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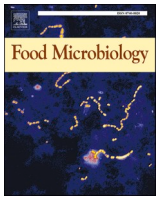

Insights into intraspecific diversity of central carbon metabolites in *Saccharomyces cerevisiae* during wine fermentation

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ABSTRACT

Saccharomyces cerevisiae is a major actor in winemaking that converts sugars from the grape must into ethanol and CO_2 with outstanding efficiency. Primary metabolites produced during fermentation have a great importance in wine. While ethanol content contributes to the overall profile, other metabolites like glycerol, succinate, acetate or lactate also have significant impacts, even when present in lower concentrations. *S. cerevisiae* is known for its great genetic diversity that is related to its natural or technological environment. However, the variation range of metabolic diversity which can be exploited to enhance wine quality depends on the pathway considered. Our experiment assessed the diversity of primary metabolites production in a set of 51 *S. cerevisiae* strains from various genetic backgrounds. Results pointed out great yield differences depending on the metabolite considered, with ethanol having the lowest variation. A negative correlation between ethanol and glycerol was observed, confirming glycerol synthesis as a suitable lever to reduce ethanol yield. Genetic groups were linked to specific yields, such as the wine group and high α-ketoglutarate and low acetate yields. This research highlights the potential of using natural yeast diversity in winemaking. It also provides a detailed data set on production of well known (ethanol, glycerol, acetate) or little-known (lactate) primary metabolites.

1. Introduction

Fermented products have today a great importance in human societies, both economically and socially. Throughout history, humans and fermentation have shared a long path: the first trace of cereal fermentation has been found in Israel and estimated to date back to 13000 B.C. (Liu et al., 2018) and the first known fermented beverage from rice, honey, and a fruit, has been traced back to 7000 B.C. in China (McGovern et al., 2004). Since then, fermentation uses have expanded into a wide diversity of processes and products, such as food, beverages or more recently biofuels. In alcoholic beverages, alcoholic fermentation is the main step of elaboration and is mostly carried out by yeasts of the *Saccharomyces* genus, especially the species *Saccharomyces cerevisiae*. A perfect example is wine, which is the result of the alcoholic fermentation of grapes or grape juice. From a technological point of view, wine fermentation is the biotransformation of glucose and fructose, existing in equal proportions in grapes, into carbon dioxide and ethanol, which imparts new characteristics to the product such as sensory qualities and stability (Sablayrolles 2008).

Alcoholic fermentation is of high technological interest as well as of metabolic importance for *Saccharomyces cerevisiae*. Through glycolysis, this biological process generates pyruvate and energy in the form of ATP. Pyruvate, which is a central metabolite, is then converted in two steps into ethanol and carbon dioxide, which ensures a rapid reoxidation of enzymatic cofactors used in glycolysis, making alcoholic fermentation the most efficient way to promptly supply energy to the cell (Bakker et al., 2001). Moreover, in typical wine making conditions, this is the only way for *S. cerevisiae* to produce ATP, respiration being repressed by the Crabtree effect or impossible due to the absence of dioxygen (De Deken, 1966; Pfeiffer and Morley, 2014). The main products of fermentation, carbon dioxide and ethanol, are by far the most produced metabolites during alcoholic fermentation and therefore in wine making (Nidelet et al., 2016). A simple way to compare these productions between species, strains or fermentation conditions is to define yield as the ratio of the quantity of metabolite produced per unit of substrate consumed. Ethanol yield in wine fermentations carried out by

Abbreviations: CCM, Carbon Central Metabolism; GM, Genetically Modified; CV, Coefficient of Variation; α−KG, α-Ketoglutarate; TCA, Tricarboxylic Acid; YAN, Yeast Assimilable Nitrogen.

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S. cerevisiae is known to reach around 0.47 g per gram of hexoses consumed, which represent 92% of the maximum theoretical yield (calculated as 1 mol of glucose producing 2 mol of ethanol) (Tilloy et al., 2015). Most of the remaining hexoses are used as a carbon source for cell multiplication and the production of other metabolites in minor concentrations, such as glycerol, acetate, succinate, acetaldehyde, lactate, etc. These metabolites represent much lower carbon fluxes, but can be of significant technological value. Glycerol, which is linked to stress resistance, can impact wine mouthfeel above a certain concentration (Albertyn et al., 1994; Noble and Bursick 1984). It has been identified as the second most produced metabolite in fermentation and as the flux with the greatest impact on ethanol production (Goold et al., 2017). Acetate, which is a way to restore redox balance in the cell and a metabolic intermediary, is a major off-flavour-linked compound and subject to legal limits (Vilela-Moura et al., 2008). It appears that yields of fermentation metabolites such as glycerol or acetate are linked to the degree of domestication of the producing strains, as these metabolites are linked to positive or unwanted wine properties (Tapia et al., 2018).

In most studies, the carbon metabolites considered are those most present in fermentation, which are final steps of metabolic pathways and therefore important markers: ethanol, glycerol, succinate, or α-ketoglutarate. However, other carbon metabolites are evoking an increasing interest, as they can deeply shape the sensorial identity of wine, in particular malic acid (Vion et al., 2023) or lactic acid (with a particular focus on yeast species other than *S. cerevisiae*, which is commonly considered as a very poor producer) (Vicente et al., 2021). Other minor metabolites are synthesised at very low concentrations. and, despite their low levels, they have a significant impact on the final fermented product, with examples including organic acids, higher alcohols and esters. Consequently, their production mechanisms have been extensively studied (Antonelli et al., 1999; Regodón Mateos et al., 2006).

For all compounds, yield values differ according to strain and fermentation conditions (oxygenation, temperature, nutrients concentrations or even presence of other microorganisms) (Du et al., 2012; Tronchoni et al., 2022) but the range of variation for ethanol remains very limited compared to that observed for biomass or other metabolites. In their work, Nidelet et al. (2016) compared 43 strains from six different ecological origins and showed that the coefficient of variation of carbon flux toward ethanol synthesis following glycolysis and alcoholic fermentation is only between 2 and 3%. In a contrasting way, yields of glycerol or acetate have a respective variation of around 10 and 30% although they represent significantly lower carbon fluxes for the cell (Camarasa et al., 2011; Nidelet et al., 2016). Generally, yields are calculated at fixed points of the fermentation, such as 80% of hexoses consumed or during the exponential phase. One of the reasons for these choices is that ethanol yield is not constant during fermentation, apart during the exponential growth phase which is the only stage with a quasi-steady state (Celton et al., 2012; Nidelet et al., 2016; Quirós et al., 2013). In order to compare yield of different strains, it is necessary to calculate it at the same quantity if hexoses consumed, which is ideally when the fermentation has been completed in a dry wine production context.

Over the past thirty years, considerable research efforts have been made to understand and influence primary metabolism, mainly with the aim of reducing wine final ethanol content. Beside physical or chemical methods, many microbial strategies have been developed to modify ethanol production during fermentation. We can cite here genetically modified yeast strains, hybrid strains or optimisation through adaptive laboratory evolution (reviewed in Varela and Varela, 2019). However, modulating the central carbon metabolism (CCM) without disturbing the cell balance still remains complex in a wine context, mostly because of the multigenic basis of the associated traits (Bro et al., 2006; Hubmann et al., 2013a; Hubmann et al., 2013b; Salinas et al., 2012). Therefore, elaborating approaches to develop *S. cerevisiae* strains with a modified primary metabolic yield in wine fermentation requires to clearly identify the diversity of central metabolism as well as its constraints and trade-offs.

