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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation

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Abstract In this study, the influence of twenty different single (i.e. 19 amino acids and ammonium sulphate) and two multiple nitrogen sources (N-sources) on growth and fermentation (i.e. glucose consumption and ethanol production) performance of *Saccharomyces cerevisiae* and of four wine-related non-*Saccharomyces* yeast species (*Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* and *Torulaspora delbrueckii*) was investigated during alcoholic fermentation. Briefly, the N-sources with beneficial effects on all performance parameters (or for the majority of them) for each yeast species were alanine, arginine, asparagine, aspartic acid, glutamine, isoleucine, ammonium sulphate, serine, valine and mixtures of 19 amino acids and of 19 amino acids plus ammonium sulphate (for *S. cerevisiae*), serine (for *L. thermotolerans*), alanine (for *H. uvarum*), alanine and asparagine (for *M. pulcherrima*), arginine, asparagine, glutamine, isoleucine and mixture of 19 amino acids (for *T. delbrueckii*). Furthermore, our results showed a clear positive effect of complex mixtures of N-sources on *S. cerevisiae* and on *T. delbrueckii* (although to a lesser extent) as to all performance parameters studied, whereas for *L. thermotolerans*, *H. uvarum* and *M. pulcherrima*, single amino acids affected growth and fermentation performance to the same extent as the mixtures. Moreover, we found groups of N-sources with similar effects on the growth and/or fermentation performance of two or more yeast species. Finally, the influences of N-sources observed for *T. delbrueckii* and *H. uvarum* resembled those of *S. cerevisiae* the most and the least, respectively. Overall, this work contributes to an improved understanding of how different N-sources affect growth, glucose consumption and ethanol production of wine-related yeast species under oxygen-limited conditions, which, in turn, may be used to, e.g. optimize growth and fermentation performance of the given yeast upon N-source supplementation during wine fermentations.

Keywords *Saccharomyces cerevisiae* · Non-*Saccharomyces* species · Nitrogen sources · Oxygen-limited fermentation

Varongsiri Kemsawasd and Tiago Viana contributed equally to this work.

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Introduction

Nitrogen deficiency is one of the major causes of stuck or sluggish fermentations during wine production (Pretorius 2000). To overcome this, a supplementary nitrogen source (N-source) is added during wine fermentations typically as ammonium salts (sulphate or phosphate). However, within recent years, more and more focus has been put into also using amino acids as N-source supplements (Gutiérrez et al. 2012). To optimize growth and fermentation performances of the specific

wine yeast used upon N-source supplementation, it is important to know how a given N-source affects both these parameters. A lot of effort has been put into understanding how N-sources affect especially the growth of the primary wine yeast *Saccharomyces cerevisiae* (Albers et al. 1996; Godard et al. 2007; Gutiérrez et al. 2012, 2013). Within the last decade, the use of non-*Saccharomyces* yeasts as starter cultures for wine fermentations has gained more and more attention (Ciani et al. 2010; Jolly et al. 2014). However, very little is known about the basic physiology of non-*Saccharomyces* yeasts, in particular regarding their growth and fermentation abilities under different N-source-conditions.

Only few attempts have been made to study the effect of N-sources on both yeast growth and fermentation performance under anaerobic conditions, and mainly, these studies only include *S. cerevisiae*. For instance, Varela et al. (2004), using a complex mixture of amino acids, showed that higher biomass concentrations led to faster fermentations, as determined by reduced fermentation time and high sugar consumption rate. In contrast, Gutiérrez et al. (2012) investigated the effect of ammonium, arginine and glutamine in a synthetic grape must on growth (i.e. μ_{max} and maximum population size) and fermentation activity (i.e. density reduction of grape must), and they found no strict correlation between growth behaviour and fermentation activity; that is good growth performance did not always result in good, but rather in bad, fermentation performance (Gutiérrez et al. 2012). These latter data highlight the fact that performance parameters, describing the N-source preference of yeasts, have to be carefully considered and clearly defined.

Even fewer studies have investigated the influence of N-source on growth and fermentation of non-*Saccharomyces* yeasts during alcoholic fermentation (Andorrà et al. 2010, 2012; Blomqvist et al. 2012). Recently, *Dekkera bruxellensis* was reported as facultatively anaerobic yeast that requires amino acid supplementation for anaerobic growth (Blomqvist et al. 2012). The higher demand for amino acids for growth in the absence of oxygen was previously announced for other non-*Saccharomyces* yeast species (e.g. *Torulaspora globosa*, *Kluyveromyces lactis*) (Merico et al. 2007). To the best of our knowledge, only one study was recently published suggesting optimized nitrogen conditions for three wine-related non-*Saccharomyces* species (*Lachancea thermotolerans*, *Metschnikowia pulcherrima* and *Issatchenkia orientalis*) (Schnierda et al. 2014). Specifically, the authors suggested the use of yeast extract as N-source in quantities to provide a minimum of 500 mg/l of yeast assimilable nitrogen (Schnierda et al. 2014). However, that study aimed at using fully aerobic bioreactors for obtaining high biomass yields of those non-*Saccharomyces* yeasts for industrial production of starter cultures in complex medium.

Grape juice contains a wide variety of N-sources, including ammonium ions and amino acids that may constitute on average 40 % and 51 to 92 %, respectively, of the yeast assimilable nitrogen (Bell and Henschke 2005). All amino acids may be present in grape juice, but typically, glutamate, proline and arginine are those found in highest amounts, whereas glycine, methionine and tyrosine are some of the amino acids that are present in low amounts (Bell and Henschke 2005). Notably, however, proline is not assimilated by *S. cerevisiae* under anaerobic conditions (Henschke and Jiranek 1993). The recommended minimum amount of elemental nitrogen required by wine yeasts for completing fermentations at a normal rate has so far only been determined for *S. cerevisiae* to be at a level of 120–140 mg N/l (Bely et al. 1990; Jiranek et al. 1995; Reed and Nagodawithana 1990).

The N-source preferences of wine yeasts have primarily been studied using various synthetic grape musts optimized for growth and fermentation of industrial and lab strains of *S. cerevisiae* (reviewed in Viana et al. 2014). These authors reported a synthetic grape must (ISA-SGM) in which *S. cerevisiae* lab strains showed fermentative profiles similar to that of a commercial wine strain in natural grape must (Viana et al. 2014). Also, the synthetic medium yeast nitrogen base (YNB) has been solely developed for the use of *S. cerevisiae* (Sherman 2002). YNB contains more or less the same vitamins and mineral salts as does ISA-SGM, although, generally, in lower concentrations. It, however, only contains glucose as carbon source, instead of equimolar concentrations of glucose and fructose, and it neither contains the major organic acids present in natural grape musts (i.e. tartaric, malic and citric acids) nor sulphur dioxide (used as a standard enological treatment).

In this study, we investigated how 19 single amino acids, ammonium sulphate and two complex mixtures of N-sources affected growth and fermentation abilities of *S. cerevisiae* and four other wine-related yeast species (*L. thermotolerans*, *Hanseniaspora uvarum*, *M. pulcherrima* and *Torulaspora delbrueckii*), during alcoholic fermentation in a modified YNB medium. The results obtained in this work should be useful, e.g. to winemakers for the optimization of their alcoholic fermentations, considering the specific N-source needs of the given wine yeast used.

Materials and methods

Yeast strains and inoculation cultures

S. cerevisiae (Saint Georges S101, Bio Springer, Maisons-Alfort, France) and four other wine-related yeast strains, i.e. *L. thermotolerans* (CBS2803), *M. pulcherrima* (CBS2251), *H. uvarum* (CBS314) and *T. delbrueckii* (CBS3085), were maintained at 4 °C on YPG agar (containing 5 g/l yeast extract, 10 g/l peptone, 11 g/l glucose monohydrate and 20 g/l agar, pH 5.6).

In preparing the inoculation cultures, cells from YPG agar were transferred to glass tubes containing 10 ml YPG and incubated with agitation (140 rpm) at 25 °C for 24 h. Subsequently, these cells were transferred to 50 ml YPG in 100 ml shake flasks to an initial concentration of 3×10^6 cells/ml and incubated with agitation (140 rpm) at 25 °C for 24 h.

Fermentations

Each yeast species was inoculated to an initial, standardized concentration of 1×10^5 cells in 90 ml of YNB synthetic medium modified for alcoholic fermentations (YNBMAF, 1.17 g/l yeast nitrogen base without amino acids and ammonium sulphate (Difco, BD, Albertslund, Denmark), 220 g/l glucose monohydrate, 10.75 g/l disodium hydrogen phosphate, 14.67 g/l citric acid monohydrate, 20 mg/l ergosterol and 840 mg/l Tween 80, pH 3.5) using a Neubauer haemocytometer. The YNBMAF was supplemented with single and multiple N-sources, as shown in Supplementary Tables S1 and S2. For each yeast species, 23 experiments were performed; i.e. 19 experiments with a single amino acid (approx. 2000 mg/l), one experiment with ammonium sulphate (Merck, Hellerup, Denmark) (approx. 2000 mg/l), one experiment with a mixture of the 19 amino acids at virtually similar concentrations (approx. 2000 mg/l in total) (MixAA), one experiment with a mixture of the 19 amino acids at virtually similar concentrations (approx. 1000 mg/l in total) and ammonium sulphate (approx. 1000 mg/l) ($MixNH_4$) and one experiment without a N-source (negative control, Neg).

The 19 L-amino acids used were (alanine, Ala; arginine, Arg; asparagine, Asn; aspartic acid, Asp; cysteine, Cys; glutamine, Gln; glutamic acid, Glu; glycine, Gly; histidine, His; isoleucine, Ile; leucine, Leu; lysine, Lys; methionine, Met; phenylalanine, Phe; serine, Ser; threonine, Thr; tryptophan, Trp; tyrosine, Tyr; and valine, Val) (Sigma-Aldrich, Brøndby, Denmark). Proline, although present in high quantity in grape must, was not tested as sole N-source in this work, since *S. cerevisiae* cannot metabolize it under anaerobic conditions (Henschke and Jiranek 1993). Moreover, recent findings seem to indicate also a low ability of proline to support growth of *D. bruxellensis*, when provided as a sole N-source (Blomqvist et al. 2012).

