

Etude de la modulation de l'acidité des vins par Saccharomyces cerevisiae par des approches génétiques et métabolomiques. Rôle de la consommation et de la production de l'acide malique au cours de la fermentation alcoolique.

Charlotte Vion

▶ **To cite this version:**

Charlotte Vion. Etude de la modulation de l'acidité des vins par Saccharomyces cerevisiae par des approches génétiques et métabolomiques. Rôle de la consommation et de la production de l'acide malique au cours de la fermentation alcoolique.. Sciences agricoles. Université de Bordeaux, 2023. Français. ⟨NNT : 2023BORD0143⟩. ⟨tel-04208534⟩

HAL Id: tel-04208534

https://theses.hal.science/tel-04208534v1

Submitted on 15 Sep 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

université de BORDEAUX

THÈSE PRÉSENTÉE POUR OBTENIR LE GRADE DE

DOCTEUR DE L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE : Sciences de la Vie et de la Santé

Spécialité OENOLOGIE

Par

Charlotte VION

Etude de la modulation de l'acidité des vins par *Saccharomyces cerevisiae* par des approches génétiques et métabolomiques. Rôle de la consommation et de la production de l'acide malique au cours de la fermentation alcoolique.

Sous la direction de : Philippe MARULLO

Soutenue le 14 juin 2023

Membres du jury :

Mme. MOING Annick, Directrice de Recherche, INRAE Bordeaux	Présidente
M. ALEXANDRE Hervé, Professeur, Université de Bourgogne (IUVV)	Rapporteur
Mme TAILLANDIER Patricia, Professeure, INP-ENSCIACET	Rapportrice
Mme. GALEOTE Virginie, Chargée de recherche, UMR SPO, Montpellier	Examinatrice
M. MARCHAL Axel, Professeur, Université de Bordeaux (ISVV)	Invité
M. MARULLO Philippe, Chargé de Recherche à BIOLAFFORT	Directeur de Thèse

université de BORDEAUX

THÈSE PRÉSENTÉE POUR OBTENIR LE GRADE DE

DOCTEUR DE L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE : Sciences de la Vie et de la Santé

Spécialité OENOLOGIE

Par

Charlotte VION

Etude de la modulation de l'acidité des vins par *Saccharomyces cerevisiae* par des approches génétiques et métabolomiques. Rôle de la consommation et de la production de l'acide malique au cours de la fermentation alcoolique.

Sous la direction de : Philippe MARULLO

Soutenue le 14 juin 2023

Membres du jury :

Mme. MOING Annick, Directrice de Recherche, INRAE Bordeaux	Présidente
M. ALEXANDRE Hervé, Professeur, Université de Bourgogne (IUVV)	Rapporteur
Mme TAILLANDIER Patricia, Professeure, INP-ENSCIACET	Rapportrice
Mme. GALEOTE Virginie, Chargée de recherche, UMR SPO, Montpellier	Examinatrice
M. MARCHAL Axel, Professeur, Université de Bordeaux (ISVV)	Invité
M. MARULLO Philippe, Chargé de Recherche à BIOLAFFORT	Directeur de Thèse

Titre : Etude de la modulation de l'acidité des vins par *Saccharomyces cerevisiae* par des approches génétiques et métabolomiques. Rôle de la consommation et de la production de l'acide malique au cours de la fermentation alcoolique

Résumé :

La hausse des températures a de nombreux impacts sur la vigne et les baies de raisin. Parmi eux, une augmentation de la teneur en sucre des baies à maturité et une diminution de leur teneur en acidité. L'adaptation des pratiques viti-vinicoles aux matrices œnologiques de demain passe, entre autres, par l'utilisation de levures de vinification mieux adaptées aux caractéristiques physico-chimiques des moûts. Il existe au sein de l'espèce *Saccharomyces cerevisiae* une grande variabilité génotypique et phénotypique impactant de nombreux traits technologiques. La capacité des levures de vinification à moduler l'acidité lors de la fermentation alcoolique est au centre de ce travail de thèse. Cette propriété technologique a été optimisée grâce à des programmes de croisement assistés à l'aide de marqueurs génétiques liés à des QTLs (*Quantitative Trait Loci*) préalablement identifiés. Des souches fortement consommatrices ou productrices d'acide malique ont été obtenues. Ces souches ont été caractérisées d'un point de vue fermentaire, métabolique et sensoriel dans plusieurs matrices œnologiques afin de les intégrer à différents itinéraires de vinification. Un état des lieux de la modulation de l'acidité d'une cinquantaine de souches du genre *Saccharomyces* a également été entrepris. Ce paysage phénotypique a été réalisé par une approche de métabolomique ciblée par RMN du proton dans différentes matrices œnologiques. Des différences phénotypiques portant sur la teneur finale en acides organiques ont été mises en évidence. Pour un sous-ensemble de souches de *S. cerevisiae* appartenant aux groupes génétiques de vin et de voile, une étude comparative de la concentration en métabolites intracellulaires a été réalisée. Ce travail a nécessité le développement d'une méthode d'extraction et de quantification par spectrométrie RMN du proton. Des *trade-off* métaboliques avec des traits d'histoire de vie ont été mis en évidence entre les deux groupes. Une dernière partie plus fonctionnelle vise à comprendre les modes de production et de consommation de l'acide malique au cours de la vinification. L'ajout dans le milieu fermenté d'acide malique marqué au ^{13}C et l'utilisation de plusieurs approches métabolomiques: LC-MS/MS, GC-MS, RMN-2D, a permis de comprendre les spécificités métaboliques de souches extrêmes. Enfin l'étude métabolomique non ciblée de plusieurs vins obtenus à partir de souches extrêmes a permis de définir des molécules clés capables de moduler l'acidité des vins.

Mots clés :

Contrôle de l'acidité des vins ; Œnologie ; Acide malique ; Sélection génétique ; Métabolomique

Title : Study of the modulation of wine acidity by *Saccharomyces cerevisiae* using genetic and metabolomic approaches. Role of consumption and production of malic acid during alcoholic fermentation.

Abstract:

The increase in temperature has many impacts on grapevines and grape berries. Among them, an increase in the sugar content of ripe grapes and a decrease in their acidity content. The adaptation of winemaking practices to tomorrow's oenological matrices requires, among other things, the use of winemaking yeasts that are better adapted to the physicochemical characteristics of musts. There is a large genotypic and phenotypic variability within the *Saccharomyces cerevisiae* species, which has an impact on many technological traits. The ability of winemaking yeasts to modulate acidity during alcoholic fermentation is the focus of this thesis. This technological property was optimized through breeding programs assisted by genetic markers linked to previously identified QTLs (*Quantitative Trait Loci*). Strains with high malic acid consumption or production were obtained. These strains were characterized from a fermentative, metabolic, and sensory point of view, in several oenological matrices, in order to integrate them into different winemaking itineraries. An overview of the acidity modulation of about 50 strains of the *Saccharomyces* genus was also undertaken. A phenotypic landscape was carried out by a targeted metabolomic approach using ^{1}H NMR in different oenological matrices. Phenotypic differences in the final organic acid content were identified. A comparative study of intracellular metabolite concentration was performed for a subset of *S. cerevisiae* strains belonging to the wine and flor genetic groups. This work required the development of an extraction and quantification method by ^{1}H NMR spectrometry. Metabolic trade-offs with life history traits have been highlighted between the two groups. A last and more functional part aimed at understanding the production and consumption modes of malic acid during the wine making process. The addition of ^{13}C-labelled malic acid to the fermented medium and the use of several metabolomic approaches: LC-MS/MS, GC-MS, 2D-NMR allowed us to understand the metabolic specificities of extreme strains. Finally, the non-targeted metabolomic study of several wines obtained from extreme strains allowed to define key molecules able to modulate the acidity of wines.

Keywords :

Wine acidity control ; Oenology ; Malic acid ; Genetic selection ; Metabolomics

Remerciements

En premier lieu, je tiens à remercier chaleureusement mon directeur de thèse, Monsieur Philippe Marullo. Merci d'avoir cru en moi en me donnant l'opportunité de réaliser cette thèse. Merci pour tes conseils, ta disponibilité et toutes les connaissances que tu as pu me transmettre. C'était un réel plaisir de travailler avec toi.

Je remercie les membres de mon jury d'avoir accepté d'évaluer mon travail. Merci au Professeur Hervé Alexandre, à la Professeure Patricia Taillandier d'avoir accepté d'être rapporteurs de ce travail. Merci également à Madame Virginie Galeote et Madame Annick Moing d'avoir accepté d'examiner ce travail, ainsi qu'au Professeur Axel Marchal d'avoir accepté de participer à ce jury.

J'exprime toute ma gratitude à Monsieur Luc Laffort pour m'avoir permis de réaliser cette thèse au sein de société Biolaffort.

Je remercie également la région Nouvelle Aquitaine pour avoir accordé un financement au projet de recherche MEGA VIP dans lequel s'intègre ma thèse.

Merci à Emilien Peltier qui m'a encadré lors de mon stage de fin d'études et qui m'a appris à appréhender et même à aimer le logiciel R. « *R a toujours raison, il fait ce que tu lui demandes. Si ce n'est pas le cas, c'est toi qui as mal codé. » E. Peltier 2019.*

Merci à toutes les personnes de la société Biolaffort avec qui j'ai eu le plaisir de travailler, d'échanger ou de trier les raisins : Enzo Barbier, Philippe Louazil, Anna-Maria Lowe-Manolis, Ana Hranilovic, Julia Capitanio, Marion Favier, Karine Gay, Chantal Mansour. Je remercie Joana Coulon de m'avoir suivie lors de cette thèse, pour ses conseils et sa gentillesse ainsi que Virginie Moine pour son aide et sa bienveillance.

Ce travail n'aurait pas pu être réalisé sans les nombreuses collaborations et échanges avec d'autres équipes scientifiques. A la base des meilleurs projets, il y a la communication, les échanges d'idées et le partage de connaissances. Merci à Carole Camarasa et Audrey Bloem de m'avoir accueillie dans leur équipe au sein de l'Unité de Recherche SPO de Montpellier. Merci pour nos échanges scientifiques et vos conseils.

Merci à Grégory Da Costa, Inès Le Mao et Tristan Richard de l'axe MIB pour m'avoir appris tout ce que je sais aujourd'hui sur la RMN.

Merci à Marie Le Scanff, Tom Estier et Axel Marchal de l'axe QIV de vous être rendus disponibles pour passer tous mes échantillons et d'avoir répondu à toutes mes questions naïves.

Merci à tous mes stagiaires Valentine Fautré, Mathilde Brambati, Bruce Richard et Faustine Rose. Votre aide au laboratoire m'a été extrêmement précieuse. Je vous souhaite à tous une le meilleur pour la suite.

Parce qu'une bonne ambiance au bureau et au laboratoire est indispensable pour avancer,

J'adresse un immense merci à mes camarades de bureau Maïtena Muro, Margaux Bernard, Laura Chasseriaud et Jules Harrouard. Merci pour votre bonne humeur au quotidien, pour votre aide amicale, c'était un plaisir de vous retrouver chaque jour. Merci également à Cécile Miot-Sertier, pour ta bonne humeur et la vie que tu donnes au laboratoire. Merci à Warren Albertin pour ta bienveillance, ton aide et ton humour.

Naturellement, merci du fond du cœur à tous les doctorants, les post-doctorants et les copains de l'unité, qui sont pour beaucoup devenus des amis. Vous avez rendu cette aventure inoubliable.

Jules, Sara, Pierre, Marion, François, Benjamin, Tom, Inès, Justine G, Xavier, Julie, Paul, Gauthier, Pauline, Adeline, Justine L, Marie C, Marie LS, Marie A, Emilio, Alicia, Edouard, Laura F, Amel, Florencia, Lisa, Manon, Margot L, Margot P, Hugo.

Merci à vous tous pour l'ambiance de travail exceptionnelle, les pauses du midi, les pauses du soir, et tous les à-côtés.

Un merci tout particulier,

À Sara, pour ton aide, tes conseils mais surtout ton amitié et ton soutien à toute épreuve pendant la rédaction de cette thèse.

À Pierre, compagnon de fortune de rédaction, merci pour ton soutien et ta générosité.

À Paul, qui aura fait le même chemin que moi à quelques jours près.

Enfin, un grand merci à ma famille et tous mes proches pour leur soutien sans faille depuis toujours. Je remercie tout particulièrement mes parents qui m'ont toujours encouragée et soutenue, dans tous mes choix de vie. Promis, cette fois ça y est, j'arrête les études.

Productions scientifiques

Publications à comité de lecture

Vion, C.; Peltier, E.; Bernard, M.; Muro, M.; Marullo, P. **Marker Assisted Selection of Malic-Consuming Saccharomyces cerevisiae Strains for Winemaking. Efficiency and Limits of a QTL's Driven Breeding Program.** J. Fungi 2021, 7, 304. https://doi.org/10.3390/jof7040304

Peltier E., Vion C., Abou Saada O., Friedrich A., Schacherer J. and Marullo P. (2021) **Flor Yeasts Rewire the Central Carbon Metabolism During Wine Alcoholic Fermentation.** Front. Fungal Biol. 2:733513. doi: 10.3389/ffunb.2021.733513

Vion C., Muro M., Bernard M., Richard B., Fautré V., Yéramian N., Masneuf-Pomarède I., Tempère S., Marullo P. **New malic acid producer strain of *Saccharomyces cerevisiae* for preserving wine acidity during alcoholic fermentation.** Food Microbiology 2022,112. doi:10.1016/j.fm.2022.104209

Vion C., Yéramian N., Hranilovic A., Masneuf-Pomarède I., Marullo P. **Exploring the Influence of Yeasts on Wine Acidity: New Insights from *Saccharomyces cerevisiae***. Will be submitted soon.

Vion C., Le Mao I., Yéramian N., Muro M., Da Costa G., Richard T., Marullo P. **Targeted ^{1}H-NMR wine analyses revealed specific metabolome signatures of yeast populations belonging to the *Saccharomyces* genus.** Submitted in Food Microbiology

Vion C., Brambati M., Da Costa G., Richard T., Marullo P. **Endo metabolomic profiling of flor and wine yeasts by ^{1}H-NMR approach.** Will be submitted soon.

Vion C., Bloem A., Valette G., Da Costa G., Richard T., Camarasa C., Marullo P. **Investigation of malic acid metabolic pathways during alcoholic fermentation using GC-MS, LC-MS and NMR derived ^{13}C-labeled data.** Will be submitted after few amendments.

Vion C., Le Scanff M., Estier T., Rose F., Richard B., Marchal A., Marullo P. **Untargeted LC-HRMS metabolomics reveals phenotypic specificities of extreme *Saccharomyces cerevisiae* strains selected for their acidification properties.** Will be submitted after few amendments.

Communications orales

Vion C., Brambati M., Le Mao I., Richard T., Bloem A., Camarasa C., Marullo P. **NMR Analysis of *Saccharomyces cerevisiae* endo-metabolome : Comparison of Yeast Strains of Different Origin during the Alcoholic Fermentation of a Grape Must.** 14th International Conference, Levure, Modèles et Outils "LMO 14"

Posters

Vion C., Muro M., Bernard M., Richard B., Fautré V. Peltier E., Le Mao I., Da Costa G., Richard T., Marullo P. ***Acidic* and *Demalic Saccharomyces cerevisiae* Strains for Managing Acidity during Alcoholic Fermentation**. The 3rd Microbiology Day 2022

Vion C., Brambati M., Le Mao I., Richard T., Bloem A., Camarasa C., Marullo P. **NMR Analysis of *Saccharomyces cerevisiae* endo-metabolome : Comparison of Yeast Strains during Alcoholic Fermentation.** The 36th International Specialized Symposium on Yeast (ISSY36)

Table des matières

Vue d'ensemble du projet et questions scientifiques soulevées ..... - 1 -

Introduction : Etdue bibliographique..... - 9 -

1. Présentation des principales étapes du processus de vinification et implication des levures œnologiques.....- 9 -

1.1. La vinification..... .....- 9 -

1.2. Les levures de vinification .....- 12 -

2. Modulation biologique de l'acidité des vins .....- 14 -

Exploring the Influence of Yeasts on Wine acidity: new insights from *Saccharomyces cerevisiae*- 15 -

Introduction.....**- 17 -**

1. The origin of the acidity in grape juices and wines **..... - 18 -**

1.1. A brief definition of wine acidity .....- 18 -

1.2. Organic acids and wine acidity .....- 19 -

2. Metabolic pathways of organic acids yeast. **..... - 21 -**

2.1. Pyruvic acid.....- 22 -

2.2. Malic acid.....- 22 -

2.3. Lactic acid .....- 24 -

2.4. Acetic acid .....- 25 -

2.5. Citric, fumaric, α-ketoglutaric, and succinic acids .....- 25 -

3. Genetic levers for controlling organic acids in wines **..... - 26 -**

3.1. Genetically modified yeast strains.....- 26 -

3.2. Natural genetic variations found in S. cerevisiae populations. .....- 28 -

4. Microbiological applications for reducing wine acidity during the alcoholic fermentation. **..... - 30 -**

4.1. Contribution of Saccharomyces cerevisiae.....- 31 -

4.2. Contribution of Schizosaccharomyces pombe .....- 32 -

4.3. Contribution of Zygosaccharomyces bailii.....- 33 -

5. Microbiological applications for enhancing wine acidity during the alcoholic fermentation. **..... - 34 -**

5.1. Malic acid production during wine fermentation .....- 34 -

5.2. Lactic acid production during alcoholic fermentation .....- 35 -

5.3. Succinic acid producing yeasts ..... *- 37 -*

6. Conclusion ..... **- 38 -**

7. Bibliography ..... **- 39 -**

Chapitre 1 : Sélection et caractérisation de souches de *Saccharomyces cerevisiae* pour la modulation de l'acidité des vins ..... - 49 -

1. Sélection assistée par marqueurs de souches consommatrices d'acide malique ..... - 49 -

1.1. Contexte et problématique de l'étude ..... - 49 -

1.2. Principaux résultats ..... - 50 -

2. Préservation de l'acidité des vins par l'utilisation de souches acidifiantes ..... - 74 -

2.1. Contexte et problématique de l'étude ..... - 74 -

2.2. Principaux résultats ..... - 74 -

3. Etude de la réponse phénotypique des souches démaliquantes et acidifiantes ..... - 89 -

3.1. Contexte et problématique ..... - 89 -

3.2. Matériels et méthodes ..... - 89 -

3.3. Résultats ..... - 92 -

3.4. Discussion ..... - 100 -

4. Evaluation sensorielle de l'impact de nouvelles souches extrêmes sur l'acidité perçue dans les vins - 105 -

4.1. Problématique ..... - 105 -

4.2. Matériels et méthodes ..... - 105 -

4.3. Résultats ..... - 108 -

5. Evaluation au chai de deux souches acidifiantes ..... - 113 -

5.1. Description de l'essai et analyse des moûts ..... - 113 -

5.2. Résultats des essais ..... - 114 -

Conclusion du chapitre ..... - 117 -

Chapitre 2 : Etude des variations métaboliques inter- et intraspécifiques de souches du genre *Saccharomyces* ..... - 119 -

1. Contexte..... - 121 -
2. Etat de l'art sur l'analyse métabolomique par RMN ^{1}H..... **- 121 -**

2.1. Principe de la RMN du proton ..... - 121 -
2.2. Applications de la métabolomique par RMN en science des aliments ..... - 123 -

3. Etude des variations métabolomiques chez des populations de levure du genre *Saccharomyces* : analyse des vins par RMN du proton ..... **- 125 -**

Targeted ^{1}H-NMR wine analyses revealed specific metabolomic signatures of yeast populations belonging to the *Saccharomyces* genus. ..... - 126 -

1. Introduction ..... **- 127 -**
2. Materials and Methods ..... **- 128 -**
2.1. Yeast strains used and culture methods..... - 128 -
2.2. Microsatellite genotyping..... - 131 -
2.3. Metabolites analysis by ^{1}H NMR..... - 132 -
2.4. Statistical analyses ..... - 132 -
4. Results ..... **- 133 -**
3.1. Genetic characterization of a panel of Saccharomyces strains ..... - 133 -
3.2. Assessment of wine metabolites variability of fermenting yeasts by targeted ^{1}H-NMR analysis.- 135 -
5. Discussion ..... **- 141 -**
5.1. ^{1}H NMR as an effective tool for analyzing wine microorganism metabolites..... - 141 -
5.2. Wine metabolome is partially impacted by the population origin of the fermenting strains.... - 143 -

Supplementary data ..... - 145 -
References - 147 -

4. Comparaison métabolique de deux populations de *Saccharomyces cerevisiae* au niveau intracellulaire : développement d'une méthode d'extraction des métabolites et analyse par RMN du proton ……- 153 -

4.1. Contexte de l'étude ..... *- 153 -*
4.2. Principaux résultats ..... *- 153 -*
1. Introduction ..... **- 155 -**
2. Materials and Methods ..... **- 156 -**
2.1. Yeast strains used and culture methods ..... *- 156 -*
2.2. Alcoholic fermentation assays ..... *- 158 -*
2.3. Biomass collection ..... *- 158 -*
2.4. Enzymatic assay ..... *- 160 -*
2.5. Statistical analyses ..... *- 160 -*
3. Results and discussion ..... **- 161 -**
3.1. Experimental design ..... *- 161 -*
3.2. Fermentation and yeast growth monitoring ..... *- 162 -*
3.3. Development and optimization of a methodology to extract intracellular metabolites from S. cerevisiae and analysis by ^{1}H-NMR. ..... *- 163 -*
3.4. Wine and flor yeast showed life history traits and metabolic trade-offs. ..... *- 166 -*
3.5. The variability of metabolites abundance is mostly characterized by complex genetic × sampling time interactions. ..... *- 169 -*
3.6. Comparison of intracellular metabolites abundance of two strains showing opposed malic acid metabolism - 172 -
Conclusion ..... **- 174 -**
Supplementary data ..... **- 175 -**
Bibliography ..... **- 178 -**

Conclusion du chapitre 2 ..... **- 183 -**

Chapitre 3 : Etude des voies métaboliques empruntées par l'acide malique lors de la fermentation alcoolique ..... - 189 -

1. Contexte et objectifs ..... - 189 -
2. Principaux résultats ..... - 189 -

Investigation of malic acid metabolic pathways during alcoholic fermentation using GC-MS, LC-MS, and NMR derived ^{13}C-labeled data ..... - 191 -

1. Introduction ..... - 192 -
2. Materials and Methods ..... - 193 -
2.1 Yeast strains used and culture methods. ..... - 193 -
2.2. Alcoholic fermentation assays ..... - 193 -
2.2.2. Alcoholic fermentation monitoring ..... - 194 -
2.3. Flow cytometry analysis ..... - 194 -
2.4. Enzymatic assay of extracellular metabolites ..... - 194 -
2.5. NMR analysis of intra and extra-cellular metabolites ..... - 195 -
2.6. Measurement of the isotopic enrichment of intracellular amino acids ..... - 196 -
2.7. Quantification of labeled extracellular organic acids by LC-MS/MS ..... - 197 -
2.8. Statistical analyses ..... - 199 -
3. Results and discussion ..... - 199 -
3.1. Rational of the study and analytical chemistry analyses ..... - 199 -
3.2. Consumption and de novo synthesis of malic acid during the alcoholic fermentation. ..... - 201 -
3.3. Most part of succinic acid produced might be derived from glyoxylate shunt. ..... - 203 -
3.4. Undirect evidence of the glyoxylate shunt as the main source of de novo malate synthesis until mid-alcoholic fermentation. ..... - 206 -
3.5. ^{13}C isotopic filiation of α-ketoglutaric acid revealed that malic acid is assimilated differentially by acidic and demalic strains. ..... - 207 -
3.6. 2D NMR approach captured additional labeled molecules that are markers of the pathways taken by ^{13}C labeled malic acid. ..... - 210 -
3.7. Isotopic enrichment of proteinogenic amino acids ..... - 210 -
3.8. Relative quantification of intracellular metabolites using a 2D NMR approach - 214 -
Conclusion ..... - 216 -
References ..... - 219 -

