OXFORD SIMB Society for Industrial Microbiology and Biotechnology

Journal of Industrial Microbiology and Biotechnology, 2022, 49, kuac025
https://doi.org/10.1093/jimb/kuac025
Advance access publication date: 12 November 2022
Food Biotechnology & Probiotics – Original Paper

The growth and metabolome of *Saccharomyces uvarum* in wine fermentations are strongly influenced by the route of nitrogen assimilation

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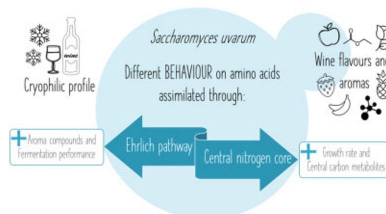
Abstract: Nitrogen is a critical nutrient in beverage fermentations, influencing fermentation performance and formation of compounds that affect organoleptic properties of the product. Traditionally, most commercial wine fermentations rely on *Saccharomyces cerevisiae* but the potential of alternative yeasts is increasingly recognised because of the possibility to deliver innovative products and process improvements. In this regard, *Saccharomyces uvarum* is an attractive non-traditional yeast that, while quite closely related to *S. cerevisiae*, displays a different fermentative and aromatic profile. Although *S. uvarum* is used in cider-making and in some winemaking, better knowledge of its physiology and metabolism is required if its full potential is to be realised. To address this gap, we performed a comparative analysis of the response of *S. uvarum* and *S. cerevisiae* to 13 different sources of nitrogen, assessing key parameters such as growth, fermentation performance, the production of central carbon metabolites and aroma volatile compounds. We observed that the two species differ in the production of acetate, succinate, medium-chain fatty acids, phenylethanol, phenylethyl acetate, and fusel/branched acids in ways that reflect different distribution of fluxes in the metabolic network. The integrated analysis revealed different patterns of yeast performance and activity linked to whether growth was on amino acids metabolised via the Ehrlich pathway or on amino acids and compounds assimilated through the central nitrogen core. This study highlights differences between the two yeasts and the importance that nitrogen metabolism can play in modulating the sensory profile of wine when using *S. uvarum* as the fermentative yeast.

Keywords: *Saccharomyces uvarum*, Metabolome, Aroma compounds, Wine fermentation, Ehrlich pathway

Graphical abstract



Depending on the pathway responsible for their catabolism, amino acids either promote growth or aroma production during fermentation by Saccharomyces uvarum.

Introduction

Nitrogen is one of the most important nutrients affecting wine fermentation. The composition and amount of nitrogen depend on the grape variety, viticultural management practices, soil, climate, and degree of ripeness (Bell and Henschke, 2005). This nutrient is present in grape juice as a complex mixture of nitrogen-containing compounds, only some of which—amino acids, ammonium, and small peptides—are available to yeast. The total amount of nitrogen that can be accessed by yeast for growth is referred to as 'Yeast Assimilable Nitrogen' (YAN) and its content in wine musts varies between 60 and 500 mg/L (Bely et al., 1990a). Two critical roles for YAN are described in *S. cerevisiae*, the quintessential yeast for producing alcoholic beverages. First, the nitrogen content of grape juice has been reported as the main factor limiting yeast growth during wine fermentation (Varela et al., 2004), with a direct impact on yeast fermentative activity and consequently on the fermentation kinetics (Bely et al., 1990b). It is generally accepted that YAN concentration below 150 mg/L affects yeast metabolism and causes stuck or sluggish fermentations (Bell and Henschke, 2005; Blateyron and Sablayrolles, 2001), which are major issues in the wine industry. The presence of residual sugars associated with stuck fermentation is not desirable in many wine styles and becomes a risk for

Received: June 10, 2022. **Accepted:** November 1, 2022.
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development of spoilage microorganisms. To face this problem, nitrogen additions in the form of di-ammonium phosphate are commonly used in wine making. Second, studies assessing the influence of the quantity and quality of the nitrogen source on yeast metabolism revealed an effect on both the formation of central carbon metabolites, including glycerol (Albers et al., 1996), and on the production of volatile compounds, which together affect mouthfeel, flavour and aroma (Gobert et al., 2019). In particular, it was reported that levels of YAN have a great impact on the formation of higher alcohols and branched acids (Rollero et al., 2015, 2017). To a certain extent, this is because the branched and aromatic amino acids, which are catabolised through the Ehrlich pathway (Hazelwood et al., 2008), are precursors of volatile compounds in *S. cerevisiae* (Fairbairn et al., 2017). In an indication of complexity, however, amino acids are not major contributors to the production of aroma compounds during wine fermentation because of their low content in grape juice with respect to the production of volatile compounds, as shown by a quantitative analysis of nitrogen metabolism in *S. cerevisiae* (Crépin et al., 2017; Rollero et al., 2017).

When considering the production of volatile compounds, it is important to recognise that nitrogen catabolic and biosynthetic pathways function in parallel, and the balance between these is coordinated by regulatory processes to generate an appropriate response to nitrogen availability (Ljungdahl and Daignan-Fornier, 2012). The α-ketoacids originated from the Ehrlich pathway and the central carbon metabolism (CCM) are significant as they are precursors of both amino acids and aromatic/branched higher alcohols and acids. They play a key role in the nitrogen network behaving as metabolic nodes, around which the flux distribution is regulated depending on anabolic requirements and nitrogen availability. Furthermore, higher alcohols and branched acids are precursors for the synthesis of esters, compounds with a positive impact on the sensory quality of wine (fruity or floral notes). The formation of acetate esters result from the esterification of higher alcohols with acetyl-CoA by the acetyltransferases Atf1 and Atf2 (Lilly et al., 2006; Verstrepen et al., 2003), and branched ethyl esters from branched acids with ethanol by Eeb1 and Eht1 (Saerens et al., 2006). Other volatile compounds that contribute to wine aroma are the medium-chain fatty acids (MCFA) ethyl esters that result from the condensation of acyl-CoA and ethanol (Saerens et al., 2006) and possibly through a mono-acyl glycerol lipase (Marullo et al., 2021). These substances are related to lipid and acetyl-CoA metabolism, therefore, the fatty acids content of grape juice impacts the production of ethyl esters (Liu et al., 2019). In addition, however, the nitrogen source has a slight impact on their production (Barbosa et al., 2009; Seguinot et al., 2018).

The nitrogen sources that compose YAN are transported and assimilated by a wide range of permeases and catabolic pathways, supporting yeast activity in different manners (Bianchi et al., 2019). Several different classifications that refer to distinct physiological mechanisms have been proposed to assess the efficiency of these nutrients to support yeast activity. First, nitrogen preference based on the efficiency to support growth has been reported in *S. cerevisiae* where the amino acids were classified as not efficient (Lys, His, and Gly), medium efficient (Val, Phe, Leu, Ile, Met, Tyr, Thr, Trp, and Pro) and highly efficient (Asn, Asp, Gln, Glu, Ser, Ala, Arg, and NH_4) to sustain growth (Fairbairn et al., 2017; Watson, 1976). This classification is related to the activity through amino acid degradation pathways fulfilling anabolic requirements. Second, the efficiency to maintain high metabolic activity during the stationary phase of fermentation has been considered to determine the yeast nitrogen preference. This concept relies on the amount of nitrogen to be added during the stationary phase of wine fermentation to maintain a high metabolic activity, protein turnover and cellular maintenance (Manginot et al., 1998). Finally, the order of consumption of nitrogen sources provided as a mixture of ammonium and amino acids has been studied in a chemically defined medium mimicking the wine context (Crépin et al., 2012). The differentiation between first consumed (Lys), early consumed (Asp, Thr, Glu, Leu, His, Met, Ile, Ser, Gln, and Phe), and late consumed (NH_4, Val, Arg, Ala, Trp, and Tyr) compounds is mainly associated to the regulation of permeases activity through NCR and SPS-sensor systems and the kinetic characteristics of transporters (Hofman-Bang, 1999; Ljungdahl, 2009).

