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Study of Some *Saccharomyces cerevisiae* Strains for Winemaking after Preadaptation at Low Temperatures

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Low-temperature fermentations (13 °C) are considered to improve wine aromatic profiles. However, because the risk of stuck and sluggish fermentations is high, these fermentations are not common. The aim of this paper was to analyze the effect of different preadaptation protocols in two commercial wine strains on the fermentation and some wine parameters. Preadaptation is understood to be the process between the rehydration of active dry yeast and the inoculation. In this study, it consisted of preparing a fermentation starter (addition of yeast grown at 25 °C) or inocula preadapted at low temperatures (as before, but grown at a fermentation temperature of 13 or 17 °C). These results were compared with those of rehydrated active dry yeast, and a commercial "cryotolerant" yeast was used as a reference. General fermentation kinetic parameters, yeast imposition, nitrogen consumption, and main wine products were analyzed. The results showed that the preadaptation of a yeast could improve the fermentation performance, although this improvement was strain-dependent. Low-temperature fermentations also had some general effects: reduction of acetic acid and fusel alcohol production and increased concentrations of glycerol. When the yeast performed better in fermentation because of preadaptation, nitrogen consumption was faster and the wine's "negative" attributes (acetic acid, fusel alcohols) were significantly reduced. Thus, in some strains, preadaptation could be an effective mechanism for improving low-temperature fermentation, which also significantly reduces detrimental wine attributes.

KEYWORDS: ***Saccharomyces cerevisiae*; ammonium; fusel alcohols; acetic acid; esters**

INTRODUCTION

It is well-known that fermentation at low temperatures (<15 °C) leads to more aromatic and paler wines (*1*, *2*). These characteristics are appreciated in white and rosé wines. Although this enological practice is well established in some traditional European processings such as Sauternes, Jura, Amarone, Vinosanto, and the second fermentation of sparkling wines (*3*, *4*), low-temperature fermentations can be problematic in musts from southern European regions. These musts typically have a high sugar content and a low nitrogen availability for yeast. In addition, at low temperatures, competition for nutrients is higher between the inoculated yeast and the non-*Saccharomyces* species (*5*). Altogether this means that fermentations can be stuck or sluggish (*6*). Low temperatures increase the duration of alcoholic fermentation, decrease the rate of yeast growth, and modify the ecology of wine fermentation (*7*). Indigenous non-*Saccharomyces* species are more likely to contribute more to fermentations conducted below 20 °C. Such ecological influences should be reflected in the chemical composition and sensory properties of the wine (*5*, *8*). Therefore, although low-temperature fermentations have interesting applications in the enological industry, they also have an adverse effect on cell growth, because they increase the yeast stress during wine production (*2*). Wine technology also makes it possible to inoculate a selected yeast so that the alcoholic fermentation can be better controlled (*9*). The inoculation of must that is to be fermented with active dry yeasts involves previous rehydration with lukewarm water (35−42 °C). This process and the inoculation of rehydrated yeasts in a colder must can involve physiological stress for inoculated yeasts, which affects the correct operation of the alcoholic fermentation (*2*). In fact, the first effect of fermentations at low temperatures is the extended lag phase (*10*). Various solutions have been proposed for solving this problem. Du Plessis (*11*) proposed that active dry yeast rehydration could be used to lower temperatures and, in this way, obtained faster beginnings of fermentation. However, success in yeast rehydration is related to the temperature of the water that prevents cell leakage (*12*). On the other hand, Argiriou et al. (*3*) preadapted cryotolerant yeast to the fermentation temperature before it was inoculated in low-temperature alcoholic fermentation.

On the molecular level, there is some evidence to show that yeast cells can adapt to stress after the temperature downshift.

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Thus, membrane lipid composition is modified to correct the changes in fluidity caused by low temperatures (*13*, *14*). In fact, the membrane fatty acid composition is highly variable and clearly influenced by such environmental factors as temperature, oxygen, nutrient limitation, and growth rate (*15*, *16*). The membrane fatty-acyl composition of yeasts, like that of many other microorganisms, changes with temperature: the lower the temperature, the more unsaturated the membrane fatty-acyl composition (*17*). Some other metabolites and enzyme activities have been shown to be modified in low-temperature fermentations such as glycerol (*18*, *19*), storage carbohydrates such as trehalose or glycogen (*10*, *20*), nitrogen assimilation and metabolism, etc.

Thus, yeast metabolism is strongly affected by temperature. Adaptation through several media and temperatures before inoculation can affect not only the ability of the cells to survive but also the final composition and quality of the wines. Accordingly, the aim of this work is to evaluate how effectively various commercial yeasts adapt to low temperatures. To make the differences more evident and relevant, we have considered two of the most widely used commercial strains in Spain, which are recommended for different kinds of fermentations (multiuse, second fermentation in sparkling wines), and a very problematic must, which is normally low in nitrogen (*21*). Thus, the main interest of the present work is to analyze if there is an improvement by yeast preadaptation on fermentation kinetics and on the final quality of wines in conditions that are known to be problematic.

MATERIALS AND METHODS

Must. Carinyena, a red variety from Penedès (Miguel Torres cellar, Vilafranca del Penedès, Spain), was used. This must is prone to difficult fermentations as shown in a previous study (*21*). After the grapes were crushed, the must was macerated in the presence of 60 mg of SO_2 L^{-1}. After 8 h, the must was drawn, and the clear must was inoculated with the different strains and conditions tested.

Yeast Strains. Three wine yeast strains were used: strain A, commercially identified as *Saccharomyces cerevisiae*; strain B, commercialized as *Saccharomyces bayanus*; and strain C, commercialized as *Saccharomyces uvarum* cryotolerant strain. However, they were all identified as *S. cerevisiae* by the RFLP-mtDNA method (*22*). The yeasts were inoculated at 2×10^6 cells mL^{-1} after the different adaptation procedures.

