**Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth
and fermentation under winemaking conditions**

Ying Su^{1†}, Pauline Seguinot^{2†}, Isabelle Sanchez³, Anne Ortiz-Julien⁴, José Maria Heras⁵, Amparo Querol¹, Carole Camarasa², José Manuel Guillamón^{1*}

¹Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), Valencia, Spain

²UMR SPO: INRA, Université Montpellier, Montpellier SupAgro, 34060, Montpellier, France; Lallemand SAS, 31700, Blagnac, France.

³UMR MISTEA: INRA, Montpellier SupAgro, 34060, Montpellier, France.

⁴Lallemand SAS, 31700, Blagnac, France.

⁵Lallemand Bio, 28521, Madrid, Spain

*Corresponding author: José M. Guillamón, guillamon@iata.csic.es
+34963900022ext: 2303

†These authors contributed equally to this work

Abstract

Wine-related non-*Saccharomyces* yeasts are becoming more widely used in oenological practice for their ability to confer wine a more complex satisfying aroma, but their metabolism remains unknown. Our study explored the nitrogen utilisation profile of three popular non-*Saccharomyces* species, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola*. The nitrogen source preferences to support growth and fermentation as well as the uptake order of different nitrogen sources during wine fermentation were investigated. While *T. delbrueckii* and *S. cerevisiae* strains shared the same nitrogen source preferences, *Metschnikowia* sp. displayed a lower capacity to efficiently use the preferred nitrogen compounds, but were able to assimilate a wider range of amino acids. During alcoholic fermentation, the non-*Saccharomyces* strains consumed different nitrogen sources in a similar order as *S. cerevisiae*, but not as quickly. Furthermore, when all the nitrogen sources were supplied in the same amount, their assimilation order was similarly affected for both *S. cerevisiae* and non-*Saccharomyces* strains. Under this condition, the rate of nitrogen source consumption of non-*Saccharomyces* strains and *S. cerevisiae* was comparable. Overall, this study expands our understanding about the preferences and consumption rates of individual nitrogen sources by the investigated non-*Saccharomyces* yeasts in a wine environment. This knowledge provides useful information for a more efficient exploitation of non-*Saccharomyces* strains that improves the management of the wine fermentation.

Keywords: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, nitrogen preference, nitrogen uptake

1. Introduction

In the past 50 years, the inoculation of selected *Saccharomyces cerevisiae* strains has become increasingly prevalent in winemaking. It is an excellent way to control alcoholic fermentation by ensuring the complete exhaustion of sugars and avoiding the formation of undesirable off-flavours. During wine fermentation, the predominant non-*Saccharomyces* yeasts in grape juice are rapidly outcompeted by *S. cerevisiae* because they poorly adapt to increasing ethanol concentrations and to low levels of dissolved oxygen (Bauer and Pretorius, 2000; Boulton et al., 1995; Bisson, 1999). Some of these non-conventional yeasts have attracted more attention as they positively modify the wine chemical composition and, consequently, improve wine flavour and bouquet. (Padilla et al., 2016). However, as a result of their low ethanol tolerance, these species are unable to achieve complete sugar consumption. Consequently, in order to take advantage of their phenotypic specificities while avoiding stuck or sluggish fermentations, they are usually combined in industry with *S. cerevisiae* in sequential or co-inoculation. In this context, both the growth and the metabolic activity of non-*Saccharomyces* yeasts may impact *S. cerevisiae* performance in mixed culture fermentation mainly due to their nutrient consumption (Medina et al., 2012; Taillandier et al., 2014).

Nitrogen availability in grape must is a key parameter for the wine fermentation progress as it acts at many levels. Firstly, nitrogen is an important nutrient for sustaining yeast growth and, for the vast majority of fermentations, it is the limiting factor for biomass production (Bell and Henschke, 2005; Martínez-Moreno et al., 2012; Mendes-Ferreira et al., 2004; Varela et al., 2004). Nitrogen also plays a key role in fermentation kinetics and fermentation duration because insufficient nitrogen source availability in must can lead to stuck or sluggish fermentation (Bely et al., 1990; Mendes-Ferreira et al., 2004). Lastly, the catabolism of nitrogen sources, together with central carbon metabolism, is involved in the synthesis of fermentative aroma precursors in wine yeasts (Barbosa et al., 2012; Bell and Henschke, 2005).

Grape musts contain a wide range of yeast assimilable nitrogen (YAN) sources, including not only amino acids and ammonium, but also urea, citrulline, ornithine and small peptides (Kevvai et al., 2016). The utilisation of these compounds during wine fermentation has been extensively described in *S. cerevisiae*. Nitrogen source quality, in terms of the capacity to support yeast growth and fermentation activity, has been evaluated in this species (Cooper, 1982; Fairbairn et al., 2017; Godard et al., 2007;

Gutiérrez et al., 2013), with major differences between poor nitrogen sources, such as tryptophan, tyrosine, threonine or methionine, and rich nitrogen sources like ammonium, glutamine or asparagine. When provided as a mixture during fermentation, some nitrogen compounds are taken up early by yeasts, such as lysine, aspartate or threonine, while others (arginine, glycine, tryptophan, etc.) are consumed later (Jiranek et al., 1995; Crepin et al., 2012; Rollero et al., 2018). Indeed many processes required for the uptake and growth in lower quality nitrogen sources are suppressed in the presence of a higher quality nitrogen source (Godard et al. 2007). Nitrogen sources are sequentially assimilated through various transporters that are regulated by two main systems: nitrogen catabolite repression (NCR) and the Ssy1p-Ptr3p-Ssy5 (SPS) system (Crépin et al., 2012; Godard et al., 2007; Ljungdahl and Daignan-Fornier, 2012). Consistently with phenotypic changes, the nitrogen source available in the medium determines the transcription level of almost 10% of all the genes in *S. cerevisiae* (Godard et al., 2007). The different regulation pathways triggered by various nitrogen sources can explain this transcriptional reprogramming.