Fermentations were carried out under oxygen-limited conditions with agitation (140 rpm) at 25 °C in 100 ml BlueCap flasks fitted with a butyl stopper and a fermentation lock in Tygon tubing containing 50 % (*v*/*v*) sterile glycerol. In all fermentation experiments, the initial pH of the medium was 3.50 ± 0.02 and the pH at the last sample point of all experiments was 3.49 ± 0.28 (data not shown). All fermentations were carried out in duplicate.

Analysis of growth and survival

Growth and survival of the yeasts were determined by plate counting. Samples were withdrawn throughout the fermentations and diluted appropriately in dilution medium (containing 8.5 g/l NaCl, 1 g/l peptone and 0.3 g/l disodium hydrogen phosphate, pH 5.6). Each yeast species was enumerated using YPG agar plates incubated at 25 °C for 2–3 days before counting.

Analysis of glucose, glycerol and ethanol

Samples were filtrated (0.45 μm) (Q-max, Knebel, Denmark) and kept at −20 °C until analysis. Glucose, glycerol and ethanol concentrations were determined using a HPLC (HP series 1100, Hewlett-Packard Company, Palo Alto USA) with an Aminex 87H column (Bio-Rad Laboratories, Hercules, USA) connected to a RI detector (HP1047A, Hewlett-Packard Company, Palo Alto, USA). The column was eluted with a degassed mobile phase containing 1 mM H_2SO_4, pH 2.75, at 30 °C and at a flow rate of 0.5 ml/min.

Yeast performance parameters

As yeast performance parameters, we chose typical oenological growth and fermentation properties, i.e. the maximum specific growth rate (μ_{max}), viability at the beginning (N_{max}) and late (N_{end}) stages of stationary phase; i.e. day 6, as well as glucose consumption (GluC) and ethanol production (EthP) after 6 days of fermentation (Fig. 1). Maximum specific growth rate was determined using the DMFit software available on the Combase website (http://www.combase.cc/index.php/en/), where μ_{max} values were obtained after the fit of the growth curves to the model proposed by Baranyi and Roberts (1994). The growth parameter N_{end} is an important checkpoint of alcoholic fermentation and yeast physiology (Viana et al. 2012) and a well-known phase where non-*Saccharomyces* cells normally die-off during alcoholic fermentations (Nissen et al. 2003). Growth, glucose and ethanol curves for representative experiments can be seen in Supplementary Figs. S1, S2 and S3.

Analysis of amino acids and ammonium sulphate

Samples were filtrated (0.45 μm) (Q-max, Knebel, Denmark) and kept at 4 °C until analysis. An initial and final concentration of amino acids was estimated by pre-column *o*-phthaldialdehyde derivatization of the amino acids followed by reversed phase HPLC separation with fluorometric detection according to the method described by Bütikofer and Ardö (1999). The analyses were carried out using an Alliance, Waters 2695 Separation Module with a Waters 996 Photodiode Array Detector and an X-Terra RP18 column, 3.5 μm (Waters Corporation, Hedehusene, Denmark), associated with

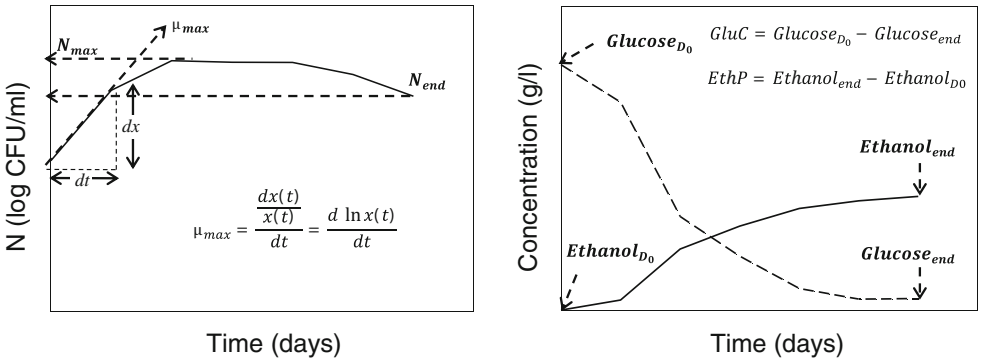


Fig. 1 Growth and fermentation performance parameters evaluated in this study for the five yeast species

Millennium 32 software, version 3.20 (Waters Corporation, Hedehusene, Denmark). The concentration of each individual amino acid was calculated as μmol/ml based on peak areas relative to Norvaline (Sigma-Aldrich, Brøndby, Denmark) internal standard, with reference to an external standard mixture of amino acids.

Ammonium sulphate was measured in the supernatant by an enzymatic assay, Ammonia Assay Kit (Sigma-Aldrich, Brøndby, Denmark) at 340 nm using Shimadzu UV-1800 (Shimadzu Corporation, Kyoto, Japan). The concentration of ammonium was determined as described by the manufacturer.

Statistical analysis

Quantitative results were expressed in matrixes of 23 media and of 5 parameters: maximum specific growth rate—μ_{max} (1/h), viability at the beginning of stationary phase—N_{max} (log CFU/ml), viability at late stages of stationary phase—N_{end} (log CFU/ml), glucose consumption—GluC (g glucose/l) and ethanol production—EthP (g ethanol/l) during fermentation. One-way ANOVA followed by multiple comparisons between groups of N-sources using least significant difference (LSD) method and Bonferroni correction of p values were made in R studio (version 2.15.3), package "agricolae" (de Mendiburu 2014). Probabilities below 0.05 were considered significant.

Heatmaps were drawn by using the function "heatmap.2" of the package "g.plots" (Warnes et al. 2012) in R studio (version 2.15.3), with rows representing media and columns each yeast species. Hierarchical clustering was applied using Euclidean distance and was expressed as a dendrogram coupled to each heatmap. The number of clusters was determined using package "NbClust" (Charrad et al. 2013) in R studio (version 2.15.3). Since parameters evaluated had different scales, data were normalized and reduced according to the formula:

$$Y_{ij} \rightarrow Z_{ij} = \frac{Y_{ij} - Y_{\mathrm{MixNH_4}}}{\sigma_{i.}}$$

where Y = raw data, Z = reduced and normalized data, i = parameter and j = N-source.

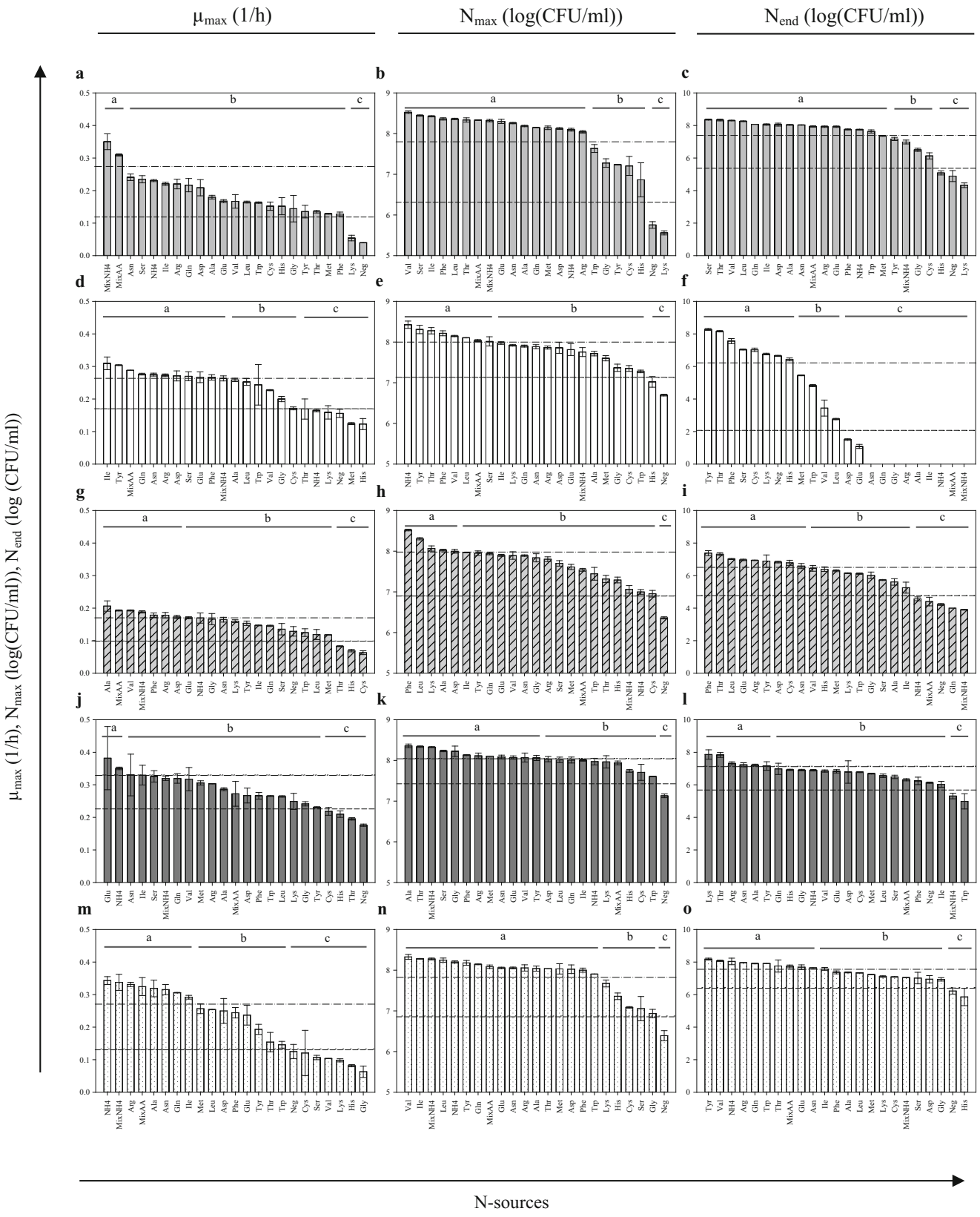
Differences between yeast species as to a specific influence of a N-source were obtained through Pearson's correlation using package "Hmisc" (Harrell et al. 2014) in R studio (version 2.15.3). Correlation coefficients (in absolute value), which were ≤0.35 were considered to represent low or weak correlations, 0.36 to 0.69 modest or moderate correlations, and 0.70 to 1.0 strong or high correlations (adapted from Taylor 1990).