Chapitre 4 : Utilisation de la métabolomique non ciblée pour la discrimination de différents groupes de souches..... - 227 -

1. Contexte et problématique..... - 227 -
2. Principaux résultats ..... - 227 -

Untargeted LC-HRMS metabolomics reveals phenotypic specificities of extreme *S. cerevisiae* strains selected for their acidification properties. ..... - 228 -

1. Introduction ..... - 229 -
2. Methods ..... - 230 -
2.1. Origin and preparation of the wine sample analyzed. ..... - 230 -
2.2. LC-HRMS acquisition ..... - 231 -
2.3. Raw data treatment and QC filtering procedure..... - 232 -
2.4. Identification and classification of molecules ..... - 233 -
2.5. Statistical analyses and graphical representations..... - 233 -
3. Results and Discussion ..... - 234 -
3.1. Experimental design ..... - 234 -
3.2. Set up of a LC-HRMS analysis on non-volatile metabolome workflow: data filtering, automatic annotation of metabolite features and identification of impacting molecules..... - 236 -
3.3. Decomposition of the metabolomic variability of four unrelated grape juices. .. - 237 -
3.4. Yeast strain, sugar, and malic acid effect on the metabolomic variability of supplemented grape juices. - 241 -
3.5. Semantic analysis of molecular signatures discriminating acidic and demalic strains. .... - 245 -
3.6. Identification of a short list of metabolic compounds discriminating acidic and demalic strains. ..... - 246 -
3.7. Experimental validation that the CompX is linked to malic acid metabolism..... - 248 -
Conclusion ..... - 250 -
Supplementary files ..... - 251 -
References..... - 253 -

Conclusion du chapitre 4 ..... - 255 -

Conclusion générale et perspectives ..... - 257 -

1. Caractérisation des souches démaliquantes vs acidifiantes ..... **- 259 -**
2. Métabolisme de l'acide malique ..... **- 260 -**
3. Techniques employées ..... **- 263 -**

Références bibliographiques ..... - 264 -

Liste des figures

Figure 1. Cadre du projet MEGA VIP. ..... - 4 -
Figure 2. Principales étapes de la vinification en rouge et en blanc. ..... - 11 -
Figure 3. Représentation schématique des différents itinéraires de vinification ..... - 49 -
Figure 4. Plan d'expérience présentant..... - 93 -
Figure 5: Positionnement des six jus de raisin naturels et des six jus supplémentés en fonction de leur quantité de sucre initiale (g/L) et concentration en acide malique initial (g/L)..... - 94 -
Figure 6: Analyse en composante principale (ACP) des trois groupes dans les douze matrices. ..... - 95 -
Figure 7. Principaux paramètres impliqués dans la ségrégation des deux types de souches. ..... - 96 -
Figure 8. Normes de réaction ..... - 99 -
Figure 9. Valeurs de pH en fonction de l'acide malique (g/L) pour les trois types de souches ..... - 102 -
Figure 10. Analyses des vins blancs et rouges. ..... - 109 -
Figure 11: Position relative des vins blancs et rouges évalués lors d'une tâche de tri dirigée sur les deux premières dimensions d'une analyse MDS. ..... - 110 -
Figure 12. Résultats de la troisième nano-vinfication. ..... - 111 -
Figure 13. Evolution de la concentration en acide malique (g/L) après encensement de *Oenococcus oeni* . - 116 -
Figure 14. Alignement des protons dans le champ magnétique externe B_0. ..... **Erreur ! Signet non défini.**
Figure 15. Séquence de base pour la spectroscopie RMN. Après l'impulsion RF, le signal est détecté pendant la durée du FID ..... - 123 -
Figure 16: Concentration en acide malique par souche pour trois milieux ayant des concentrations croissantes d'acide malique initial. ..... - 262 -
Figure 17: Volcano plot..... - 264 -

Liste des tableaux

Tableau 1. Souches utilisées..... - 90 -
Tableau 2. Caractéristiques oenologiques des 12 matrices de l'étude..... - 91 -
Tableau 3. Part de variance expliquée par le pH initial, sucre initial ou l'interaction pH initial : Sucre initial sur les différents composés et paramètres cinétiques en fonction du type de souche ..... - 98 -
Tableau 4. Caractéristiques des moûts utilisés pour les nano-vinifications ..... - 106 -
Tableau 5. Souches utilisées pour les nano-vinifications..... - 106 -
Tableau 6. Détails des essais et composition des moûts ..... - 114 -
Tableau 7. Paramètres œnologiques des vins en fin de fermentation alcoolique. Les valeurs mise en avant en bleu gras sont celles qui diffèrent de la souche témoins pour chaque vinification. ..... - 115 -
Tableau 8. Paramètres chimiques des vins rouges post-FML en lien avec l'acidité..... - 115 -

Abréviations

ACP : Analyse en Composante Principale / PCA : Principal Component Analysis

ADN : Acide Désoxyribonucléique / DNA : Desoxyribonucleic Acid

ANOVA : Analysis Of Variance

AT : Acidité Total / TA : Total Acidity

AV : Acidité Volatile / VA : Volatile Acidity

CV : Coefficient de Variation / Coefficient of Variation

DACP : ACP Discriminante / DAPC : Discriminant PCA

FA : Fermentation Alcoolique / AF : Alcoholic Fermentation

FCa : Forimate de Calcium

FID : Free Induction Decay

FML : Fermentation Malo Lactique / MLF : Malo Lactic Fermentation

GC-MS : Gaz Chromatography – Mass Spectrometry

GM : Genetically Modified

GSD : Global Spectra Deconvolution

HPLC : High Performance Liquid Chromatography

HSQC : Heteronuclear Single Quantum Coherence

Hz : Hertz

LC-MS : Liquid Chromatography – Mass Spectrometry

LM : Linear Model

MAC : Malic Acid Consumed

MDS : Multidimensional Scaling

pH : Potentiel Hydrogène

QC : Quality Control

QTL : *Quantitative Trait Locus*

RF : Radio Frequence

RMN : Résonnance Magnétique Nucléaire / NMR : Nuclear Magnetic Resonance

TAP : Titre Alcoolémique Potentiel

TAV : Titre Alcoolémique Volumique

TSP : Acide triméthylsilylpropanoïque

YPD : Yeast Peptone Dextrose

Vue d'ensemble du projet et questions scientifiques soulevées

Le travail présenté dans ce manuscrit a été réalisé au sein de l'unité de recherche UMR 1366 Œnologie de l'Université de Bordeaux à l'Institut des Sciences de la Vigne et du Vin (ISVV), plus précisément au sein de l'axe Microorganismes (diversité, métabolismes, sélection). Cette thèse de doctorat a été financée par la société Biolaffort dans le cadre d'une convention industrielle de formation par la recherche (CIFRE). Les objectifs de cette thèse s'inscrivent en grande partie dans le projet MEGA-VIP (Métabolomique Et Génétique des levures Acidifiantes Vinification et Incidence sur le PH) financé par la région Nouvelle-Aquitaine. Ce projet de transfert de technologie vise à développer des itinéraires de vinification innovants pour maitriser l'acidité des vins dans un contexte de changement climatique. Le périmètre du projet MEGAVIP et ses quatre principaux axes sont présentés dans la figure 1. Ce projet est basé sur la découverte précédente de variations génétiques des levures permettant de moduler le métabolisme des acides organiques. Un des principaux attendus du projet est la sélection de levains industriels appartenant à l'espèce *Saccharomyces cerevisiae* capables de moduler de façon tangible le pH des vins pendant la fermentation alcoolique (Axe 1). Un autre aspect applicatif est de définir le périmètre des conditions d'application de ces nouveaux levains et d'explorer l'impact de ces souches sur le développement d'autres espèces d'intérêt telles que les bactéries lactiques et *Lachancea thermotolerans* (Axe 2). Au-delà de cette partie appliquée, le projet MEGAVIP vise à mieux comprendre les leviers génétiques et métaboliques qui caractérisent de telles souches. La compréhension des mécanismes génétiques sous-jacents nécessitait la mise en place de protocoles d'ingénierie génétique par l'outil CRISPR/CAS9 (Axe 3). La compréhension des mécanismes métaboliques nécessitait le développement de méthodes d'investigation biochimiques non disponibles au laboratoire (Axe 4).

Lors de mon travail de thèse, j'ai contribué à l'avancée du projet MEGAVIP dans ces quatre axes comme indiqué dans la figure 1. J'ai été accompagnée par plusieurs scientifiques et personnels techniques qui m'ont permis de produire plusieurs articles scientifiques au cours de ces trois années de thèse. Afin de prioriser la rédaction de ces articles, j'ai choisi de présenter mes travaux de recherche sous forme d'une thèse dite « sur articles ». La rédaction de six articles a permis de couvrir une partie importante de la littérature scientifique nécessaire pour nourrir les questions fondamentales et les aspects méthodologiques de mon travail. Deux ont déjà été publiés, deux autres ont été soumis récemment et deux doivent faire l'objet d'amendements supplémentaires. Le manuscrit présenté est composé d'une introduction, de quatre chapitres traitant des différentes questions soulevées et il se termine par une conclusion générale. Afin d'apporter au lecteur une présentation générale du contexte œnologique, une première **partie introductive** est composée d'une brève présentation du processus de vinification et de la place des levures en œnologie, ainsi que d'une

revue bibliographique sur la modulation biologique de l'acidité des vins. Cette revue sera prochainement soumise dans un journal scientifique.

La première partie englobe la totalité de l'Axe 1 du projet MEGAVIP, c'est-à-dire la sélection génétique de souches de *S. cerevisiae* non génétiquement modifiées présentant des propriétés extrêmes vis-à-vis de la modulation de l'acidité des vins. Dans cette partie, des souches ont été sélectionnées par des approches de croisement pilotées par l'utilisation de marqueurs génétiques. Ce travail original de sélection a nécessité la manipulation de nombreux marqueurs QTLs permettant d'obtenir des levures aux caractéristiques extrêmes. Les aspects techniques du croisement des souches et leur caractérisation génétique ont été pris en charge par le personnel permanent de la société Biolaffort (Maitena Muro et Margaux Bernard).

Sur le plan expérimental, j'ai principalement réalisé la caractérisation phénotypique des souches permettant de valider leurs phénotypes extrêmes. L'obtention d'un vaste jeu de données phénotypiques et génétiques pour des individus appartenant à plusieurs pedigrees a permis de poser des questions sur la pénétrance des QTLs lors de programmes de croisements multi-allélique. J'ai également travaillé à caractériser ces souches extrêmes d'un point de vue phénotypique dans différentes matrices représentant un large éventail de conditions œnologiques. Les aptitudes fermentaires et les propriétés œnologiques basiques des vins obtenus à partir de ces souches ont été caractérisées par des analyses sensorielles. L'impact de la forte consommation d'acide malique par certaines levures sur la fermentation malolactique a également été abordée (article 1). Ces expérimentations s'inscrivent dans l'axe 2 du projet MEGAVIP. Une partie d'entre elles ont été réalisées avec l'aide de deux étudiants de Master 2, Bruce Richard et Faustine Rose, que j'ai encadrés en 2022 et 2023. Enfin, cette partie se termine par une évaluation du potentiel œnologique de deux souches extrêmes estimé lors d'essais préliminaires au chai. Ce corpus d'expérimentations est rédigé en français. Il permet de soulever différentes questions scientifiques en lien avec le comportement des levures extrêmes sélectionnées. La question de la robustesse des souches à différents environnements a permis de définir les matrices préférentielles à l'expression des caractères acidifiants et démaliquants des souches. La question de l'impact sensoriel sur les vins produits avec de telles souches a également été abordée. Ces premiers travaux d'analyses sensorielles sur les souches ont eu pour objectif d'évaluer si les différences d'acidité des vins sont perceptibles à la dégustation.

La deuxième partie vise à développer une méthode de spectroscopie par RMN en collaboration avec nos collègues de l'axe MIB (« Molécules d'Intérêt Biologique ») de l'unité de recherche. Cet objectif méthodologique répond au besoin de caractériser plus finement les produits fermentés et s'inscrit dans l'axe 4 du projet MEGAVIP. Dans le cadre de cette thèse je me suis intéressée à l'exploration de la diversité métabolique de souches de levures du genre *Saccharomyces* dans le contexte de la fermentation alcoolique

du jus de raisin. Cette variabilité métabolique a été étudiée en lien avec le degré d'acidité des matrices fermentées. Pour cela, une méthode d'extraction et de dosage des métabolites intracellulaires de *S. cerevisiae* par RMN du proton a été mise au point au laboratoire. Par ailleurs, les méthodes d'analyses des vins déjà disponibles ont permis de caractériser de nombreux échantillons vinifiés avec des souches de levures sélectionnées pour leurs différences. Ces approches de métabolomique ciblée m'ont permis de mesurer les concentrations intra et extra cellulaires d'une vingtaine de composés chimiques issus du métabolisme central de la levure. Ce travail avait pour but de rechercher l'existence d'éventuelles relations entre les concentrations de composés observés dans le milieu extracellulaire et leur concentration intracellulaire. La question de l'existence de *trade-off* métaboliques en lien avec la sélection génétique de souches sélectionnées pour un phénotype extrême a également été posée. En intégrant la dimension génétique de l'origine des souches, j'ai également pu établir un état des lieux de la variabilité métabolomique de différentes souches et populations de levures, posant la question de l'existence de marqueurs métaboliques spécifiques à des populations ou des espèces. Cette seconde partie comporte un premier article soumis (article 3) et un second qui sera soumis prochainement (article 4).

La troisième partie de cette thèse comporte un volet plus fonctionnel. En effet, la question centrale de cette partie était de mieux définir et comprendre les voies métaboliques impliquées dans la consommation et la production de l'acide malique dans les conditions de la fermentation alcoolique chez *S. cerevisiae*. Cette partie fait l'objet d'un article (article 5) qui sera soumis après la réalisation d'expérimentations fonctionnelles complémentaires. Ce dernier traite des voies de consommation et production de l'acide malique grâce à l'utilisation d'une expérience de marquage au ^{13}C sur deux souches aux comportements extrêmes vis-à-vis du métabolisme de l'acide malique. Ces souches sont issues des programmes de sélection génétique réalisés dans la première partie. Différentes méthodes analytiques ont été combinées afin de recueillir des informations sur différents types de composés au niveau extra- et intracellulaires : LC-MS/MS, GC-MS, RMN 2D et RMN du proton. L'ensemble des informations recueillies a permis d'établir de nouvelles hypothèses sur le rôle de l'acide malique dans le métabolisme central de deux souches de *S. cerevisiae* ainsi que sur les voies de production des acides organiques. Des questions relatives au transport de ces acides dans et hors de la cellule ont également émergé de ces expériences.

Enfin, une **quatrième et dernière partie** présente un article (article 6) réalisé en collaboration avec nos collègues de l'axe QIV « Qualité et Identité des Vins » de notre unité de recherche. Ce travail est basé sur l'utilisation de métabolomique non ciblée (LC-HRMS) ayant pour objectif de discriminer des vins produits à partir de souches productrices ou consommatrices d'acide malique. L'utilisation de cette méthode a permis d'identifier des composés métaboliques en lien avec le niveau de production d'acide malique par les levures au cours de la vinification. Pour des raisons de propriété intellectuelle, ces composés ne sont pas

communiqués (le dépôt d'un brevet est actuellement à l'étude). Par conséquent, l'article en question ne présente pas sa forme définitive et sera soumis ultérieurement.

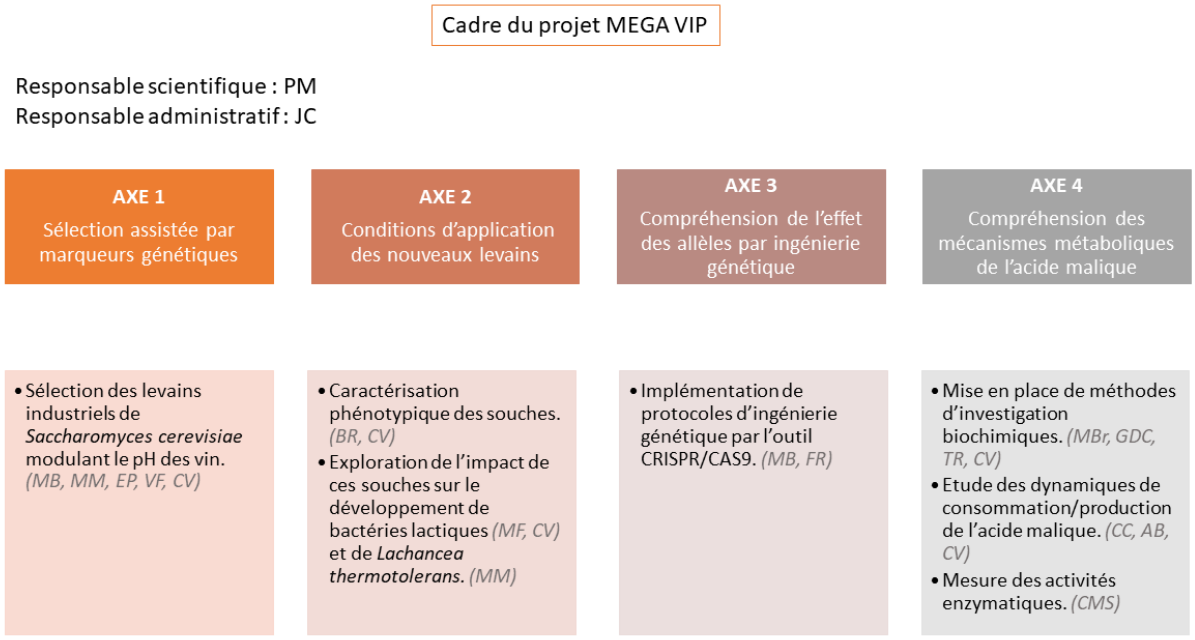


Figure 1. Cadre du projet MEGA VIP. Présentation des principales missions du projet MEGAVIP en 4 axes, ainsi que les acteurs impliqués (MB : Margaux Bernard, assistante de recherche Biolaffort, MBr : Mathilde Brambati stagiaire Master 2 Biolaffort, JC : Joana Coulon, responsable R&D Microbiologie Biolaffort, GDC : Grégory Da Costa, Maitre de conférence UB, VF : Valentine Fautré : stagiaire Master 2 UB, MF : Marion Favier, ingénieure de recherche Biolaffort, PM : Philippe Marullo, chargé de recherche Biolaffort, CMS : Cécile Miot-Sertier, technicienne de recherche, MM : Maitena Muro, chargée de projets Biolaffort, EP : Emilien Peltier, post-doctorant UB, BR : Bruce Richard, stagiaire Master 2 Biolaffort, TR : Tristan Richard, professeur UB, FR : Faustine Rose : stagiaire Master 2 Biolaffort, CV : Charlotte Vion).

Introduction : Etude bibliographique

Introduction : Etude bibliographique

Au cours de ce travail, je me suis particulièrement concentrée sur l'étude du métabolisme de l'acide malique par les levures de vinification. Cet effort a conduit à la rédaction d'une revue sur la modulation de l'acidité des vins par les levures de vinification qui reprend l'essentiel des connaissances biochimiques et microbiologiques nécessaires à comprendre le contexte de mon travail. Afin d'apporter aux lecteurs quelques notions plus générales sur l'œnologie, cette revue est précédée d'une première partie qui présente sommairement les étapes clés de la vinification et les principales caractéristiques technologiques des levures de vinification.

1. Présentation des principales étapes du processus de vinification et implication des levures œnologiques.

1.1. La vinification

Les différents styles de vin résultent de la variété des cépages utilisés, des pratiques agriculturales et des conduites œnologiques qui englobent la gestion des fermentations et des étapes pré- et post-fermentaires. La différence entre les vins blancs et les autres types de vins (rouge, rosé) tient à l'étape de macération. Les principales étapes de la vinification sont présentées dans la figure 2. Les raisins utilisés pour la vinification proviennent de l'espèce *Vitis vinifera*, au sein de laquelle de nombreux cépages se distinguent par des caractéristiques biochimiques (Ribéreau-Gayon *et al*., 2006). Les vendanges sont réalisées à la main ou à la machine selon les règlementations d'AOC lorsque le raisin a atteint sa maturité œnologique optimale. Cette maturité dépend du cépage, des conditions environnementales et du type de vin souhaité (Ribéreau-Gayon *et al*., 2006). Les raisins sont ensuite généralement triés et éraflés pour séparer les baies des rafles.

1.1.1. Spécificités des vins rouges

Le jus et les parties solides sont encuvés et laissés en macération afin d'extraire les composés phénoliques et aromatiques des baies. La durée et la température de cette macération dépendent du style de vin désiré (Şener, 2018). La macération peut être intensifiée par des opérations de pigeage ou remontage. La fermentation alcoolique s'opère à la fois dans une matrice liquide (le jus de raisin) et sur une matrice solide (le chapeau), constituée des pellicules qui recouvrent le jus. Le moût peut être sulfité, ensemencé avec des micro-organismes sélectionnés et dans certain cas corrigé chimiquement selon la réglementation (chaptalisation, ajout d'acide, nutrition œnologique…). Il peut aussi fermenter spontanément. Une fois que le niveau d'extraction en composés phénoliques souhaité est atteint, le « vin de goutte » est écoulé, le marc décuvé et pressé. Le vin résultant de la presse des composants solides est appelé « vin de presse ». Les vins de goutte et de presse peuvent être assemblés ou élevés séparément.

1.1.2. Spécificités des vins blancs

Dans le cas des vins blancs, les grappes sont généralement pressées entières. Le moût pressé est ensuite mis à débourber, les lies se déposent sous l'effet du froid et l'action d'enzymes. Le jus clair est encuvé avec quelques lies fines qui sont gardées afin d'apporter un complément nutritionnel aux levures. S'en suit la fermentation alcoolique (FA) en phase liquide.

1.1.3. Etapes de la vinification commune à tous les vins

A la suite de la fermentation alcoolique, une seconde fermentation d'origine bactérienne peut avoir lieu, il s'agit de la fermentation malolactique (FML). Cette désacidification naturelle peut être spontanée ou déclenchée par l'inoculation de bactéries lactiques. Selon le type de vins, cette dernière est recherchée ou doit être au contraire évitée. D'autres transformations adviennent ensuite durant l'élevage. Dans le cas d'un élevage en barrique, le bois de chêne est fréquemment utilisé. Cette matrice complexe contient des composés extractibles plus ou moins disponibles selon la qualité de la barrique. Durant l'élevage, les lies peuvent être retirées ou à l'inverse activement réintégrées selon le profil organoleptique recherché. Cette étape est appelée le bâtonnage. Lors de l'élevage, le vin est ouillé pour compenser la part évaporée (« part des anges »). Le vin est ensuite soutiré pour être embouteillé. Des assemblages peuvent être faits. Le vin peut être clarifié par l'utilisation de colles qui provoquent la floculation des particules en suspension et aide à l'étape de filtration. Il peut également être passé au froid pour précipiter les cristaux d'acide tartrique. La filtration permet de rendre le vin limpide et d'éliminer les micro-organismes qui pourraient induire une nouvelle fermentation en bouteille.