In recent years, particular attention has been paid to so-called non-conventional or non-traditional yeasts as they offer outstanding alternatives for the innovation of fermented products and the improvement of processes (Drumonde-Neves et al., 2021; Fleet, 2008; Jolly et al., 2022; Pretorius, 2020). *Saccharomyces uvarum,* a close relative of *S. cerevisiae,* is one of the non-traditional yeasts associated with low ethanol production and strong aromatic intensity because of the high production of acetate esters (Stribny et al., 2016). *S. uvarum* is also a cryotolerant yeast meaning that it displays a good fermentation performance at low temperatures (12°C20°C) (Almeida et al., 2014; Zhang et al., 2015). These interesting traits make this species very attractive for cider (González Flores et al., 2017) and wine production. In fact, some strains have already been commercialized and hybrids have been generated for winemaking and the brewing industry (Gamero et al., 2013; Gibson et al., 2017). To date, information on *S. uvarum* metabolism has mainly focused on CCM, showing a high capacity of this species to produce glycerol and succinate, as well as an interesting system of acetate production-consumption to maintain the cryotolerant metabolism (Henriques et al., 2021; Minebois et al., 2020a). The characteristics of nitrogen metabolism and its relationship with the formation of volatile compounds are not well-documented in this species, with only one study reporting a moderate impact of nitrogen availability on fermentative capacities (Su et al., 2019). Nevertheless, such knowledge is essential for the managed use of *S. uvarum* as a credible alternative to *S. cerevisiae* for the modulation of the aroma profile of wine.

This study provides a comprehensive picture of the impact of nitrogen sources on *S. uvarum* behaviour during fermentation and explores the metabolic specificities of this yeast. The nitrogen preference of *S. uvarum* in terms of efficiency to support growth under oenological conditions was first determined. Then, the impact of the nitrogen source on fermentation performance, production of central carbon metabolites and volatile compounds during wine fermentation was characterised. The integrated analysis of the data set provides new insights on the relationships between the catabolism of nitrogen substrates and the metabolome in *S. uvarum* and provides some explanations for the distinctive phenotypic traits of this species. This research offers crucial fundamental knowledge that will facilitate informed use of *S. uvarum* to modulate the sensory profile of wine.

Material and methods

Strains and pre-cultures

The strains used in this study are listed in Table 1. The strains were reactivated in YPD broth (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L). The pre-cultures were grown in YPD at 28°C for 16 h, then centrifuged 5 min at 4500 r/min. The pellet was suspended in Yeast Nitrogen Base (YNB) media (glucose 20 g/L) without ammonium and amino acids for 4 h at 28°C to exhaust the nitrogen

Table 1. Strains used in this study.

Strain	Environment	Geographical origin	Reference
S. uvarum MTF3098/BMV58	Wine	Valencia, Spain	Velluto BMV58™ (Gamero et al., 2013)
S. uvarum CBS395/CLIB251	Fruits: Juice of *Ribes nigrum*	The Netherlands	Pulvirenti et al., 2000
S. uvarum OS24/CBS7001/MCYC623	Insect Mesophylax adopersus	Avila, Spain	Kellis et al., 2003
S. uvarum OS472/A4	End of wine fermentation Sauvignon Blanc	Marlborough, New Zealand	Zhang et al., 2015
S. cerevisiae EC1118	Wine	Champagne, France	Novo et al., 2009
S. cerevisiae CEN.PK113-7D	Laboratory	-	Nijkamp et al., 2012
S. cerevisiae S288c	Laboratory	-	Mortimer and Johnston, 1986

Table 2. Nitrogen conditions tested in micro-fermentations and comparative synthetic wine must (MS) fermentations. The amount of YAN was 200 mg/L in synthetic must (MS) and 1059.48 mg/L in minimal media (MM); * g/L solubility limit of amino acid; † mix of amino acids as described by Bely et al., (1990a).

			Concentration of nitrogen compound	
Nitrogen condition	Nomenclature	Number of assimilated nitrogen atoms per molecule	Micro-fermentations in MM (g/L)	Fermentations in MS (g/L)
Ammonium sulfate	NH_4	2	5	0.94
Methionine	Met	1	11.29	2.13
Phenylalanine	Phe	1	12.5	2.36
Asparagine	Asn	2	5	0.94
Aspartate	Asp	1	4.5 *	1.90
Valine	Val	1	8.87	1.67
Isoleucine	Ile	1	9.93	1.87
Leucine	Leu	1	9.93	1.87
Glutamine	Gln	2	5.53	1.04
Glutamate	Glu	1	7.5 *	2.10
MS 200 mg/L YAN	MS200	N/A	N/A	Ammonium chloride 0.216, mix of amino acids 6.16 mL/L †

reserves. The cells were washed with 0.9% NaCl saline solution to remove all nitrogen residues and then resuspended in the test media without the nitrogen source. The cell suspension was further used to inoculate fermentations.

Micro-fermentations in minimal media

The micro-fermentations were done in microplates with seven strains (Table 1). The minimal media (MM) components were glucose 20 g/L, KH_2PO_4 3 g/L, $MgSO_4.7H_2O$ 0.5 g/L, vitamin mix, and trace elements adapted from Verduyn media (Verduyn et al., 1992). YAN was provided in excess, at the same concentration (1059 mg/L) but in 10 different forms, ammonium or amino acids as sole nitrogen sources (Table 2). For the microplate fermentations, 20 μL of pre-culture suspension at OD_{600} of ~0.5 was added to 180 μL of fresh medium (~0.05 A_{600}) in each well. This experiment was done with three biological replicates of each condition using one blank per medium. The 96 flat wells microplate was incubated in the microplate reader CLARIOStar®^{Plus} (BMG LABTECH, Germany) at 20°C. The OD_{600} was measured for 60 cycles of 1 hour (24 flashes/cycle) with continual double orbital shaking (500 r/min) between measurements.

Alcoholic fermentations in grape must

Fermentations in synthetic and natural grape must were conducted with *S. uvarum* MTF3098 and *S. cerevisiae* EC1118. Synthetic grape must (MS) was used to mimic the composition of grape juice allowing the variation of the nitrogen source. Two natural musts, which have a mixture of nitrogen sources, were also used. The MS was prepared following the composition of Bely et al., (1990a) with some adjustments. The base MS contained glucose 100 g/L, fructose 100 g/L, malic acid 6 g/L, citric acid 6 g/L, KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.25 g/L, $CaCl_2.2H_2O$ 0.155 g/L, NaCl 0.2 g/L, phytosterols 5 mg/L and trace elements and vitamins. YAN was provided at 200 mg/L as 11 different forms listed in Table 2. The pH of the MS was set to 3.3 with NaOH. The two natural white grape musts were Chardonnay (pH 3.78, 210 g/L sugar, 200 mg/L YAN) and Maccabeu (pH 3.5, 235 g/L sugar, 104 mg/L YAN) provided by the experimental unit INRAE Pech Rouge. The MS and natural musts were pasteurised for 20 min and then oxygenated by bubbling air for 30 min before inoculation.

Batch fermentations were performed in 330 mL fermenters containing 250 mL of medium, equipped with fermentation locks to avoid the entry of oxygen and allow CO_2 release. The initial population in each fermenter after inoculation was 5×10^5 cells/mL (corresponding to ~0.04 A_{600} for *S. uvarum* and ~0.05 A_{600} for *S. cerevisiae*). The fermentations were carried out in biological triplicates at 20°C with continuous magnetic stirring (230 r/min) with an automated robotic system (PhenOFerm LabServices, Breda, Netherlands) capable of moving the fermenter from its location on the stirring plate to a precision balance (Duc et al., 2020). The fermentation progress was monitored by CO_2 release (g/L), weighing the fermenters every hour. A custom-developed Labview

application automatically calculated the amount of CO_2 released from the weight loss and the CO_2 production rate (g/L/h), which were determined based on polynomial smoothing (Sablayrolles et al., 1987). Samples for metabolome analysis were collected at 60 g/L of CO_2 produced and at the end of fermentation (CO_2 production rate lower than 0.05 g/L/h) by centrifuging 25 mL of medium (3500 g, 5 min, 4°C) to remove cells and storing the supernatant at −20°C.

