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Nitrogen metabolism in three non-conventional wine yeast species: A tool to modulate wine aroma profiles

Stéphanie Rollero [a], Audrey Bloem [b], Jeanne Brand [a], Anne Ortiz-Julien [c], Carole Camarasa [b], Benoit Divol [a,*]

[a] South African Grape and Wine Research Institute, Faculty of AgriSciences, Stellenbosch University, Stellenbosch, South Africa
[b] UMR SPO, INRA, SupAgroM, Université de Montpellier, Montpellier, France
[c] Lallemand SAS, Blagnac, France

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ABSTRACT

The positive impact of certain non-*Saccharomyces* yeasts on the aromatic profile of wines has been well documented in literature and their industrial use in association with *S. cerevisiae* is now recommended. Competition between non-*Saccharomyces* species and *Saccharomyces cerevisiae* for various nutrients, especially nitrogen sources, greatly impacts the production of aroma compounds. In this study, we further explored the impact of different nitrogen nutrition strategies on the production of carbon and sulphur volatile compounds of three non-*Saccharomyces* strains, namely *Pichia burtonii, Kluyveromyces marxianus, Zygoascus meyerae* sequentially inoculated with *S. cerevisiae* in Sauvignon blanc and Shiraz grape musts. Nitrogen additions were implemented according the specific requirement of each species. At the end of fermentation, we observed specific metabolic signatures for each strain in response to the nature of the nitrogen source suggesting strain-specific metabolic fluxes present. Overall, these results confirmed and further explored the interconnection between nitrogen sources and aroma metabolism (including that of higher alcohols, fatty acids, esters and volatile sulphur compounds), and their variations according to species and the nature of the nitrogen source. The knowledge generated provides new insights to modulate the aroma profile of wines produced with non-*Saccharomyces* species.

1. Introduction

Winemaking is a complex biochemical process resulting from the degradation of grape juice sugars into ethanol and CO_2 by yeasts and mainly by the *Saccharomyces cerevisiae* species. To avoid stuck and sluggish fermentations as well as the production of off-flavors, a common practice in winemaking is to inoculate the must with selected active *S. cerevisiae* dry yeasts. Despite the advantages of this practice to control the fermentation process, some winemakers - under consumer demand- believe that wine produced by *S. cerevisiae* only lacks flavor complexity compared to spontaneous fermentations (Padilla et al., 2016). Indeed, although *S. cerevisiae* is the main microorganism during alcoholic fermentation, a wide diversity of other yeast species (often referred to as non-*Saccharomyces* yeasts) is observed during the early stages of spontaneous fermentations (Raymond Eder et al., 2017).

Over the past twenty-odd years, numerous studies have demonstrated the positive impact of non-*Saccharomyces* yeasts on wine quality, leading to a growing interest in the use of these species in enology. These yeasts can be used to achieve specific objectives such as lowering the ethanol content, preventing wine spoilage and increasing the production of specific compounds and enzymes (Bergler et al., 2020; Ciani et al., 2010; Englezos et al., 2018; Renault et al., 2016; Rollero et al., 2018a; Seguinot et al., 2020a). However, the majority of non-*Saccharomyces* yeasts are not able to ferment all the sugars present in must, which requires the addition of *S. cerevisiae* to complete alcoholic fermentation. It is in this context that co-inoculation or sequential inoculation strategies have been developed in order to take advantages of the metabolic characteristics of non-*Saccharomyces* yeasts without running risks of stuck fermentations and wine spoilage (Hu et al., 2019; Nisiotou et al., 2018). During co-inoculation/sequential inoculation of non-*Saccharomyces* yeasts with *S. cerevisiae*, various types of interactions between yeasts take place, especially competition for nutrient resources such as nitrogen (Gobert et al., 2019; Rollero et al., 2018a; Seguinot et al., 2020b).

* Corresponding author.
E-mail address: divol@sun.ac.za (B. Divol).

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Nitrogen sources, a limited resource under enological conditions, is essential for yeast growth and the fermentation process. The nitrogen requirements and the order of assimilation of nitrogen substrates are strain-dependent, a phenomenon which directly leads to competition for nitrogen sources during sequential inoculation (Crépin et al., 2012; Gobert et al., 2017; Rollero et al., 2018a). Moreover, the presence of non-*Saccharomyces* yeasts in high cell numbers affects nutrient availability for *S. cerevisiae* (Rollero et al., 2018a; Seguinot et al., 2020b). In practice, to overcome nitrogen deficiency in grape musts, winemakers generally supplement with Yeast Assimilable Nitrogen (YAN), mainly ammonium and amino acids, to reach 150 mg/L of YAN concentration (Bely et al., 1990). Moreover, many aroma compounds excreted by the yeasts during fermentation are directly produced from and influenced by nitrogen metabolism such as higher alcohols, acetate esters, ethyl esters and sulphur compounds which participate in the organoleptic complexity of wine (Gobert et al., 2017; Rollero et al., 2018b; Seguinot et al., 2020a; Su et al., 2020). Nevertheless, the metabolic pathways leading to the compounds are well known (with some exceptions such as those leading to volatile sulphur compounds), while the influence of the yeast genetic background and that of the environmental conditions still require further investigations. Indeed, further work is required to better understand the effects of nitrogen nutrient addition on aroma compounds production during co-inoculation and sequential inoculation, which can allow an appropriate management of nitrogen and the improvement of wine quality.

In a previous work, the nitrogen assimilation profile of three non-*Saccharomyces* yeasts (*Kluyveromyces marxianus, Zygoascus meyerae* and *Pichia burtonii*) was reported (Rollero et al., 2018a). We showed that these yeasts can produce wines with added complexity but competition for nutrients with *S. cerevisiae* may impair fermentation (Rollero et al., 2018a). These results, like most published in literature were obtained using a synthetic grape juice medium, mimicking enological conditions. Real grape juices and the environmental conditions governing fermentation in these media are much more complex. The aim of this study was to evaluate the impact of grape variety (Shiraz and Sauvignon blanc musts) and nitrogen supplementation on yeast growth, fermentation performances as well as sugar and nitrogen metabolism of the three non-*Saccharomyces* yeasts previously selected, during sequential inoculation with *S. cerevisiae*.

2. Materials and methods

2.1. Yeast strains and preculture conditions

The fermentations were performed with the commercial wine strain *Saccharomyces cerevisiae* Lalvin EC1118® (Lallemand SA, Montreal, Canada) and three non-*Saccharomyces* yeasts isolated from South African grape juices (IWBT collection, Stellenbosch, South Africa), namely *Kluyveromyces marxianus* IWBT Y885, *Zygoascus meyerae* IWBT Y826 and *Pichia burtonii* IWBT Y951. The cryopreserved yeast cultures were thawed at room temperature and streaked on Yeast Peptone Dextrose (YPD) agar (Biolab-Merck, Modderfontein, South Africa). Starter cultures of all yeast strains were prepared by inoculating a single colony into 5 mL YPD broth for each strain. The cultures were incubated at 30 °C on a test tube rotating wheel for 24 h. These starter cultures were used to inoculate YPD precultures at an initial cell density of 1×10^6 cells/mL which were incubated at 30 °C with shaking (125 rpm) for 9 h. Prior to inoculation, the yeasts were transferred to one final preculture medium consisting of Yeast Nitrogen Base without nitrogen sources (Difco Laboratories) supplemented with 20 g/L of glucose at 30 °C with shaking (125 rpm). They were incubated for 4 h (*P. burtonii*), 6 h (*K. marxianus*) or 8 h (*Z. meyerae* and *S. cerevisiae*). This aimed to starve the cells for nitrogen.

Sequential mixed cultures were performed with the inoculation of one of the non-*Saccharomyces* yeasts 48 h before *S. cerevisiae*. A pure culture with only *S. cerevisiae* was also carried out. All the strains were inoculated from the preculture at 1×10^6 cells/mL.

2.2. Fermentation conditions

Experiments were conducted using a Sauvignon blanc (Franschhoek Cellar, Franschhoek, South Africa) and a Shiraz grape juice (Beau Belle wine estate, Stellenbosch, South Africa) from 2017 vintage. The main characteristics of the juices are presented in Table 1.

After grape crushing and destemming, 30 mg/L of SO_2 was added in the form of potassium metabisulfite in both white and red grape musts. The white grapes were pressed with a pneumatic press and the juice was clarified overnight at 15 °C with 2.5 mL/hL of Rapidase® Clear (Oenobrands, Montpellier, France). The clarified juice was distributed in 4.5-L glass bottles and alcoholic fermentation was performed at 15 °C.

