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The utilisation of nitrogenous compounds by commercial non-*Saccharomyces* yeasts associated with wine

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ABSTRACT

Commercial wine fermentation is commonly conducted by inoculated strains of *Saccharomyces cerevisiae*. However, other non-*Saccharomyces* yeast species have recently become popular co-inoculants. Co-inoculated yeast species compete with each other for nutrients, and such competition may impact fermentation kinetics and aroma production. Understanding the specific nutrient requirements of non-*Saccharomyces* strains therefore is essential to better characterize the competitive potential of each strain, and to support rational decision making for nutrient supplementation during wine making. This study investigated the nitrogen source preference of commercial non-*Saccharomyces* yeasts by conducting pure culture and sequential culture fermentations in synthetic grape musts with adjusted nitrogen contents. Amino acid and ammonium uptake varied between yeast species. *Lachancea thermotolerans* and *Torulaspora delbrueckii* assimilated more nitrogen at a faster rate than *Pichia kluyveri* and *Metschnikowia pulcherrima*. Significant variation in amino acid preference between species was observed. Sequential fermentations confirmed the more competitive behaviour of *L. thermotolerans* and *T. delbrueckii*, with consequences for fermentation kinetics and aroma production. Furthermore, the data suggest that declining populations of non-*Saccharomyces* yeasts release nitrogen and supports the activity of *S. cerevisiae*. The data provide the most detailed assessment of nitrogen utilisation by the investigated yeast strains in a wine environment.

1. Introduction

Yeasts are primarily responsible for alcoholic wine fermentation. The species best adapted to the wine fermentation environment is *Saccharomyces cerevisiae*. This species is in most cases barely detectable in grape juice, but rapidly dominates the other yeast species during spontaneous fermentation and is responsible for completion of alcoholic fermentation (Aranda et al., 2011). Until recently, other yeast species, although naturally dominant in freshly pressed grape juice at the start of fermentation, were mostly considered of little interest or as potential spoilage yeasts. However, over the past decades, it has been reported that many of these species show desirable properties such as the release of flavour and aroma compounds which positively contribute to the organoleptic complexity of the final wine (Andorrá et al., 2012; Whitener et al., 2015; Liu et al., 2016; Lachance, 2016). As a consequence, several strains representing species such as *Torulapora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima* and *Pichia kluyveri* have been commercially released in the past decade. These strains are co-inoculated together with *S. cerevisiae* since they are unable to ferment to dryness on their own. However, while the metabolic networks that govern nutrient preferences and flavour- and aroma-relevant outputs of *S. cerevisiae* during wine fermentation have been extensively investigated and described (Styger et al., 2011; Rollero et al., 2015, 2017), relatively little corresponding information exists about the non-*Saccharomyces* species (Kemsawasd et al., 2015; Rollero et al., 2018a). Such information however is essential to manage potential fermentation problems such as competition for nutrients between inoculated species and to achieve desirable organoleptic profiles from co-inoculated fermentations.

Nitrogenous compounds are essential for yeast growth and also serve as precursors for the production of aromatic compounds. In wine, the nitrogenous compounds that can be utilised by yeast are referred to as yeast assimilable nitrogen (YAN). Free amino nitrogen (FAN), viz. all amino acids except proline, as well as ammonium ions contribute the majority of YAN (Mendes-ferreira et al., 2011). Proline, a proteinogenic amino acid, is excluded from YAN as *S. cerevisiae* is not able to metabolise it under anaerobic conditions (Henschke and Jiranek, 1993). Low YAN concentrations can lead to stuck fermentations and increased H_2S formation, while high YAN concentrations may increase turbidity, promote microbial instability and favour the production of off-flavours

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(Beltran et al., 2004; Mendes-ferreira et al., 2011; Burin et al., 2015; Sturgeon et al., 2013).

Data show that assimilable nitrogen is taken up within the first few days of alcoholic fermentation by the dominant fermenting yeast strain (Tesnière et al., 2015). In *S. cerevisiae*, amino acids are assimilated and catabolised in a specific order of preference, with some such as glutamic acid and glutamine supporting better yeast growth and fermentative activity (Fairbairn et al., 2017). Besides their role in supporting growth, amino acids also serve as direct percursors to a large number of wine aroma impact metabolites that are produced by yeast during fermentation, and the amino acid composition of grape juice can be directly linked to the aromatic profiles of finished wines (Henschke and Jiranek, 1993; Mendes-ferreira et al., 2011; Sturgeon et al., 2013; Fairbairn et al., 2017; Dos Santos et al., 2015).

While amino acid preference and impact on growth and aroma metabolism have been well researched for *S. cerevisiae*, little information is available for amino acid uptake and utilisation in non-*Saccharomyces* yeasts. The few studies that have been published have primarily investigated the competition for nitrogen sources between some non-*Saccharomyces* yeast and *S. cerevisiae* (Medina et al., 2012; Lleixá et al., 2016; Gobert et al., 2017; Rollero et al., 2018b), and some data on the sequential up-take of amino acids have been described (Gobert et al., 2017). However, the conditions used in these studies did not establish nitrogen perferences since the concentrations of amino acids in the media – either real must or synthetic must with a wine-like amino acid composition - were unbalanced. Such knowledge would however be critical for wine-makers to optimise the use of non-*Saccharomyces* yeasts and achieve desired organoleptic outcomes. This is particularly important in the context of co- or sequential inoculation of the non-*Saccharomyces* yeasts strains since the high cell density associated with inoculation can lead to competition for nutrients and altered aroma production (Medina et al., 2012; Lleixá et al., 2016; Gobert et al., 2017; Rollero et al., 2018b).

Beyond the question of preference for nitrogen sources and the impact on fermentation performance, the effect of nitrogen composition on aroma compound production in non-*Saccharomyces* yeasts has not been researched extensively. The limited number of reports demonstrate that the initial concentration of assimilable nitrogen has an effect on the concentrations of various aroma compounds produced by non-*Saccharomyces* yeasts. At low initial YAN, aroma compound production by *L. thermotolerans*, *T. delbrueckii*, *M. pulcherrima* and *P. kluyveri* resulted in higher concentrations of esters and lower concentrations of fatty acids and higher alcohols (de Koker, 2015). Low YAN levels also resulted in higher acetic acid production and, for sequential fermentations, increased levels of organic acids and their corresponding esters. The study also confirmed higher propanol and butanol production for *L. thermotolerans* than for *S. cerevisiae* (Gobbi et al., 2013; de Kock, 2015). Moreover, *M. pulcherrima* in sequential fermentation with *S. cerevisiae* promoted volatile fatty acid production. Sequential *P. kluyveri* and *S. cerevisiae* fermentations promoted high ester levels. However, no clear links to nitrogen consumption could be established in these studies.

This study investigated the nitrogen preferences and order of uptake of nitrogen sources for various commercial non-*Saccharomyces* yeast strains as well as the potential for competition between *S. cerevisiae* and non-*Saccharomyces* yeasts. *S. cerevisiae* was used as a reference species and the results were compared with previous findings in literature. The effects of nitrogen on fermentation and growth kinetics were also investigated as well as the link between nitrogen sources and aroma compound release.