Analytical methods

The yeast population at different fermentation times was quantified with a Coulter counter (BECKMAN®) and by measuring optical density (A_{600}) with a spectrophotometer. Growth kinetics data was analysed using R Studio software, version 1.3.1093 (RStudio Team, 2020). The area under the curve (AUC) was calculated with GrowthCurver package (Sprouffske and Wagner, 2016) as a measure of growth kinetics comprising the lag phase length, growth rate, final time and maximum biomass produced. The AUC metric has been used in previous studies comparing yeast growth depending on the nitrogen source (Su et al., 2020). This parameter represents the area defined by the x-axis, y-axis, growth curve, and time (48 h) that summarizes information of the lag phase, consumption rate and maximum consumption percentage. The boxplots and growth curves were made using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA).

The methods for measuring metabolites and volatiles during the wine fermentations were done as previously described (Rollero et al., 2015). Metabolites such as glucose, fructose, ethanol, glycerol and organic acids were determined by High-Performance Liquid Chromatography (HPLC) on a Phenomenex Rezex ROA column (HPLC HP1100 Infinity, Agilent Technologies). The samples treatment for HPLC consisted of dilution of 200 μL of sample in H_2SO_4 0 005 N solution, followed by purification with centrifugation in the case of MS and filtration for natural musts. The column elution was done with H_2SO_4 0 005 N at 60°C, with 0.6 mL/min flow rate, and a refractive index detector. From the same samples, the measurement of volatile compounds was performed by gas chromatography/mass spectrometry (GC-MS) with a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu, Columbia, USA), coupled to a HP 5973 mass spectrometry detector (HP, now Agilent Technologies, Santa Clara, CA, USA). The liquid extraction of 5 mL supernatant supplemented with 10 μL of a solution of deutared standards (100 μg/L in ethanol) was done with dichloromethane CH_2Cl_2, where the organic phase was recovered, dried, and concentrated with nitrogen gas before injection. The compounds were separated using a 30 m × 0.25 mm Phenomenex fused silica capillary column ZB-WAX, 0.25 μm film thickness (Agilent Technologies, Santa Clara, California, USA), using helium as carrier gas with a flow rate of 1 mL/min in constant flow mode and a linear velocity of 36 cm/s and with a temperature gradient between 40 and 220°C. The measured aromatic compounds were higher alcohols, esters and fatty acids. Calibration curves were built for each compound, from reference solutions prepared in alcoholic medium (12% ethanol, 6 g/l malic acid, pH3.3) and treated as samples. The metabolome data was graphed using GraphPad Prism v8.0.2.

Statistical analysis

Statistical tests for growth analysis and metabolites production were made using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA). Two-way ANOVA was performed for heatmaps with correction for multiple comparisons using Tukey's test, where a p-value < 0.05 was considered significant. The raw data for both growth and the metabolome at different conditions are available on Zenodo DOI:10.5281/zenodo.6627770.

To get an overview of correlation of the nitrogen source and metabolites produced, a principal component analysis (PCA) was performed for each group of volatile compounds using the R Studio software (Supplementary figure 1).

Results

Nitrogen preference in *S. cerevisiae* and *S. uvarum* species

To evaluate nitrogen preference, we monitored the growth of three *S. cerevisiae* strains and four *S. uvarum* wine strains (Table 1) during micro-fermentations conducted in MM varying the nitrogen source. The area under the curve (AUC) values, which provide an overall view of the efficiency of the nitrogen source to support growth, were compared between species (Fig. 1a). Both species have the same growth capacity in most of the nitrogen sources, namely, good growth on ammonium, aspartate and glutamine, intermediate behaviour on phenylalanine, leucine, valine and glutamate, and poor growth on isoleucine. On the other hand, methionine and asparagine showed different efficiency to support growth between the two species. *S. uvarum* strains grew efficiently in methionine while *S. cerevisiae* strains had poor growth. In contrast, growth in asparagine was low for *S. uvarum* but very high for *S. cerevisiae*. However, the nitrogen preference can also be strain specific (Fig. 1b): for example, *S. cerevisiae* CEN.PK113-7D showed a slower growth than the other *S. cerevisiae* strains in aspartate, while the lowest growth on isoleucine and methionine was observed with strains EC1118 and S288c, respectively. Regarding *S. uvarum,* strain CBS472 showed a different nitrogen preference than other *S. uvarum* strains, most notably a higher growth capacity on isoleucine than on leucine.

Effect of the nitrogen source on fermentation kinetics and profile

To further understand how the nitrogen source affects the fermentation profile in *S. uvarum*, the representative wine strains *S. uvarum* MTF3098 and *S. cerevisiae* EC1118 (used as reference) were grown in two natural musts and eleven synthetic grape juices with different nitrogen sources (Table 2). Sluggish profiles were observed during fermentations with *S. uvarum* but not with *S. cerevisiae* under the same conditions (Fig. 2a). Overall, *S. cerevisiae* displayed higher fermentation performances than *S. uvarum*. This is illustrated with the considerably shorter time required to complete the fermentation for *S. cerevisiae,* where it ranged from 234377 h, compared to *S. uvarum,* where the range was between 401 and 477 h depending on the nitrogen source (Fig. 2b). In line with these observations, the maximum fermentation rate (Rmax) was in general lower in *S. uvarum* (0.51 g/L/h in asparagine to 0.75 g/L/h in leucine) than in *S. cerevisiae* (0.7 g/L/h in phenylalanine and 0.99 g/L/h in glutamine) (Fig. 2c, Supplementary Table 1). The largest differences between the two strains were found in glutamine and asparagine fermentations, with 56 and 45% of variation, respectively. In methionine and isoleucine, *S. uvarum* had lower Rmax and fast decrease of fermentation rate that caused increase of total fermentation time. Surprisingly, *S. cerevisiae* displayed a longer lag phase than *S. uvarum* in most of nitrogen sources, except for asparagine. The lag phase of *S. uvarum* strongly