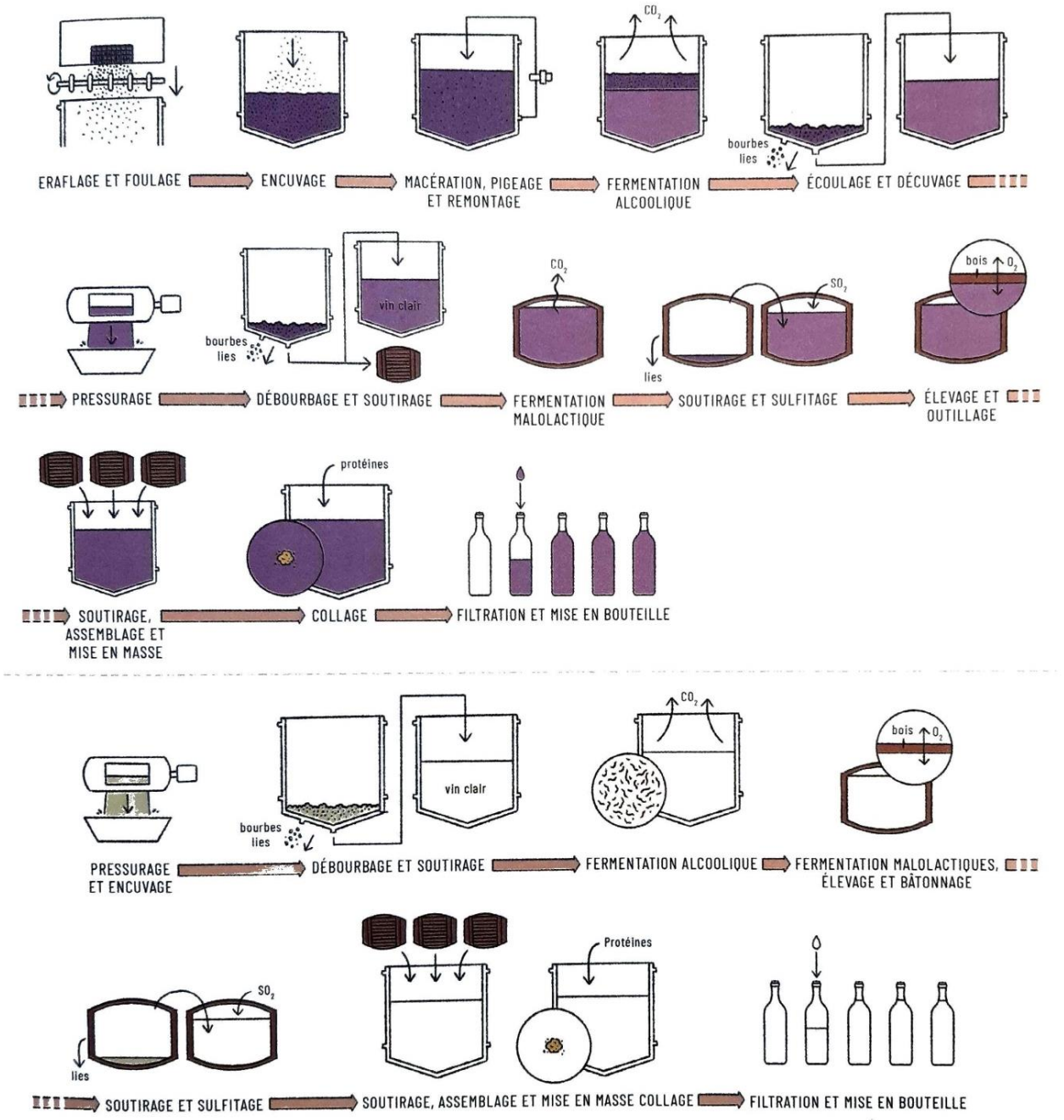


Figure 2. Principales étapes de la vinification en rouge et en blanc. Image issue de « Mille Vignes – Penser le vin de demain » Par Pascaline Lepeltier (Lepeltier, 2022).

1.2. Les levures de vinification

1.2.1. Principaux genres rencontrés en œnologie

La fermentation alcoolique du moût de raisin fait intervenir des levures dites de vinification. Parmi les milliers d'espèces de levures identifiées, seulement 15 correspondent à des levures participant de manière plus ou moins régulière à cette transformation microbiologique (Ribéreau-Gayon *et al*., 2006). Traditionnellement, le vin était produit grâce à l'action des levures naturellement présentes sur les baies de raisin et dans l'environnement du chai. Ces levures appartiennent généralement aux genres *Aureobasidium, Candida, Cryptococcus, Debaryomyces, Dekkera, Issatchenkia, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Saccharomycodes, Schizosaccharomyces, Sporidiobolus, Torulaspora* et *Zygosaccharomyces* (Fleet *et al*., 2002; Ribéreau-Gayon, P., Dubourdien, D., Donéche, 2006). Sur la baie de raisin, la levure fermentaire *Saccharomyces cerevisiae* n'est que rarement isolée (Mortimer and Polsinelli, 1999). En revanche, elle se développe très rapidement dès qu'elle entre en contact avec le jus de raisin grâce à ses excellentes propriétés fermentaires. Ces levures sont bien adaptées à la fermentation alcoolique des jus de raisin. En effet, elles sont capables de fermenter d'importantes quantités de sucre et produisent peu d'acidité volatile (Albertin *et al*., 2011; Camarasa *et al*., 2011). Elles sont également capables d'assimiler les acides aminés, les vitamines du moût et les stérols, lipides nécessaires à sa croissance (Legras *et al*., 2018).

La sélection de bons ferments est une préoccupation ancienne. Empiriquement, les vignerons utilisaient des « pieds de cuve », c'est-à-dire des levains préparés à partir d'un moût de raisin choisi et fermenté à part pour multiplier les levures indigènes (Gonzalez and Morales, 2022). Ces levains étaient ensuite utilisés pour ensemencer les autres cuves. Actuellement, la stratégie communément utilisée consiste à inoculer le moût avec des levains sélectionnés, sous la forme de Levures Sèches Actives (LSA). Cette pratique a émergé dans les années 70 pour réduire la phase de latence et assurer une fermentation complète des moûts (Bauer and Pretorius, 2000). Les « pieds de cuve » sont aujourd'hui encore utilisés par certains vignerons produisant des vins « natures » ou travaillant en biodynamie, qui n'autorisent pas l'utilisation de LSA. Certains viticulteurs estiment que les levures indigènes transmettent toutes les subtilités et caractéristiques du terroir et du millésime. Ces considérations restent cependant non démontrées scientifiquement à ce jour.

1.2.2. Le marché des levures œnologiques

Le marché des levures œnologiques est en croissance constante depuis plusieurs décennies, notamment dans les pays néo-viticoles. Jusque dans les années 1970, la majorité des fermentations était « spontanées ». Aujourd'hui, à l'inverse, la production mondiale a majoritairement recours à l'ensemencement. Il existe un grand nombre de souches de levures sélectionnées. En France, plus de 350 produits étaient disponibles à la vente en 2020. En réalité le nombre de souches est inférieur (environ 150) car il existe de nombreux distributeurs de produits œnologiques qui partagent les mêmes références. Le choix de la souche à utiliser

dépend du type de levurage, (lancement, reprise de fermentation, prise de mousse…), de la nature du moût, du style de vin recherché, de la température de fermentation, de ses besoins en azote, de sa résistance à l'éthanol et à la pression osmotique induite par la concentration initiale en sucres. L'utilisation de souches commerciales peut, dans certains cas, permettre aux producteurs de répondre aux nouveaux défis de l'œnologie moderne et d'accroitre la variabilité et la qualité des vins en adéquation avec la demande du marché (Querol *et al.*, 2018).

La plupart des levures commerciales disponibles appartiennent à l'espèce *S. cerevisiae*. Cette espèce est de loin la plus fréquemment ensemencée pour réaliser la fermentation alcoolique des moûts de raisins. Cependant, l'utilisation d'autres levures présentant des caractéristiques technologiques spécifiques émerge depuis une décennie avec la promesse de répondre aux défis de la filière viti-vinicole. Ces levures appartiennent principalement à trois espèces (*Lachancea thermotolerans, Torulaspora delbrueckii, Metschnikowia pulcherrima*) et plusieurs souches ont été proposées pour diminuer naturellement le taux d'éthanol des vins, corriger leur acidité, modifier leur palette aromatique, ou sont utilisées comme agents de biocontrôle (Simonin *et al.*, 2018).

1.2.3. Variabilité génétique et technologique des souches œnologiques (S. cerevisiae)

Au sein de l'espèce *Saccharomyces cerevisiae*, il existe une très forte variabilité génétique qui a été mise en évidence par de nombreux travaux de génétique et de génomique. De tels travaux ont montré que des variations génétiques structurent des groupes de souches isolées dans différentes niches écologiques (Aa *et al*., 2006). Ainsi, les activités humaines utilisant empiriquement les levures lors de la production de vin, de pain ou de bière ont favorisé la différenciation de groupes génétiques en fonction des processus de fermentation. Ces activités ont participé à l'émergence de populations génétiques distinctes (Coi *et al*., 2017; Peter *et al*., 2018; Sicard and Legras, 2011).

La performance fermentaire des souches œnologiques de *S. cerevisiae* reflète leur bonne adaptation au jus de raisin qui est un milieu de culture particulièrement sélectif. Cependant, au sein de ce groupe génétique, il existe une grande variabilité technologique au regard de leur activité fermentaire et leur contribution aromatique. Les souches œnologiques se distinguent par leur taux de fermentation maximal (Marullo *et al.*, 2006), leur capacité à consommer rapidement le fructose (Guillaume *et al.*, 2007), leur besoin en azote (Jiranek *et al.*, 1995; Julien *et al*., 2000; Taillandier *et al*., 2007), leur résistance aux températures élevées (Marullo *et al.*, 2009) et basses (García-Ríos *et al*., 2017). Les souches de levures œnologiques présentent également de fortes différences dans leur capacité à consommer et produire certains métabolites issus du métabolisme central. L'acide acétique (Giudici and Zambonelli, 1992; Marullo *et al*., 2004; Vilanova *et al*., 2007), le glycérol (Gardner *et al.*, 1993; Remize *et al.*, 2003), l'acide malique (Delcourt *et al.*, 1995; Yéramian *et al.*, 2007) et le SO_2 (Divol *et al.*, 2012) sont les plus connus.

La variabilité technologique des souches de *S. cerevisiae* est également très importante sur le plan

sensoriel et de très nombreux travaux ont permis de caractériser le rôle essentiel des levures dans la production de composés volatiles et non volatiles des vins. Concernant les molécules odorantes, les levures contribuent à la production de centaines de composés qui peuvent être regroupés en trois principales catégories : les arômes fermentaires, les arômes variétaux ainsi que les défauts olfactifs. Les molécules constituant les arômes fermentaires sont issues du métabolisme des sucres et des acides aminés. Les principaux sont les alcools supérieurs, esters et certains acides gras sous forme libre ou estérifiée par l'éthanol (Ribéreau-Gayon *et al.*, 2006). Plusieurs revues bibliographiques sont disponibles sur le sujet (Carpena *et al*., 2021; Lambrechts and Pretorius, 2000; Styger *et al.*, 2011). Les arômes variétaux sont issus du cépage. Certains d'entre eux sont sous forme de précurseurs glycosylés ou S-conjugués libérés par l'action du métabolisme des levures (Ribéreau-Gayon, P., Dubourdien, D., Donéche, 2006). Il a été démontré que différentes souches de levures révélaient mieux que d'autres le potentiel aromatique dans les moûts (Murat *et al.*, 2001; Tomašević *et al.*, 2023). Enfin, la levure réduit le sulfate inorganique en ions sulfides (H_2S). Au cours de cette assimilation, les levures peuvent exporter dans le milieu de l'H_2S, qui présente une odeur d'œuf pourri, et du SO_2 en quantités fortement variables selon les souches (Mendes-Ferreira *et al*., 2009).

Cette importante variabilité entre souches a largement été exploitée pour sélectionner des levains performants en exacerbant des traits phénotypiques d'intérêt pour différents itinéraires de vinification (Marullo *et al*., 2009, 2006; Romano *et al*., 1985; Vion *et al*., 2021).

2. Modulation biologique de l'acidité des vins

Cette sous-partie a fait l'objet d'une revue bibliographique qui situe le contexte œnologique général de l'acidité des vins ainsi que des moyens microbiologiques qui peuvent être utilisés pour maîtriser ce paramètre œnologique majeur.

Exploring the Influence of Yeasts on Wine Acidity: New Insights from *Saccharomyces cerevisiae*

Vion Charlotte[1,2#], Nadine Yeramian[3#], Ana Hranilovic[1], Isabelle Masneuf-Pomarède[2], Marullo Philippe[1,2]

[1]Biolaffort, Bordeaux, FRANCE

[2] UMR 1366 Œnologie, Université de Bordeaux, INRAE, Bordeaux INP, BSA, ISVV

[3]Microbiology Division, Department of Biotechnology and Food Science, Faculty of Science-University of Burgos, Spain

[#]These authors contributed equally to this work

Abstract

Climate change is strongly affecting the winemaking sector. In particular, it is driving decreases in the acidity of the finished wines due to lower malic acid levels in the grapes. Wine-related microorganisms can greatly affect the organic acid content in wines as they are able to metabolize or synthesize different acids. Major advances in biochemistry, ecophysiology and molecular biology led to the selection of numerous yeast strains presenting specific oenological properties, including acidity modulation. The yeast *Saccharomyces cerevisiae* is the most extensively studied species, harboring both malic acid consuming and malic acid producing strains that are of interest for different vinification itineraries. Indeed, yeast-derived acidification of wines can be achieved via malic acid production by *S. cerevisiae*, and as described more recently via lactic acid production by *Lachancea thermotolerans*. Co-fermentations of these two species lend themselves as promising tools to manage wine acidity while ensuring fermentation competition and wine quality. Deacidification of wines via malic acid consumption is relevant in cooler winemaking regions, and/or for shortening of the malolactic fermentation and thereby increasing wine stability. This review delivers an in-depth overview of the effect of various oenologically relevant yeasts on wine acidity, with a special focus on the latest findings on novel (de)acidifying *S. cerevisiae* strains.

Contents

Introduction. ..... **- 17 -**

1. The origin of the acidity in grape juices and wines ..... **- 18 -**

1.1. A brief definition of wine acidity ..... *- 18 -*

1.2. Organic acids and wine acidity ..... *- 19 -*

2. Metabolic pathways of organic acids yeast. ..... **- 21 -**

2.1. Pyruvic acid ..... *- 22 -*

2.2. Malic acid ..... *- 22 -*

2.2.1. Malic acid production pathways ..... - 22 -

2.2.2. Malic acid degradation pathways ..... - 24 -

2.3. Lactic acid ..... *- 24 -*

2.4. Acetic acid ..... *- 25 -*

2.5. Citric, fumaric, α-ketoglutaric, and succinic acids ..... *- 25 -*

3. Genetic levers for controlling organic acids in wines ..... **- 26 -**

3.1. Genetically modified yeast strains ..... *- 26 -*

3.1.1. Lactic acid overproduction ..... - 26 -

3.1.2. Malic acid degradation ..... - 27 -

3.1.3. Malic acid transformation in lactic acid ..... - 27 -

3.1.4. Malic acid overproduction ..... - 28 -

3.2. Natural genetic variations found in yeast populations. ..... *- 28 -*

4. Microbiological applications for reducing wine acidity during the alcoholic fermentation. ..... **- 30 -**

4.1. Contribution of Saccharomyces cerevisiae ..... *- 31 -*

4.2. Contribution of Schizosaccharomyces pombe ..... *- 32 -*

4.3. Contribution of Zygosaccharomyces bailii ..... *- 33 -*

5. Microbiological applications for enhancing wine acidity during the alcoholic fermentation ..... **- 34 -**

5.1. Malic acid production during wine fermentation ..... *- 34 -*

5.2. Lactic acid production during alcoholic fermentation ..... *- 35 -*

5.2.1. The lactic producing species *L. thermotolerans* ..... - 35 -

5.2.2. The contribution of other yeast species ..... - 36 -

5.3. Succinic acid producing yeasts ..... *- 37 -*

6. Conclusion ..... **- 38 -**

7. Bibliography ..... **- 39 -**

Introduction

Climate change is a direct consequence of global warming and represents the greatest challenge that humanity is facing. Steady increases in carbon dioxide and other human-made emissions accentuate the greenhouse effect, with a direct rise of earth temperature which drastically impacts many agricultural productions (IPCC 2020). Climate is crucial to the concept of *terroir*; therefore, its modification largely affects the development and the quality of grapes (Dubourdieu 2004; van Leeuwen and Darriet 2016). The variations in climatic conditions lead to an advanced phenology (Duchêne and Schneider 2005; van Leeuwen and Darriet 2016) with a subsequent maturation phase coinciding with the warmer summer periods. This trend shortens the grape ripening season which may not be compatible with the production of high-quality wines (van Leeuwen and Darriet 2016), especially in continental regions (Webb, Whetton, and Barlow 2008).

The increases in temperatures affect multiple compositional parameters of grapes, including higher sugar concentrations (Coombe 1987; Jones 2007; Nistor *et al*. 2018; Poudel *et al*. 2009), minor synthesis of anthocyanins (Arrizabalaga *et al.* 2018; Coombe 1987; Downey, Dokoozlian, and Krstic 2006; Poudel, Koyama, and Goto-Yamamoto 2020) and decreases of titratable acidity (Arrizabalaga *et al.* 2018; Coombe 1987; van Leeuwen and Darriet 2016; Mira de Orduña 2010; Nistor *et al*. 2018; Poudel *et al.* 2009) due mainly to lower malate concentrations (Iland and Coombe 1988). In turn, the resulting wines have higher alcohol content (Duchêne and Schneider 2005; van Leeuwen and Darriet 2016; Mira de Orduña 2010) and altered aroma composition and sensorial properties (Bureau, Razungles, and Baumes 2000). Acidity is of primary importance for wine balance and its overall sensory profile, as reviewed (Chidi, Bauer, and Rossouw 2018; Conde *et al.* 2007; Frost, Harbertson, and Heymann 2017). Moreover, insufficient acidity in grapes and wines negatively impacts their microbial stability. Indeed, sulfur dioxide (SO_2) is commonly added to wines as a strong reducing agent and antioxidant (Divol, Du Toit, and Duckitt 2012). Several studies showed that low pH facilitates the SO_2 uptake into yeast cells (Macris and Markakis 1974) and increases the abundance of the molecular SO_2, its most antimicrobial and antioxidant form (Divol, Du Toit, and Duckitt 2012). Therefore, at higher pH, the antioxidant and antimicrobial effect of this compound is lower, and its increased additions are required to reach the same level of effectiveness. This practice, however, may not be compatible with increasing consumer demands for wines with lower SO_2 content. (Vally and Misso 2012).

Thus, grape growers and winemakers seek multidisciplinary solutions to adapt their viticultural and oenological practices to preserve the overall quality of grapes and resulting wines (S. Dequin *et al.* 2017; Duchêne and Schneider 2005; van Leeuwen and Darriet 2016; Merloni *et al.* 2018; Webb, Whetton, and Barlow 2008). Several chemical and biological solutions to modulate acidity can be applied before, after or during alcoholic fermentation (AF).

The most common method for chemical deacidification consists of adding calcium ($CaCO_3$) or potassium carbonate (K_2CO_3) to wine, to induce reaction with tartaric acid and precipitation as either potassium bitartrate or calcium bitartrate. Wine acidity can also be corrected via blending strategies with the grape juice/wine of different acidity levels (Comuzzo and Battistutta 2019). Acidification is most commonly achieved by addition of tartaric acid, the strongest organic acid in wine with the highest impact on the pH. Other organic acids such as lactic acid, malic acid, or citric acid can be used as acidulants. Recently, fumaric acid was also authorized as a wine additive, but only to inhibit the malolactic fermentation (RESOLUTION OIV-OENO 581A-2021). For a review of physiochemical methods for acidity adjustment in wine, see H. Volschenk, van Vuuren, and Viljoen-Bloom (2006).

Modern winemaking seeks to limit the external inputs in wines, and biological approaches for managing wine acidity are thus preferred. Wine acidity is modulated by various wine-related microorganisms, in particular by yeasts during AF and lactic acid bacteria during malolactic fermentation (MLF). During MLF, the malic acid is converted into lactic acid and CO_2 via the activity of the **malolactic enzyme** (**MLE EC 4.1.1.101**) found in some lactic acid bacteria belonging to the genera *Oenococcus, Lactobacillus,* and *Pedicoccus* as reviewed by (Sumby, Grbin, and Jiranek 2014). During AF, yeasts can either acidify or deacidify the medium. Both these aspects will be developed in this review, with a special emphasis on the yeasts capable of preserving the wine acidity.

Firstly, the key organic acids that play a role in wine acidity will be briefly overviewed. Secondly, the metabolic origin and the pathways involved in the biosynthesis and catabolism of organic acid will be presented. Finally, the fourth and fifth sections will be dedicated to biological deacidification and acidification of wines, respectively.

1. The origin of the acidity in grape juices and wines

1.1. A brief definition of wine acidity

The acidity in wine can be defined by two main parameters: the pH and the Titratable Acidity (TA) (Ribereau-Gayon *et al.* 2006). pH is the measure of free protons concentration of a solution, calculated as $pH = - \log[H^+]$, while TA refers to the concentration of titratable H_3O^+ ions in wine. In hydro-alcoholic solutions like wine, weak organic acids are partially dissociated, and their dissociation degree is represented by their pKa. Lower the pKa, stronger the dissociation and then the concentration of H_3O^+ ions in the solution. Typical values for white wine are pH of 3.0-3.4 and TA of 6-9 g/L as tartaric acid and for red wine, pH of 3.3-3.7 and TA of 5-8 g/L as tartaric acid. The TA is a good proxy for the perceived sourness in wine, while pH is weakly correlated with the sourness perception (Plane, Mattick, and Weirs 1980). In practice, goals of achieving low wine pH avoiding excessively high TA (and thus sourness) are often competing. Besides pH and TA, another parameter of the oenological importance is the buffer capacity. The buffer

capacity can be defined as the ability of a solution to maintain a stable pH upon addition of a strong acid or base. This property is directly correlated with the concentration of weak acids and their conjugate bases. Thus, wines with higher TA have higher buffering capacity.

Acidity is a key parameter of wine quality and has a complex impact on taste, aroma, and mouthfeel (Frost, Harbertson, and Heymann 2017). Too acidic wines are perceived by consumers as sour and too sharp. Conversely, wines with too little acidity are described as flabby, flat, with less defined aromas and flavors (Robinson 2006). In addition, acidity contributes to 'freshness', a feature sought by consumers in modern wines. Acidity of wine will maintain its freshness while also modifying its flavor components and its color (Conde *et al*. 2007). Freshness is a complex sensory attribute that requires a correct balance between various components, including acidity, ethanol, and fruitiness. Wine acidity also have a direct impact on color since pH directly impacts anthocyanins absorbance (Sims and Morris 1985). Finally, more acidic wines are generally considered as having increased aging potential (Fowles 1992), thus, controlling wine acidity is a key factor for various components of wine quality.

1.2. Organic acids and wine acidity

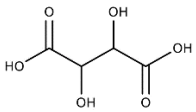
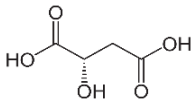
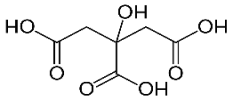
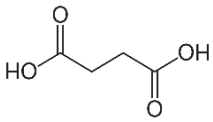
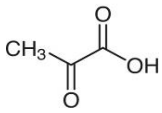
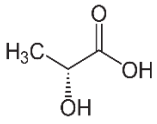
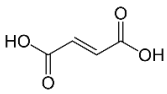
The main organic acids contributing to wine TA are presented in Table 1. Two of them, tartaric acid and malic acid contribute up to 90% of the titratable acidity of grape juices and wines. Tartaric acid is a marker of *Vitis vinifera* species (Ribereau-Gayon *et al.* 2006) and is quantitatively the main organic acid of grape juice and wine. The isomer of tartaric acid found in the grape is the L (+) form. Its concentration varies between 2 and 10 g/L (Chidi, Bauer, and Rossouw 2018). Malic acid takes its name from the apple (*malus* in Latin), in which it is present in high concentration. Mature grapes contain between 2 and 6.5 g/L of malic acid (Chidi, Bauer, and Rossouw 2018). This C4-dicarboxylic organic acid takes three acid-base forms: malic acid (H2M), hydrogen malate (HM) or malate (M). In grape juice and wine, the protonated forms are predominant (pK_{a1} = 3.40) while malate is mostly found in cytosolic conditions (pK_{a2} = 5.11). Malic acid has two stereoisomeric forms (L and D) but only the L-isomer exists naturally.