2. Materials and methods

2.1. Yeast species used

Five commercial wine yeast strains were used in this study as described in Table 1. *Saccharomyces cerevisiae* Lalvin® EC1118 (Lallemand, Blagnac, France) was used as a control in pure culture fermentations and sequentially inoculated with non-*Saccharomyces* yeast strains, namely *Pichia kluyveri* Viniflora® FootZen™ (Chr. Hansen, Hørsholm, Denmark), *Torulaspora delbrueckii* Biodiva™ TD291 (Lallemand), *Metschnikowia pulcherrima* Flavia® MP346 (Lallemand) and *Lachancea thermotolerans* Viniflora® Concerto™ (Chr. Hansen).

2.2. Preculture and fermentation conditions

Precultures were carried out initially by inoculating a single colony of each yeast into 5 mL Yeast Extract Peptone Dextrose (YPD) broth (Merck (Pty) Ltd, Modderfontein, South Africa) and growing the cells at 30 °C on a test tube rotator for approximately 24 h 50 µL of this preculture was then re-inoculated into 5 mL fresh YPD broth and grown for approximately 24 h at 30 °C on the test tube rotator. Cells were then inoculated from this preculture into 200 mL YPD broth at $OD_{600\ nm}$ 0.1 and grown at 30 °C with shaking at 120 rpm until the cells reach mid-exponential phase (ca. 9 h). A nitrogen starvation preculture was then conducted in a Yeast Nitrogen Base medium (20 g/L glucose, 1.7 g/L YNB base without amino acids and ammonium (Difco Laboratories)). The whole 200 mL YPD broth preculture was centrifuged at 2795 *g* for 5min and the supernatant was discarded. The cells were resuspended in 0.9% NaCl solution and centrifuged at 2795 *g* for 5 min. The supernatant was removed and the cells were resuspended in the YNB medium. The cells were left in the YNB medium at 25 °C with shaking at 120 rpm until growth stopped as determined by optical density readings. Once growth plateaued, the yeast were deemed starved of nitrogen and were then inoculated into synthetic grape must medium in which the fermentations were performed.

Three different synthetic grape must media were prepared in order to test the effects of different nitrogen sources and concentrations. Table 2 shows the differing nitrogen source concentrations for each treatment. Pure cultures of each yeast strain were inoculated alone, or sequentially with *S. cerevisiae*, into three synthetic musts with nitrogen source levels as shown in Table 2. In the sequential fermentations, *S. cerevisiae* was inoculated 48 h after the non-*Saccharomyces* yeast (in order to replicate typical wine-making practices) and another treatment was added. The latter entailed filtering out the non-*Saccharomyces* yeast before *S. cerevisiae* inoculation. All fermentations were carried out in triplicate and at 25 °C with shaking at 120 rpm.

Table 3 indicates the concentrations of other components present in the synthetic grape must medium. These concentrations were constant over treatments. Concentrations are adapted from Henschke and Jiranek (1993).

2.3. Sampling and fermentation kinetics monitoring

Fermentations were performed in 200 mL medium in 350-mL Erlenmeyer flasks fitted with a fermentation locks filled with water to maintain anaerobiosis. 20-mL samples were withdrawn from the fermentations in a laminar flow hood for chemical and microbiological analyses at the time points of 0 h, 2 h, 6 h, 18 h, 24 h, 48 h and endpoint (which is when fermentation ceased as no more weight loss was quantified, regardless of residual sugar level) for treatments 1 and 2, while only time points 0 and 48 h where sampled for treatment 3. Amino acid concentration analysis was performed at all time points until 48 h. Samples were centrifuged at 2516 g for 5 min and the supernatant was filtered through a 0.22-µm membrane (Starlab Scientific, Cape Town, South Africa). The samples were frozen at −20 °C until amino acid, glucose, fructose and ammonium analysis could be performed. Glucose, fructose and ammonium concentrations as well as accumulated weight loss were monitored throughout fermentation in order to determine the fermentation kinetics and analysis was performed using Enzytec Fluid (Roche, R-biopharm) kits E5140, E5120 and E5390 respectively. Fermentations were stopped when weight losses became negligibly low. Plate counts on YPD agar (Merck (Pty)

Table 1
Yeast species and strains used in this study.

Yeast species	Strain/Commercial name	Region of isolation/institution	Manufacturer
Saccharomyces cerevisiae	Lalvin® EC1118	Champagne, France	Lallemand
Pichia kluyveri	Viniflora® FootZen™	Auckland University, New Zealand	Chr. Hansen
Torulaspora delbrueckii	Biodiva™ TD291	North America	Lallemand
Metschnikowia pulcherrima	Flavia® MP346	Universitad de Santiago de Chile	Lallemand
Lachancea thermotolerans	Viniflora® Concerto™	Greece	Chr. Hansen

Table 2
Individual nitrogen source concentrations per treatment. Total YAN for each treatment was 200 mg/L. Treatment 1: Amino acids present at equal assimilable nitrogen levels (calculated in terms of nitrogen equivalents considering the nitrogen content of each amino acid), with ammonium. Treatment 2: Amino acids present at equal assimilable nitrogen levels, without ammonium. Treatment 3: grape-must like nitrogen source concentrations.

Nitrogen source	Treatment 1 pure cultures (mg/L)	Treatment 2 pure cultures (mg/L)	Treatment 3 pure cultures, sequential cultures, sequential cultures + filtering (mg/L).
Alanine	47.77	63.69	96.87
Arginine	23.29	31.06	249.58
Asparagine	35.38	47.17	35.33
Aspartic acid	71.43	95.24	29.67
Cysteine	64.94	86.58	8.73
Glutamine	39.06	52.08	336.85
Glutamic acid	78.95	105.26	80.29
Glycine	40.32	53.76	12.22
Histidine	27.68	36.90	21.82
Isoleucine	70.09	93.46	21.82
Leucine	70.09	93.46	32.29
Lysine	39.06	52.08	11.34
Methionine	79.79	106.38	20.94
Phenylalanine	88.24	117.65	25.31
Proline	61.48	81.97	408.41
Serine	56.39	75.19	52.36
Threonine	63.56	84.75	50.61
Tryptophan	54.74	72.99	119.56
Tyrosine	97.40	129.87	12.22
Valine	62.50	83.33	29.67
Ammonium chloride	189	0	306.67

Table 3
Components of synthetic grape must medium.