Unlike *S. cerevisiae*, very little is known about the physiology and behaviour of non-*Saccharomyces* yeasts during wine fermentation, and their consumption profiles and preferences for amino acids have been poorly explored. In previous studies, Kemsawasd et al. (2015) studied the influence of pure nitrogen sources on the growth, glucose consumption and ethanol production of four wine-related non-*Saccharomyces* yeast species, and revealed that the effect of nitrogen sources on yeast growth and fermentation performance is species-specific. Furthermore, Gobert et al. (2017) and Rollero et al. (2018) investigated the nitrogen source uptake order by wine-related non-*Saccharomyces* strains while fermenting natural and synthetic grape musts, respectively. Both studies highlighted the importance of monitoring nutrient concentration during sequential fermentations with *S. cerevisiae* to avoid stuck or sluggish fermentations. Consequently, a comprehensive analysis of the efficiency of amino acids, used as the sole nitrogen source, to sustain the growth and fermentation performances of non-*Saccharomyces* species can be regarded as valuable research. This information would be essential for implementing the sequential or co-fermentation of these species with *S. cerevisiae*.

For this purpose, four commercially available non-*Saccharomyces* strains, which belong to the species *Torulaspora delbrueckii*, *Metschnikowia fructicola* and *Metschnikowia pulcherrima* were chosen to explore their nitrogen source management under

fermentation and growth conditions. This study generally highlights the differences between the *S. cerevisiae* and non-*Saccharomyces* species in single nitrogen source preferences and the assimilation order of each compound in mixed nitrogen sources. Furthermore, the lack of relationship between the sequence of consumption of amino acids and their capacity to support growth are highlighted, regardless of species.

2. Material and Methods

2.1 Strains and media

All the yeast strains herein used were provided by Lallemand Inc. (France). These strains are *S. cerevisiae* Lalvin QA23[®] (SC), *Torulaspora delbrueckii* Biodiva[®] (TD), *Metschnikowia pulcherrima* Flavia[®] (MP), *Metschnikowia fructicola* Gaia[®] (MFa) and *Metschnikowia fructicola* LYCC7706 (MFb).

Yeast extract peptone dextrose (YPD) medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) and SD medium (20 g/L glucose, 0.17 g/L yeast nitrogen base with 0.23 g/L NH₄Cl) were used for yeast propagation.

A synthetic grape must (SM) base without nitrogen was prepared based on the recipe of Bely et al. (1990) with some modifications. This medium contains 200 g/L of sugar as a mixture of 50% glucose, 50% fructose and 5 mg/L of phytosterols. The concentrations of organic acids, minerals and vitamins were the same as those described by Su et al. (2019). The concentration of the different nitrogen sources was adjusted to specific levels, and detailed concentrations are explained in the following sections. When small volume (growth experiment) was needed, synthetic must was sterilised by filtration through 0.22 µm pore-size membrane filters (Thermo scientific). When large volume was needed (fermentation experiment), synthetic must was sterilised by steam pasteurisation for 15 min.

2.2 Determination of the growth rate, lag phase length and biomass yield in different nitrogen sources

The growth of the four non-*Saccharomyces* strains and one *S. cerevisiae* control strain in the different sole nitrogen sources was determined by the 96-well microtiter plate screening method, as described by Warringer and Blomberg (2003), with some modifications. In all, 27 individual nitrogen sources, including amino acids and ammonium, were used at two concentrations: 140 mg/L and 200 mg/L of YAN (Table 1).

As a control fermentation, a mixed nitrogen source was used, which was made up of a mixture of 70% of amino acids and 30% of ammonium chloride (Table 2).

To assess the growth parameters, yeast cells were first pre-cultured in YPD medium overnight before being transferred to SD medium, which contains only 60 mg/L YAN, to be cultivated for 24 h to eliminate the influence of the nitrogen-rich YPD medium. Cells were washed and inoculated into SM with different nitrogen sources at a density of 10^6 yeast cells/mL. 250 μ L of inoculated SM were dispensed to each microtiter plate well. Each condition was assayed in triplicate. Microtiter plates were incubated in a SPECTROstar Nano[®] microplate reader (BGM Labtech, Offenburg, Germany) at 25°C with 500 rpm orbital shaking. Growth curves were monitored by recording the increase in optical density (OD) at wavelength 600 nm every 30 minutes. The growth parameters (lag phase λ , maximal specific growth rate $\mu_{\max}$) were deduced from the growth curve by directly fitting OD measurements *versus* time to the Gompertz equation proposed by Zwietering et al. (1990), which takes the following expression:

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

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t ; $D = \ln(OD_{\infty}/OD_0)$ is the OD value reached with OD_{∞} as the asymptotic maximum. R script (statistical software R, v.3.0) was used to facilitate the calculations of $\mu_{\max}$ and λ with the Gompertz equation.

2.3 Fermentation kinetic experiment

Fermentations were conducted with synthetic must containing sole nitrogen sources with four non-*Saccharomyces* strains and one *S. cerevisiae* control strain. Two nitrogen concentrations, 140 mg/L and 200 mg/L, were used, and the detailed concentration of each nitrogen source is shown in Table 1. Fermentations were carried out in 15 mL glass tubes closed with a filter tip to allow the release of CO₂. Tubes were kept at 24°C with continuous magnetic stirring (260 rpm). Fermentations were followed by monitoring released CO₂ by weighing tubes every 4 h. The experiment was carried out in duplicate. This measure was automatically taken by a robotic device (Bloem et al., 2018).

2.4 Nitrogen source uptake order during alcoholic fermentation

Alcoholic fermentations were carried out at 25°C with a mixture of nitrogen sources to disclose the uptake order of the different nitrogen sources during fermentation. Firstly, fermentations were conducted with SM140 and SM300 (synthetic must of 140 and 300

mg/L YAN), whose composition is described in Table 2. Out of the two *M. fructicola* strains, only MFa (Gaia) was used for assessing the nitrogen uptake order. Samples were taken every 4 h until 48 h of fermentation. Fermentations were also conducted with SM300E in which an equal amount of each nitrogen sources (in terms of YAN) was supplied. For the SM300E fermentations, the *T. delbrueckii* and the *M. pulcherrima* strains, together with the control *S. cerevisiae*, were included in the experiment. Samples were taken every 4 h until 24 h. Yeast cells were removed from samples by centrifugation. The residual nitrogen in samples was analysed by UHPLC equipped with a UV detector (Thermo Scientific, MA, USA). The HPLC analysis method was based on Gómez-Alonso et al. (2007), but with some modifications. Samples were derivatised by diethylethoxymethylenemalonate (DEEMM). An accucore[®] C18 (Thermo scientific) LC column was used to separate amino acids.

2.5 Statistical analysis

All the experiments were carried out at least in duplicate. To study the growth on different media, heatmaps were plotted by Mev MultiExperiment Viewer, hierarchical clustering was performed with Euclidean distance metrics and group clustering was based on group averages (average linkage). The area under the curve (AUC) was calculated by the R statistical software, v. 3.0., with the growth curver package (Sprouffske, 2018).

