Fermentative behaviour and competition capacity of cryotolerant *Saccharomyces* species in different nitrogen conditions

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ABSTRACT

The selection of yeasts with low nitrogen requirement is a current need in winemaking. In this work, we analysed nitrogen requirements of strains belonging to the cryotolerant species *S. uvarum*, *S. eubayanus* and *S. kudriavzevii*, in order to evaluate their potential for conducting the fermentation of low nitrogen content grape musts. Our result demonstrated that *S. eubayanus* is the species less influenced by the increasing nitrogen concentrations in both growth and fermentation conditions. Strains showing the best behaviours, *S. eubayanus* NPCC 1285 and *S. uvarum* NPCC 1317, were selected to be tested in mixed cultures with *S. cerevisiae* T73 at different temperatures (12 °C, 20 °C and 28 °C) in synthetic grape must with different nitrogen concentrations (60, 140 and 300 mg/L YAN). The cryotolerant strains dominated the fermentations carried out at 12 °C while *S. cerevisiae* prevailed at 28 °C independently from the nitrogen concentration. At intermediate temperature, 20 °C, *S. eubayanus* mono and mixed cultures showed the best fermentative behaviour especially with low and intermediate nitrogen concentration. In summary, cryotolerant *Saccharomyces* species, particularly *S. eubayanus*, could be interesting tools to avoid fermentations stucks caused by low nitrogen content in grape musts.

1. Introduction

Nitrogen is a key nutrient during wine fermentation, affecting both fermentation kinetics and the formation of wine aroma. It is the major limiting nutrient for growth under oenological conditions. Although alternative nitrogen sources, such as oligopeptides, amides, biogenic amines and nucleic acids, can be found and might constitute a substantial nitrogen resource in grape juice (Ough, 1991; Henschke and Jiranek, 1993; Perry et al., 1994; Marsit et al., 2015), yeast assimilable nitrogen (YAN) is mainly composed of ammonium and amino acids (Henschke and Jiranek, 1993). Different factors including grape variety, geographical origin, climate conditions and some technological processes affect the YAN content in musts and thus the fermentation kinetics (Butzke, 1998; Dubois et al., 1996; Nicolini et al., 2004). Previous works have determined that, in general, a minimum of 140 mg/L

of YAN is required for yeast to complete alcoholic fermentation (Bell and Henschke, 2005; Bely et al., 1990; Butzke, 1998). Nevertheless, it strongly depends on the yeast species developing during the fermentation process.

Saccharomyces cerevisiae is the main yeast species used in the wine industry. Its favoured characteristics such as high fermentation efficiency, high ethanol tolerance and consistency of wine quality help *S. cerevisiae* to maintain its dominant position (Mas et al., 2016). However, the context of the worldwide Oenology has altered with the climate change, which affects the grape composition and ends up with grape musts with low nitrogen and high sugar concentrations (van Leeuwen and Darriet, 2016). This situation gives the wine industry a big challenge to meet consumers' preference for wines with lower alcohol and fruitier aromas. One of the oenological practices applied by winemakers is to use lower fermentation temperatures, as far as

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10–12 °C to preserve aroma compounds in wines (Beltran et al., 2006; Alonso del Real et al., 2017). However, low-temperature fermentation produced similar metabolic and transcriptional effects to those obtained in nitrogen-limited fermentations (Beltran et al., 2006; Pizarro et al., 2008). Both conditions decreased biomass yield and fermentation rate during wine fermentation. This is not unexpected, because low temperature produces a rigidification of the plasma membrane that impairs the activity of some permeases, reducing the transport of nutrients (Beltran et al., 2006). Thus, low-temperature influences the quantity and the quality of yeast nitrogen requirements. In summary, the lack of nitrogen, the high sugar concentration, and the low fermentation temperature have made a very complicated harsh condition for yeasts and often causes the main fermentative problems.

In this context, strains belonging to cryotolerant *Saccharomyces* species, showing good adaptation at low temperature, lower nitrogen requirements and lower ethanol yields, may play an important role in wine fermentations. *Saccharomyces uvarum*, *Saccharomyces kudriavzevii* and *Saccharomyces eubayanus* have been identified as natural cryotolerant species in the genus. In particular, *S. uvarum* has been isolated from both natural habitats (Almeida et al., 2014; Bing et al., 2014; Gayevskiy and Goddard, 2016; Rodríguez et al., 2014) and fermentation environments including wines, ciders and apple chichas (for a review see Rodríguez et al., 2016). The fermentation profile of *S. uvarum* is different from that shown by *S. cerevisiae*. This species has a shorter lag phase than *S. cerevisiae* at low fermentation temperature (around 13 °C). Comparing with *S. cerevisiae*, it also produces higher amounts of glycerol and lower amounts of ethanol (Castellari et al., 1994; Masneuf-Pomarède et al., 2010), and it generates a differential aromatic profile, particularly characterised by a higher production of 2-phenylethanol which gives a very pleasant rose-like floral odour (Bertolini et al., 1996; Masneuf-Pomarède et al., 2010; Origone et al., 2018). These different traits support the great potential of *S. uvarum* being widely used in wine industry at low temperature fermentation. The other two cryotolerant species, *S. kudriavzevii* and *S. eubayanus*, have only been found in natural habitats, although it has been demonstrated that they contribute to ferment beverages through their presence as part of the genome of chimeric strains with other *Saccharomyces* species, predominantly *S. cerevisiae* (González et al., 2006; González et al., 2007; Libkind et al., 2011).

Many studies have been carried out to explore the nitrogen demanding character of *S. cerevisiae* both phenotypically and genotypically (García-Ríos et al., 2014; Gutiérrez et al., 2012, 2013, 2016). However, there are few studies about the nitrogen requirement of the cryotolerant species of the genus. It has only been described that *S. uvarum* strains have lower nitrogen requirement than *S. cerevisiae* (Masneuf-Pomarède et al., 2010), which suggests the putative competitiveness of this species during specific wine fermentations.

In our study, we phenotypically analysed the combined effect of temperature and nitrogen availability on the fermentation behaviour of cryotolerant yeast strains. Firstly, Nitrogen requirements of a set of *S. uvarum* and *S. eubayanus* strains recently isolated from Patagonia as well as *S. kudriavzevii* strains from natural habitats in Spain were studied. Secondly, Competition assays have also been evaluated by selected cryotolerant strains against *S. cerevisiae* in different conditions of temperature and nitrogen content in order to find out the optimal condition for the better implantation of these cryotolerant species.

2. Materials and methods

2.1. Strains and media used in the study

Thirty-two strains belonging to *S. uvarum*, *S. eubayanus* and *S. kudriavzevii* species, isolated from natural (Lopes et al., 2010; Rodríguez et al., 2014) and fermentative (Rodríguez et al., 2017) environments were used in this work. *S. uvarum* BMV58 and CECT12600 and *S. cerevisiae* T73 were also used for comparative purpose.

Yeast extract peptone dextrose (YPD) medium (2% glucose, 2% peptone, 1% yeast extract) was used for yeast propagation.