In this context, our study presents the outcomes of a screening applied to 51 strains from different origins in order to identify the range of variability in primary fermentation metabolite yields under laboratory wine-like conditions among the species *S. cerevisiae*.

2. Materials and methods

2.1. Strains

51 strains of *S. cerevisiae* were used (see information in supplementary data (S1)).

Strains were selected considering results from precedent works in the laboratory, with the aim to maximise diversity in fermentation profiles (Camarasa et al., 2011; Legras et al., 2018; Nidelet et al., 2016). EC1118 was chosen as a reference strain to estimate block effect. Genetically modified (GM) and laboratory strains evolved for precise CCM traits were also included. Strains were conserved at −80 °C in 20% glycerol YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and cultivated on YPD agar plate (YPD + 20 g/L agar).

2.2. Genetic groups constitution

Strains from various genetic backgrounds, but all linked to fermented beverages, are represented in the set. 37 out of 51 were sequenced as part of previous works (Akao et al., 2011; Eder et al., 2018; Fay and Benavides, 2005; Liti et al., 2009; Marsit et al., 2015; Novo et al., 2009; Schacherer et al., 2009). To classify and organise intraspecific diversity, two works were used to define the following genetic groups: wine, rum, West African, sake and flor (Legras et al., 2018; Peter et al., 2018). Strains without information were labelled as “Unknown”. A supplementary group, labelled as “Miscellaneous”, was used to assemble those strains with mosaic, very singular or unclassifiable genomes, but was not used as a consistent group like the others.

2.3. Fermentation conditions

Fermentation conditions were chosen to ensure a quick and complete alcoholic fermentation. One colony was grown overnight in YPD medium as pre-culture. Then 10^6 cells/ml of this pre-culture were inoculated in 280 ml fermenters. A synthetic medium that mimics grape must was used for fermentation, based on the work of Bely et al. (1990). This medium contained, per litre: 90 g glucose, 90 g fructose, 6 g citric acid, 6 g DL-malic acid, 750 mg KH_2PO_4, 500 mg K_2SO_4, 250 mg $MgSO_4.7H_2O$, 155 mg $CaCl_2.2H_2O$, 200 mg NaCl, 4 mg $MnSO_4.H_2O$, 4 mg $ZnSO_4.7H_2O$, 1 mg $CuSO_4.5H_2O$, 1 mg KI, 0.4 mg $CoCl_2.6H_2O$, 1 mg H_3BO_3, 1 mg $(NH_4)_6Mo_7O_{24}$, 20 mg myo-inositol, 2 mg nicotinic acid, 1.5 mg calcium pantothenate, 0.25 mg thiamine-HCl, 0.25 mg pyridoxine and 0.003 mg biotin. 425 mg/L of yeast assimilable nitrogen (YAN) was added as a mixture of amino acids and ammonium containing, per litre: 460 mg NH_4Cl, 612 mg L-proline, 505 mg L-glutamine, 179 mg L-tryptophan, 145 mg L-alanine, 120 mg L-glutamic acid, 78 mg L-serine, 76 mg L-threonine, 48 mg L-leucine, 45 mg L-aspartic acid, 45 mg L-valine, 38 mg L-phenylalanine, 374 mg L-arginine, 33 mg L-histidine, 33 mg L-isoleucine, 31 mg L-methionine, 18 mg L-glycine, 17 mg L-lysine, 18 mg L-tyrosine and 13 mg L-cysteine. Anaerobic growth factors were added as a solution to reach the following concentrations in the medium: 0.05% v/v Tween 80; 15 mg/L of ergosterol and 0.0005% v/v oleic acid. The pH was adjusted to 3.3 with 10 M NaOH. Fermenters filled with medium were heat-sterilised (100 °C, 10 min) and cooled down to 28 °C before inoculation.

Fermentations were carried out at 28 °C with agitation. The fermenter’s weight was monitored twice daily to follow fermentation progress. The measured weight loss directly corresponded to the production and emission of carbon dioxide from glucose consumption. Fermentations carried at the same time represented a fermentation

block. Three replicates were performed for each strain (except LMD17, LMD37, LMD39, performed in six replicates due to their use in a parallel project and EC1118 performed in duplicate per block, i. e. 28 replicates in total).

2.4. Metabolite analysis

Fermentation metabolites concentrations were measured using high performance liquid chromatography (HPLC) as described in Deroite et al. (2018) and analysing chromatograms with OPEN LAB 2X software. Fermentation samples were centrifuged 5 min at 3500 rpm at 4 °C and kept at −18 °C. Before analysis, samples were diluted to 1/6 with 0.0025 mol/L H_2SO_4 and then centrifuged 5 min at 13000 rpm at 4 °C. The supernatant was kept at −18 °C until analysis. The HPLC method allowed the measurement of concentrations of glucose, fructose, ethanol, glycerol, acetate, succinate, α-KG, lactate and malate. Analyses were performed in duplicate and the mean was calculated for each sample and used in results analysis.

Quantification was made with a Rezex ROA column (Phenomenex, Torrance, California, USA) set at 60 °C on an HPLC equipment (HPLC 1260 Infinity, Agilent Technologies, Santa Clara, California, USA). It was resolved isocratically with 0.0025 mol/L H_2SO4 at a flow rate of 0.6 mL/min. Concentrations of acetate, malate, lactate and α-KG were measured with a UVmeter at 210 nm and other compounds with a refractive index detector.

For each fermentation, two measurements were taken: in the must before fermentation (for each block) and at the end of fermentation. All analyses were conducted on finished fermentation, i.e. when combined fructose and glucose concentration fell below 3 g/L, or when fermenter weight remained constant for 24h.

Yield was calculated for each metabolite as follows:

$$Y_{metabolite} = \frac{C_{metabolite}}{C_{glucose+fructose;initial} - C_{glucose+fructose;final}}$$

Concentrations were expressed in g/L or mg/L, leading to yields expressed in g/g or mg/g. When necessary, yield values were expressed as follows: mean ± standard error.

Malate being both produced and consumed during fermentation, the final concentration measured was used to calculate a difference with the initial theoretical malate concentration (here 6 g/l).

2.5. Statistical analysis

Statistical analysis was performed using R studio software (version: April 1, 1106). Multiple R packages were used to carry out data analysis and visualisation: “tidyverse” (1.3.2), “FactoMineR” (2.6), “factoextra” (1.0.7), ”ggpubr” (0.4.0), “viridis” (0.6.2) “GGally” (2.1.2)

EC1118 was used in each fermentation block in order to evaluate a possible block effect (i.e. differences between reference values from different fermentation blocks). It was estimated using the following model:

$$Y_{lm} = \mu + Block_l + E_{lm}$$

With: Y the phenotype of EC1118 for the block l (1–14) and the replicate m (1–2). μ represent the mean of the considered phenotype and E the residual error, with $E \sim N(0, \sigma^2)$.