The CO₂ production curves were smoothed on the same time vector using the cellGrowth package, version 1.22.0 (Gagneur and Neudecker, 2012). These data were clustered by employing the Euclidean distance between CO₂ points at the same time, and by the Ward aggregation method. The number of clusters was set at seven. A principal component analysis (PCA) was carried out with the FactoMineR package (Le et al., 2008).

3. Results

3.1 Influence of the single nitrogen source on the yeast growth rate, lag phase length and biomass yield

The lag phase, growth rate ($\mu_{\max}$) and maximum OD (OD_{max}) of four non-*Saccharomyces* and one *S. cerevisiae* strains were compared during growth in 27 different single nitrogen sources (140 or 200 mg/L YAN). The heatmap, which summarises the comparison of the growth parameters depending on the nitrogen sources, the concentrations and strains (Figure 1A), revealed that the herein used nitrogen sources hierarchically clustered into three categories, namely good, intermediate and poor nitrogen sources. Generally, the

good nitrogen sources supported a relatively shorter lag phase time, a higher maximum growth rate and a higher maximum OD. Conversely, the poor nitrogen sources only supported weak, or even no, strain growth. NH_4Cl , glutamate, glutamine, aspartate, asparagine, GABA, arginine, alanine, leucine and valine were classified as good nitrogen sources for both *S. cerevisiae*, as previously reported (Godard et al., 2007; Gutiérrez et al., 2013), and the non-*Saccharomyces* species: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola*. Similarly, cysteine, cytosine, citrulline, lysine, glycine, tyrosine and histidine poorly sustained the growth of all the strains. Surprisingly according to our results, proline, which has always been considered an unassimilable nitrogen source for *S. cerevisiae* strains under anaerobic conditions, was clustered in the intermediate nitrogen sources because the non-*Saccharomyces* strains, especially the *Metschnikowia* strains, efficiently used proline for growth.

A further analysis of the dataset revealed marked differences in the growth rate (Figure 1B) and the lag phase time (Figure 1C), depending on both the nature of the nitrogen source and the yeast species. The *Metschnikowia* strains displayed poorer growth performance for most nitrogen sources and a narrower variance in their growth characteristics according to the nitrogen source. However, the *Metschnikowia* strains exhibited greater growth capacity than *S. cerevisiae* in some nitrogen sources, such as isoleucine, glycine, threonine, histidine, citrulline and proline. The *Metschnikowia* and *T. delbrueckii* strains were able to use lysine as a sole nitrogen source, whereas *S. cerevisiae* was unable to metabolise it. On the contrary, *S. cerevisiae* grew much faster than the *Metschnikowia* strains in aspartate, asparagine, glutamine, glutamate, GABA, arginine and urea. *T. delbrueckii* took an intermediate position with higher μ_{max} than the *Metschnikowia* strains, but a lower μ_{max} than *S. cerevisiae* in the richer nitrogen sources. To summarise these specific growth traits, it was concluded that *S. cerevisiae* had a higher μ_{max} than the non-*Saccharomyces* strains when grown on good nitrogen sources, while the non-*Saccharomyces* strains displayed better growth performances than *S. cerevisiae* on the poorest nitrogen sources (proline, cysteine, lysine and histidine).

Interestingly enough, although the non-*Saccharomyces* strains, especially the *Metschnikowia* strains, showed less growth capabilities than *S. cerevisiae*, the non-*Saccharomyces* strains displayed approximately a 10-hour shorter lag phase than *S. cerevisiae* for more than half the single nitrogen sources. Only under the mix-N condition

did the lag phase time of *S. cerevisiae* substantially lower and reach a similar value to the non-*Saccharomyces* strains.

Two YAN concentrations (140 mg/L and 200 mg/L), which correspond to the nitrogen levels commonly found in natural grape must, were assayed for each pure nitrogen source in our study. No substantial differences were observed for either the lag phase time or the maximum growth rate between both concentrations for most nitrogen sources (Suppl. Figures 1-2), except for the poorer nitrogen sources (lysine, glycine, histidine, proline and tyrosine). The increased concentration of these poorer nitrogen sources yielded an increase in $\mu_{\max}$ and a reduction in the lag phase. Finally, as expected, a higher maximum OD value was obtained with 200 mg/L YAN than with 140 mg/L YAN for almost all the nitrogen sources (Suppl. Figure 3).

3.2 Capability of nitrogen sources to sustain fermentation

In parallel, four fermentation parameters were assessed: the fermentation lag phase, the maximal fermentation rate, the time to reach this maximum and the ability of strains to complete fermentation. A wide range of fermentation profiles was obtained from the five strains (Figure 2). By applying Ward's agglomerative hierarchical clustering procedure to compare the profiles at 200 mg/L YAN, seven representative clusters of specific behaviours were differentiated (Figure 2). Clusters 1, 2 and 3 collected most of the *S. cerevisiae* and *T. delbrueckii* fermentations, characterised by high fermentation rates from 0.55 g/L/h to 0.99 g/L/h, which allowed fermentation to be completed in less than 350 h. These three clusters were differentiated by lag phase length, which was shorter in Cluster 1 (a mean lag phase of 18.8 h), intermediate for Cluster 2 (mean value of 44.5 h), but much longer in Cluster 3 (a mean lag phase of 80.5 h). Clusters 4, 5 and 6 grouped the fermentations in which sugars were not exhausted. All these conditions corresponded to the cultures carried out with *Metschnikowia* sp., apart from the *S. cerevisiae* and *T. delbrueckii* fermentations on tryptophan and adenine, respectively. Under these conditions, fermentation activity was moderate, and a sharp drop in the fermentation rate occurred after approximately 50 h of fermentation. The average duration of the lag phases was shorter than in Clusters 1-3, and varied from 16.8 h to 29.3 h. This reduction in the fermentation lag phases agrees with the above-mentioned shorter growth lag phases for *Metschnikowia* sp. Finally, Cluster 7 grouped the conditions that were unable to support efficient fermentation, in which the lag phase was long and the fermentation rate was very

slow. Histidine and cysteine were the only identified amino acids as being unable to support fermentation for all four species.

