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Nitrogen modulation of yeast fitness and viability during sparkling wine production

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ABSTRACT

In the production of sparkling wine by the traditional method a second fermentation inside the bottle is required. To survive in such conditions of high ethanol concentration and high pressure, yeast cells must previously undergo an acclimation process. In this study, we investigated the role of the nitrogen composition during the acclimation phase by measuring growth and fermentative parameters through the second fermentation process. We used eight *Saccharomyces cerevisiae* strains of different origin to determine the impact of yeast genetic background on the efficiency of the acclimation process. The nitrogen source used in the acclimation media had a strong impact on yeast growth during this phase, but also affected significantly fermentation kinetics during the second fermentation. The yeast strain origin mostly affected the second fermentation kinetics. Surprisingly, the use of a medium rich in amino acids that are precursors of fusel alcohols, although triggered slow growth during the acclimation phase, it increased yeast viability and fitness through the second fermentation. Overall, we demonstrated how the nitrogen composition of the acclimation media impacts on yeast fitness and viability. The modification of the nitrogen composition during this phase is proposed as a tool to optimize yeast performance during the second fermentation.

1. Introduction

Microbial acclimation occurs naturally and allows the microbial community to adjust its metabolism to the ever-changing environment. Furthermore, microorganism acclimation is used in several biotechnological processes in the food industry that require an inoculum adapted to a given stress, in order to maintain its metabolic activity in the stressful conditions, e.g. to optimize the leavening activity of the yeast in the bakery industry (Zamani et al., 2008) or to ensure the successful fermentation of ice wines (Kontkanen et al., 2004). In enology, sparkling wine production involves the use of an acclimated inoculum (pied-de-cuve) to ensure the successful completion of the process. Its production involves two fermentation steps. The first alcoholic fermentation transforms the grape must into the base wine. The second step consists of the transformation of the base wine into sparkling wine due to a second fermentation that, in the traditional method, takes place inside the bottle. This second fermentation begins after bottling the base wine and the "liqueur de tirage", which contains sucrose (20–24 g/l), adjuvant (usually bentonite, to promote the flocculation and later removal of the yeast cells) and an acclimated yeast inoculum (Buxaderas and López-Tamames, 2003; Carrascosa et al., 2011). In the bottle, the yeast population must face several environmental stresses, including the increasing CO_2 pressure, the high ethanol content of the base wine and the low temperatures of fermentation. To overcome these restrictive conditions, the yeast population must be properly acclimated before being inoculated into the base wine. The acclimation process usually involves the culture of the desired yeasts in diluted wine that contains increasing concentrations of ethanol and sugar. Kunkee and Ough (1966) analyzed the effect of the acclimation process on the growth and fermentation capability of *Saccharomyces cerevisiae* during the second fermentation. These authors reported that the acclimation of yeast in a base wine, either under or without CO_2 pressure, is essential for a quick and efficient completion of the second fermentation. Other studies analyzed the effect of aeration during the pied-de-cuve, the use of wine as the adaptation medium and the quantity of inoculum on the fermentation development

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(Juroszek et al., 1987; Laurent and Valade, 2007; Monk and Storer, 1986).

The role of nitrogen in wine production has been extensively studied, and it is known to affect growth and biomass production, fermentation kinetics and even the organoleptic characteristics of the final product (reviewed by Bell and Henschke (2005)). The role of nitrogen composition in the ability of yeast to overcome stressful conditions, such as fermenting high sugar musts, has also been recently analyzed (Martínez-Moreno et al., 2012). However, nitrogen preferences and utilization diverge among different yeast strains (Brice et al., 2014b; Gutiérrez et al., 2012), partly due to their genetic variations (Brice et al., 2014a; Ibstedt et al., 2014; Jara et al., 2014). The selected yeast strain has also a clear effect on both the fermentation kinetics (Martí-Raga et al., 2015) and the organoleptic characteristics of the final product, mostly due to its autolytic capacity (reviewed by Alexandre and Guilloux-Benatier (2006)).

The nitrogen concentration of the base wine will strictly depend on both the grape must nitrogen content and how much is consumed by yeast during the first fermentation. Our recent work (Martí-Raga et al., 2015) demonstrated that although the nitrogen content of the base wines is highly variable (17–75 mg N/l), a correlation between second fermentation kinetics and the nitrogen content could only be established for those base wines containing less than 30 mg N/l. Furthermore, we demonstrated that the nitrogen taken up during the pied-de-cuve is enough to cover the yeast nitrogen requirements during the second fermentation, independently of the nitrogen content of the base wine, and clearly affects the development of the fermentation, with strain-dependent nutrient preferences. Thus, the aim of this study is to understand how different sources of nitrogen in the pied-de-cuve can modulate yeast strain viability and fitness during the second fermentation. In order to determine also strain-specific requirements for given forms of nitrogen sources, several nitrogen mixtures, as well as several yeast strains of different natural origin, were used in the whole process. We propose variations on the nitrogen composition of the acclimation media as a tool to optimize yeast fitness for a given biotechnological process, such as sparkling wine production.

2. Materials and methods

2.1. Yeast strains used and propagation media

Eight *S. cerevisiae* yeast strains were used in this study, most of them being wine strains. However, to better capture the phenotypic variability of *S. cerevisiae* species two strains of distillery and one isolated from oak bark were also included. These strains were either wine starters supplied by the Laffort company (F10 and Spark) or diploid monosporic clones derived from different origins, such as wine (VL3, GN and SB), distillery (A24 and 294) or oak bark (OS104) (Supplementay Table S1). Before the experiments, yeast cells were cultivated 24 h at 25 °C in YNB medium (2% glucose, 0.17% YNB *w/o* amino acids and ammonium) supplemented with 140 mg N/l of ammonium phosphate.