		Amount/L
Carbon Sources	Glucose	115 g
	Fructose	115 g
Acids	KH Tartrate	2.5 g
	L-Malic acid	3 g
	Citric acid	0.2 g
Salts	Potassium hydrogen phosphate	1.14 g
	Magnesium sulfate heptahydrate	1.23 g
	Calcium chloride dehydrate	0.44 g
Trace elements	Manganese(II) chloride tetrahydrate	200 µg
	Zinc chloride	135 µg
	Iron(II) chloride	30 µg
	Copper(II) chloride	15 µg
	Boric acid	5 µg
	Cobalt(II) nitrate hexahydrate	30 µg
	Sodium molybdate dehydrate	25 µg
	Potassium iodate	10 µg
Vitamins	Myo-inositol	100 mg
	Pyridoxine hydrochloride	2 mg
	Nicotinic acid	2 mg
	Calcium pantothenate	1 mg
	Thiamine hydrochloride	0.5 mg
	PABA.K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic acid	0.2 mg
Anaerobic factors	Ergosterol	10 mg
	Tween 80	0.5 mL
Nitrogen Sources	As described in Table 2	

Ltd, Modderfontein, South Africa) and $OD_{600\ nm}$ measurements were made throughout fermentation in order to monitor growth kinetics for treatments 1 and 2. Plates were incubated for 3–4 days at 30 °C after which colonies were counted. For treatment 3, GC-FID was performed in addition to the other analyses at 48 h and endpoint. In the sequential inoculations, *S. cerevisiae* (also nitrogen-starved) was inoculated 48 h after non-*Saccharomyces* yeast inoculation. Sequential inoculations were carried out with both yeasts present after 48 h, as well as first filtering out the non-*Saccharomyces* yeast before inoculating *S.cerevisiae* at 48 h.

2.4. Major volatile compound analysis (adapted from Louw et al. (2009))

The concentrations of various major volatile compounds (Isoamyl alcohol, isobutanol, phenylethanol, propanol, 2-phenylethyl acetate and isoamyl acetate) were measured using gas chromatography fitted with a flame ionisation detector. A J&W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, DE) with dimensions 60 m length × 0.32 mm internal diameter × 0.5 µm was used for analysis in a Hewlett-Packard 6890 Plus GC (Agilent) equipped with a split/splitless injector. Analytical standards of the different compounds were run individually to determine the retention times to allow identification and standard curves were established to allow quantification. Sample preparation was performed as follows: 5 mL of each sample (samples were filtered and centrifuged to ensure that no yeast cells were present for analysis) were placed into glass vials after which 100 µL of the internal standard (4-methyl-2-pentanol) was added to each sample. 1 mL diethyl ether was then added to the vials and the vials were placed in an ultrasonic bath for 5 min (for high sugar samples, magnetic stirrer bars were used for agitation instead of the ultrasonic bath in order to prevent an emulsion due to sugar and solvent reaction). The vials were centrifuged at 1789 g for 3 min (high sugar samples were spun at 447 g). GC-FID vials were prepared with sodium sulfate to absorb water. The top layers (separation visible) of samples were placed into inserts which were in turn placed into the GC-FID vial. These samples were then analysed.

2.5. Amino acid analysis using HPLC

High performance liquid chromatography (HPLC) was used for individual amino acid analysis of samples (based on a method described in Henderson and brooks (2010) with some injection and derivatisation modifications). Derivatisation used iodoacetic acid (Sigma Aldrich) for cysteine, o-phthaldialdehyde (Sigma-Aldrich) for primary amino acids and fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) for secondary amino acids. Sarcosine (Sigma Aldrich) and norvaline (Sigma Aldrich) as internal standards were spiked before derivatisation for each sample. A Poroshell HPH-C18 (4.6 mm, 150 mm, 2.7 µm) column (Agilent), preceded by a UHPLC guard 3 PK Poroshell HPH-C18 guard column (Agilent), was used for analysis. One-millilitre samples were filtered and centrifuged to ensure that no yeast cells were present for analysis. Analytical standards of each amino acid (LAA21-1 KT, Sigma-

Aldrich) were used to determine retention times and standard curves were established for quantification within the dynamic range.

2.6. Statistics

Tukey test analyses were performed on RStudio (https://www.rstudio.com/products/rstudio/download/) to determine statistical significance between treatments and strains.

3. Results

3.1. Pure culture fermentations with amino acids at the same assimilable nitrogen levels

3.1.1. Ensuring nitrogen starvation of yeast before final inoculation

In order to accurately monitor the uptake and release of nitrogenous compounds in fermentation medium, yeasts were starved of nitrogen before inoculation, as typically performed in industry during the preparation of active dry yeasts.

Results in the nitrogen-less medium showed that the growth of *P. kluyveri* and *M. pulcherrima* populations stopped after approximately 4 h, whereas that of *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* populations only stopped after approximately 8 h (Fig. S1). At this time point (i.e. 4 h or 8 h depending on the yeast species), the yeasts were deemed starved of nitrogen. They were then inoculated into the fermentation medium.

3.1.2. Fermentation kinetics, yeast population dynamics and consumption of nitrogen in pure culture fermentations

Pure culture fermentations were conducted by inoculation from the nitrogen starvation medium into synthetic grape must-like medium containing amino acids present at equal assimilable nitrogen levels (calculated in terms of nitrogen equivalents considering the nitrogen content of each amino acid) with (treatment 1) or without (treament 2) the presence of ammonium. The ammonium treatment was included to assess the impact of this nitrogen source on general nitrogen metabolism, since ammonium is the most commonly used nutrient to adjust nitrogen levels in commercial wine fermentations.

While *S. cerevisiae* fermented the sugars present in the media to dryness, none of the fermentations carried out by the non-*Saccharomyces* yeasts could achieve a similar outcome (Figs. 1 and S2). The presence of ammonium had no major impact on the rate and the level of CO_2 release. No significant differences were observed for residual sugar concentration at the end of fermentation between treatments with and without ammonium for each species. All yeast strains investigated was consumed glucose faster than fructose, thereby showing their glucophilic nature (Fig. S2). Similar sugar consumption rates were observed for *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* within the first 48 h. *P. kluyveri* and *M. pulcherrima*, however, showed slower consumption rates. CO_2 release followed the same pattern. Total CO_2 release was the highest for *S. cerevisiae*, followed by *L. thermotolerans* and *T. delbrueckii*, which showed identical CO_2 release over time (Fig. 1). *P. kluyveri* showed a significantly slower and lower CO_2 release, followed by *M. pulcherrima* which released the least CO_2. CO_2 release was proportional to sugar consumption for the different yeasts tested (Fig. 1 and Table S1).

Yeast growth was monitored regularly during the first 48 h and again at the end of fermentation. Plate counts showed that some yeast species behaved similarly over the course of the fermentations with only minor differences between species. (Fig. S3).

Ammonium consumption occurred at different rates depending on yeast species. *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* consumed all ammonium (below 5 mg/L remaining) by 18 h. *P. kluyveri* ammonium consumption was complete at 24 h and the slowest consumption of ammonium was by *M. pulcherrima* which took longer than 48 h (Fig. 2).

The uptake of individual amino acids was monitored over time (Fig. 3). The uptake of a large portion of amino acid began within 6 h after inoculation and the majority of amino acids started being taken up within 18 h. Strain as well as treatment (i.e. presence or absence of ammonium) differences were observed with regard to time of initial uptake of amino acids (Fig. 3 and Table S2). Initial uptake by *M. pulcherrima* and *P. kluyveri* was delayed for a large number of amino acids, while *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* started taking up most amino acids early on. Ammonium appeared to accelerate amino acid uptake significantly in *T. delbrueckii* and *M. pulcherrima* but did not impact uptake by *S. cerevisiae* and *L. thermotolerans*, but interestingly, glycine was released during fermentation by *P. kluyveri*. When *M. pulcherrima* fermented with ammonium, no uptake of glycine and threonine was detected.