To further investigate the effect of the different nitrogen sources on the fermentation performance of the four yeast species, three quantitative parameters, which describe fermentation kinetics (lag phase duration, maximal fermentation rate and time to reach this maximum; Figure 3) were compared by a PCA analysis. The first principal component explained 76% of the variance in the dataset on the basis of lag phase duration and the time to reach the maximal fermentation rate. A group of individuals (blue circle), which corresponded mainly to the fermentations done with cysteine and histidine as the nitrogen source, for which the four species' activity was very limited, was clearly distinguished from the other conditions according to these two parameters. A clear differentiation was also observed on the second axis between the fermentation parameters obtained with *T. delbrueckii* and *S. cerevisiae* (light green circle) on the one hand, and with *Metschnikowia sp.* on the other hand (yellow circle). The fermentations performed by the *Metschnikowia* strains had a low R_{max} , but a shorter lag phase. *T. delbrueckii* and *S. cerevisiae* achieved better performances with higher fermentation rates. These strains were also characterised by a wider variety of profiles depending on the amino acid, with differentiation based mainly on the fermentation rate (Figure 3). For example, *T. delbrueckii* and *S. cerevisiae* both had a low fermentation rate on cytosine and glycine. However, *S. cerevisiae* obtained a high fermentation rate for adenine, whereas *T. delbrueckii* proved less effective on this medium. Overall, most of the amino acids that efficiently supported fermentation were comparable for the five strains (Table 3). These amino acids included alanine, aspartate, asparagine, GABA, glutamate, ammonium and urea.

Furthermore, even if *T. delbrueckii* and *S. cerevisiae* similarly responded to the majority of amino acids, some differences can be highlighted between these species (Suppl. Figures 4-5, Table 3). In general, *S. cerevisiae* had higher fermentation rates than *T. delbrueckii*, especially on adenine, glycine, tryptophan, tyrosine, valine and serine. Conversely, *T. delbrueckii* fermented more efficiently than *S. cerevisiae* on lysine, and exhibited a shorter lag phase and a higher fermentation rate on citrulline and ornithine. No significant differences in fermentation performances were observed between *M. pulcherrima* and *M. fructicola*, apart from the more efficient fermentation on lysine for *M. fructicola*.

Finally, the nitrogen concentration (140 or 200 mg/L of YAN) did not impact the classification of nitrogen sources. Higher maximal fermentation rates were obtained for higher nitrogen concentrations, except for the fermentation of *T. delbrueckii* on adenine, and the fermentation of *S. cerevisiae* on cysteine and histidine (Suppl. Figure 4). Lag phases were longer on the medium containing the lower nitrogen concentration (140 mg/L of YAN), especially for the non-*Saccharomyces* yeasts (Suppl. Figure 5).

3.3 Correlation between the growth and fermentation activity of each strain by using a single nitrogen source

The correlation between maximum growth rate $\mu_{\max}$ and maximum fermentation rate $R_{\max}$ by using different nitrogen sources at 200 mg/L YAN was calculated (Figure 4). A generally good correlation was observed between both parameters. However, this correlation was better for the *Metschnikowia* strains than for *S. cerevisiae* and *T. delbrueckii*. For the last two strains, the two parameters were well correlated when the $\mu_{\max}$ values were under 0.1 h^{-1} . Only for the amino acids that supported poor growth, the fermentation capacity was limited by biomass (a linear correlation between $\mu_{\max}$ comprised between 0 and 0.1 h^{-1}). When using the richer nitrogen sources, *S. cerevisiae* and *T. delbrueckii* showed higher growth rates ($\mu_{\max}$ higher than ≈ 0.1), but $R_{\max}$ reached a constant value around 0.95 g/L/h for *T. delbrueckii* and 1.1 g/L/h for *S. cerevisiae*. As the maximum growth rates were lower than 0.1 h^{-1} for the *Metschnikowia* strains with all the nitrogen sources, a linear correlation was observed between $R_{\max}$ and $\mu_{\max}$ for these strains.

For all the strains, some specific nitrogen sources did not show the aforementioned correlation between $\mu_{\max}$ and $R_{\max}$. They were mainly poor nitrogen sources like Cys, His and Ade, which supported neither good growth nor high fermentation speed. Special cases were observed for *T. delbrueckii*, with Cys sustaining high $\mu_{\max}$, but very low $R_{\max}$, or Ser showing low $\mu_{\max}$, but high $R_{\max}$.

3.4 Nitrogen sources utilisation efficiency by different strains during alcoholic fermentation.

To explore the sequential utilisation of different nitrogen sources by each yeast strain during synthetic must fermentation, fermentations were carried out with SM140 and SM300, which respectively corresponded to intermediate and high nitrogen availability conditions (Table 2). As the behaviour of two *Metschnikowia fructicola* strains was similar, only one of them (MFa Gaia) was included. To gain a clearer view of the nitrogen

consumption profiles in each strain and for each fermentation condition, the AUC of each individual nitrogen source was calculated, which represents information about the time to start consumption, the maximum consumption rate and the maximum consumption percentage (Figure 5A). Thus the nitrogen sources with higher AUC values could be considered an earlier and more complete consumed compound. Conversely, a low AUC value represented slow and partial nitrogen source consumption. The box plots in Figure 5B-C represent a general distribution of the AUC values of 19 nitrogen sources for the different strains in SM140 and SM300 for 24 h of fermentation. These plots clearly show a quicker and better utilisation efficiency of most nitrogen sources by *S. cerevisiae* and *T. delbrueckii* than by the *Metschnikowia* strains. These AUC values were ranked to represent the nitrogen sources uptake order by each strain (Figure 6A-B). Each species showed a specific order of assimilation of the nitrogen substrates. However, nitrogen concentration barely influenced this order of assimilation. This result illustrated that, without changing the proportion of the different nitrogen sources, increasing the nitrogen concentration did not significantly influence the nitrogen source uptake order. The representation of the AUC values on a heatmap hierarchically clustered the nitrogen sources into four categories (Figure 6D). Firstly, nitrogen sources lysine, leucine, isoleucine, histidine, methionine, glutamate, glutamine, and valine were the most rapidly consumed by all the studied strains. Of these, lysine was the first nitrogen source to be consumed in most cases. Of the early consumed nitrogen sources, differences appeared in the order of uptake in distinct strains. Remarkably, Ile, Gln and Val were more quickly depleted by the *Metschnikowia* strains, whereas His and Glu were poorly or slowly consumed by *T. delbrueckii*. It is noteworthy that all these early consumed amino acids were transported to yeast cells through SPS regulated permeases. The second group of nitrogen sources consumed at a moderate speed was made up of Tyr, NH₄, Arg, Ala, Phe, Thr, Ser, and Asp. Once again, specific differences were observed among the studied strains for this group of amino acids. *T. delbrueckii* consumed Asp and Ser more quickly than the other strains. *S. cerevisiae* and *T. delbrueckii* also showed a higher uptake speed for Phe than the *Metschnikowia* strains. The third group was formed by Gly and Trp, which were the last amino acids to be consumed, despite the fact that the *Metschnikowia* strains consumed Trp earlier than other amino acids. Finally, Pro was clustered alone and represented the nitrogen source that was practically not consumed under any condition (only partial consumption by *T. delbrueckii* in SM140 was detected).