Fermentation Conditions. Laboratory scale fermentors (750 mL) were sealed with a cotton cap, and semianaerobiosis conditions were assumed. The fermentations were carried out at the usual cellar temperature of 17 °C and at a low temperature of 13 °C in a Hot−Cold incubator (Selecta, Barcelona, Spain) without stirring. The experimental trials were carried out in duplicate.

Adaptation Conditions. Three different conditions were monitored for each strain (strains A and B) and for each fermentation temperature (17 and 13 °C): (i) dry activated yeast cells were rehydrated at 10% (w/v) in 1% (w/v) glucose at 37 °C for 30 min according to the manufacturer's procedures; (ii) cells were rehydrated as before and then subcultivated three times at the fermentation temperatures; (iii) cells were rehydrated as in (i) and then subcultivated three times at 25 °C (fermentation starter effect). The cryotolerant strain was used as a control to compare results, and it was only rehydrated only before it was inoculated in the must.

The subcultures were carried out in liquid YEPD medium (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract) in 250 mL Erlenmeyer flasks, in an agitated incubator (Selecta). The glucose concentration was 2% as this concentration was needed to sustain growth, instead of 1% as in the rehydration. The first flask was inoculated with 2×10^6 cells mL^{-1} of rehydrated yeast. At the end of the exponential phase determined by the absorbance at 600 nm, another flask was inoculated with a small volume to obtain 2×10^6 cells mL^{-1}. The process was repeated, and then the Carinyena must (700 mL) was inoculated with 2×10^6 cells mL^{-1}. The must was supplemented with 0.2 g L^{-1} ammonium diphosphate and 0.2 g L^{-1} Fermaid K (Lallemand Inc., Montreal, Canada).

Sampling and Yeast Growth Measurement. Samples from a Carinyena must fermentation were collected after the fermentors had been stirred for 5 min on alternate days. Growth was determined by plating a suitable dilution of inoculated must on YEPD medium (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract, 20 g L^{-1} agar) and counting the colony-forming units (CFU). Incubation time was ~2−3 days at 28 °C.

Identification of Yeast Strains. Samples were taken at the end of fermentation. Aliquots (100 μL) of several dilutions were plated on YEPD agar medium and incubated at 28 °C for 2 days. Thirty colonies from each sample were analyzed randomly. The *Saccharomyces* and non-*Saccharomyces* species were separated on both lysine and YEPD selective culture media (*23*). The *Saccharomyces* group was analyzed by mtDNA restriction analysis using Querol et al.'s method (*22*). The other yeasts were analyzed by rDNA restriction analysis using Guillamón et al.'s method (*24*). Samples for microbiological analysis were taken at the beginning of the fermentation (after inoculation, day 1), at the middle of fermentation (MF, day 9 at 13 °C and day 6 at 17 °C), and at the end of fermentation (END, residual sugars <0.2%).

Must Analysis. The titratable acidity, expressed as grams of tartaric acid, total sulfur dioxide content, and pH were determined in the must according to OIV methods (*25*). The sugar concentrations were measured using the modified Rebelein method (*26*). During the fermentation, sugar consumption was estimated by the weight loss produced by the CO_2 released. This method was validated by using the enzymatic method to analyze the sugar content of several samples (glucose-fructose kit; Boehringer Mannheim, Mannheim, Germany). The maximum fermentation rates were determined by calculating the slopes of the linear part of the fermentation kinetics (sugar content against time). The ethanol content was measured by near-infrared spectrophotometry using the InfraAnalyzer 400 (Technicon, Dublin, Ireland) (*27*). The ammonium content of the must and wine was quantified using an enzymatic method (Boehringer Manheim). The yeast available nitrogen (YAN) was measured according to the Sorensen method (*28*) using formaldehyde. Amino acids were quantified according to the modified ninhydrin method (*6*) using glycine as standard, so the results are expressed in terms of milligrams of of Gly per liter. Acetic acid, acetaldehyde, glycerol, and succinic acid were determined using enzymatic kits from Boehringer Manheim.

Fusel alcohols were determined according to Bertrand's method (*29*). Analytical GC was carried out on a Hewlett-Packard 4890A connected to a Hewlett-Packard Integrator 3393A equipped with a flame ionization detector (Agilent Technologies, Wilmington, DE). The wine sample (1 μL) was injected (split 1:30) into a CP-Wax 57CB column of 50 m × 0.25 mm and 0.2 μm phase thickness (Chrompack, Middelburg, The Netherlands). The temperature program went from 30 °C (5 min) at 200 °C (20 min) at 3 °C min^{-1}. Injector and detector temperatures were 220 and 280 °C, respectively. The carrier gas was helium at 1 mL min^{-1}. Fusel alcohols were identified and quantified by comparison with the corresponding standards.

To determine volatile fatty acids, the ethyl esters of fatty acids, and fusel alcohol acetates, we used the protocol proposed by Lema et al. (*30*) as modified by Torija et al. (*14*). Analytical GC was carried out on a Hewlett-Packard 4890A connected to a Hewlett-Packard Integrator 3393A equipped with a flame ionization detector (Agilent). In this analysis, 50 mL of wine, 100 μL of internal standards (0.92 mg L^{-1} 1-nonanol; 3.22 mg L^{-1} heptanoic acid, and 2.90 mg L^{-1} heptadecanoic acid) and 200 μL of 85% orthophosphoric acid were extracted with 4 mL of hexane/diethyl ether (1:1). After 5 min of shaking, the organic phase was recovered. The extraction was repeated with 2 mL of solvent mixture. Once the organic phases were totally recovered, 2 μL was injected (splitless, 1 min) into an FFAP-HP column of 30 m × 0.25 mm and 0.25 μm phase thickness (Agilent). The temperature program went from 50 to 240 °C (15 min) at 4 °C min^{-1}. The injector and detector temperatures were 240 °C, and the carrier gas was helium at 1 mL min^{-1}. Volatile compounds were identified and quantified by comparison with standards.