A block effect was observed on EC1118 data. This was corrected by calculating a variation factor on EC1118 metabolite values. The correction coefficient was calculated as the difference between a phenotype value of a block and the first block (chosen arbitrarily as reference). This correction (raw value - correction coefficient) was then applied to all variables. The block effect being eliminated, yields can be expressed with the following models: (1) for the strain effect and (2) for the genetic group effect. Results of these tests are available as supplementary information.

$$Y_{ik} = \mu + S_i + E_{ik} \quad (1)$$

$$Y_{jk} = \mu + G_j + E_{jk} \quad (2)$$

With: Y the phenotype (yield for a given metabolite) corrected for block effect for the strain i (1–51), the genetic group j (1–5) and for the replicate k (1–28). μ represent the mean of the considered phenotype, S the effect of the strain i, G the effect of the genetic group j and E the residual error, with $E \sim N(0, \sigma^2)$.

To express the yield variation for a metabolite among a group of strains, the variation coefficient was used (Albatineh et al., 2014). A correction according to the number of strains in a group was applied, allowing us to compare groups of different sizes. The correction was applied as follows:

$$CV_{corr} = \frac{\sigma}{\mu} \times \left(1 - \frac{1}{4(n-1)} + \frac{1}{n}\left(\frac{\sigma}{\mu}\right)^2 + \frac{1}{2(n-1)^2}\right) \times 100$$

With, for a group of strains and a metabolic yield: μ the mean, σ the standard deviation, n the size of the group and CV_{corr} the corrected coefficient of variation, expressed as percentage.

The coefficient of variation was calculated for ethanol, glycerol, acetate, succinate, α-KG and lactate yields (malate being excluded).

2.6. Comparison with other screening works

For the four main metabolites considered (ethanol, glycerol, acetate and succinate), we compared the results of the present work with significant datasets previously obtained. Data were acquired online or directly from the authors. These data compared different sets of strains in similar conditions but with different analysis timing (resumed in Table 1). To overcome these differences, a normalised yield value was calculated as follows:

$$Y_{norm} = \frac{(Y - \mu)}{\mu}$$

With: Y the yield of a metabolite for a designated strain, μ the mean of all strains and Y_{nom} the normalised yield. For the data set produced in the present work, normalisation was performed with and without genetically modified or laboratory evolved strains (*i. e.* strains LMD41, LMD45, 5074, LMD13 and LMD14).

3. Results

We present the results obtained for 51 strains following the fermentation of a synthetic grape must. They were chosen according to their capacity to complete a wine laboratory fermentation and their use in previous works. Moreover, we aimed to have various genetic backgrounds (wine, rum, sake, flor, west African and or unclassifiable

Table 1
List of screening works on CCM in fermentation used as comparison.

Reference	Number of strains used	Fermentation conditions	Sampling time
Camarasa et al. (2011)	72	Synthetic grape must (240 g/L glucose and fructose, 200 mg/L YAN), 28 °C	75% of hexoses consumed
Legras et al. (2018)	57	Synthetic grape must (240 g/L glucose and fructose, 200 mg/L YAN), 28 °C	75% of hexoses consumed
Nidelet et al. (2016)	45	Synthetic grape must (240 g/L glucose and fructose, 200 mg/L YAN), 28 °C	11 g of CO_2 produced
This work	51 (46 with only wild-type strains)	Synthetic grape must (180 g/l glucose and fructose, 425 mg/L YAN), 28 °C	End of fermentation (all hexoses consumed)

strains) for a better representation of intraspecific diversity. Additionally, a set of industrial distillery and bioethanol strains was added (LMD40, LMD43, LMD44 and LMD46). They are not sequenced yet but bring information on strains used in conditions where ethanol is the main production target. Concentrations were measured for 7 compounds linked to the central carbon metabolism (CCM): ethanol, glycerol, succinate, acetate, α-KG, lactate and malate, determined by HPLC. After correction of the block effect (see Material & Method), strains were compared to each other. Among our set of strains, two are genetically modified: LMD41 and LMD45 (confidential genetic constructions) and three have been obtained using adaptive laboratory evolution methods: 5074, LMD13, LMD14 (Cadière et al., 2011; Tilloy et al., 2014). All these strategies aimed to modify CCM during fermentation. These strains were used as controls for their metabolic productions. Consequently, they were withdrawn from PCA because their metabolism does not represent a natural variation within the species. For variation and correlation analysis, the 46 other strains were placed in a group termed "wild-type" and compared with the values obtained from the total data set.

All strains were able to entirely consume glucose and fructose in the must within 5 days (data not shown). In addition to the overall analysis, separate results for each metabolite are available as supplementary information.

3.1. Comparison of variation of metabolic yields

To compare yields between metabolites and between strain groups, the coefficient of variation (CV) was calculated for each metabolite (Fig. 1). Malate, as an initial component of the synthetic grape must, is both produced and consumed during fermentation. Moreover, its final delta outcomes can be positive or negative depending on strains; consequently, the application of CV as a descriptive parameter for malate becomes impractical due to its versatile behaviour in our experimental context.

The CV was corrected to allow comparison of groups of different sizes (described in Material & Method). For better readability, we chose to represent only the wine and rum groups as they include more strains. The full figure, with all major genetic groups, is available as supplementary information (S8).

Among all metabolites examined, ethanol presented the lowest yield variation with a variation coefficient of 1.8 % when all strains were considered. When only wild-type strains were taken into account, the coefficient of variation was even lower, dropping to 0.8 %. The wine and rum groups exhibited similar values. Other metabolites displayed a more important variation among our selection of strains, with a peak for α-KG around 86% (when all strains are considered). Overall, variation was higher when considering all strains. Acetate was the only exception, with a similar coefficient of variation both for the whole set and wine group (both ~46%).

3.2. Correlations between metabolic yields

Pearson's coefficients were used to compare all metabolites with one another and evaluate possible correlations. The malate delta corresponds to the difference between its initial concentration and the concentration observed at the end of fermentation. Results, represented as a correlogram, are available in Figs. 2 and 3, respectively for the whole set of strains and for wild-type strains only.

The strongest correlation spotted in the whole data set is a negative relation between glycerol and ethanol yields ($r_{all} = -0.931$) (Fig. 2). However, this correlation seems driven by the extreme phenotype of modified and evolved strains, as it is still present but weaker in the wild-type only dataset ($r_{wild\text{-}type} = -0.589$) (Fig. 3). Other significant correlations ($|r| > 0.300$) were identified in both sets for other metabolites. This includes positive correlations between glycerol and succinate yields, lactate yield and malate delta, glycerol and α-KG yields and also negative correlations between acetate and succinate yields, acetate and α-KG yields, lactate and α-KG yields and α-KG yield and malate delta.

Most of the observed correlations apply to both the whole data set and the subset of wild-type strains. However, there are a few notable exceptions. In particular, two negative correlations, both involving ethanol, are significant in the whole data set, but are not apparent in the subset of wild-type strains. These negative correlations involve ethanol and succinate yields, as well as ethanol and α-KG yields. In addition, there is a positive correlation between α-KG and succinate yield that is only significant in the whole data set. Conversely, a positive correlation between succinate and lactate yield stands out in the wild-type subset but is weaker when all strains are considered. These distinctions between the two subsets can be directly attributed to the specific modifications and adaptations present in the genetically modified and evolved strains.