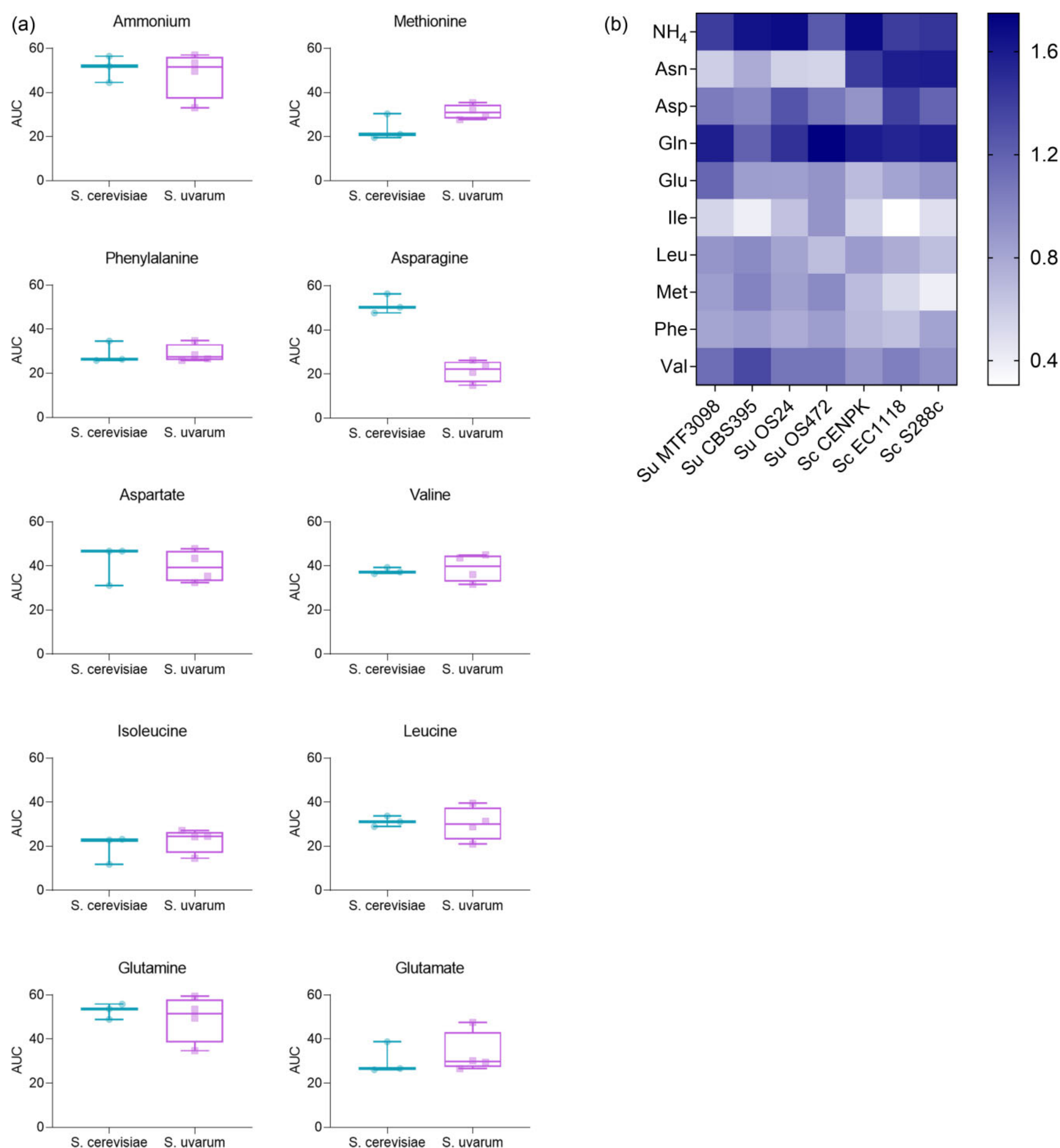

Fig. 1 Growth efficiency in different nitrogen sources. Micro-fermentations were performed in minimal media supplemented with ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln) and glutamate (Glu) as a sole source of nitrogen. Fermentations were done at 20°C for 48 h and growth was determined using the area under the curve (AUC) method. (a) Area under the curve (AUC) of *S. cerevisiae* and *S. uvarum* species in different nitrogen sources. The evaluated strains were *S. cerevisiae* (CEN.PK113-7D, EC1118, S288c) and *S. uvarum* (MTF3098, CBS395, OS24, OS472). (b) Growth of *S. uvarum* (Su) and *S. cerevisiae* (Sc) strains expressed in AUC. Values were normalized with the mean of all strains for each nitrogen source. This allowed the classification of the strains according to their capacity to use a nitrogen compound. The highest AUC represents more efficient growth while low AUC indicate difficulty to assimilate the nitrogen source and sustain growth.

depended on the nitrogen source, being >50 h in Ehrlich pathway amino acids and <50 h in nitrogen compounds assimilated through the central nitrogen metabolism, except for asparagine (80 h).

In addition to growth, fermentation performance was assessed as the capacity to completely deplete sugar, a trait of interest to the wine industry. The two species differed in their capacity to deplete sugars. At the end of fermentation, *S. cerevisiae* exhausted sugars in most of the conditions, except from Maccabeu and phenylalanine. Conversely, *S. uvarum* depleted sugars only when isoleucine or leucine were used as the sole nitrogen source. Low amounts of residual sugars (<5 g/L), mainly in the form of

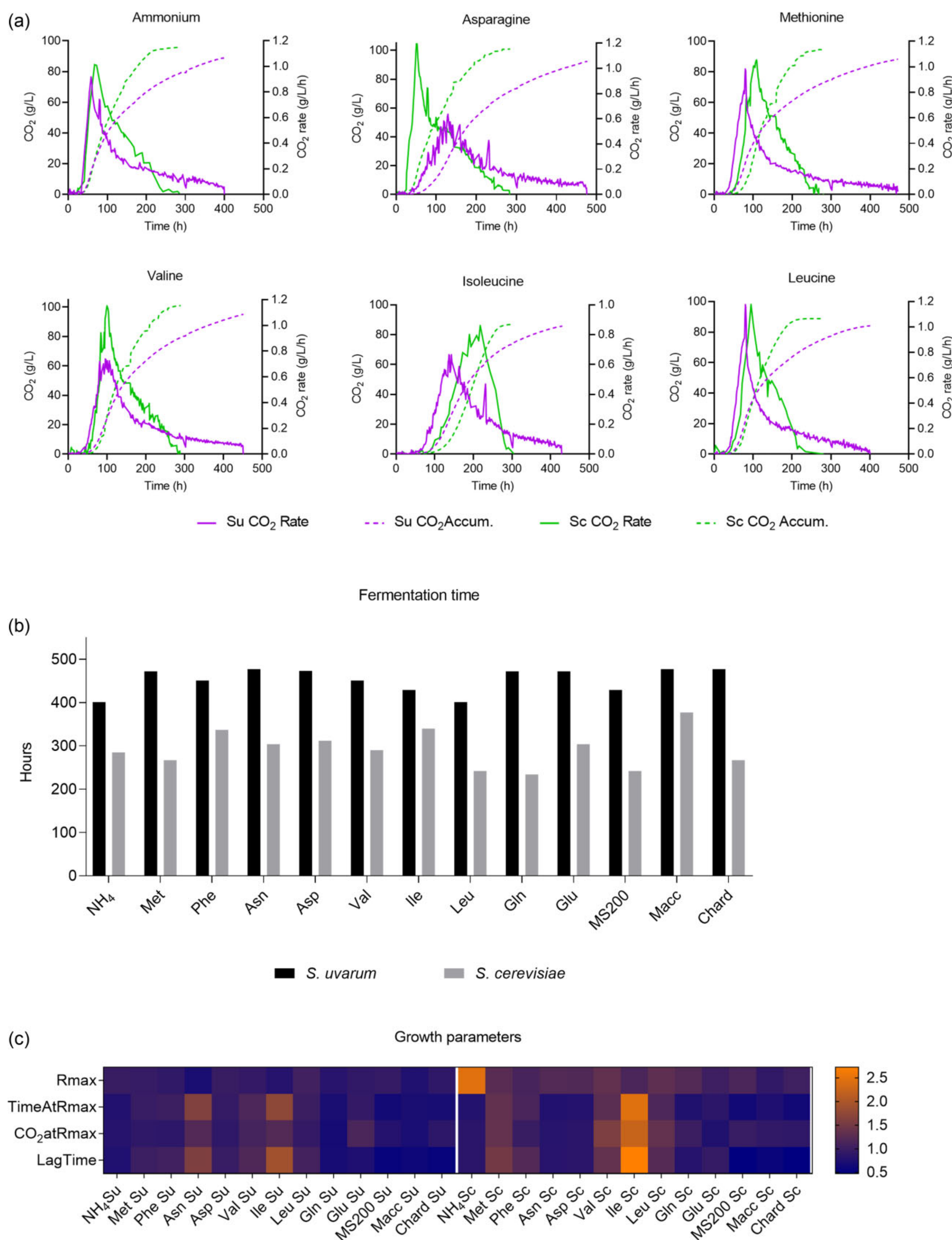

Fig. 2 Growth kinetics of S. *uvarum* MTF3098 (Su) and S. *cerevisiae* EC1118 (Sc). Fermentations were conducted in synthetic must (MS) in 11 different nitrogen conditions and two natural grape juices, at 20°C until the CO_2 production rate was lower than 0.05 g/L/h. (a) Fermentation profile on ammonium, asparagine, methionine, valine, isoleucine, and leucine. The accumulated CO_2 is expressed in g/L and the CO_2 production rate is expressed in g/L/h. (b) Fermentation time expressed in hours in 13 different nitrogen conditions: ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu), MS200, Maccabeu (Macc), and Chardonnay (Chard). (c) Heatmap of the growth parameters of S. *cerevisiae* and S. *uvarum* depending on the nitrogen sources. Fermentation rate (Rmax), time passed until reaching Rmax (TimeAtRmax), CO_2 produced at the Rmax point (CO_2atRmax), duration of the lag phase (LagTime). The values were normalized with the mean of each parameter among the strains and all the conditions tested.