Table 1. Chemical Composition of Carinyena Must

	must
sugar (g L^{-1})	225
titratable acidity (g of tartaric acid L^{-1})	5.5
pH	3.35
total SO_2 total (mg L^{-1})	32
YAN[a] (mg of N L^{-1})	118
YAN ammonium	37,3
YAN amino acids	80,7
YAN[b] (mg of N L^{-1})	176
YAN ammonium	97,2
YAN amino acids	78,8

[a] Must. YAN, yeast assimilable nitrogen. [b] Must after addition of 0.2 g L^{-1} diammonium phosphate and 0.2 g L^{-1} Fermaid K.

Statistical Analysis. The statistical analyses were carried out using the three-way MANOVA test at a significance level of $p \leq 0.05$ in a Statistica for Windows 4.2 program (Statsoft, Inc., 1993).

Principal component analysis (PCA) was performed with the Statistica package as before. A data matrix was structured with 28 samples representing wines and 6 variables, which were maximal fermentation rates (*R*), initial consumption of amino acids (ICAA), acetic acid (AcA), fusel alcohols (FA), fusel alcohol acetates (FAA), and medium-chain fatty acids (MCFA).

RESULTS AND DISCUSSION

The balance between the sugar content and the titratable acidity of Carinyena must (**Table 1**) indicated that grapes were harvested at a good maturation time. This and the Carinyena cultivar characteristics (*6*) yielded a low yeast assimilable nitrogen (YAN) content as seen for a concentration that could be considered under the minimum quantity for a reasonable fermentation (140−150 mg L^{-1}) (*31*). This limitation was corrected by adding yeast nutrients, especially diammonium phosphate. Among the nutrients, we also added Fermaid K, which is a widely used adjuvant of fermentation mostly composed by yeast extract, vitamins, and some ammonium diphosphate, all of which are needed in the case of problematic fermentations such as the ones in Carinyena musts (*21*). These musts have shown to be very problematic when the vinification proceeds as a rosé wine (that is, starting with a short maceration with skins, ∼8 h, and later the must is drawn and fermented at a controlled temperature, such as 17 °C), showing stuck or sluggish fermentations (*21*).

Fermentation Kinetics and Effectiveness of the Inocula. The parameters of fermentation kinetics (**Table 2**) vary among yeast strains. The fastest yeast strain at both temperatures was strain B. The cryotolerant strain, on the other hand, had the lowest fermentative capacity at 13 °C, yet was very similar to the other strains at 17 °C. However, at 13 °C this strain reached a maximum viable population size of 10^8 cells mL^{-1} (data not shown). Thus, it is evident that the cryotolerant strain has limited performance in musts with these high sugar and low nitrogen contents.

To improve the low-temperature fermentations, inocula were prepared in several ways. Results from **Table 2** and **Figure 1** show that the preparation of strain A did not affect fermentation kinetics. However, the preadapted inocula at the fermentation temperature of strain B led to better fermentation kinetics than the other inocula preparations: rehydrated dry active yeast and cells grown at the usual cellar temperature (fermentation starter). This improvement shortened the total length of the fermentation, especially at 13 °C. To a lesser extent, the preadaptation increased the maximum fermentation rates. Nevertheless, this increase was only significant at 17 °C and similar to the increase caused by fermentation starter groups.

The evolution of non-*Saccharomyces* and *Saccharomyces* yeast populations during fermentation (**Table 3**) showed that non-*Saccharomyces* initially predominated in a ratio of almost 1:10 in most fermentations, but were then replaced by *Saccharomyces* spp., which are more tolerant to ethanol and more competitive for growth in media with high sugar concentrations (*32*). There are three possible explanations for a high non-*Saccharomyces* population in Carinyena must. First, the harvest of the Carinyena grapes is one of the latest in our area (end of September or beginning of October), and so the probability of non-*Saccharomyces* yeast contamination on the berry surface is higher, as is described in *Botrytis*-affected grapes (*33*). However, the presence of *Saccharomyces* species on the berry surface is rare (*34*). Second, when the Carinyena harvest enters the winery, the machinery that processes the grapes may contaminate it with non-*Saccharomyces* yeast that is present because of previous processing of grapes that had ripened earlier (*1*). Finally, rosé wine production requires must to be macerated with grape skins for a minimum of 8 h before commercial yeast is inoculated. This favors the juice enrichment of non-*Saccharomyces* yeast (*1*). These three phenomena can occur all together and result in a high initial non-*Saccharomyces* population in the must. The following non-*Saccharomyces* species were identified in must according to the frequency of presence: *Hanseniaspora uvarum*, *Candida stellata*, *Pichia fermentans*, *Candida incommunis*, and *Pichia angophorae*. Although this is a very well defined starting situation, it should be pointed out that non-*Saccharomyces* species (in particular *Candida stellata*) were present in considerable quantitative populations throughout most of the fermentations and were even detected at the end. *Hanseniaspora uvarum* and *Candida stellata* were the main non-*Saccharomyces* species at the start of fermentation (days 0 and 1). It has been reported that these species grow well in juices with high concentrations of sugar and at low temperatures [10−15 °C (*35*)]. These conditions are similar to

Table 2. Effect of Preadaptation on the Maximum Fermentation Rates (*R*) (Grams of Sugar per Liter per Day) and Total Fermentation Time (*T*) (Days)[a]