The individual consumption of each nitrogen source is shown in Supplementary Figures 6 and 7. All the nitrogen sources were completely depleted by the *S. cerevisiae* strain within the first 24 h of both fermentations, except for proline, which was not consumed at all within the studied time frame. With the *T. delbrueckii* strain, most nitrogen sources were consumed within the first 48 h during both fermentations, with some exceptions. Trp and Gly were partially consumed in SM300 (60% and 30%, respectively) and Pro was partially consumed (48%) in SM140, but was not consumed at all in SM300 within the studied time frame. For both *Metschnikowia* strains, the depletion of different nitrogen sources was clearly slower. In SM300, only lysine, the two branched-chain amino acids (BCAA) leucine and isoleucine and glutamine were almost fully consumed (more than 90%). *M. pulcherrima* consumed more than 90% of valine, the third BCAA, but only 50% of this amino acid was depleted by *M. fructicola*. This could indicate a specific preference of *M. pulcherrima* for this amino acid compared to *M. fructicola*, but more strains should be studied to confirm this hypothesis. For both *Metschnikowia* strains, glutamate, glutamine, histidine and methionine were moderately utilised, with consumption exceeding 50% in 48 h. Proline and glycine were not consumed within the first 48 h.

3.5 Influence of the initial concentration on the nitrogen source uptake order

To assess the influence of the initial concentration of the different nitrogen sources on the uptake order, fermentations were carried out by the strains of *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* on a synthetic grape must with an equal amount (in YAN terms) of each nitrogen source and a final YAN concentration of 300 mg/L (SM300E; Table 2). Strangely enough, the equal proportion of each nitrogen source in the final YAN substantially changed the general uptake profile compared to SM300. By comparing the distribution of the AUC values (Figure 5C-D), the first point to be underlined is that, unlike the results of the SM300 fermentations, no major differences were observed in the general nitrogen source consumption efficiency for the three strains. The analysis of the distribution of the consumed nitrogen sources showed a delayed uptake of some nitrogen sources by *S. cerevisiae* (Suppl. Figure 8) because several amino acids had only been partially consumed after 24 h, but they had all been completely depleted in SM300 after 20 h (except Pro). Interestingly, these amino acids poorly sustained yeast growth and fermentation (Trp, Gly and His), whereas SM300E seemed to favour the uptake of many

amino acids for the *M. pulcherrima* and *T. delbrueckii* strains, with more amino acids completely depleted after 24 h (Suppl. Figure 8).

The SM300E AUC values were also represented on a heatmap (Figure 6E). To better compare them with SM140 and SM300, the uptake order is shown in Figure 6C. NH_4Cl and Arg were the fastest nitrogen sources to be consumed by the *S. cerevisiae* and *T. delbrueckii* strains in SM300E, while these nitrogen sources were ranked from position 13 to 15 in SM300 (Figure 6B). We should bear in mind that these compounds are the most abundant nitrogen sources in SM300 and their concentrations sharply drop in SM300E. Although both NH_4Cl and Arg were also rapidly depleted by the *M. pulcherrima* strain, Gln and Glu were preferentially consumed by it. Another major difference between SM300E and SM300 was observed for Leu and Ile as their consumption was significantly delayed in the SM300E fermentations by the three strains. Valine was more quickly exhausted by the *M. pulcherrima* strain when nitrogen sources were provided in similar amounts. All these BCAAs were provided in bigger quantities in SM300E. Lys was also one of the quickest amino acids to be consumed in the three strains, but took longer than NH_4Cl and Arg depletion. For proline, all the strains showed the assimilation of this amino acid in SM300E (*T. delbrueckii* even completely depleted it), but it was not assimilated in SM300.

4. Discussion

Mixed culture fermentations of the non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* involve different synergic or antagonistic interactions between species and strains, including competition for nutrients, especially nitrogen sources, which play an significant role in winemaking (Rollero et al., 2018). Consequently, understanding the nitrogen resource management by non-*Saccharomyces* yeasts is essential to modulate the implementation of different species in sequential inoculations. This study provides a comprehensive overview of the nitrogen assimilation of four non-*Saccharomyces* industrial strains by comparing their nitrogen source preferences in terms of not only growth and fermentation performances, but also of their consumption sequence of these nitrogen sources. During cultures with good nitrogen sources, *T. delbrueckii* displayed high growth and fermentation rate, similar to the performance of *S. cerevisiae*. This could be due to the close phylogenetic relationship between those two species (Kurtzman and Robnett, 2003). Both these species displayed a high degree of specialisation in their nitrogen preferences, with wide variability in the characteristic parameters of their

capacity to grow and complete wine fermentation depending on the nitrogen source. Based on their efficiency to support growth, as measured by the generation time, amino acids and ammonium have been previously classified as “preferred”, “intermediate” and “non-preferred” nitrogen sources for lab and wine *S. cerevisiae* strains (Cooper et al., 1982, Godard et al., 2007; Gutiérrez et al., 2013). Interestingly, the growth characteristics of both commercial *S. cerevisiae* and *T. delbrueckii* strains are consistent with this classification, apart from some specific amino acids likely reflecting differences in the genetic background between both species. For example, *T. delbrueckii* exhibited substantial growth in a lysine medium, whereas *S. cerevisiae*, in which the key enzymes involved in the lysine degradation pathways have not yet been identified (Bhattacharjee, 1992; Neshich et al., 2012), was unable to grow when using this amino acid as the sole nitrogen source. On the contrary, glutamine, glutamate and aspartate, which are key elements of the central core of nitrogen metabolism, supported *S. cerevisiae* growth more efficiently than that of *T. delbrueckii*.