Results

Influence of nitrogen sources on each yeast species

To classify N-sources as 'good', 'intermediate' or 'bad' for each species as to each parameter, the data (shown in Figs. 2, 3 and 4) were submitted to a classification method based on ranking distribution. As such, a range was established through the difference between the maximum and the lowest absolute values for each parameter, for each species. The so-called good N-sources were those that belonged to the top 25 % (in Table 1 represented with 'green' colour) of the range, whereas the bottom 25 % (in Table 1 represented with 'red' colour) referred to bad N-sources. N-sources that were placed in the 50 % region in between good and bad were classified as intermediate (in Table 1 represented

Fig. 2 Growth performance parameters; i.e. maximum specific growth rate (μ_{max}, 1/h), viability at the beginning of stationary phase (N_{max}, log(CFU/ml)) and viability at late stages of stationary phase; i.e. day 6 (N_{end}, log(CFU/ml)), of **a**–**c** *S. cerevisiae*, **d**–**f** *L. thermotolerans*, **g**–**i** *H. uvarum*, **j**–**l** *M. pulcherrima* and **m**–**o** *T. delbrueckii*. Fermentations were performed in YNBMAF supplemented with different N-sources under oxygen-limited conditions at 25 °C. All fermentations were performed in duplicate. Mean values are presented and *error bars* represent standard deviations. *Threshold lines* were established to select N-sources that belonged to the top 25 %; i.e. those above the *dashed-dotted line* were considered as 'good' N-sources, and to the bottom 25 %; i.e. those below the *dashed line* were considered as 'bad' N-sources as to each parameter. The N-sources displayed in between those two lines were classified as 'intermediate'. Groups of N-sources indexed with different letters were significantly different ($p < 0.05$)



with ‘white’ colour). This classification of N-sources was statistically validated using one-way ANOVA and LSD method (Figs. 2, 3 and 4). Table 1 summarizes the influence of different N-sources regarding each parameter studied. Figures 2, 3

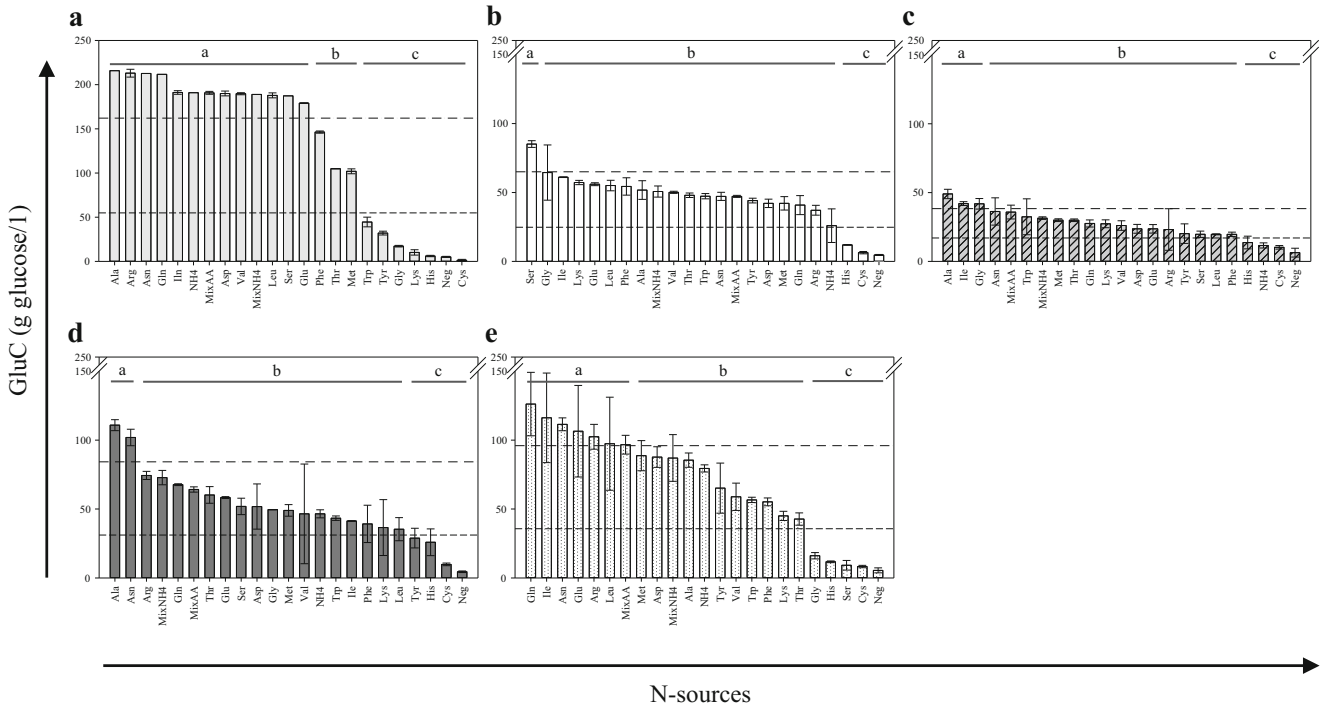


Fig. 3 Glucose consumption after 6 days of fermentation (GluC, g glucose/l) of **a** *S. cerevisiae*, **b** *L. thermotolerans*, **c** *H. uvarum*, **d** *M. pulcherrima* and **e** *T. delbrueckii* from fermentations in YNBMAF supplemented with different N-sources under oxygen-limited conditions at 25 °C. All fermentations were performed in duplicate. Mean values are presented and *error bars* represent standard deviations. *Threshold lines* were established to select N-sources that belonged to the top 25 %; i.e. those above the *dashed-dotted line* were considered as 'good' N-sources, and to the bottom 25 %; i.e. those below the *dashed line* were considered as 'bad' N-sources as to glucose consumption. The N-sources displayed in between those two lines were classified as 'intermediate'. Groups of N-sources indexed with different letters were significantly different ($p < 0.05$)

and 4 show in details the specific influence of different N-source supplementations on the growth and fermentation performance of each yeast species.

Oxygen-limited growth

As to μ_{max} of *S. cerevisiae*, almost all N-sources belonged to the intermediate group, with the exception of the two complex mixtures of N-sources that were considered as good and of Lys and Neg that were, oppositely, considered as bad (Table 1 and Fig. 2a). The μ_{max} of *S. cerevisiae* in MixAA and $MixNH_4$ fermentations (0.35 and 0.31/h, respectively) were remarkably higher in comparison with those supplemented with single N-sources (e.g. 0.24/h with Asn, which was the highest μ_{max} for a single N-source supplementation) (Fig. 2a). This result led us to reinforce the clear positive effect of complex mixtures of N-sources on *S. cerevisiae*, previously observed and reported either using commercial or laboratorial strains of *S. cerevisiae* (Albers et al. 1996; Martínez-Moreno et al. 2012). On the contrary and as already mentioned, Lys and Neg had the opposite effect on *S. cerevisiae* μ_{max} (0.05 and 0.04/h, respectively) (Table 1 and Fig. 2a). This result confirms also Lys as a bad single N-source for this species (Thomas et al. 1994) and the absence of N-sources (Neg) as a harsh environment for this species to grow and to ferment. Regarding N_{max}, the majority of N-sources (i.e. Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, Met, MixAA, $MixNH_4$, NH_4, Phe, Ser, Thr and Val) supported *S. cerevisiae* cells to reach high cell viability at the beginning of stationary phase (with N_{max} above 7.8 log CFU/ml) (Table 1 and Fig. 2b). On the contrary, Lys and Neg did not support N_{max} (with N_{max} below 6.3 log CFU/ml, respectively). Considering the cell viability at late stages of stationary phase (N_{end}), and apart from Lys and Neg, also His did not sustain this parameter (with N_{end} below 5.3 log CFU/ml) (Table 1 and Fig. 2c). However, a large number of N-sources were good for *S. cerevisiae* cells to remain viable at late stages, such as Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, MixAA, Met, NH_4, Phe, Ser, Thr, Trp and Val (with N_{end} above 7.4 log CFU/ml) (Table 1 and Fig. 2c).

Many N-sources (Arg, Asn, Asp, Gln, Glu, Ile, MixAA, MixNH4, Phe, Ser and Tyr) were good for *L. thermotolerans* as to sustaining high μ_{max} (with μ_{max} above 0.27/h), whereas His, Lys, Met, NH_4, Neg and Thr were the conditions responsible for lower μ_{max} values (with μ_{max} below 0.12/h) (Table 1 and Fig. 2d). Regarding viability, two conditions were deleterious for N_{max}, His and Neg, with values of 7.0 and 6.7 log CFU/ml, respectively (Table 1 and Fig. 2e). On the contrary,