Overall, *L. thermotolerans* and *T. delbrueckii* showed similar consumption patterns and took up most of the amino acids fully within 48 h, whereas *P. kluyveri* and *M. pulcherrima* did not take up the majority of amino acids fully (Fig. 3 and Table S2). In fact, *M. pulcherrima* and *P. kluyveri* initially released several amino acids before subsequent uptake occurred, suggesting that the starvation medium had led to the accumulation and storage of amino acids in these yeast.

S. cerevisiae took up all amino acids independently of the presence of ammonium with the exception of Pro. The amino acids that were consumed the earliest (within 18 h) were Asp, Glu, Arg and Lys. All other amino acids were fully taken up within 24 h, with the exceptions of Asn and Gly which were fully consumed by 48 h. Interestingly, while not impacting amino acid uptake, the presence of ammonium resulted in a measurable release of several amino acids Glu, Gln, His, Thr, Arg, Trp, Phe and Ile later on during fermentation.

Ammonium also had little impact on the total consumption of amino acids in *P. kluyveri* and *M. pulcherrima.* Arg, Met, Leu and Lys were fully consumed by both yeasts, while *P. kluyveri* also fully consumed His and Ala, and *M. pulcherrima* Glu, Ser, Gln and Ile, suggesting different uptake patterns or nitrogen source preferences between the two species. The only impact of ammonium was that in its absence, His, Val and Ile were now also fully consumed by both yeasts.

3.2. Pure and sequential culture fermentations with amino acids at concentrations mimicking those of an average grape must

3.2.1. Fermentation kinetics, consumption of nitrogen and aroma compounds production

Fermentations in synthetic grape must with a nitrogen composition that mimics an average grape must were carried out. This medium was either only inoculated with pure cultures of non-*Saccharomyces* yeasts, or was fermented sequentially when *S. cerevisiae* was inoculated into these fermenting musts after 48 h later, a strategy regularly employed in industry. In addition, a third experiment was carried out where juice partially fermented by the non-*Saccharomyces* yeast was filtered after 48 h to eliminate cells and then inoculated with *S. cerevisiae*. The latter experiment was performed in order to assess whether the different non-*Saccharomyces* yeasts can serve as a source of nitrogen for *S. cerevisiae*, especially in the case of yeast species that deplete the fermenting grape juice from nitrogen sources prior to the inoculation of *S. cerevisiae*.

Fig. 4 shows the fermentation data for the single and co-inoculated fermentations and Table S1 the concentrations of residual sugars when fermentation ceased. As a reference, fermentation of *S. cerevisiae* alone reached dryness rapidly and released almost 100 g/L CO_2 (Fig. 4). Fermentation patterns of single non-*Saccharomyces* species were similar to those described previously, suggesting that the more grape-like amino acid composition did not provide better fermentation support.

P. kluyveri and *M. pulcherrima* pure culture fermentations proceeded slowly and ended with the highest residual sugar levels. When *S. cerevisiae* was sequentially co-inoculated into these cultures, glucose was entirely consumed in both filtered and unfiltered sequential fermentations. Fructose was almost consumed entirely in these fermentations,

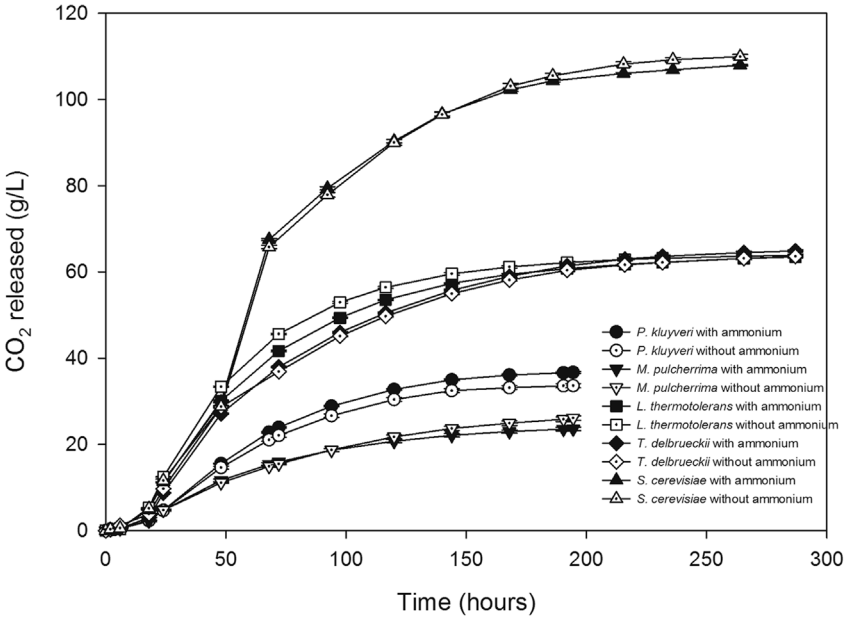

Fig. 1. Fermentation kinetics of different pure culture yeast species in synthetic grape juice-like medium with all nitrogen sources at the same nitrogen-equivalent concentration, with and without the presence of ammonium.

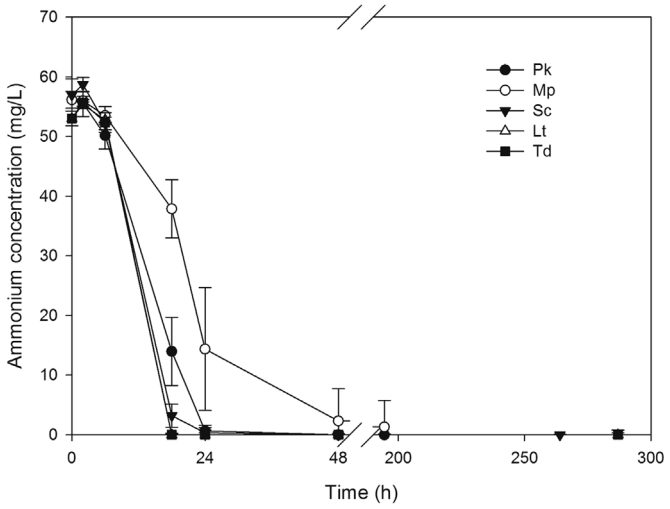

Fig. 2. Ammonium consumption of pure culture fermentations. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*.

suggesting nevertheless that the presence of these two species impacted somewhat on the ability of *S. cerevisiae* to complete the fermentation. In the fermentations with *L. thermotolerans* and *T. delbrueckii*, significant differences between the different fermentations became apparent. In the case of *L. thermotolerans*, none of the fermentations reached dryness. Only the unfiltered sequential *T. delbrueckii/S. cerevisiae* and *L. thermotolerans/S. cerevisiae* fermentation managed to nearly achieved this threshold. The highest residual glucose and fructose concentrations were observed in the filtered sequential fermentations, indicating that *S. cerevisiae* did not find sufficient nutrients in these filtered media to proceed efficiently. Since the unfiltered sequential fermentations consumed significantly more of the sugar than both the filtered and the single species fermentations, the data clearly suggest that release of nutrients by the non-*Saccharomyces* yeast provides essential support for *S. cerevisiae* in these conditions.