Previous studies (Prior et al, 2019; Barbosa et al, 2018) have revealed the poor fermentation activity of the *Metschnikowia* strains in synthetic and natural grape musts. In the present study, the phenotypic traits of the *Metschnikowia* strains also stood out from those of the other two species as they were unable to complete wine fermentation, regardless of the nature of the nitrogen source, and they generally exhibited comparatively low growth and fermentation rates. Compared to the *T. delbrueckii* and *S. cerevisiae* species, the characteristics of the *Metschnikowia* strains were less influenced by the nature of nitrogen sources, even though these compounds had been differentiated between preferred and non-preferred nitrogen sources. Consequently, their fitness during growth and fermentation on poor nitrogen sources, including lysine, histidine and threonine, was better than that of *S. cerevisiae*. Notably, *Metschnikowia* strains are able to use proline more efficiently than *S. cerevisiae*. The presence of dissolved oxygen during microplate cultivations may favour proline consumption. However, the growth conditions were the same for both non-*Saccharomyces* and *S. cerevisiae*. Thus, our results indicate that proline might be differently metabolised by these species. This observation also indicates that the nitrogen quality plays a more relevant role in controlling the growth of the *S. cerevisiae* and *T. delbrueckii* yeasts than that of the *Metschnikowia* strains. Finally, the *Metschnikowia* strains were characterised by a shorter lag phase compared to the *T. delbrueckii* and *S. cerevisiae* species for most nitrogen

sources. This shorter lag phase, a fermentation trait during the wine fermentation process to which very little attention has been paid (Novo et al., 2003), confers the *M. pulcherrima* and *M. fructicola* species a great competitive advantage for establishing in grape juice and starting fermentation, despite the rise in ethanol and oxygen depletion favouring the progressive imposition of *S. cerevisiae*. Our previous observations indicated that, in mixed culture fermentations, increasing the abundance of some non-preferred nitrogen sources could favour the competitiveness of *Metschnikowia* strains. These considerations may be particularly relevant for winemaking as the metabolic activities of the non-*Saccharomyces* species, including *M. pulcherrima* and *M. fructicola*, have been reported to impart a positive impact on the sensory quality of wines (Ruiz et al., 2018).

The literature reported that good growth fitness is not necessarily associated with the good fermentation performance of a particular yeast (Crépin et al., 2012; Gutiérrez et al., 2013; Kemsawasd et al., 2015; Prior et al., 2019; Su et al., 2019). In our study, very good correlations between these two parameters were observed for the *Metschnikowia* strains in most individual nitrogen sources. However, this correlation only remained for the nitrogen sources with $\mu_{\max}$ values below 0.1 h^{-1} in the case of the *S. cerevisiae* and *T. delbrueckii* strains. In these strains, the most preferred nitrogen sources clearly improved the growth rate, but this growth did not impact the fermentation rate. When fermentations were carried out in good nitrogen sources, YAN concentration may become the main limiting factor for the yeast carrying capacity, which in turn limited the maximum fermentation rate.

The four commercial wine species consumed the available nitrogen in synthetic must with an assimilation sequence that was generally conserved between strains. The differentiation between the early (including Leu, Met, Ile, Thr, Glu, His) and late (ammonium, Ala, Arg and Tyr) consumed amino acids, reported for a collection of 14 *S. cerevisiae* strains (Crépin et al., 2012), *P. kluyveri*, *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* (Prior et al., 2019), also applied to all the yeasts that were herein characterised. In *S. cerevisiae*, this nitrogen source ranking is explained mainly by two molecular mechanisms: the Ssy1p-Ptr3p-Ssy5 (SPS) system, which regulates the permeases involved in transporting early assimilated amino acids, and the nitrogen catabolite repression (NCR) system, which controls the uptake of late consumed nitrogen compounds (Beltran et al., 2004; Godard et al., 2007; Ljungdahl, 2009; Crépin et al.,

2012). However, some exceptions to this general pattern were evidenced in the non-*Saccharomyces* strains, which are worth pointing out in the winemaking context as they could result in the limited availability of a specific nitrogen compound to support *S. cerevisiae* growth in mixed or sequential fermentations. In particular, aspartate and serine were consumed earlier by *T. delbrueckii* than other strains, while isoleucine, valine, glutamine and tryptophan were exhausted more quickly during fermentation with the *Metschnikowia* species. Given the similarity of the nitrogen source uptake profiles between the *S. cerevisiae* and non-*Saccharomyces* strains, we hypothesised that similar regulatory mechanisms like NCR and SPS could also be present in the *T. delbrueckii* and *Metschnikowia* strains. Lleixà et al. (2018) recently demonstrated that the NCR mechanism also operated in *Hanseniaspora vineae*. Furthermore, changes in the concentration of nitrogen sources also affected the consumption sequence of N-containing molecules differentially between the *S. cerevisiae* and non-*Saccharomyces* strains. Compared to the concentrations usually found in natural grape must (used in SM, Bely et al., 1990), when all the nitrogen sources were supplied at the same concentration, *S. cerevisiae* slowed down the uptake of many nitrogen sources, whereas the non-*Saccharomyces* strains more efficiently took up these compounds, with the consequent reduced fitness advantage of *S. cerevisiae* over other species. This observation likely reflects the adaptation of *S. cerevisiae* wine strains to grape must environments in order to efficiently use a scarce nitrogen resource. It also indicates that the competitiveness of the different species during co- or sequential fermentations can be modulated by modifying nitrogen source compositions and concentrations. The uptake of arginine and ammonium was most significantly affected by their concentration in SM. The delayed consumption of these nitrogen sources under wine fermentation conditions can be explained by their high concentration in grape juice, combined with a repressed uptake by NCR. Nonetheless, the uptake of some nitrogen sources was not affected by their availability in the medium, like lysine, which was early consumed in both media, or tryptophan and proline, which were exhausted later regardless of the nitrogen resource composition. With lysine, this behaviour could be related to the kinetic properties of major lysine transporter Lyp1p, which allows the fast and efficient uptake of this compound (Bianchi et al., 2016). In a population of *S. cerevisiae* wine strains, a positive correlation has been reported between the growth efficiency and uptake rate of nitrogen provided as a mixture of ammonium and amino acids (Gutiérrez et al., 2012). This can be extended to the species employed herein with the strains displaying the least growth

capacity (*Metschnikowia* strains) with the lowest nitrogen consumption rate, and *vice versa*. However, no correlation was found between the uptake order of the different nitrogen sources and their capacity to support growth and fermentation activity as some of the most rapidly consumed nitrogen sources poorly sustained yeast growth. Conversely, some late consumed sources supported good growth and fermentation capacity. The most paradigmatic example is the early consumption of lysine, although this amino acid poorly supports the growth of the non-*Saccharomyces* strains or is unable to sustain *S. cerevisiae* growth. Conversely, alanine was consumed late, but is considered a preferred nitrogen source because it efficiently sustains the growth and fermentation of wine yeast species. The differences between these two classifications can be explained by the fact that the sequence of assimilation of nitrogen sources is controlled by the regulation of the permeases involved in transporting these molecules, while different mechanisms underlie the efficiency of nutrients to sustain growth. Hence the carbon derivatives resulting from the catabolism of most preferred nitrogen sources produces C-compounds that are directly assimilable by the central carbon metabolism, while the transamination of less efficient sources leads to the formation of keto-acids, which are converted into higher alcohols (Godard et al., 2007; Gutiérrez et al., 2013).