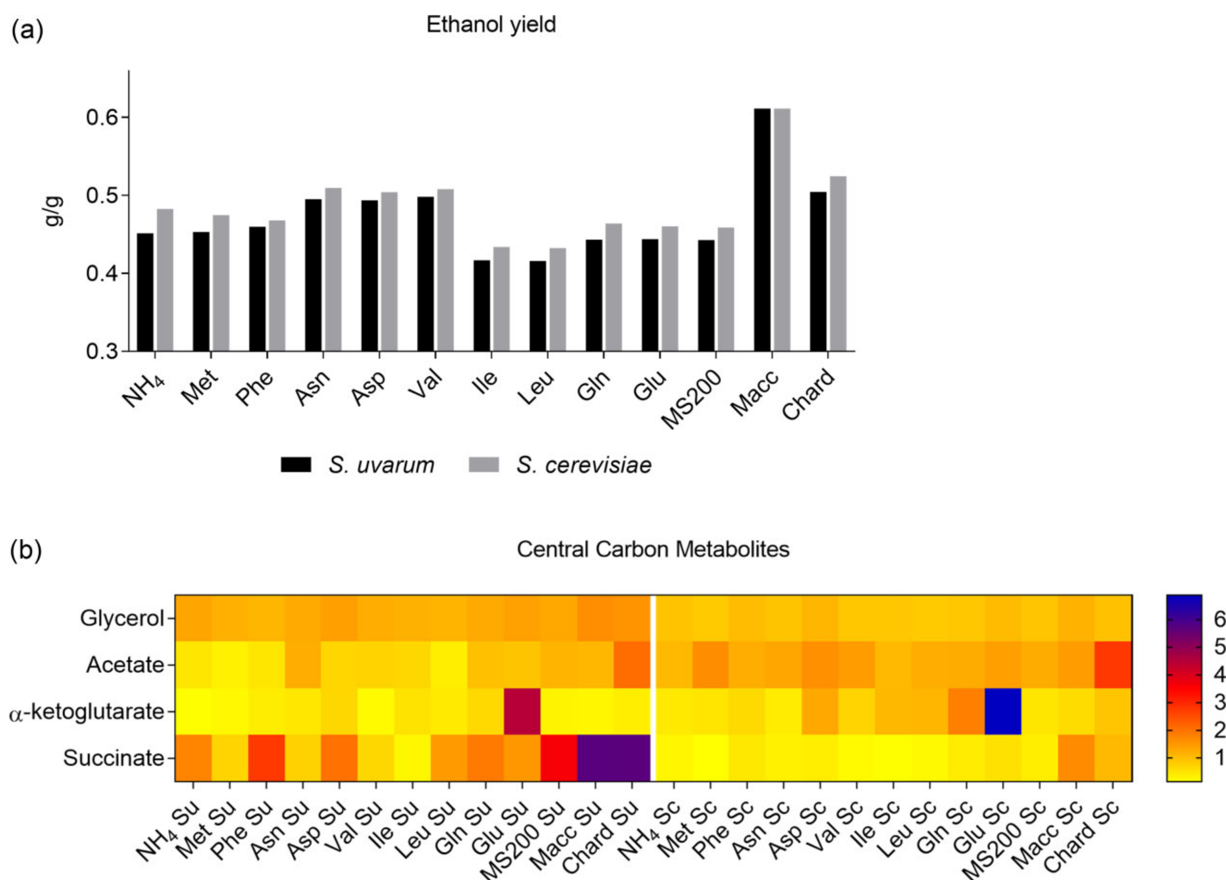

Fig. 3 Production of central carbon metabolites by *S. uvarum* and *S. cerevisiae* when growing on different nitrogen sources. Fermentations were conducted with *S. uvarum* (Su) and *S. cerevisiae* (Sc) in 13 different nitrogen conditions: ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu), MS200, Maccabeu (Macc), and Chardonnay (Chard). (a) Ethanol yield of *S. uvarum* and *S. cerevisiae* calculated from biological triplicates, expressed as grams of ethanol produced per grams of sugar consumed. (b) Heatmap of the final production of central carbon metabolites (g/L) normalized with the mean of each compound among all the nitrogen conditions and strains.

fructose, were found during fermentations with ammonium, valine, phenylalanine, and MS200, intermediate levels with glutamate and methionine, and high concentrations (>11 g/L) on glutamine, aspartate, and asparagine (Supplementary material). Hence, the source of nitrogen inversely influenced *S. uvarum* growth and fermentation performance. The amino acids assimilated through the central nitrogen core efficiently support growth but left residual sugar at the end of wine fermentations, whereas Ehrlich amino acids fully used all sugars but only supported slower growth.

Central carbon metabolites during wine fermentation

The physiological behaviour of *S. uvarum* during wine fermentation with different nitrogen sources was investigated by comparing the profile of central carbon metabolites between the species. It is immediately apparent that the ethanol production by *S. cerevisiae* was higher than that of *S. uvarum* in all conditions (Fig. 3a). Taking the synthetic medium MS200 as an example, *S. cerevisiae* produced 91.6 g/L ethanol (11.6% volume) while *S. uvarum* produced 86.6 g/L (11%). The ethanol yield, arising because of conversion of sugars to ethanol, varied depending on nitrogen source in a similar pattern for both yeasts, showing higher ethanol yields when growing on asparagine, aspartate, and valine and lower yields on leucine and isoleucine.

S. uvarum was also differentiated from *S. cerevisiae* when central carbon metabolites were considered (Fig. 3B). The most notable differences were increased production of glycerol and succinate, and lower formation of acetate and α-ketoglutarate (α-KG) in *S. uvarum*. In addition, an important effect of the nitrogen source on the formation of these metabolites was observed, except for glycerol in which the variation to the mean, calculated with data from the other nitrogen conditions, was <15% in both species and thus nitrogen effects were subtle. We found that the use of glutamate resulted in 10-fold increase of α-KG production by *S. uvarum* relative to the mean. The increase factor was 1.67 times for glutamine and aspartate. α-KG formation by *S. cerevisiae* was also largely induced by glutamate and, in a lesser extent, by glutamine and aspartate. Furthermore, the production of succinate was widely affected by the nitrogen source, with an 8.6 variation factor between the lowest and the highest levels of production by *S. uvarum*. Three groups of nitrogen source were distinguished, showing low (1 g/L in isoleucine), intermediate (~2 g/L in valine, methionine and asparagine) and high succinate production (from 47.5 g/L in the other nitrogen compounds). Unexpectedly, the response of succinate production to the nitrogen source by *S. cerevisiae* was very different, with a variation factor among all conditions of only 3.7. Moreover, the production of succinate