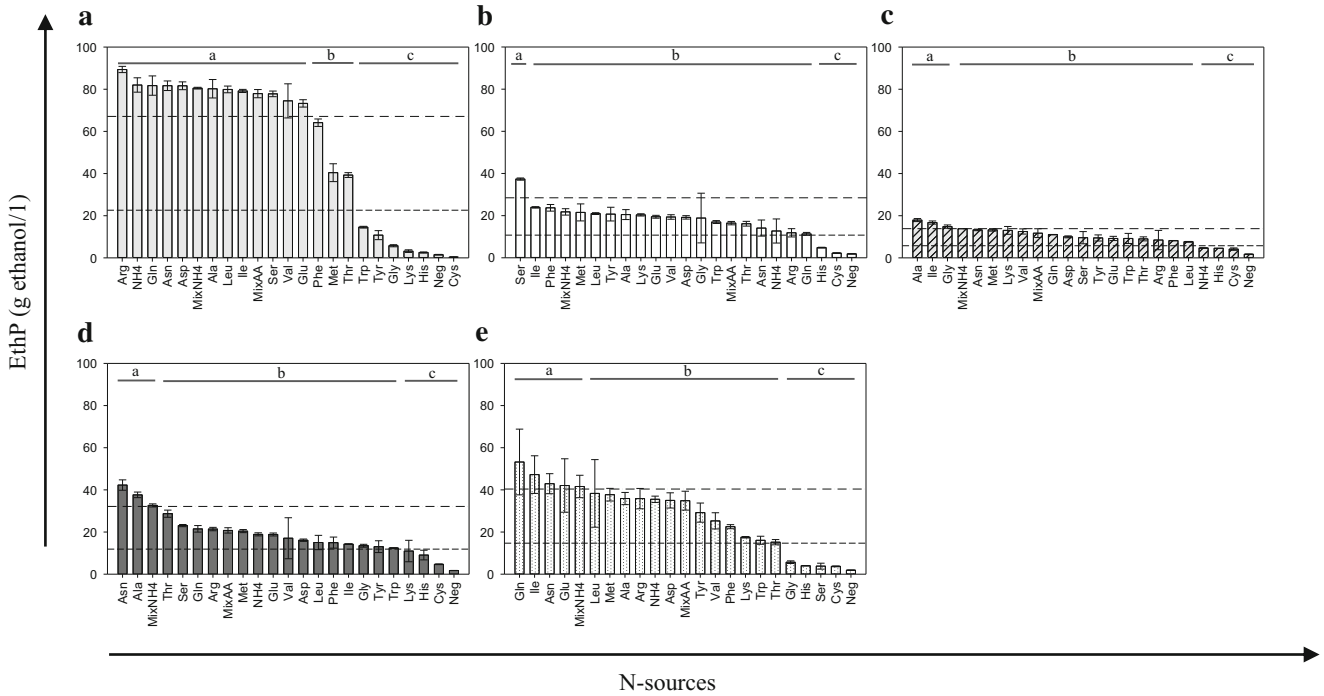


Fig. 4 Ethanol production after 6 days of fermentation (EthP, g ethanol/l) of **a** *S. cerevisiae*, **b** *L. thermotolerans*, **c** *H. uvarum*, **d** *M. pulcherrima* and **e** *T. delbrueckii* from fermentations in YNBMAF supplemented with different N-sources under oxygen-limited conditions at 25 °C. All fermentations were performed in duplicate. Mean values are presented and *error bars* represent standard deviations. *Threshold lines* were established to select N-sources that belonged to the top 25 %; i.e. those above the *dashed-dotted line* were considered as 'good' N-sources, and to the bottom 25 %; i.e. those below the *dashed line* were considered as 'bad' N-sources as to ethanol production. The N-sources displayed in between those two lines were classified as 'intermediate'. Groups of N-sources indexed with different letters were significantly different ($p < 0.05$)

Leu, MixAA, NH_4, Phe, Ser, Thr, Tyr and Val were the N-sources supporting high cell viability of this species at the beginning of stationary phase (with N_{max} above 8.0 log CFU/ml) (Table 1 and Fig. 2e). *L. thermotolerans* cell viability at late stages of stationary phase (N_{end}) was severely impaired for a high number of N-source supplementations (Ala, Arg, Asn, Asp, Gln, Glu, Gly, Ile, MixAA, $MixNH_4$ and NH_4) (Table 1 and Fig. 2f). On the other hand, Cys, His, Lys, Neg, Phe, Ser, Thr and Tyr supported N_{end} (with N_{end} above 6.2 log CFU/ml) (Table 1 and Fig. 2f).

For *H. uvarum*, Ala, Arg, Asp, MixAA, $MixNH_4$, Phe and Val promoted high μ_{max} (with μ_{max} above 0.17/h), whereas Cys, His and Thr had the opposite effect on μ_{max} (with μ_{max} below 0.09/h) (Table 1 and Fig. 2g). Regarding N_{max}, Ala, Asp, Leu, Lys and Phe supported high cell viability at the beginning of stationary phase (with N_{max} above 8.0 log CFU/ml), whereas Neg was the only condition that did not support *H. uvarum* N_{max} (Table 1 and Fig. 2h). As to N_{end}, Arg, Asn, Asp, Cys, Glu, Leu, Phe, Thr and Tyr supported this growth parameter (with N_{end} above 6.5 log CFU/ml) (Table 1 and Fig. 2i). With the opposite effect, Gln, MixAA, $MixNH_4$, Neg and NH_4 were responsible for the lower N_{end} observed (with N_{end} below 4.8 log CFU/ml) (Table 1 and Fig. 2i).

Regarding *M. pulcherrima*, Glu and NH_4 were the good N-sources as to sustaining high μ_{max} (0.38 and 0.35/h, respectively) (Table 1 and Fig. 2j). On the contrary, Cys, His, Neg and Thr were responsible for decreasing μ_{max} (with μ_{max} below 0.33/h). Only Neg did not allow a high N_{max} of *M. pulcherrima* (7.1 log CFU/ml), whereas Ala, Arg, Asn, Glu, Gly, Met, $MixNH_4$, Phe, Ser, Thr, Tyr and Val clearly improved N_{max} (with N_{max} above 8.0 log CFU/ml) (Table 1 and Fig. 2k). Finally, at late stages of stationary phase, Ala, Arg, Asn, Lys, Thr and Tyr had a positive effect on the maintenance of cell viability (with N_{end} above 7.1 log CFU/ml), whereas $MixNH_4$ and Trp had a negative effect, lowering N_{end} (5.3 and 5.0 log CFU/ml, respectively) (Table 1 and Fig. 2l).

Considering *T. delbrueckii*, Ala, Arg, Asn, Gln, Ile, MixAA, $MixNH_4$ and NH_4 sustained high μ_{max} (with μ_{max} above 0.27/h), whereas Cys, Gly, His, Lys, Neg, Ser and Val had the opposite effect (with μ_{max} below 0.13/h) (Table 1 and Fig. 2m). A considerably large number of N-sources (Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, Met, MixAA, $MixNH_4$, NH_4, Phe Thr, Trp Tyr and Val,) were responsible for promoting high cell viability at the beginning of stationary phase (with N_{max} above 8.0 log CFU/ml), whereas only Neg impaired N_{max} (6.4 log CFU/ml) (Table 1 and Fig. 2n). Finally, Arg, Asn, Glu, Gln, MixAA, NH_4, Trp, Thr, Tyr and Val

Table 1. Overview of the influence of N-sources on oxygen-limited yeast growth, glucose consumption and ethanol production

N-source	*S. cerevisiae*					*L. thermotolerans*					*H. uvarum*					*M. pulcherrima*					*T. delbrueckii*				
	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP	μ_{max}	N_{max}	N_{end}	GluC	EthP
Ala																									
Arg																									
Asn																									
Asp																									
Cys																									
Gln																									
Glu																									
Gly																									
His																									
Ile																									
Leu																									
Lys																									
Met																									
MixAA																									
$MixNH_4$																									
Neg																									
NH_4																									
Phe																									
Ser																									
Thr																									
Trp																									
Tyr																									
Val																									

'Good' N-sources are indicated with green, 'bad' with red and 'intermediate' with white. N-sources that were exhausted before the final day of fermentation, and therefore not included in our conclusions, are indicated with grey. The studied growth and fermentation parameters were maximum specific growth rate (μ_{max}), viability at the beginning (N_{max}) and late (N_{end}) stages of stationary phase, i.e. day 6, as well as glucose consumption (GluC) and ethanol production (EthP) after 6 days of fermentation

promoted the maintenance of cell viability at late stages of stationary (with N_{end} above 7.6 log CFU/ml), whereas His and Neg lowered N_{end} (5.8 and 6.2 log CFU/ml) (Table 1 and Fig. 2o).

N-sources that had a positive effect on all three growth parameters (i.e. μ_{max}, N_{max} and N_{end}) in each species, were MixAA for *S. cerevisiae*, Phe, Ser and Tyr for *L. thermotolerans*, Asp and Phe for *H. uvarum* and Arg, Asn, Gln, MixAA and NH_4 for *T. delbrueckii*. None of the N-sources had a positive effect on all three growth parameters of *M. pulcherrima*. Regarding *S. cerevisiae* growth (μ_{max}, N_{max} and N_{end}), Cys, Gly, His and Tyr did not particularly support any of the three parameters evaluated (Table 1 and Fig. 2). The weak support of Cys, Gly and His on *S. cerevisiae* growth is in accordance with previously published reports (Cooper 1982; Ljungdahl and Daignan-Fornier 2012). In fact, Cys, Gly and His did not sustain growth very well of all non-*Saccharomyces* species studied, with few exceptions (Table 1). Ala sustained high values of N_{max} and N_{end} of *S. cerevisiae* and *M. pulcherrima*, as well as μ_{max} and N_{max} of *H. uvarum* and *T. delbrueckii*. However, for *L. thermotolerans*, Ala only had an intermediate effect on μ_{max} and N_{max} and was not good as to N_{end}. Met had an intermediate effect on almost all three growth parameters of all species and was not good for *L. thermotolerans* as to μmax. It, however, favoured N_{max} of *S. cerevisiae*, *M. pulcherrima* and *T. delbrueckii* as well as N_{end} of *S. cerevisiae*. Trp, in general, had intermediate and negative effects on all three growth parameters of all species. Exceptions were noticed for N_{max} (*T. delbrueckii*) and N_{end} (*S. cerevisiae* and *T. delbrueckii*), on which this N-source had a positive effect. Interestingly, the complex mixture of N-sources $MixNH_4$ was classified as intermediate by *S. cerevisiae* and by *T. delbrueckii* but did not assure the maintenance of high cell viability at late stages of stationary phase (N_{end}) of *L. thermotolerans*, of *H. uvarum* and of *M. pulcherrima* (Table 1 and Fig. 2). This loss of viability was most pronounced in *L. thermotolerans* (Fig. 2f).

Oxygen-limited glucose consumption

For *S. cerevisiae*, a wide number of N-sources supported glucose consumption, specifically Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, MixAA, $MixNH_4$, NH_4, Ser and Val (Table 1 and Fig. 3a), with glucose exhaustion occurring after 5–6 days of fermentation (Supplementary Fig. S2, data not shown). Contrarily, Cys, Gly, His, Lys, Neg, Trp and Tyr were bad N-source conditions as to GluC (Table 1 and Fig. 3a).

For *L. thermotolerans*, only Ser supported high GluC (with a total consumption of 85 g/l glucose) (Table 1 and Fig. 3b). On the contrary, Cys, His and Neg had a negative effect on this parameter (with GluC below 25 g/l glucose) (Table 1 and Fig. 3b).

Regarding *H. uvarum*, Ala, Gly and Ile promoted GluC (with GluC above 38 g/l glucose), whereas Cys, His, Neg and NH_4 had the opposite effect (with GluC below 17 g/l glucose) (Table 1 and Fig. 3c).

For *M. pulcherrima*, Ala and Asn sustained high GluC (with GluC above 84 g/l glucose), whereas Cys, His, Neg and Tyr did not support this parameter (with GluC below 31 g/l glucose) (Table 1 and Fig. 3d).