5. Conclusions

Nitrogen source preference and the metabolism of wine-related non-*Saccharomyces* species are very important for the wine industry. This work thoroughly studied the nitrogen preferences and its utilisation profile of a *T. delbrueckii* and three *Metschnikowia* commercial strains. Regardless of the nitrogen source, clear differences were observed between the *T. delbrueckii* and *Metschnikowia* strains. The *T. delbrueckii* strain showed a similar growth character and fermentation performance to *S. cerevisiae*, whereas the *Metschnikowia* strains exhibited a much lower growth rate and less fermentation activity. However, the *Metschnikowia* strains were able to grow much better than *S. cerevisiae* in the so-called poor nitrogen sources. This result, together with a shorter lag phase than *S. cerevisiae*, can confer these species a great competitive advantage for starting fermentation. In addition, the initial proportion of nitrogen sources in the medium has important influence on their assimilation order, especially for arginine and ammonium, and consequently on the fitness of the species. The non-*Saccharomyces* strains had a similar nitrogen source uptake profile with *S. cerevisiae*, which indicates that similar nitrogen regulation systems may also exist in the non-*Saccharomyces* species

during alcoholic fermentation. Overall, these results point out that an appropriate management of the nitrogen nutrition during mixed fermentation is required for an optimal use of the non-*Saccharomyces* species during winemaking.

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Table 1. Concentration of each individual nitrogen sources in synthetic must

	Assimilable nitrogen	Molecular mass (g/mol)	Concentration (g/L) for 200 mg/L of YAN	Concentration (g/L) for 140 mg/L of YAN
Ala	1	89.1	1.273	0.891
NH ₄ Cl	1	53.5	0.764	0.535
Arg	3	174.2	0.830	0.581
Asp	1	133.1	1.901	1.331
Cys	1	121.2	1.731	1.212
Glu	1	147.1	2.102	1.471
Gln	2	146.1	1.044	0.731
Gly	1	75.1	1.072	0.751
His	1	155.2	2.217	1.552
Ile	1	131.2	1.874	1.312
Leu	1	131.2	1.874	1.312
Lys	2	146.2	1.044	0.731
Met	1	149.2	2.132	1.492
Phe	1	165.2	2.360	1.652
Pro	1	115.1	1.645	1.151
Ser	1	105.1	1.501	1.051
Thr	1	119.1	1.702	1.191
Trp	1	204.2	2.918	2.042
Tyr	1	181.2	2.588	1.812
Val	1	117.2	1.674	1.172
Ade	1	135.1	1.930	1.351
Cyt	1	111.1	1.587	1.111
GABA	1	103.1	1.473	1.031
Orn	2	168.6	1.204	0.843
Asn	2	132.1	0.944	0.661
Cit	2	175.2	1.251	0.876
Urea	2	60.1	0.429	0.300

Table 2. Concentration of each individual nitrogen source in synthetic must MS140, MS300 and MS300E.

	MS140		MS300		MS300E	
	Concentration (g/L)	YAN (g/L)	Concentration (g/L)	YAN (g/L)	Concentration (g/L)	YAN (g/L)
Ala	0.048	0.008	0.103	0.016	0.095	0.015
Arg	0.123	0.030	0.264	0.064	0.062	0.015
Asp	0.015	0.002	0.031	0.003	0.143	0.015
Cys	0.004	0.000	0.009	0.001	0.130	0.015
Glu	0.040	0.004	0.085	0.008	0.158	0.015
Gln	0.166	0.032	0.357	0.068	0.078	0.015
Gly	0.006	0.001	0.013	0.002	0.080	0.015
His	0.011	0.001	0.023	0.002	0.166	0.015
Ile	0.011	0.001	0.023	0.002	0.141	0.015
Leu	0.016	0.002	0.034	0.004	0.141	0.015
Lys	0.006	0.001	0.012	0.002	0.078	0.015
Met	0.010	0.001	0.022	0.002	0.160	0.015
Phe	0.013	0.001	0.027	0.002	0.177	0.015
Pro	0.202	0.000	0.432	0.000	0.123	0.000
Ser	0.026	0.003	0.055	0.007	0.113	0.015
Thr	0.025	0.003	0.054	0.006	0.128	0.015
Trp	0.059	0.008	0.127	0.017	0.109	0.015
Tyr	0.006	0.000	0.013	0.001	0.194	0.015
Val	0.015	0.002	0.031	0.004	0.126	0.015
NH ₄ Cl	0.151	0.040	0.324	0.085	0.057	0.015

708 **Table 3.** Classification of nitrogen sources depending on their ability to sustain
 709 fermentation.

	<i>T. delbrueckii</i>		<i>Metschnikowia sp.</i>		<i>S. cerevisiae</i>	
++	Ala	Urea	Ala	NH ₄	Ala	Met
	Arg	Leu	Arg	Ser	Arg	NH ₄
	Asn	Met	Asn	Urea	Asn	Phe
	Asp	NH ₄	Asp		Asp	Ser
	Cit	Orn	GABA		GABA	Tyr
	GABA	Phe	Gln		Gln	Urea
	Gln		Glu		Glu	Val
	Glu		Leu		Leu	
+	Cyt	Ile	Ade	Orn	Cyt	Trp
	Gly	Pro	Cit	Pro	Gly	
	Lys		Gly	Thr	Pro	
	Ser		Lys	Val	Thr	
	Thr		Phe	Trp	Ade	
	Trp		Tyr		Cit	
	Tyr		Ile		Ile	
	Val		Met		Orn	
-	Ade		Cys		Cys	
	Cys		Cyt		His	
	His		His		Lys	