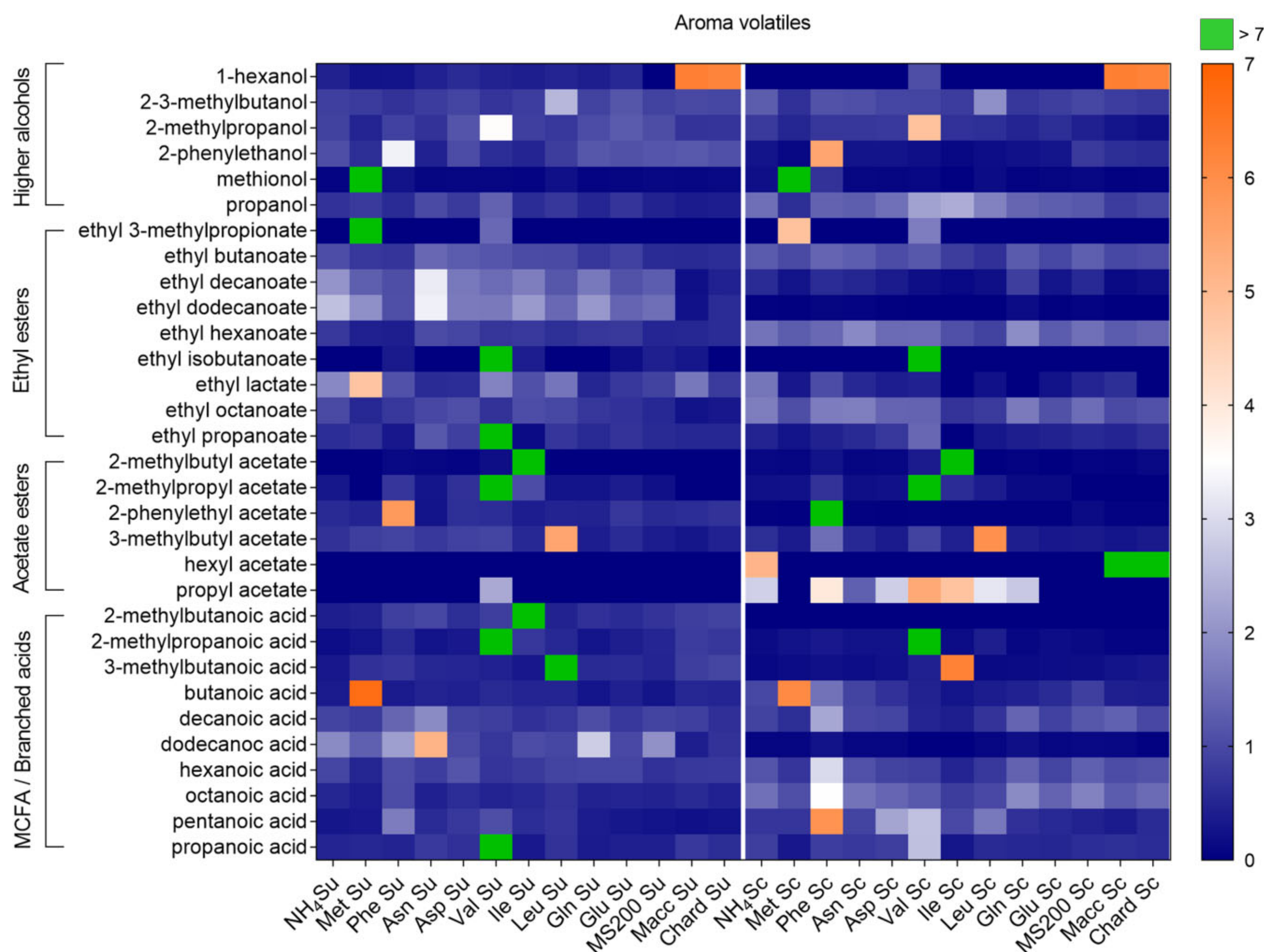

Fig. 4 Heatmap summarizing the final production of volatile compounds by *S. uvarum* growing on different nitrogen sources. *S. uvarum* (Su) and *S. cerevisiae* (Sc) fermentations were achieved using 13 different nitrogen conditions providing 200 mg/L of YAN as ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu) as a sole, and MS200, Maccabeu (Macc) and Chardonnay (Chard). Final concentration expressed in mg/L normalized with the mean of each volatile compound produced by both strains in all the nitrogen conditions tested. Green colour represents values >7.

by *S. cerevisiae* was low during methionine and isoleucine fermentation, intermediate when valine, leucine, and ammonium were used and high on the rest nitrogen sources, reflecting a different clustering of nitrogen sources in their ability to promote succinate formation than that of *S. uvarum*. Our results showed that, in general, *S. uvarum* produced lower amounts of acetate but with a higher variability depending on nitrogen conditions (from 0.100.35 g/L) compared to *S. cerevisiae* (from 0.300.48 g/L). Similar to the formation of succinate, the amino acids triggering the highest acetate production differed between the two species: glutamine and asparagine for *S. uvarum,* and methionine and aspartate for *S. cerevisiae*. Acetate production on methionine was very interesting with opposite effects seen: acetate levels were lowest (of all the single nitrogen sources) in *S. uvarum* but highest in *S. cerevisiae*. Finally, it is noteworthy that the production of central carbon metabolites by *S. uvarum* using a mixture of nitrogen compounds was in the range of variation found using a unique nitrogen source, except for glycerol (overproduced in natural grape juice) and succinate (overproduced in natural and synthetic grape juice). During fermentation on Chardonnay must, acetate was substantially overproduced by both strains.

Influence of the nitrogen source on the volatile compounds production

To evaluate the influence of the nitrogen source on the aroma profile of *S. uvarum* MTF3098 and *S. cerevisiae* EC1118, the concentrations of volatile compounds at the end of fermentation were compared (Fig. 4). A principal component analysis (PCA) revealed that both species and nitrogen source affected their production during fermentation (Supplementary figure 1).

Medium chain fatty acids (MCFA) and MCFA ethyl esters

MCFA from C4 to C12 are produced through the elongation of the carbon chain by the addition of C2 units from acetyl-CoA. For this analysis, the overall MCFA production is reported in μM of acetyl-CoA equivalents, which reflects the pathway activity. In most nitrogen sources, *S. uvarum* produced less MCFA than *S. cerevisiae* (Fig. 5). First, the total MCFA formation by *S. uvarum* was affected by the nitrogen source, with low production on valine, isoleucine, leucine, glutamine, glutamate, aspartate, and ammonium (range from 300470 μM), moderate production on phenylalanine (562 μM) and high production on asparagine and methionine (724 and 723 μM, respectively). The MCFA production profile in S.