	A				B				C			
	13 °C		17 °C		13 °C		17 °C		13 °C		17 °C	
	R	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*
not preadapted	18.0 ± 0.5a	19	30.6 ± 0.2b	13	20.7 ± 0.5**c**	19	32.5 ± 0.5d	13	16.3 ± 0.2a	31	29.0 ± 0.2b	13
preadapted	16.0 ± 0.2a	19	30.4 ± 1.1b	13	21.6 ± 0.8c	15	34.8 ± 0.1e	11				
fermentation starter	17.8 ± 0.5a	19	30.0 ± 0.1b	13	21.1 ± 0.1c	19	33.8 ± 0.1de	13				

[a] A, B, and C represent the three different strains of *S. cerevisiae* tested and commercialized *S. cerevisiae* (A), *S. bayanus* (B), and *S. uvarum* cryotolerant strain (C). Each value represents the average ± standard deviation of duplicate determinations of two parallel fermentations. Statistically different groups (a–e) were established according yeast strain, fermentation temperature, and modality of inoculum preparation.

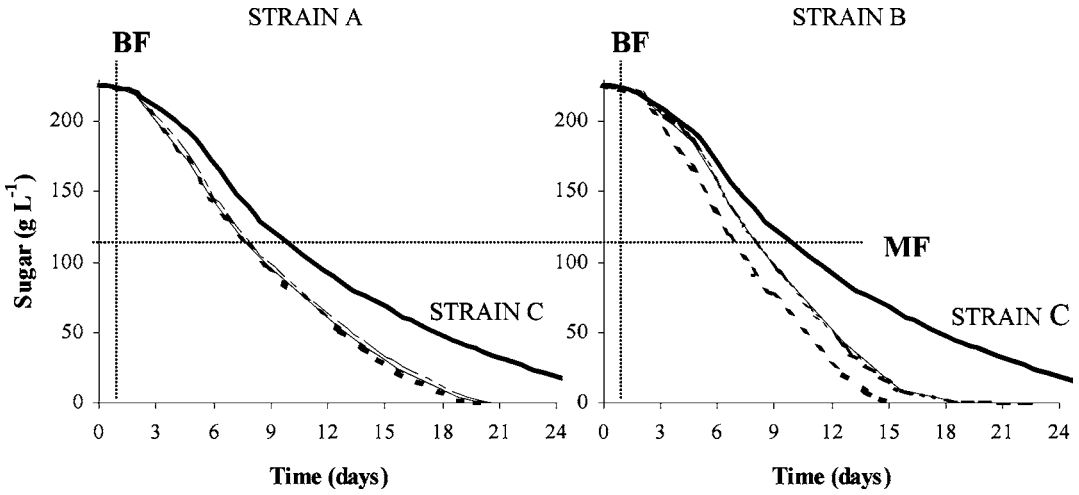

Figure 1. Evolution of fermentation at 13 °C with strains A–C: strain A or B, preadapted (– –), fermentation starter (– - –), not preadapted (—); strain C, cryotolerant strain (**—**). BF and MF are beginning and middle fermentation points, when samples were taken for analysis (**Table 3**).

Table 3. Percentage of *S. cerevisiae* Imposition at the Beginning, Middle, and Final Fermentation[a]

	A			B			C
	not pre-adapted	pre-adapted	fermentation starter	not pre-adapted	pre-adapted	fermentation starter	not pre-adapted
13 °C							
begin	17	8	13	17	42	15	7
mid	93	83	95	82	53	86	85
end	100	93	100	100	100	92	100
17 °C							
begin	24	46	32	17	42	15	5
mid	83	87	74	75	87	94	88
end	88	93	80	100	100	100	100

[a] Each value represents the average of two parallel fermentations.

ours. In fact, it can be seen that at 13 °C it generally takes longer than at 17 °C for the *Saccharomyces* strains to become dominant in the fermentation. In all cases, the *Saccharomyces* strains present during the fermentations were the inoculated ones.

Nitrogen Metabolism during Fermentation. It is well-known that nitrogen availability and metabolism is one of the key components of both fermentation development and final wine quality. As seen in **Table 4**, nitrogen compounds are taken up by *S. cerevisiae* during the initial stages of growth and probably stored in the vacuole until needed (*36*). Generally, ammonium and YAN disappear from the medium in the first few days of the fermentation (*37*), although at low temperatures this usually takes longer (*10*, *21*), probably because of fermentation performance. Ammonium disappeared more quickly in the fermentations inoculated with strain A than in those inoculated with strain B or C. **Table 2** shows that the fermentation rate with strain A is not maximum, so it seems that these parameters are not related. The preadaptation inocula for strain B was observed to have an effect, although the rates of consumption of ammonium at 17 °C were similar in all fermentations. It is interesting to note that although each strain may have a different pattern for ammonium uptake and utilization, which gives no indication of the fermentation performance, in our case it seems that the fastest uptake in a given strain after different treatments could be an indicator. However, this is based on only one observation, and further studies are required if it is to be confirmed.

