
ICV D 80	Rhône, France
ICV D 254	Languedoc, France
IOC 18-2007	Not Known
K1M ICV-INRA	Languedoc, France
Lalvin BM 45	Sangiovese, Italy
Lalvin EC1118	Champagne, France
Lalvin QA23	Portugal
Lalvin Cy 3079	Bourgogne, France
Levuline BRG	Not Known
Maurivin AWR12	Bordelais, France
Maurivin PDM	Champagne, France
Uvaferm 228	France
Uvaferm ALB	Not Known
Uvaferm CS2	Alsace, France
Uvaferm L 2056	Rhône, France
Uvaline arôme	Loire, France
Uvaline BL	Champagne, France
Uvaline CVR	Not Known
Vitilevure Chardonnay	Languedoc, France
Vitilevure Sauvignon	Sauvignon, France
Zymaflore F10	Bordelais, France
Zymaflore F15	Gironde, France
Zymaflore VL1	Gironde, France
Zymaflore VL2	Burgundy, France
Zymaflore VL3	Gironde, France

Table 3

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

Table 4

Commercial yeast strains recovered in each vineyard over the 3 years studied

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

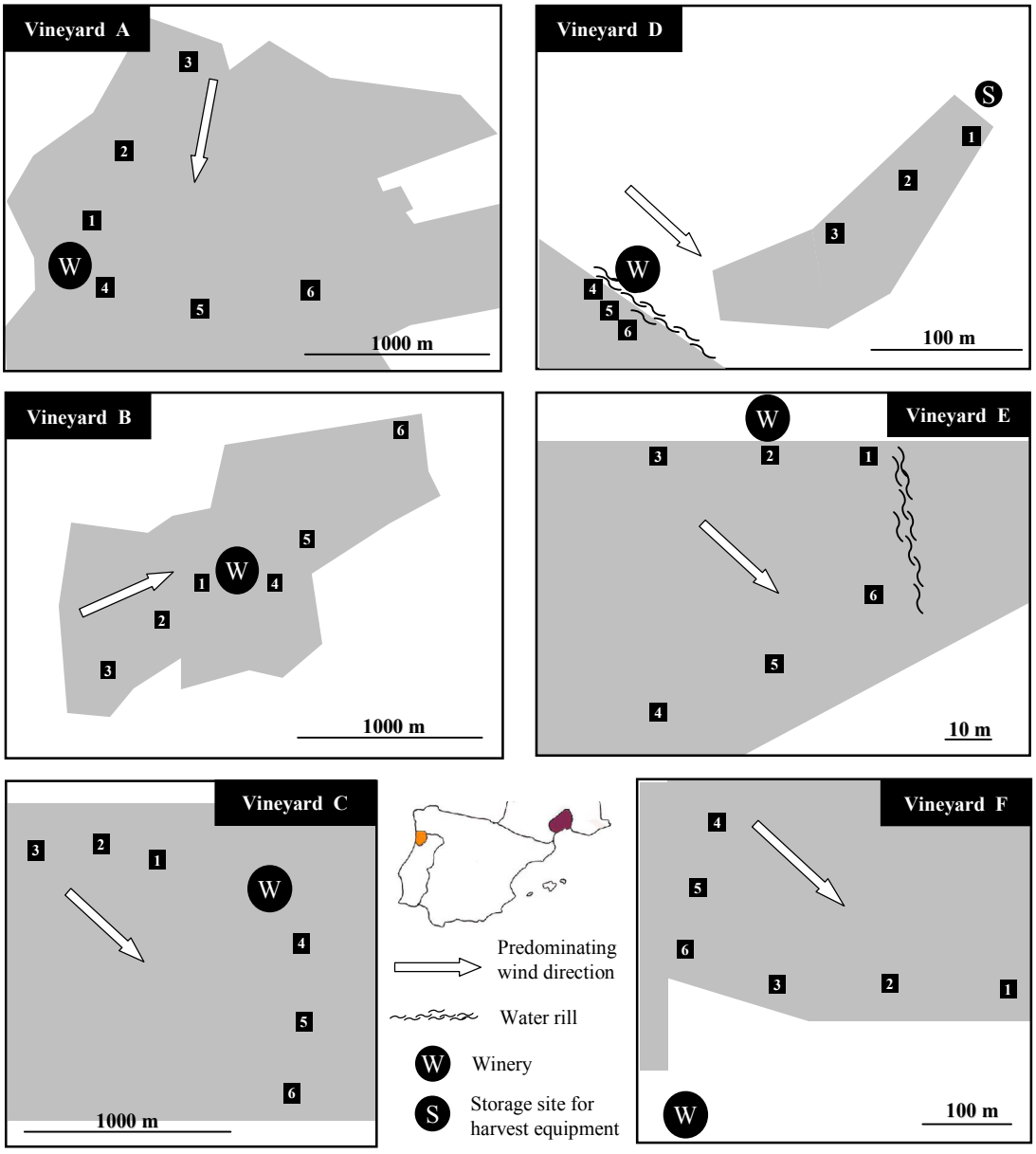
*Commercial yeasts initially isolated in the same region