2.2. Media for the pied-de-cuve and second fermentation

The experiment consisted of two successive steps: the adaptation (48 h) and the proliferation phases (96 h). Both steps were performed in a chemically defined media simulating base wine. This synthetic base wine contained sucrose 50 g/l, tartaric acid 4 g/l, L-citric acid 0.5 g/l, malic acid 0.5 g/l, sodium acetate 0.134 g/l, YNB *w/o* amino acids and ammonium 1.7 g/l and glycerol 4.0 g/l. The pH was adjusted to 3.3 with potassium hydroxide. The ethanol concentration was adjusted to 6 and 8% (v/v) for the adaptation and proliferation stages, respectively. In the adaptation phase, the nitrogen source used was 20 mg N/l diammonium phosphate. For the proliferation phase, six different nitrogen mixtures were used with a final concentration of 60 mg N/l (Table 1). The first three mixtures (I, Mu, and BW) attempted to emulate the common industrial practices, containing diammonium phosphate (I) or the nitrogen content of grape must (Mu) or base wine (BW). The three other nitrogen mixtures (O, Ar and AG) were experimental compositions used for investigating the effect of several organic nitrogen sources. The mixture O contained the average organic nitrogen content found in nitrogen-rich fermentation activators. The mixture Ar contained five amino acids that are precursors of aromatic compounds and showed endurance properties of yeast during the alcoholic fermentation when used as the sole source of nitrogen (Martínez-Moreno et al., 2012). The mixture AG combined the amino acids present in Ar with amino acids of quick uptake (glutamine/glutamate). This mixture was only used for the experiment on yeast viability in the second fermentation.

The second fermentation was conducted using natural base wine (ethanol 10.11% (v/v), pH = 3.1, YAN = 30.8 mg N/l), kindly donated by Juve & Camps (Penedés, traditional mixture of Macabeu, Xarel·lo and Parellada grape varieties) supplemented with 22 g/l sucrose.

2.3. Growth parameters for the pied-de-cuve

At the end of the adaptation step of pied-de-cuve (48 h, 25 °C), yeast growth was measured (OD_{600} readings) and yeast strains were inoculated (OD_{600} = 0.2) into a fresh synthetic base wine for the proliferation phase (96 h, 25 °C). During the proliferation stage of pied-de-cuve cell growth was monitored in 96-well microcultivation plates using a POLARstar Omega (BMG Labtech, Offenburg, Germany). Culture plates were shaken every 30 min for 60 s prior to the OD_{600} measurement. The well position on the microplate was randomized, and six replicates were run for each condition. Data from the microplate reader were transformed with the polynomial curve $y = -0.0018^{*}x^3 + 0.1464^{*}x^2 + 0.7757^{*}x + 0.0386$ to correct the nonlinearity of the optical recording at higher cell densities. The polynomial curve was obtained following the protocol described by (Warringer and Blomberg, 2003).

Growth kinetic data were fitted using a modified 3PL model to include an evaporation coefficient. The incorporation of the evaporation coefficient into the model allowed us to rectify the steady increase of the OD_{600} value due to the evaporation of the media that occurs once the stationary plateau is reached. The final equation was as follows,

$$N_t = \frac{KN_0 e^{rt}}{K + N_0(e^{rt} - 1)} + \text{evap}^{*}t$$

Here, *Nt* is the OD_{600} at time *t*, *K* is the maximum population (maximum OD_{600} reached), N_0 is the initial OD_{600} value, *r* is the growth rate and *evap* is the evaporation coefficient. The fitting of the data allowed us to extract *K* (OD units) and *r* (OD/h). We also calculated time-related parameters, such the lag phase (*lag*, h) (Fig. 1A).

2.4. Second fermentation in bottles: measurement of the fermentation kinetics

The kinetics of CO_2 production during the second fermentation were measured in a separate experiment. Yeasts were adapted following the previously described pied-de-cuve protocol. To obtain sufficient volume to inoculate the base wine, the pied-de-cuve was conducted using sterile vessels of 100 ml. To confirm

Table 1
Nitrogen mixtures used in the study. The quantity of each amino acid is expressed in mg N/l.

	Inorganic (I)	Must (Mu)[a]	Base wine (BW)[b]	Organic (O)	Aromatic (Ar)	Aromatic enriched (Ag)
Ammonium	60	17.7	4.14	0	0	0
Asp	0	0.66	2.22	3.6	0	0
Glu	0	1.68	2.94	5.4	0	11.34
Ser	0	1.5	0	0	0	0
Gln	0	14.04	4.68	0	0	10.98
His	0	0.42	1.14	0	0	0
Gly	0	0.48	1.68	0	0	0
Thr	0	1.32	1.14	4.2	20.94	13.26
Arg	0	13.02	9.84	3.66	0	0
Ala	0	3.36	4.38	19.62	0	0
Tyr	0	0.24	0	0	0	0
Cys	0	0.36	0	0	0	0
Val	0	0.78	3.9	4.2	11.58	7.2
Met	0	0.42	2.52	4.2	0	0
Trp	0	1.74	0	0	0	0
Phe	0	0.48	2.64	4.32	7.62	4.8
Ile	0	0.48	3.9	0	7.68	4.8
Leu	0	0.78	3.54	4.8	12.18	7.62
Lys	0	0.48	5.28	6	0	0
GABA	0	0	0.48	0	0	0
Asn	0	0	5.16	0	0	0
Orn	0	0	0.42	0	0	0

[a] The composition of the Mu mixture corresponds to that described in Beltran et al. (2004).
[b] The composition of the BW mixture results from averaging the nitrogen content of several base wines (Martí-Raga et al., 2015).