As far as the amino acid metabolism is concerned (**Table 5**), it can be seen that the rates of amino acid disappearance were similar in low-temperature fermentations inoculated with strains A and B when not preadapted. The uptake of cryotolerant strain C was reduced at 13 °C, whereas the initial uptake for preadapted strain B was fast. As in the case of ammonium, this fast uptake of nitrogen could be related to a better fermentation performance. The strain B fermentation starter also showed an increased consumption of amino acid but to a lesser extent. This observation shows that inoculating a fermentation starter (yeast in growth) may have a positive effect on the rate of consumption of amino acids and also that an additional effect exists to

Table 4. Ammonium Contents (Milligrams per Liter) during Fermentations at 13 and 17 °C under Different Inoculum Conditions[a]

	A			B			C
	not pre-adapted	preadapted	fermentation starter	not pre-adapted	preadapted	fermentation starter	not pre-adapted
13 °C							
must	125	125	125	125	125	125	125
5	0.5 ± 0.4	0.7 ± 0.1	3.3 ± 2.3	40 ± 17	0.5 ± 0.1	40 ± 17	49 ± 5
9	0.5 ± 0.3	0.8 ± 0.5	0.9 ± 0.6	0.4 ± 0.5	0.2 ± 0.2	0.2 ± 0.1	0.4 ± 0.1
15	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.3
end	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.2	0.7 ± 0.1	6 ± 0.1	0.9 ± 0.3
17 °C							
must	125	125	125	125	125	125	125
2	22 ± 0.1	21 ± 0.2	21 ± 0.3	22 ± 0.2	21 ± 0.2	20 ± 1.5	19 ± 3.3
6	0.6 ± 0.3	0.7 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.9 ± 0.0
14	0.4 ± 0.0	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.0	1.1 ± 0.5	0.4 ± 0.2	0.5 ± 0.1
end	1.5 ± 0.9	1.1 ± 0.0	1.3 ± 0.0				1.4 ± 0.5

[a] Each value represents the average ± standard deviation of duplicate determinations of two parallel fermentations.

Table 5. Amino Acid Contents in the Must–Wine during Fermentation at 13 and 17 °C (Milligrams of Gly per Liter) under Different Inoculum Conditions[a]

	A			B			C
	not pre-adapted	preadapted	fermentation starter	not pre-adapted	preadapted	fermentation starter	not pre-adapted
13 °C							
must	543	543	543	543	543	543	543
5	115 ± 8.8	120 ± 14	119 ± 3.2	128 ± 9.8	77 ± 2.6	90 ± 9.5	165 ± 7.7
9	36 ± 1.2	36 ± 17	47 ± 11	33 ± 9.6	32 ± 2.8	30 ± 3.4	38 ± 6.3
15	37 ± 3.9	29 ± 0.1	29 ± 0.1	22 ± 2.3	20 ± 0.4	23 ± 1.3	42 ± 2.6
end	37 ± 1.7	35 ± 1.3	37 ± 6.2	33 ± 9.2	34 ± 11	22 ± 0.2	39 ± 8.5
17 °C							
must	543	543	543	543	543	543	543
2	226 ± 29	148 ± 9.0	173 ± 44	223 ± 16	40 ± 4.0	132 ± 41	53 ± 24
6	43 ± 2.0	32 ± 7.0	38 ± 1.4	26 ± 1.3	30 ± 4.2	35 ± 5.0	30 ± 0.7
end	39 ± 7.8	42 ± 11	46 ± 2.0	48 ± 2.7	41 ± 7.2	54 ± 2.8	52 ± 2.8

[a] Each value represents the average ± standard deviation of duplicate determinations of two parallel fermentations.

Table 6. Effect of Preadaptation on the Final Composition of Wines[a]

	A			B			C
	not pre-adapted	preadapted	fermentation starter	not pre-adapted	preadapted	fermentation starter	not pre-adapted
13 °C							
ethanol (% v/v)	14.2 ± 0.0	13.8 ± 0.1	13.8 ± 0.4	13.4 ± 0.6	13.8 ± 0.0	13.9 ± 0.4	13.2 ± 0.0
glycerol (g L^{-1})	10.1 ± 1.0	10.8 ± 2.0	7.9 ± 2.0	9.8 ± 1.3	10.4 ± 2.0	8.3 ± 0.4	8.5 ± 0.2
succinic acid (g L^{-1})	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.8 ± 0.0
acetic acid (mg L^{-1})	1026 ± 64	930 ± 21	939 ± 77	948 ± 33	340 ± 56	758 ± 41	1211 ± 45
acetaldehyde (mg L^{-1})	12 ± 0.3	13 ± 1.6	16 ± 0.4	32 ± 5.5	15 ± 2.4	23 ± 0.3	22 ± 0.6
ethyl acetate (mg L^{-1})	80 ± 0.9	72 ± 11.2	70 ± 2.1	66 ± 10.5	59 ± 16.1	56 ± 13.4	55 ± 7.5
17 °C							
ethanol (% v/v)	14.7 ± 0.4	13.9 ± 0.0	14.2 ± 0.0	14.0 ± 0.3	14.2 ± 0.0	13.5 ± 0.4	14.2 ± 0.6
glycerol (g L^{-1})	8.8 ± 0.2	9.1 ± 0.3	9.1 ± 1.2	9.7 ± 0.8	8.9 ± 0.2	6.7 ± 0.2	12.9 ± 0.3
succinic acid (g L^{-1})	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	0.9 ± 0.0
acetic acid (mg L^{-1})	1188 ± 38	1002 ± 32	1020 ± 74	911 ± 9.9	678 ± 28	702 ± 34	1650 ± 5.0
acetaldehyde (mg L^{-1})	16 ± 2.7	13 ± 2.2	28 ± 9.8	59 ± 6.7	45 ± 6.7	52 ± 1.3	19 ± 1.1
ethyl acetate (mg L^{-1})	65 ± 11.4	56 ± 9.7	65 ± 12.1	75 ± 8.3	56 ± 18.4	64 ± 2.4	77 ± 14.3

[a] Significance from MANOVA (the values of wines obtained of cryotolerant strain inoculation are not considered): **strain**, ethanol, glycerol, succinic acid, acetic acid, and acetaldehyde; **temperature**, ethanol, glycerol, acetic acid, and acetaldehyde; **inocula preparation**, acetic acid and acetaldehyde; **strain** × **temperature**, glycerol and acetaldehyde; **strain** × **inocula preparation**, ethanol, acetic acid, and acetaldehyde; **temperature** × **inocula preparation**, acetic acid; **strain** × **temperature** × **inocula preparation**, glycerol and acetic acid.