The concentrations of organic acids in grapes are influenced by many factors, including grape variety, ripening stage, climatic conditions, soil potassium levels, plant nutrition, and the canopy management (Fowles 1992; Ruhl 1989). For example, in warm climate, malic acid can decrease to below 1 g/L, contributing to insufficient TA and excessive pH at harvest (Buttrose, Hale, and Kliewer 1971; Kliewer 1964; H. Volschenk, van Vuuren, and Viljoen-Bloom 2006). Several reviews (Ford CM. 2012; Hernân Gerós, Chaves, and Delrot 2012; H. Volschenk, van Vuuren, and Viljoen-Bloom 2006) focus on the metabolism and the composition of organic acids in grapes, which is beyond the scope of this review.

Both tartaric and malic acid formed in grapes early in the growing season, but their behaviour during ripening and winemaking differs. Tartaric acid is synthesised during initial berry cell division and remains

stable throughout the berry ripening process. It is not metabolized during winemaking but can be lost through physiochemical mechanisms like precipitation. Malic acid is present at very high concentrations prior to veraison, but it is actively metabolized during berry ripening, and is significantly impacted by the microbial activity, as described in the following sections of this review. Organic acids can be partially or totally consumed during the successive fermentation steps that transform grape must in wine. In turns, yeast and bacteria metabolisms produce other organic acids such as succinic acid, lactic acid, malic acid, citric acid, and acetic acid, that modulate wine acidity.

Table 1: Organic acids in grape must and wine

Acid	Structure	pK_a in water*	Typical concentrations in grape juice (g/L)	Typical concentrations in wine (g/L)	Source**
Tartaric		2.98; 4.34	2-10	2-10	G
Malic		3.40; 5.11	1-7	0.5-7	G, Y
Citric		3.13; 4.76; 6.4	0.1-0.7	0.1-0.8	G, Y
Succinic		4.21; 5.64	0	0.5-1.5	Y
Pyruvic		2.4	0	0.01-0.5	Y
Lactic		3.86	0	0-3	LAB, Y
Acetic		4.76	0	0.1-0.5	Y, LAB, AAB
Fumaric		3.03; 4.44	0-0.1	0-0.1	G,Y

**Polyprotic acids have one pK_a for each -COOH group. The pK_a values in water are slightly different to those in wines, as they are affected by ethanol concentration, ionic strength, and temperature. As a rule of thumb, the first pK_a of an organic acid is 0.10-0.15 units higher in wine than water and 0.10-0.15 units lower for second pK_a.*

*** G: grape, Y: yeast, LAB: lactic acid bacteria, AAB : acetic acid bacteria*

2. Metabolic pathways of organic acids yeast

Organic acids constitute branch points of many catabolic routes. Pyruvic acid is the end point of glycolysis while citrate, malate, fumarate, and succinate are the main metabolites of tricarboxylic acid (TCA) and glyoxylate cycles. Organic acids are building blocks involved in amino acids and fusel alcohols biosynthesis. Moreover, due to their different oxidoreductive levels, their metabolism plays a central role in the oxidoreductive homeostasis. Interestingly, most organic acids participate in metabolic reactions in cytosol, peroxisome, and mitochondrial matrix, which are catalyzed by specific isoforms. This compartmentalization, as well as the existence of membrane shuttle systems, add complexity to our understanding of metabolic flux of these compounds. As previously reviewed, the transfers between yeast compartments play an essential role in the homeostasis of oxidoreductive cofactors ($NAD(P)^+$/NAD(P)H) into the mitochondrial matrix (Bakker *et al.* 2001). The complex interconnection of organic acids is outlined out in the Figure 1, representing the central metabolism map of the model species *Saccharomyces cerevisiae* that has been widely investigated. Interestingly, *Schizosaccharomyces pombe* and *Lachancea thermotolerans* species, which participate in the AF, have particular metabolic features in their malate and lactate metabolism (blue and green inserts, respectively). The following paragraphs highlight the general biochemical and enzymatic aspects of organic acid metabolism, which are crucial for understanding the biological variations during the winemaking process. Each enzymatic reaction is described by its EC identifier as well as by the name of the *S cerevisiae* protein(s).

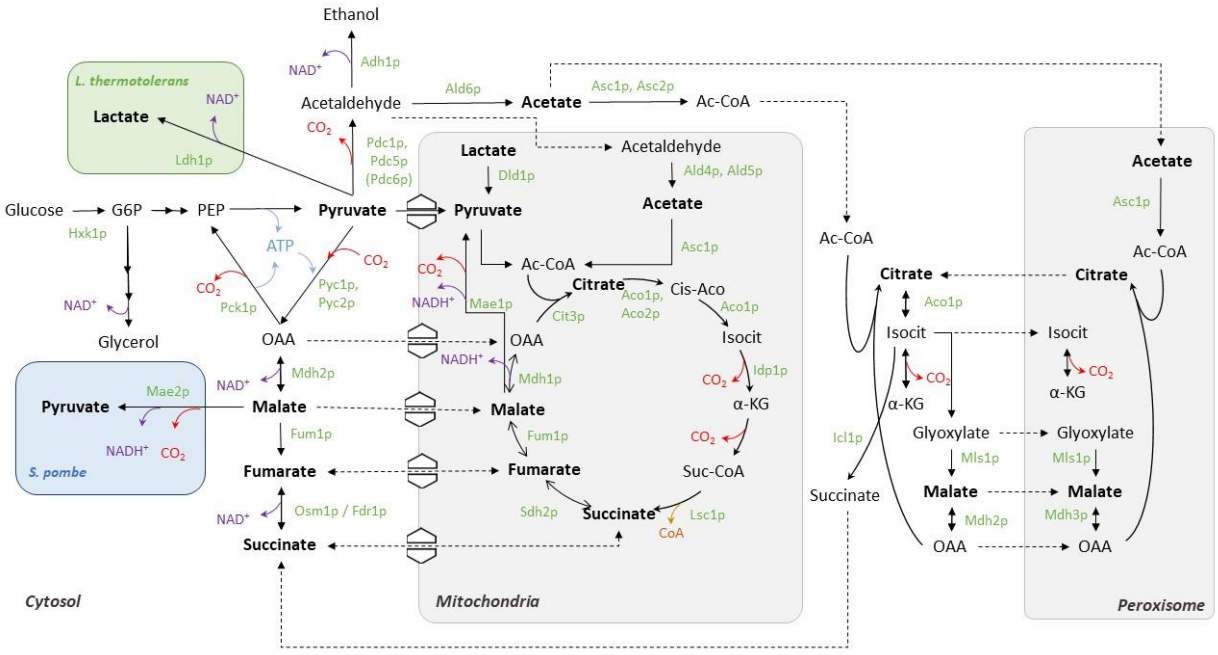


Figure 1: Interconnection of organic acids in the central carbon metabolism of *S. cerevisiae.*

2.1. Pyruvic acid

The pyruvic acid is the end-product of glycolysis and is produced by the irreversible dephosphorylation of phosphoenol-pyruvate (PEP) by the pyruvate kinase (Cdc19p/Pyk2p **EC 2.7.1.40**). In presence of oxygen, this acid is carried in the mitochondrial matrix and incorporated in the TCA cycle by the pyruvate dehydrogenase complex (Pdh-cpx **EC 1.2.4.1**) where it is fully oxidized through the respiration chain that provides energy (ATP) to the cell. Pyruvate can also be decarboxylated in acetaldehyde by the cytosolic pyruvate decarboxylase. Acetaldehyde is then converted in acetate which results in the production of cytosolic acetyl-CoA that plays a role in the biosynthesis of fatty acids during alcoholic fermentative. This shunt is known as the pyruvate dehydrogenase by-pass (Pronk, Jack T; Steensma, H. Yde; Van Dijken 1996; Remize, Andrieu, and Dequin 2000). In hypoxic conditions, ATP is only produced by glycolysis and must be constantly reduced for regenerating the oxidized form (NAD^+) which is essential for the ongoing of glycolysis. The cytosolic reduction of pyruvate may provide NAD^+ by different metabolic routes. In higher eucaryotes, pyruvate is decarboxylated and reduced in lactic acid, which also occurs for some yeast species (see below). Alternatively, pyruvate follows the pathway of AF, which is a common feature of fermenting yeast species. Briefly, during AF pyruvate is decarboxylated and then reduced to ethanol by the subsequent actions of pyruvate decarboxylase (Pdc1p/Pdc5p. **EC 4.1.1.72/43**) and cytosolic alcohol dehydrogenase (Adh1p: **EC 1.1.1.1**). Alternatively, pyruvate can be reduced to malate (via oxaloacetate) or oxidized to citrate, isocitrate and a-ketoglutarate through the oxidative branch of the TCA cycle. Pyruvate is therefore the origin of all organic acids wine, as discussed below. This also explains its very low concentration level at the end of the fermentation.

2.2. Malic acid

2.2.1. Malic acid production pathways

In fungi, malic acid is produced from pyruvate by taking four main routes.

(1) In presence of oxygen, malic acid is produced in the mitochondrial matrix from fumarate which is in turn formed by the succinate dehydrogenase complex (SDH-cpx, **EC 1.3.5.1**). These steps belong to the oxidative branch of the TCA cycle. The conversion of fumarate to malate is catalyzed by the activity of fumarase (Fum1p, **EC 4.2.1.2**) which has much higher affinity for fumarate than for malate (Pines *et al.* 1996). Interestingly, this enzyme can be located both in the cytosol and the mitochondrial matrix, which dependents of the glyoxylate shunt activity (Regev-rudzki *et al*. 2009). Therefore, it is difficult to discriminate between the cytosolic and the mitochondrial production of succinate and fumarate from malate.

(2) During the alcoholic fermentation, the TCA cycle is split in two branches (oxidative and reductive) at the level of SDH-cpx due to the lack of oxygen as final electron acceptor. However, C4 organic acids (malate, fumarate, and succinate) can still be produced in the mitochondria from oxaloacetate by the

reductive branch of TCA (Camarasa, Grivet, and Dequin 2003; Clement *et al.* 2013). This pathway requires the reduction of oxaloacetate into malate by the mitochondrial isoform of the malate dehydrogenase (Mdh1p, **EC 1.1.1.37**). In *S. cerevisiae*, this enzyme has a low Km for both malate and oxaloacetate and is active in both directions (Pines *et al.* 1996, 1997; Steffan and McAlister-Henn 1992).

(3) The third route is the cytosolic production of C4 organic acids that follows a parallel path to the reductive branch of TCA. Since oxaloacetate is exclusively produced by the cytosolic pyruvate carboxylase activity (Pyc1p/Pyc2p **EC 6.4.1.1**), C4 acids are derived from cytosolic oxaloacetate when glucose is the sole carbon source. This anabolic reaction is essential for gluconeogenesis and plays a decisive role in biosynthesis of aspartate on fermentable carbon source (Stucka *et al.* 1991). The presence of a cytosolic malate dehydrogenase (Mdh2p **EC 1.1.1.38**) allows the direct reduction of oxaloacetate in malate without any mitochondrial transport. In *S. cerevisiae*, the cytosolic isoform has a strong affinity for oxaloacetate (Km = 0.07 mM) and controls malic acid production (Pines *et al.* 1997). This cytoplasmic reaction can provide an alternative pool of NAD^+ at the beginning of alcoholic fermentation, supplementing the NAD^+ generation by the glycerol biosynthesis during the glycerol-pyruvic fermentation (Á. Benito *et al.* 2015). The activity of cytosolic malate dehydrogenase is negatively regulated by glucose at the transcriptional and post transcriptional level (Hung *et al.* 2004; Minard and McElister-Henn 1994), and the role of this route is this route is thus minor role in high gravity matrices. However, cytosolic Mdh2p isoform is routinely quantified during alcoholic fermentation (Blein-Nicolas *et al*. 2015) by proteomics and its role on malic acid homeostasis remains to be clarified.

(4) The fourth production route of malic acid involves the condensation and acetyl-CoA and glyoxylate, catalyzed by the malate synthase (Mls1p **EC 2.3.3.9**). Although the glyoxylate cycle is involved in utilization of lipidic sources in peroxisome, this protein subject to glucose catabolic repression is also localized in the cytoplasm in presence of ethanol (Kunze *et al.* 2002) and has been quantified by proteomics during AF (Blein-Nicolas *et al.* 2015).

The secretion of malate into the extracellular media remain poorly documented. (Salmon 1987) reported that the export of malic acid depends on an active transporter. However, the mechanisms triggering the expulsion of malic acid outside the cell are poorly documented. Salmon provided preliminary evidence of a malic efflux dependent on glucose, but such transporter was never characterized despite several efforts (Casal *et al.* 2008).

2.2.2. Malic acid degradation pathways

During the vinification, malic acid is partially degraded by fermenting yeasts. First, malic acid can be converted in another C4 organic acids via the glyoxylate, and TCA cycles as described above. In addition, malate may be assimilated as a carbon source by the malic enzyme. Yeasts decarboxylate malic acid into pyruvic acid by the NAD-dependent malic enzyme (Mae1p, EC : 1.1.1.38) (Boles, De Jong-Gubbels, and Pronk 1998). This enzyme requires divalent cations (Mn^{2+} or Mg^{2+}) as cofactors and may have different compartmentation according to the yeast species. In *Sz. pombe*, the decarboxylation of malic acid occurs in the cytosol and the Km of malic enzyme has a strong affinity to malic acid (Km = 3.2 mM). In *S. cerevisiae*, the enzyme is located in the mitochondria and exhibits a much higher Km (50 mM) (Saayman and Viljoen-Bloom 2006).

2.3. Lactic acid

Lactic acid is a monoprotic acid (pK_a 3.86) that is mostly produced by the malolactic enzyme of bacteria. Its concentration range in wine mostly depends on the malolactic fermentation which is not the purpose of this review. The species *S cerevisiae* do not produce significative amount of lactic acid since this organic acid is mostly consumed to produce pyruvate in respiratory conditions (Lodi and Ferrero 1993). In contrast, other fermenting yeast such as *L. thermotolerans* can produce high amount of lactic acid through the direct transformation of pyruvate by the cytosolic lactate dehydrogenase (Ldh1p/Ldh3p EC 1.1.1.27). The molecular mechanisms underlying lactic acid biosynthesis at the expense of ethanol or any other metabolite in *L. thermotolerans* are still poorly understood, as well as the genetic basis of a high inner-strain variation in this trait (Banilas, Sgouros, and Nisiotou 2016; Hranilovic *et al.* 2018). Based on the whole genome sequence of the strain type (Souciet *et al*. 2009), *L. thermotolerans* possesses three Ldhp and two Adhp paralogues. The expression of these genes was recently analyzed in a study delivering the initial information on molecular mechanisms of differential lactic acid production in *L. thermotolerans* (Sgouros *et al.* 2020). This revealed up-regulation of *LDH2* in high-lactate producing strains, with no further differences in expression of other genes (i.e., *LDH1*, *LDH3*, *ADH1* and *ADH2*) at the early stationary phase (Sgouros *et al*. 2020).

Moreover, it is unclear whether formation of lactic acid from pyruvate due to the inherent *LDH* activity serves to replenish oxidized NAD^+ depleted through glycolysis, which is in yeasts primarily achieved through alcoholic fermentation. However, while ethanol can leave the cell by passive diffusion, lactic acid has to be actively transported at the expense of ATP, as it is at high intercellular pH present in a dissociated form (Sauer *et al*. 2010). To maintain the proton motive force and the intercellular pH, protons must be exported via the plasma membrane H^+-ATPase at the expense of one ATP per proton. Though, the exact mechanisms are still unknown, the export of lactate (*i.e.*, dissociated anion) could also be ATP-dependent (Sauer *et al*. 2010). Once exported, lactic acid is at low extracellular pH present in its protonated

form and can thus permeate the cell membrane via passive diffusion, perpetuating the energy-requiring cycle (Sauer *et al.* 2010). Recycling of NADH via lactic acid pathway therefore appears to be more costly for the cell compared to the ethanol pathway. The physiological and/or evolutionary benefits of the simultaneous accumulation of ethanol and lactic acid are unclear, but this strategy might be useful to outcompete microorganisms that co-exist in a same niche, comparable to the 'make-accumulate-consume' strategy in *S. cerevisiae* (Hagman *et al.* 2013). Altogether, this invites further research on central carbon metabolism in *L. thermotolerans*, particularly on the regulatory framework of redox balance, through studies purposely designed to quantify the microbial growth and evolution of metabolites in conjunction with transcriptomics.

2.4. Acetic acid

Acetic acid is the main volatile acid in wine and is a byproduct of microbial metabolism. It is considered as an undesirable compound and constitutes an organoleptic default in wine at high concentration. Except in the case of wine spoilage by acetic acid bacteria, acetic acid is mostly produced by fermenting yeasts at the beginning of alcoholic fermentation in amounts ranging from 200 to 600 mg/L (Vilela-Moura *et al*. 2011).

The metabolic pathway of acetate under anaerobic conditions of grape juice's acetic acid is mostly produced by the pyruvate dehydrogenase bypass which reroutes part of acetaldehyde in acetate by the main cytosolic isoform of **aldehyde dehydrogenase** (**Ald6p EC 1.2.1.3**) (D. Postma *et al.* 2022; Remize, Andrieu, and Dequin 2000). Acetic acid formed is then transformed into Acetyl-CoA by the **acetyl-CoA synthetase** (**Acs1p EC 6.2.1.1**). The resulting acetyl-CoA might be used in fatty acids biosynthesis or enter in the mitochondria with a further oxidation in the tricarboxylic cycle. The mitochondrial isoenzyme Aldp5 is also implicated in acetate formation in oenological conditions (Saint-Prix, Bönquist, and Dequin 2004).

Acetic acid production can be partially linked to glycerol production in specific conditions (Eglinton *et al*. 2002). Remarkably high sugar concentration (>300 g/L) triggers an overproduction of glycerol by yeasts in response to the osmotic stress (Blomberg 2000). This glycerol synthesis leads to an overflow of oxidated NAD. This response is coupled with an overproduction of acetic acid due to the overexpression of *ALD2* and *ALD3* genes, regenerating NADH (Navarro-Aviño *et al.* 1999). The coupled biosynthesis of glycerol and acetate is transitory and depends on proteins synthesis (Albertyn, Hohmann, and Prior 1994).

2.5. Citric, fumaric, α-ketoglutaric, and succinic acids

The TCA acids are typical by-products of AF detected in wines in variable concentrations During AF, succinate can be formed via both the branches of the TCA cycle: (1) the oxidative branch of the TCA pathway, or (2) by the TCA reductive pathway via fumarate reductase. In the first case, the TCA cycle proceeds from oxaloacetate via malate to succinate but does not progress any further as the SDH complex is not functional during AF (Wales, Cartledge, and Lloyd 1980). Additional succinate is formed by oxidative

decarboxylation of α-ketoglutarate when glutamate is present in the medium (Camarasa, Grivet, and Dequin 2003). Instead of being produced by the TCA pathway, succinic acid can also be synthetized from isocitrate by the glyoxylate shunt. This reaction is catalyzed by isocitrate lyase (Icl1p **EC 4.1.3.1**) (Fernandez, Moreno, and Rodicio 1992). However, enzyme is induced by growth on ethanol and repressed by growth on glucose (Raab and Lang 2011) and thus might play a minor role during the alcoholic fermentation (Klerk 2010).

Fumarate is an intermediary of the TCA cycle and can be formed by the reductive pathway and catalyzed by the fumarate synthase (Fum1p **EC 4.2.1.2**) that has both mitochondrial and cytosolic localization (Wu and Tzagoloff 1987). Citrate is part of the TCA cycle and can be formed by the condensation of oxaloacetate and acetyl-CoA. This reaction is catalyzed by the citrate synthase (Cit1p **EC 2.3.3.1**) (Kim, Rosenkrantz, and Guarente 1986). Its transcription is subject to glucose repression (Rosenkrantz *et al*. 1994). *CIT1* has a paralog: *CIT2* which is the peroxisomal isoenzyme involved in glyoxylate cycle. It also catalyzes the condensation of oxaloacetate and acetyl-CoA to form citrate. In the TCA cycle, citrate is converted in cis-aconitate then isocitrate into α-ketoglutarate by the aconitase (Aco1p **EC 4.2.1.3**) (Gangloff, Marguet, and Lauquin 1990) followed by the isocitrate dehydrogenase (Idp1p **EC 1.1.1.42**). This conversion of citrate to α-ketoglutarate is also possible in the cytosol as the Aco1p localization is dual. In addition, *IDP1* has a paralog, *IDP2* which is the cytosolic isoenzyme (D. Postma *et al*. 2022).

3. Genetic levers for controlling organic acids in wines

3.1. Genetically modified yeast strains

In the last decades, several attempts have been made to modulate the acidity by using genetically modified yeasts. These tentative mostly focused on the modulation of lactic and malic acids and some of them were applied at the industrial scale.

3.1.1. Lactic acid overproduction

Advances were made to genetically engineer *S. cerevisiae* strains for increased lactate yields for the oenological use. Such strains were obtained by implementing the heterologous expression of the *L-LDH* gene of *Lactobacillus casei*, controlled by the yeast Adh1p promoter (Sylvie Dequin and Barre 1994). This resulted in simultaneous conversion of glucose to both ethanol and lactate in YNB medium (5% glucose, pH 5), with up to 20% of the glucose transformed into L-lactate (Sylvie Dequin and Barre 1994). In a follow-up study, eight commercial wine starters were engineered for lactic acid production and characterized under oenological condition (S Dequin, Baptista, and Barre 1999). Depending on the strain, lactic acid levels in a synthetic grape juice-like medium ranged from 1.6 to 4.1 g/L, whereas the corresponding parental strains

formed less than 0.2 g/L of lactic acid (S Dequin, Baptista, and Barre 1999). The matrix-derived impact on final lactate yields was further trialed using the strain that produced the largest amounts of this metabolite. Wines obtained from seven grape musts contained between 2.6 and 8.6 g/L of lactic acid, highlighting the impact of grape juice composition in the pathway. The final acidity was affected by the lactic acid concentration but also the buffering capacities of each grape juice. For example, lactic acid concentration of 5.7 g/L decreased the pH of one wine by 0.11 and another by 0.36 units. Despite the slower CO_2 production rate, extent of the engineered strain remained unaffected, as did the volatile acidity production. The acidified wines also showed up to 0.25% v/v lower ethanol content compared to the control strain as a result of partial carbon diversion from ethanol to lactate (S Dequin, Baptista, and Barre 1999). Because lactic acid serves as a final electron sink, its formation results in reduction of equimolar amounts of alcohol without affecting the intracellular redox balance. This is of additional value given that the wines which are deficient in acidity often contain overly high ethanol levels. However, given that the concentrations of lactic acid required to decrease ethanol content by 1% v/v exceed 15 g/L, any major decreases via this strategy are likely to impart excessive acidity to wines (Tilloy *et al.* 2015).

3.1.2. Malic acid degradation

In *S. cerevisiae*, malic acid degradation is incomplete due to several factors. The transport of this acid into the cell is inefficient (Salmon, Vezinhet, and Barre 1987) and the activity of its malic enzyme is moderate due to its mitochondrial localization and its high Km value (reveiwed by (Saayman and Viljoen-Bloom 2006)).To overcome these limitations Heinrich Volschenk *et al.* (1997) proposed the heterologous expression of the genes *mae1* and *mae2* of *Sz. pombe* by a genetic engineering approach. These genes encode for a transmembrane malic acid transporter (J Grobler et al. 1995) and a cytosolic malic isoform (Viljoen *et al*. 1994), respectively. This GM *S. cerevisiae* strain degraded up to 8 g/L of malic acid, greatly exceeding *S. cerevisiae* malate depletion rate (0 to 3 g/L) (H. Volschenk *et al*. 2001) and avoiding off flavors produced by *Sz. pombe*.