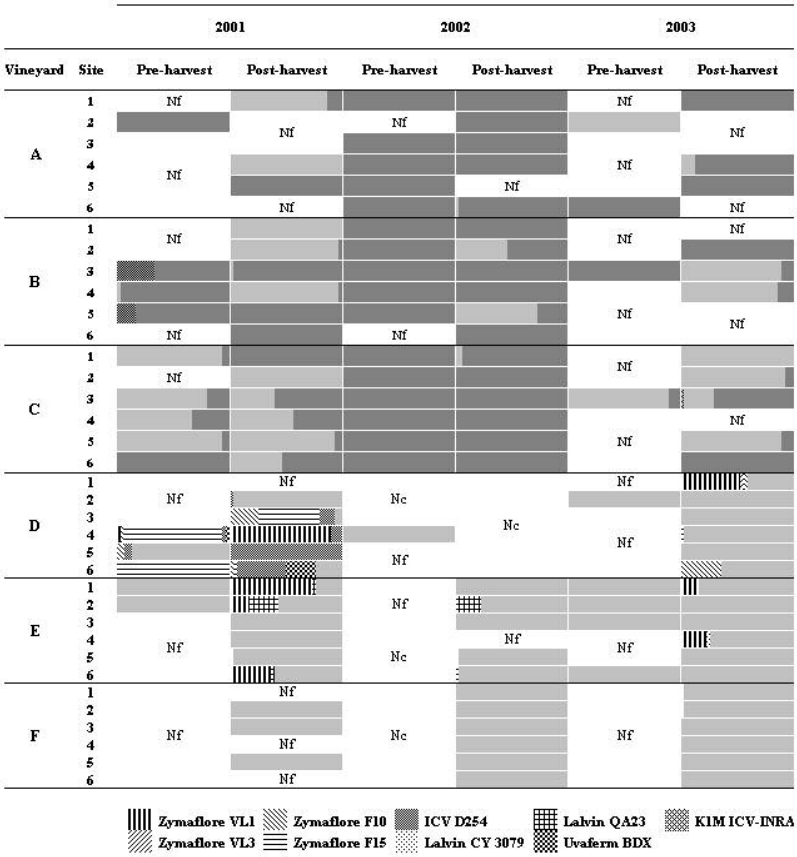
Table 5

Origin of 296 strains with genetic patterns identical to commercial yeast strains used in the wineries

Commercial wine strains	Nb strains with identical genetic pattern	Wineries were these strains are used	Vineyard were these strains are collected	Utilization level during the 3 years
Zymaflore VL1	99	D, E, F	D, E, F	+++
Zymaflore F15	74	D	D	+
ICV D254	68	A, B, C, D*	B**, D	++
Zymaflore F10	24	D	D	++
Lalvin QA23	19	A	E**	+
Uvaferm BDX	9	D*	D	-
K1M ICV-INRA	1	A, B, C	C	+++
Zymaflore VL3	1	C, D	D	+
Lalvin CY 3079	1	D, E, F	E	+

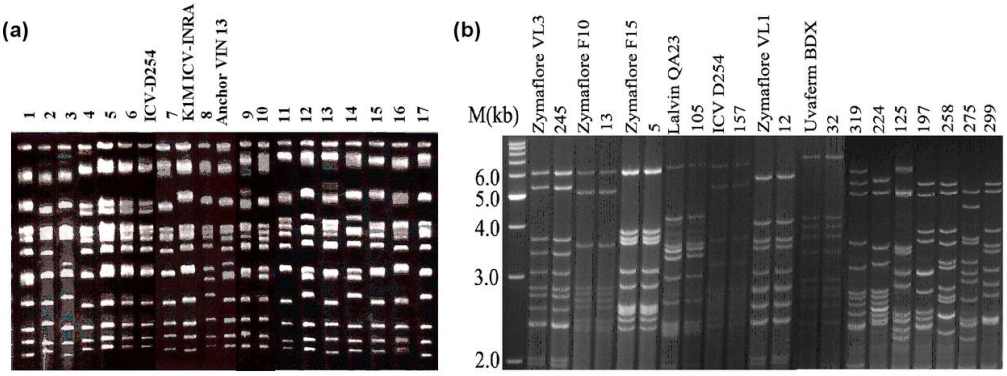
*Used before the study **Isolated in the same region