preadapt inocula to the temperature of fermentation. This preadaptation effect was also detected at 17 °C for strain B and to a lesser extent for strain A. It should also be pointed out that the rate of consumption of amino acids of the cryotolerant strain improved notably at 17 °C. The importance of a fast uptake of nitrogen could be related to the differential uptake of the nitrogen sources. In fact, none of them are taken up at the same time or at the same rate. It is well-known that *S. cerevisiae* can use different nitrogen sources for growth, but not all nitrogen sources support growth equally well. Thus, different rates of nitrogen uptake can change the selection of nitrogen sources, and it is known that nitrogen sources enable the best growth by a mechanism called nitrogen catabolite repression (NCR) (*38*, 39). The priority use of good nitrogen sources such as glutamine, asparagine, or ammonium, which occur at the very beginning of the fermentation (*36*), could be related to a good fermentation performance. Intracellular amino acids could be free to the media after yeast autolysis at the end of fermentation, which could explain the final increase of amino acids in the media in some fermentations (*36*).

Analysis of the Main Compounds in the Final Wine. Table 6 shows the main fermentation metabolites. As general remarks of these metabolites can be pointed out the high concentrations of acetic acid, which have been previously observed in the fermentations of this variety and probably are related to the long survival of non-*Saccharomyces* yeast. Also, the concentrations of this compound were reduced at low temperatures, as previously observed (*21*). It is clear that the presence of high concentrations of acetic acid are clearly deleterious for the quality of the wine, as they are much higher than the perception threshold [800 mg L^{-1} (*40*)]. Thus, this parameter and its reduction could be a good indicator for the effect of the preadaptation. In fact, the first important observation is that strain C is the highest producer of acetic acid. In the case of strains A and B, the adaptation protocols considerably reduced the production of acetic acid, which could be related to the redox equilibrium once the yeast has begun to ferment (*1*, *41*). This effect is very strong when the general fermentation also improves, such as in the case of strain B, which yields the only "acceptable" concentrations of acetic acid at both fermentation temperatures.

Overall, and after the statistical treatment (MANOVA), it is clear that the yeast strain and fermentation temperature influenced all of the main parameters in the final wine composition, as generally agreed (*1*). However, this effect is modulated by the adaptation period, as the aforementioned effect on acetic acid made clear. In fact, although strain A clearly yields more ethanol and glycerol than the other two strains at 13 °C, the

Table 7. Effect of Preadaptation on the Aromatic Profiles of Wines (Milligrams per Liter)[a]

	A			B			C
	not pre-adapted	preadapted	fermentation starter	not pre-adapted	preadapted	fermentation starter	not pre-adapted
13 °C							
fusel alcohols	436 ± 29	518 ± 25	339 ± 25	509 ± 8.4	338 ± 11	401 ± 20	358 ± 32
fatty acids	9.20 ± 0.10	7.37 ± 1.85	7.10 ± 0.55	5.34 ± 0.10	5.97 ± 0.34	8.47 ± 0.35	9.03 ± 0.38
fatty acid esters	5.61 ± 0.16	4.13 ± 2.12	4.01 ± 4.65	2.63 ± 0.16	7.50 ± 0.07	4.27 ± 0.70	8.21 ± 0.82
fusel alcohol acetates	1.19 ± 0.03	1.50 ± 0.14	0.60 ± 0.67	0.88 ± 0.03	1.22 ± 0.05	1.39 ± 0.09	0.50 ± 0.03
17 °C							
fusel alcohols	641 ± 3.8	601 ± 54	615 ± 18	628 ± 25	622 ± 15	530 ± 77	507 ± 35
fatty acids	7.13 ± 0.83	6.25 ± 0.34	8.04 ± 0.22	6.22 ± 0.21	4.79 ± 0.09	7.29 ± 0.73	3.91 ± 0.29
fatty acid esters	11.28 ± 5.48	4.41 ± 0.71	4.46 ± 0.28	2.78 ± 0.93	2.72 ± 0.03	4.35 ± 0.33	2.50 ± 0.11
fusel alcohol acetates	1.19 ± 0.72	1.89 ± 0.03	1.72 ± 0.07	1.16 ± 0.10	1.50 ± 0.17	1.44 ± 0.07	0.66 ± 0.00

[a] Significance from MANOVA (the values of wines obtained of cryotolerant strain inoculation are not considered): **strain**, fusel alcohols and fatty acids; **temperature**, fusel alcohols; i**nocula preparation**, fusel alcohols and fatty acids; **strain** × **temperature**, fusel alcohols; **strain** × **inocula preparation**, fusel alcohols and fatty acids; **temperature** × **inocula preparation**, fusel alcohols; **strain** × **temperature** × **inocula preparation**, fusel alcohols and fatty acids.

yields are similar when the strains are subject to a preadaptation protocol. In general, it is clear that the reduction in acetaldehyde and acetic acid is one of the main advantages of fermentation at low temperatures. Finally, the inocula preparation had a direct and significant influence on the contents of acetaldehyde and acetic acid. Preadapted inocula yielded lower amounts of acetaldehyde and acetic acid than when only a fermentation starter was used. Overall, the inocula previously grown under aeration (preadapted inocula and fermentation starter) yield wines with lower contents of ethanol (not significant), acetic acid, and acetaldehyde and a greater content of glycerol (not significant). The greater activation of the glyceropyruvic fermentation at the beginning, described for the yeast inoculum growing under aeration to the detriment of the alcoholic fermentation (*1*), would explain these results. Also, glycerol concentration increases have been reported in wines when higher temperatures (*7*) are used in fermentations, yet the difference between 13 and 17 °C is too small for these changes to be detected. As expected for the levels of acetic acid and ethanol, the amount of ethyl acetate is rather high, although there were not significant differences due to the treatments of the yeast strains.