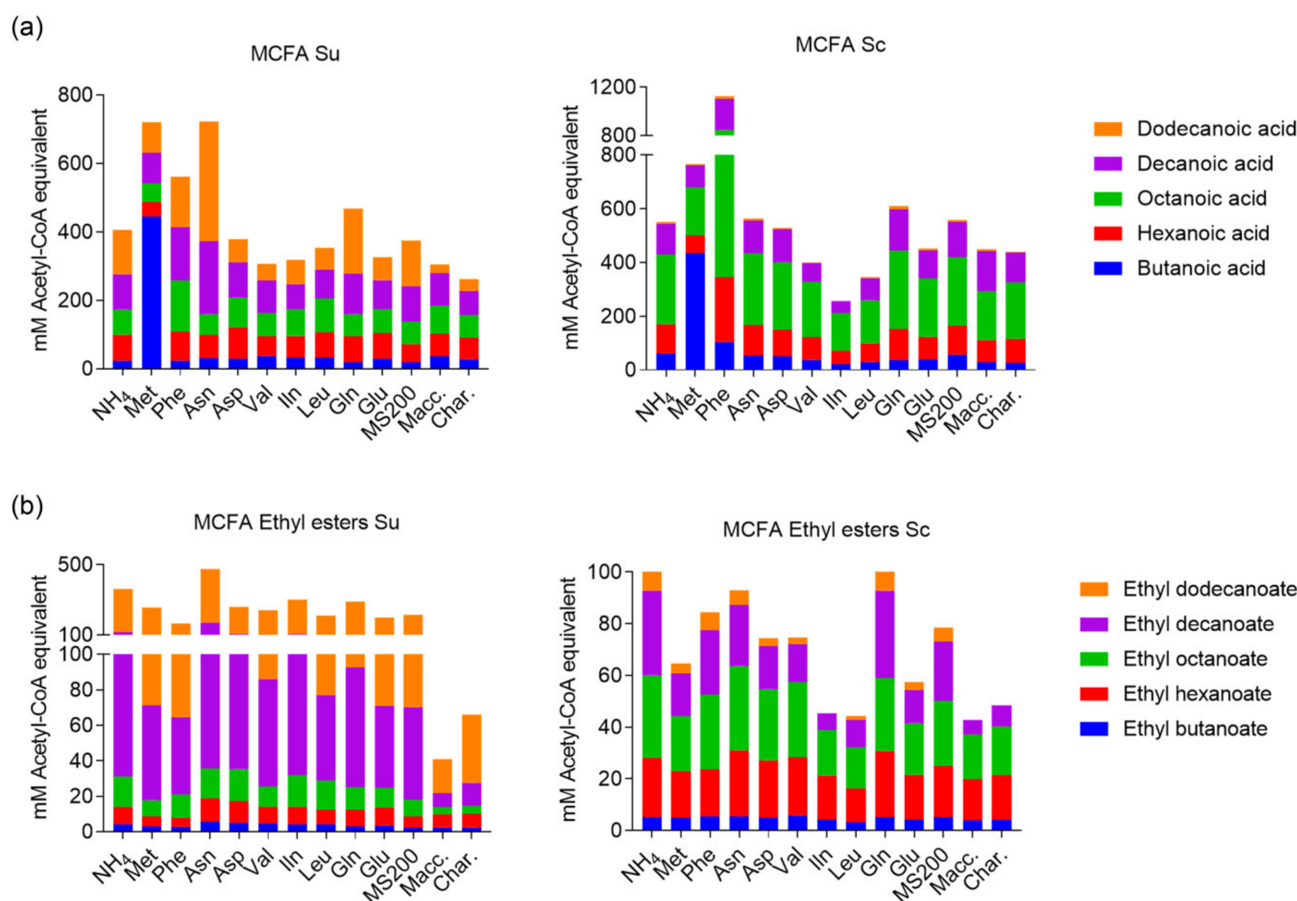

Fig. 5 Final production of MCFA and MCFA ethyl esters by *S. uvarum* and *S. cerevisiae* depending on the nitrogen source. Fermentations were conducted using 13 different nitrogen conditions: ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu), MS200, Maccabeu (Macc), and Chardonnay (Chard). Concentration expressed in μM acetyl-CoA equivalent considering the number of acetyl-CoA units required for the synthesis of each compound (2,3,4,5,6 C-units for butanoic, hexanoic, octanoic, decanoic, and dodecanoic acids, respectively). (a) Medium chain fatty acids (MCFA). (b) MCFA Ethyl esters.

uvarum was also influenced by the nitrogen source. During fermentations with valine, isoleucine, leucine, phenylalanine, glutamate, aspartate, and ammonium the MCFA profile was characterised by a low amount of butanoic acid compared to the other MCFAs. The profile of MCFAs formation obtained with *S. cerevisiae* under the same conditions was quite different, with a major contribution of octanoic acid. The use of methionine as sole nitrogen source triggered the overproduction of butanoic acid by both strains. Finally, in asparagine and glutamine, decanoic, and dodecanoic acids were overproduced specifically by *S. uvarum*.

Formation of MCFA ethyl esters was slightly altered by the nitrogen source in a species-dependent way. In *S. uvarum,* the production of ethyl decanoate and ethyl dodecanoate accounted for 84 and 91% of the total production. In *S. cerevisiae*, however, ethyl hexanoate, ethyl octanoate and ethyl decanoate represented between 80 and 88.4% of the total MCFA ethyl esters, with limited production of ethyl butanoate and ethyl dodecanoate. The variation in the total MCFA ethyl esters was low in *S. uvarum,* generally comprising between 8 and 11.9 mg/L, with slightly decreased production on phenylalanine (6.5 mg/L) and higher production on ammonium (14.2 mg/L) and asparagine (18.7 mg/L). Likewise, the nitrogen source had a weak impact on the formation of MCFA ethyl esters in *S. cerevisiae*, showing low concentrations in Ehrlich pathway amino acids (methionine, isoleucine, and leucine) and higher concentrations in nitrogen sources assimilated through the central nitrogen metabolism (ammonium, glutamine, and asparagine). Higher alcohols and their esters derivatives

Higher alcohols and their acetate esters derivatives directly related to the reductive branch of the Ehrlich pathway were differently produced by the two yeast species, and the particular influence of some nitrogen precursors on the production of some volatile compounds was evident. Focusing first on the species effect, comparing the average production for all the amino acids apart from the Ehrlich amino acid precursor, we found overproduction of phenylethanol (4-fold), phenylethyl acetate (17-fold) and 2-methylpropanol (isobutanol) (1.4-fold), and underproduction of methionol (0.7-fold) and propanol (0.5-fold) in *S. uvarum* compared to *S. cerevisiae*. Other volatile compounds in this category like 2-methylpropyl acetate (isobutyl acetate), 3-methylbutanol (isoamyl alcohol) and 3-methylbutyl acetate (isoamyl acetate) were produced in similar concentrations by both strains.

As anticipated, the production of higher alcohols and their acetate ester derivatives was considerably promoted when their specific precursor amino acid was provided as a sole nitrogen source. In *S. uvarum*, increase factors ranging from threefold (isoamyl alcohol in presence of leucine compared to the mean of all the other conditions) to 90-fold (methionol in presence of methionine) were found for higher alcohols, and comprised between 7.5-fold (isoamyl acetate in leucine) to 17.7-fold (isobutyl acetate in valine)

for acetate esters. In addition, however, some less expected variations in the production of higher alcohols and acetates by *S. uvarum* were triggered by changes of the nitrogen source, like the twofold increase of methionol production (compared to the mean) resulting from the use of phenylalanine or leucine as sole nitrogen source. Another surprising observation was the higher production of phenylethanol and 2-methylpropanol in the presence of glutamate, glutamine, ammonium, and aspartate but lower production with the Ehrlich amino acids (apart from phenylalanine and valine, for phenylethanol and 2-methylpropanol, respectively).

Higher acids from branched chain amino acids

Overall, branched-chain carboxylic acids and their ethyl esters derivatives were overproduced by *S. uvarum* compared to *S. cerevisiae*. These compounds derive from the oxidation of the aldehyde intermediates of the Ehrlich pathway, and as seen for the higher alcohols, their formation was promoted when their respective precursor amino acid was provided for growth. Indeed, the production of 2-methylbutanoic, 3-methylbutanoic and 2-methylpropanoic acid was around 20-times increased compared to the mean and their ethyl esters formation was only observed when isoleucine, leucine and valine were used as sole nitrogen sources, respectively. Otherwise, basal production of 2- and 3-methylbutanoic acids (1.72 mg/L) by *S. uvarum* was found, without a substantial effect of the nitrogen source (<40% variation to the mean). Notably, however, 2-methylpropanoic acid was the only branched acid produced by *S. uvarum* that was modulated by the nitrogen sources other than its direct Ehrlich precursor amino acid (valine). Its formation was increased compared to the mean in the presence of isoleucine, leucine, and phenylalanine and conversely decreased on ammonium, glutamine, asparagine, and methionine.

Discussion

In recent years, the distinctive phenotypic traits of the non-*S. cerevisiae* yeasts have led to increased interest in considering these as promising alternatives to meet the current challenges of the winemaking sector. New, more sustainable and environmentally-friendly production strategies have to be developed to cope with global warming and indeed to respond to changes in consumer requirements. More widespread and efficient use of these species is restricted, however, because of insufficient knowledge of their metabolic behaviour and no clear understanding of the similarities and differences to *S. cerevisiae*. In this context, we investigated the preferences for nitrogen sources and the effects of these nutrients on the fermentative performances and on the orientation of metabolism in *S. uvarum*.

As a component of proteins and nucleic acids, nitrogen is an essential nutrient for any microorganism and the efficiency of its assimilation strongly shapes their growth capacity and their activity. In *S. cerevisiae*, nitrogen sources related to the central nitrogen metabolic core support growth more efficiently than amino acids of the Ehrlich pathway (Ljungdahl and Daignan-Fornier, 2012; Magasanik and Kaiser, 2002). In this study, a similar classification was demonstrated for *S. uvarum*, however, important differences were found between the two species in their ability to grow using methionine and asparagine as sole nitrogen source. In *S. cerevisiae*, methionine assimilation is achieved through the Ehrlich pathway and the poor growth compared to other nitrogen sources has been explained by either a low efficiency of the enzymes involved in this metabolic route to retrieve nitrogen for *de novo* synthesis of amino acids (Gutiérrez et al., 2013), or by an imbalance between enzyme activities resulting in intracellular accumulation of the toxic intermediate 3-methylthiopropionaldehyde (Che et al., 2020; Deed et al., 2019). The relatively better growth of *S. uvarum* on methionine suggests higher efficiency of the transaminases Aro8p and Aro9p or more fine-tuned regulation of the enzymes in this species. Regarding asparagine, the better growth of *S. cerevisiae* could possibly be attributed to the presence of *ASP3* in some strains, but that cannot be the full explanation since, like *S. uvarum, S. cerevisiae* EC1118 lacks this gene (Coral-Medina et al., 2022; League et al., 2012). It is also noteworthy that outliers to these species-level findings can be observed at the strain level.