3.1.3. Malic acid transformation in lactic acid

Most red wines and some white ones are subject to MLF. This secondary fermentation has some disadvantages such as unpredictability of its occurrence and control during vinification (Bousbouras and Kunkee 1971; Kunkee 1991). Hence, several studies attempted to consume malic acid via *S. cerevisiae* during alcoholic fermentation to avoid the need of MLF.

The introduction of the malolactic enzyme in *S cerevisiae* was proposed by different teams by cloning the malolactic gene *MLES* of *Lactococcus lactis* (V Ansanay *et al.* 1993; Denayrolles, Aigle, and Lonvaud-Funel 1995). However, the transformation of malate into lactate was incomplete (Virginie Ansanay *et al.* 1996) due to the lack the pump for malic acid uptake in *S. cerevisiae*. To overcome this, different strains of

S. cerevisiae co-expressing the malic transporter encoded by the gene *mae1* of *Sz. pombe* and the *Lactococcus lactis* malolactic gene *MLES* were proposed (Bony *et al.* 1997; Heinrich Volschenk *et al*. 1997). The combined action of these enzymes led to successful and complete malolactic fermentation by yeast without the use of lactic bacteria.

As an attempt to include at the industrial scale the MLF step in the alcoholic fermentation process, the ML01 strain has been genetically modified to conduct the malolactic fermentation (Husnik *et al.* 2007). This genetically modified wine yeast was a "*Prise de Mousse"* strain. It contains the malate transporter gene (*MAE1*) from *Sz. pombe* and the malolactic gene (*MLEA*) from *Oenococcus oeni*. It is capable to decarboxylate up to 9.2 g/L of malate to equimolar amounts of lactate during the alcoholic fermentation. Sensory analyses reported that it would be suitable for winemaking.

3.1.4. Malic acid overproduction

The inability to use genetically modified yeast in industrial fermentations limited the implementation of genetic engineering strategies for managing wine acidity. Interestingly GM strains has been created to overproduce malic acid in a non-oenological context. (Zelle *et al.* 2008) proposed to achieve efficient malate production by improving the following cytosolic pathway: conversion of glucose to pyruvate trough the glycolysis, followed by carboxylation of pyruvate to oxaloacetate (by Pyc2p) and reduction of oxaloacetate to malate (by the cytosolic isoenzyme Mdh2p). They evaluated the impact of three genetic modifications: (i) overexpression of the native pyruvate dehydrogenase encoded by *PYC2*, (ii) high expression of an allele of *MDH3* of which the encoded malate dehydrogenase was retargeted to the cytosol. Indeed, *MDH3* encodes the peroxisomal isoenzyme of the malate dehydrogenase but Mdh3p will be used preferentially over the cytosolic Mdh2p because the latter is subject to catabolite inactivation which is undesirable for cultivation on glucose. Finally, (iii) the *Sz. pombe* malate transporter was expressed in the *S. cerevisiae* strain. The cumulative effect of the three genetic modifications was stronger than a single modification and the resulting engineered strain produced up to 59 g/L of malic acid.

3.2. Natural genetic variations found in *S. cerevisiae* populations

Recent studies focused on elucidating the natural variation in the production of organic acids by fermenting *S. cerevisiae* strains in the oenological context using the quantitative genetics approaches. Several QTLs were linked to the variation of succinate production located on the chromosome IV, VI, XI, XV, XIII and XIV (Ambroset *et al*. 2011; Eder *et al.* 2020; Salinas *et al*. 2012). For succinic acid production, the impact of two genes *FLX1* (Chr IX QTL) and *MDH2* (Chr XV QTL) were experimentally validated. *FLX1* encodes a transporter of flavin adenine dinucleotide (FAD) across the mitochondrial membrane that could modulate the activity of the succinate dehydrogenase. *MDH2* encodes the cytosolic malate dehydrogenase involved in malate/oxalacetate interconversion that play a role in glyoxylate cycle. Recently,

the genetic determinism of malic acid has been also investigated in multi environmental QTL-mapping program (Peltier *et al.* 2021). The percentage of malic acid consumed by a wide population yeast strain has been calculated (MAC%). Eleven QTLs linked to malic acid consumption have been identified (Peltier *et al.* 2021; Vion *et al.* 2021). For six of them a gene affecting the variation of MAC% among progeny were validated by functional genetics experiments. The genes *MAE1*, *PYC2*, and *SDH2* are directly related to the malic acid, pyruvic acid, and oxaloacetate metabolism and their position on the metabolic map are figured out Figure 2. *MAE1* encodes the mitochondrial malic enzyme, *PYC2* encodes an isoform of pyruvate kinase, and *SDH2* the catalytic subunit of the succinate dehydrogenase complex. Interestingly, the gene *MAE1* carries a single nonsynonymous allelic variation $MAE1^{I605V}$ that has been previously described to modify the production of branched ethyl esters with are directly connected to the malic acid catabolism (Eder *et al.* 2018). In addition, two other genes *PMA1* and *PNC1* have a role on proton and NAD^+/$NADH,H^+$ homeostasis. Finally, the gene *YBL036c*, encodes for a putative alanine racemase with connection with the mitochondrial pyruvate pool. Interestingly, most of the allelic forms of QTLs involved in malic acid consumption were derived from the same parental strain. Phylogenomic analyses demonstrated that those alleles were derived to flor yeast genome (Peltier *et al.* 2021) that constitutes a specific genetic group of wine yeasts (Coi *et al*. 2017). Flor yeasts are adapted to survive in harsh environment, depleted of sugar and enriched in ethanol. Recently, we demonstrated that compare to other *S. cerevisiae* strains, the yeast population is able to consume a greatest fraction of malic acid present in grape juice (Vion *et al.* 2023).

The expression and the contribution of the different QTLs mapped for malic acid consumption have been investigated in breeding programs aiming to control the level of malic acid at the end of the AF. First, a marker assisted selection of malic-consuming strains was achieved demonstrating that individuals carrying a high proportion of *enhancer* alleles consumed statistically more malic acid than those carrying a proportion of *preserver* alleles. Although, each allele had a small impact on the final MAC% value their cumulative effect strongly impacts the MAC% (Vion *et al.* 2021). Second, malic producer strains were obtained by crossing together strains consuming low amounts of malic acid. After two cycles of segregation and selection, individuals producing up to 3.5 g/L of malic acid at the end of the alcoholic fermentation were obtained. These extreme strains were significantly enriched in *preserver* alleles (Vion *et al.* 2022).

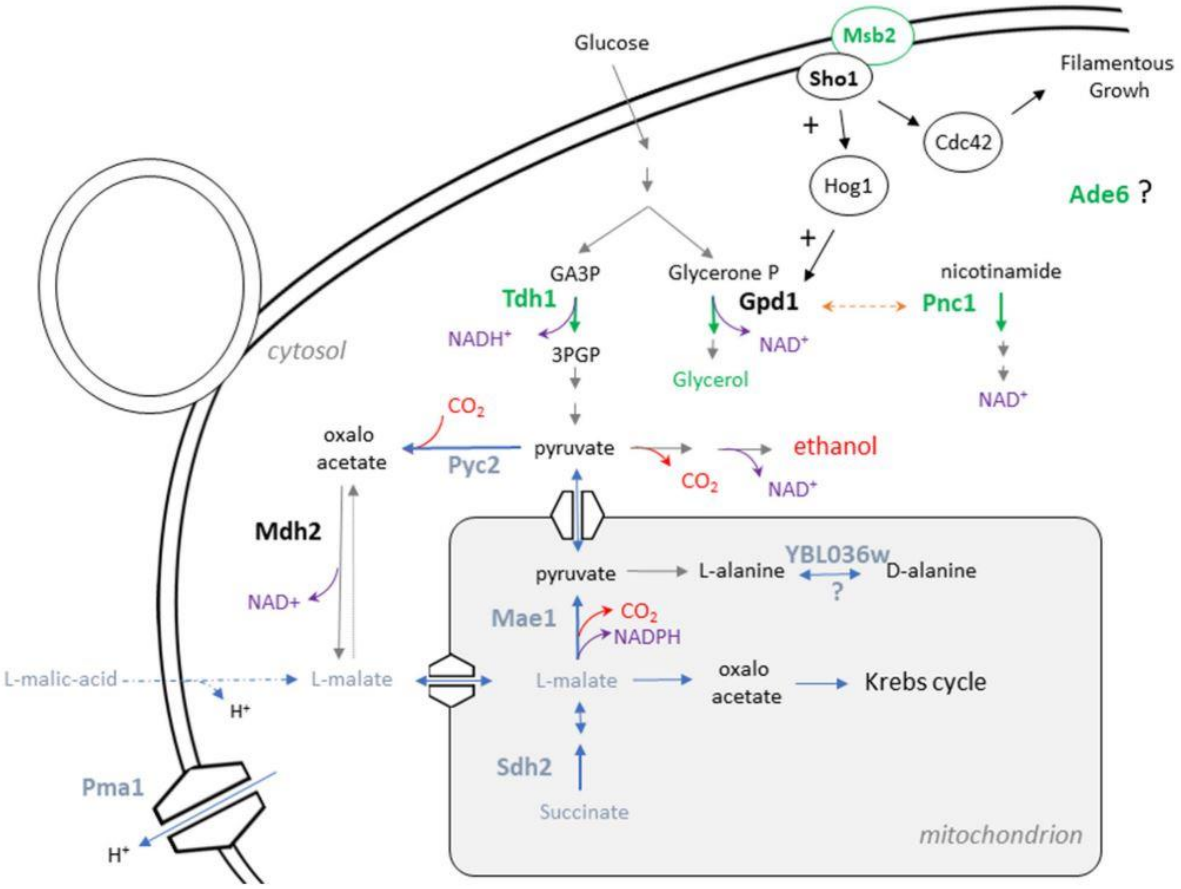


Figure 2: Metabolomic map of *S. cerevisiae*. Genes impacting glycerol production are represented in green while gene impacting *MAC%* are presented in blue. Figure from (Peltier *et al.* 2021).

4. Microbiological applications for reducing wine acidity during the alcoholic fermentation

The deacidification of wine may be necessary for maintaining a good sensorial balance between acid and sweet taste. In red wines, it is used for two main reasons: (1) to facilitate the beginning of MLF since LAB are inhibited by low pH (Davis *et al.* 1985; Wibowo *et al.* 1985); (2) to impact the sensory perception of wines, as high acidity may cause excessive sourness and negatively impact other wine sensory parameters (e.g., astringency) (Sowalsky and Noble 1998). Since tartaric acid is not metabolized by yeasts (Gao and Fleet 1995), the reduction of acidity during the alcoholic fermentation is due to the capability of fermenting yeast to consume malic acid. This degradation significantly modifies the TA and wine pH. As recently demonstrated, the amount of malic acid consumed by yeast depends on many genetic factors that have been presented in the previous section for *Saccharomyces cerevisiae*. In addition, major differences exist between yeast species that are mostly due to three biochemical features: (i) the presence of a specific transporter in

the cell, (ii) the affinity of the malic enzyme for the malic acid, and (iii) the cellular location of the malic enzyme. In this section, the technological specificities of three yeasts species that have been used for reducing wine acidity will be discussed as well as their respective use for winemaking.

4.1. Contribution of *Saccharomyces cerevisiae*

Several studies investigated the ability of *S. cerevisiae* strains to consume malic acid during the alcoholic fermentation. Some strains have been reported to consume up to 45 % of malic acid, while other strains have a role conserving acidity and consume little or no malic acid (Delcourt *et al.* 1995; Peltier *et al.* 2018, 2021; Rankine 1966; Redzepovic *et al.* 2003; Taillandier, P.; Ramon Portugal, F.; Seiller, I.; Favarel, J.L.; Nepveu, F.; Strehaiano 1999). More recently, strains have been selected to consume around 70 % of malic acid (Vion *et al.* 2021). Selection of these strains based on genetic markers enable the use of *S. cerevisiae* for better managing wine acidity. Such strains have proven to facilitate malolactic fermentation by reducing wine malic acid concentration and increasing its pH (Vion *et al.* 2021). The natural variability of *Saccharomyces* strains regarding consumption of malic acid in different grape juices has been investigated for genetically distinct populations (Vion *et al.* 2023). Flor yeast population consumed significantly more malic acid than wine and fruit populations. This higher consumption might be regarded as a sign of the adaptation of these yeasts to grow in harsh media with depleted sugars and with high ethanol concentrations. This property might be due to complex genetic regulation and adaptation as suggested by the recent findings presented in the previous section. Indeed, flor yeasts has been described as having a shift of metabolism to an oxidative one when sugar is depleted (David-Vaizant and Alexandre 2018). Hence, flor yeasts might be able to consume more malic acid at the end of fermentation than wine yeasts. To our knowledge, no study showed a consumption higher than 80% of initial malic acid or less 0.5 g/L of malic acid remaining after fermentation by a strain of *S. cerevisiae* regardless of the initial medium.

However, *S. cerevisiae* remains considered as poor metabolizer of extracellular malate compared to other species due to the lack of active transport of malate through the membrane (Salmon 1987) and weak malate dehydrogenase (Mdh2p) affinity for malate. In addition, the *S. cerevisiae* malic enzyme (Mae1p) characterized by (Fuck, Stärk, and Radler 1973) is localized in the mitochondrion which is dysfunctional during fermentation. This further contributes to the poor ability of *S. cerevisiae* to degrade extracellular malic acid (Minard and Mcalister-henn 1991; Salmon 1987). Also, this enzyme has a low affinity for malate (Km = 50 mM). It has been described that malic acid enters the cell under its undissociated form (H2M), by simple diffusion (Salmon 1987). Malic acid has two pKa (pK_{a1} = 3.40 and pK_{a2} = 5.11) while grape juice's pH is around 3.2-4. Extracellular malic acid can be found mostly in its undissociated (H2M) and mono-dissociated (HM) forms. Once it enters the cell, it takes its deprotonated configuration (M). A proton pump ensures the exit of H^+ and helps maintaining an intracellular pH around 5-6. As malic acid enters by

diffusion, it is its undissociated form that enters the cell, which represents about 50% of the total malic acid available in a grape juice at pH 3.5. As low pH values enhance the H2M/HM ratio, more di-protonated is consumed, triggering the deacidification of the medium. This explains why more malic acid is consumed in grape juice with higher acidity levels. For all these reasons, *S. cerevisiae* therefore consumes less malic acid than other yeasts such as *Z. bailii* or *Sz. pombe*.

Malic acid consumption by *S. cerevisiae* yeasts is dependent on environmental factors such as grape juice pH, initial concentration in malic acid or concentration in assimilable nitrogen (Delcourt *et al.* 1995; Vilanova *et al.* 2007). The degradation of malic acid by *S. cerevisiae* is strongly influenced by the medium. Several studies indicate that a high initial malic acid concentration leads to its greater consumption (Delcourt *et al*. 1995; Taillandier, P.; Ramon Portugal, F.; Seiller, I.; Favarel, J.L.; Nepveu, F.; Strehaiano 1999; Vion *et al*. 2022) with malic acid production repressed in what would normally be malic acid-producing yeasts (Farris, Fatichenti, and Deiana 1989; Yéramian, Chaya, and Suárez Lepe 2007). However, Redzepovic *et al.* (2003) found no difference in malic acid consumption between two media with 3 g/L and 8 g/L of initial malic acid. Malic acid degradation is also favored by a low biotin content (Salmon, Vezinhet, and Barre 1987; Schwartz and Radler 1988), and by an adequate glucose concentration (Delcourt *et al.* 1995) (i.e., not too high, since glucose inhibits the active transport of malate outside the cell (Salmon 1988)). Finally, low pH promotes the consumption of malate (Delcourt *et al.* 1995; Ramon-Portugal *et al*. 1999) since malic acid enters the cell under its undissociated form, by simple diffusion. Finally, the addition of thiamine also facilitates malic acid decomposition by *S. cerevisiae* (Carre, Lafon-lafourcade, and Bertrand 1983).

4.2. Contribution of *Schizosaccharomyces pombe*

The genus *Schizosaccharomyces* is encompasses four related species (*Sz. japonicus, Sz. octosporus, Sz. cryophilus,* and *Sz. pombe*) (Hironori 2014; Vaughan Martini 1991); this last being particularly efficient for malic acid consumption. *Sz. pombe* is mostly isolated from tropical regions and from high sugar habitats (Jeffares 2018) but is rarely detected in winemaking conditions because it is outcompeted by *S. cerevisiae* (Yokotsuka *et al*. 1993). This species is characterized by its ability to completely metabolize malic acid from grapes. This specific feature is due to the action of a constitutive active malic acid transporter encoded by *mae2* gene (Jandre Grobler *et al*. 1995).

The incorporated malic acid is decarboxylated to pyruvic acid by the malic enzyme (in presence of the NAD^+ and one of the divalent cations Mn^{2+} or Mg^{2+}) (Osothsilp and Subden 1986). The high affinity of malic enzyme for its substrate (Km 3.2 mM) and its cytosolic localization contribute to a stronger efficiency of malo-ethanolic fermentation respective to *S. cerevisiae*. The resulting pyruvate follows the alcoholic fermentation pathway producing ethanol and CO_2. This pathway is known as the malo-ethanolic fermentation in which one molecule of malic acid is fermented into one molecule of ethanol and two

molecules of CO_2 in anaerobic conditions (H. Volschenk, van Vuuren, and Viljoen-Bloom 2003). In *Sz. Pombe*, both malic acid transporter and malic enzyme activities are induced by the presence of malic acid in the medium (Osothsilp and Subden 1986).

Several authors have proposed to add *Sz. pombe* in grape juices either for partial or complete consumption of malic acid as an alternative to MLF (S. Benito et al. 2012; Ciani et al. 2009; Redzepovic *et al.* 2003). The proposed itineraries involve pure culture fermentations of *Sz. pombe*, their co-cultures with *S. cerevisiae* or, as described more recently, with *L. thermotolerans* (Benito *et al*. 2015)

One strain of *Sz. pombe* is available commercially in an immobilized form (Suárez-Lepe *et al*. 2012) for a controlled biological deacidification process in which *S. cerevisiae* achieved the fermentation using almost all the sugar available while the immobilized *Sz. pombe* cells used malic acid (Ciani *et al.* 2009). Despite the advantages of deacidifying wines with *Sz. pombe*, its use has been limited, because the production of off-flavors and/or off odors (Benito *et al.*, 2012; Dharmadhikari & Wilker, 1998), or a loss in typicity and fruitiness (Carre *et al.*, 1983; Dharmadhikari & Wilker, 1998; Redzepovic *et al.*, 2003). Wines fermented with *Sz. pombe* present higher sulfur dioxide SO_2 (Dharmadhikari & Wilker, 1998; Snow & Gallander, 1979), 2,3-butanediol (Carre *et al.*, 1983), acetaldehyde (Benito *et al.*, 2012; Carre *et al*., 1983; Dharmadhikari & Wilker, 1998) ethyl acetate (Carre *et al*., 1983; Dharmadhikari & Wilker, 1998) and hydrogen sulfide levels (Rankine, 1966). In addition, they also present high acetic acid production levels (Benito *et al.*, 2012; Dharmadhikari & Wilker, 1998; Snow & Gallander, 1979) and consequently high volatile acidity. Only a small percentage of *Sz. pombe* have shown a moderate volatile acidity (Domizio *et al.*, 2017). These major drawbacks explain why the industrial use of this species in a winemaking context has been very limited.

4.3. Contribution of *Zygosaccharomyces bailii*

Zygosaccharomyces bailii is fructophilic yeast which can degrade high concentration of malic acid during AF (Baranowski and Radler 1984). This species is considered a spoilage organism in the food industry because of its strong resistance to weak organic acids, chemical preservatives (sulfites, sorbic acid), ethanol, and high sugar concentrations (Martorell *et al.* 2007; Radler *et al*. 1993; Sousa *et al*. 1996). In wineries, different studies reported the use this species in mixed fermentation with *S. cerevisiae* (Escribano-Viana *et al.* 2019; Escribano *et al.* 2018; Garavaglia *et al.* 2015). *Z. bailii* preferably degrades fructose, followed by glucose; malic acid is only degraded during the glucose degradation step. Even though, most of malate is oxidatively decarboxylated to pyruvate by malic enzyme, while a small fraction is reduced by fumarase and fumarase reductase (Kuczynski and Radler 1982).

The malic enzyme of *Z. bailii*, which was characterized by (Kuczynski and Radler 1982), is constitutive (Baranowski and Radler 1984) and has a notable affinity for malate (K_m=10 mM) (Kuczynski and Radler 1982). In addition, this yeast possesses a protein that transports L-malate inside the cell, and

which is induced by glucose and inactivated by fructose (Baranowski and Radler 1984). These properties allow *Z. bailii* to metabolize large amount of malic acid in the presence of glucose. Nevertheless, the consumption of malate by *Z. bailii* seems to depend on the glucose concentration of the medium and is reduced in the presence of both high and low levels of glucose (Rodriguez and Thornton 1990). According to the latter authors, in the presence of fructose, malate can enter into *Z. bailii* cells by simple diffusion. Moreover, the lower the pH, the greater the consumption of malate, which seems to indicate that malate is transported into the cell in an undissociated form (Salmon 1988).

Z. bailii alone is not used as a starter for vinification. However, the use of multi-starters for fermentations including a strain of *S. cerevisiae* and a non-*Saccharomyces* yeast are more and more studied for several purposes such as biological deacidification, but also bio-protection, and to confer aroma complexity to wines. In this view, active dried yeasts of *Z. bailli* has become available (Ciani *et al.* 2009).

5. Microbiological applications for enhancing wine acidity during the alcoholic fermentation

The main purpose of acidifying wines with organic acids is to increase the TA and to decrease the pH, which might be necessary to keep the freshness of a wine. The indirect aims are to enhance and stabilize the color and the tannin structure of the wine, and to prevent microbial spoilage. An adequate acidity preserves the wine over time, allows the reduction of sulfur dioxide content and enables microbiological stabilization.

Acid-producing yeasts are generally less common because of their slightly lower rate of multiplication and growth, compared to non-acid-producing yeasts (Kuczynski and Radler 1982). For this reason, acid-producing strains are rarely dominant in natural yeast populations of grape must. Nevertheless, if a sufficiently large population of acid-producing strains is inoculated in the must, they could become dominant and increase the acidity of the resulting wine.

5.1. Malic acid production during wine fermentation

Pioneer studies indicate that some yeast species were able to anabolize malic acid in oenological conditions (Dakin 1924). The ability of *S. cerevisiae* to produce malate in an oenological context has been poorly documented. Earlier studies reported that concentrations of 1 g/L can be reached under optimal conditions of pH and temperature (Battacharajee, J.K; Maragoudakis, M.E. Strassmorn 1968; Farris, Fatichenti, and Deiana 1989; Yéramian, Chaya, and Suárez Lepe 2007) in wine making conditions. Recently, malic acid producing *S. cerevisiae* strains were selected for preserving wine acidity during alcoholic fermentation. Such strains were able to produce up to 3.5 g/L of malic acid and decreasing the wine pH up 0.5 units compared to fermentations conducted with malic consuming strains (Vion *et al.* 2022). Cryotolerant yeasts such as *Saccharomyces uvarum* tend to produce more malic acid than *S. cerevisiae*

(Coloretti *et al.* 2002; Fatichenti *et al*. 1984; Schwartz and Radler 1988) which is related to their psychrophilic properties. This feature is mostly shared by hybrids between *S. cerevisiae* and *S. uvarum* (Origone *et al.* 2018) that have been proposed as a solution for coping for both acidity drop and high sugar levels in grape juices. The recent comparison of *S cerevisiae and S. uvarum* strains confirmed the important malic acid production of this former species (Vion *et al.* 2023).