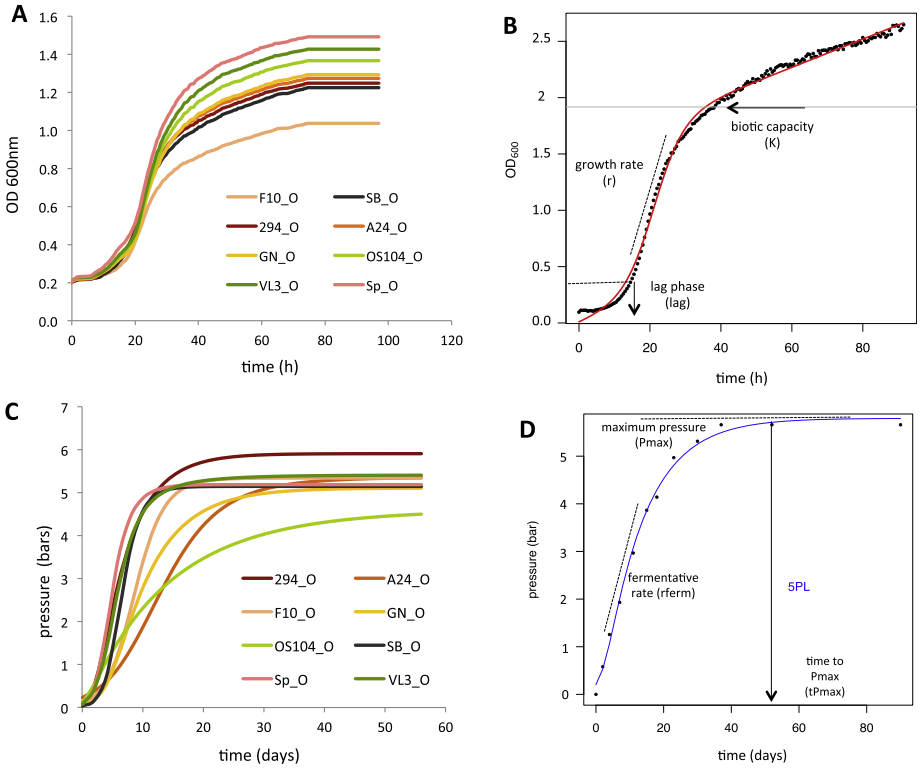

Fig. 1. Kinetic curves obtained during the study. (A) Growth kinetic curve at acclimation phase for all the strains, using O as nitrogen source. The kinetic curves represented correspond to the adjusted data. (B) Modeling of the growth kinetic curve. Black points represent the raw data, the red line represents the adjusted data and the gray signals the correction applied for the evaporation effect. The parameters extracted from the curve were: maximum population (K) corrected for the evaporation phenomenon, growth rate (r) and lag phase time (lag). (C) Fermentation kinetic curve during the second fermentation for all the strains, using O in the acclimation phase. (D) Modelization of the fermentation kinetic curve. The parameters extracted were: maximum pressure (Pmax), fermentation rate (rFerm) and time needed to reach the maximum pressure (tPmax). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that the growth pattern was not affected by the container (microplate or vessels), the growth during the proliferation phase of the pied-de-cuve was followed for three strains (VL3, A24 and SB), using I and Ar in the acclimation media (Fig. S1). At the end of the proliferation phase, yeasts were inoculated at 0.2 OD ($2*10^6$ cells/ml) into natural base wine supplemented with sucrose (22 g/l). Bentonite (30 mg/l) was used as a ridding agent. The mixture was introduced into bottles (750 ml) that were hermetically closed. Each condition was run in triplicate. The second fermentation took place at 16 °C. The CO_2 production in the bottle

was monitored over time by measuring the pressure inside the bottle with an aphrometer (L.sensor.CO2, L PRO SRL, Camisano Vicentino, Italy). This technique allows the pressure inside a bottle to be followed in a non-invasive way. The values were normalized according to the temperature using Henry's law constant and expressed as pressure (bar, 10^{-5} bar = 1 Pa) at 10 °C. The fermentation kinetics data were fitted using the 5 PL model (Gottschalk and Dunn, 2005).

$$P_t = P\max + \frac{P_0 - P\max}{\left[1 + \left(\frac{t}{ip}\right)^{r}\right]^{m}}$$

where P_t is the pressure (bars) inside the bottle at time t (days), P_0 is the initial pressure inside the bottle, *Pmax* is the maximum pressure achieved (bars), r is the maximum fermentative rate (bar/day), ip is the inflexion point between the initial pressure and the maximum fermentative rate (days) and m controls the asymmetry of the curve. From the fitted data, we extracted three parameters of interest: the maximal pressure reached, *Pmax* (bar); the maximum fermentation rate, *rFerm* (bar/days); and the time needed to finish the second fermentation, *tPmax* (days) (Fig. 1B).