A synthetic grape must (SM), similar to natural grape must, but with a defined composition, was used in our study to determine yeast growth and fermentation characteristics. The SM was prepared as described by Riou et al. (1997), but with some modifications. The SM contains 200 g/L reducing sugar (100 g/L glucose and 100 g/L fructose), malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L, KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4$ 0.25 g/L, $CaCl_2$ 0.16 g/L, NaCl 0.2 g/L, trace elements ($MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH_4)_6Mo_7O_{24}$ 1 mg/L), vitamins (myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid 2 mg/L, thiamine hydrochloride 0.25 mg/L, pyridoxine hydrochloride 0.25 mg/L and biotine 0.003 mg/L). The composition of nitrogen source in the SM is 40% ammonium and 60% amino acids, as described in Beltran et al. (2004). The composition of amino acids in 1 L amino acid stock is L-tyrosine 1.5 g, L-tryptophan 13.4 g, L-isoleucine 2.5 g, aspartic acid 3.4 g, glutamic acid 9.2 g, L-arginine 28.3 g, L-leucine 3.7 g, L-threonine 5.8 g, glycine 1.4 g, L-glutamine 38.4 g, L-alanine 11.2 g, L-valine 3.4 g, L-methionine 2.4 g, L-phenylalanine 2.9 g, L-serine 6 g, L-histidine 2.6 g, L-lysine 1.3 g, L-cysteine 1.5 g and L-proline 46.1 g, which corresponded to 13.75 g/L assimilable nitrogen. The final pH of the SM was adjusted to 3.3 with sodium hydroxide. The SM was sterilised by filtration through 0.22 μm pore size membrane filter (Thermo scientific).

Fermentation medium was inoculated with a yeast overnight preculture to reach an OD_{600nm} of approximately 0.1. Preliminary experiments have been carried out to relate cell counts, by using Neubauer chamber (Brand GMBH, Germany), with OD value. The result showed that, for the different species used in our study, $OD_{600nm} = 0.1$ approximately equals to 1×10^6 cells/mL (data not shown).

2.2. Strain screening based on nitrogen requirement

2.2.1. Growth character screening

S. uvarum, *S. eubayanus* and *S. kudriavzevii* strains were screened by both growth and fermentation character under different nitrogen concentrations. The growth character was determined by microtiter plate screening method as described by Warringer and Blomberg (2003) with some modifications. 96 well microtiter plates were used. Each well was filled with 250 μL of synthetic must of different nitrogen concentrations and with 10^6 yeast cells/mL. Growth curves were monitored by recording the increase of optical density (OD) at wavelength 600 nm. The microtiter plates were incubated in SPECTROstar Nano® microplate reader (BGM Labtech, Offenburg, Germany) at 25 °C with 500 rpm orbital shaking. The optical density of each well was measured every 30 min until the growth reached the stationary phase. The growth characters of 32 strains were screened under 12 nitrogen concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 300 mg/L YAN). All the nitrogen conditions were assayed by triplicate. The growth character of each strain under different nitrogen condition can be calculated by directly fitting OD measurements versus time to the Gompertz equation proposed by Zwietering et al. (1990), which has the following expression:

$$y = D * \exp\{-\exp[((\mu_{max} * e)/D) * (\lambda - t) + 1]\}$$

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D = \ln(OD_\infty/OD_0)$ is the OD value reached with OD_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h). R code (statistical software R, v.3.0) was used to facilitate calculating μ_{max} and λ with Gompertz equation.

2.2.2. Fermentation character screening

The fermentation character was screened by microscale fermentation method. The fermentations were carried out in 15 mL tubes with 10 mL synthetic must. The screw caps of the tubes were not completely

tightened in order to allow CO_2 release. Synthetic must was modified with three different nitrogen concentrations which were 60, 140 and 300 mg/L YAN. These values correspond to nitrogen limited, standard and excessive conditions (Bely et al., 1990). The fermentations were followed by CO_2 production which can be represented by the weight loss of the fermentation tubes. Three tubes filled with 10 mL SM without inoculation are used as triplicate controls for the evaporation weight loss (EWL). The fermentation tubes were weighted around every 12 h. The weight loss (WL) can be simply calculated as: $WL = W_0 − W_t − EWL$. The fermentations were considered as finished when WL stops to increase. Lag phase time (λ) and maximum fermentation rate (V_{max}) were calculated by applying the weight loss data into Gompertz model as described in Section 2.2.1.

2.3. Competition fermentation

In order to determine the influence of nitrogen concentration and fermentation temperature on the yeast population in a mixed culture, competition fermentation was carried out between *S. cerevisiae* strain T73 and one of the non-*cerevisiae* strains. The strains used for fermentation were first propagated in liquid YPD media overnight and then transferred to SD media (2% glucose, 0.017% yeast nitrogen base) with 230.8 mg/L NH_4Cl (corresponding to 60 mg/L YAN) in order to eliminate the influence of YPD nitrogen-rich media.

Fermentations were carried out in 100 mL bottles with 80 mL synthetic must of three nitrogen concentrations (60, 140 and 300 mg/L YAN). All the fermentations were inoculated with 2×10^6 cells/mL. In the case of mix-culture fermentations, 1×10^6 cells/mL of each strain was inoculated. In order to observe the influence of temperature on competition capacity of each strain, the fermentations were carried out at three temperatures: 12, 20 and 28 °C, representing low, medium and high temperature respectively. The fermentation process is monitored by measuring the density of the media (g/L), after a slight centrifugation at 5.000 rpm for 5 min for cell removal and using a portable densitometer (Mettler Toledo, USA). The fermentation was considered as finished when the density reaches 0.998 g/L (Gutiérrez et al., 2012). Mono-culture fermentations were also carried out as references.

2.4. Strain population dynamics in competition fermentation

As *S. uvarum* and *S. eubayanus* are less heat tolerant than *S. cerevisiae*, we confirmed the incapability of *S. uvarum* and *S. eubayanus* to grow on at 37 °C, whereas they perfectly grow at 30 °C in YPD (data not shown). On the other hand, the colony forming ability of *S. cerevisiae* strains is the same at 30 °C and 37 °C. The percentage of strains belonging to *S. cerevisiae* or to non-*cerevisiae* can be simply determined by plating the sample on two YPD plates and incubated at 30 °C and 37 °C. CFUs were counted after 2 days of incubation. The CFU number on the 37 °C plate represents the population of *S. cerevisiae* in the mixed culture and the CFU difference between the two plates represents the population of non-*cerevisiae* strain in the mixed culture.

At the end of the competition experiment, fermentation samples were taken and analysed for the main wine chemical parameters including glucose, fructose, ethanol, glycerol and main organic acids. The samples were first centrifuged and diluted 3 times with deionized water, then filtrated through 0.22 mm pore size nylon filters (Micron Analitica, Spain). The analysis was carried out on a UHPLC (Thermo Scientific, ultimate 3000) equipped with a refraction index detector and a UV–visible detector (Thermo Scientific). The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 45 °C. The metabolites were separated by a HyperREZ XP Carbohydrate H+ 8 mm column (Thermo Scientific) and their concentration was calculated by using external standards.