3.2.2. Nitrogen consumption and aroma compounds produced

A comparison of the percentage uptake of amino acids at 48 h between treatments with and without ammonium, as well as with grape-must like nitrogen concentrations is displayed in Table S3. *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* behaved similarly to each other with regard to amino acid uptake and had taken up the majority of amino acids fully by 48 h. These yeasts behaved very differently to *P. kluyveri* and *M. pulcherrima* however as these yeasts displayed much lower amino acids consumption profiles, similarly to the previous observations.

For *S. cerevisiae*, fermentations with grape must-like amino acid concentrations had similar percentage amino acid uptake levels at 48 h as the amino acids all at the same assimilable nitrogen concentration with the presence of ammonium. Some exceptions can however be noted, where the former treatment showed lower percentage uptake of the amino acids Asn, Gly, Arg and Pro. With regard to *L. thermotolerans* fermentations, all three treatments showed similar percentage uptake of amino acids at 48 h except for Glu, Asn (compared to treatment without ammonium), Gly, Ala, Val, Trp and Pro for the treatment with grape must-like amino acid concentrations for which lower percentage uptake was observed. For *T. delbrueckii*, lower uptakes for the grape must-like nitrogen treatment was found for the amino acids Asn, Gly and Pro.

For *P. kluyveri* fermentations, the most marked differences between treatments with amino acids at the same assimilable nitrogen level and grape must-like nitrogen concentrations are the following: For grape must-like nitrogen concentrations, His was taken up at half of the observed percentage uptake for the treatments with amino acids at the same assimilable nitrogen levels, regardless of the presence of ammonium. Gly was taken up in the grape-must like nitrogen treatment whereas a release was observed for the other treatment. Arg, Ala and Pro percentage uptake were however less for this treatment compared to the other two treatments. *M. pulcherrima* fermentations had lower percentage uptakes for the grape must-like nitrogen concentration treatment compared to the other treatments for the amino acids Asp, Glu, Asn, Ser, Gln, Thr, Arg, Ala and Pro.

Ammonium consumption is shown in Table 4. *P. kluyveri* and *M. pulcherrima* showed high levels of ammonium at 48 h, whereas for *L. thermotolerans, T. delbrueckii* and *S. cerevisiae*, ammonium is mostly or all consumed at 48 h, therefore there was no ammonium left in the medium at the time of *S. cerevisiae* inoculation. At the end of fermentation, pure cultures of *P. kluyveri* and *M. pulcherrima* showed residual ammonium, however all ammonium was consumed in all the other

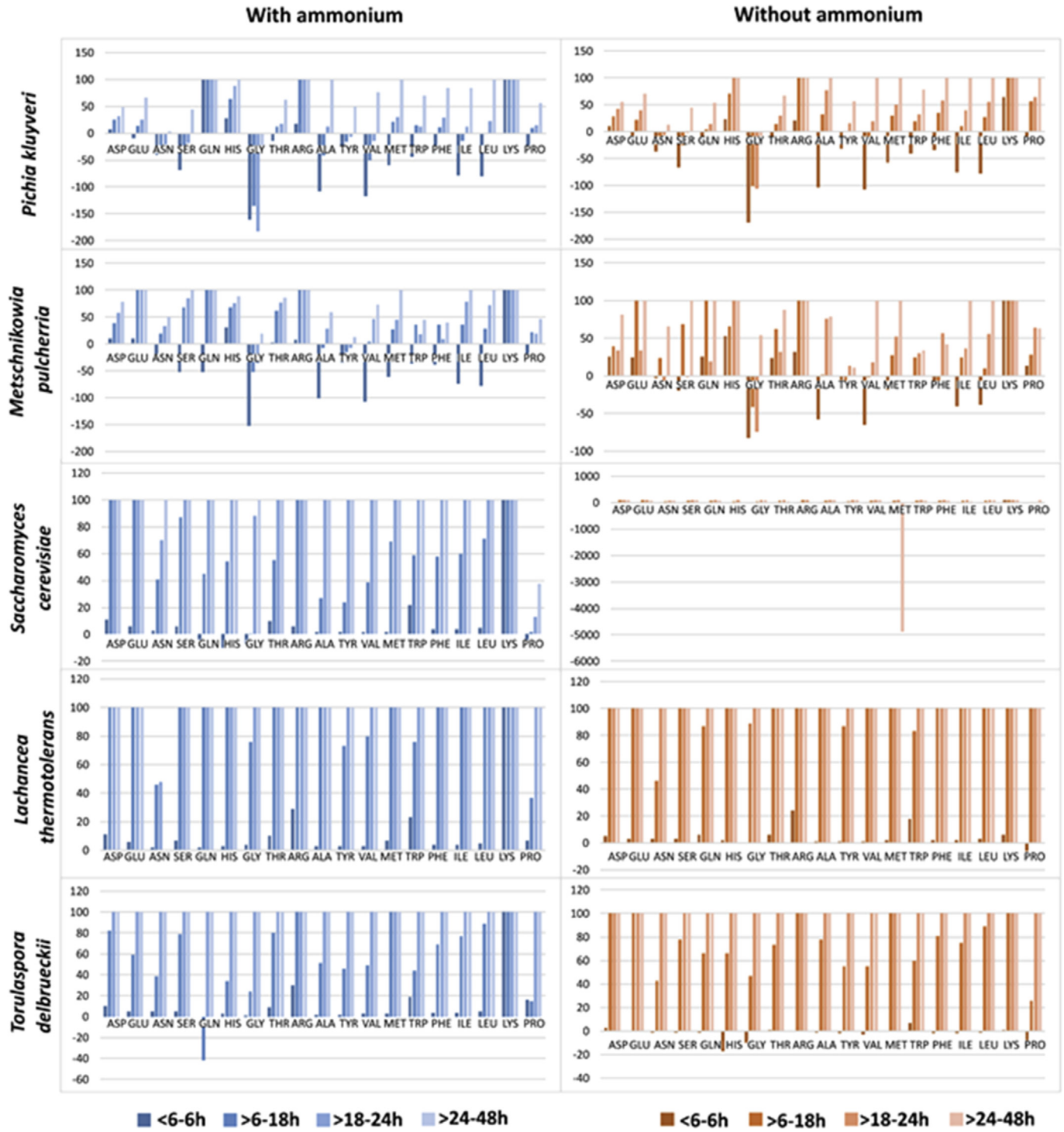

Fig. 3. Kinetics of amino acid percentage uptake. Percentage uptake of 90% and above was considered fully taken up (i.e. 100%). Negative values indicate amino acid release.

fermentations.

3.2.3. Uptake of amino acids catabolised through the Ehrlich pathway and their corresponding higher alcohol production

A general higher percentage uptake for the amino acids Leu, Val, Phe, Ser and Thr (some of the amino acids involved in the Ehrlich pathway) was observed for *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* when compared with *P. kluyveri* and *M. pulcherrima* (Table 5). Leucine was fully consumed by all yeasts by 48 h. *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* consumed all of these amino acids with the exception of valine for *L. thermotolerans*. *P. kluyveri* consumed only leucine and phenylalanine fully, while *M. pulcherrima* consumed only leucine and valine fully.