2.5. Second fermentation in microplates: measurement of the cell viability

The cell concentration and viability during the second fermentation were also estimated for a longer period of time, by performing the second fermentation on a microplate scale. Six yeast strains (A24, GN, SB, Spark, VL3, F10) were grown using I, Ar and AG as the nitrogen sources for the proliferation phase of the pied-de-cuve. After the growth, the yeast were inoculated into the base wine and distributed into several microplates. One microplate contained three replicates of the same condition at random positions and six blank wells used to detect contaminations. Microplates were then closed in a hermetic recipient with an anaerobiosis bag to ensure anaerobic conditions and maintained at 16 °C. One recipient was opened at each selected time-point (0, 6, 14, 39 and 61 days) to collect data during the second fermentation. The yeast population and viability during the second fermentation were monitored using a flow cytometer (Quanta SC MPL, Beckman Coulter, Fullerton, California). Samples were diluted in McIlvaine buffer (0.1 M citric acid, 0.2 M sodium phosphate dibasic, pH = 4.0). Propidium iodide (0.3% v/v) was added to stain the dead cells according to (Zimmer et al., 2014).

2.6. Statistical analyses

All statistical and graphical analyses were conducted using the R program (R Development Core Team, 2011). The variation of each trait was estimated by an analysis of variance (ANOVA) following the mixed model: $Z = \mu + media_j + strain_i + position_k + media_j{*}strain_i + \varepsilon_{ijk}$, where Z is the experimental trait measured, *media* is the media effect (j = 1…5), *strain* is the strain effect (i = 1…8), *position* is the effect of the position in the microplate (center or border, k = 1, 2), *media***strain* is the interaction effect of the *strain* and *media* factors and ε is the residual error. In the case of the effect of the position factor being significant, the effect was corrected using the tools included in the *ber* package (Giordan, 2013) before proceeding with the analysis. Post-hoc analyses were conducted using the L-Duncan and effects test included in the *agricolae* (De Mendiburu, 2014) and *effect* (Fox, 2003) packages, respectively.

Correlations between variables (mean of every replicate, data corrected for position effect) were performed using Spearman's rank correlations (ρ), and p-values were corrected for multiple testing using the Bonferroni correction included in the *psych* package (Revelle, 2015).

3. Results

3.1. Nitrogen source used during the pied-de-cuve modulates growth kinetics

We first quantified the effect of the nitrogen source on yeast fitness during the pied-de-cuve. Eight strains of different origin were cultivated in five nitrogen sources: Inorganic nitrogen (I), Must (Mu), Base wine (BW), Aromatic precursors (Ar) and Organic nitrogen (O). The growth curves (OD *vs.* time) obtained during the proliferation phase were fitted with a logistic model to extract the lag phase length (*lag_PC*), the growth rate (*r_PC*), and the maximum population (*K_PC*), as shown in Fig. 1A and B.

The analysis of variance (Table 2) revealed that both factors considered in the study, strain and media, as well as the interaction between them, had a significant impact on the maximum population, growth rate and lag phase time. The nitrogen source used in the pied-de-cuve explained most of the variance observed: up to 48%, 46%, and 36% for K_PC, r_PC, and *lag_PC*, respectively. The rest of the variance was mostly explained by the strain for r_PC (34%) and by the interaction between the strain and the nitrogen source for *K_PC* and *lag_PC*. These results are illustrated in Fig. 2 by comparing the mean values of *K_PC* and *r_PC* obtained for each strain in each medium. The nitrogen source effect was accentuated when using the experimental nitrogen sources Ar and O. In contrast, the nitrogen sources reproducing industrial practices (I, BW, Mu) showed lower levels of variation in both their *K_PC* and *r_PC* (Fig. 2B and D). Interestingly, strain OS104 behaved differently from the other strains, especially in its response to being acclimated with Ar and O. This strain was isolated from a natural biotope without a direct relation with any fermentation process. All together, these results demonstrate that the nitrogen source used during the pied-de-cuve strongly impacts yeast growth in a strain dependent manner. The origin of a strain may be important to fully understand its response to a given nitrogen source; however, a higher number of strains from different origins should be used in order to test this hypothesis. We also verified the suitability of using microplates as a technological platform to conduct the pied-de-cuve by comparing the growth obtained in microplates and in vessels. The growth parameters obtained when using vessels and microplates are positively correlated (Supplementary Fig. S1).

3.2. Second fermentation performance

The effect of different acclimation media and yeast strains on the development of the second fermentation was also analyzed by monitoring the CO_2 production inside the bottles over time. The conditions used in this experiment were similar to those found in cellars for sparkling wine production by the traditional method. The pressure data collected were fitted to compute the maximum pressure *Pmax*, the fermentation rate *rFerm*, and the time needed to finish second fermentation *tPmax* (Fig. 1C and D). The analysis of variance conducted for all the parameters extracted revealed that strain is the factor that explains most of the variance observed (44.74%, 50.43% and 76% for *Pmax*, *rFerm* and *tPmax*, respectively) (Table 2). For these parameters, the *nitrogen source***strain* interaction also plays a relevant role. Indeed, *Pmax* varies greatly depending on the strain and media used, going from 4.60 to 5.92 (Fig. 3A). Strain OS104 (oak derivate) had the lowest fermentation capacity and was only able to finish the second fermentation (*Pmax*>5 bars) when using the mixture Ar in the pied-de-cuve. Furthermore, some enological strains caused stuck fermentations,

Table 2
Results of the analysis of variance applied for the growth during the pied-de-cuve, the growth during the second fermentation and the second fermentation kinetics. The parameters listed in the table are: K_PC, biotic capacity in the pied-de-cuve; r_PC, growth rate in the pied-de-cuve and lag_PC, lag phase in the pied-de-cuve. The parameters extracted for the second fermentation kinetics are: Pmax, maximum pressure achieved; rferm, fermentative rate and tPmax, time needed to reach the maximum pressure. In the table, the percentage explained by each factor and the significance of each factor (*, p-value < 0.05; **, p-value < 0.01, *** p-value < 0.001) are indicated.