Vineyard A
Vineyard D
Vineyard B
Vineyard E
Vineyard C
Vineyard F
W
S
1000 m
100 m
10 m
Predominating wind direction
Water rill
Winery
Storage site for harvest equipment



Global composition of the yeast communities isolated from each site at the six wineries during the pre- and post-harvest sampling campaigns over the three years. The motifs show the presence of commercial yeasts, light grey indicates other Saccharomyces strains and dark grey the non-Saccharomyces strains. Nf: no fermentation; Nc: not collected.

190x254mm (96 x 96 DPI)



Examples of molecular fingerprinting of commercial yeast and natural isolates (indicated by numbers). (a) Chromosomal profiles of commercial yeast and natural isolates from France. Profiles 4, 5 and 6 are identical to ICV-D254. (b) mtDNA RFLP profiles of commercial yeast and natural isolates from Portugal. Profiles 245, 13, 5, 105, 157, 12 and 32 are identical to Zymaflore VL3, F10, F15, Lalvin QA23, ICV-D254, Zymaflore VL1 and Uvaferm BDX respectively.

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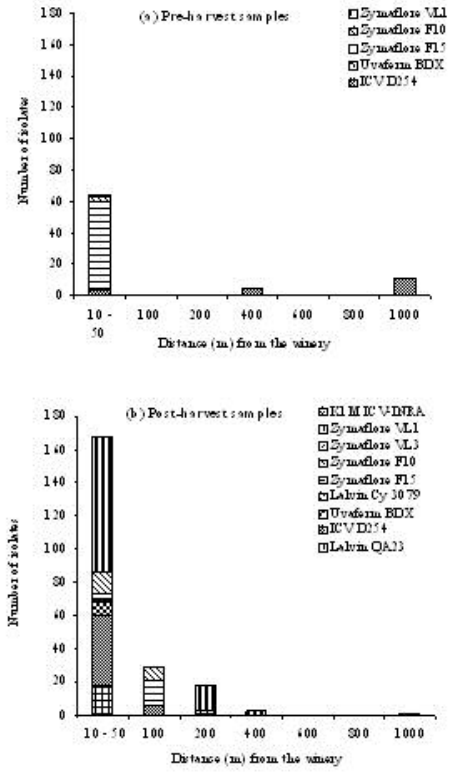
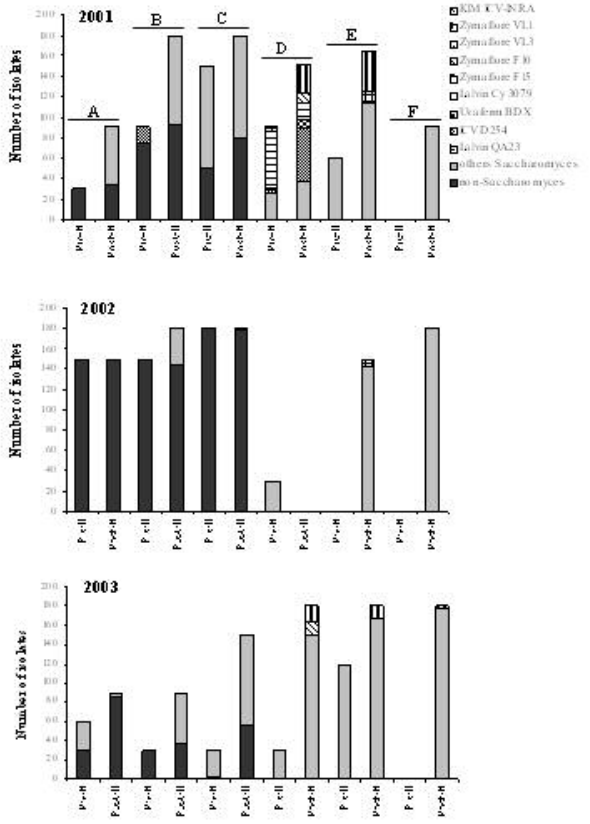
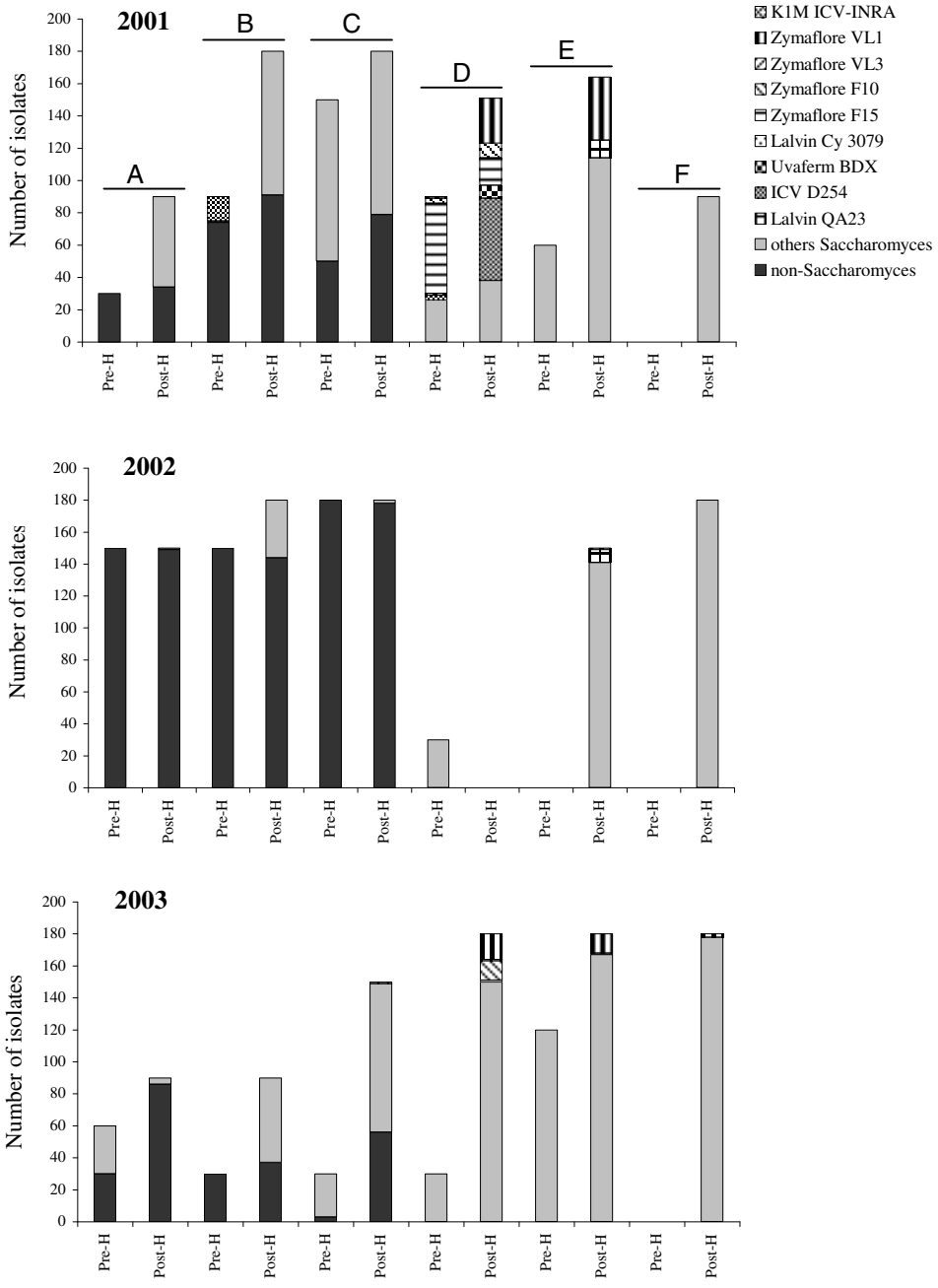


Overall (three years) distribution of commercial yeast strains according to the distance from the wineries in pre-harvest (a) and in post-harvest (b) campaigns.
190x254mm (72 x 72 DPI)



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.
190x254mm (72 x 72 DPI)


2001
2002
2003
A
B
C
D
E
F
Number of isolates
Pre-H
Post-H
K1M ICV-INRA
Zymaflore VL1
Zymaflore VL3
Zymaflore F10
Zymaflore F15
Lalvin Cy 3079
Uvaferm BDX
ICV D254
Lalvin QA23
others Saccharomyces
non-Saccharomyces