3.3. Global analysis and hierarchical clustering

To obtain an overall view of our data set, a Principal Component Analysis (PCA) was performed with yields values for key metabolites: ethanol, glycerol, acetate, succinate, lactate and α-ketoglutarate (α-KG) and with malate delta which corresponds to the difference with the initial concentration (Fig. 4). This analysis allowed us to position strains

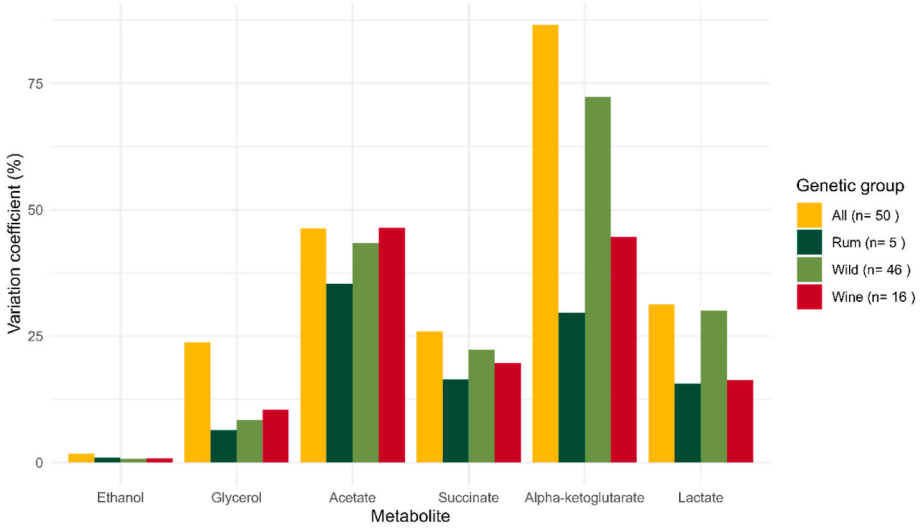

Fig. 1. Variation coefficient of each metabolite for all strains (n = 51), wild-type strains (n = 46), wine strains (n = 16) and rum strains (n = 5).

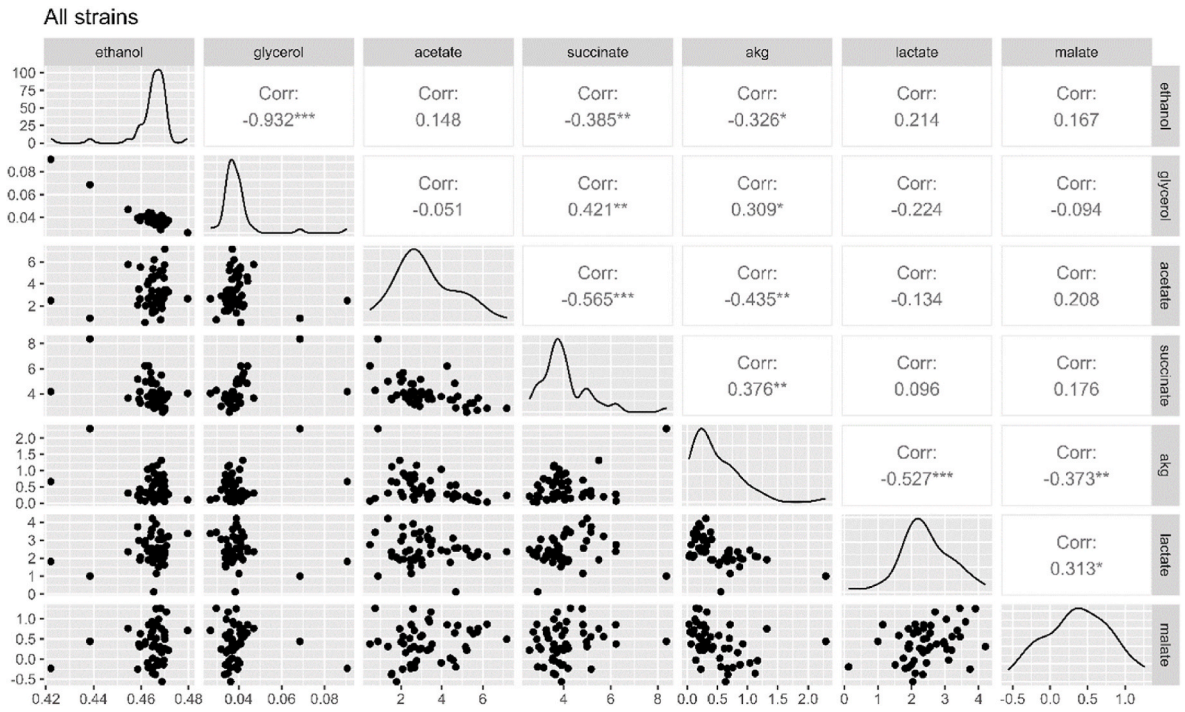

Fig. 2. Pearson's correlation matrix between all metabolic data for the whole set of strain (n = 51).

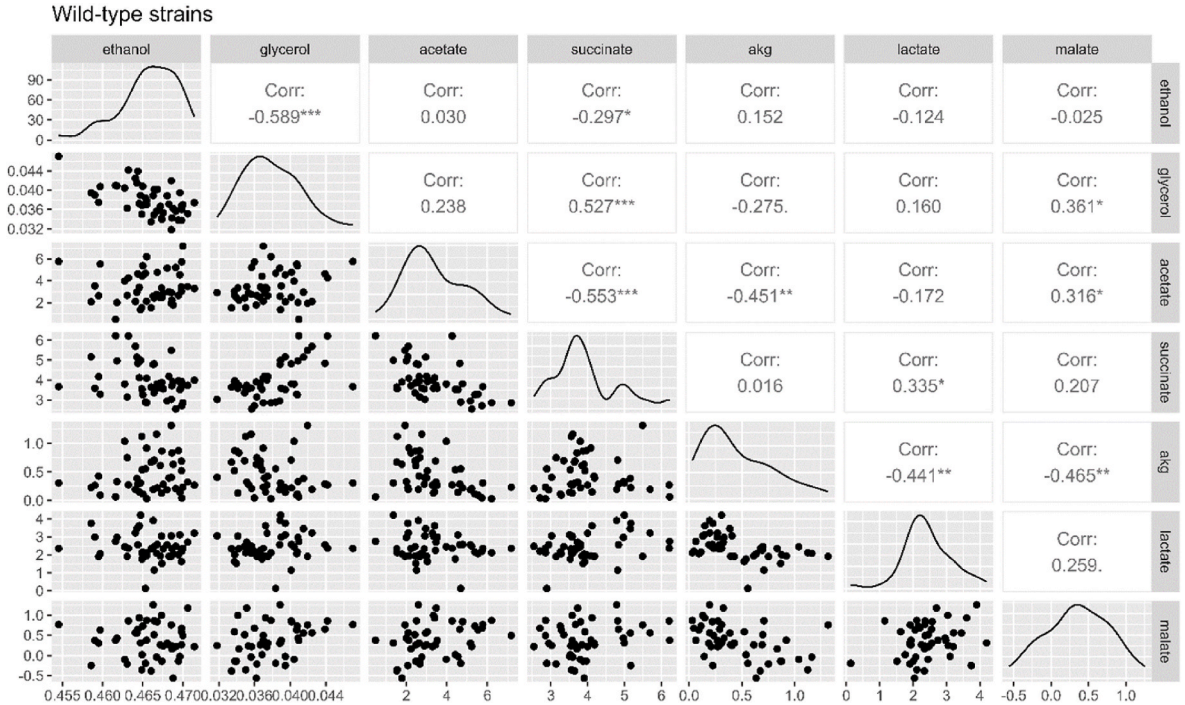

Fig. 3. Pearson's correlation matrix between all metabolic data for only wild-type strains (n = 46).