Aromatic Compounds in Final Wines. As far as the aromatic profiles of wines are concerned (**Table 7**), it can be observed that the fusel alcohol content increased significantly at 17 °C whatever the strains and the preadaptation conditions were. The fact that the concentrations found in our conditions were higher could be the consequence of the continuous presence of non-*Saccharomyces* species, which have been reported as high producers of fusel alcohol in mixed cultures (*42*). The fermentation at 13 °C also reduced the negative organoleptic impact of these substances, especially when strain B was preadaptated at low temperatures. No significant changes were observed in the formation of fusel alcohol acetates.

On the other hand, the contents of medium-chain fatty acids (C6−C12) and their corresponding ethyl esters were influenced not only by the fermentation temperature but especially and significantly by the yeast strain and its preadaptation as reported by Torija et al. (*14*). The most important result was the effect of preadapted strain B at 13 °C on the higher concentration of fatty acid ethyl esters, which are positive for the organoleptic aspects of wine. However, our data also show that preadaptation of yeast A, irrespective of temperature, actually decreased the sensorially beneficial effects of fatty acid ethyl esters, showing that the benefits of preadaptation are strain specific.

Table 8. Correlation Matrix of the Variables Used for the PCA[a]

	FA	FAA	AcA	MCFA	R	ICAA
FA	1.000					
FAA	+0.620	1.000				
AcA	+0.197	−0.117	1.000			
MCFA	−0.188	+0.200	−0.042	1.000		
R	+0.777	+0.429	+0.007	−0.476	1.000	
ICAA	+0.678	+0.197	+0.229	−0.508	+0.881	1.000

[a] FA, fusel alcohols; FAA, fusel alcohol acetates; AcA, acetic acid; MCFA, medium-chain fatty acids; R, maximum fermentation rates; ICAA, initial consumption of amino acids.

The results of PCA (**Table 8** and **Figures 2** and **3**) showed the relationship between some parameters and fermentation products and their incidence on the representation of the wines obtained. Three significant factors, which accounted for 88% of the total variance, were chosen. Of the correlation matrix results, the maximum fermentation rate (*R*) was closely related to the initial consumption of amino acids (ICAA) (+0.881) and also to the fusel alcohol contents (+0.777). Furthermore, the production of MCFA seemed to be negatively related to ICAA (−0.508), and the formation of acetic acid was unrelated to any parameters. When the plane consisting of one and two component factors was used, two clusters were formed that separated wines according to the fermentation temperature: on the left are the wines obtained at 13 °C and on the right are the wines obtained at 17 °C (**Figure 2**). However, two wines (repeated conditions, numbered as 27 and 28) from the cryotolerant strain fermentation were more separated than the others because their acetic acid contents were very high. **Figure 3** shows the projections of the original variables and the wines belonging to the training set on the factorial plane formed by component factors 1 and 3. In this case, two wines (9 and 10) from the preadapted strain B fermentation (repeated condition) were distinguished from the others by the low production of acetic acid.

Conclusions. The results showed that the preadaptation of a yeast can improve the fermentation performance, although this improvement is strain dependent. The general effects of low-temperature fermentations were also observed: lower acetic acid and fusel alcohol production and higher concentrations of glycerol. When the yeast performed better in fermentation due to preadaptation, nitrogen was consumed more quickly and