2.5. Statistical analysis

All the experiments were carried out at least in triplicate. Physiological data were analysed by the Sigma Plot 13.0 software and the results are expressed as mean and standard deviation. Significance was determined by analysis of variance (ANOVA) using the Statistica, version 7.0, software package. The statistical level of significance was set at a p value of ≤ 0.05 with a Tukey HSD test. Multi-factorial ANOVA based on the percentage of *S. cerevisiae* in the mixed culture fermentation was also carried out (Statistica, version 7.0) in order to analyse the significance of the influences of temperature (12, 20 and 28 °C), nitrogen concentration (60, 300 mg/L YAN), and the type of mixed culture (*S. cerevisiae* + *S. eubayanus*, *S. cerevisiae* + *S. uvarum*). The data group of fermentation carried out at 20 °C with 140 mg/L YAN was not included in the multi-factorial ANOVA, since it is the unique group with 140 mg/L YAN. Phenotypic data were fitted to the modified Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the function in the R statistical software, v.3.0. Hierarchical clustering used in heatmap plot was performed by MeV MultiExperiment Viewer with Euclidean distance metrics and group clustering was based on group averages (average linkage).

3. Results

3.1. Nitrogen requirements of S. eubayanus, S. uvarum and S. kudriavzevii in pure cultures

Nitrogen requirements of a set of 32 non-conventional cryotolerant *Saccharomyces* yeast strains including *S. uvarum* (17 different strains), *S. eubayanus* (13 different strains) and *S. kudriavzevii* (2 different strains) were evaluated in synthetic grape must at 25 °C. During the first stage, yeast growth was evaluated by OD increases in microtiter plates containing synthetic must with different total nitrogen concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 300 mg/L YAN). Growth curves were fitted to modified Gompertz equations for all yeast strains and conditions (a total of 1260 growth curves, including the three reference strains). After calculating by Gompertz equation, μ_{max} (maximum growth rate) and λ (lag phase time) values were successfully obtained for all analysed strains and conditions and their absolute values are exhibited in Supplementary material Tables 1 & 2. Lag phase time was mainly strain-dependent and increasing nitrogen concentration did not show significant differences on λ value of each strain. In order to easily detecting different nitrogen requirement among analysed species and strains, a heatmap analysis was carried out with μ_{max} data (Fig. 1). For this analysis, the μ_{max} value obtained for each strain was normalised by mean value of all strains at each particular nitrogen concentration. Values lower and higher than the average are indicated in green and red respectively in the heatmap (Fig. 1). This heatmap is hierarchically clustered into four clusters. The first cluster is made up of the strains with the highest μ_{max}, which mostly belong to *S. eubayanus*, with the exception of two *S. uvarum* strains (NPCC1317 and NPCC1323), which were isolated from apple chicha. The second cluster is formed by a group of strains with higher or similar growth rate than the average value of all strains (red to black colour). These strains were mostly isolated from *A. araucana* tree (Wild), which is a gymnosperm endemic of the lower slopes of the Chilean and Argentinian south-central Andes, typically above 1000 m of altitude (Rodríguez et al., 2014). The third group was represented by strains mostly with lower μ_{max} than the average (black to green colour). This cluster is mainly made up of *S. uvarum* isolated from wild and fermentative environments (chicha and cider) and, curiously, the three wine strains, two *S. uvarum* references BMV58 and CECT12600, and the control *S. cerevisiae* T73, are included in this third cluster, showing similar interspecific growth features. The fourth group includes two strains of *S. kudriavzevii*, showing the lowest growth rate of all the tested strains.

In order to evaluate if the behaviour evidenced in yeast growth is
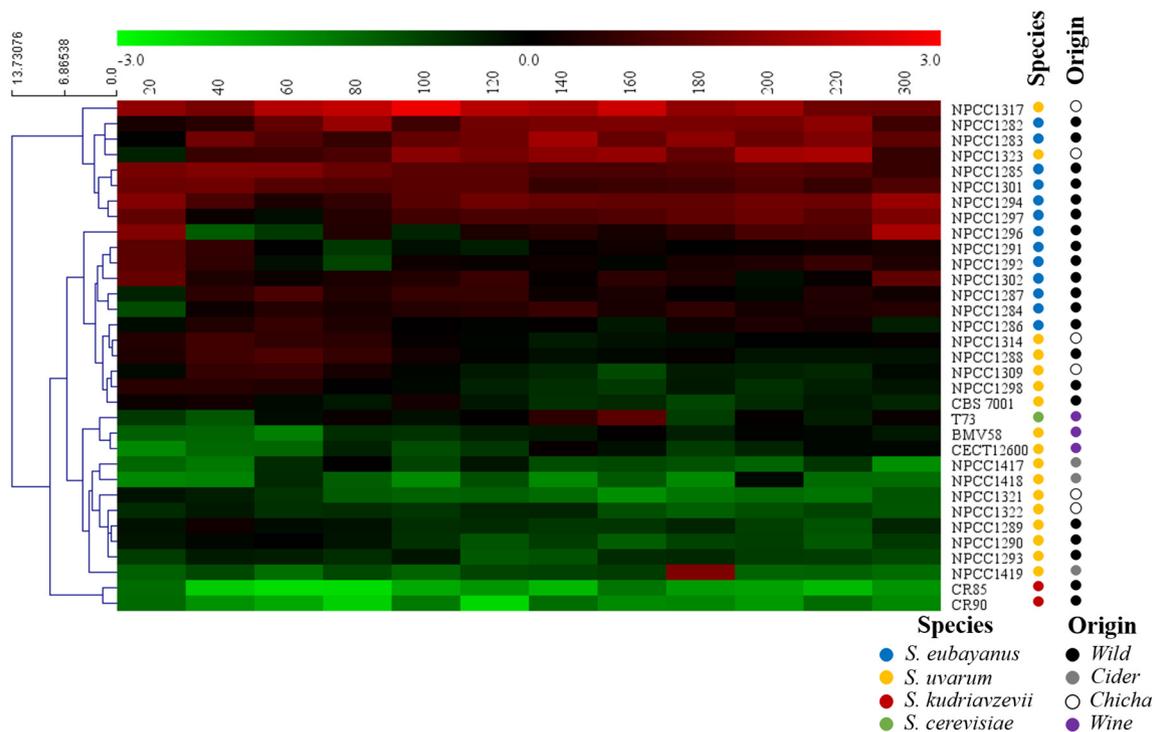
Fig. 1. Heatmap representation of μ_{max} values of the analysed strains at increasing nitrogen concentrations. Each line corresponds to a strain (indicated at the right of the Figure) and each column to a particular nitrogen concentration (indicated at the top of the Figure). The colour key bar at the top indicates μ_{max} value relative to the average for each particular nitrogen concentration: values higher than the average are in red and values lower than the average are in green. Hierarchical clustering is showed on the left. Colour dots on the right of the Figure indicate both species identity and origin of the strains. The statistical significance is shown in Supplementary material Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
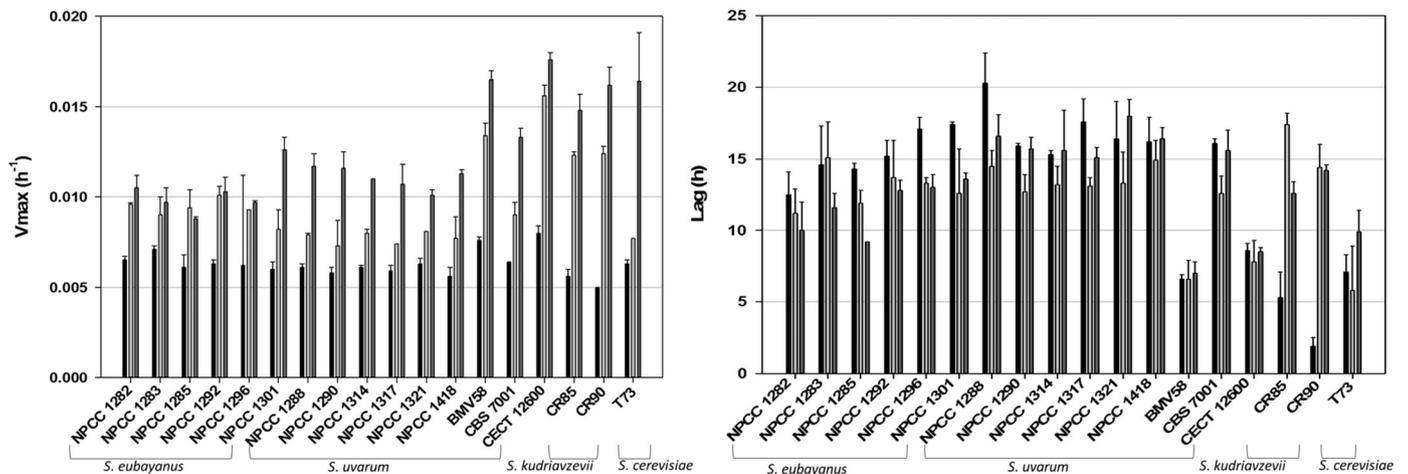
Fig. 2. (A) Maximum fermentation velocity and (B) lag stage of strains from several origins at fermentation performed at different YAN concentrations (■ 60 mg/L, ▨ 140 mg/L and ▨ 300 mg/L). The statistical significance is shown in Supplementary material Table 3.