The red grape must was distributed in 10-L buckets and alcoholic fermentation was performed at 25 °C. It was fermented with the skins until the end of alcoholic fermentation; the skin cap was punched down twice a day. Upon reaching dryness and after pressing, each wine was transferred into glass bottles equipped with fermenter lock and the lactic acid bacterium *Oenococcus oeni* VP41 (Lallemand, Montreal, Canada) was inoculated (at 1 g/hL) to perform malolactic fermentation at 20 °C.

At the end of alcoholic or malolactic fermentation (for Sauvignon blanc and Shiraz wines, respectively), SO_2 was added in the form of potassium metabisulfite in order to obtain a similar free SO_2 concentration (20 mg/L) in all bottles. Following the post-fermentation cold stabilisation process (15 days at −4 °C), the final wines were bottled in 750-mL glass bottles with screw caps and stored at 15 °C. All fermentations were performed in triplicates.

2.3. Nitrogen additions

As a consequence of the results obtained in Rollero et al. (2018a), for some fermentations where *Z. meyerae* or *K. marxianus* were sequentially inoculated with *S. cerevisiae*, nitrogen sources (diammonium phosphate (DAP), mixture of various amino acids (Sigma-Aldrich, Saint-Louis, MO, USA) or FermaidO®® (Lallemand SAS, Montreal, Canada)) were added at the same time as the inoculation of *S. cerevisiae*. With *Z. meyerae*, 40

Table 1
Grape juice characteristics (a) and nitrogen source concentration (in mgN/L) (b) a.b.

	Sugars (g/L)	pH	Total acidity (g/L)	Yeast Assimilable Nitrogen (mg/L)	Free SO_2 (mg/l)
Sauvignon blanc	195	3.18	7.19	200	13
Shiraz	225	3.7	3.5	320	11

	Sauvignon blanc	Shiraz
ASP	2.68	3.66
GLU	5.15	9.16
ASN	2.68	5.43
SER	4.55	7.36
GLN	42.23	58.17
HIS	4.56	6.50
GLY	1.43	3.14
THR	4.08	6.55
ARG	59.96	71.30
ALA	11.07	17.09
GABA	35.56	48.93
TYR	0.75	1.46
VAL	2.69	3.87
MET	1.28	2.15
TRP	10.05	18.23
PHE	3.04	6.23
ILE	1.92	4.05
LEU	2.08	10.88
LYS	1.97	5.05
NH4	68.99	76.17

mg N/L were added as FermaidO®®. With *K. marxianus*, four different forms of nitrogen amounting to 70 mg N/L were performed: (i) FermaidO®®, (ii) DAP, (iii) glutamine (240 mg/L) + glutamate (240 mg/L), (iv) leucine (80 mg/L), isoleucine (80 mg/L), valine (75 mg/L), threonine (75 mg/L), phenylalanine (100 mg/L), tyrosine (115 mg/L), tryptophan (130 mg/L) and methionine (90 mg/L).

2.4. Quantification of ammonium by enzymatic assays

To quantify the ammonium concentration, 400 μL of filtered sample was enzymatically analyzed (Enzytec™ Fluid Ammonia, Id-No: 5390, R-BiopharmAG, Germany) using the Arena 20XT (Thermo Fisher Scientific, Waltham, MA) which makes use of automated spectrophotometric readings to determine the concentrations of the various compounds.

2.5. Quantification of individual amino acids

Amino acids quantification was performed by high performance liquid chromatography (HPLC), Agilent 1100 (Agilent Technologies, Santa Clara, CA, USA) by pre-column derivatization and fluorescence detection based upon a method previously described (Henderson and Brooks, 2010) with some modifications to the derivatization and injection. A Poroshell HPH-C18 column (4.6 mm length x 150 mm internal diameter, 2.7 μm particle size; Agilent Technologies) was used following derivatization of the amino acids. Derivatization was performed using three different reagents: iodoacetic acid (Sigma Aldrich) for cysteine, o-phthaldialdehyde (OPA, Sigma Aldrich) for primary amino acids and fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) for secondary amino acids. Internal standards norvaline (Sigma Aldrich) and sarcosine (Sigma Aldrich) were spiked to each sample prior to derivatization. One milliliter of each filtered sample was analyzed.

2.6. Quantification of carbon metabolites

The amount of sugars and organic acids were quantified by High Performance Liquid Chromatography (HPLC) with the use of an Aminex HPX-87H (300 × 7.8 mm) column, at 55 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.5 mL/min as exactly described by Eyéghé-Bickong et al. (2012). Peaks were detected and quantified using Agilent RID and UV detectors in tandem. HPChemstation software was used for data analysis, expressing metabolite quantities in g/L.

2.7. Quantification of major volatile compounds

The quantification of major volatiles (i.e. a selection of higher alcohols, acetate esters, fatty acids, and fatty acid ethyl esters) was carried out by gas chromatography equipped with a flame ionization detector (GC-FID) using the Agilent GC System HP 6890 Series (Agilent Technologies) as described previously (Louw et al., 2009) with minor modifications. Five millilitres of each of the filtered samples were used with 100 μL of 4-methyl-2-pentanol (internal standard). Diethyl ether (1 mL) was added to the mixture which was then placed in an ultrasonic bath for 5 min to extract the volatile compounds. Thereafter, the samples were centrifuged at 4000 *g* for 3 min. Sodium sulphate was added to remove any water from the non-polar layer. HP Chemstation software was used for data analysis.

2.8. Analysis of sulphur compounds

2.8.1. Chemicals

The following sulphur compounds studied were purchased from Sigma Aldrich: ethyl mercaptan (EtSH) [75−08-1], dimethyl sulfide (DMS) [75−18-3], diethyl sulphide (DES) [352−93-2], thiophene (TP) [110−02-1], dimethyl disulfide (DMDS) [624−92-0], ethyl thioacetate (ETA) [625−60-5], diethyl disulfide (DEDS) [110−81-6], 3-(methylthio)-propanal (MAL) [3268−49-3], 2-mercaptoethanol (ME) [60−24-2], 2-methyl-tetrahydrothiophen-3-one (MTHTP) [13679-85-1], 2-(methylthio)-ethanol (2MTE) [5271−38-5], ethyl 3-(methylthio)-propanoic acid (E3MTP) [13327-56-5], 3-(methylthio)-propyl acetate (3MTPAc) [16630-55-0], 3-mercapto-1-propanol (3 MP) [19721-22-3], 3-(methylthio)-1-propanol (ME) [505−10-2], 3-(ethylthio)-1-propanol (ETP) [18721-61-4], 4-(methylthio)-1-butanol (MTB) [20582-85-8], 3-(methylthio)-propanoic acid (3MTPA) [646−01-5]. L-malic acid was obtained from Sigma Aldrich and ethanol was from VWR (Fontenay-sous-Bois, France).

2.8.2. Synthetic wine

The synthetic wine was made by dissolving 6 g/L of L-malic acid in 1 L of 12% v/v ethanol solution, and the pH was adjusted to 3.3 with 1 M NaOH.

2.8.3. Sulphur standards and internal standard preparation

Standard solutions of 10 g/L were individually prepared in cooled ethanol (−20 °C) and stored at −20 °C. An internal standard solution was made by dissolving 100 mg/L (w/w) of thiophene in ethanol and stored at −20 °C.

2.8.4. Calibration of standard curves

Dilutions were made with synthetic wine. 5 mL of synthetic wine containing different concentrations of sulphur compounds and 10 μL of internal standard solution were placed in 15-mL pyrex tube with a Teflon cap (Supelco, Bellefonte, PA, USA). The calibration samples are prepared as the samples. The standard curve for individual sulphur compounds was built up by plotting the sulphur response ratio of target compound and the internal standard against the concentration ratio.

2.8.5. Samples preparation

10 μL of internal standard diluted in ethanol (100 mg/L) was added to the samples (5 mL) in a 15-mL pyrex tube with a Teflon cap. One milliliter of dichloromethane was added, and the mixture was shaken for 20 min on shaking table at 150 rpm. The samples were centrifuged for 5 min at 3000 *g* at 4 °C. The organic phase was collected in a 4-mL vial and a second extraction with 1 mL of dichloromethane was performed on the aqueous phase. The organic phase was added to the same 4-mL vial containing the organic phase from the first extraction. The organic phase was dried with anhydrous Na_2SO_4 and was transferred to a vial. The sample was evaporated under nitrogen flux to a final volume of 0.5 mL and transferred to an insert in a vial.