in relation to one another while providing insights on the impact of their genetic background. PCA was performed using wild-type strains only, to avoid biases induced by genetically modified and evolved strains. Hierarchical Clustering on Principal Components (HCPC) was also carried out on these results, allowing us to define three clusters of strains (Fig. 4). This number of clusters was chosen because it is the smallest that best represents the distribution.

The wine strains group was quite homogenous and mainly located in cluster 1: 13 out of 16 wine strains are included. Moreover, strains LMD12, LMD38 and LMD39, which are commercialised for wine fermentation, are clustered with wine genetic strains. L1528, located in cluster 2, was still relatively close to cluster 1 in representation of dimension 1 and 2 (61.7% of variance explained). The wine group seems mainly driven by the high α-KG and low acetate yields, with high malate delta (except for the strain DBVPG1373). Most of flor strains (3 out of 4), including EC1118, were also located in cluster 1. Sake, rum and West African groups did not display any consistency in clustering and were scattered among clusters 2 and 3. However, rum strains, and

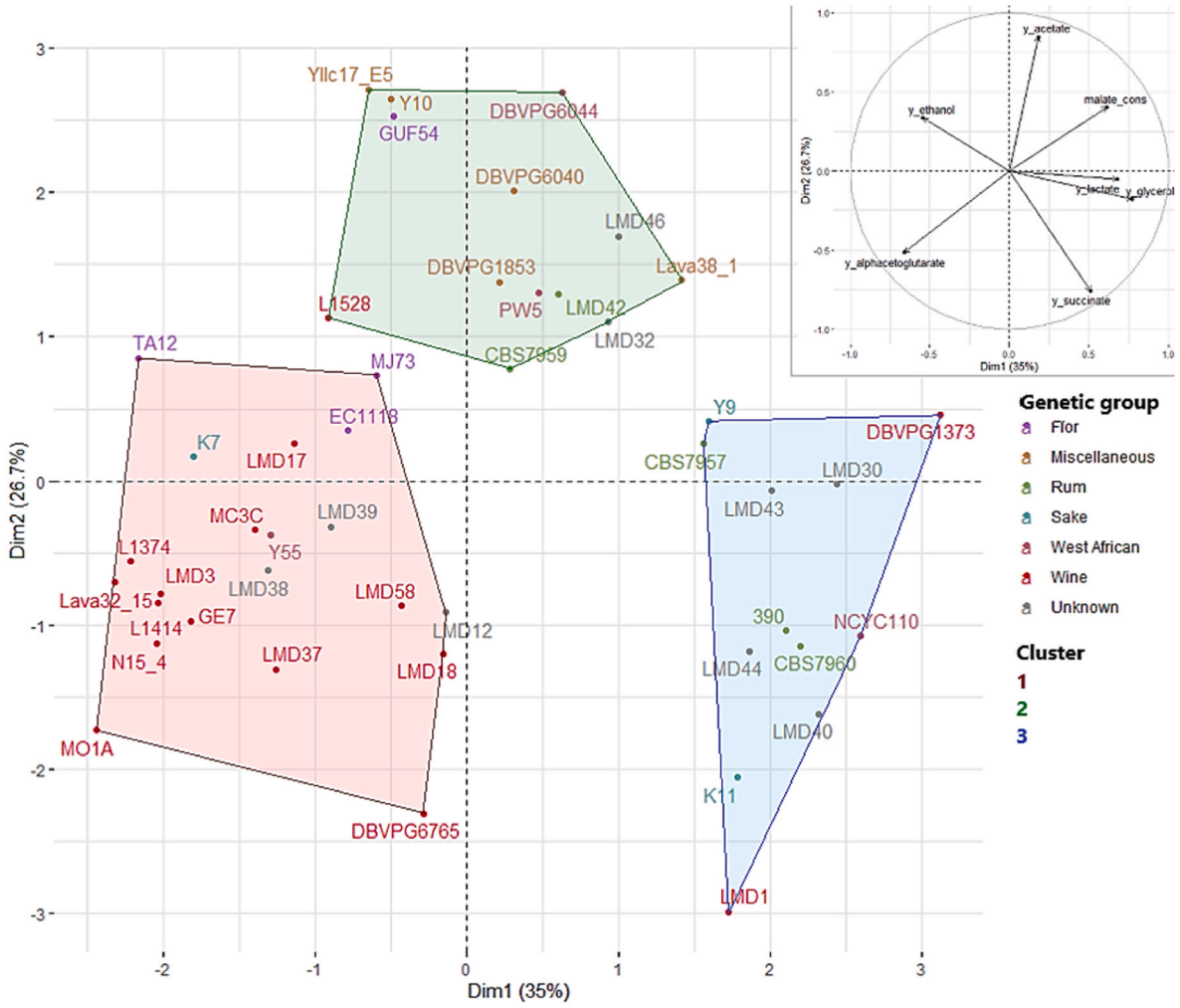

Fig. 4. Representation of PCA on wild-type strains for ethanol, glycerol, acetate, succinate, α-KG and lactate yields and malate final difference with initial concentration, individual and variable plot, with HCPC clustering (Dimension 1 and 2, 61.7% of variance explained). *Coloured points represent strain, tinted by genetic origin. Three clusters have been define: 1 (red), 2 (green) and 3 (blue)*

unsequenced strains used in distillery conditions (LMD40, LMD43, LMD44 and LMD46) were relatively close in the representation, mainly driven by lactate and glycerol yields.

Finally, previously observed correlations between metabolites were still apparent and clear drivers of the clustering, such as the strong negative relation between α-KG yield and final malate delta.

3.4. Comparison with other works

Data obtained with the present screening strategy were compared with others arising from similar screening works (Camarasa et al., 2011; Legras et al., 2018; Nidelet et al., 2016). Data were normalised to consider only the relative distribution among a set. With this aim, the present work was included twice: first with all the strains and secondly with normalisation performed after withdrawing the modified strains (Fig. 5).

All four metabolites showed a similar distribution, with ethanol always the less diversified yield and succinate and acetate displaying the most diversity. Glycerol showed an intermediary distribution. For all metabolites, divergent strains could be observed.

For ethanol, distribution seemed more limited in the data set exposed in this work, compared to the data set from the work of Nidelet et al. (2016), the two others being intermediary. Interestingly, this hierarchisation can be linked to the sampling time: the earlier the sampling, the higher the ethanol yield diversity among all strains. Ethanol production is not constant during fermentation, therefore we hypothesise that differences, even if they are low, appear during earliest fermentation phases, like growth phase.