also observed in the fermentation performance of the yeasts, we selected strains showing high, low and intermediate nitrogen requirements to be evaluated in fermentations with low (60 mg/L YAN), high (300 mg/L YAN) and intermediate (140 mg/L YAN) nitrogen concentrations. The strains with high nitrogen requirements were selected among those showing low μ_{max} values at low nitrogen concentrations and high μ_{max} values at high nitrogen concentrations according to the normalised data from Fig. 1 (*S. eubayanus* NPCC 1296, 1301 and *S. uvarum* NPCC 1321, 1418). Conversely, the strains with low nitrogen requirements were selected among those showing high μ_{max} values at low nitrogen concentrations and also high (or average) μ_{max} values at high nitrogen concentrations (*S. eubayanus* NPCC 1282, 1283, 1285 and *S. uvarum* NPCC 1288, 1314, 1317). *S. eubayanus* strain NPCC 1292 and

S. uvarum strain NPCC 1290, showing intermediate nitrogen requirements, were also evaluated. For comparative purposes, we additionally included the two *S. kudriavzevii* strains CR85 and CR90, the *S. uvarum* reference strains BMV58, CBS 7001 and CECT 12600 and the *S. cerevisiae* reference strain T73.

Weight loss data due to CO_2 release were calculated by Gompertz equation and parameters V_{max} (representing the maximum rate of CO_2 production) and λ_{CO2} (representing the time required to start the fermentation) were obtained for each strain at three nitrogen conditions: 60, 140 and 300 mg/L (Fig. 2 and Supplementary material Table 3). Interestingly, contrary to the expected, the strains selected for their high or low nitrogen requirements showed similar fermentative behaviour in each nitrogen concentration. Generally, the increasing nitrogen

concentration enhanced the V_{max}, but with different intensity in the various group of strains. *S. eubayanus* strains were less affected by the increasing nitrogen concentration, showing similar values in V_{max} obtained at both 140 mg/L and 300 mg/L. The V_{max} of *S. uvarum* Patagonian strains (NPCC strains) improved greatly along with increasing nitrogen concentration, especially high value was observed at 300 mg/L. The greatest influence of nitrogen concentration on V_{max} was observed for the wine *S. uvarum* and *S. kudriavzevii* strains, which showed around twice V_{max} values at high nitrogen concentrations compared with that observed at 60 mg/L YAN (Fig. 2A).

Regarding the time needed to start fermentation (Fig. 2B; λ_{CO2}), *S. eubayanus* and *S. uvarum* mostly shortened this lag period as nitrogen concentration increased. Conversely, the two *S. kudriavzevii* strains showed a negative correlation between nitrogen concentration and the time needed for starting fermentation, which showed higher λ_{CO2} values at intermediate and high nitrogen concentrations. Finally, the shortest λ_{CO2} was observed in the wine strains *S. uvarum* BMV58, CECT12600 and *S. cerevisiae* T73, regardless the nitrogen concentration. This is of great significance for the reason that, it evidences that regardless of the species or the nitrogen concentration, the isolating origin of the strains is the most influencing factor for a good adaptation to the growth medium.

3.2. Competition tests S. cerevisiae vs. S. eubayanus/S. uvarum

For their industrial application potential, one *S. eubayanus* strain (NPCC 1285) and one *S. uvarum* strain (NPCC 1317), showing the best performance at low nitrogen concentrations (especially evident in growth assays) were selected to perform mixed culture fermentation with *S. cerevisiae* reference strain T73, in order to evaluate their competition capacity in synthetic grape musts with different nitrogen concentrations. We evaluated, in the first stage, the competition at both low and high nitrogen content (60 mg/L and 300 mg/L YAN). Moreover, due to the well-known differential temperature preference of the yeasts involved in the competitions, two different fermentation temperatures (28 °C and 12 °C) were evaluated for the mixed cultures. For comparative purposes, monoculture fermentations were also carried out with the three strains involved in the competitions.

Our result showed that both fermentation temperature and nitrogen concentrations have significant influences on the fermentation kinetics and on the competitiveness of the strains. At 28 °C, most of the fermentations were completed with both nitrogen concentrations (only *S. eubayanus* monoculture was unable to end up fermentation). However, the low YAN concentration yielded sluggish fermentations (Fig. 3A–B). Conversely, at 12 °C, the combination low temperature and low YAN resulted in unfinished fermentations for all the tested monocultures or mixed cultures (Fig. 3C). At low temperature but high YAN concentration, *S. uvarum* and *S. eubayanus* monocultures showed the highest fermentation activity and the shortest fermentation times (Fig. 3D). The higher fermentation activity for *S. cerevisiae* at 28 °C and for *S. eubayanus*/*S. uvarum* at 12 °C generally correlated with higher CFU counts (Fig. 4). Regardless YAN concentration, the cryotolerant species showed greater final population size at 12 °C. Conversely, only *S. eubayanus* produced more cells than *S. cerevisiae* at 28 °C and low YAN.