	Growth during pied-de-cuve			Second fermentation kinetics		
	(K_PC)	(r_PC)	(lag_PC)	(Pmax)	(rferm)	(tPmax)
Strain	1.93% **	34.16% ***	7.87% ***	44.74% ***	50.43% ***	76.50% ***
Nitrogen source	48.41% ***	46.16% ***	36.21% ***	26.25% ***	10.03% ***	0.82% *
Strain × nitrogen source	34.32% ***	14.42% ***	33.11% ***	20.42% ***	28.59% ***	16.58% ***

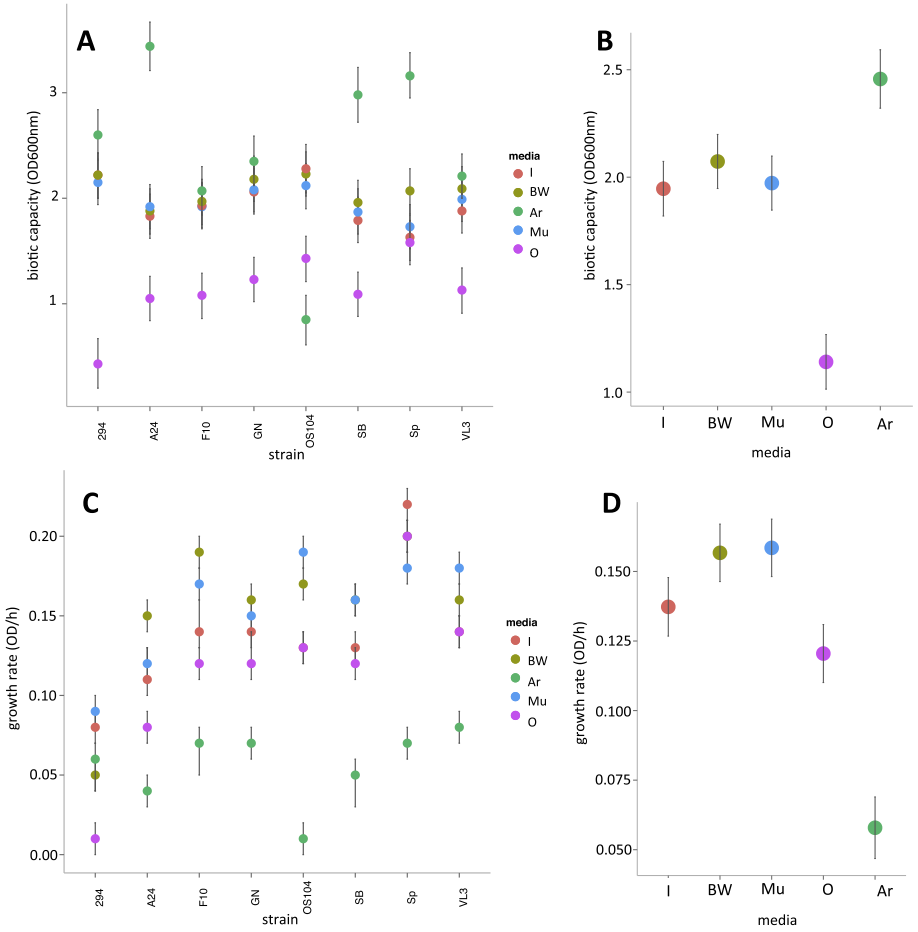

Fig. 2. Parameters extracted from the growth curves during the acclimation phase for all the strains and nitrogen mixtures used in the study (I, inorganic; BW, base wine; Mu, must; Ar, aromatic; O, organic). (A). Maximum population achieved at the end of the acclimation phase. Each value represented is the mean of 6 replicates and the error bars are the confidence intervals ($\alpha = 0.05$) (B) Mean of the maximum population achieved at the end of the acclimation phase for each media considering all the strains (n = 48). Error bars are the confidence intervals ($\alpha = 0.05$). (C) Growth rate of each strain in each media during the acclimation phase, each value is the mean of six replicates and the error bars are the confidence intervals ($\alpha = 0.05$). (D) Mean of the growth rate achieved for all the strains (n = 48) using each nitrogen source in the acclimation phase, error bars are the confidence intervals ($\alpha = 0.05$).

depending on the media used in the pied-de-cuve. This was the case for GN acclimated with ammonia (I). The time needed to reach the final pressure also showed a huge variation, ranging from 10.6 to 43.4 days (Fig. 3B). This variation was mostly explained by the strain used to ferment (76.50%), however, the interaction between strain and nitrogen source is not negligible (16.58%), indicating that the nitrogen used to acclimate the yeasts affected their tPmax in a strain dependent manner. As an example, when acclimated with Ar, the strains A24, GN, OS104 and VL3 had their lowest *tPmax*, and 294, F10, SB and SP their highest *tPmax*, thus demonstrating the specific yeast strain nitrogen requirements. The slowest strain was GN, with an average fermentation time of 37.5 days, while the fastest strain was Sp, which needed only 12.8 days to finish the second fermentation. This strain was especially selected and commercialized for its good performance in the production of sparkling wine.