2.8.6. Gas chromatography/mass spectrometry

Samples were analyzed with an Agilent 7890A GC system gas chromatograph (Agilent Technologies) equipped with a Gerstel Multi-Purpose Sampler MPS-2XL (Gerstel, Mülheim an der Ruhr, Germany) used in liquid mode, and coupled to an Agilent 5975C mass spectrometry detector (Agilent Technologies). The instrument was controlled with the Gerstel MAESTRO software and the data analyzed with Chemstation software (Agilent Technologies). The gas chromatograph was fitted with a 30 m × 0.25 mm fused silica capillary column ZB-WAX, 0.25-μm film thickness (Phenomenex, Torrance, CA, USA). The carrier gas was helium, linear velocity 36 cm/s, flow rate 1.0 mL/min in constant flow mode. The initial oven temperature was 40 °C for 3 min. The temperature was increased by 4 °C/min until it reached 220 °C, and was held at this temperature for 20 min. The injector and the transfer line were held at 250 °C. The sample volume injected was 2 μL, and the splitter, at 10:1, was opened after 30 s. The focus liner (Agilent Technologies) was deactivated and tapered with glass wool (2–4 mm). The mass spectrometer quadrupole temperature was set at 150 °C, the source was set at 230 °C, and the transfer line was held at 250 °C. For quantification, mass spectra were recorded in Selected Ion Monitoring (SIM) mode with positive ion electron impact at 70 eV. The ions monitored in SIM runs are shown Table S1. The ion in bold for each compound was typically used for quantification, the other ions were used as qualifiers.

Both major volatile and sulphur compounds were analyzed at the end of alcoholic fermentation, in both white and red wines (i.e. before malolactic fermentation in the red wines) in order to compare both types of wine (red and white) in the same conditions. Malolactic fermentation was only performed in the red wines to facilitate the sensory evaluation (see paragraph 2.9 below). Indeed, red wines containing malic acid are typically difficult to taste because the sensory panel tend to focus on this "fault".

2.9. Sensory analysis: preference ranking

For the sensory analysis, the conditions with *K. marxianus* without nitrogen addition were not included because of the presence of residual sugars in these samples (Table S5). The number of consumers that performed the test was 95 for Sauvignon blanc wines and 93 for Shiraz wines.

A sensory laboratory equipped with individual computerized tasting booths, controlled lighting and temperature conditions (20 ± 2 °C) secluded from extraneous noise was used during the consumer preference testing.

Wine samples were served in international ISO standard tasting glasses (ISO NORM 3591, 1977), coded with random three-digit codes and served in a randomized manner to the consumers according to a Williams Latin Square design.

During the preference ranking tests consumers had to rank the wines from the most liked to the least liked. The value one was assigned to the most liked wine. The data was captured electronically with 23″ touch screen computers using Compusense cloud (Compusense Inc., Guelph, Canada). Rank sums were computed by adding the rank-values for a wine across all the consumers. Subsequently the most preferred wine had the lowest rank sum. Significant differences between wines in terms of consumer preference was determined through multiple comparison of the rank sums using the "Expanded tables for multiple comparison procedures in the analysis of ranked data" (Newell and MacFarlane, 1987).

2.10. Statistical analyses

All conditions were performed in triplicate. The values were presented as means ± SD. The differences between treatments were determined using analysis of variance (One-way ANOVA with treatment as the factor) with the R software, version 3.2.3 (http://cran.r-project.org/). The differences were considered significant if the p-values were equal or less than 0.05. For each parameter, normality of residual distributions and homogeneity of variance were studied using standard diagnostic graphics; no violation of the assumptions was detected. As the effect was significant at a p-value threshold of 0.05, all pairwise comparisons for agitation speed were tested using Tukey's honestly significant difference (HSD) test.

The hierarchical clustering was carried out with the ape package version 5.3 (Paradis and Schliep, 2019) with Euclidean distance and the complete agglomerative method.

3. Results

Sauvignon blanc and Shiraz fermentations were carried out using *K. marxianus, P. burtonii* or *Z. meyerae* in sequential fermentation with *S. cerevisiae* (added 48 h after the non-*Saccharomyces* species). Furthermore, on the basis of previous works (Rollero et al., 2018a), some nitrogen additions (40 or 70 mg N/L for *Z. meyerae* and *K. marxianus*, respectively) were performed at the time of *S. cerevisiae* inoculation, in the form of FermaidO® (for *K. marxianus* and *Z. meyerae*), as well as DAP (a commonly used source of nitrogen in the wine industry), glutamate and glutamine (the key amino acids of nitrogen metabolism in yeasts) or a mixture of amino acids catabolized through the Ehrlich pathway (*K. marxianus*). Fermentation using *S. cerevisiae* in pure culture was performed separately as a control. In the latter fermentation, *S. cerevisiae* was inoculated at time 0 and not at the same time as in the other fermentations in order to mimic an inoculation in an industrial setting. Furthermore, Binati et al. (2020) recently showed that inoculating *the S. cerevisiae* control fermentations at time 0 or at 48h did not result in any significant difference in the final outcome.

3.1. Fermentation kinetics and nitrogen consumption

Overall, the variations observed in the fermentation performances according to the inoculation scenario and the nitrogen nutrition were similar in Shiraz and Sauvignon blanc. All fermentations reached dryness in around 300 h (Sauvignon blanc) and around 168 h (Shiraz) except those carried out with *K. marxianus* in sequential inoculation with *S. cerevisiae* without adding nitrogen. The latter fermentations stopped with 30 g/L of residual sugars in Sauvignon blanc and, although these ran to dryness in Shiraz, but were slower than the other modalities and finished in 192 h (Fig. 1).

As a rule, all the sequential fermentations took longer than *S. cerevisiae* pure fermentations. Interestingly, a 24- to 72-h extended lag phase was observed during fermentations with an early inoculation of *P. burtonii* and *Z. meyerae,* which may explain, at least in part, the increase in the duration of fermentation (Table SD2). In these fermentations, the nitrogen uptake for the first 48 h of fermentation remained very low apart from arginine (*Z. meyerae*) and GABA (*Z. meyerae* and *P. burtonii*) (Tables SD3 and SD4, Fig. 2). While most of the nitrogen sources were depleted by *S. cerevisiae* during the same timeframe, the nitrogen consumption within the first 48 h was comprised between 29 mg N/L (Sauvignon blanc) and 34 mg N/L (Shiraz) and 24 mg N/L (Sauvignon blanc) and 73 mg N/L (Shiraz) for *P. burtonii* and *Z. meyerae*, respectively. Moreover, adding 40 mg N/L nitrogen as FermaidO® during *Z. meyerae/S. cerevisiae* sequential fermentation resulted in a shorter fermentation duration, without affecting the fermentation rate (Fig. 1, Table SD2).

Regarding the fermentation with *K. marxianus* sequentially inoculated with *S. cerevisiae,* a fermentative activity (i.e. CO_2 release or sugar consumption) was observed during the first 48 h although not as strong as that of *S. cerevisiae* alone, together with a substantial consumption of nitrogen (up to 99 mg N/L). Furthermore, the fermentation rate measured after *S. cerevisiae* addition was lower than that of *S. cerevisiae* pure culture (Fig. 1, Table SD2) for both the natural musts. With nitrogen supplementations, the final nitrogen consumption and the fermentation rate were substantially increased, and the fermentations were able to reach dryness regardless of the nature of the nitrogen source added (Table SD2, Fig. 1).

3.2. Formation of compounds from central carbon metabolism

The production of the main compounds deriving from the central carbon metabolism was assessed for each modality at the time of *S. cerevisiae* inoculation and at the end of alcoholic fermentation.