Fig. 5 displays the production of some of the higher alcohols which can be produced via the Ehrlich pathway at 48 h. Higher propanol concentrations were observed for the yeast strains which took up 100% of Threonine and Serine, namely *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*. Statistical analysis shows that for isoamyl alcohol, the concentrations at 48 h were statistically significant for *P. kluyveri* compared to *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*, which in

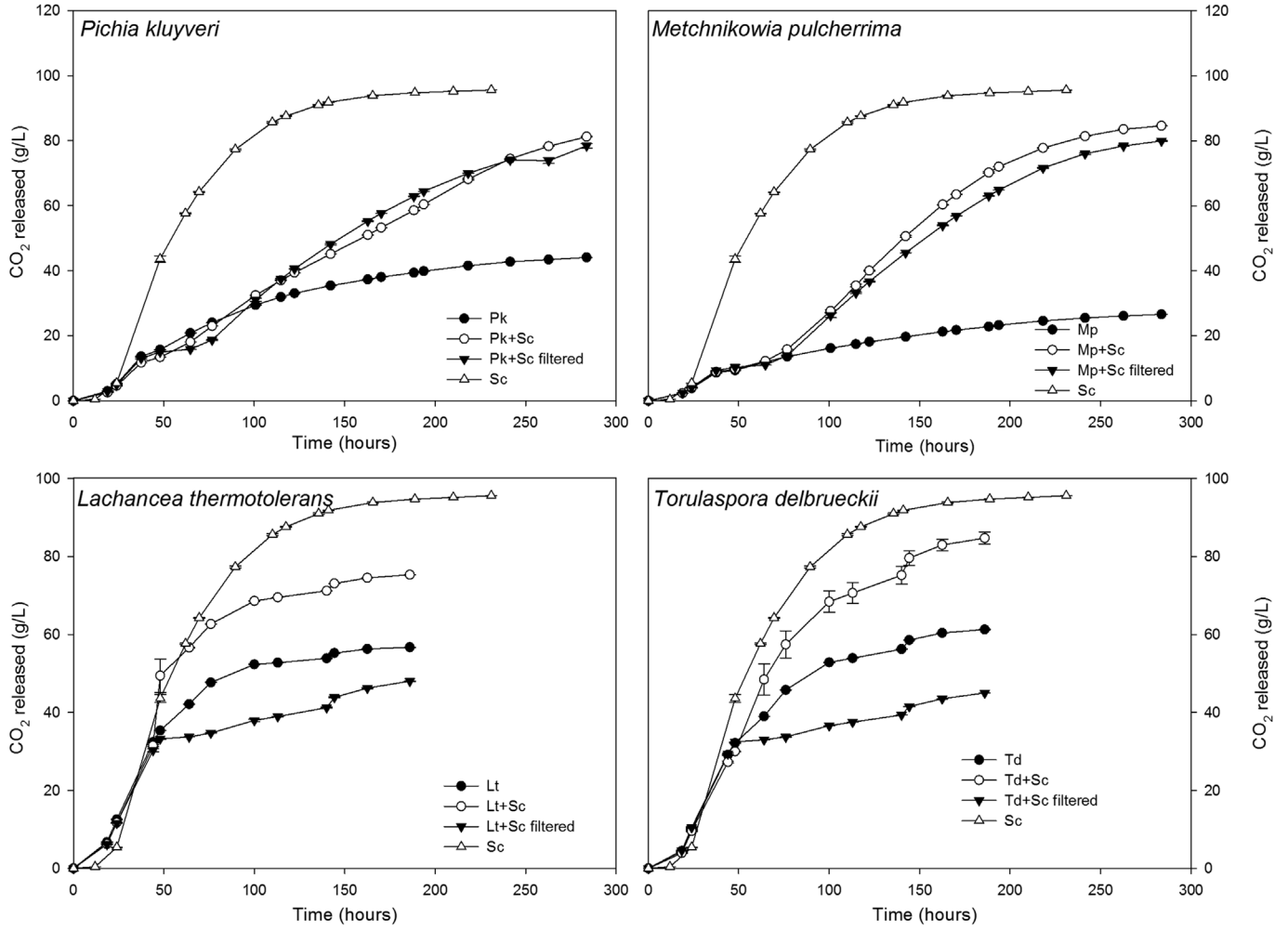

Fig. 4. Fermentation kinetics of pure culture and sequential fermentations. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*. Pk + Sc, Mp + Sc, Lt + Sc, Td + Sc: Sequential fermentations. Pk + Sc filtered, Mp + Sc filtered, Lt + Sc filtered, Td + Sc filtered: Sequential fermentations, however non-*Saccharomyces* yeast were filtered out before *S. cerevisiae* inoculation.

Table 4
Residual ammonium concentrations. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*.

Yeast strain and treatment	Residual ammonium at 48 h (mg/L)	Residual ammonium at endpoint (mg/L)
Pk	47.0 ± 1.6	43.2 ± 2.3
Pk + Sc	49.0 ± 5.5	0 ± 0.1
Pk + Sc filtered	47.9 ± 5.5	0 ± 0.3
Mp	59.8 ± 4.8	56.6 ± 5.5
Mp + Sc	59.6 ± 6.5	0 ± 0.4
Mp + Sc filtered	55.0 ± 3.2	0 ± 0.3
Lt	0 ± 0.3	0 ± 0.3
Lt + Sc	0 ± 0.6	0 ± 0.2
Lt + Sc filtered	4.7 ± 7.7	0 ± 0.1
Td	0 ± 0.5	0 ± 0.3
Td + Sc	0 ± 0.3	0 ± 0.1
Td + Sc filtered	0 ± 0.3	0 ± 0.5
Sc	0 ± 0.1	0 ± 0.2

turn grouped as not significantly different to each other, whereas *M. pulcherrima* showed no significant differences when compared to any of the other yeast strains. For isobutanol, the only significant differences were between *M. pulcherrima* and *S. cerevisiae*. Phenylethanol concentrations were statistically different for *M. pulcherrima* when compared to *P. kluyveri* and *S. cerevisiae*, whereas propanol showed statistically significantly higher levels produced by *S. cerevisiae*, followed by

Table 5
Percentage uptake of amino acids involved in the Ehrlich pathway at 48 h (before sequential inoculation). An uptake of 90% or higher was recorded as 100% taken up. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*.