4. Discussion

Saccharomyces cerevisiae has already been the subject of multiple studies on its metabolism, with comparison between different strains and links between phenotype and genotype unveiled. Accordingly, this work on a diverse set of strains allows a broad view of primary metabolic diversity. Our results confirm significant variations that exist among different *S. cerevisiae* strains in terms of primary metabolites yields. The diversity of these variations is not uniform and depends on the specific metabolite considered. In this work, multiple correlations were confirmed (such as the well-known glycerol and ethanol negative correlation). Other correlations, between minor and less studied metabolites, were established as well, such as the positive link between glycerol and succinate, lactate and malate delta, glycerol and α-KG or the negative correlation between acetate and succinate, and between α-KG, lactate and malate delta. If most of the correlations are significant with or without evolved and genetically modified strains, slight differences appear between these two data sets.

Moreover, this methodology affords a greater precision in metabolic yield assessment, corroborating existing data obtained in the last years and generating a robust and standardised dataset that can be reused in

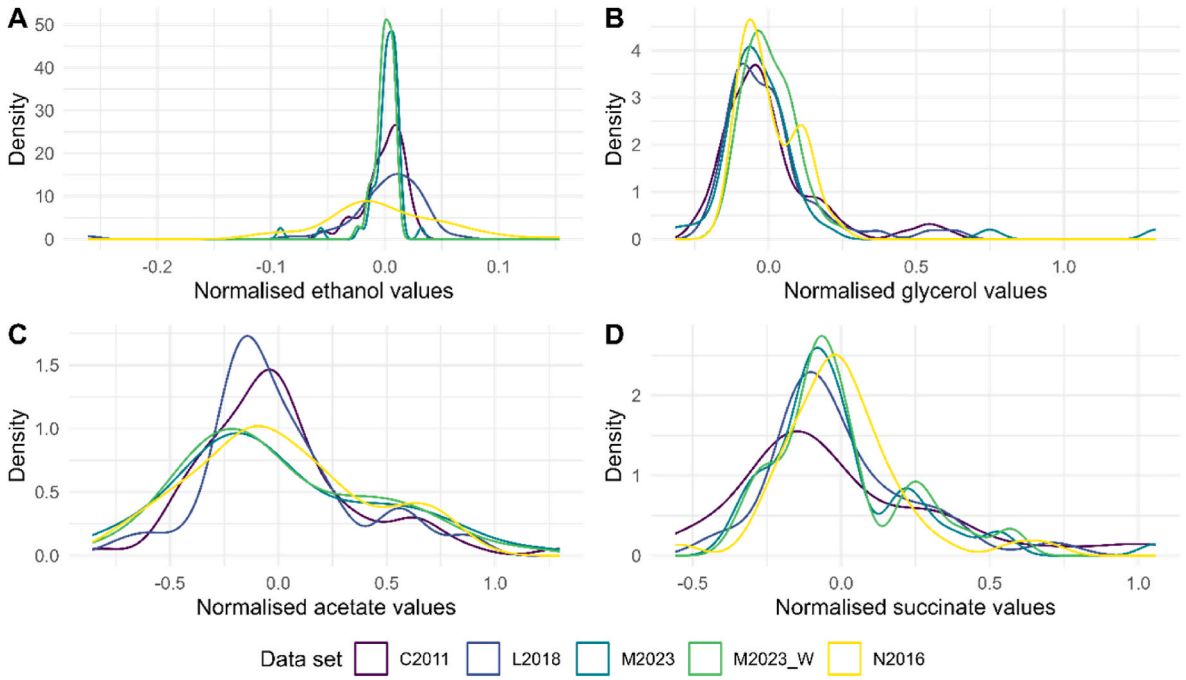

Fig. 5. Distribution of ethanol (**A**), glycerol (**B**), acetate (**C**) and succinate (**D**) yields in 5 data sets. ***C2011***: *Camarasa et al. (2011)*; ***N2016***: *Nidelet et al. (2016)*; ***L2018***: *Legras et al. (2018)*; ***M2023***: *this work, all strains;* ***M2023_W***: *this work, wild-type strains only*.

other studies on yeast metabolism. It enables yields to be precisely defined in a wine-like context, using a synthetic grape must with metabolite assessment at fermentation final stage. Lastly, it provides for the first time a screening of S. cerevisiae strains on lactate production in a wine-like context.

To simplify the discussion on metabolic yield results and their connections, a map of carbon central metabolism in oenological conditions is available in Fig. 6.

The data set gathers 51 strains, including five evolved or modified strains for specific features linked to carbon metabolism. Although these strains were discarded for overall analysis, their behaviour in terms of metabolism was considered as control. We observed that the two strains displaying the highest glycerol yield and the lowest ethanol yield among all strains were LMD14 and 5074, both obtained following an adaptive evolution aiming to reduce their ethanol production while enhancing glycerol production (Tilloy et al., 2014). At the other end of the spectrum, LMD41, modified to enhance ethanol production while impeding glycerol production, exhibited the highest value of ethanol yield and the lowest for glycerol. Finally, the last GM strain LMD45 showed the second lowest acetate and glycerol yields, which is consistent with its

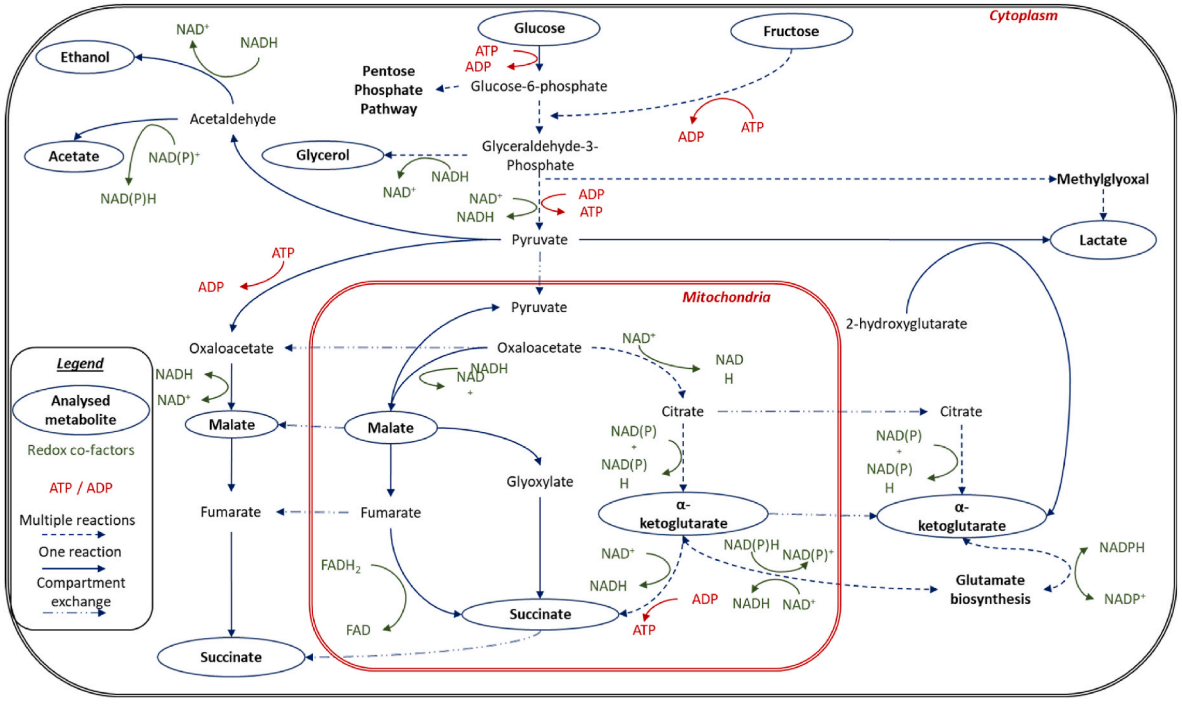

Fig. 6. Simplified central carbon metabolism of *S. cerevisiae* in oenological conditions.