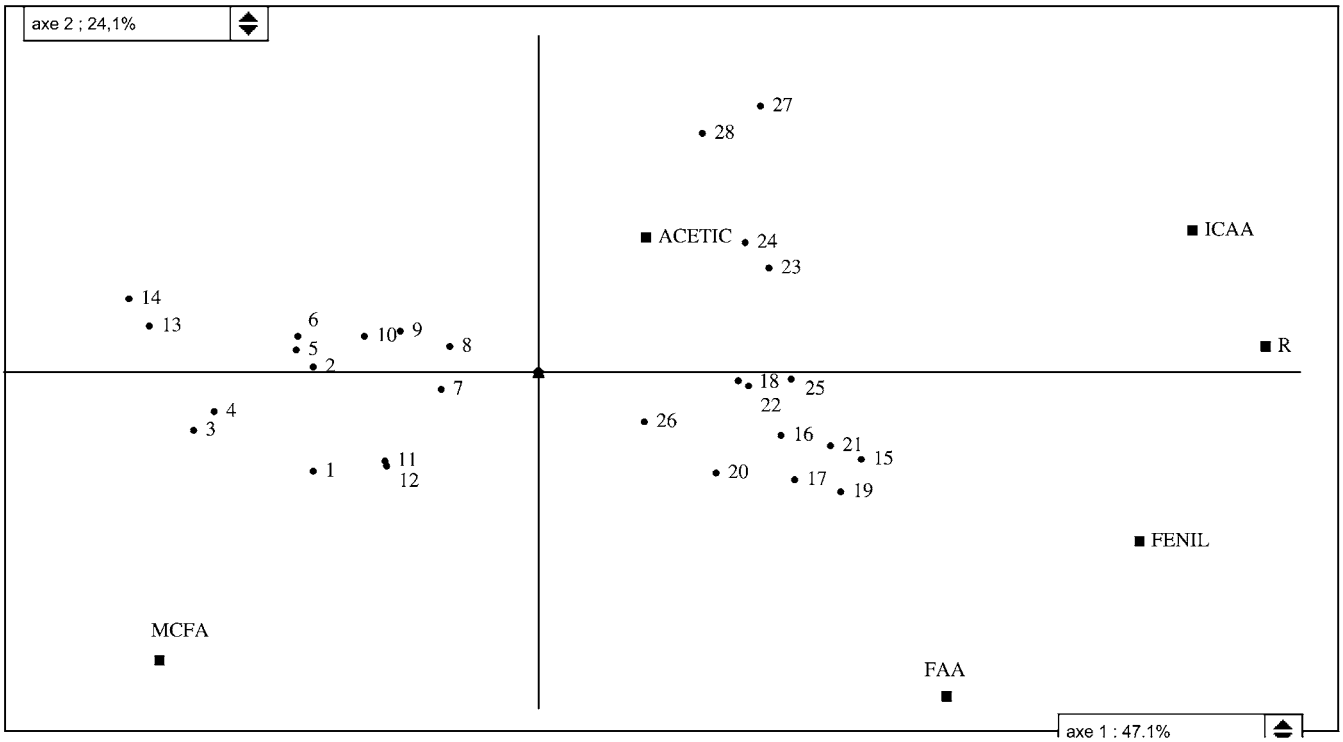

Figure 2. Projection of the variables and wines onto the plane formed by the first and second principal components. Numbers represent individual wines. ACETIC, acetic acid concentration; ICAA, initial composition of amino acids; *R*, maximal fermentation rate; FENIL, fusel alcohols; MCFA, medium-chain fatty acids; FAA, fusel alcohol acetates.

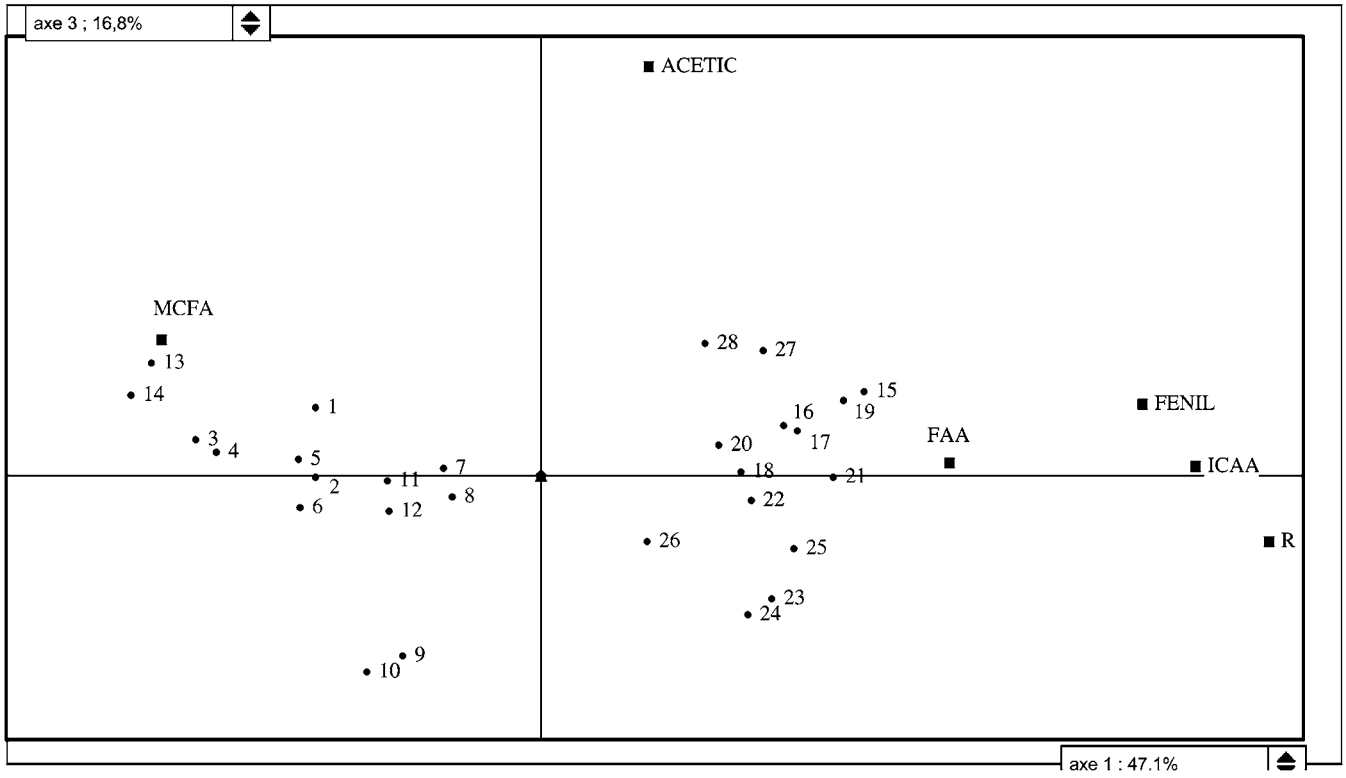

Figure 3. Projection of the variables and wines onto the plane formed by the first and third principal components. Numbers represent individual wines. ACETIC, acetic acid concentration; ICAA, initial composition of amino acids; *R*, maximal fermentation rate; FENIL, fusel alcohols; MCFA, medium-chain fatty acids; FAA, fusel alcohol acetates.

"negative" wine attributes (acetic acid, fusel alcohols) were significantly reduced. Thus, in some strains, preadaptation could be an effective mechanism for improving low-temperature fermentations. The main conclusion to be drawn from this paper is that an easy preadaptation protocol of some strains could lead to a considerable improvement in both the low-temperature (13 °C) fermentation kinetics and the chemical (and organoleptic) characteristics of final wines.

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