Considering *T. delbrueckii*, Asn, Arg, Gln, Glu, Ile, Leu and MixAA supported high GluC (with GluC above 96 g/l glucose) (Table 1 and Fig. 3e). Contrarily, Cys, Gly, His, Neg and Ser had a negative effect on GluC, promoting the consumption of below 36 g/l glucose (Table 1 and Fig. 3e).

Within the non-*Saccharomyces* species, *T. delbrueckii* showed the highest ability to consume glucose (maximum of 126 g/l for Gln—Fig. 3e), whereas *H. uvarum* was the least efficient (maximum of 49 g/l for Ala—Fig. 3c).

As can be deduced from the above, Cys, His and Neg did not favour glucose consumption in any of the alcoholic fermentations.

Oxygen-limited ethanol production

For *S. cerevisiae*, several N-sources (Ala, Arg, Asn, Asp, Gln, Ile, MixAA, $MixNH_4$, NH_4, Ser and Val) allowed this species to produce ethanol to final concentrations above the threshold (with EthP above 67 g ethanol/l) established to classify N-sources as good for this specific parameter and species. Contrarily, Cys, Gly, His, Lys, Neg and Tyr were classified as bad, with EthP below 23 g ethanol/l (Table 1 and Fig. 4a).

Regarding *L. thermotolerans*, only Ser was classified as a good N-source (with EthP above 28 g ethanol/l), whereas Cys, His and Neg led to the production of low concentrations of ethanol (with EthP below 11 g ethanol/l) (Table 1 and Fig. 4b).

For *H. uvarum*, Ala, Gly and Ile promoted the highest EthP values for this species (with EthP above 14 g ethanol/l), whereas Cys, His, Neg and NH_4 had the opposite effect (with EthP below 6 g ethanol/l) (Table 1 and Fig. 4c).

Considering *M. pulcherrima*, Ala, Asn and $MixNH_4$ increased EthP (with EthP above 32 g ethanol/l), whereas Cys, His, Lys and Neg decreased EthP (with EthP below 12 g ethanol/l) (Table 1 and Fig. 4d).

Regarding *T. delbrueckii*, Asn, Gln, Ile and $MixNH_4$ sustained high EthP (with EthP above 40 g ethanol/l), whereas Cys, Gly, His, Neg and Ser had the opposite effect (with EthP below 12 g ethanol/l).

Comparison of oxygen-limited growth, glucose consumption and ethanol production of each yeast species

The complex mixture of amino acids; i.e. MixAA, was the only N-source supplementation that had a positive effect on all parameters studied (i.e. on μ_{max}, N_{max}, N_{end}, GluC and EthP) for *S. cerevisiae* (Table 1). Contrarily, for this species, Lys was the only N-source that impaired all parameters (Table 1). Regarding the non-*Saccharomyces* species, Ser (for *L. thermotolerans*) and both Asn and Gln (for *T. delbrueckii*) were the N-sources that improved all parameters, whereas no N-source had a negative effect on all parameters (Table 1).

Remarkably, for *S. cerevisiae*, Ala, Arg, Asn, Asp, Gln, Ile, MixAA, NH_4, Ser and Val had a positive effect on N_{max}, N_{end}, GluC and EthP. $MixNH_4$ was a good N-source for this species as to all parameters with the exception of N_{end}. This list adds to those good N-sources for *S. cerevisiae* previously presented by different authors (Gutiérrez et al. 2013; Ljungdahl and Daignan-Fornier 2012).

Phe, Ser and Tyr sustained all three growth parameters (i.e. μ_{max}, N_{max} and N_{end}) of *L. thermotolerans* and also GluC and EthP (for the specific case of Ser). For *H. uvarum*, Asp and Phe sustained high μ_{max}, N_{max} and N_{end}, whereas only Ala had a positive effect on μ_{max}, N_{max}, GluC and EthP. As to *M. pulcherrima*, Ala and Asn had both a positive effect on N_{max}, N_{end}, GluC and EthP, while Thr and Tyr sustained high cell viability (i.e. high N_{max} and N_{end}). $MixNH_4$ had a positive effect on both N_{max} and EthP. Finally, for *T. delbrueckii*, Arg, Asn, Gln and MixAA supported high growth (μ_{max}, N_{max}, N_{end}) and GluC, while NH_4 sustained high growth (μ_{max}, N_{max}, N_{end}) (Table 1). Ile and $MixNH_4$ had also a remarkable positive effect on supporting μ_{max}, N_{max}, EthP (both N-sources) and GluC (for the specific case of Ile) of *T. delbrueckii* (Table 1).

Not surprisingly, the absence of supplementation with N-source, Neg, imposed the harshest condition for yeast growth and fermentation (Table 1), leading to stuck and sluggish fermentations in all alcoholic fermentations (data not shown). Interestingly, the effect of Neg on the growth of non-*Saccharomyces*, although negative, was not as severe in absolute values as observed for *S. cerevisiae* (with μ_{max} within the range of 0.12–0.18/h and N_{max} of 6.4–7.1 log CFU/ml) (Fig. 2).

Regarding N-sources that, for each yeast species, had a positive effect on both fermentation performance parameters (i.e. GluC and EthP), from Table 1, we could observe for *S. cerevisiae* several N-sources with these effects (i.e. Ala, Arg, Asn, Asp, Gln, Ile, MixAA, $MixNH_4$, NH_4, Ser and Val). For *L. thermotolerans*, only Ser improved both parameters; for *H. uvarum*, Ala, Gly and Ile were the N-sources with these beneficial effects; for *M. pulcherrima*, Ala and Asn improved both parameters and, finally, for *T. delbrueckii*, Asn, Gln and Ile sustained high GluC and EthP together.

Moreover, both mixtures of amino acids (i.e. $MixNH_4$ and MixAA) did not show any particular positive effect, as to all parameters studied, on three of the non-*Saccharomyces* species tested (i.e. *L. thermotolerans*, *H. uvarum* and *M. pulcherrima*) in comparison with single amino acid supplementations. Contrarily, for *S. cerevisiae* and, to a lesser extent, for *T. delbrueckii*, $MixNH_4$ and MixAA had a pronounced positive effect on all parameters studied, in comparison with single amino acid supplementations (Table 1).

Comparison of nitrogen source influence profiles of different yeast species

Oxygen-limited growth

Heatmaps were built to compare the N-source influence profiles as to oxygen-limited growth parameters (i.e. μ_{max}, N_{max} and N_{end}) of the different yeast species (Fig. 5). The results shown in the heatmaps were built from data reduced and normalized to the corresponding measure in the medium supplemented with the most complex mixture of N-sources (i.e. the $MixNH_4$ supplementation).

Cluster analysis grouped N-sources into three major groups based on their effect on μ_{max} for the tested species (Fig. 5a). Cluster 1 (Lys, Neg, His, Thr and Cys) included the N-sources and the absence of N-source supplementation responsible for lowering μ_{max} of all species, as compared with the reference ($MixNH_4$). Cluster 2 (Ala, Arg, Asn, Asp, Glu, Gln, Ile, Met, MixAA and NH_4) grouped the N-sources that supported moderate μ_{max} of all the non-*Saccharomyces* yeasts studied. The N-sources of cluster 3 (Gly, Leu, Phe, Ser, Trp, Tyr and Val) did not support μ_{max} of *S. cerevisiae* and of *T. delbrueckii*, whereas for *L. thermotolerans*, *H. uvarum* and *M. pulcherrima*, they had a moderate effect on μ_{max}.

Regarding N_{max} (Fig. 5b), cluster analysis grouped N-sources into three main groups: Cluster 1 (Ala, Arg, Asn, Asp, Glu, Gln, Ile, Leu, Met, MixAA, NH_4, Phe, Thr, Tyr and Val) assembled the N-sources that, compared to the reference, moderately and highly supported N_{max} of *L. thermotolerans* and of *H. uvarum*, respectively. N-sources of cluster 1 had a mildly deleterious effect on N_{max} of *M. pulcherrima*, though the N-source effect pattern was similar to *S. cerevisiae*; Cluster 2 (Cys, Lys, Gly, His, Ser and Trp,) included the N-sources that poorly supported N_{max} of *S. cerevisiae*, of *L. thermotolerans*, of *M. pulcherrima* and of *T. delbrueckii* and highly supported N_{max} of *H. uvarum* (with exception of Cys); Cluster 3 (Neg), in which the lowest value of N_{max} was observed for all species.

Considering N_{end} (Fig. 5c), N-sources were also clustered into three groups. Clusters 1 (Ala, Arg, Asn, Asp, Gln, Glu, Gly, Ile, Leu, MixAA, NH_4 and Val) and 2 (Cys, Met, Phe, Thr, Trp, Tyr and Ser) grouped the N-sources that for *S. cerevisiae* and *T. delbrueckii* promoted a comparable N_{end} (with the reference), with only minor differences. Furthermore, N-sources of clusters 1 and 2 were responsible for enhancing the maintenance of N_{end} of *H. uvarum* and of *M. pulcherrima*. *Lachancea thermotolerans* remained viable only upon supplementation with N-sources from cluster 2, whereas for N-sources of cluster 1, the viability of this species decreased considerably (Fig. 5c). Cluster 3 (His, Lys and Neg) grouped N-sources and negative control that, on *S. cerevisiae* and on *T. delbrueckii*, did not support viability at the end of fermentation, lowering N_{end} (the only exception was observed for Lys in *T. delbrueckii*).

Within non-*Saccharomyces* species, only moderate correlations of N-source influences on μ_{max} were noticed (e.g. between *L. thermotolerans* and *H. uvarum*, $r = 0.59$, $p < 0.01$). Regarding N_{max}, a strong correlation was identified between *L. thermotolerans* and *T. delbrueckii* ($r = 0.74$, $p < 0.01$) and moderate correlations were also observed between *L. thermotolerans* and *M. pulcherrima* ($r = 0.68$, $p < 0.01$) and between *H. uvarum* and *M. pulcherrima* ($r = 0.67$, $p < 0.01$). For N_{end}, no relevant correlations were found within non-*Saccharomyces* species (Supplementary Table S3).