It first appeared that neither the inoculation procedure nor the management of nitrogen nutrition significantly affected the final ethanol content of the wines, comprised between 102 and 105 g/L for Sauvignon blanc and 103 and 107 g/L for Shiraz (Tables SD5 and SD6). Similarly, the content of acetic and succinic acids in wines did not vary substantially according to the inoculation protocol or the addition of nitrogen. Compared to *S. cerevisiae* pure culture, only a slight increase in the final concentration of acetic acid, from 0.56 to 0.69 g/L and from 0.45 to 0.66 g/L for Shiraz and Sauvignon blanc wines, respectively, was observed when *K. marxianus* was sequentially used, while the acetic acid content in Shiraz decreased by 20% as a consequence of an early inoculation with *P. burtonii* (Fig. 3). Regarding the other organic acids, a substantial formation of citric acid was observed between 48 h and the end of fermentation, especially during Shiraz fermentation, irrespective of the fermentation conditions (inoculation, nitrogen nutrition)

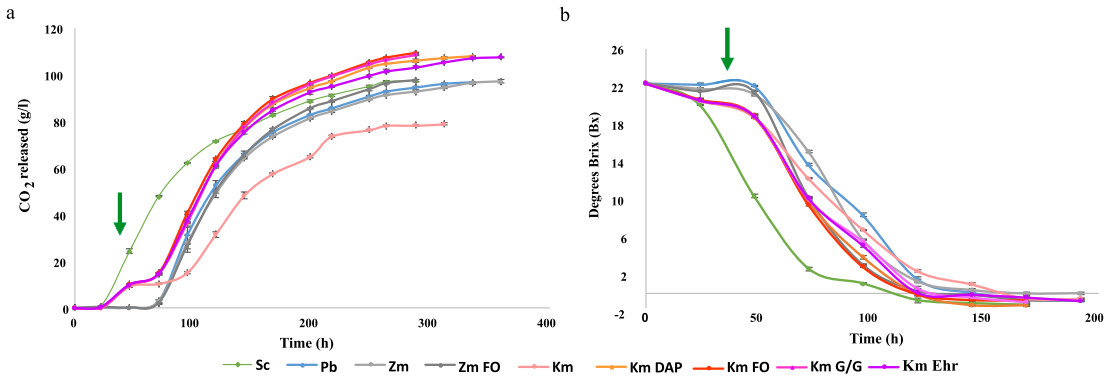

Fig. 1. Fermentation kinetics in Sauvignon blanc (a) and Shiraz (b).

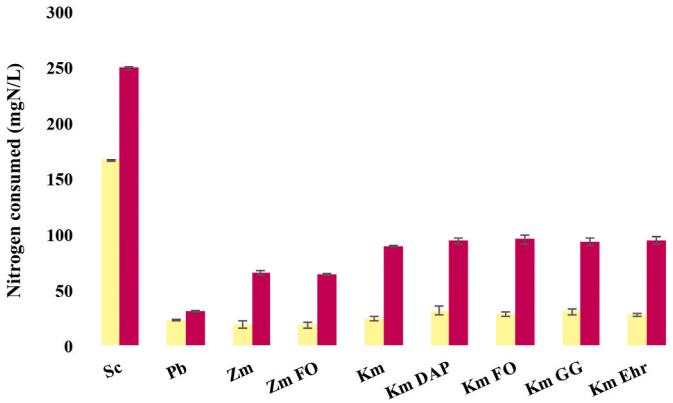

Fig. 2. Consumption of nitrogen (in mgN/L) after 48 h of fermentation in Sauvignon blanc (yellow) and Shiraz (purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Tables SD5 and SD6). The final concentration of citric acid in the wines tended to decrease slightly during sequential fermentations. As an example, the citric acid content in Sauvignon blanc wines obtained by sequential fermentation varied from 0.38 g/L to 0.41 g/L while the concentration in wine produced using *S. cerevisiae* pure culture was 0.46 g/L. With regard to malic acid, only a slight decrease during Shiraz sequential fermentations with *P. burtonii* (1.36 g/L ± 0.09) and *Z. meyerae* (1.59 g/L ± 0.28) compared with *S. cerevisiae* (2.06 g/L ± 0.10) was noteworthy (Tables SD5 and SD6).

Regarding the production of glycerol, different behaviours were evidenced depending on the nature of grape juice (Fig. 3, Table SD5 and SD6). During the Sauvignon blanc fermentations, the production of glycerol within the first 48 h by the non-*Saccharomyces* species was very low compared with *S. cerevisiae*, but without affecting the final production of this compound, comprised between 6.4 and 6.7 g/L for all the fermentation conditions. Conversely, using Shiraz grape juice, the yields of glycerol production from glucose after 48 h of fermentation of *K. marxianus, P. burtonii* and *Z. meyerae* (80, 208 and 139 mg/g of consumed glucose) were higher than that of *S. cerevisiae* (64 mg/g of consumed glucose) (Tables SD5 and SD6). As an expected outcome, the final glycerol content of Shiraz wines produced using a sequential inoculation appeared higher (between 9.5 and 10.2 g/L) than using *S. cerevisiae* in pure culture (8.2 g/L).

3.3. Production of volatile compounds

The comparative analysis of the concentration of volatile compounds measured at the end of each alcoholic fermentation revealed that the production of a number of these molecules was modulated by the species used in sequential fermentation in interaction with the type of grape juice.

First, the important decrease in the formation of propanol during Shiraz sequential fermentation with *P. burtonii, Z. meyerae* and *K. marxianus* compared with *S. cerevisiae* pure culture (by 51%, 34% and 46%, respectively) was not observed in Sauvignon blanc (Fig. 4, Tables SD7 and SD10). Relevant to this, the yields of propanol production from consumed glucose measured after 48 h displayed a lower variability between species on Sauvignon blanc (between 0.34 mg/g of consumed glucose for *P. burtonii* and 0.54 mg/g of consumed glucose for *S. cerevisiae*) than on Shiraz (between 0.41 mg/g of consumed glucose for *Z. meyerae* and 0.92 mg/g of consumed glucose for *S. cerevisiae*).

A decrease in the final content of isobutanol and isoamyl alcohol in wines produced in the sequential fermentations with non-*Saccharomyces*

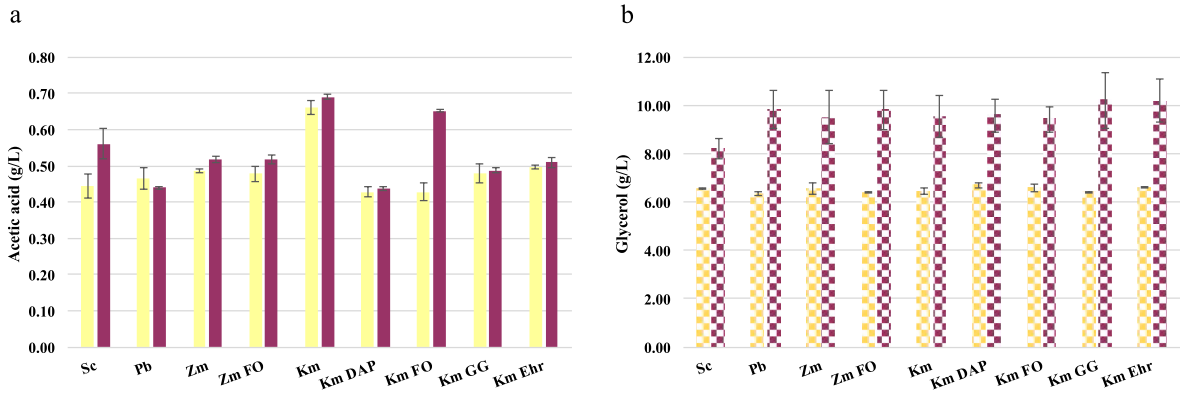

Fig. 3. Production of acetic acid (a) and glycerol (b) at the end of fermentation in Sauvignon blanc (yellow) and Shiraz (purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

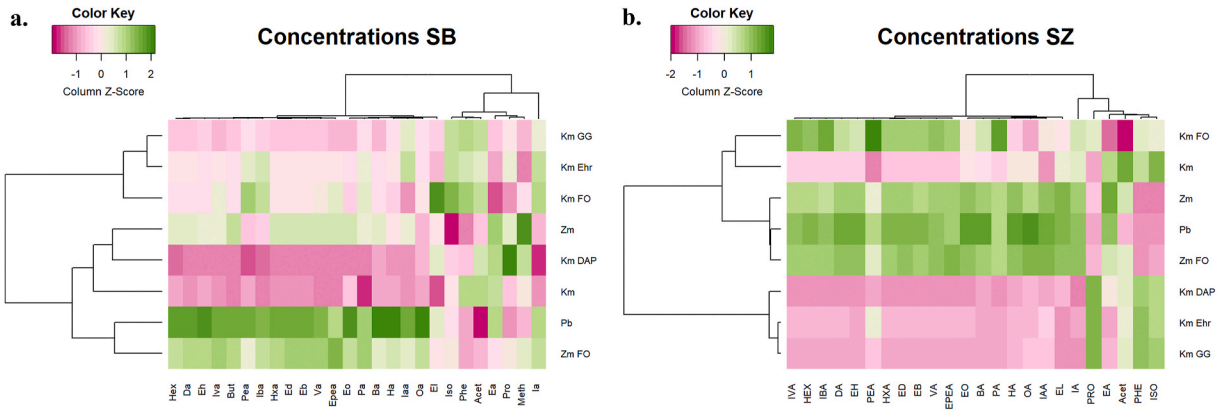

Fig. 4. Relative production of 26 volatile compounds between non-*Saccharomyces* yeast species and nitrogen treatments at the end of fermentation in Sauvignon blanc (a) and Shiraz (b).