modifications aiming to reduce fermentation by-product synthesis. These features clearly correspond to the already known characteristics of the selected strains, for which they have been modified or evolved, and thus validate our methodology. The medium used in this study is a very close imitation of grape must, perfectly suited to study wine strain metabolism, but also suitable to every strain able to ferment a complex medium with high sugar concentrations (Bely et al., 1990). Fermentation duration being dependent on nitrogen level and temperature, our fermentations were carried out at 28 °C with a must containing a relatively low concentration of sugars (from a winemaking point of view) and a high concentration of usually limiting nutrients (assimilable nitrogen, vitamins or anaerobic growth factors). This ensured a rapid and complete hexose conversion to ethanol (Rollero et al., 2015).

In overview, if we compare metabolites with one another, great differences in yield exist. Ethanol was the most produced compound, with a yield ten-fold higher than glycerol, whose yield was itself ten-fold higher than acetate yield. α-KG had the lowest yield values but still close to acetate. It should be noted that substantial yield variations were observed among strains for each metabolite, underlining the suitability of our experimental conditions to effectively discriminate strains on the basis of their primary metabolite yields.

However, variations among yields differed depending on the considered metabolite. Ethanol and glycerol are the most produced metabolites during alcoholic fermentation. Here, ethanol, with a variation coefficient inferior to 2%, showed a remarkably low variation. This level of variation was even lower when considering only wild-type strains. By contrast, glycerol yield exhibited a higher degree of variability, with a coefficient of variation around 25%. The same variation ranking can be observed in Nidelet et al. (2016) results, obtained in a similar medium using 43 strains (including 20 in common with our set), with ethanol being the most constant flux, followed by glycerol and then acetate, succinate and α-KG as the most variable. For the first four metabolites, these observations were confirmed by our normalised comparison and can also be found in other different works on CCM: indeed, Tronchoni et al. (2022), performed a screening in wine-like media in aerobic conditions using 25 *S. cerevisiae* wine strains. Ethanol yields in their study were lower, consistent with aerobic conditions, but the range of variation was very close to our results. It should be noted that no substantial differences were observed, and significant variations were only evident between the extreme values. Another comparable screening can be found in the work of Nieuwoudt et al. (2006) on 15 strains (commercial or not) and 19 hybrids using both natural and synthetic laboratory must. On both media, similar results were obtained: a higher range of variation was observable for glycerol than for ethanol. Similarly, Hubmann et al. (2013) performed a relevant screening on 52 beer and distillery strains of *S. cerevisiae* for their ethanol and glycerol yields, on a YPD-like medium. All these data present a greater diversity among strains for glycerol than for ethanol.

Furthermore, our data confirm the negative correlation observed in prior studies between ethanol and glycerol productions or yields, with a stronger link when extreme values from modified and evolved strains (strains 5074, LMD14 and LMD41) are considered. These values clearly drive the correlation in the whole set, but this negative correlation is still significant in the wild-type strains set, and supports the results of redox balancing between the two metabolites (Goold et al., 2017). Glycerol, considering its concentration and variation range and the strong negative correlation with ethanol yield, is confirmed to be the best candidate to orient carbon fluxes in the cell toward ethanol production. Surprisingly, no relation between genetic groups and glycerol or ethanol yields was found in our data. This goes against previous observations stating that wine strains are high glycerol producers compared to other groups (Camarasa et al., 2011). Nevertheless, it is worth noting that, in the study of Camarasa et al. (2011), groups were based on their environmental origin whereas ours were based on genetic origin. These two origins do not always match (as an example, strain Y55 used to be classified as a laboratory strain isolated from a wine environment, but Liti et al. (2009) showed that this strain is in fact closer to a West African genetic lineage).

Acetate is responsible for major off-flavours in wine, and so is subjected to regulatory limits (Paraggio and Fiore, 2004; Vilela-Moura et al., 2008). Our data revealed substantial variation in acetate yield and significant links with strains from different genetic groups. Specifically, strains from the wine group displayed a very low acetate yield, significantly lower than those from the West African group. This phenotype can be seen as a domestication footprint as it is most likely a direct consequence of selection for low acetate production during wine fermentation, while West African strains are known to have been less subjected to domestication (Warringer et al., 2011).

Succinate was produced in minor concentrations compared to ethanol or glycerol and its production appears negatively correlated with ethanol and consistently positively with glycerol, with whom it shares a similar variance (all strains considered). One explanation to this observation could be linked to the redox balance (Fig. 6). Indeed, NADH used in glycerol production needs to be regenerated afterwards. In the meantime, production of succinate by the oxidative branch of TCA produces NADH while this branch is also the most subject to variation (Camarasa et al., 2003) (Fig. 6).

Another metabolite, α-KG, provided interesting results, especially with its link to specific genetic groups. Wine strains showed a higher yield than other groups, while rum strains showed a lower yield. This particularity can be directly explained by the strong relation between this metabolite and nitrogen metabolism. Indeed, α-KG is mainly used in the cell for ammonium uptake and subsequent glutamate synthesis (Fig. 6). However, in grape must (and also in the synthetic must used in this study), where glutamate is abundant, α-KG is not fully utilised and is instead released into the environment (Avendaño et al., 1997; DeLuna et al., 2001; Camarasa et al., 2003; Magyar et al., 2014). Glutamate synthesis consumes the NADPH cofactor, which must be regenerated. One way to produce NADPH is via $NADP^+$ reduction occurring during conversion of acetaldehyde to acetate (Saint-Prix et al., 2004) (Fig. 6). Moreover, the wine strain group displayed a low acetate yield on average (if we exclude DBVPG1373 which shows abnormal values compared to the rest of the group). In the work of Nidelet et al. (2016), it was observed that acetate flux in fermentation is negatively correlated to biomass synthesis and that biomass is positively correlated to α-KG, revealing a negative link between α-KG and acetate. Despite the lack of data on biomass, the negative correlation between acetate and α-KG on our set seems to corroborate with these results. Moreover, succinate and α-KG were negatively correlated in the whole data set, but not when considering only wild-type strains. This correlation was mainly driven by strain LMD14, which has been developed using adaptive evolution for high glycerol and low ethanol production (Tilloy et al., 2014). This strain is also known for its high organic acid production, here confirmed and likely a consequence of a high TCA activity (Camarasa et al., 2003).