Amino acid → Yeast ↓	Leu	Val	Phe	Ser	Thr
Pk	100	87	100	59	77
Mp	100	100	35	50	59
Sc	100	100	100	100	100
Lt	100	87	100	100	100
Td	100	100	100	100	100

L. thermotolerans and *T. delbrueckii*. *P. kluyveri* only produced isobutanol at 48 h.

At the end of fermentation, it could be seen that sequential fermentations with *P. kluyveri* and *M. pulcherrima* generally showed higher production of higher alcohols when compared to fermentations with *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* (Fig. 6). The only statistically significant differences regarding strain (regardless of treatment) were found for *M. pulcherrima* for isobutanol and phenylethanol. Statistically significant differences between treatments were found for a number of compounds. In general, sequential fermentations resulted in higher alcohol concentrations compared to pure culture fermentation. For *L. thermotolerans* and *T. delbrueckii*, the non-filtered sequential

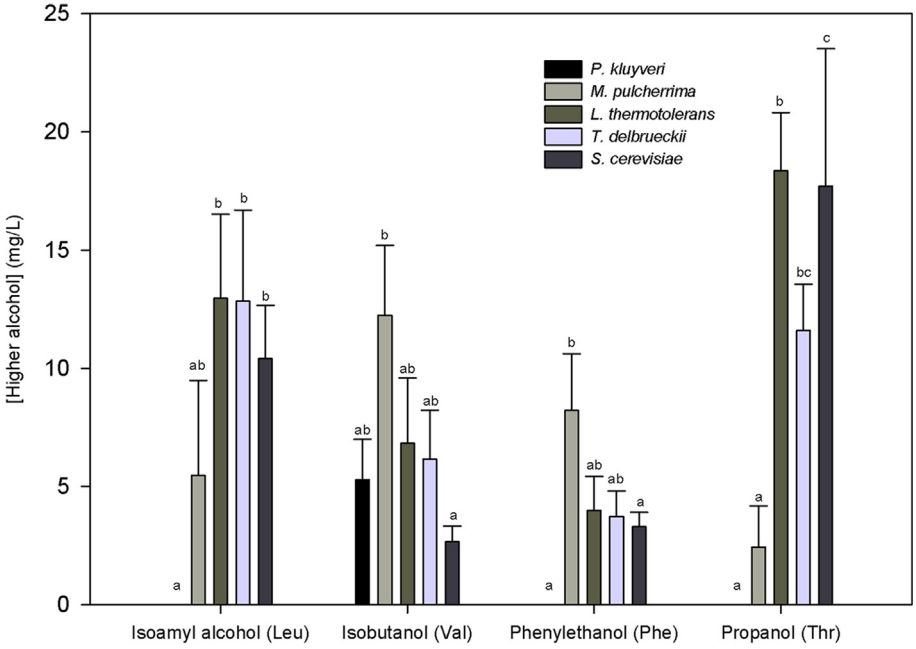

Fig. 5. Higher alcohol production at 48 h from corresponding amino acids which can be catabolised via the Ehrlich pathway. a,b,c: show statistical significance within a compound over strain. Amino acids which are catabolised through the Ehrlich pathway are indicated in brackets next to their corresponding higher alcohols.

fermentations resulted in the highest production of higher alcohols. For isoamyl alcohol, isobutanol and phenylethanol, higher alcohol concentrations produced by fermentation with pure *S. cerevisiae* showed similar levels to *L. thermotolerans* and *T. delbrueckii*, whereas for propanol, levels were more similar to sequential fermentations with *P. kluyveri* and *M. pulcherrima*. Some corresponding acetate esters were also measured. One of the most noteworthy acetate ester productions was that of 2-phenylethyl acetate, formed from phenylethanol by *P. kluyveri*, which produced 2.1 mg/L, 2.2 mg/L and 0.9 mg/L of this compound in pure, sequential and filtered sequential fermentations, respectively. No other yeast produced this compound above detectable levels. Isoamyl acetate was produced in fermentations containing *P. kluyveri* and pure culture *M. pulcherrima* fermentations only. Concentrations produced were 1.0 mg/L, 1.6 mg/L, 0.6 g/L and 0.06 mg/L for pure *P. kluyveri*, sequential *P. kluyveri*, filtered sequential *P. kluyveri* and pure *M. pulcherrima* fermentations respectively.

4. Discussion

4.1. General fermentation kinetics and links with nitrogen uptake

In this study, the nitrogen use of 5 wine-relevant yeast species was investigated under different conditions. *L. thermotolerans* and *T. delbrueckii* showed higher cell counts than *M. pulcherrima* and *P. kluyveri*

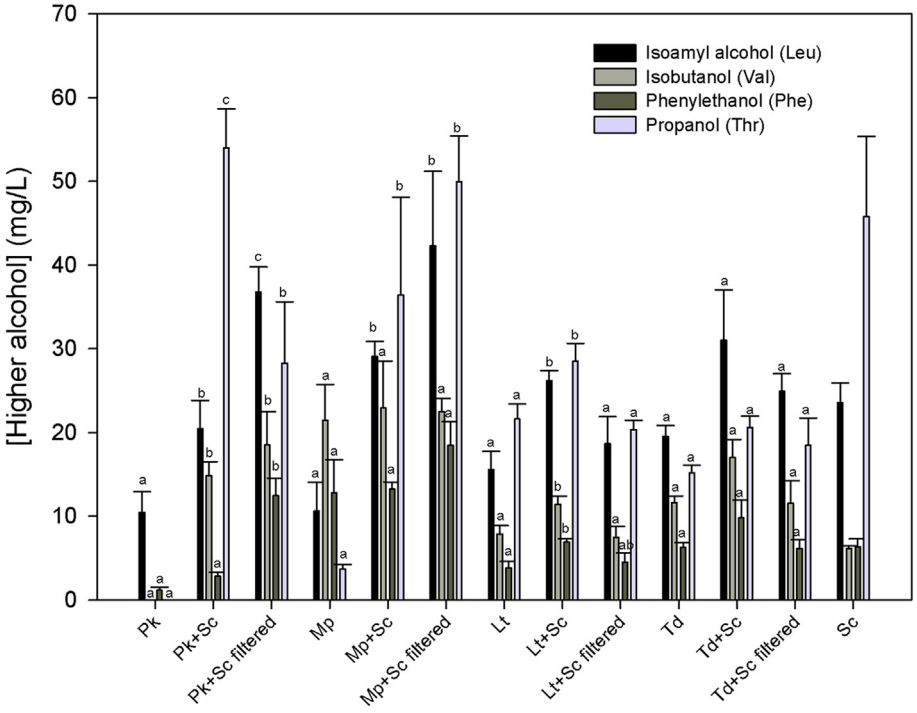

Fig. 6. Concentration of higher alcohols at endpoint. Pk: *P.kluyveri*; Mp: *M. pulcherrima*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sc: *S. cerevisiae*. Pk + Sc, Mp + Sc, Lt + Sc, Td + Sc: Sequential fermentations. Pk + Sc filtered, Mp + Sc filtered, Lt + Sc filtered, Td + Sc filtered: Sequential fermentations, however non-*Saccharomyces* yeast were filtered out before *S. cerevisiae* inoculation. a, b, c: show statistical significance within each strain between treatments.

(Wang et al., 2016), also correlating with higher uptake of nitrogen sources (Guttiérez et al., 2016). The latter study also demonstrated that nitrogen source uptake began before the onset of growth, which is confirmed in the current study. In our conditions, ammonium was fully consumed by all yeast species but its uptake was much faster in *L. thermotolerans, T. delbrueckii* and *S. cerevisiae* than in *P. kluyveri* and *M. pulcherrima*. Furthermore, most nitrogen source uptake began earlier in the stronger fermentation strains than in *M. pulcherrima* and *P. kluyveri*, thus confirming the link between nitrogen uptake and fermentation ability previously reported in literature (Ribéreau-Gayon et al., 2006).

4.2. Nitrogen source preference

4.2.1. Response of yeast to the presence of ammonium

The presence of ammonium favoured the amino acid uptake in *T. delbrueckii* and *P. kluyveri*.