species compared to *S. cerevisiae* pure culture was observed (Fig. 4, Table SD7 and SD10). Overall, this decrease was more pronounced in Shiraz than in Sauvignon blanc. This decrease could be mainly attributed to the large differences between the two cultivars observed in the *S. cerevisiae* pure culture fermentations (final isobutanol concentration of 29 and 74 mg/L in Sauvignon blanc and Shiraz, respectively). The range of variation was also species- and fusel alcohol-dependent. Compared to *S. cerevisiae* in pure culture, a greater decrease in isobutanol formation was observed when *Z. meyerae* was used in sequential fermentations (−70%) while an early inoculation with *K. marxianus* resulted in the most important decrease in isoamyl alcohol formation (−79%). Finally, a positive impact of nitrogen addition on the formation of these molecules was evidenced when *Z. meyerae* and *K. marxianus* were sequentially used with *S. cerevisiae* (Fig. 4, Tables SD7 and SD10). During *K. marxianus/S. cerevisiae* sequential fermentation, for which nitrogen has been provided in different forms, the effect depended on the nature of the N-compound added. On both fermenting grape juices, adding amino acids involved in the Ehrlich pathway was the most efficient way to increase the production of isobutanol (+20%) and isoamyl alcohol (+15%) (compared to less than 10% increase when DAP or Glutamate/Glutamine were supplemented). Moreover, an important increase (+62%) in isoamyl alcohol in Shiraz wines produced by sequential fermentation with *K. marxianus* resulted from the addition of FermaidO®, a commercial nutrient formulation which contains amino acids involved in the Ehrlich pathway amongst other nutrients. With respect to the fusel acids counterparts of these higher alcohols (propionic, isobutyric and isovaleric acids), their production decreased (Shiraz) or remained unchanged when sequential fermentations were carried out (Fig. 4., Tables SD8 and SD11). The nitrogen had only a slight incidence on these productions, apart from the addition of FermaidO® during *K. marxianus/S. cerevisiae* sequential fermentation in Shiraz that resulted in the recovery of almost all the fusel acids production of *S. cerevisiae* pure culture.

The production of phenylethanol varied according to grape variety, inoculation method and nitrogen nutrition (Fig. 4, Table SD7 and SD10). During Shiraz fermentations, a substantially lower content in phenylethanol was observed in wines produced using *Z. meyerae* or *P. burtonii* sequentially with *S. cerevisiae* (from 51.6 mg/L (*S. cerevisiae* pure culture) to 24.6 and 23.8 mg/L, respectively) while it was not affected in wines produced with *K. marxianus* (49.3 mg/L). Furthermore, adding nitrogen during sequential fermentation with *K. marxianus* and *S. cerevisiae* resulted in an increase in phenylethanol formation, by 37–53%. Using Sauvignon blanc, the differences in phenylethanol production were less pronounced, but similar patterns were observed. This lower production of phenylethanol in Sauvignon blanc compared to Shiraz could be tentatively correlated with the difference in initial phenylalanine concentrations between the two cultivars (3.04 mg N/L in Sauvignon vs 6.23 mg N/L in Shiraz). However, it is worth noting a 36% increase of the phenylethanol production when *K. marxianus* was inoculated early compared to *S. cerevisiae* pure culture, which was not modulated by providing additional nitrogen (Fig. 4, Tables SD7 and SD10).

Regarding acetate esters, their production was generally increased as a result of sequential fermentations compared to *S. cerevisiae* pure culture, up to 2.5 times (phenylethyl acetate, sequential Sauvignon blanc fermentation with *K. marxianus*), with a positive effect of nitrogen addition in Shiraz medium that was not observed in Sauvignon blanc (Fig. 4, Table SD7 and SD10). The major exception to this general pattern was related to the formation of phenylethyl acetate during Shiraz fermentations, which was strongly decreased when sequential inoculations were performed (from 2.5 to 0.7 mg/L with *Z. meyerae* or *P. burtonii*). A decrease was also detected during *K. marxianus/S. cerevisiae* fermentation (1.8 mg/L), but in this case, adding nitrogen allowed to restore and improve the phenylethyl acetate production at levels higher than those of the control fermentation (2.5 mg/L): from 3.8 mg/L with DAP, FermaidO® and glutamate/glutamine additions to 5.0 mg/L with addition of amino acids involved in the Ehrlich pathway including phenylalanine (Fig. 4, Tables SD7 and SD10).

Finally, no significant impact of both the inoculation modality and the nitrogen nutrition was observed on the formation of medium chain fatty acids and their ethyl ester derivatives during Sauvignon blanc fermentation (Fig. 4a, Table SD8). On the contrary, compared to pure cultures, the final contents of these classes of volatile compounds in Shiraz wines were greatly increased when sequential fermentations were achieved, irrespective of the non-*Saccharomyces* species, with up to 10-fold increase factors in the case of ethyl octanoate (Fig. 4b, Table SD11). The only point to be highlighted as regards the incidence of nitrogen nutrition on the formation of medium-chain fatty acids and their ethyl ester derivatives was the major negative effect of the addition of FermaidO®.

3.4. Formation of sulphur compounds

The production of 15 volatile sulphur compounds (VSCs) was measured at the end of alcoholic fermentation, for each modality. First, important differences in the profiles of VSCs were observed according to the nature of the grape juice (Shiraz versus Sauvignon blanc) (Fig. 5a and b, Tables SD9 and SD12). During fermentations on Shiraz, the production of the main sulphur compounds synthetized from methionine intermediate (i.e. methional, methionol, acid-3-methylthiopropanoic, ethyl-3-methylthio propanoate, S-methyl-thioacetate and 4-methylthiobutanol) was more important than that measured on Sauvignon blanc, except for 3-methylthiopropyl acetate (Fig. 5a and b, Table SD9 and SD12). In contrast, Sauvignon blanc