In addition to strain variability, fermentation conditions can largely influence malic acid production. The oenological conditions are in fact not be optimal for malate synthesis. High pH (around 5), low initial malic acid content, and low yeast-assimilable nitrogen (YAN) concentrations were instead found to promote the production of malate by *S. cerevisiae* (Salmon, Vezinhet, and Barre 1987; Schwartz and Radler 1988; Yéramian, Chaya, and Suárez Lepe 2007). Despite suboptimal conditions, some yeast strains can anabolize malic acid during AF (Dakin 1924; Farris, Fatichenti, and Deiana 1989; Fatichenti *et al*. 1984; Pronk, Jack T; Steensma, H. Yde; Van Dijken 1996; Schwartz and Radler 1988; Whiting 1976). In general, malic acid production is greater when the initial level of malic acid in grapes are low (Davaux 2001; Ramon-Portugal *et al*. 1999; Vion *et al*. 2022; Yéramian, Chaya, and Suárez Lepe 2007). Recently, Vion *et al*. (2022) demonstrated that a high production level of malic acid partially negatively affects the fermentation performance of acidifying strains. This finding suggested a phenotypic trade-off between fermentation completion and malic acid production.

5.2. Lactic acid production during alcoholic fermentation

Lactic acid is a microbially-derived wine acid, and a permitted oenological acidulant under most regulations (Waterhouse, Sacks, and Jeffery 2016). It is often described as ‘soft’ and ‘mild’ acid, unlike ‘green’ and ‘harsh’ which are more often used to describe malic and tartaric acids. However, the pertinence of such attributes remains elusive. It is particularly unclear whether ‘softer’ acidity perception of lactic acid simply reflects partial deacidification of wine via malolactic fermentation. Despite such ambiguities, acidification by lactic acid has certain advantages. It is not lost by precipitation, as is the case of tartaric acid, due to solubility of both potassium and calcium salts. It is also not prone to microbial degradation (Waterhouse, Sacks, and Jeffery 2016).

5.2.1. The lactic producing species L. thermotolerans

The yeast *L. thermotolerans* is an occasional constituent of grape/wine microbiome, but it is also found in a range of other natural anthropic habitats worldwide (Freel *et al*. 2014; Hranilovic *et al.* 2017). Population of *L. thermotolerans* is differentiated in relation to both geographic origin and the ecological niche of isolation (Hranilovic *et al*. 2017), and such differentiation is reflected on the phenotypic level, in terms of the oenological performance of the strain (Hranilovic *et al*. 2018). The metabolic hallmark of *L. thermotolerans* is L-lactic acid production concomitant to alcoholic fermentation. The maximum reported

concentrations are 16.6 g/L (Banilas, Sgouros, and Nisiotou 2016), which by far exceed those recorded for any non-GM yeast, however, this trait is highly strain dependent (Banilas, Sgouros, and Nisiotou 2016; Hranilovic *et al.* 2018). For example, final levels of lactic acid formed in fermentations of the same grape juice by 94 different *L. thermotolerans* strains ranged between 1.8 to 12 g/L (Hranilovic *et al.* 2018). In mixed cultures of *L. thermotolerans* and *S. cerevisiae*, applicable for 'dry' wine production, levels of lactic acid production depend on the *L. thermotolerans* strain but also on the yeast inoculation regimes. Due to the antagonistic activity of *S. cerevisiae* towards *L. thermotolerans*, mediated by mechanisms of cell-cell contact and secretion of antimicrobial peptides (Kemsawasd *et al*. 2015), co-inoculations generally lead to lower extent of lactic acid production compared to the sequential inoculations (Gobbi *et al.* 2013; Kapsopoulou *et al*. 2007; Sgouros *et al*. 2020). In the latter, longer delay of *S. cerevisiae* inoculation results in higher metabolic contribution *of L. thermotolerans*. For example, (Kapsopoulou *et al*. 2007) reported 0.18 g/L of lactic acid produced in co-inoculated fermentation modality. A tenfold increase (1.8 g/L) was recorded when inoculation with *S. cerevisiae* was postponed for one day, whereas two and three days if inoculation delay resulted in production of 4.28 g/L and 5.13 g/L of lactic acid respectively (Kapsopoulou *et al*. 2007). In terms of acidity modulation, *L. thermotolerans* strains are also capable of partial degradation of up to 20% of malic acid and their acetic acid production is low and rather invariant (Hranilovic *et al*. 2018). The final wine pH in *L. thermotolerans* modalities can decrease by about 0.5 units (Banilas, Sgouros, and Nisiotou 2016; Morata *et al.* 2019), which represents a dramatic acidification capacity. Depending on the strain and fermentation conditions, these wines also contained either comparable or up to 1.6% v/v less ethanol compared to their respective *S. cerevisiae* monocultures (Gobbi *et al.* 2013; Kapsopoulou *et al.* 2007; Sgouros *et al*. 2020). Lower ethanol content in line with partial diversion of carbon flux from ethanol to lactic acid, but precise studies on carbon flux in different *L. thermotolerans* strains are still lacking.

5.2.2. The contribution of other yeast species

Under oenological conditions, *S. cerevisiae* strains produce very little (if any) lactic acid. This occurs via reduction of pyruvate by NAD-dependent D and L LDHs in mitochondria, and the lows yields of 0.2-0.4 g/L are thought to be related to their inefficiency during fermentation (Sylvie Dequin and Barre 1994). However, research on this phenomenon is dated (Whiting 1976) and potentially lack modern day scientific scrutiny. The information on the ability of yeasts other than *L. thermotolerans* to produce lactic acid is limited and little effort was out into systematic screenings for this trait (Sauer *et al.* 2010). An agar plate-based assay 'LASSO' was developed (Witte, Krohn, and Emis C. 1989) for detection of lactic acid production and used to screen a collection of 100 yeast strains. Only two strains were capable to produce lactic acid, and they were both identified as *L. thermotolerans*. This assay was revisited only recently and modified to a liquid format (225 µL) for multi-well plates (Osburn *et al.* 2018). In a study focusing on selection of yeasts for sour-style beer production without the use of LAB, strains of five species were able

to produce lactic acid (Osburn *et al*. 2018). Besides *L-thermotolerans*, they belonged to *L. fermentati*, *Hanseniaspora vinae*, *Schizosaccharomyces japonicus* and *Wickerhamomyces anomalus* (Osburn *et al.* 2018). All these species were, to a certain degree, evaluated for their winemaking potential (Domizio *et al.* 2018; Medina *et al*. 2013; Padilla, Gil, and Manzanares 2018; Porter, Divol, and Setati 2019), but, to the best of our knowledge, without delivering any striking result regarding lactic acid or wine acidity modulation. One exception is *Sz. japonicus*, which was reported to decrease the total acidity in wine in both pure and co-cultures with *S. cerevisiae* due to its ability to degrade malic acid (Domizio *et a*l. 2018). To date, the most extensive characterization of lactic acid production by yeasts other than *L. thermotolerans* is available for *L. fermentati*. Final lactic acid concentrations in beers produced by *L. fermentati* depended on the strain and fermentation conditions, with maximal values of 1.6 g/L (Bellut *et al*. 2019; Bellut, Krogerus, and Arendt 2020; Osburn *et al*. 2018). Lower inoculation rate in combination with increased fermentation temperatures boosted lactic acid production, as did higher initial glucose concentrations (Bellut, Krogerus, and Arendt 2020). Comparison of whole genome sequences of strains with differential lactic acid production revealed a mutation in a low lactic acid production strain resulting in a premature stop codon in a homologue *S. cerevisiae JEN1* (Bellut, Krogerus, and Arendt 2020). This gene encodes for a monocarboxylate transporter involved in the export of lactic acid, thus offering tentative explanation for different lactic acid production capacities of studies *L. fermentati* strains. This further highlighted the poor knowledge on lactic acid biosynthesis by yeasts.

5.3. Succinic acid producing yeasts

Succinic acid is the weakest wine acid (pKa1 = 4.18 and pKa2 = 5.23). Although it is absent in grapes, it is the main carboxylic acid produced by yeasts during alcoholic fermentation, mainly during exponential growth (Thoukis, Ueda, and Wright 1965). It is found at levels ranging from a few milligrams to 2 g/L (Whiting 1976). This organic acid was described as salty, slightly bitter, and very desiccating (Peynaud 1981).

Yeast strain strongly influence succinic acid production (Thoukis, Ueda, and Wright 1965). For example, the cryotolerant strain *S. uvarum* produces larger amount of succinic acid than non-cryotolerant strains (Dakin 1924; Farris, Fatichenti, and Deiana 1989). *S. uvarum* produces between 1-2 g/L of succinic acid, whereas *S. cerevisiae* produces 0.5 to 1.5 g/L during alcoholic fermentation. (Taillandier, P.; Ramon Portugal, F.; Seiller, I.; Favarel, J.L.; Nepveu, F.; Strehaiano 1999) reported that the higher the concentration of malic acid in the must, the greater the production of succinic acid during alcoholic fermentation, suggesting that it is produced via the reduction of malate. However, a positive correlation was found between high malic acid production and succinic acid production (Vion *et al.* 2022) which suggests that the production of both acids might be partially coupled. Its production is stimulated at low TA and pH between 4-4.4 (Thoukis, Ueda, and Wright 1965); however, pH variation between 3 and 3.8 did not significantly

affect the succinate production (Whiting 1976). In addition, the formation of succinic acid increases with nitrogen concentrations up to 500 mg/L. Succinic acid production also increases with temperature in range 10-30°C but diminishes after 40°C (Shimazu and Waranabe 1981). A linear correlation exists between glucose concentration (up to 8%) and the formation of succinic acid, independent of the nitrogen source. Finally, Shimazu and Waranabe (1981) indicated that *S. cerevisiae* produces considerably more succinic acid when SO_2 is absent of the medium.

6. Conclusion

Wine acidity has a great contribution to the wine quality. The emergence of acidity management yeast strains for winemaking will undoubtedly help the wine industry to meet the challenges it is facing due to climate change. Modern winemaking is mostly focused on acidification to improve the sensorial perception of wines in warm areas. De-acidification can nonetheless present an interest for reduce the acidity in colder regions as well as to shorten the malo-lactic fermentation by lowering malic acid content at the end of the AF. The large panel of yeasts usable for winemaking allows a multitude of vinification itineraries depending on the climate, *terroir*, and type of wine desired. Some trends combine several of these microorganisms during alcoholic fermentation to correct acidity and stabilize the wine from a microbiological point of view. Sequential inoculations of *S. cerevisiae* with another yeast species are of great interest for winemaking practices in warm areas and the number of studies focusing on this subject is still scarce but increasing in the recent years. The acidity of wine is a key component of its overall quality. With climate change posing a significant challenge to the winemaking industry, the emergence of yeast strains for wine acidity management has become an essential tool for winemakers. While modern winemaking trends often require biological acidification to enhance the sensory characteristics of wines produced in warmer regions, de-acidification can also be of interest in cooler regions or to shorten or even avoid the MLF. Mixed culture fermentations of specific non-*Saccharomyces* yeasts and *S. cerevisiae* represent an increasingly popular approach to correct acidity and ensure microbial stability of wines. The diverse range of yeast starters can therefore allow the pursuit of specific vinification strategies tailored to the climate, terroir, and desired wine style.

7. Bibliography

Albertyn, Jacobus, Stefan Hohmann, and Bernard A. Prior. 1994. "Characterization of the Osmotic-Stress Response in *Saccharomyces cerevisiae*: Osmotic Stress and Glucose Repression Regulate Glycerol-3-Phosphate Dehydrogenase Independently." *Current Genetics* 25(1): 12–18.

Ambroset, Chloé *et al.* 2011. "Deciphering the Molecular Basis of Wine Yeast Fermentation Traits Using a Combined Genetic and Genomic Approach." *G3: Genes|Genomes|Genetics* 1(4): 263–81.

Ansanay, V, S Dequin, B Blondin, and P Barre. 1993. "Cloning, Sequence and Expression of the Gene Encoding the Malolactic Enzyme from *Lactococcus Lactis*." *FEBS letters* 332(1–2): 74–80.

Ansanay, Virginie *et al*. 1996. "Malolactic Fermentation by Engineered *Saccharomyces cerevisiae* as Compared with Engineered *Schizosaccharomyces pombe*." *Yeast* 12(3): 215–25.

Arrizabalaga, Marta *et al*. 2018. "Tempranillo Clones Differ in the Response of Berry Sugar and Anthocyanin Accumulation to Elevated Temperature." *Plant Science* 267: 74–83.

Bakker, Barbara M. *et al*. 2001. "Stoichiometry and Compartmentation of NADH Metabolism in *Saccharomyces cerevisiae*." *FEMS Microbiology Reviews* 25(1): 15–37.

Banilas, Georgios, Georgios Sgouros, and Aspasia Nisiotou. 2016. "Development of Microsatellite Markers for *Lachancea thermotolerans* Typing and Population Structure of Wine-Associated Isolates." *Microbiological Research* 193: 1–10.

Baranowski, K, and F Radler. 1984. "The Glucose-Dependent Transport of L-Malate in *Zygosaccharomyces bailii*." *Antonie van Leeuwenhoek* 50: 329–40.

Battacharajee, J.K; Maragoudakis, M.E. Strassmorn, M. 1968. "Identification of Malic Acid from Yeast." *J. Bacteriol.* 95: 495–97.

Bellut, Konstantin *et al*. 2019. "Investigation into the Potential of *Lachancea fermentati* Strain KBI 12.1 for Low Alcohol Beer Brewing." *Journal of the American Society of Brewing Chemists* 77(3): 157–69. https://doi.org/10.1080/03610470.2019.1629227.

Bellut, Konstantin, Kristoffer Krogerus, and Elke K. Arendt. 2020. "*Lachancea fermentati* Strains Isolated from Kombucha: Fundamental Insights, and Practical Application in Low Alcohol Beer Brewing." *Frontiers in Microbiology* 11(April): 1–21.

Benito, Ángel, Fernando Calderón, Felipe Palomero, and Santiago Benito. 2015. "Combine Use of Selected *Schizosaccharomyces pombe* and *Lachancea thermotolerans* Yeast Strains as an Alternative to the Traditional Malolactic Fermentation in Red Wine Production." *Molecules* 20(6): 9510–23.

Benito, Santiago *et al*. 2012. "New Applications for *Schizosaccharomyces pombe* in the Alcoholic Fermentation of Red Wines." *International Journal of Food Science and Technology* 47(10): 2101–8.

Blein-Nicolas, Mélisande *et al*. 2015. "A Systems Approach to Elucidate Heterosis of Protein Abundances in Yeast." *Molecular and Cellular Proteomics* 14(8): 2056–71.

Blomberg, Anders. 2000. "Metabolic Surprises in *Saccharomyces cerevisiae* during Adaptation to Saline Conditions: Questions, Some Answers and a Model." *FEMS Microbiology Letters* 182(1): 1–8.

Boles, Eckhard, Patricia De Jong-Gubbels, and Jack T. Pronk. 1998. "Identification and Characterization of *MAE1*, the *Saccharomyces cerevisiae* Structural Gene Encoding Mitochondrial Malic Enzyme." *Journal of Bacteriology* 180(11): 2875–82.

Bony, M. *et al*. 1997. "Metabolic Analysis of *S. cerevisiae* Strains Engineered for Malolactic Fermentation." *FEBS Letters* 410(2–3): 452–56. http://dx.doi.org/10.1016/S0014-5793(97)00637-6.

Bousbouras, George E, and Ralph E Kunkee. 1971. "Effect of PH on Maloc-Lactic Fermentation in Wine."

Bureau, Sylvie M, Alain J Razungles, and Raymond L Baumes. 2000. "The Aroma of Muscat of Frontignan Grapes: Effect of the Light Environment of Vine or Bunch on Volatiles and Glycoconjugates." *Journal of the Science of Food and Agriculture*.

Buttrose, M S, C R Hale, and W M Kliewer. 1971. "Effect of Temperature on the Composition of 'Carbernet Sauvignon' Berries." *American Journal of Enology and Viticulture*.

Camarasa, Carole, Jean Philippe Grivet, and Sylvie Dequin. 2003. "Investigation by 13C-NMR and Tricarboxylic Acid (TCA) Deletion Mutant Analysis of Pathways of Succinate Formation in *Saccharomyces cerevisiae* during Anaerobic Fermentation." *Microbiology* 149(9): 2669–78.

Carre, E, Suzanne Lafon-lafourcade, and A Bertrand. 1983. "Desacidification Biologique Des Vins Blancs Secs Par Fermentation De L'Acide Malique Par Les Levures." *Connaissance Vigne Vin* 17: 43–53.

Casal, Margarida, Sandra Paiva, Odília Queirós, and Isabel Soares-Silva. 2008. "Transport of Carboxylic Acids in Yeasts." *FEMS Microbiology Reviews* 32(6): 974–94.

Chidi, B.S., Bauer F.F., and Rossouw D. 2018. "Organic Acid Metabolism and the Impact of Fermentation Practices

on Wine Acidity : A Review." *South African Journal of Enology & Viticulture* 39(2).
Ciani, Maurizio, Francesca Comitini, Ilaria Mannazzu, and Paola Domizio. 2009. "Controlled Mixed Culture Fermentation: A New Perspective on the Use of Non-*Saccharomyces* Yeasts in Winemaking." *FEMS Yeast Research* 10(2): 123–33.
Clement, T. *et al*. 2013. "Metabolic Responses of *Saccharomyces cerevisiae* to Valine and Ammonium Pulses during Four-Stage Continuous Wine Fermentations." *Applied and Environmental Microbiology* 79(8): 2749–58.
Coi, A. L. *et al*. 2017. "Genomic Signatures of Adaptation to Wine Biological Ageing Conditions in Biofilm-Forming Flor Yeasts." *Molecular Ecology* 26(7): 2150–66.
Coloretti, Fabio *et al*. 2002. "The Effect of DL-Malic Acid on the Metabolism of L-Malic Acid during Wine Alcoholic Fermentation." *Food Technology and Biotechnology* 40(4): 317–20.
Comuzzo, Piergiorgio, and Franco Battistutta. 2019. Red Wine Technology *Acidification and PH Control in Red Wines*. Elsevier Inc. http://dx.doi.org/10.1016/B978-0-12-814399-5.00002-5.
Conde, Carlos; *et al*. 2007. "Biochemical Changes throughout Grape Berry Development and Fruit and Wine Quality." *Food*: 157–80.
Coombe, B. G. 1987. "Influence of Temperature on Composition and Quality of Grapes." *Acta Horticulturae*.
D. Postma, Eline *et al*. 2022. "Top-Down, Knowledge-Based Genetic Reduction of Yeast Central Carbon Metabolism." *mBio*: 1–6.
Dakin, B Y H D. 1924. "The Formation of L-Malic Acid as a Product of Alcoholic Fermentation by Yeast." *Journal of Biological Chemistry*.
Davaux, F. 2001. "L'acidité Du Mout Au Vin: D'autres Alternatives à l'acidification Par l'acide Tartrique." *Comment maitriser l'acidité du vin*: 23–27. https://www.vignevin-sudouest.com/publications/itv-colloque/documents/COLLOQUE_Comment-maitriser-acidite-vin-integral.pdf.
David-Vaizant, Vanessa, and Hervé Alexandre. 2018. "Flor Yeast Diversity and Dynamics in Biologically Aged Wines." *Frontiers in Microbiology* 9(SEP): 1–16.
Davis, C R *et al.* 1985. "Practical Implications of Malolactic Fermentation: A Review." *American Journal of Enology and Viticulture* 36.
Delcourt, F., P. Taillandier, F. Vidal, and P. Strehaiano. 1995. "Influence of PH, Malic Acid and Glucose Concentrations on Malic Acid Consumption by *Saccharomyces cerevisiae*." *Applied Microbiology and Biotechnology* 43(2): 321–24.
Denayrolles, M, M Aigle, and A Lonvaud-Funel. 1995. "Functional Expression in *Saccharomyces cerevisiae* of the *Lactococcus lactis MleS* Gene Encoding the Malolactic Enzyme." *FEMS microbiology letters* 125(1): 37–43.
Dequin, S. *et al*. 2017. "How to Adapt Winemaking Practices to Modified Grape Composition under Climate Change Conditions". Journal International des Sciences de la Vigne et du Vin, 51.
Dequin, S, E Baptista, and P Barre. 1999. "Acidification of Grape Musts by *Saccharomyces cerevisiae* Wine Yeast Strains Genetically Engineered to Produce Lactic Acid." *Am. J. Enol. Vitic* 50.
Dequin, Sylvie, and Pierre Barre. 1994. "Mixed Lactic Acid-Alcoholic Fermentation by *Saccharomyces cerevisiae* Expressing the *Lactobacillus casei* L(+)-LDH." *Nature Biotechnology*. http://www.nature.com/naturebiotechnology.
Divol, Benoit, Maret Du Toit, and Edward Duckitt. 2012. "Surviving in the Presence of Sulphur Dioxide: Strategies Developed by Wine Yeasts." *Applied Microbiology and Biotechnology* 95(3): 601–13.
Domizio, Paola *et al*. 2018. "Evaluation of the Yeast *Schizosaccharomyces japonicus* for Use in Wine Production." *American Journal of Enology and Viticulture* 69(3): 266–77.
Downey, Mark O., Nick K. Dokoozlian, and Mark P. Krstic. 2006. "Cultural Practice and Environmental Impacts on the Flavonoid Composition of Grapes and Wine: A Review of Recent Research." *Am. J. Enol. Vitic*. https://www.researchgate.net/publication/275832356.
Dubourdieu, Denis. 2004. "Influence of Climate, Soil, and Cultivar on Terroir Influence of Climate, Soil, and Cultivar on Terroir." *Am. J. Enol. Vitic* 55: 3. https://www.researchgate.net/publication/279554723.
Duchêne, Eric, and Christophe Schneider. 2005. "Grapevine and Climatic Changes: A Glance at the Situation in Alsace." *Agron. Sustain. Dev*: 93–99.
Eder Matthias *et al.* 2018. "QTL Mapping of Volatile Compound Production in *Saccharomyces cerevisiae* during Alcoholic Fermentation." *BMC Genomics* 19(1): 1–19.
Eder Matthias *et al*. 2020. "QTL Mapping of Modelled Metabolic Fluxes Reveals Gene Variants Impacting Yeast Central Carbon Metabolism." *Scientific Reports* 10(1): 2162. https://doi.org/10.1038/s41598-020-57857-3.
Eglinton, Jeffrey M. *et al*. 2002. "Decreasing Acetic Acid Accumulation by a Glycerol Overproducing Strain of *Saccharomyces cerevisiae* by Deletiong the *ALD6* Aldehyde Dehydrogenase Gene." *Yeast* 19(4): 295–301.
Escribano-Viana, Rocío *et al*. 2019. "Effect of the Sequential Inoculation of Non-*Saccharomyces*/*Saccharomyces* on the Anthocyans and Stilbenes Composition of Tempranillo Wines." *Frontiers in Microbiology* 10(APR): 1–10.

Escribano, R. *et al*. 2018. "Wine Aromatic Compound Production and Fermentative Behaviour within Different Non-*Saccharomyces* Species and Clones." *Journal of Applied Microbiology* 124(6): 1521–31.

Farris, Ga, F Fatichenti, and P Deiana. 1989. "Incidence De La Temperature Et Du PH Sur La Production D'Acide Malique Par *Saccharomyces cerevisiae.* " Journal International des Sciences de la Vigne et du Vin, 23

Fatichenti, Fabrizio, Giovanni Antonio Farris, Pietrino Deiana, and Salvatore Ceccarelli. 1984. "Malic Acid Production and Consumption by Selected of *Saccharomyces cerevisiae* under Anaerobic and Aerobic Conditions." *Applied Microbiology and Biotechnology* 19(6): 427–29.