However, absence of ammonium also promoted the uptake of some specific amino acids, including Pro for *S. cerevisiae*, Val, Ile and Phe for *P.* kluyveri, His and Val for *M. pulcherrima*, Asn for *L. thermotolerans* and Asp and Glu for *T. delbrueckii*, most likely to compensate for the relative lack of easy to use nitrogen sources. Val and Asn are usually taken up later by low-affinity SPS permeases and thus the absence of ammonium possibly led to their earlier activation (Schreve et al., 1998; Crépin et al., 2012).

4.2.2. Order of uptake of amino acids

The amino acids consumed by *S. cerevisiae* within 18 h were Asp, Glu, Arg and Lys. These were also previously determined as early consumed amino acids in conditions when nitrogen source levels were adjusted to equal amounts (Crépin et al., 2012). Differences in rate of uptake were observed for Arg and ammonium when different nitrogen sources levels were used (Crépin et al., 2014). These early amino acids were also deemed preferred sources in a previous study (Jones and Pierce, 1964), however arginine uptake rates were found to be dependent on yeast strain. Lys was the most rapidly consumed amino acid for both treatments for *S. cerevisiae* which is confirmatory of literature (Crépin et al., 2012).

The most rapid uptakes observed for *P. kluyveri* were those of Lys and Arg, and for *M. pulcherrima* were those of Lys, Arg, Glu and Gln. This rapid uptake of Lys is similar to that observed for *S. cerevisiae*. As a sole nitrogen source, lysine does not promote the growth of *S. cerevisiae*, however it has been able to promote the growth of various non-*Saccharomyces* yeasts when provided as a single nitrogen source including *Pichia* species and *M. pulcherrima* (Walters et al., 1953). In the media adjusted for equivalent nitrogen concentrations, Arg was provided in much lower concentrations than usually found in grape must-like media, possibly explaining its rapid uptake. Rapid Glu and Gln uptake can be attributed to their central role in yeast nitrogen metabolism, since these amino acids are essential for ammonium fixation and many transamination reactions, playing a central role in the *de novo* synthesis of other amino acids (Ljungdahl et al., 2012).

Interestingly, *M. pulcherrima* and *P. kluyveri* also released a large amount of amino acids before uptake began which suggests that the response to the stresses of inoculation of these yeasts led to a different behaviour compared to the other yeasts tested.

All amino acids were fully taken up for both treatments for *L. thermotolerans* (except Asn as mentioned above), with the majority of amino acids being taken up within 18 h. Thus, this yeast has an especially fast uptake rate, even compared to *S. cerevisiae*. This is contrary to data reported previously (de Koker, 2015) that *S. cerevisiae* took up amino acids the most rapidly. A major difference between the two studies is that fermentations were agitated, and differences in nitrogen uptake linked to agitation have been recently reported (Rollero et al., 2018c). *T. delbrueckii* also fully consumed all amino acids for both treatments, but the majority of amino acids were fully taken up by 24 h.

4.3. Competition for nitrogen sources and correlation with aroma compound production (grape must-like nitrogen source concentrations)

4.3.1. Competition for nitrogen sources and its effect on fermentation ability

M. pulcherrima and *P. kluyveri* took up the lowest amount of nitrogen sources under grape must-like concentrations, leaving 54 and 41 mg/L YAN respectively in the must at the time of *S. cerevisiae* inoculation. These YAN levels were found to be adequate for fermentation after the inoculation of *S. cerevisiae*, although significantly below the values generally considered as safe, already suggesting a significant release of additional nitrogen sources by these yeast during further fermemtaion progress. *L. thermotolerans* and *T. delbrueckii* took up the majority of nitrogen sources (leaving only 6 and 2 mg/L YAN respectively) before *S. cerevisiae* inoculation. Sequential inoculations (no filtering) showed improved sugar consumption and CO_2 release compared to pure cultures although final residual sugar concentrations were higher than those of the sequential fermentations with *M. pulcherrima* and *P. klyuveri*. This suggests significant competition for nutrients, and/or other cellular interactions which were not monitored in this study. Competityion for nutrients is however the most likely explanation for the poor performance of *S. cerevisiae* in the filtered medium. These data clearly suggest that the non-*Saccharomyces* yeast species release significant amounts of nitrogen and probably other nutrients, supporting the growth and fermentation of *S. cerevisiae*.

4.3.2. Aroma compound production and the link with amino acid uptake and competition

At 48 h, the only higher alcohol to show a correlation with the uptake of its corresponding amino acid was propanol. *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*, assimilated the highest levels of threonine and produced the highest levels of propanol. High propanol levels produced by *L. theromotolerans* have also been found previously (de Koker, 2015). The correlation between amino acid uptake and propanol produced is further strengthened by another study (Mouret et al., 2014) which found that propanol was not connected to the central carbon metabolism and its production rather directly correlated with the phase of nitrogen consumption, initial nitrogen concentration and intracellular threonine catabolism. The other higher alcohols tested, however, can be produced either via the central carbon metabolism or the Ehrlich pathway (Rollero et al., 2017; Crépin et al., 2017). For instance, *M. pulcherrima*'s high production of phenylethanol compared to the other yeasts is consistent with results reported (Padilla et al., 2016), however this yeast's low uptake of phenylalanine suggests that this compound's production largely originates from the central carbon metabolism.

At end point, *M. pulcherrima* and *P. kluyveri* filtered sequential fermentations generally produced the highest higher alcohol and acetate ester levels, which can possibly be attributed to the fact that the non-*Saccharomyces* yeasts were no longer available to consume nitrogen, thus promoting the increased aroma compound production by *S. cerevisiae* (as competition for nitrogen sources was not particularly evident) and/or that their production originated from carbon metabolism. In most cases for *P. kluyveri* and *M. pulcherrima* sequential fermentations, more higher alcohols were produced compared to the pure culture of *S. cerevisiae* indicating that these yeasts remained active producing most of the higher alcohols. This is further suggested by propanol production since a similar production is observed for sequential fermentations and pure *S. cerevisiae. P. kluyveri* was the only yeast to produce 2-phenylethyl acetate in this study and literature confirms this yeast's high capacity for the production of esters (Padilla et al., 2016). *P. kluyveri* also showed the highest production of isoamyl acetate, which is a known attribute of this yeast, as reviewed previously (Padilla et al., 2016). Fermentations with *L. thermotolerans* and *T. delbrueckii* showed low aroma compound production in general compared to the other yeasts tested, possibly due to the competition for nitrogen sources as previously determined, however when comparing fermentations

treatments within these yeast species, non-filtered sequential fermentation showed the most favourable results, which correlated with their previously determined more favourable fermentation kinetics. It was previously found that *L. thermotolerans* contributed to elevated 2-phenylethanol concentration while in sequential fermentation with *S. cerevisiae* (Benito et al., 2016), however the concentrations of this compound found in this study for the same treatment were low compared to the other yeast strains. *L. thermotolerans*'s characteristically high 2-phenylethyl acetate production levels were also not confirmed for the yeast strain in the current study compared to that found previously (Benito et al., 2016). These contradictions could possibly be a result of different strains being used. *T. delbrueckii* has also been noted as a high ester producer (Padilla et al., 2016), however no significant acetate ester production was noted in this study for the esters tested. This is possibly also due to strain variation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.002.

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