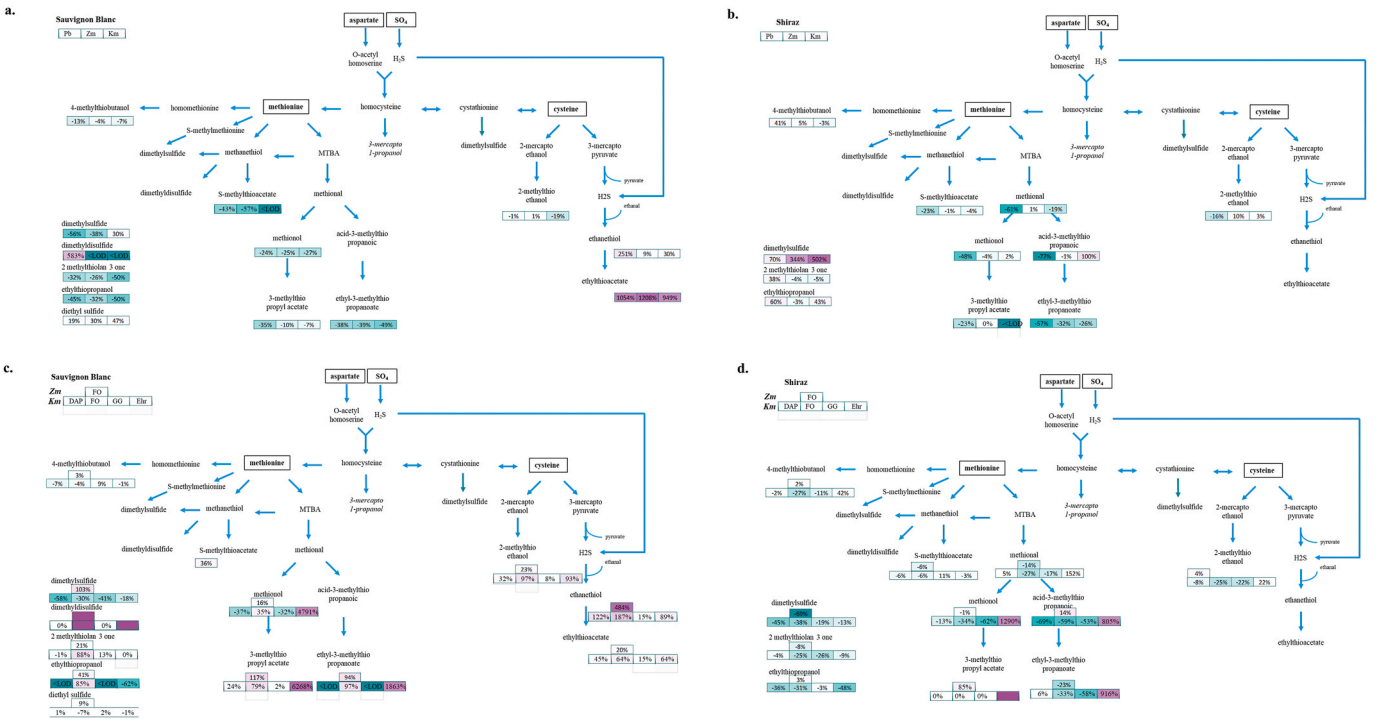

Fig. 5. Sulphur compound production at the end of fermentation in Sauvignon blanc (a,c) and Shiraz (b,d).

fermentations were characterised by an important formation of dimethyl sulphide, diethyl sulphide and dimethyl disulphide that was not observed on Shiraz, apart from a low formation of dimethyl sulphide (Fig. 5a and b, Tables SD9 and SD12). Furthermore, the formation of ethanethiol and ethylthioacetate from cysteine was favoured on Sauvignon blanc, at the expense of 2-methylthioethanol, while on Shiraz, 2-methylthioethanol was the only produced VSC deriving from cysteine catabolism.

The comparative analysis of the VSCs final concentrations also revealed that conducting sequential fermentation greatly modified the profile of production of these molecules, according to the non-*Saccharomyces* species inoculated and the grape juice fermented. During Sauvignon blanc fermentation, similar variations were observed using *P. burtonii, Z. meyerae* or *K. marxianus* in sequential fermentation with *S. cerevisiae*, starting with a huge increase in the formation of metabolites deriving from the combination of ethanol with H_2S, ethanethiol (by a factor up to 3.5 for *P. burtonii*) and ethylthioacetate (by a factor up to 13 for *Z. meyerae*) (Fig. 5a and b, Tables SD9 and SD12). The production of diethyl sulphide was also increased for all the species, but in a lesser extent (from 19% to 47%). The formation of 2-methylthioethanol, originating from the catabolism of cysteine, was not significantly affected, with only a decrease by 20% during sequential fermentation involving *K. marxianus*. Conversely, the production of all the molecules derived from methionine catabolism and other VSCs (2-methylthiolan-3-one, ethylthiopropanol) was usually decreased, with a degree depending on both the strain and the compound (Fig. 5a and b, Tables SD9 and SD12). For example, the final content of wines in ethyl-3-methylthiopropanoate was decreased by 40% during sequential fermentation with *P. burtonii* and *Z. meyerae* and up to 50% with *K. marxianus* and the formation of *S*-methylthioacetate was entirely removed only when *K. marxianus* was sequentially used with *S. cerevisiae*. Finally, it was noteworthy that a production of dimethyldisulphide was only observed during *P. burtonii/S. cerevisiae* sequential fermentation (Fig. 5a and b, Tables SD9 and SD12). A quite different pattern of variations in VSCs production according to the used species was observed during Shiraz fermentations (Fig. 5a and b, Tables SD9 and SD12). Under these conditions, the formation of 2-methylthioethanol was only slightly affected during *P. burtonii/S. cerevisiae* fermentation (−16%). The formation of VSCs related to methionine catabolism was strongly dependent on the non-*Saccharomyces* strain used in combination with *S. cerevisiae*. With *P. burtonii,* the formation of all these metabolites was substantially reduced, from 23% (S-methylthioacetate) to 77% (3-methylthiopropanoic acid). An early inoculation with *Z. meyerae* did not significantly affect the final content in methionine-derived VSCs of Shiraz wines, apart from ethyl-3methylthioproanoate, reduced by 32% (Fig. 5a and b, Tables SD9 and SD12). Finally, fermentations for which *K. marxianus* was sequentially used with *S. cerevisiae* were characterised by opposite variations focused on metabolites deriving from methional, with a strong increase in the formation of 3-methylthiopropionic acid (53 mg/L compared with 26 mg/L during *S. cerevisiae* pure culture) while the formation of 3-methylthiopropyl acetate was abolished (under LOD) (Fig. 5a and b, Tables SD9 and SD12). Species-dependent higher production was observed for 2-methylthiolan-3-one (+38% during *P. burtonii/S. cerevisiae* fermentation) and for ethylthiopropanol (increased by 60 and 43% when *P. burtonii* and *Z. marxianus* were sequentially used with *S. cerevisiae*, respectively) (Fig. 5a and b, Tables SD9 and SD12).

Nitrogen addition triggered a differential response in terms of VSCs formation, depending on the strains, the nature of the N-compound provided, and the grape juice considered (Fig. 5c and d, Tables SD9 and SD12). Adding nitrogen with *S. cerevisiae* during Sauvignon blanc sequential fermentation with *Z. meyerae* or *K. marxianus* resulted in an increased formation of ethanethiol, ethylthioacetate and 2-methylthioacetate (Fig. 5c and d, Tables SD9 and SD12). The most important variations were observed with FermaidO® or Ehrlich amino acids supplementations, up to a 4.8- and 1.8-fold increase in ethanethiol production observed adding FermaidO® during *Z. meyerae* or *K. marxianus* sequential fermentation with *S. cerevisiae*, respectively (Fig. 5c and d, Tables SD9 and SD12). Regarding the formation of methionine-derived metabolites, as expected, an increase was shown when the medium was supplemented with FermaidO® (by 35%–117%, depending on the VSCs) or especially with the Ehrlich amino acids mixture (including 600 μM methionine), which resulted in increases ranging from 20 times (ethyl-3-methylthiopropanoate) to 64 times (3-

methylthiopropyl acetate) (Fig. 5c and d, Tables SD9 and SD12). In addition, the formation of 2-methylthiolan-3-one and ethylthiopropanol were specifically increased by FermaidO® addition (up to 80%), regardless the strain used sequentially with *S. cerevisiae,* and remained unchanged or decreased for the other nutrition procedures (Fig. 5c and d, Tables SD9 and SD12). Conversely, the response to nitrogen addition in terms of dimethyl sulphide production depended only on the species, with an increase by a factor 2 when *Z. meyerae* was used and a decrease ranging from 18% to 58% during *K. marxianus/S. cerevisiae* sequential fermentation (Fig. 5c and d, Tables SD9 and SD12).

A decrease in the formation of VSCs was usually observed as a consequence of nitrogen addition during Shiraz sequential fermentation, irrespective of the N-compound added. As an example, the formation of dimethylsulphide decreased from 13% to 69%, and very low final concentrations of 3-methylthiopropyl acetate, below the limit of detection, were measured in wines produced by implementing nitrogen nutrition management (Fig. 5c and d, Tables SD9 and SD12). However, two main exceptions must be pointed out. First, as reported for Sauvignon blanc fermentations, adding a mixture of Ehrlich amino acids had a huge impact on the production of all the methionine-derived VSCs, with factors of increase ranging from 9 to 39 times. Then, when *Z. meyerae* was sequentially inoculated with *S. cerevisiae*, an increase in both 3-methylthiopropanoic acid and 3-methylthiopropyl acetate was observed as a consequence of FermaidO® supplementation (Fig. 5c and d, Tables SD9 and SD12).

3.5. Preference ranking

All wines were subjected to sensory evaluation during which the tasting panel was requested to perform a preference ranking. The wine obtained from the fermentation of Sauvignon blanc by *K. marxianus* supplemented with the Ehrlich amino acid mixture stood out since this white wine was significantly the least preferable to the panel. This is likely due to the higher levels of sulphur compounds, such as DES, ETA, DMDS, 2MTE, E3MTP and ME, that occurred in these wines. Indeed, the difference between rank sums exceeded the critical value of 105 as determined from the "Expanded tables for multiple comparison procedures in the analysis of ranked data" (Newell and MacFarlane, 1987). The higher the rank sum, the less the wine was preferred by the panel. The other white wines were not significantly different from each other since the rank sum differences remained below the critical value (Table 2a).