Fernandez, Ernestina, Fernando Moreno, and Rosaura Rodicio. 1992. "The *ICL1* Gene from *Saccharomyces cerevisiae*." *European Journal of Biochemistry* 204(3): 947–1189.

Ford CM. 2012. "The Biochemistry of Organic Acids in the Grape." In *Biochem Grape Berr.*, eds. Hernâni Gerós, Manuela Chaves, and Serge Delrot, 67–88.

Fowles, G. W.A. 1992. "Acids in Grapes and Wines: A Review." *Journal of Wine Research* 3(1): 25–41.

Freel, Kelle C., Anne Friedrich, Jing Hou, and Joseph Schacherer. 2014. "Population Genomic Analysis Reveals Highly Conserved Mitochondrial Genomes in the Yeast Species *Lachancea thermotolerans*." *Genome Biology and Evolution* 6(10): 2586–94.

Frost, Scott C., James F. Harbertson, and Hildegarde Heymann. 2017. "A Full Factorial Study on the Effect of Tannins, Acidity, and Ethanol on the Temporal Perception of Taste and Mouthfeel in Red Wine." *Food Quality and Preference* 62: 1–7.

Fuck, E, G Stärk, and F Radler. 1973. "Äpfelsäurestoffwechsel Bei *Saccharomyces*." *Arch. Mikrobiol*: 223–31.

Gangloff, S., D. Marguet, and G. Lauquin. 1990. "Molecular Cloning of the Yeast Mitochondrial Aconitase Gene (*ACO1*) and Evidence of a Synergistic Regulation of Expression by Glucose plus Glutamate." *Molecular and cellular Biology* 10(7): 3551–61.

Gao, C, and G H Fleet. 1995. "Degradation of Malic and Tartaric Acids by High Density Cell Suspensions of Wine Yeasts". *Food Microbiology*, 12

Garavaglia, Juliano *et al*. 2015. "Evaluation of *Zygosaccharomyces bailii* BCV 08 as a Co-Starter in Wine Fermentation for the Improvement of Ethyl Esters Production." *Microbiological Research* 173: 59–65. http://dx.doi.org/10.1016/j.micres.2015.02.002.

Gerós, Hernân, Manuela M. Chaves, and Serge Delrot. 2012. "The Biochemistry of the Grape Berry." *The Biochemistry of the Grape Berry*.

Gobbi, Mirko *et al*. 2013. "*Lachancea thermotolerans* and *Saccharomyces cerevisiae* in Simultaneous and Sequential Co-Fermentation: A Strategy to Enhance Acidity and Improve the Overall Quality of Wine." *Food Microbiology* 33(2): 271–81. http://dx.doi.org/10.1016/j.fm.2012.10.004.

Grobler, Jandre, Florian Bauer, Ron E. Subden, and Hendrik J.J. Van Vuuren. 1995. "The *Mae1* Gene of *Schizosaccharomyces pombe* Encodes a Permease for Malate and Other C4 Dicarboxylic Acids." *Yeast* 11(15): 1485–91.

Hagman, Arne, Torbjörn Säll, Concetta Compagno, and Jure Piskur. 2013. "Yeast 'Make-Accumulate-Consume' Life Strategy Evolved as a Multi-Step Process That Predates the Whole Genome Duplication" ed. Cecile Fairhead. *PLoS ONE* 8(7): e68734. https://dx.plos.org/10.1371/journal.pone.0068734 (March 9, 2021).

Hironori, Niki. 2014. "*Schizosaccharomyces japonicus*: The Fission Yeast Is a Fusion of Yeast and Hyphae." *Yeast* 31: 83–90.

Hranilovic, Ana *et al*. 2017. "The Evolution of *Lachancea thermotolerans* Is Driven by Geographical Determination, Anthropisation and Flux between Different Ecosystems." *PLoS ONE* 12(9): 1–17.

Hranilovic, Ana *et al*. 2018. "Oenological Traits of *Lachancea thermotolerans* Show Signs of Domestication and Allopatric Differentiation." *Scientific Reports* 8(1): 14812. http://www.nature.com/articles/s41598-018-33105-7.

Hung, Guo Chiuan *et al.* 2004. "Degradation of the Glyconeogenic Enzymes Fructose-1,6-Bisphosphatase and Malate Dehydrogenase Is Mediated by Distinct Proteolytic Pathways and Signaling Events." *Journal of Biological Chemistry* 279(47): 49138–50.

Husnik, John I *et al*. 2007. "Functional Analyses of the Malolactic Wine Yeast ML01." *Am. J. Enol. Vitic* 58(Maicas 2001): 1.

Iland, P. G., and B. G. Coombe. 1988. "Malate, Tartrate, Potassium, and Sodium in Flesh and Skin of Shiraz Grapes During Ripening: Concentration and Compartmentation." *American Journal of Enology and Viticulture* 39(1).

IPCC. 2020. International Encyclopedia of Geography: People, the Earth, Environment and Technology *Climate Change and Land*.

Jeffares, Daniel C. 2018. "The Natural Diversity and Ecology of Fission Yeast." *Yeast* 35(3): 253–60.

Jones, Gregory V. 2007. *Climate Change: Observations, Projections, and General Implications for Viticulture and Wine Production*. Jones.

Kapsopoulou, K., A. Mourtzini, M. Anthoulas, and E. Nerantzis. 2007. "Biological Acidification during Grape Must Fermentation Using Mixed Cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*." *World Journal of Microbiology and Biotechnology* 23(5): 735–39.

Kemsawasd, Varongsiri *et al*. 2015. "Cell-to-Cell Contact and Antimicrobial Peptides Play a Combined Role in the Death of *Lachanchea thermotolerans* during Mixed-Culture Alcoholic Fermentation with *Saccharomyces cerevisiae*." *FEMS Microbiology Letters* 362(14): 1–8.

Kim, Kwang-Soo, Mark S. Rosenkrantz, and Leonard Guarente. 1986. "*Saccharomyces cerevisiae* Contains Two Functional Citrate Synthase Genes." *Molecular and Cellular Biology*: 1936–42.

Klerk, Jean-Louis De. 2010. "Succinic Acid Production by Wine Yeasts."

Kliewer, W. Mark. 1964. "Influence of Environment on Metabolism of Organic Acids and Carbohydrates in *Vitis Vinifera*. I. Temperature ." *Plant Physiology* 39(6): 869–80.

Kuczynski, J T, and F Radler. 1982. "The Anaerobic Metabolism of Malate of *Saccharomyces bailii* and the Partial Purification and Characterization of Malic Enzyme." *Archives of Microbiology* 131: 266–270.

Kunkee, Ralph E. 1991. "Some Roles of Malic Acid in the Malolactic Fermentation in Wine Making." *FEMS Microbiology Letters* 88(1): 55–71.

Kunze, Markus *et al*. 2002. "Targeting of Malate Synthase 1 to the Peroxisomes of *Saccharomyces cerevisiae* Cells Depends on Growth on Oleic Acid Medium." *European Journal of Biochemistry* 269: 915–22.

van Leeuwen, Cornelis, and Philippe Darriet. 2016. "The Impact of Climate Change on Viticulture and Wine Quality." *Journal of Wine Economics* 11(1): 150–67.

Lodi, T, and I Ferrero. 1993. "Isolation of the DLD Gene of *Saccharomyces cerevisiae* Encoding the Mitochondrial Enzyme D-Lactate Ferricytochrome c Oxidoreductase." *Molecular & general genetics : MGG* 238(3): 315–24.

Macris, Basil J., and Pericles Markakis. 1974. "Transport and Toxicity of Sulphur Dioxide in *Saccharomyces cerevisiae Var Ellipsoideus*." *Journal of the Science of Food and Agriculture* 25(1): 21–29.

Martorell, Patricia *et a*l. 2007. "Physiological Characterization of Spoilage Strains of *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* Isolated from High Sugar Environments." *International Journal of Food Microbiology* 114(2): 234–42.

Medina, K. *et al*. 2013. "Increased Flavour Diversity of Chardonnay Wines by Spontaneous Fermentation and Co-Fermentation with *Hanseniaspora vineae*." *Food Chemistry* 141(3): 2513–21. http://dx.doi.org/10.1016/j.foodchem.2013.04.056.

Merloni, Eva, Luca Camanzi, Luca Mulazzani, and Giulio Malorgio. 2018. "Adaptive Capacity to Climate Change in the Wine Industry: A Bayesian Network Approach." *Wine Economics and Policy* 7(2): 165–77.

Minard, Karyl I., and Lee McElister-Henn. 1994. "Glucose-Induced Phosphorylation of the *MDH2* Isoenzyme of Malate Dehydrogenase in *Saccharomyces cerevisiae*." *Archives of Biochemistry and Biophysics*: 302–9.

Minard, Karyl I, and L E E Mcalister-henn. 1991. "Isolation, Nucleotide Sequence Analysis , and Disruption of the *MDH2* Gene from *Saccharomyces cerevisiae* : Evidence for Three Isozymes of Yeast Malate Dehydrogenase." *Molecular and Cellular Biology* 11(1): 370–80.

Mira de Orduña, Ramón. 2010. "Climate Change Associated Effects on Grape and Wine Quality and Production." *Food Research International* 43(7): 1844–55.

Morata, Antonio *et al*. 2019. "*Lachancea thermotolerans* as a Tool to Improve PH in Red Wines from Warm Regions." *European Food Research and Technology* 245(4): 885–94. http://dx.doi.org/10.1007/s00217-019-03229-9.

Navarro-Aviño, Juan P. *et al*. 1999. "A Proposal for Nomenclature of Aldehyde Dehydrogenases in *Saccharomyces cerevisiae* and Characterization of the Stress-Inducible *ALD2* and *ALD3* Genes." *Yeast* 15(10 A): 829–42.

Nistor, E., A. G. Dobrei, A. Dobrei, and D. Camen. 2018. "Growing Season Climate Variability and Its Influence on Sauvignon Blanc and Pinot Gris Berries and Wine Quality: Study Case in Romania (2005-2015)." *South African Journal of Enology and Viticulture* 39(2): 196–207.

Origone, Andrea Cecilia *et al*. 2018. "*Saccharomyces cerevisiae* × *Saccharomyces uvarum* Hybrids Generated under Different Conditions Share Similar Winemaking Features." *Yeast* 35(1): 157–71.

Osburn, Kara *et al*. 2018. "Primary Souring: A Novel Bacteria-Free Method for Sour Beer Production." *Food Microbiology* 70: 76–84. https://doi.org/10.1016/j.fm.2017.09.007.

Osothsilp, C., and R. E. Subden. 1986. "Isolation and Characterization of *Schizosaccharomyces pombe* Mutants with Defective NAD-Dependent Malic Enzyme." *Canadian Journal of Microbiology*.

Padilla, Beatriz, Jose V. Gil, and Paloma Manzanares. 2018. "Challenges of the Non-Conventional Yeast *Wickerhamomyces anomalus* in Winemaking." *Fermentation* 4(3).

Peltier, Emilien *et al*. 2018. "Wine Yeast Phenomics: A Standardized Fermentation Method for Assessing Quantitative Traits of Saccharomyces Cerevisiae Strains in Enological Conditions." *PloS one*: 1–23.

Peltier Emilien *et al*. 2021. "Flor Yeasts Rewire the Central Carbon Metabolism During Wine Alcoholic Fermentation." *Frontiers in Fungal Biology* 2(October).

Peynaud, Emile. 1981. *Connaissance Et Travail Du Vin*. 2nd ed. ed. Bordas. Paris.

Pines, O. *et al.* 1996. "The Cytosolic Pathway of L-Malic Acid Synthesis in *Saccharomyces cerevisiae*: The Role of Fumarase." *Applied Microbiology and Biotechnology* 46(4): 393–99.

Pines, O., S. Shemesh, E. Battat, and I. Goldberg. 1997. "Overexpression of Cytosolic Malate Dehydrogenase (*MDH2*) Causes Overproduction of Specific Organic Acids in *Saccharomyces cerevisiae*." *Applied Microbiology and Biotechnology* 48(2): 248–55.

Plane, R., LR Mattick, and LD Weirs. 1980. "An Acidity Index for the Taste of Wines." *American Journal of Enology and Viticulture* 31: 258–65.

Porter, Tristan Jade, Benoit Divol, and Mathabatha Evodia Setati. 2019. "*Lachancea* Yeast Species: Origin, Biochemical Characteristics and Oenological Significance." *Food Research International* 119(October 2018): 378–89. https://doi.org/10.1016/j.foodres.2019.02.003.

Poudel, Puspa Raj, Kazuya Koyama, and Nami Goto-Yamamoto. 2020. "Evaluating the Influence of Temperature on Proanthocyanidin Biosynthesis in Developing Grape Berries (*Vitis Vinifera L.*)." *Molecular Biology Reports* 47(5): 3501–10.

Poudel, Puspa Raj, Ryosuke Mochioka, Kenji Beppu, and Ikuo Kataoka. 2009. "Influence of Temperature on Berry Composition of Interspecific Hybrid Wine Grape 'Kadainou R-1' (*Vitis ficifolia Var. Ganebu* × *V. vinifera* 'Muscat of Alexandria')." *Journal of the Japanese Society for Horticultural Science* 78(2): 169–74.

Pronk, Jack T; Steensma, H. Yde; Van Dijken, Johannes P. 1996. "Pyruvate Metabolism in *Saccharomyces cerevisiae*." *Yeast* 12: 1607–33.

Raab, Andreas M., and Christine Lang. 2011. "Oxidative versus Reductive Succinic Acid Production in the Yeast *Saccharomyces cerevisiae*." *Bioengineered Bugs* 2(2).

Radler, F., S. Herzberger, I. Schonig, and P. Schwarz. 1993. "Investigation of a Killer Strain of *Zygosaccharomyces bailii*." *Journal of General Microbiology* 139(3): 495–500.

Ramon-Portugal, F. *et al.* 1999. "Kinetics of Production and Consumption of Organic Acids during Alcoholic Fermentation by *Saccharomyces cerevisae*." *Food Technology and Biotechnology* 37(4): 235–40.

Rankine, B. C. 1966. "Decomposition of L-malic Acid by Wine Yeasts." *Journal of the Science of Food and Agriculture* 17(7): 312–16.

Redzepovic, S. *et al.* 2003. "Differential Malic Acid Degradation by Selected Strains of *Saccharomyces* during Alcoholic Fermentation." *International Journal of Food Microbiology* 83(1): 49–61.

Regev-rudzki, Neta, Emil Battat, Israel Goldberg, and Ophry Pines. 2009. "Dual Localization of Fumarase Is Dependent on the Integrity of the Glyoxylate Shunt." *Molecular Microbiology* 72(March): 297–306.

Remize, F., E. Andrieu, and S. Dequin. 2000. "Engineering of the Pyruvate Dehydrogenase Bypass in *Saccharomyces cerevisiae*: Role of the Cytosolic Mg2+ and Mitochondrial K+ Acetaldehyde Dehydrogenases Ald6p and Ald4p in Acetate Formation during Alcoholic Fermentation." *Applied and Environmental Microbiology* 66(8): 3151–59.

Ribereau-Gayon, Pascal, Y. Glories, A. Maujean, and Denis Dubourdieu. 2006. "Handbook of Enology Volume 2 The Chemistry of Wine Stabilization and Treatments."

Robinson, J. 2006. *The Oxford Companion to Wine*. Oxford Uni. ed. J. Robinson.

Rodriguez, Susan B., and Roy J. Thornton. 1990. "Factors Influencing the Utilisation of L-Malate by Yeasts." *FEMS Microbiology Letters* 72(1–2): 17–22.

Rosenkrantz, Mark *et al*. 1994. "Distinct Upstream Activation Regions for Glucose-Repressed and Derepressed Expression of the Yeast Citrate Synthase Gene *CIT1*." *Current Genetics* 25(3): 185–95.

Ruhl, EH. 1989. "Effect of Potassium and Nitrogen Supply on the Distribution of Minerals and Organic Acids and the Composition of Grape Juice of Sultana Vines." *Australian Journal of Experimental Agriculture* 29(1): 133.

Saayman, M., and M. Viljoen-Bloom. 2006. "The Biochemistry of Malic Acid Metabolism by Wine Yeasts – A Review." *South African Journal of Enology & Viticulture* 27(2): 113–22.

Saint-Prix, Florence, Linda Bönquist, and Sylvie Dequin. 2004. "Functional Analysis of the ALD Gene Family of *Saccharomyces cerevisiae* during Anaerobic Growth on Glucose: The NADP+-Dependent Ald6p and Ald5p Isoforms Play a Major Role in Acetate Formation." *Microbiology* 150(7): 2209–20.

Salinas, Francisco *et al*. 2012. "The Genetic Basis of Natural Variation in Oenological Traits in *Saccharomyces cerevisiae*." *PloS one* 7(11): e49640.

Salmon, J. M. 1987. "L-Malic-Acid Permeation in Resting Cells of Anaerobically Grown *Saccharomyces cerevisiae*." *BBA - Biomembranes* 901(1): 30–34.

Salmon, J.M. 1988. "Métabolisme de l'acide Malique Chez La Levure." In *Application à l'oenologie Des Progrès Récents En Microbiologie et En Fermentation.*, ed. OIV. Paris, 388.

Salmon, J.M., F. Vezinhet, and P. Barre. 1987. "Anabolic Role of L-Malic Acid in *Saccharomyces cerevisiae* in Anaerobiosis during Alcoholic Fermentation ." *FEMS Microbiology Letters* 42(2–3): 213–20.

Sauer, Michael, Danilo Porro, Diethard Mattanovich, and Paola Branduardi. 2010. "16 Years Research on Lactic Acid Production with Yeast–Ready for the Market?" *Biotechnology and Genetic Engineering Reviews* 27(1): 229–56. https://pubmed.ncbi.nlm.nih.gov/21415900/ (March 8, 2021).

Schwartz, H., and F. Radler. 1988. "Formation of L(-)Malate by *Saccharomyces cerevisiae* during Fermentation." *Applied Microbiology and Biotechnology* 27(5–6): 553–60.

Sgouros, Georgios *et al*. 2020. "Molecular Characterization and Enological Potential of a High Lactic Acid-Producing *Lachancea thermotolerans* Vineyard Strain." *Foods* 9(5).

Shimazu, Y, and M Waranabe. 1981. "Effects of Yeast Strains and Environmental Conditions on Formation of Organic Acids in Must during Fermentation." *Fermentation Technology* 59(1): 27–32.

Sims, C. A., and J. R. Morris. 1985. "A Comparison of the Color Components and Color Stability of Red Wine from Noble and Cabernet Sauvignon at Various PH Levels." *American Journal of Enology and Viticulture* 36(3).

Souciet, Jean Luc *et al*. 2009. "Comparative Genomics of Protoploid Saccharomycetaceae." *Genome Research* 19(10): 1696–1709. https://pubmed.ncbi.nlm.nih.gov/19525356/ (March 8, 2021).

Sousa, Maria João, Letícia Miranda, Manuela Côrte-Real, and Cecília Leão. 1996. "Transport of Acetic Acid in *Zygosaccharomyces bailii*: Effects of Ethanol and Their Implications on the Resistance of the Yeast to Acidic Environments." *Applied and Environmental Microbiology* 62(9): 3152–57.

Sowalsky, Richard A., and Ann C. Noble. 1998. "Comparison of the Effects of Concentration, PH and Anion Species on Astringency and Sourness of Organic Acids." *Chemical Senses* 23(3): 343–49.

Steffan, J. S., and L. McAlister-Henn. 1992. "Isolation and Characterization of the Yeast Gene Encoding the MDH3 Isozyme of Malate Dehydrogenase." *Journal of Biological Chemistry* 267(34): 24708–15.

Stucka, Rolf, Sylvie Dequin, Jean Michel Salmon, and Carlos Gancedo. 1991. "DNA Sequences in Chromosomes 11 and VII Code for Pyruvate Carboxylase Isoenzymes in *Saccharomyces cerevisiae*: Analysis of Pyruvate Carboxylase-Deficient Strains." *MGG Molecular & General Genetics* 229(2): 307–15. https://link.springer.com/article/10.1007/BF00272171 (March 8, 2021).

Suárez-Lepe, J. A. *et al*. 2012. "Oenological Versatility of *Schizosaccharomyces* Spp." *European Food Research and Technology* 235(3): 375–83.

Sumby, Krista M., Paul R. Grbin, and Vladimir Jiranek. 2014. "Implications of New Research and Technologies for Malolactic Fermentation in Wine." *Applied Microbiology and Biotechnology* 98(19): 8111–32.

Taillandier, P.; Ramon Portugal, F.; Seiller, I.; Favarel, J.L.; Nepveu, F.; Strehaiano, P. 1999. "Effet de Cinq Souches de Levures Sur l'évolution de l'acidité Pendant La Fermentation Alcoolique." *Revue Francaise d'Oenologie* 178: 18–24.

Thoukis, G -, M Ueda, and D Wright. 1965. "The Formation of Succinic Acid During Alcoholic Fermentation." *American Journal of Enology and Viticulture* 16.

Tilloy, Valentin, Axelle Cadière, Maryam Ehsani, and Sylvie Dequin. 2015. "Reducing Alcohol Levels in Wines through Rational and Evolutionary Engineering of *Saccharomyces cerevisiae*." *International Journal of Food Microbiology* 213: 49–58. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.027.

Vally, Hassan, and Neil L.A. Misso. 2012. "Adverse Reactions to the Sulphite Additives." *Gastroenterology and Hepatology from Bed to Bench* 5(1): 16–23.

Vaughan Martini, Ann. 1991. "Evaluation of Phylogenetic Relationships among Fission Yeast by NDNA/NDNA Reassociation and Conventional Taxonomic Criteria." *Yeast*.

Vilanova, M. *et al*. 2007. "Assimilable Nitrogen Utilisation and Production of Volatile and Non-Volatile Compounds in Chemically Defined Medium by *Saccharomyces cerevisiae* Wine Yeasts." *Applied Microbiology and Biotechnology* 77(1): 145–57.

Vilela-Moura, Alice *et al.* 2011. "The Impact of Acetate Metabolism on Yeast Fermentative Performance and Wine Quality: Reduction of Volatile Acidity of Grape Musts and Wines." *Applied Microbiology and Biotechnology* 89(2): 271–80.

Viljoen M.; R.E. Subden; A Krizus, and H J Van Vuuren. 1994. "Molecular Analysis of the Malic Enzyme Gene (Mae2) of *Schizosaccharomyces pombe*." *Yeast (Chichester, England)* 10(5): 613–24.

Vion Charlotte *et al*. 2021. "Marker Assisted Selection of Malic-Consuming *Saccharomyces cerevisiae* Strains for Winemaking. Efficiency and Limits of a QTL's Driven Breeding Program." *Journal of Fungi*,7,304.

Vion Charlotte *et al*. 2022. "New Malic Acid Producer Strains of *Saccharomyces cerevisiae* for Preserving Wine Acidity during Alcoholic Fermentation." *Food Microbiology* 112.

Vion Charlotte *et al*. 2023. "Targeted 1H-NMR Wine Analyses Revealed Specific Metabolome Signatures of Yeast Populations Belonging to the *Saccharomyces* Genus." *submitted*.

Volschenk, H., M. Viljoen-Bloom, R. E. Subden, and H. J.J. Van Vuuren. 2001. "Malo-Ethanolic Fermentation in Grape Must by Recombinant Strains of *Saccharomyces cerevisiae*." *Yeast* 18(10): 963–70.

Volschenk, H., H. J.J. van Vuuren, and M. Viljoen-Bloom. 2003. "Malo-Ethanolic Fermentation in *Saccharomyces* and *Schizosaccharomyces*." *Current Genetics* 43(6): 379–91.