In terms of strain composition in mixed culture fermentations, temperature of fermentation determined the competitiveness of the different strains and nitrogen did not have any impact on their implantation. At 28 °C, *S. cerevisiae* completely dominated the two mixed-culture fermentations, with implantation percentages higher than 60% in all samples only after 6 h of fermentation (Fig. 5A–B). Conversely, at 12 °C, the cryotolerant yeasts *S. eubayanus* and *S. uvarum* dominated the mixed fermentations. Nevertheless, this implantation was not absolute, with percentages of *S. cerevisiae* around 10–30% at the end of the fermentations (Fig. 5C–D).

As 12 °C is an extreme low fermentation temperature to conduct

fermentation in the wineries, we decided to test the selective effect of a milder temperature for yeast development. Thus, we subsequently evaluated the same mono and mixed cultures at 20 °C. In this case, we also considered to include, additionally, an intermediate nitrogen concentration of 140 mg/L. In this assay, since the influence of the temperature has been reduced, a strong effect of nitrogen concentration on fermentation capacity, and hence, in the competition capacity of the yeast species involved in the mixed cultures was observed (Fig. 6). The implantation percentages of *S. cerevisiae* at 300 mg/L YAN were around 60–90%, whereas the same at 60 mg/L YAN was only 40–50%, independently from the analysed mixed culture (*S. cerevisiae* + *S. uvarum* or *S. cerevisiae* + *S. eubayanus*). Moreover, at this high nitrogen concentration, the imposition of *S. cerevisiae* is stronger competing with *S. eubayanus* than with *S. uvarum*. Interestingly, with 140 mg/L YAN, the implantation of *S. cerevisiae* kept around 50–60% throughout the fermentation (Fig. 6A). This is of great importance, since under this condition, most of the fermentations were able to finish (Fig. 6B), and the implantation of the cryotolerant strains remain considerable. Furthermore, the monoculture of *S. eubayanus* also showed the fastest fermentation activity. As at 12 °C, none of the strains finished the nitrogen-limited fermentations (60 mg/L YAN) and the cryotolerant strains exhibited higher CFU counts than *S. cerevisiae* monocultures, regardless of the nitrogen concentration (Fig. 6B–C).

Based on the percentage of *S. cerevisiae* at the end of mixed culture fermentation, a multi-factorial ANOVA was performed (Table 1). In the multi-factorial ANOVA, the effects of three parameters have been compared: temperature (12 °C, 20 °C and 28 °C), nitrogen (60 mg/L and 300 mg/L) and the cryotolerant strain used in the mixed culture (*S. cerevisiae* + *S. eubayanus* and *S. cerevisiae* + *S. uvarum*). According to the results, temperature (T), nitrogen concentration (N) and the combination of T and N had significant and strong influences on the competitiveness of *S. cerevisiae* in the mixed culture; regardless of the cryotolerant species used in combination with *S. cerevisiae*. When combining all the three parameters together, the impact is also significant with p value lower than 0.05.

The analysis of main chemical parameters at the end of fermentations has been carried out for fermentations at 20 °C. As mentioned above, no culture was able to complete the fermentation at low nitrogen concentration (60 mg/L), with the presence of high values of residual sugars in the final wines (Supplementary Fig. 1A). Since not all the fermentations were completed, the production of ethanol and glycerol is shown as yield produced per gram of sugar (glucose + fructose) consumed (Supplementary Fig. 1B–C). No significant differences were observed in ethanol yield among different nitrogen concentrations and different strains. On the contrary, *S. eubayanus* and *S. uvarum* strains have much higher glycerol yield than *S. cerevisiae*, especially with low nitrogen concentration 60 mg/L YAN. Nitrogen concentration does not have big influence on glycerol yield by *S. cerevisiae*.

4. Discussion

4.1. Growth and fermentative performance are variable according to species and life history of yeasts in monocultures

It has been described that the nitrogen requirement during wine fermentation is a strain-specific feature (Gutiérrez et al., 2012). We demonstrated, for the first time, the existence of a differential behaviour in nitrogen requirement among strains of the cryotolerant species *S. uvarum*, *S. eubayanus* and *S. kudriavzevii*. The effect of different nitrogen concentrations was evidenced in both yeast growth and fermentation rate of the strains. However, a good growth performance was not necessarily associated with a good fermentation performance of a particular yeast, i.e. *S. eubayanus* or *S. uvarum* strains, with the highest and the lowest μ_{max} growth values, showed no differences in their maximum fermentation rate evaluated by CO_2 release. Gutiérrez et al. (2012) and Kemsawad et al. (2015) have also reported this lack of
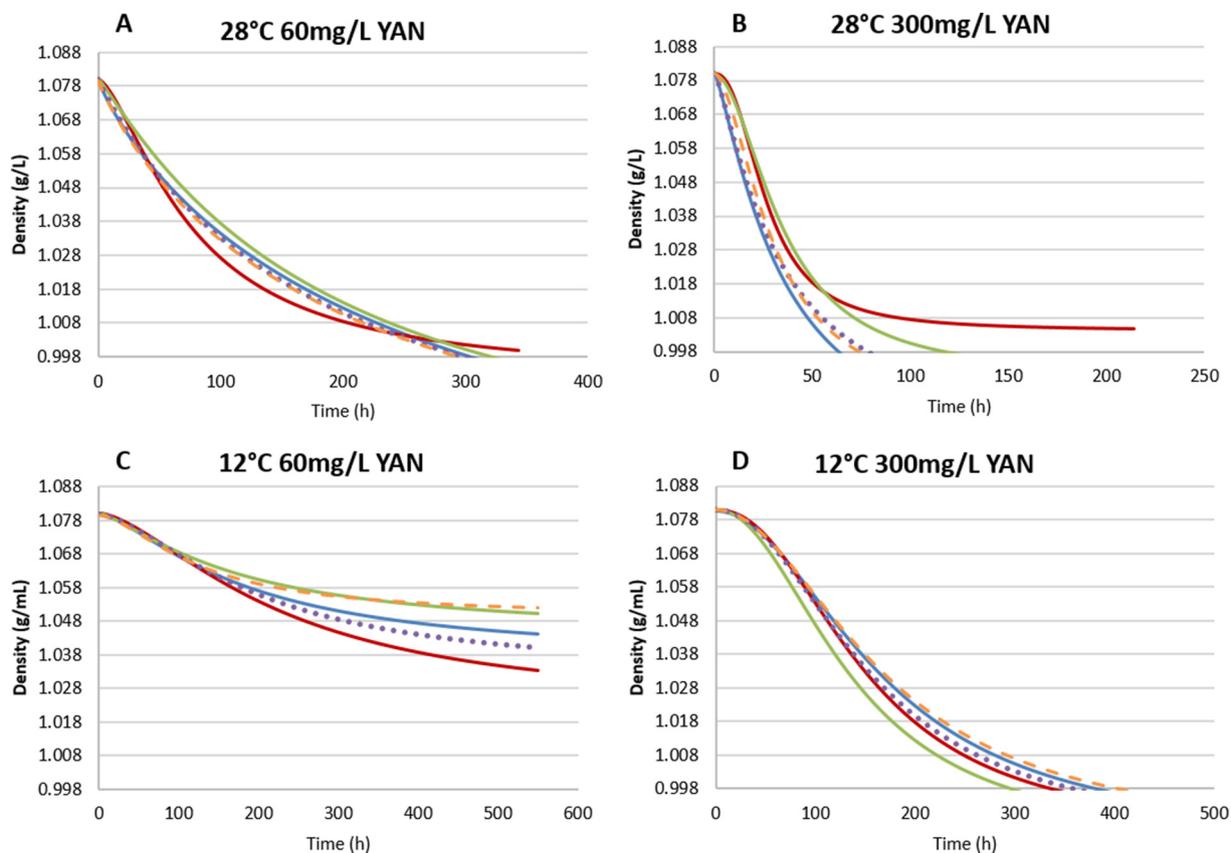
Fig. 3. Fermentation kinetics at 28 °C 60 mg/L YAN (A), 28 °C 300 mg/L YAN (B), 12 °C 60 mg/L YAN (C) and 12 °C 300 mg/L YAN (D) ▬ *S. cerevisiae*, ▬ *S. eubayanus*, ▬ *S. uvarum*, ••• *S. eubayanus + S. cerevisiae*, ▬ ▬ *S. uvarum + S. cerevisiae*.