Another organic acid of interest in the context of winemaking is lactate. Although most of it is produced during malolactic fermentation by bacteria (Volschenk et al., 2006), a fraction of this organic acid can be produced by yeast during alcoholic fermentation, but has not been extensively studied. Yet, lactate production in *S. cerevisiae* displayed a wide range of diversity, with significant links with genetic groups. Wine and flor strains tended to be low producers of lactate whilst rum and sake strains were high producers. Additionally, all strains ungrouped but used in distillery conditions (LMD40, LMD44, and GM strains LMD45 and LMD41) ranked among the best producers (available in supplementary information). These findings enforce the idea of a connection between the rum group and lactate production. However, if diversity was indeed present, measured concentrations were by far lower than those observed in wine fermentation with other yeasts such as *Lachancea thermotolerans* that can reach up to 12 g/L (Vicente et al., 2021). Contrary to *Lachancea* yeasts, which possess lactate dehydrogenase dedicated enzymes (LDH), lactate in *S. cerevisiae* is produced in small amounts from pyruvate reduction by residual lactate dehydrogenases

(coded by *DLD1*, *DLD2* and *DLD3* genes), Dld3p being responsible for the major part of LDH activity in anaerobic conditions (Fig. 6). This activity is coupled to α-KG production from 2-hydroxyglutarate and pyruvate (Becker-Kettern et al., 2016). The present data set showed a negative correlation between lactate and α-KG. This can be explained by the multiple paths available leading to α-KG synthesis, especially in nitrogen metabolism, but also by the production, upstream of glycolysis, of lactate via methylglyoxal degradation. This toxic metabolite is produced from glycolysis intermediary (trioses phosphates) and can be degraded through the glyoxalase pathway. In this respect, our fermentation conditions, with quite high hexose concentrations, entailed a high glycolytic flux that can cause a methylglyoxal production harmful to the cell (Martins et al., 2001; Stewart et al., 2013). This link between glycolytic flux and lactate can also be a plausible hypothesis for the high lactate production of the rum group. These strains are used in fermentations with harsh conditions, especially high temperature, which increases oxidative stress. Human selection for rapid fermentation, in a productivity-focused purpose, would also lead to an increase of glycolytic flux and consequently an increase of methylglyoxal formation (Stewart et al., 2013). The high lactate production would therefore be seen as an adaptation to these stress conditions inherent to the rum or bioethanol environment.

Finally, malate stands in a particular situation in wine fermentation. It is present in relatively high concentration in the synthetic grape must used (6 g/L) and it can be both consumed and produced during fermentation. Therefore, its analysis as a final concentration gives only an indication on the final balance resulting from the whole metabolic changes (Vion et al., 2023). This led to the identification of strains resulting in positive malate delta (equivalent to production, mostly rum and west African strains), and others, mainly wine strains, displaying no or little negative malate delta (equivalent to consumption). This result is counterintuitive since malate is naturally present in the natural environment of wine strains. In the whole set, malate consumption never exceeded 1 g/l (see supplementary information), which is consistent with previous results obtained in a large-scale study on *S. cerevisiae* (Yéramian et al., 2007). Final malate concentration is found to be negatively correlated to α-KG content, meaning that higher malate production or lower consumption was associated with lower α-KG production.

Overall, tendencies in our data set are consistent with conclusions drawn in other publications, including those used to select our set of strains (Camarasa et al., 2011; Nidelet et al., 2016; Legras et al., 2018). Specifically, strains belonging to the West African genetic group (including strains from palm wine and other traditional African beverages making processes) and the flor group have been identified as notable producers of high acetate and low succinate. The diversity in acetate levels within our dataset was found to be significant, exceeding that of glycerol or ethanol, in line with the findings reported by Tronchoni et al. (2022).

Moreover, the individual metabolite approach highlighted interesting correlations. PCA also allowed to visualise all these correlations combined, which makes it a good tool to group strains according to their metabolic features. For wine strains, metabolic clusters on the PCA matched with the genetic groups except for a few strains. Rum strains and unsequenced strains used in distillery conditions (LMD40, LMD43, LMD44 and LMD46) were grouped in cluster 2 and 3 by Hierarchical Clustering on Principal Components, but relatively close in dimension 1 and 2 of PCA representation, suggesting a phenotypic proximity linked to the environment. Genome sequencing is necessary to conclude that the four commercial distillery strains belong to the rum group. The 3 strains from the sake group were scattered in the PCA representation, without any common features appearing. This high phenotypic diversity has already been highlighted in the past for other traits in the sake population (Warringer et al., 2011).

The ability to complete a wine-like fermentation is strongly linked to domestication and genetic origin (strains from bread or from natural environments such as oak trees are most of the time unable to perform a wine-like alcoholic fermentation (Camarasa et al., 2011; Legras et al., 2018; Tapia et al., 2018)); yet, a complementary set of strains, wider and more balanced between genetic groups, could provide more diversity and strengthen our analysis of natural yield variations. Strains from other anthropogenic origins (such as beer or cider) were not included here. However they should be considered in an extended study, after checking that they can complete a full alcoholic fermentation in wine conditions. Moreover, for strains from genetic groups other than wine, a synthetic grape must represents conditions very far from their usual environment. Despite this, our methodology provides keys to identify strains with good potentialities for wine fermentation.

Our study confirms precedent observations but also provides a robust comparative methodology and an easily useable data set obtained on 51 strains from various genetic backgrounds. Experimental conditions allowed a medium-throughput screening with a good balance between the number of strains and a high accuracy enabling the identification of traits with low variation. This screening helps to define and confirm the existing phenotypic variations for wine fermentation products among the *S. cerevisiae* species and sets the potential of improvement for these traits. It also provides information on lactate production in *Saccharomyces cerevisiae*, which shows a poor ability, however with a significant diversity and links with genetic origins. Nevertheless, the set of metabolites considered is limited, without any data on notable negative or positive aromatic metabolites which exhibit a greater diversity among *S. cerevisiae* strains, even in homogenous genetic groups. Completing this analysis with additional information on other metabolite production or consumption would strengthen the clustering and allow a broader view on metabolic differences. In the meantime, it would reveal patterns of interaction between pathways (nitrogen metabolism or lipid biogenesis for instance). To conclude, the present screening answers the main initial questions: some diversity, weak but significant, exists in ethanol yield among the *S. cerevisiae* species. Larger fluxes, such as ethanol or glycerol, are the most constraint and not linked to genetic origins, while, by contrast, smaller fluxes show larger variations and clear links with genetic origin. This represents improvement potentials of wine strains for these characteristics with non-GM methods (such as adaptive laboratory evolution, positive selection or breeding). If the two major produced metabolites, ethanol and glycerol, are linked in their production, yields of minor metabolites are more related to the genetic background of strains which is shaped by selection in a defined environment. Beyond confirming results observed in the last years with a robust and standardised method, our work also provides insights on little-studied metabolites with high technological potential in wine fermentation.

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CRediT authorship contribution statement

Ludovic Monnin: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Thibault Nidelet:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jessica Noble:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Virginie Galeote:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

Data, scripts, code, and supplementary information availability.

Data sets, R scripts and all supplementary informations are available on the following link: **10.5281/zenodo.10529112**.

Data availability

The link of the archive of all data and code used is in the manuscript

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Further reading

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