3.3. Correlation between pied-de-cuve and second fermentation

The results presented above illustrate that both growth and fermentative kinetics are affected by the nitrogen source used in the acclimation phase. This effect is strongly modulated by the

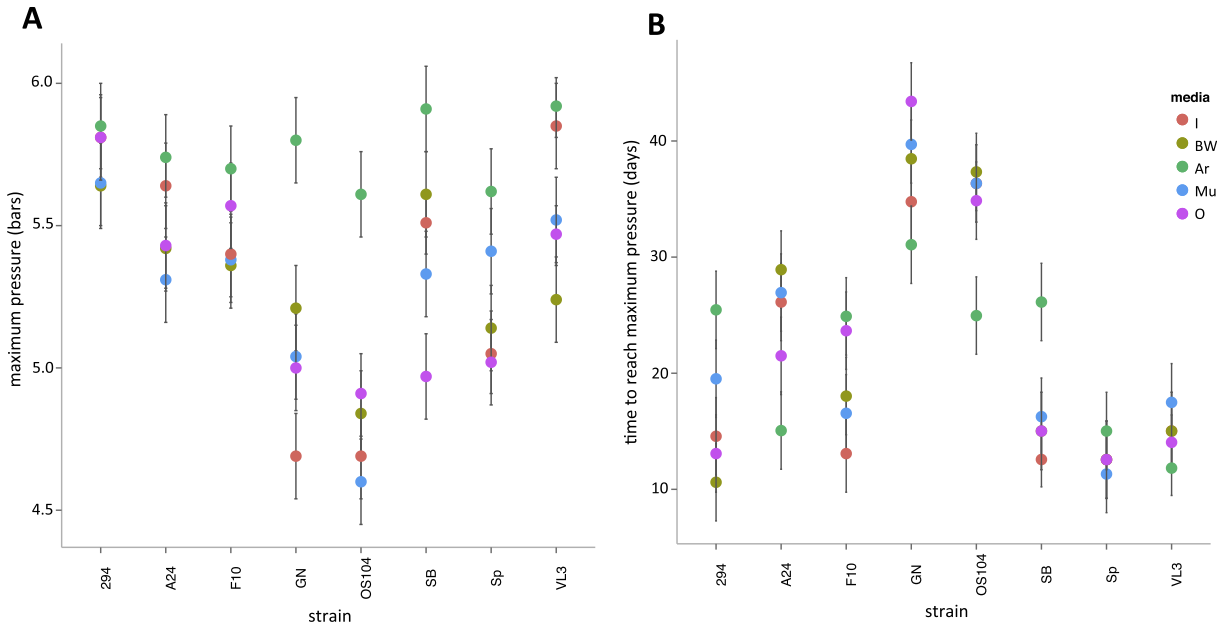

Fig. 3. Parameters extracted from the fermentation kinetics exhibited by each strain after being acclimated with different nitrogen sources. Each value is the mean of three replicates and error bars represent confidence intervals ($\alpha = 0.05$). (A) Maximum pressure achieved at the end of the fermentation. (B) Time needed to finish the second fermentation.

genetic nature of the strain used. To establish the relation between the pied-de-cuve and the second fermentation performance, a correlation study was carried out using Spearman's test. Fig. 4 illustrates one of the most striking correlations: slower growth rates during the pied-de-cuve led to higher maximum pressures during the second fermentation. All strains are evenly distributed in the graph, with the exception of OS104, which does not appear to follow the pattern of the other strains. Furthermore, when adapted with the mixture of amino acids that are aromatic precursors (Ar), all the strains (including OS104) clustered at the upper part of the graph where the highest pressures are achieved. These results indicate that the use of a poor nitrogen source during the acclimation process (e.g., Ar), may increase the yeast viability and, consequently, the fermentation activity during sparkling wine production.

3.4. Viability through the second fermentation

To test whether the nitrogen source used in the pied-de-cuve has an impact on the yeast viability through the second fermentation, three nitrogen sources were used during the acclimation process: (I) inorganic nitrogen (quick uptake and growth), (Ar) an amino acid mixture containing precursors of aromatic compounds (slow uptake and growth), and (AG) an amino acid mixture that combines both amino acids of quick uptake (glutamine/glutamate) and the amino acids presents in Ar. Six strains were used in the study, all the wine yeast strains and only one from distillery. After inoculating the acclimated cells into the base wine, the yeast growth and viability were monitored during the second fermentation.

The viable cell concentration reached its highest value when the

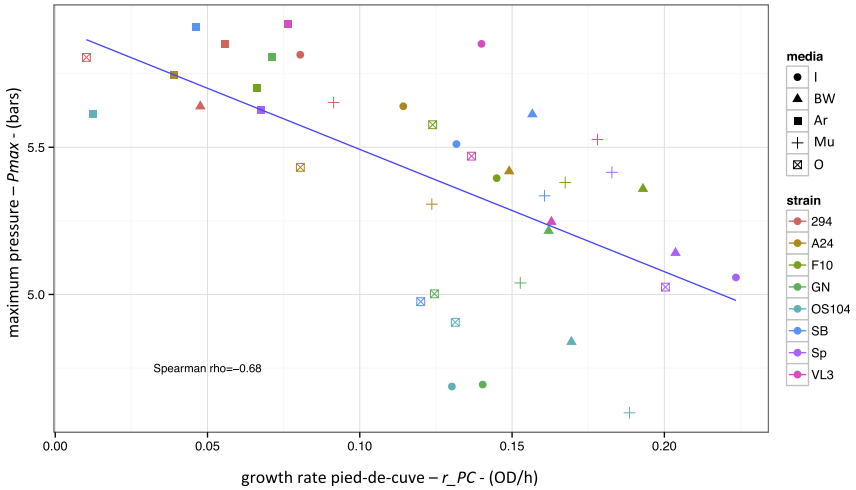

Fig. 4. Correlation between the growth rate during the acclimation phase, and the maximum pressure achieved at the end of the fermentation for all the conditions considered in the study. Each colour represents one strain and each media is represented by a different symbol.