correlation between growth and fermentation behaviour. In the study of Gutiérrez et al. (2012), the nitrogen requirements of four commercial *S. cerevisiae* wine strains were analysed under growth and fermentation conditions. One of the strains, which showed the poorest growth capacity, had the best fermentation performance. Kemsawasd et al. (2015) investigated the influence of different nitrogen sources on growth and fermentation activity of *S. cerevisiae* and four wine-related non-*Saccharomyces* yeast species (*Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* and *Torulaspora delbrueckii*), and they have also concluded that a good growth capacity does not always result in a good fermentation behaviour. Therefore, the two parameters, growth and fermentation performance, must be evaluated

in order to understand the yeast performance during alcoholic fermentation.

Interestingly, most of the strains identified as *S. eubayanus* showed higher μ_{max} values in microplate assays than those belonging to *S. uvarum* species, for all different nitrogen concentrations tested. Even *S. uvarum* strains isolated from the same natural habitats as *S. eubayanus* (*A. araucana* trees) showed lower μ_{max} values, similar to those observed for the *S. uvarum* strains isolated from fermentative environments. This observation evidences a clear species-specific behaviour, potentially associated with a particular ecological strategy. The species-specific effect of nitrogen on both growth rate and fermentation performance was also reported by Kemsawasd et al. (2015) for a set of non-
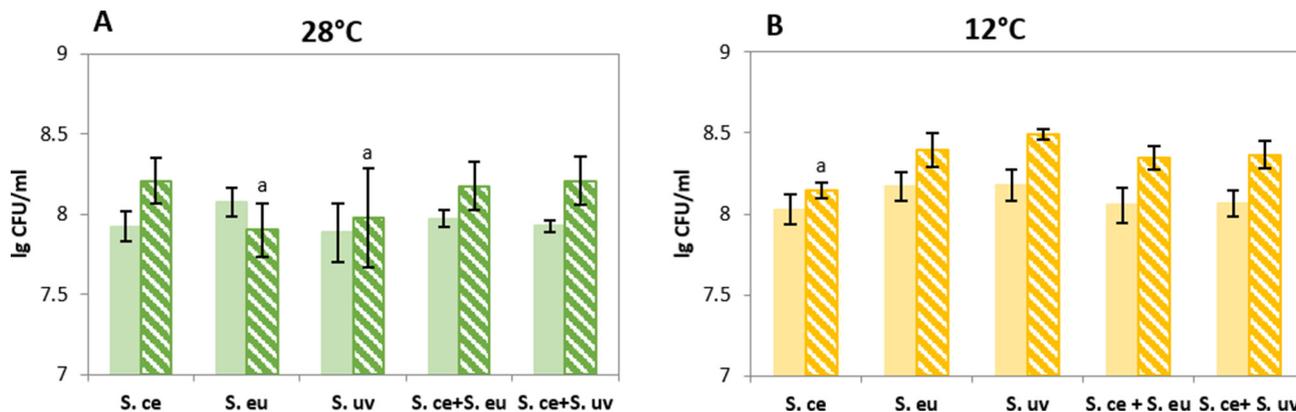

Fig. 4. Maximum population during stationary phase of fermentation at 28 °C (A) and 12 °C (B). Light green (28 °C) and light yellow (12 °C) bars represent 60 mg/L YAN. Dark green (28 °C) and dark yellow (12 °C) bars with diagonal lines represent 300 mg/L YAN. One-way ANOVA was performed within each nitrogen concentration. Letters on top of the bars indicate significant difference (p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
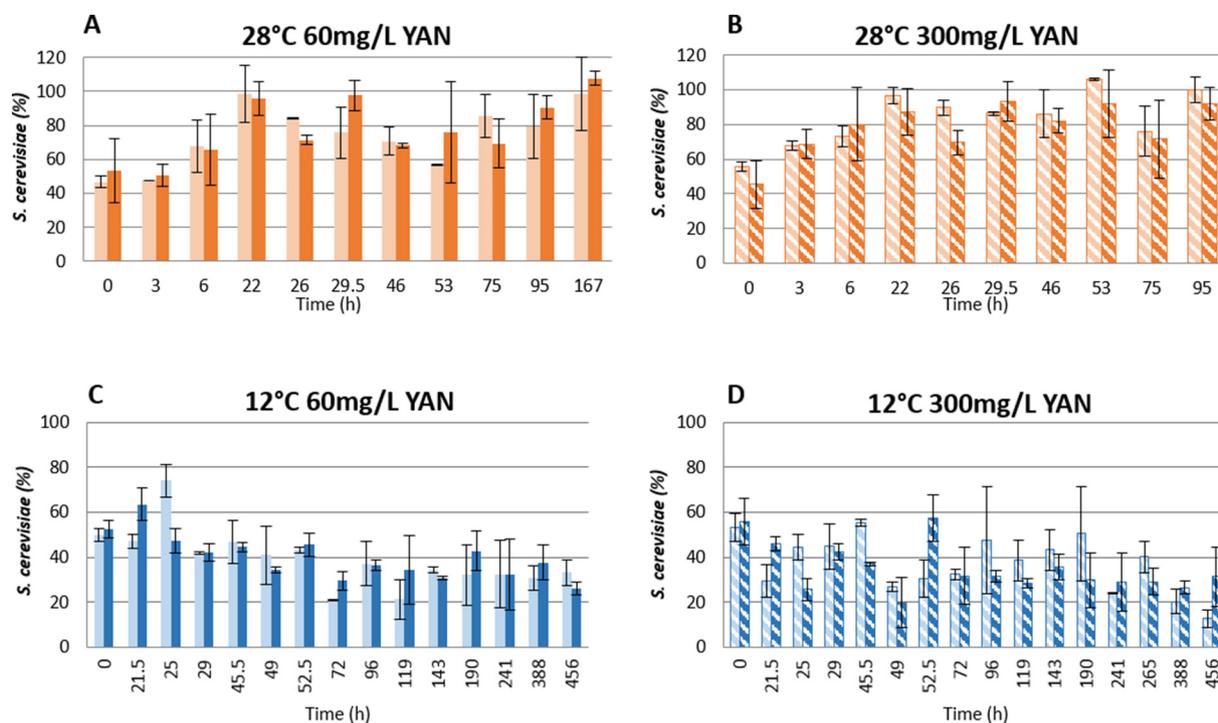
Fig. 5. *S. cerevisiae* growth evolution in mixed culture fermentations at 28 °C 60 mg/L YAN (A), 28 °C 300 mg/L YAN (B), 12 °C 60 mg/L YAN (C) and 12 °C 300 mg/L YAN (D). Bars in light colour represent *S. eubayanus* + *S. cerevisiae* and bars in dark colour represent *S. uvarum* + *S. cerevisiae*. Bars with diagonal lines show the value of 300 mg/L YAN. Bars without patterns show the value of 60 mg/L YAN.