Comparing *S. cerevisiae* and non-*Saccharomyces* species, a moderate correlation was established between *S. cerevisiae* and *T. delbrueckii* for μ_{max} ($r = 0.59$, $p < 0.01$). The majority of the supplemented N-sources enhanced, in a similar way, the ability of *S. cerevisiae* and *T. delbrueckii* cells to remain viable at the beginning and at the end of fermentation (N_{max} $r = 0.65$, $p < 0.01$; N_{end} $r = 0.67$, $p < 0.01$) (Supplementary Table S3).

Oxygen-limited glucose consumption

Heatmaps were also constructed to distinguish the patterns of N-source influences on glucose consumption (GluC) for the different yeast species (Fig. 6). Once again, the data shown for each species in the heatmap were reduced and normalized to those obtained with $MixNH_4$ for the given species.

Cluster analysis of GluC grouped N-sources and negative control into three groups (Fig. 6). Cluster 1 (Cys, His and Neg) did not support glucose consumption in any of the five species, as compared with the reference. Cluster 2 (Ala, Asn, Arg, Asp, Gln, Glu, Ile, Leu, MixAA, NH_4 and Ser) grouped N-sources that conferred moderate/high GluC of *S. cerevisiae* and *T. delbrueckii* (the only exception observed with Ser for

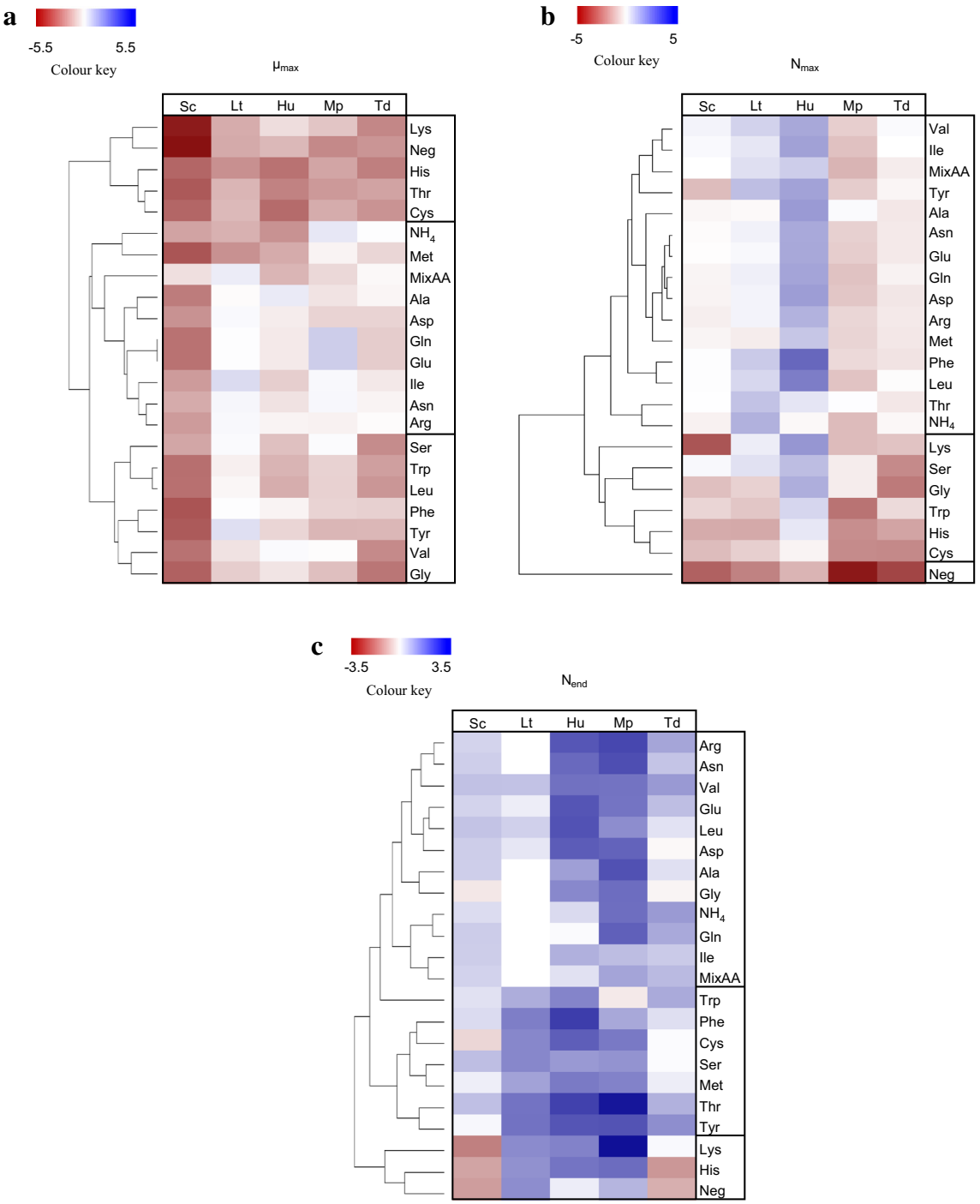


Fig. 5 Growth phenotypic variation of the five yeast species from fermentations in YNBMAF with different N-source supplementations under oxygen-limited conditions at 25 °C. Three growth parameters were considered: **a** Maximum specific growth rate, (μ_{max},1/h), **b** viability at the beginning of stationary phase (N_{max}, log(CFU/ml)) and **c** viability at late stages of stationary phase; i.e. day 6 (N_{end}, log(CFU/ml)). Fermentations were performed in duplicate. Each *row* of the heatmap indicates a particular N-source, and each *column* represents a specific yeast species: *S. cerevisiae*, Sc; *L. thermotolerans*, Lt; *H. uvarum*, Hu; *M. pulcherrima*, Mp and *T. delbrueckii*, Td. Mean values were reduced and normalized to those from the reference ($MixNH_4$). Regarding the colour key, *red* and *blue* represent low and high value, respectively, in comparison with the reference value. Hierarchical clustering is represented by dendrograms combined with each heatmap, in order to group the N-sources according to the Euclidean distance

T. delbrueckii). Cluster 3 (Gly, Lys, Met, Phe, Thr, Trp, Tyr and Val) grouped N-sources that showed a moderate/deleterious effect on GluC in *L. thermotolerans* and *H. uvarum*. Also, cluster 3 grouped N-sources that led to a more pronounced decrease of GluC of *S. cerevisiae*, *M. pulcherrima* and *T. delbrueckii* (Fig. 6).

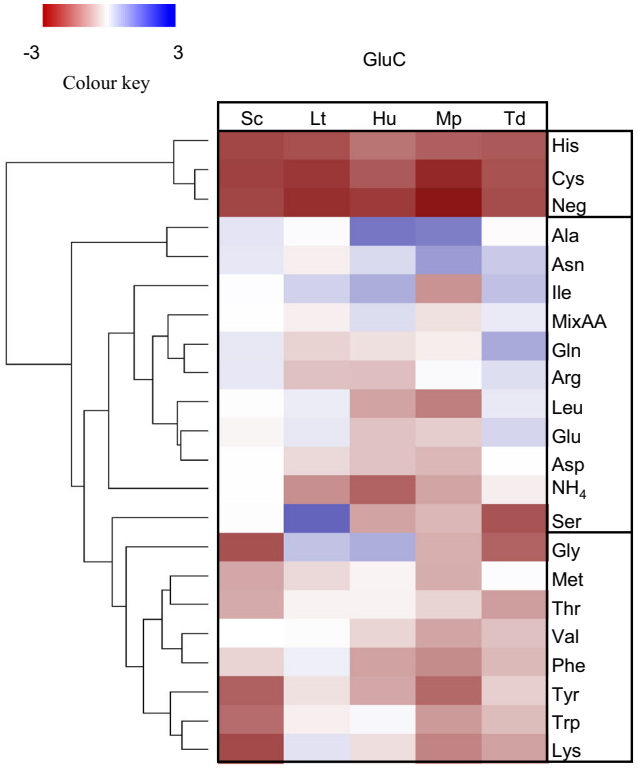


Fig. 6 Glucose consumption phenotypic variation (g glucose/l) of the five yeast species after 6 days of fermentation in YNBMAF with different N-source supplementations under oxygen-limited conditions at 25 °C. Fermentations were performed in duplicate. Each *row* of the heatmap indicates a particular N-source, and each *column* represents a specific yeast species: *S. cerevisiae*, Sc; *L. thermotolerans*, Lt; *H. uvarum*, Hu; *M. pulcherrima*, Mp and *T. delbrueckii*, Td. Mean values were reduced and normalized to those from the reference ($MixNH_4$). Regarding the colour key, *red* and *blue* represent low and high value, respectively, in comparison with the reference value. Hierarchical clustering is represented by dendrograms combined with each heatmap, in order to group the N-sources according to the Euclidean distance

Within non-*Saccharomyces* species, *H. uvarum* and *M. pulcherrima* showed a strong correlation ($r = 0.70$, $p < 0.01$) between the patterns of N-source influences on GluC, while moderate correlations where found between *H. uvarum* and *L. thermotolerans* ($r = 0.58$, $p < 0.01$) and between *M. pulcherrima* and *T. delbrueckii* ($r = 0.59$, $p < 0.01$) (Supplementary Table S3).

Comparing *S. cerevisiae* and non-*Saccharomyces* species, *T. delbrueckii* exhibited a strong correlation with *S. cerevisiae* as to GluC ($r = 0.75$, $p < 0.01$) (Supplementary Table S3). A moderate/strong correlation was also found between *S. cerevisiae* and *M. pulcherrima* ($r = 0.69$, $p < 0.01$) (Supplementary Table S3).

Oxygen-limited ethanol production

Cluster analysis of the heatmap for the ethanol production (EthP), built up from reduced and normalized data to the $MixNH_4$ experiments as mentioned before, grouped N-sources into three groups (Fig. 7):

Cluster 1 (Cys, His, Neg) grouped the sources that did not support EthP of all tested species. Cluster 2 (Gly, Lys, Ser, Thr, Trp, Tyr) included N-sources that had moderate/deleterious effect on EthP of *L. thermotolerans* and of *H. uvarum* (with the exception of Ser for *L. thermotolerans* and Gly, in a less extent, for *H. uvarum*, whose positive effect was noticed on both species). N-sources of cluster 2 showed also a pronounced deleterious effect on EthP of *S. cerevisiae*, *M. pulcherrima* and of *T. delbrueckii*. Cluster 3 (Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, Met, MixAA, NH_4, Phe, Val) grouped N-sources that induced less deleterious, and therefore more moderate effect in all species in general, and in particular in *S. cerevisiae* and *T. delbrueckii*. The majority of N-sources of cluster 3 negatively affected EthP of *M. pulcherrima* (exceptions were observed with the supplementations of Ala and Asn).