strains were adapted with Ar. This increase on the viable cell concentration was observed at 39 days, and was significant for all strains except for F10 (Fig. 5A). The effect of the nitrogen source was tested by an analysis of variance for the total cell concentration and the cell mortality at each time point (Fig. 5B and C). The nitrogen source used in the pied-de-cuve had a significant impact on the total cell concentration and the cell mortality at 39 days, and the highest cell concentration and lowest yeast mortality at 39 days were obtained when acclimating yeast cells using Ar. We could not detect any differences in the total cell concentration or yeast mortality between the cells that had been adapted using AG or I. Surprisingly, the total cell concentration of the yeast cells acclimated using Ar decreased between 39 and 60 days, probably due to changes in the yeast cell size and morphology interfering with the flow cytometry measure. The use of Ar in the proliferation phase allowed the yeast cells to reach the end of the fermentation with more viable cells, probably due to an extension of their chronological lifespan.

4. Discussion

Yeast acclimation is a common practice in several industries, among them oenology. In sparkling wine production by the traditional method, the pied-de-cuve is conducted with the aim of obtaining a sustainable inoculum to conduct the second fermentation (Carrascosa et al., 2011). In a previous work (Martí-Raga et al., 2015) we have demonstrated that the nitrogen taken up during the pied-de-cuve can cover the nitrogen requirements of the second fermentation and modulate the second fermentation kinetics, with strain-dependent nutrient preferences. In this work, we investigated how variations on the nitrogen composition of the acclimation media can modulate yeast strain viability and fitness during the second fermentation, and impact an economically relevant fermentation process, such as sparkling wine production.

To analyze a large set of strains and media conditions, we monitored cell growth using a microplate reader, which is a technique commonly used in phenomics approaches (Warringer and Blomberg, 2003). We developed a modified 3 PL model (Lord, 1980) to overcome the evaporation phenomenon observed when culturing yeast in a medium with ethanol.

As a first result, we showed that the nitrogen source used in the acclimation phase has a major effect on both the pied-de-cuve and the second fermentation. Several authors have reported the effect of the nitrogen source on the growth of yeast, and it is widely accepted that the nitrogen concentration and composition in the media greatly affect the biomass production, yeast growth and metabolic production (Bell and Henschke, 2005). Furthermore, we have observed that the use of either Ar or O, which includes a mixture of amino acids supporting slow growth (Godard et al., 2007), magnifies the differences within and between the strains,

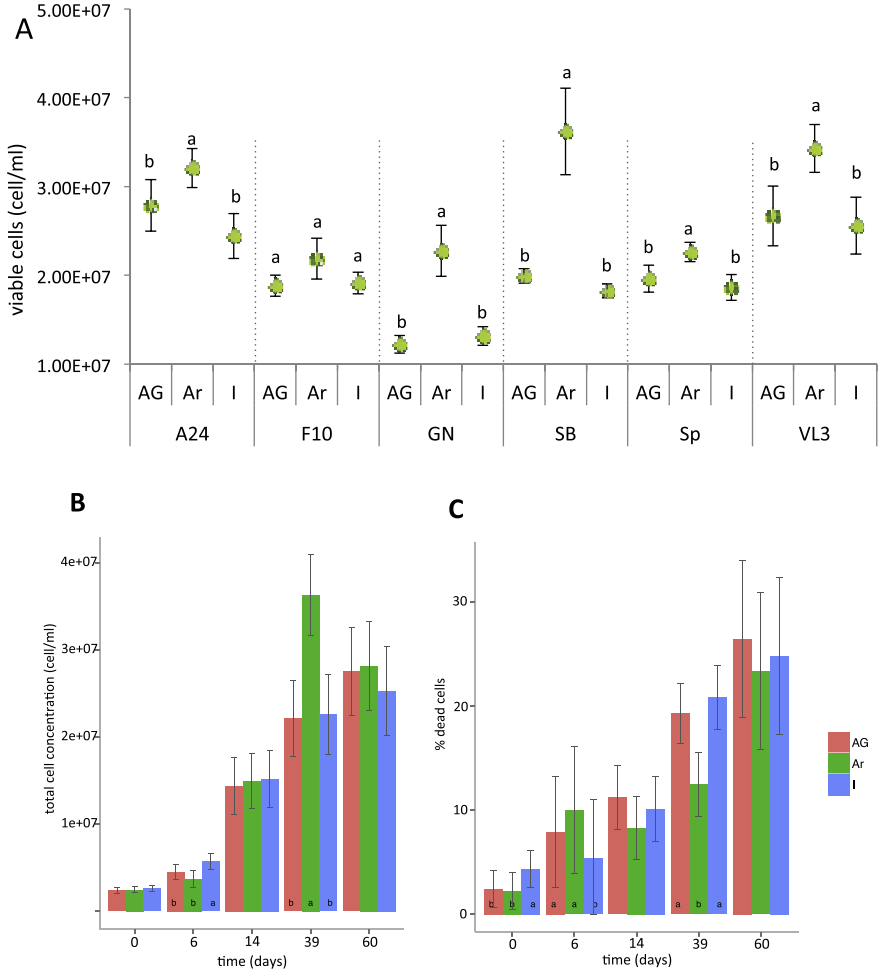

Fig. 5. Analysis of cell viability through the second fermentation. (A) Viable cells at 39 days of fermentation. Each value represented is the mean of each triplicate. Error bars are the confidence intervals ($\alpha = 0.05$). Different letters indicate significantly differences due to the nitrogen source used to acclimate yeast cells. (B) Results of the post-hoc analysis for the total cell concentration at each time point. (C) Results of the post-hoc analysis for the percentage of mortality through the second fermentation.

especially for the maximal population. These results are in accordance with the results obtained by Gutiérrez et al. (2013), who reported that the poorer the nitrogen source, the higher the phenotypic variability between strains.