Saccharomyces species including *Lachancea thermotolerans* and *Metschnikowia pulcherrima*. The authors suggested that some of the differences between the responses of non-*Saccharomyces* species and *S. cerevisiae* could be related to genetic differences shaped by human activity (domestication) of *S. cerevisiae*. Spor et al. (2009) also observed that populations harboured different strategies depending on their ecological niches. These authors found that forest and laboratory strains reach a large carrying capacity (population size) and a small cell size in fermentation, but they have a low reproduction rate in respiration and produce lower quantities of ethanol, suggesting that they store cell resources rather than secreting secondary products. Contrarily, the industrial strains of this species reproduce slowly, reach a small carrying capacity but have a big cell size in fermentation and a high reproduction rate in respiration with higher glucose consumption rates. These two contrasted behaviours were metaphorically defined by the authors (Spor et al., 2009) as "ant" and "grasshopper" strategies of resource utilisation. Similar results were obtained in this work when compared with different strains of *Saccharomyces* non-*cerevisiae* species isolated from diverse habitats. We observed that independently from the nitrogen concentration, both the highest μ_{max} in microplates assays and the highest CFU numbers in competition experiments were observed for *S. eubayanus* strains isolated from natural habitats. It is interesting to note that for the specific case of *S. uvarum* species, including strains from natural habitats and fermentative environments, previous phylogenetic analyses have evidenced the existence of at least two different populations with low genetic flux among the strains of this species, isolated from natural habitats in Patagonia and named Southamerica-A and Southamerica-B/Holartic (Almeida et al., 2014). On the other hand, all strains isolated from fermented beverages in the same region belonged to the Southamerica-A/Holartic subpopulation (Almeida et al., 2014; Rodríguez et al., 2017). This data also supports the fact that the origin, more than the phylogenetics relationships, are involved in the physiological response of the yeast to the analysed culture conditions. In previous work carried out in our laboratory with a set of *S. uvarum* and *S. eubayanus* strains including those used in this work, we

also evidenced clear physiological differences between strains from natural and fermentative habitats (González Flores et al., 2017; Origone et al., 2017).

The effect of nitrogen content on both the growth and fermentative capacity of the evaluated yeasts is a complex issue. Although several works have evaluated the chemical composition of grape must in different winemaking areas, the lack of information about the same in natural habitats makes the evaluation of this phenomenon difficult. Strikingly, the strongest effect of increasing nitrogen concentrations on the maximum fermentative rate was observed for strains associated with industrial fermentations (*S. cerevisiae* T73, *S. uvarum* BMV58 and CECT 12600) as well as for strains strictly associated with natural habitats (*S. kudriavzevii* CR85 and CR90). This was observed for pure culture fermentations with 60, 140 and 300 mg/L YAN. However, the same assay carried out with *S. eubayanus*, the other species associated only with natural habitats, demonstrated only a slight difference in this parameter at increasing nitrogen concentrations. These results suggest that both the yeast species and their ecological life-history are involved in the response strategy of yeasts to this environmental factor. This phenomenon became relevant for the development of mixed cultures for winemaking as it has been demonstrated to enhance the organoleptic complexity of wines. This fact, added to the tendency to produce wines at low temperature as well as to produce more aromatic wines, makes the study of *Saccharomyces* non-*cerevisiae* cryotolerant yeast strains an interesting topic for research, especially in mixed cultures with *S. cerevisiae*. The competition ability of several *S. cerevisiae* strains under different nitrogen concentration has been evaluated (García-Ríos et al., 2014; Lemos Junior et al., 2017; Vendramini et al., 2017); however, little is known about the competition ability between *S. cerevisiae* versus other species from the *Saccharomyces* genus.

4.2. Low temperature and low nitrogen concentration favour Saccharomyces non-cerevisiae implantation in mixed cultures

The implantation capacity of two *Saccharomyces* non-*cerevisiae*
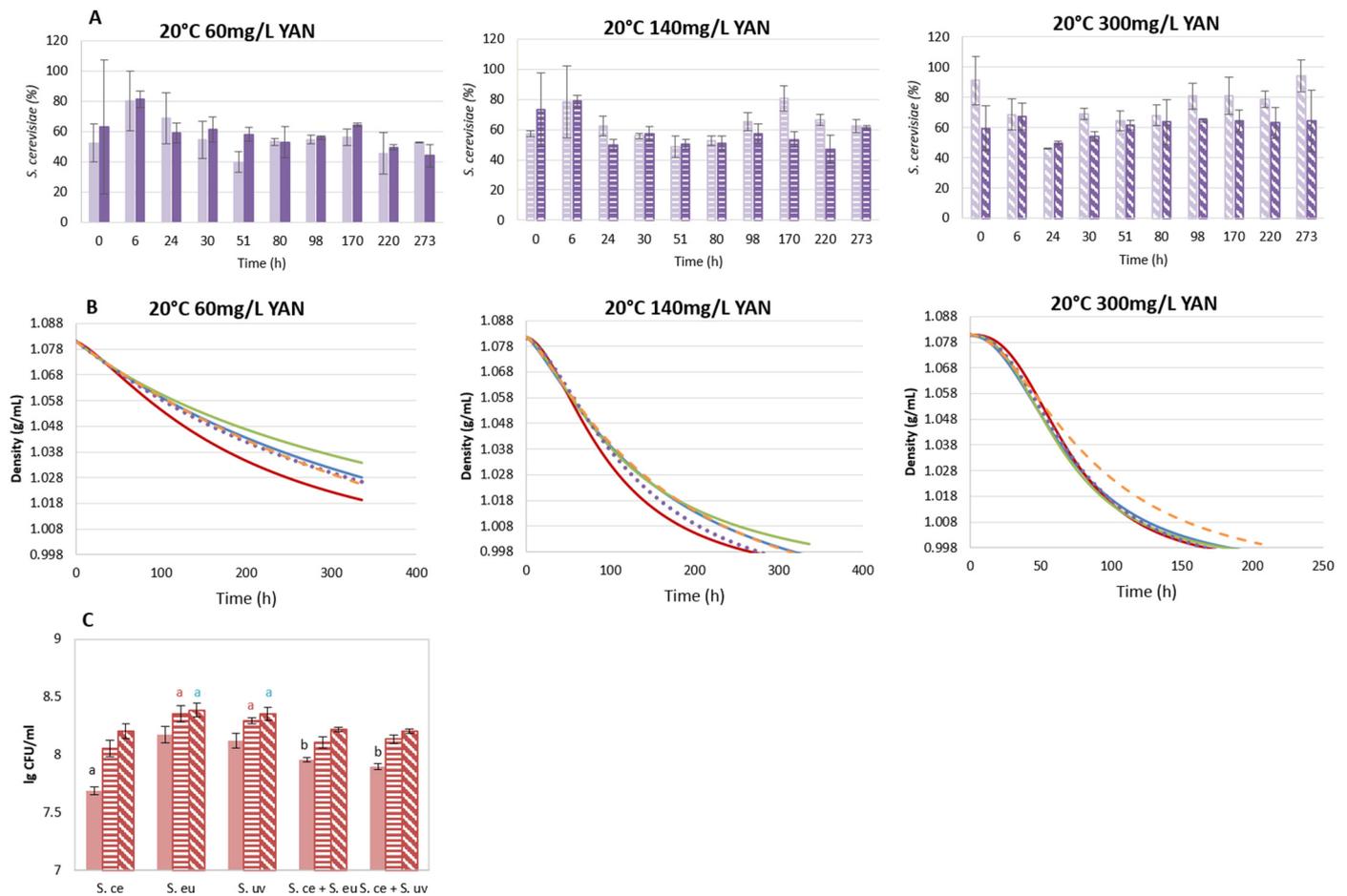
Fig. 6. (A) *S. cerevisiae* growth evolution in mixed culture fermentations. Light purple bars represent *S. eubayanus* + *S. cerevisiae* and dark purple bars represent *S. uvarum* + *S. cerevisiae*. 60, 140 and 300 mg/L of YAN are represented by bars without patterns, bars with horizontal lines, and bars with diagonal lines, respectively. (B) Fermentation kinetics at 20 °C: ▬ *S. cerevisiae*, ▬ *S. eubayanus*, ▬ *S. uvarum*, ▪▪▪ *S. eubayanus* + *S. cerevisiae*, ▬ ▬ *S. uvarum* + *S. cerevisiae*. (C) Maximum population during stationary phase of fermentation. Pink bars without pattern represent 60 mg/L YAN. Red bars with horizontal lines represent 140 mg/L YAN and red bars with diagonal lines represent 300 mg/L YAN. One-way ANOVA was performed within each nitrogen concentration. Letters on top of the bars indicate significant difference (p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strains (*S. eubayanus* NPCC 1285 from natural habitats and *S. uvarum* NPCC 1317 from apple chicha), which were selected according to their performance in fermentations with low nitrogen concentrations, was evaluated in mixed cultures with the wine commercial *S. cerevisiae* strain T73 at different temperatures.