Within non-*Saccharomyces* species, *H. uvarum* and *L. thermotolerans* ($r = 0.56$, $p < 0.01$), *H. uvarum* and *M. pulcherrima* ($r = 0.55$, $p < 0.01$), *H. uvarum* and *T. delbrueckii* ($r = 0.45$, $p < 0.05$), as well as *M. pulcherrima* and *T. delbrueckii* ($r = 0.49$, $p < 0.05$) showed moderate correlations between the N-source influence patterns as to EthP (Supplementary Table S3).

Finally, comparing *S. cerevisiae* and non-*Saccharomyces* species, moderate and strong correlations for EthP were found between *S. cerevisiae* and *M. pulcherrima* ($r = 0.62$, $p < 0.01$) and *S. cerevisiae* and *T. delbrueckii* ($r = 0.73$, $p < 0.01$), respectively (Supplementary Table S3).

Discussion

In this study, we have used YNBMAF; i.e. a buffered YNB medium (pH 3.5), to which we have added 200 g/l glucose and anaerobic growth factors, as the base model medium, mimicking grape juice. Although YNBMAF and grape juice have rather different compositions, we find comparable doubling times of *S. cerevisiae* in our study using YNBMAF with $MixNH_4$ (2.0 ± 0.19 h) and in that of Viana et al. (2014) using a synthetic grape must (1.9 ± 0.12 h) under similar fermentation conditions (i.e. 25 °C and 120–140 rpm orbital shaking). Furthermore, considering the different initial glucose contents of the media in the two studies, rather similar glucose exhaustion times by *S. cerevisiae* are found; i.e. 120–144 h in YNBMAF with $MixNH_4$ (200 g/l initial glucose) and 105 h in the synthetic grape must (125 g/l initial glucose) (Viana et al. 2014). Together, these results sustain the use of YNBMAF as a grape juice model substrate.

In literature, two different approaches are commonly used when assessing nitrogen effects on yeast physiology: (1) addition of a comparable level of total elemental nitrogen within

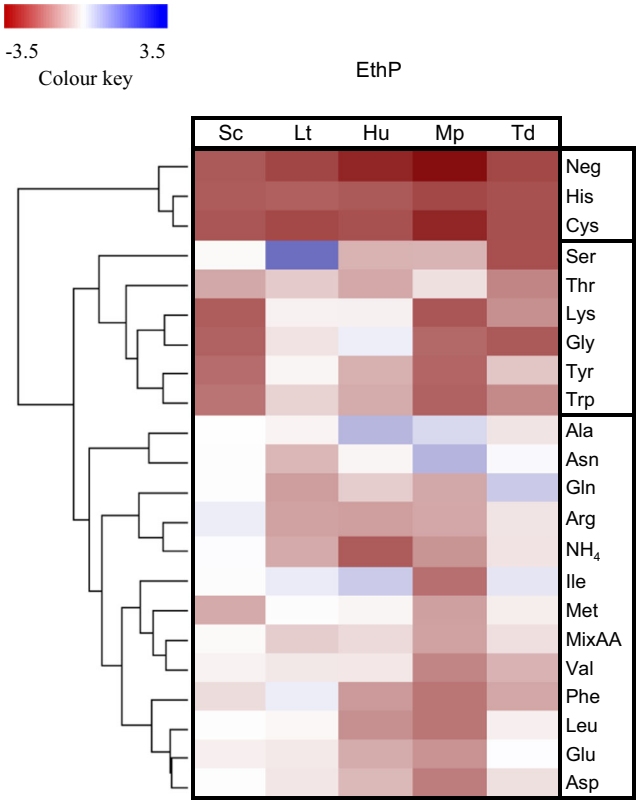


Fig. 7 Ethanol production phenotypic variation (g ethanol/l) of the five yeast species after 6 days of fermentation in YNBMAF with different N-source supplementations under oxygen-limited conditions at 25 °C. Fermentations were performed in duplicate. Each *row* of the heatmap indicates a particular N-source, and each *column* represents a specific yeast species: *S. cerevisiae*, Sc; *L. thermotolerans*, Lt; *H. uvarum*, Hu; *M. pulcherrima*, Mp and *T. delbruecki*, Td. Mean values were reduced and normalized to those from the reference ($MixNH_4$). Regarding the colour key, *red* and *blue* represent low and high value, respectively, in comparison with the reference value. Hierarchical clustering is represented by dendrograms combined with each heatmap, in order to group the N-sources according to the Euclidean distance

the different N-sources (Godard et al. 2007; Gutiérrez et al. 2012) and (2) addition of a comparable level of the different N-sources as whole molecules (Albers et al. 1996; Blomqvist et al. 2012). In our study, we used the latter approach supplementing media with an initial concentration of ≈2000 mg/l of each N-source, thus rendering an amount of elemental nitrogen in each medium above the recommended minimum level for *S. cerevisiae* as to completion of fermentation at a normal rate (120–140 mg N/l—Bely et al. 1990; Jiranek et al. 1995; Reed and Nagodawithana 1990). This amount of nitrogen did not constitute a limitation factor in the majority of experiments. Exceptions were only noticed for *S. cerevisiae* (Glu, Leu, Met, Phe and Trp), for *L. thermotolerans* (Met and Trp), for *M. pulcherrima* (Trp) and for *T. delbrueckii* (Glu and Met), where the N-source was exhausted before the final day of fermentation (i.e. day 6) (data not shown). Even though the rapid consumption may suggest a positive effect of those specific N-sources on the given yeasts, we have not included any of the data from nitrogen-limited fermentations in our conclusions due to the fact that nitrogen limitation influences yeast physiology (Brice et al. 2014; Parrou et al. 1999), thereby rendering non-comparable results. Also, although the elemental nitrogen ranges from 176 to 673 mg N/l in our experiments (Supplementary Table S1), we believe that these values are in a range where the difference in N-concentrations will not by itself affect the growth and fermentation performance of the yeasts (Gutiérrez et al. 2012).

It is well known that absence of N-source decreases the fermentation capacity of *S. cerevisiae* and leads to incomplete alcoholic fermentations (Bell and Henschke 2005; Bisson 1999; Boulton et al. 1996). In this study, the absence of N-source supplementation (Neg) had also a negative impact on the majority of the parameters evaluated (in particular, in N_{max}, GluC and EthP) for all species, confirming that it constitutes a harsh growth and fermentation condition common to different yeast species. However, non-*Saccharomyces* species, in particular *L. thermotolerans* and *M. pulcherrima*, could remarkably grow and maintain cell viability at late stages of stationary phase (i.e. above 6 log CFU/ml) under this condition of N-source absence. One explanation of this phenomenon may be that these non-*Saccharomyces* species are able to undergo cryptic growth after the induction of autolysis of part of the population and consequently utilize cell lysis products for further growth. The concept of cryptic growth is well described for prokaryotes (Banks and Bryers 1990; Mason and Hamer 1987), and autolysis has been generally correlated with increasing levels of extracellular ammonia under harsh growth conditions (White et al. 2002). In fact, it seems that non-*Saccharomyces* species can grow on lysis products, as they have been reported of being able to produce acid proteases in media that have proteins as the sole N-source. This was observed for *Candida albicans* (Banerjee et al. 1991; Dabas and Morschhäuser 2008) and more recently for *M. pulcherrima* and *C. apicola* (Reid et al. 2012), whereas the available data for *S. cerevisiae* seems to indicate that secretion of proteases is not an ability commonly attributed to this species (Alexandre et al. 2001).

Godard et al. (2007) studied the gene expression of nitrogen-regulated genes in a prototrophic *S. cerevisiae* lab strain (∑1278b) and basically classified N-sources into two groups; i.e. good (Ala, Arg, Asn, Asp, Gln, Glu, NH_4 and Ser) and bad (Ile, Leu, Met, Thr, Trp and Tyr), based on (i) supporting/not supporting high μ_{max}, (ii) activating/inactivating the nitrogen catabolite repression (NCR) regulatory pathway and (iii) inactivating/activating the general amino acid control (GAAC) regulatory pathway. It has been suggested that the good N-sources are readily integrated into the central carbon metabolism, whereas the bad N-sources (mainly constituting branched-chain amino acids, aromatic amino acids and methionine) are subject to conversion into non-

catabolizable and, perhaps even, growth-inhibitory fusel alcohols (Godard et al. 2007; Ljungdahl and Daignan-Fornier 2012). If we consider only experiments with single N-sources (i.e. excluding those with MixAA, $MixNH_4$ and Neg), we find, with a few exceptions, a rather similar list of good N-sources for *S. cerevisiae* as to supporting high μ_{max}, namely Arg, Asn, Gln, Ile, NH_4 and Ser (data not shown). Since the fermentations in Godard et al. (2007) were aerobic and ours were oxygen-limited, and since these authors used another strain than we did, these data indicate that Arg, Asn, Gln, NH_4 and Ser promote high growth rates in *S. cerevisiae*, regardless of the amount of oxygen available and the strain used. The fact, however, that we do not find Ala, but rather Ile, on the good list regarding μ_{max} could, in turn, be due to the different fermentation conditions and/or *S. cerevisiae* strains used in the two studies. Using the same approach, we find also a similar list of bad N-sources for *S. cerevisiae* as to supporting low μ_{max}, namely Met, Thr and Tyr (data not shown). The only exception is observed for Phe, which is included in this group of bad N-sources in our study and was classified as intermediate in Godard et al. (2007). Again, different fermentation conditions and/or *S. cerevisiae* strains used in the two studies may be the cause(s) of this discrepancy.

The only study that, to our best knowledge, investigated the effect of single N-sources on anaerobic growth (i.e. number of cell divisions) of a non-*Saccharomyces* species (*Dekkera bruxellensis*) highlighted the importance of Ala, Arg, Asn, His and Lys (Blomqvist et al. 2012). In our study and from this list, Ala and Lys are favourable for *H. uvarum* as to N_{max} and Ala, Arg, Asn for *M. pulcherrima* and *T. delbrueckii*. In contrast, for *L. thermotolerans*, these N-sources do not show any particular positive effect regarding N_{max}. Together, these data demonstrate that each N-source has a specific impact on oxygen-limited growth of non-*Saccharomyces* yeasts, stressing the importance of characterizing the effect of a given N-source on the growth of a particular yeast species to be used in a certain alcoholic fermentation.