There were substantial differences in the formation of central carbon metabolites between the species. The higher production of succinate and glycerol and low production of ethanol in *S. uvarum* has been reported before (Minebois et al., 2020a; Sipiczki, 2008) and is suggested to be a strategy of cryotolerant yeasts to resist low temperatures (Gamero et al., 2013; Lopez-Malo et al., 2013). The high glycerol production, for instance, is explained by the ability of cryotolerant strains to direct carbon flux towards glycerol, whereas *S. cerevisiae* orients the carbon flux towards ethanol production (Arroyo-López et al., 2010). These data also reflect different partitioning of carbon fluxes in the central carbon metabolic network and likely, different strategies for adapting to stresses and constraints of fermentation. *S. uvarum* synthetises glycerol and succinate to ensure energy production and the maintenance of the redox balance, which are essential for growth, while *S. cerevisiae* achieves this through ethanol production. In *S. uvarum*, the highest production of glycerol and succinate was found in compounds from the nitrogen metabolic core, which promote more efficient growth resulting in increased anabolic requirements and *de novo* synthesis of building blocks. Consequently, a variable demand in energy and redox balance management may explain nitrogen source-dependent differences in the formation of central carbon metabolites.

Focusing on the pattern of acetate production, substantial variations were found between the species, most notably lower secretion in *S. uvarum* across all conditions. It has been recently reported that the production of acetate, taking place during the first part of fermentation, is followed by progressive consumption of this compound in *S. uvarum*, but not in *S. cerevisiae* (Minebois et al., 2020a). Furthermore, higher levels of intracellular acetyl-CoA were observed in *S. uvarum* compared to *S. cerevisiae* during wine fermentation (Henriques et al., 2021; Minebois et al., 2020b). There was also a clear impact of the nitrogen source on the formation of acetate in *S. uvarum*, with less secreted acetate when Ehrlich amino acids were used as sole nitrogen source. Under these conditions, growth/fermentation was slower, but the actual fermentation performance as determined by complete sugar utilisation was good. An indication emerging from these studies of *S. uvarum*-specific management of the acetate metabolic node was reinforced by our findings on the production of acetate-related metabolites.

Compared to *S. cerevisiae*, as well as lower levels of acetate production in *S. uvarum,* there was also a lower amount of excreted total MCFA (as acetyl-CoA equivalent) and an enrichment in C10 and C12 MCFA and their ethyl esters at the expense of compounds with a shorter carbon chain (C4, C6, C8). Taken together, these observations suggest that, in *S. uvarum*, intracellular acetate is mainly directed to the production of MCFAs via acetyl-CoA (Krivoruchko et al., 2015). The reduced levels of secreted shorter MCFAs could indicate their incorporation in membranes to improve plasticity and stress tolerance, rather than excretion or conversion to ethyl esters as is seen in *S. cerevisiae*. This specific trait

could be related to the cryophilic phenotype of this species, as an adaptation mechanism to low temperature. Supporting this hypothesis, it was reported that the cryophilic yeast *S. kudriavzevii* has a different lipid composition of the membrane from *S. cerevisiae*, with higher percentage of MCFAs and shorter fatty acids chain (Tronchoni et al., 2012), which could contribute to membrane fluidity and stress tolerance (Mannazzu et al., 2008). Moreover, the high production of C10 and C12 MCFA ethyl esters by *S. uvarum* was observed regardless the nitrogen source. As fatty acids provide the precursors for MCFA ethyl esters, this may be related to lipid metabolism, for example via differences in the regulatory enzymes Mgl2 and Yju3 (Marullo et al., 2021). Alternatively, differential expression, activity or substrate specificity of the ethanol acyltransferases Eeb1 and Eht1 (Saerens et al., 2006) may provide the explanation.

S. uvarum produced higher amounts of 2-methylpropanol, phenylethanol and phenylethyl acetate than *S. cerevisiae* regardless of the nitrogen source. There are several possible explanations for this. *S. uvarum* is reported to have a higher flux through the pentose phosphate pathway, which would potentially result in more availability of the shikimate precursor erythrose-4-phosphate (Minebois et al., 2020a, 2020b). Alternatively, it is possible that decarboxylation of phenylpyruvate is preferentially catalysed by Aro10 rather than competing decarboxylases (Pdc1, Pdc5, and Pdc6) (Deed et al., 2019). The recent identification of a new *ARO80* allele coding for a more efficient transcriptional activator of *ARO9* and *ARO10* in *S. uvarum* and *S. kudriavzevii*, could support this hypothesis (Tapia et al., 2022). It is also worth noting that Ehrlich volatiles can be synthesised from amino acid catabolism or from the CCM, since α-ketoacids are nodes in both processes. While the highest levels of Ehrlich volatiles are seen when growing on the cognate amino acid (e.g. 8 mM isoamyl alcohol in presence of 14 mM leucine), there is also clear evidence of synthesis via CCM. For example, when growing on a mixture of amino acids the concentration of isoamyl alcohol (2.8 mM) exceeds what could have been provided by the available leucine (0.17 mM) by more than a factor of 10. Consequently, it can be concluded that, as with *S. cerevisiae* (Crépin et al., 2017), CCM plays a major role in supplying precursors for synthesis of volatile compounds during fermentation on natural or synthetic grape must. Furthermore, the promotion of fusel acids formation in *S. uvarum* may be explained by a more efficient activity of aldehyde dehydrogenase (ALD) enzymes (Boer et al., 2007; Vuralhan et al., 2005) or by differences in the redox balance (NAD+/NADH + pools) between the two species.

In conclusion, through the examination of associations between the nature of nitrogen source and the fermentation performance of *S. uvarum*, we demonstrate both similarities and differences to *S. cerevisiae*. We determined that the specificities of *S. uvarum* in the efficiency of asparagine and methionine to support growth compared to *S. cerevisiae*, is likely to be related to its genetic background. Moreover, with integrative data analysis we concluded that the nature of nitrogen source has an impact on *S. uvarum* growth and consequently on the requirements for energy and maintenance of the redox balance. The distinctive profile of central carbon metabolites and volatile compounds of *S. uvarum* compared to *S. cerevisiae,* highlighted its unique distribution of carbon flux in the metabolic network to fulfil anabolic requirements. These peculiarities are exacerbated depending on the nitrogen source, in relation to the efficiency of amino acids and ammonium to support growth and fermentation. This is essential knowledge that should be considered in order to exploit the phenotypic potential offered by *S. uvarum* in winemaking and more widely in the food and beverage industry.

Acknowledgements

The authors would like to acknowledge the International Centre for Microbial Resources (CIRM) for providing *S. uvarum* strains. Thanks to Pascale Fernandez-Valle, Marc Perez, Faiza Macna, Valerie Nolleau, and Teddy Godet (INRAE, UMR Sciences pour l'oenologie, Montpellier, France) for helpful technical assistance.

Supplementary material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Funding

This study was funded by the European Union Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie actions [Grant Agreement No. 764 927] as part of the YEASTDOC European Joint Doctoral Programme.

Author contribution

A. C. M., J. M., and C. C. conceived and designed research. A. C. M. conducted experiments. A. C. M. and C. C. analysed data. The first draft of the manuscript was written by A. C. M. and C. C. with inputs from J. M. All authors read and approved the final manuscript.

Conflict of interest

The authors have no relevant financial or non-financial interest to disclose.

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