Regarding the red wines, the results were more complicated to interpret. The wines made with *K. maxianus* supplemented with the mixture of Ehrlich amino acids differed significantly from those made with *S. cerevisiae*, *P. burtonii*, *Z. meyerae* and *K. marxianus* supplemented with Glutamate and Glutamine (rank sums exceeded the critical value of 101.8), but not from those made with *Z. meyerae* supplemented with FermaidO®, *K. marxianus* supplemented with DAP or FermaidO® (Table 2b). Nevertheless, similarly to the white wines, the Shiraz wines originating from the fermentations by *K. marxianus* and supplemented with the mixture of Ehrlich amino acids were least preferred.

4. Discussion

This study was conducted to further investigate metabolisms of oenological relevance in three selected non-*Saccharomyces* yeasts during sequential fermentations with *S. cerevisiae* in real grape juice, in comparison with the results obtained by Rollero et al. (2018a) in synthetic grape juice.

The different fermentations carried out in Sauvignon blanc and Shiraz exhibited overall kinetics similar to those previously reported by Rollero et al. (2018a), thereby confirming that the results obtained in synthetic grape juice can reliably mimic fermentation kinetics in red and white grape juices. If the temperature (15 °C for Sauvignon blanc vs 25 °C for Shiraz) was certainly the main driver of differences in fermentation duration, the potential impact of the large difference in the initial concentration of yeast assimilable nitrogen between the two cultivars cannot be fully excluded.

Following the inoculation of *S. cerevisiae* 48 h after the other yeast species, fermentation kinetics accelerated, thereby demonstrating that *S. cerevisiae* rapidly took over the fermentations except for *K. marxianus* without addition of nitrogen. In this scenario, the fermentations did not reach dryness. A similar outcome was reported by Rollero et al. (2018a) most likely as a result of the large consumption of nitrogenous compounds by *K. marxianus* within the first 48 h prior to *S. cerevisiae* inoculation (Tables SD2 and SD3). In addition, the latter authors also reported that, under similar fermentation conditions, *K. marxianus* population persisted much longer than the other non-*Saccharomyces* yeast species considered in this study (Rollero et al., 2018a). This probably delayed the release of nitrogenous compounds through autolysis for *S. cerevisiae* to ferment to dryness. The nutrient supplementations suggested by Rollero et al. (2018a) were successful in ensuring fermentation completion with no sluggish fermentation, thereby confirming the two types of competitions previously reported: a competition for nitrogen with *K. marxianus* and a competition for another nutrient with *Z. meyerae*. Indeed, *Z. meyerae* did not consume much YAN from the medium (Tables SD2 and SD3) and the amount left

Table 2
Preference ranking results for Sauvignon blanc (a) and Shiraz (b) a.b.

	Sc	Pb	Zm	Zm FO	Km DAP	Km FO	Km GG	Km Ehr	Rank sum
Sc	0	0	2.5	6.5	58	44.5	36	192.5	385
Pb	0	0	2.5	6.5	58	44.5	36	192.5	385
Zm	2.5	2.5	0	4	55.5	42	33.5	190	387.5
Zm FO	6.5	6.5	4	0	51.5	38	29.5	186	391.5
Km DAP	58	58	55.5	51.5	0	13.5	22	134.5	443
Km FO	44.5	44.5	42	38	13.5	0	8.5	148	429.5
Km GG	36	36	33.5	29.5	22	8.5	0	156.5	421
Km Ehr	192.5	192.5	190	186	134.5	148	156.5	0	577.5
	Sc	Pb	Zm	Zm FO	Km DAP	Km FO	Km GG	Km Ehr	Rank sum
Sc	0	21.5	1.5	67.5	51.5	95.5	9.5	152	369
Pb	21.5	0	23	46	30	74	12	130.5	390.5
Zm	1.5	23	0	69	53	97	11	153.5	367.5
Zm FO	67.5	46	69	0	16	28	58	84.5	436.5
Km DAP	51.5	30	53	16	0	44	42	100.5	420.5
Km FO	95.5	74	97	28	44	0	86	56.5	464.5
Km GG	9.5	12	11	58	42	86	0	142.5	378.5
Km Ehr	152	130.5	153.5	84.5	100.5	56.5	142.5	0	521

The conditions in red are significantly different from the others, >101.8 (critical value)Sc: *S. cerevisiae*; Pb: P. burtonii; Zm: Z. meyerae; Km: K. marxianus FO: with addition of FermaidO®; DAP: with addition of DAP; GG: with addition of glutamine and glutamate; Ehr: with addition of amino acids involved in Ehrlich pathway.

in the medium after 48h allowed *S. cerevisiae* to ferment to dryness. Yet, while the addition of FermaidO® increased the overall YAN concentration minimally, it resulted in an acceleration of the overall fermentation kinetics, thereby suggesting that this acceleration was facilitated by the addition of nutrients other than nitrogenous compounds present in FermaidO® (e.g. vitamins, lipids).

The quantification of a large number of metabolites revealed no major differences for compounds directly originating from Central Carbon Metabolism (CCM), apart from glycerol. However, marked differences were observed in the production of aroma compounds such as higher alcohols, esters and sulphur compounds, according to the fermentation scenario (grape cultivar, inoculated yeasts and nitrogen supplementation). Among the different factors of these scenarios, the initial YAN composition, which differed greatly between the two cultivars (Table 1), played a critical role, but the extent of the differences in terms of metabolite production, could be directly correlated to the non-*Saccharomyces* yeast used, thereby suggesting a different management of certain nutrients, especially amino acids, as recently reported (Rollero et al., 2019; Seguinot et al., 2020a).

Overall, the production of propanol was higher in Shiraz than in Sauvignon blanc, most certainly in direct connection with the initial YAN concentration (200 vs 320 mg/L in Sauvignon blanc vs Shiraz) since propanol has been previously identified as a marker of the overall availability in nitrogen compounds (Mouret et al., 2014; Seguinot et al., 2018). Indeed, although most higher alcohols produced by yeasts originate from a dual source (sugars and specific amino acids), propanol only originates from the metabolism of threonine and indirectly from that of aspartate and asparagine, direct precursors of threonine, as well as that of serine which may be catabolized into glycine then threonine. Yet, all these amino acids were present in much higher concentrations in Shiraz than in Sauvignon blanc (increase factor between 37% and 100%, Table 1). Yeast species variability was also observed with the non-*Saccharomyces* yeasts producing less higher alcohols than *S. cerevisiae*, in line with the consumption kinetics of amino acids precursors of propanol (and that of the overall sugar consumption). This species variability was also reported previously (Rollero et al., 2018). Coincidently, glycerol production varied similarly to that of propanol. The production of glycerol is mostly driven by redox homeostasis (i.e. maintenance of the NAD^+:NADH ratio) and therefore to the extent of yeast growth, which is in turn related to nitrogen availability (Varela et al., 2004). Furthermore, the yields of production of glycerol from glucose are strain/species-dependent, the non-*Saccharomyces* species showing generally a higher capacity to produce glycerol than *S. cerevisiae* (Englezos et al., 2018; Seguinot et al., 2020b; this study). In Sauvignon blanc, the non-*Saccharomyces* yeasts consumed very small amounts of nitrogen within the first 48h. This resulted in a limited impact on *S. cerevisiae*'s metabolism in terms of nitrogen availability and glycerol production. However, in the Shiraz fermentations, the consumption of nitrogen by the non-*Saccharomyces* yeasts was greater and glycerol was produced during the first 48 h of fermentation by these species with higher yields of production from glucose. As a result, the glycerol concentration in wines obtained by sequential Shiraz fermentation were higher than that of wines produced using *S. cerevisiae* pure cultures.