We observed that the high phenotypic variation in the second fermentation kinetics is mostly due to the strain used. The effect of the yeast strain on the development of alcoholic fermentation has been extensively studied in oenology and wine yeast strains have been specifically selected for their fermenting abilities (Pretorius, 2000; Fleet, 2008). Recently, the strain ability to overcome several stress sources, such as high ethanol concentration, low pH and high glycerol content, has been proposed as a method to select strains suitable for conducting the second fermentation (Borrull et al., 2015). Martí-Raga et al. (2015) studied the effect of the strain on the development of the second fermentation as well as on the ability to maintain its fermentative capacity when changing several environmental conditions. The study revealed that not only different strains present different fermentation profiles but also that each strain showed different levels of adaptation to changes in the fermentation.

The relation between the acclimation process and the second fermentation was also considered in the present study. We have been able to detect an inverse correlation between the growth rate in the proliferation phase and the maximum pressure achieved. Thus, the strains growing at a slower rate are able to achieve higher pressures inside the bottle, meaning that they are more likely to complete the second fermentation process. Our results indicate that a slower growth during the acclimation phase results in a better acclimation to the stressful environment of the second fermentation. The same was observed in other stressful conditions, such as low temperature fermentations, in which the yeast cells grow at a lower rates but are able to maintain their viability during a longer period of time (Beltran et al., 2008, 2006; Torija et al., 2003). Furthermore, it is widely accepted that a quick exposure of the yeast to a mild stress source results in a better chance of survival in future stressful conditions (reviewed by Estruch (2000)). This survival rate has been positively correlated with a slow growth (Lu et al., 2009; Zakrzewska et al., 2011).

Our previous study (Martí-Raga et al., 2015) showed that nitrogen used in the pied-de-cuve is able to modulate the fermentation kinetics in a strain dependent manner and that nitrogen uptake during the acclimation phase is enough to cover the nitrogen requirements of the second fermentation. In the present study we have been able to find a physiological explanation to this phenomenon. We have shown that yeasts are able to maintain their viability a longer period of time when using poor nitrogen sources (like Ar) in the acclimation phase. This phenotype was lost with the addition of Glu/Gln to the mixture or with the use of ammonia on the acclimation phase. Furthermore, all the strains, independently of their origin, achieved its highest pressure when using Ar in the acclimation process. Overall, the use of Ar in the acclimation phase seems to be beneficial for long term viability, and the yeast capacity to completely consume the sugars present, but only affects their fermentative rate in a strain-dependent manner. Martínez-Moreno et al. (2012) used the same nitrogen formulation in a synthetic grape must, and their results showed that the use of Ar improved the cell viability, vitality and sugar consumption in particular stressful conditions, such as high sugar must (280 g/l). The higher viability achieved when using Ar, which included only amino acids supporting slow growth, could be explained either by an additional specific stress response to the poor nitrogen source or by the extension of the yeast chronological life span (CLS). Godard et al. (2007) analyzed the yeast transcriptomic response when yeast was grown on each individual amino acid and did not detect a specific stress response activated due to the nitrogen source used. Additionally, it is known that the CLS of the yeast is influenced by the media composition: the growth on ammonia as the sole nitrogen source for yeast shorten the CLS (Santos et al., 2013, 2012), and removing the preferred amino acids from the media increases the CLS Powers et al. (2006). Alvers et al. (2009) have determined that the growth of yeast in Ile, Leu, Thr and Val (single amino acid media) − all of them included in Ar − resulted in an extension of the CLS. In agreement with these results, we have observed that the use of Ar in the acclimation phase might extend the CLS and allows yeast cells to remain viable for a longer period of time and, subsequently, consume more sugars (achieving higher pressure inside the bottle). This phenotype was clearly lost when using ammonia or adding preferred amino acids (Glu/Gln) to the mixture.

Altogether, the nitrogen source used in the pied-de-cuve appears to play a key role in the development of the second fermentation due to its effect on the yeast viability. In a long fermentation, such as in the production of sparkling wine, we were able to increase the yeast viability at the middle-end of the fermentation using a specific nitrogen source in the acclimation media. This might be a critical requirement and a useful tool to ensure the successful completion of the second fermentation. Although the genetic background of the strain used for the second fermentation is the main cause of variation during the second fermentation, we have been able to improve yeast viability, independently of its genetic background, using a slow growth promoting nitrogen source in the acclimation phase. Thus, for those strains with problems to overcome stressful conditions, but still with other interesting enological properties, the use of Ar could increase their viability, ensuring the completion of the second fermentation. The higher viability may delay the beginning of the autolytic process and affect the final product characteristics, consideration that should be further studied.

Overall, the present study reflects how the nitrogen source used during the acclimation of yeast cells can affect their fitness and viability, and determine the successful completion of the required process, such as the second fermentation for sparkling wine production.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.10.009.

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