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Figure legend

Figure 1. (A) Growth parameters of the different strains grown in synthetic must with only one nitrogen source. Each row on the heatmap represents the sole nitrogen source used for growth. Three growth parameters, lag phase time, maximum growth rate ($\mu_{\max}$) and maximum OD ($OD_{\max}$), and two nitrogen concentrations, 140 mg/L and 200 mg/L, are compared on the heatmap. For each growth parameter, data were normalised by the mean value of all the nitrogen sources within the same strain and at the same nitrogen concentration. Hierarchical clustering was done based on Euclidean distance. The colour scheme from blue to yellow represents the normalised value from low to high. Clustering generally separates the nitrogen sources into three clades, which represent good nitrogen sources, intermediate nitrogen sources and poor nitrogen sources. **(B)** Maximum growth rate and **(C)** lag phase of the different strains when grown on synthetic must with 200 mg/L of the sole nitrogen sources. Box plots demonstrate the distribution of all the nitrogen sources for each strain. Dot plots show the value of each sole nitrogen source by specific strains. SC: *S. cerevisiae*; TD: *T. delbrueckii*; MP: *M. pulcherrima*; MFa: *M. fructicola* (Gaia); MFb: *M. fructicola* (LYCC7706).

Figure 2. Clustering of the fermentation profiles obtained on 27 media containing only one N source with the five studied strains. The means of the fermentation parameters were calculated for each cluster. **R_{max}**: Maximum fermentation rate (g CO₂/L/h). **T_{lag}**: lag phase time (h) **FC**: fermentation completion. **H**: high, **M**: medium, **L**: low

Figure 3. Principal component analysis of the fermentation parameters obtained with the five strains in the 27 media. **R_{max}**: Maximum fermentation rate; **T_{lag}**: lag phase time; **PtR_{max}**: time to reach R_{max}. Labels indicate the strain and the nitrogen source available in the medium. Points are coloured depending on the strain; SC: purple, TD: orange, MP: green, MFa: red and MFb: blue. Labels of overlapping points are shifted over or under the points following the dashed lines. The conditions form three groups, highlighted by the light green, yellow and dark blue confidence ellipses (confidence level of 0.95).

Figure 4. Pearson's correlation analysis between the maximum growth rate ($\mu_{\max}$) and the maximum fermentation rate ($R_{\max}$) for each strain. Linear regression (black line) is displayed. The squared Pearson correlation coefficient (R^2) and the p -value (P) are provided in this figure.

Figure 5. (A) AUC provides the summary information of the lag phase, consumption rate and maximum consumption percentage. AUC values of the different strains with (B) SM140, (C) SM300 and (D) SM300E.

Figure 6. Ranking of the nitrogen source uptake order of (A) SM140, (B) SM300 and (C) SM300E. (D) Heatmap of the AUC values of SM140 and SM300. (E) Heatmap of the AUC values of SM300E. The nitrogen sources with a low ranking number indicate that they were quickly consumed during alcoholic fermentation; Conversely, the nitrogen sources with a high ranking number indicate that they were slowly consumed or were not assimilated within the first 48 h of fermentation. The colour key from green to yellow represents the AUC value from low to high. Data were normalised by the mean value of each strain. Hierarchical clustering was done by Euclidean distance metrics.

Supplementary Figure 1. Comparison of the lag phase times for each strain grown in the different nitrogen sources at two concentrations: 140 mg/L nitrogen (blue bars) and 200 mg/L nitrogen (orange bars).

Supplementary Figure 2. Comparison of the maximum growth rates ($\mu_{\max}$) for each strain grown in the different nitrogen sources at two concentrations: 140 mg/L nitrogen (blue bars) and 200 mg/L nitrogen (orange bars).

Supplementary Figure 3. Comparison of the maximum OD reached for each strain grown in the different nitrogen sources at two concentrations: 140 mg/L nitrogen (blue bars) and 200 mg/L nitrogen (orange bars).

Supplementary Figure 4. Comparison of the lag fermentation phase duration and maximal fermentation rate for *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* between 140 and 200 mg/L YAN.

Supplementary Figure 5. Percentage of nitrogen source uptake for 48 h of fermentation in SM140.

Supplementary Figure 6. Percentage of nitrogen source uptake for 48 h of fermentation in SM300.

Supplementary Figure 7. Percentage of nitrogen source uptake for 24 h of fermentation in SM300E.

SC: Ala, Arg, Asn, Asp, GABA,
Gln, Glu, Met, NH₄, Phe, Ser, Tyr, Ure
TD: Ala, Arg, Asn, Asp, Cit, GABA, Gln,
Glu, Leu, Met, NH₄, Orn, Phe, Ure

MP: Ade, Ala, Arg, Asn, Asp, Cit, GABA, Gln, Glu, Gly, Iso,
Leu, Lys, Met, NH₄, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, Ure, Val
MFa: Ade, Ala, Arg, Asn, Asp, Cit, GABA, Gln, Glu, Gly, Iso,
Leu, Lys, Met, NH₄, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, Ure, Val
MFb: Ade, Ala, Arg, Asn, Asp, Cit, GABA, Gln, Glu, Gly, Iso,
Leu, Lys, Met, NH₄, Orn, Phe, Pro, Ser, Thr, Trp, Ure, Val

SC 200mg/L YAN

TD 200mg/L YAN

MP 200mg/L YAN

MFa 200mg/L YAN

MFb 200mg/L YAN

19	19	19	19
18	17	14	14
17	18	17	16
16	12	11	12
15	13	12	13
14	11	15	17
13	14	18	18
12	15	7	10
11	9	4	3
10	5	10	9
9	10	16	15
8	3	13	11
7	6	9	8
6	7	2	2
5	2	8	7
4	8	6	6
3	1	1	1
2	16	5	5
1	4	3	4

19	19	17	19
18	17	11	12
17	18	19	17
15	10	16	15
13	14	12	13
14	15	14	16
16	12	18	18
12	13	5	8
11	9	4	4
9	7	10	10
7	8	13	14
10	3	15	11
5	5	9	9
4	6	2	2
3	2	7	6
6	11	6	5
1	1	1	1
8	16	8	7
2	4	3	3

19	19	15
17	15	17
16	14	14
15	12	12
2	2	4
1	1	3
14	17	19
13	16	13
11	13	1
9	9	6
8	11	18
6	5	10
7	6	7
10	10	9
5	4	8
4	8	2
3	3	5
18	18	16
12	7	11

SC lag phase

SC SM140

TD SM140

MP SM140

MFa SM140

SC SM300

TD SM300

MP SM300

MFa SM300

Ala Arg Asp Glu Gln Gly His Ile Leu Lys
Met NH4Cl Phe Pro Ser Thr Trp Tyr Val