Our results indicate that, as expected, increased temperature improves the advantage of *S. cerevisiae*, whereas, conversely, lowered temperature reduces its competitiveness, favouring cryotolerant strains, independently from the nitrogen concentration. This phenomenon was first reported by Arroyo-López et al. (2011) and Salvadò et al. (2011) for competition experiments between different yeast strains, and recently evidenced by Alonso del Real et al. (2017) for competitions between one strain of *S. eubayanus*, *S. uvarum* or *S. kudriavzevii* and *S.*

cerevisiae. In our work, we also evidenced that temperature is the most important parameter governing the implantation of both *S. eubayanus* and *S. uvarum* in mixed cultures with *S. cerevisiae*. However, we observed a better competition capacity of our strain *S. eubayanus* NPCC 1285 against *S. cerevisiae* T73 at 20 °C. This fact was particularly observed with an intermediate nitrogen concentration (< 140 mg/L). This could be due to the fact that the strains used in competition fermentation were selected because of their low nitrogen requirement, according to the assays previously discussed.

Several other factors can be related to the competitive capacity of a yeast strain including competition for space and for other specific nutrients, oxygen affinity, production of antagonist compounds as killer toxins or other diffusible molecules, among others (Fleet, 2003). In the

Table 1
F-values and p-values for percentage of *S. cerevisiae* at the end of mix-culture fermentation obtained by a multi-factorial ANOVA.

	Sources of variation						
	Temp (T)	N concentration (N)	Mix-culture[a] (M)	T * N	T * M	N * M	T * N * M
F value	386.80	9.97	0.10	31.76	3.26	0.18	6.02
p value[b]	p < 0.001	p < 0.01	0.76	p < 0.001	0.076	0.68	p < 0.05
DF	2	1	1	2	2	1	2

[a] There are two mix-culture type which are *S. eubayanus* + *S. cerevisiae* and *S. uvarum* + *S. cerevisiae*.
[b] The data is considered as significant when p value is < 0.05.

same way, Pérez-Torrado et al. (2017) demonstrated that both cell-to-cell contact and differential sulphite production and resistance are important factors that determine the dominance of one *S. cerevisiae* strain over another. Additionally, Cheraiti et al. (2005) observed that acetaldehyde production and redox interactions are involved in competitions between *S. cerevisiae* and *S. uvarum*.

A particular strategy that seems to adopt *S. eubayanus*, and, to a lesser extent, *S. uvarum*, is a significantly higher CFU number than in *S. cerevisiae* at lower temperatures. This phenomenon was observed in most of the analysed conditions in monocultures (both microplates and competition assays). These differences were more evident at lower nitrogen concentrations, i.e. *S. cerevisiae* evidenced an increase in its population size directly proportional to the nitrogen content in the medium while this response was not in the same magnitude for *S. eubayanus*. Natural environments are usually nutrient poor; in this context, yeast strains able to grow in the presence of low nitrogen concentrations are selected, and this could be the situation for *S. eubayanus*. This species seems not to be able to respond efficiently to increasing nitrogen concentrations. Contrarily, it has been argued that *S. cerevisiae* does not show adaptations to any particular habitat, but rather an ability to survive in a wide range of conditions (Goddard and Greig, 2015) which is consistent with its life history of nomadic generalist that inhabits diverse niches (Jouhten et al., 2016; Liti et al., 2009). Interestingly, it has also been demonstrated that one genomic region that shows strong differentiation between the oak and wine European populations of *S. cerevisiae* correspond to a gene coding for a transporter for oligopeptides that can act as nitrogen sources in wines (Marsit et al., 2015). Additionally, Treu et al. (2014) evidenced that gene sequences involved in nitrogen metabolism are more variable among *S. cerevisiae* strains from vineyard than in industrial strains, suggesting a different aptitude in nitrogen uptake and management. Altogether, these data indicate that nitrogen metabolism is strongly dependent on the origin of the yeast strains in both *S. cerevisiae* and *Saccharomyces* non-*cerevisiae* yeasts. The exploitation of these differential metabolic behaviours could be of interest for the development of mixed cultures for rational winemaking.

5. Conclusions

The results obtained in this work evidenced for the first time that both nitrogen content in must and fermentation temperature are important factors involved in the competition of *S. eubayanus* and *S. uvarum* in mixed culture fermentations with *S. cerevisiae*. Moreover, *S. eubayanus* in mono and mixed culture with *S. cerevisiae* showed the best fermentation performance in synthetic must, at low nitrogen concentration and low fermentation temperature. The employment of this kind of mixed cultures can become a strategy for winemakers to overcome the problem of nitrogen-limited musts.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.11.020.

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Competing interests

The author declares that there are no competing interests regarding the publication of this paper.

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