Here, we provide for the first time the specific good N-sources of four non-*Saccharomyces* species (*L. thermotolerans*, *M. pulcherrima*, *H. uvarum* and *T. delbrueckii*) as to improving growth (i.e. μ_{max}, N_{max} and N_{end}) and fermentation (i.e. GluC and EthP) performance. For *L. thermotolerans*, Phe, Ser and Tyr sustain all three growth performance parameters as well as, in the case of Ser, both fermentation performance parameters. Regarding *H. uvarum*, Asp and Phe have a positive effect on all three growth performance parameters and Ala on μ_{max}, N_{max}, GluC and EthP. As to *M. pulcherrima*, Ala and Asn have a positive effect on N_{max}, N_{end} and both fermentation performance parameters. Finally, for *T. delbrueckii*, Arg, Asn, Gln and MixAA support high growth performance (μ_{max}, N_{max}, N_{end}), and for Asn and Gln, also both high GluC and EthP. Ile sustains high μ_{max}, N_{max}, GluC and EthP, while NH_4 promotes high μ_{max}, N_{max} and N_{end}). These results indicate that the influence of N-sources on both growth and fermentation performances under alcoholic fermentations is species dependent.

Our results show that, for *S. cerevisiae*, both $MixNH_4$ and MixAA promote higher μ_{max} than single amino acid supplementations. In fact, in *S. cerevisiae*, the complex mixture of amino acids, MixAA, is the only N-source that has a positive effect on all parameters studied (i.e. on μ_{max}, N_{max}, N_{end}, GluC and EthP). This beneficial effect of complex N-sources has already been reported on growth and fermentation rate of *S. cerevisiae* (Thomas and Ingledew 1990, 1992) and confirmed later in fermentations performed under anaerobic conditions (Albers et al. 1996; Bell and Henschke 2005; Martínez-Moreno et al. 2012). This might be explained by the fact that *S. cerevisiae* cells are prompt to grow and to ferment when multiple amino acids are available, inclusively inducing a reduction of amino acid synthesis (Albers et al. 1996). Interestingly, for three of the non-*Saccharomyces* species tested (i.e. for *L. thermotolerans*, *H. uvarum* and *M. pulcherrima*), our results demonstrate that they prefer single amino acids (as to all parameters studied) to the same or higher extent than mixtures of amino acids. The only exception is noticed for *T. delbrueckii*, where mixtures of amino acids have a positive effect, as also observed for *S. cerevisiae*, although to a lesser extent. Further studies should be addressed to elucidate the regulation of growth and fermentation of non-*Saccharomyces* species on mixtures of amino acids, as it seems to be less strictly regulated in these species than in *S. cerevisiae*.

An important outcome of our work is the finding of specific N-sources that have similar effects on a specific growth or fermentation parameter of two or more yeast species. For example, we can group N-sources that have a moderate effect on μ_{max} of all non-*Saccharomyces* yeasts (Ala, Arg, Asn, Asp, Gln, Glu, Ile, Met, MixAA and NH_4), that moderately and highly support N_{max} of *L. thermotolerans* and *H. uvarum* (Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, Met, MixAA, NH_4, Phe, Thr, Tyr and Val), that sustain high N_{end} of *H. uvarum* and *M. pulcherrima* (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Met, MixAA, NH_4, Phe, Ser, Thr, Tyr and Val), that are responsible of conferring moderate/high glucose consumption in *S. cerevisiae* and *T. delbrueckii* (Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, MixAA, NH_4 and Ser) and that have a moderate effect on ethanol production of *S. cerevisiae* and *T. delbrueckii* (Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, Met, MixAA, NH_4, Phe and Val). In addition, Cys, Gly and His are associated with poor growth and/or fermentation performances of almost all species. These findings indicate that, within each group of N-sources, similar physiological mechanisms underlying transport and/or metabolism of the specific amino acids may exist among the given yeast species. Furthermore, for each group, the results may be used for optimizing

the specific growth or fermentation performance parameter in alcoholic fermentations including mixtures of the given yeast species.

When comparing the patterns of N-source influences on the different parameters for the different non-*Saccharomyces* species, and apart from similarities found between *S. cerevisiae* and *T. delbrueckii*, only few strong correlations exist. These results basically demonstrate that different N-sources indeed affect growth and fermentation performances of different yeast species differently. In fact, the yeast species resembling *S. cerevisiae* the least in this respect is *H. uvarum*, especially regarding μ_{max}, N_{max}, GluC and EthP (Supplementary Table S3). These results may reflect the different capacity of yeast species to adapt to distinct environments. A good N-source may have a more efficient uptake rate that will result in fast growth and fermentation in that environment, leading to fast N-source exhaustion for competition effects. Thus, the influence of a N-source on growth and fermentation of a certain yeast species appears as a key parameter to account for variation in life-history traits.

As mentioned above, *T. delbrueckii* is the non-*Saccharomyces* species, of the four tested, with more similar N-source influence profiles as compared with *S. cerevisiae*. This may be due to the fact that these two yeast species have the closest phylogenetic relationship, when comparing *S. cerevisiae* with the non-*Saccharomyces* yeast species tested (Kurtzman and Robnett 2003). *Torulaspora delbrueckii* (previously named as *Saccharomyces rosei* or *Saccharomyces roseus*), besides belonging to the group of species that did not undergo whole-genome duplication (Merico et al. 2007), has been undergoing genetic changes to adapt to the environments that recently were associated with this species (Albertin et al. 2014). Moreover, these genetic changes have been strongly shaped by human activities (e.g. oenology, bakery, distillery, dairy industry, etc.), as they have also been with *S. cerevisiae*, constituting therefore an alternative model system of yeast domestication (Albertin et al. 2014).

Some non-*Saccharomyces* species (in particular *H. uvarum*) are reported as showing lower ethanol yields (i.e. ethanol produced per glucose consumed) than *S. cerevisiae* while fermenting grape juice under anaerobic conditions (Ciani et al. 2006; Gobbi et al. 2014). This is one of the reasons why non-*Saccharomyces* species are included in the strategies of reducing ethanol content in wines (Heux et al. 2006; Kutyna et al. 2010), also by taking advantage of the different distribution of the Crabtree effect throughout wine yeasts (Gonzalez et al. 2013). For the majority of N-sources tested, our results show relatively similar ethanol yields of the different wine-related yeast species (i.e. similar slopes of the linear regressions in Supplementary Fig. S4), thereby slightly disagreeing with the above mentioned results found in literature. Remarkably, among the data depicted in Supplementary Fig. S4, it can also be found that by using specific single N-sources, e.g. Tyr, Cys or Met, all non-*Saccharomyces* species have higher ethanol yields than *S. cerevisiae* (data not shown). This finding may indicate that the physiological mechanisms underlying transport and/or metabolism of those N-sources (i.e. of Tyr, Cys and Met) is conserved among non-*Saccharomyces* species. It may furthermore suggest that other sources than glucose are canalized into ethanol in those species. These sources could be, e.g. N-sources, as suggested by Freese et al. (2011). Finally, it should be noted that the putative impacts of N-source addition on redox balance, and thereby on glycerol production and ethanol yield, of yeasts under anaerobic conditions, as discussed, e.g. by Albers et al. (1996) and Blomqvist et al. (2012), will not be considered here due to the fact that our experimental setup was not strictly anaerobic but rather oxygen-limited. Taken together, our results highlight the importance of studying the full set of N-sources, if diminishments or improvements of the fermentation performance of non-*Saccharomyces* yeasts are to be achieved.

Previously, Varela et al. (2004) have shown that an increase in viable cell concentration promoted by a given N-source is correlated with higher fermentation rates. Our data confirm this finding under some conditions for *S. cerevisiae* (Ala, Arg, Asn, Asp, Gln, Ile, MixAA, NH_4, Ser and Val), *L. thermotolerans* (Ser), *M. pulcherrima* (Ala and Asn) and *T. delbrueckii* (Asn and Gln), where high N_{max} and N_{end} values correlate with high GluC and EthP. Under all other N-source conditions for these yeasts, and under most conditions for *H. uvarum*, a good growth performance does not necessarily result in a good fermentation performance of the particular yeast. These data agree with those of Gutiérrez et al. (2012), and they demonstrate that it is important to not only study the effect of an N-source on growth but also to consider its influence on fermentation, if an increased understanding of the yeast performance under alcoholic fermentation is wanted. The mechanisms underlying this lack of correlation between growth and fermentation in some yeast species under some N-source conditions need further investigation.

Finally, it should be stressed that we in this work have only studied one strain within each of the five yeast species, and although we have found differences among species, we are fully aware that variability may also occur within yeast species; i.e. among strains belonging to the same species, as already observed for *S. cerevisiae* (Georis et al. 2009; Godard et al. 2007; Gutiérrez et al. 2012; Magasanik and Kaiser 2002). Thus, care must be taken when extrapolating the data from the strains used in our study to other strains.

In conclusion, this study establishes for the first time the specific N-source influence on growth and/or fermentation performances of five wine-related yeast species during alcoholic fermentation. Succinctly, the N-sources that improve all performance parameters (or the majority of them) for each

yeast species are: Ala, Arg, Asn, Asp, Gln, Ile, MixAA, $MixNH_4$, NH_4, Ser and Val (for *S. cerevisiae*), Ser (for *L. thermotolerans*), Ala (for *H. uvarum*), Ala and Asn (for *M. pulcherrima*) and Arg, Asn, Gln, Ile and MixAA (for *T. delbrueckii*). These results reveal that the effect of N-sources on yeast growth and fermentation performance is species-specific and is dependent also on the N-source. Moreover, we find groups of N-sources with similar effects on the growth and/or fermentation performance of two or more yeast species. Finally, *T. delbrueckii* has virtually the same N-source influence profile as *S. cerevisiae*, whereas *H. uvarum* has the most different pattern. The results of this work contribute to an increased understanding of the influence of N-sources on growth and fermentation abilities of specific wine yeasts and may be used by, e.g. winemakers for the optimization of N-source addition to their alcoholic fermentations, considering the specific needs of the given wine yeast used.

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Compliance with ethical standards

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Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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