Vast differences were observed in the production of phenylethanol, isobutanol and isoamyl alcohols between yeast species. Cultivar differences were also observed, in correlation with the different initial YAN concentrations previously mentioned. These can most probably be attributed to differences in metabolic fluxes, since these higher alcohols originate from the catabolism of both sugars and amino acids, as already mentioned. Indeed, as previously reported, *K. marxianus*' metabolic fluxes differ from those of *S. cerevisiae* with regard to the catabolism of certain amino acids (Rollero et al., 2019). For instance, the low production of isoamyl alcohol in the fermentations carried out by *K. marxianus* and *S. cerevisiae* mixed culture can likely be attributed to the limited contribution of the CCM in the production of this higher alcohol in *K. marxianus* compared to *S. cerevisiae* (Rollero et al., 2019). These metabolic fluxes are likely to differ from species to species, explaining the differences observed in this study but further work is required to confirm this hypothesis. Furthermore, the addition of nitrogen resulted in an increase in the final concentration of these fermentative aroma compounds, which was dependent on the nature of the nitrogenous compounds added. Nevertheless, the addition of a specific nitrogen precursor was not directly proportional to that of the corresponding higher alcohol (Clement et al., 2013). With regard to phenylethanol, the substantial production by *K. marxianus* similar to that of *S. cerevisiae* was previously reported (Rollero et al., 2019, 2018a), although in the 2 species, the ratio of phenylethanol originating from sugar and phenylalanine metabolism differs (Rollero et al., 2019). In *K. marxianus*, it mostly derives from the catabolism of phenylalanine, which explains why the production of phenylethanol is greater in Shiraz than in Sauvignon blanc (3.04 mgN/L phenylalanine in Sauvignon blanc vs 6.23 mgN/L in Shiraz) and also why the increase is significant when Ehrlich amino acids were supplemented. The increase in the production of phenylethanol when other amino acids were added is likely due to the presence of phenylalanine in the complex nutrient formulation FermaidO® or a probable higher biomass production, especially for the pair glutamate/glutamine, as previously reported for *S. cerevisiae* (Gutiérrez et al., 2012). These comments related to phenylethanol could be extended to phenylethyl acetate, which is constantly overproduced by *K. marxianus* (Rollero et al., 2019, 2018a,b). Nevertheless, the final concentrations of acetate esters, medium chain fatty acids and their corresponding ethyl esters did not follow a generic specific pattern highlighted above for higher alcohols (Tables SD4 and SD5). This could be tentatively explained by the fact that their production responds to different mechanisms or substrates (e.g. availability of acetyl-coA).

To the best of the authors' knowledge, this is the first study to report so extensively on the production of volatile sulphur compounds (VSCs) in wine, especially in non-*Saccharomyces* yeasts. VSCs production profiles were, as with most volatile compounds, highly variable depending on the grape juice used for fermentation. The Shiraz wines were overall characterised by a higher production of Ehrlich derivatives originating from the catabolism of methionine, probably in connection to the higher initial concentration of methionine in the Shiraz grape must, almost twice as high as in Sauvignon blanc. However, Sauvignon blanc was characterised by a large production of dimethyl sulphide, diethyl sulphide and dimethyl disulphide. These enhanced concentrations of sulphur compounds (especially diethyl sulphide whose concentration was much higher than its sensory detection threshold) were negatively perceived by the sensory evaluation panel. Indeed, dimethyl sulphide smells like onion, asparagus and corn (odour threshold: 10–160 μg/L in wine); diethyl sulphide like onion, garlic and cooked vegetables (odour threshold: 0.93–18 μg/L) and dimethyl disulphide like onion and cabbage (odour threshold: 20–45 μg/L) (Mestres et al., 2000). It is likely that they even overshadowed possible differences in preference between the treatments. In order to better apprehend these differences, treatments that generated off-flavours (as observed in the chemical analysis) should be excluded from the sensory evaluation. A drastic difference in the production of DMDS between Shiraz and Sauvignon blanc was observed regardless of the yeast species. The lower concentration of DMDS in the Shiraz wines compared to the Sauvignon blanc wines is likely due to the much higher pH occurring in Shiraz (3.7 vs 3.18 in Sauvignon blanc). Indeed, Lu et al. (2018) showed that the diethyl disulphide:ethanethiol ratio was pH-dependent, this ratio decreasing as pH increases. A similar mechanism could be proposed for the DMDS: DMS ratio, explaining the differences in DMDS production between Shiraz and Sauvignon blanc fermentations.

These findings are consistent with previous studies reporting differences in the production of VSCs depending on both the fermentation media and the used *S. cerevisiae* strain (Kinzurik et al., 2016; Patrignani et al., 2016).

The production of VSCs was highly impacted by the presence of non-*Saccharomyces* yeasts.

Indeed, a high production of sulphur compounds originating from H_2S but a lower production of methionine Ehrlich derivatives were evidenced during sequential fermentation combining *P. burtonii*, *Z. meyeriae* or *K. marxianus* with *S. cerevisiae*, thereby overall showing a different sulphur management in these species compared to *S. cerevisiae*. The high production of VSCs originating from H_2S metabolism was even higher when nutrients were added to the fermentations. This could be linked to a higher production of H_2S and its derivatives from sulphates and in turn reveal either a lower requirement in sulphur than *S. cerevisiae* or an excessive uptake of sulphates, both resulting in a higher production of ethylthioacetate, possibly as a means to detoxify excess H_2S intracellularly. This hypothesis could also apply to the production of VSCs originating from methionine metabolism. Indeed, the addition of methionine (directly as part of the mixture of Ehrlich AA or indirectly as part of the complex FermaidO® nutrient formulation) resulted in an increase in the production of Ehrlich-derived VSCs.

5. Conclusion

This study confirmed the key role of yeast nitrogen metabolism on the final composition of wine. Indeed, the tight management of the yeast assimilable nitrogen (i.e. its amount and composition) is a potentially powerful tool to modulate wine aroma profiles, as revealed by the chemical analyses. However, the sensory analysis demonstrated that, as expected, high concentrations of sulphur compounds, as an outcome of the presence of methionine in the amino acid mixture supplemented, are not desirable. This study also confirmed that metabolic fluxes vary between yeast species, thereby resulting in different outcomes, but the factors driving these fluxes in different yeast species and strains are not yet fully understood. Despite the negative sensory outcome of the production of high concentrations of sulphur compounds, novel insights into yeast sulphur metabolism, which remains poorly characterised, emerged from this study. Further research should be conducted to fully unravel these metabolic fluxes and the factors (i.e. genetic background, environmental conditions) driving them in order to better exploit the yeast potential.

Acknowledgements

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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.2020.103650.

Sc: *S. cerevisiae*; Pb: *P. burtonii*; Zm: *Z. meyerae*; Km: *K. marxianus*.

FO: with addition of FermaidO®; DAP: with addition of DAP; GG: with addition of glutamine and glutamate; Ehr: with addition of amino acids involved in Ehrlich pathway. The arrow indicates the time of *S. cerevisiae* addition in sequential inoculations.

Sc: *S. cerevisiae*; Pb: *P. burtonii*; Zm: *Z. meyerae*; Km: *K. marxianus*.

FO: with addition of FermaidO®; DAP: with addition of DAP; GG: with addition of glutamine and glutamate; Ehr: with addition of amino acids involved in Ehrlich pathway.

Sc: *S. cerevisiae*; Pb: *P. burtonii*; Zm: *Z. meyerae*; Km: *K. marxianus*.

FO: with addition of FermaidO®; DAP: with addition of DAP; GG: with addition of glutamine and glutamate; Ehr: with addition of amino acids involved in Ehrlich pathway.

Sc: *S. cerevisiae*; Pb: *P. burtonii*; Zm: *Z. meyerae*; Km: *K. marxianus*.

FO: with addition of FermaidO®; DAP: with addition of DAP; GG: with addition of glutamine and glutamate; Ehr: with addition of amino acids involved in Ehrlich pathway.

Pro: propanol, Ia: isoamyl alcohol, Iso: isobutanol, Phe: phenylethanol, Meth: methionol, Hex: hexanol, Ea: ethyl acetate, Iaa: isoamyl acetate, Hxa: hexyl acetate, Pea: phenylethyl acetate, Pa: propanoic acid, Iba: isobutyric acid, Iva: isovaleric acid, Va: valeric acid, El: ethyl lactate, Epea: Ethyl phenylacetate, Ba: butyric acid, Ha: hexanoic acid, OA: octanoic acid, DA: decanoic acid, Eb: ethyl butyrate, Eh: ethyl hexanoate, Eo: Ethyl octanoate, Ed: ethyl decanoate, Acet: acetoin.

a,b: impact of yeast species (non-*Saccharomyces* yeasts vs *S. cerevisiae*).

c,d: impact of nitrogen addition on the production of sulphur compounds.

Legends are displayed in the top left corner of each panel.

Sc: *S. cerevisiae*; Pb: *P. burtonii*; Zm: *Z. meyerae*; Km: *K. marxianus*.

FO: with addition of FermaidO®; DAP: with addition of DAP; GG: with addition of glutamine and glutamate; Ehr: with addition of amino acids involved in Ehrlich pathway.

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