Volschenk, H., H.J.J. van Vuuren, and M. Viljoen-Bloom. 2006. "Malic Acid in Wine: Origin, Function and Metabolism during Vinification." *South African Journal of Enology & Viticulture* 27(2).

Volschenk, Heinrich *et al*. 1997. "Engineering Pathways for Malate Degradation in *Saccharomyces cerevisiae*." *Nature Biotechnology* 15(3): 253–57.

Wales, D. S., T. G. Cartledge, and D. Lloyd. 1980. "Effects of Glucose Repression and Anaerobiosis on the Activities and Subcellular Distribution of Tricarboxylic Acid Cycle and Associated Enzymes in *Saccharomyces carlsbergensis*." *Journal of General Microbiology* 116(1): 93–98.

Waterhouse, Andrew L., Gavin L. Sacks, and David W. Jeffery. 2016. Understanding Wine Chemistry *Understanding Wine Chemistry*.

Webb, Leanne B., P. H. Whetton, and E. W.R. Barlow. 2008. "Climate Change and Winegrape Quality in Australia." *Climate Research* 36(2): 99–111.

Whiting, By G C. 1976. "Organic Acid Metabolism Of Yeasts During Fermentation Of Alcoholic Beverages — A REVIEW." *J. Inst. Brew.* 82: 84–92.

Wibowo, D *et al.* 1985. "Occurrence and Growth of Lactic Acid Bacteria in Wine: A Review." *American Journal of Enology and Viticulture* 36(4).

Witte, V.; U.; Krohn, and C; Emis C. 1989. "Characterization of Yeasts with High L [+] -Lactic Acid Production : Lactic Acid Specific Soft-Agar Overlay (LASSO) and TAFE-Patterns." 29: 707–16.

Wu, M., and A. Tzagoloff. 1987. "Mitochondrial and Cytoplasmic Fumarases in *Saccharomyces cerevisiae* Are Encoded by a Single Nuclear Gene *FUM1*." *Journal of Biological Chemistry* 262(25): 12275–82.

Yéramian, N., C. Chaya, and J. A. Suárez Lepe. 2007. "L-(-)-Malic Acid Production by *Saccharomyces* Spp. during the Alcoholic Fermentation of Wine." *Journal of Agricultural and Food Chemistry* 55(3): 912–19.

Yokotsuka, Koki, Atsushi Otaki, Akihiko Naitoh, and Hideo Tanaka. 1993. "Controlled Simultaneous Deacidification and Alcohol Fermentation of a High-Acid Grape Must Using Immobilized Yeasts, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*." *American Journal of Enology and Viticulture* 44(4).

Zelle, Rintze M. *et al.* 2008. "Malic Acid Production by *Saccharomyces cerevisiae*: Engineering of Pyruvate Carboxylation, Oxaloacetate Reduction, and Malate Export." *Applied and Environmental Microbiology* 74(9): 2766–77.

Chapitre 1 : Sélection et caractérisation de souches de *Saccharomyces cerevisiae* pour la modulation de l'acidité des vins

Chapitre 1 : Sélection et caractérisation de souches de *Saccharomyces cerevisiae* pour la modulation de l'acidité des vins

1. Sélection assistée par marqueurs de souches consommatrices d'acide malique

1.1. Contexte et problématique de l'étude

Les levures de l'espèce *Saccharomyces cerevisiae* présentent une grande variabilité génotypique et phénotypique. L'implémentation de programmes de croisement ayant pour but de sélectionner des souches technologiquement innovantes présente un intérêt direct pour la filière viti-vinicole, qui pratique, dans la grande majorité des cas, une inoculation avec des levains commerciaux. L'utilisation de souches fortement consommatrices d'acide malique peut s'appliquer à deux itinéraires de vinifications en particulier. Premièrement, la vinification des vins rouges inclue nécessairement une étape de fermentation malolactique (FML) réalisée par des bactéries lactiques telle que *Oenococcus oeni*. Cette étape clé du processus de vinification s'avère parfois problématique. L'utilisation de souches de levures abaissant fortement le niveau d'acide malique lors de la fermentation alcoolique pourrait faciliter le déroulement de la FML de ces vins. Deuxièmement, de telles souches pourraient permettre de compenser un niveau d'acide malique trop élevé dans les vins blancs des régions septentrionales et dans les blancs vinifiés à partir de cépages particulièrement acides comme le Manseng. Une représentation plus large des itinéraires de vinification à explorer dans ce projet est décrite sur la figure 3. Ces itinéraires mettent en jeu une autre espèce de levure, *Lachancea thermotolerans* qui produit des quantités importantes d'acide lactique.

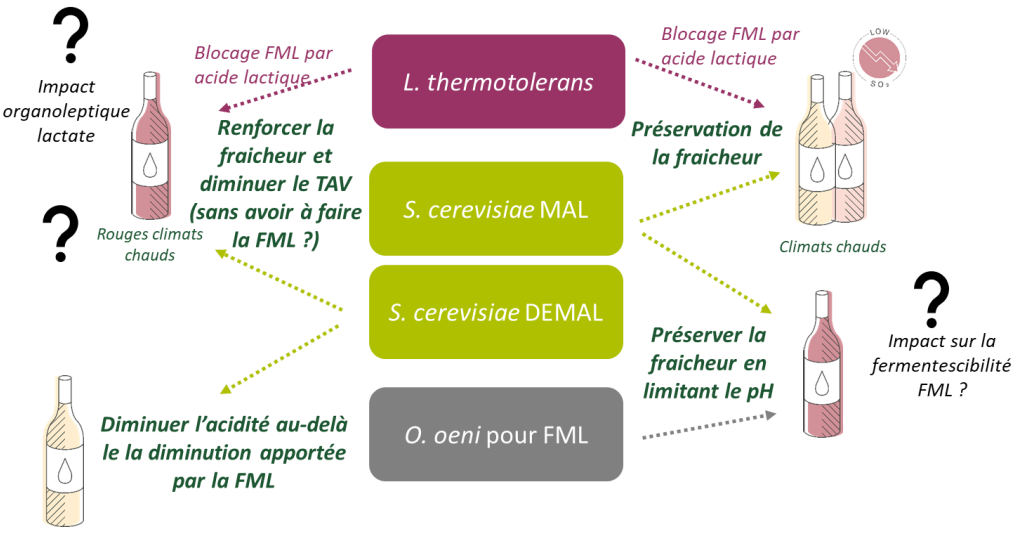


Figure 3. Représentation schématique des différents itinéraires de vinification faisant intervenir les différents microorganismes du projet. Source : Joana Coulon Société Biolaffort.

Ce premier chapitre présente un travail de sélection assistée par marqueurs génétiques pour améliorer la capacité de consommation d'acide malique de *S. cerevisiae* dans les moûts de raisin lors de la fermentation alcoolique. Lors de mon arrivée au laboratoire, un travail de cartographie génétique portant sur une dizaine de locus avait été réalisé et un panel de souches présentant un génotype optimal avait été isolé sans réaliser de fermentations (Emilien Peltier et Maitena Muro, projet SESAM2). L'efficacité de cette sélection a donc été évaluée et une caractérisation phénotypique des souches sélectionnées a été effectuée dans différentes matrices œnologiques. L'impact de l'utilisation de ces souches sur le déroulement de la FML a également été testé. Ces travaux ont été réalisés dans le cadre de mon stage de fin d'études d'élève ingénieur à l'ENSTBB (mars – septembre 2019). Ils ont contribué à la rédaction d'un article scientifique rédigé lors de ma première année de thèse. Ce premier article intitulé « *Marker assisted selection of malic-consuming Saccharomyces cerevisiae strains for winemaking. Efficiency and limits of a QTL's driven breeding program* » est paru en avril 2021 dans *Journal of Fungi*.

1.2. Principaux résultats

Cette sous-partie fait l'objet d'un article scientifique. La conception des expériences a été réalisée par Philippe Marullo (PM), Emilien Peltier (EP) et moi-même. Les expériences de sporulation et isolation des clones, d'extraction d'ADN et de génotypage ont été réalisées par Margaux Bernard et Maitena Muro. J'ai pour ma part participé à la réalisation des fermentations alcooliques et malolactiques, effectué des dosages enzymatiques et traité les données générées, notamment au moyen d'analyses de variances permettant d'étudier l'impact des facteurs abiotiques sur la consommation d'acide malique chez les souches de *S. cerevisiae* sélectionnées. Ces expérimentations et traitements de données ont été effectués dans le cadre de mon stage de fin d'étude, sous la supervision de PM et EP. Enfin, j'ai participé à la rédaction et à la relecture du manuscrit avec PM et EP.

Journal of Fungi

MDPI

Article

Marker Assisted Selection of Malic-Consuming *Saccharomyces cerevisiae* Strains for Winemaking. Efficiency and Limits of a QTL's Driven Breeding Program

Charlotte Vion [1,2,†], Emilien Peltier [1,2,3,†], Margaux Bernard [1,2], Maitena Muro [1,2] and Philippe Marullo [1,2,*]

[1] Unité de Recherche Œnologie EA 4577, USC 1366 INRAe, Bordeaux INP, ISVV, Université de Bordeaux, 33882 Villenave d'Ornon, France; charlotte.vion@u-bordeaux.fr (C.V.); epeltier@unistra.fr (E.P.); margaux.bernard@u-bordeaux.fr (M.B.); maitena.muro@u-bordeaux.fr (M.M.)
[2] Biolaffort, 33000 Bordeaux, France
[3] CNRS, GMGM UMR 7156, Université de Strasbourg, 67000 Strasbourg, France
* Correspondence: philippe.marullo@u-bordeaux.fr
† Authors had the same contribution to this work.

Abstract: Natural *Saccharomyces cerevisiae* yeast strains exhibit very large genotypic and phenotypic diversity. Breeding programs that take advantage of this characteristic are widely used for selecting starters for wine industry, especially in the recent years when winemakers need to adapt their production to climate change. The aim of this work was to evaluate a marker assisted selection (MAS) program to improve malic acid consumption capacity of *Saccharomyces cerevisiae* in grape juice. Optimal individuals of two unrelated F1-hybrids were crossed to get a new genetic background carrying many "malic consumer" loci. Then, eleven quantitative trait loci (QTLs) already identified were used for implementing the MAS breeding program. By this method, extreme individuals able to consume more than 70% of malic acid in grape juice were selected. These individuals were tested in different enological matrixes and compared to their original parental strains. They greatly reduced the malic acid content at the end of alcoholic fermentation, they appeared to be robust to the environment, and they accelerated the ongoing of malolactic fermentations by *Oenococcus oeni*. This study illustrates how MAS can be efficiently used for selecting industrial *Saccharomyces cerevisiae* strains with outlier properties for winemaking.

Keywords: wine yeast; malic acid; pH; breeding; malolactic fermentation; marker assisted selection

check for updates

Citation: Vion, C.; Peltier, E.; Bernard, M.; Muro, M.; Marullo, P. Marker Assisted Selection of Malic-Consuming *Saccharomyces cerevisiae* Strains for Winemaking. Efficiency and Limits of a QTL's Driven Breeding Program. *J. Fungi* **2021**, *7*, 304. https://doi.org/10.3390/jof7040304

Academic Editor: Antonis Rokas

Received: 2 March 2021
Accepted: 12 April 2021
Published: 15 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The yeast *Saccharomyces cerevisiae* is involved in many biotechnological processes including bioethanol, brewery, bakery and wine making [1]. This species displays a wide genetic diversity that generates a high phenotypic variability for many traits of interest. In the context of wine fermentation, the selection of industrial starters is particularly relevant since yeast modulates the final quality of the product [2,3]. Indeed, numerous studies have demonstrated that commercial *S. cerevisiae* strains are different regarding their ability to complete the alcoholic fermentation, their fermentation kinetics and their contribution to the final composition of volatile and non-volatile compounds [4–9].

The wine industry has developed basic genetic selection for making the best of phenotypic variability and proposing optimal starters. Indeed, clonal selection [10] and yeast breeding programs [9,11,12] are nowadays widely used for developing new wine starters; for documented reviews see [2,13,14]. Beside this applicative aspect, the genetic basis of trait variability has been studied for several decades by forward [15–27] and reverse [5,28–30] genetic approaches allowing the detection of numerous genes and allelic forms that possibly impact phenotypes of interest. Recently, we reviewed the phenotypic impact of 153 genes controlling traits of industrial interest [31]. The identification of such allelic variations and the steady decreasing cost of genotyping methods paved the avenue

for combining marker assisted selection (MAS) with classical breeding (cross and segregation). Despite its very efficient use in plant selection [32] and animal breeding [33], MAS has been poorly used in order to optimize fungi. Indeed, only few authors implemented this approach for accelerating breeding programs [34–38]. Generally, alleles used had a high penetrance and controlled a large part of phenotypic variation. However, most of allelic variations have a low penetrance and a small effect on the phenotype [39]. This is particularly true when MAS is implemented in outbred backgrounds [40] due to modifier alleles that drastically reduce QTL expressivity. The aim of this work is to evaluate MAS efficiency for improving a complex quantitative trait in a four-parental genetic background which represents an applied case of yeast breeding program.

We focused our investigation on the L-malic acid consumption by *S. cerevisiae* during the alcoholic fermentation, that is a highly variable trait among wine strains (Peltier 2018). The final concentration of this organic acid, originally present in grape, strongly contributes to the acidic pH of wine, which has a significant impact on its organoleptic qualities. Therefore, the control of its consumption is important for the choice of winemaking routes. *S. cerevisiae* is unable to consume all the L-malic acid of grape juice due to the lack of an active transporter [41]. Indeed, *S. cerevisiae* strains consuming more than 50% of total L-malic present in a grape juice were never reported [42,43]. During the winemaking process, malic acid can be transformed in L-lactic acid by wine-specific lactic bacteria *Oenococcus oeni* [44]. This biotransformation step, called malolactic fermentation (MLF), mostly occurs in red wines after or during the alcoholic fermentation. A part of L-malic acid can be also consumed by the malo-ethanolic fermentation (MEF) as well as by the fumarase activity [42,45].

Recently, the genetic determinism of malic acid consumption by *Saccharomyces cerevisiae* has been partially unraveled [46]. Numerous quantitative trait loci (QTL) related to the malic acid consumption were precisely mapped in the SBxGN background. Additional loci controlling the consumption of malic acid in *S. cerevisiae* have been also reported [47]. The functional exploration of these QTLs reveals the role of seven genes (*MAE1*, *PMA1*, *PYC2*, *PNC1*, *YBL036c*, *PTC5*, and *SDH2*) by reciprocal hemizygosity assay [46,47]. In this present work, a large-scale breeding program was implemented for addressing how much, the rational use of MAS can be implemented for boosting malic acid consumption during the wine alcoholic fermentation. First, optimal individuals of two unrelated F1-hybrids were crossed in order to get a new genetic background carrying many "malic consumer" loci. Eleven unlinked QTLs previously identified were used for implementing a MAS program. By this method, optimal individuals consuming more than 70% of the malic acid of grape juice were obtained. Such individuals were tested in different enological matrixes; they lower the malic acid content at the end of the alcoholic fermentation and accelerate the ongoing of inoculated malolactic fermentations.

2. Materials and Methods

2.1. Yeast Strain Used and Culture Methods

Yeast (*Saccharomyces cerevisiae*) were propagated on YPD 2% (1% peptone, 1% yeast extract, 2% glucose) at 28 °C in both liquid and plate cultures (2% agar). Long term storage at −80 °C was achieved by adding one volume of glycerol to YPD 2% overnight cultures. The main yeast strains used were described in the Table 1 and are derived from the two F1-hybrids (M2xF15 and SBxGN) used in previous QTL mapping studies [26,48]. All the strains are homozygous for the *HO* locus and are therefore diploids. The strains GS-28b and GS-41b are meiotic spore clones of SBxGN while FM-8d is a meiotic spore clone of M2xF15. These three strains were used as allele donors in a breeding program aiming to fix in few individuals most of the alleles promoting the consumption of malic acid during the alcoholic fermentation. The pedigree of the strains generated is shown in Table 1.

Table 1. Main *Saccharomyces cerevisiae* strains used.

Strain	Description	Reference	WDCM-791 (CRB ISVV, Bordeaux)
M2	parental strain, a meiotic spore clone from Enoferm M2 (Lallemand, Canada)	[26]	CRBO L2010
F15	parental strain, a meiotic spore clone from Zymaflore F15 (Laffort, France)	[26]	CRBO L2011
GN	parental strain, a meiotic spore clone from Zymaflore VL1 (Laffort, France)	[48]	CRBO L2002
SB	parental strain, a meiotic spore clone from Zymaflore BO213 (Laffort, France)	[48]	CRBO L2001
M2xF15	F1-hybrid (M2 x F15)	[26]	CRBO L2005
SBxGN	F1-hybrid (SB x GN)	[48]	CRBO L2003
GS-28b	meiotic clone of SBxGN	this work	CRBO L2012
GS-41b	meiotic clone of SBxGN	this work	CRBO L2013
FM-8d	meiotic clone of M2xF15	this work	CRBO L2014
FMGS-1	F1-hybrid (GS-28b x FM-8d)	this work	CRBO L2015
FMGS-1-647	meiotic clone of FMGS-1, malic acid consumer strain	this work	CRBO L2016
FMGS-2	F1-hybrid (GS-41b x FMGS-1-647)	this work	CRBO L2017
FMGS-1-889	meiotic clone of FMGS-1, malic acid consumer strain	this work	CRBO L2018
FMGS-1-215	meiotic clone of FMGS-1, Malic acid consumer strain	this work	CRBO L2019
FMGS-2-107	meiotic clone of FMGS-2, Malic acid consumer strain	this work	CRBO L2020
FMGS-2-265	meiotic clone of FMGS-2, Malic acid consumer strain	this work	CRBO L2021

2.2. Spore Mating and Purification

Since all the strains are homothallic, F1-hybrids (FMGS-1 and FMGS-2) were obtained by spore to spore mating on YPD 2% using a routine method previously described [9]. After few hours, zygotes (trilobed shaped) were observed and manually isolated; their heterozygous genotype was verified by Mass Array genotyping (see below). Spore purification was carried out by using the hydrophobic properties of the spore cell wall. Each F1-hybrids was sporulated by cultivating 10^9 cells in 5-mL of potassium acetate 1% (ACK) during three days at 24 °C. This high-density spore culture was digested with cytohelicase (10 mg/mL) for 2 h at 28 °C under permanent shaking. Spore preparation was then washed twice (de ionized water) and the pellet was recovered in 100 µL in one centrifuge tube (1.5 mL). The suspension was vigorously vortexed for 2 min and the suspension was removed by pipetting. The remaining mixture of spores and cells was purified by adding/removing 1 mL of de ionized water two times. The remaining spores were unstacked from the tube wall by adding 1 mL of sterile Nonidet P40 (0.01%). The purification rate obtained ranged between 85 and 95% of spore clones. The purified spores were then sonicated (30 min), incubated 2 h on YPD (28 °C) and plated on several Petri dishes in order to obtained more than 1000 isolated colonies per preparation.

2.3. Cell Culture and DNA Extraction in Microplates

Each single colony picked up from YPD-agar plate was cultivated in 96-Well microplate (coated with gas-permeable sheets (BREATHseal^{TM}, Greiner Bio-one, Courtaboeuf, France) allowing CO_2 production. Genomic DNA was extracted by using a Li-Ac SDS protocol previously adapted for microplate handling [49]. Broadly, 5×10^6 cells (200 µL of overnight cultures) were centrifuged and incubated with 50 µL of 200 mM LiAc/1% SDS at 70 °C for 5 min. Genomic DNA was extracted by mixing cell lysates with 150 µL of pure ethanol and vortexed for 15 s. After a brief spin (2 min, 4000 rpm) the pellet was washed with 70% ethanol and was the solubilized in 200 µL of milliQ water at 60 °C for 5 min. Some liquid handling steps were assisted by the robotic platform NxP (Beckman Coulter, Villepinte, France).

2.4. Mass ARRAY Genotyping

Genotyping was mostly carried out by using Mass ARRAY technology (Agena Bioscience, San Diego, CA, USA), which allows large multiplex SNP detection as previously described [50]. Less than 15 ng of DNA were used for genotyping the strain. The Online Resource 1 lists the identity of wanted SNP that possibly enhance the malic acid consumption according to a wide QTL mapping study recently published. SNPs were encoded according to the international nomenclature [51]. The genotypes of each parental strain founder (M2, F15, SB, GN) and the sequence of primers used were also provided. Primers were designed using the tool MassARRAY® Assay Design (v4.0.0.2) in order to amplify short fragments (<115 bases) with a molecular weight differences ranging between 16 and 80 Da. By this method, 11 bi allelic loci were simultaneously tracked corresponding to the following QTLs (*II_661*, *IV_31*, *IV_360*, *IV_414*, *VII_427*, *VII_480*, *XI_382*, *XI_631*, *XII_53*, *XV_491*, *XV_1052*) which control malic acid consumption in the SBxGN background. The SNP calling was carried out by the MassARRAY® System suite with an average SNP calling of 94.3%. Control strains (SB, GN, F15, M2) were genotyped several times giving in any case the identical SNP calling. All clones showing a fully homozygous genotype and 10 or more loci assigned were included in the analysis, which corresponds to 593 and 797 clones for FMGS-1 and FMGS-2, respectively.

2.5. Alcoholic Fermentation Assays

2.5.1. Grape Must

The grape musts Merlot 2015 (M15) and Sauvignon Blanc 2016 (SB16) used were provided by Vignobles Ducourt (Ladaux, France) and stored at −20 °C. Grape musts were sterilized by membrane filtration (nitrate cellulose 0.45 µm, Millipore, France). In order to test the phenotypic robustness of some relevant strains, these musts were spiked with different amounts of L-malic acid and nitrogen source (Table S2). The mixture of amino acid used mimics the average composition of organic sources in a grape wine as previously described [12]. The composition of the different grape musts in fermenting sugar, nitrogen sources and malic acid content as well their pH are listed in Table S2.

2.5.2. Alcoholic Fermentation Monitoring

Small-volume alcoholic fermentations were implemented in screwed vials fermentations according to the general procedure described in [52]. Rapidly, 20 mL-screwed vials (Thermo Fisher Scientific, Bordeaux, France) were filled with 12 mL of filtered grape must and were tightly closed with screw cap-magnetic (Agilent Technologies, hdsp cap 18 mm PTFE/sil 100 pk, Les Ulis, France) perforated by hypodermic needles (G26–0.45 × 13 mm, Terumo, Shibuya, Tokyo, Japan) for allowing the CO_2 release. Vessel was inoculated by 2.10^6 viable cell.mL^{-1} precultured in liquid YPD for 24 h. Cellular concentration and viability was estimated by flow cytometry using a Cell Lab Quanta apparatus (Beckman Coulter, Villepinte, France) as described by Zimmer et al. (2014). Fermentation took place at 24 or 28 °C in shaken vials by using an orbital shaker (SSL1, Stuart, Vernon Hills, IL, USA) at 175 rpm. Fermentation kinetics were estimated by monitoring manually (2–3 times

per day) the weight loss caused by CO_2 release using a precision balance with automatic weight recording (LabX system, Mettler Toledo, Viroflay, France). The amount of CO_2 released according to time was modeled by local polynomial regression fit [52] allowing the estimation of the time necessary to reach the maximum CO_2 produced.

2.6. Malolactic Fermentations Monitoring

At the end of the alcoholic fermentation, vials were placed for 24 h at 4 °C for settling yeast lees. Sedimented part were then solidified by soaking vials in a thin layer of liquid nitrogen. Thus, yeasts were eliminated in order to avoid lees impact on MLF and to reproduce enological-like conditions. Ten mL of wine were transferred into 10 mL cylindrical vials (Thermo Fisher Scientific, Bordeaux, France, ref: 11981523), closed with screw caps (Agilent Technologies, hdsp cap 18 mm PTFE/sil 100 pk, Les Ulis, France). The wines were inoculated with Lactoenos® B7 Direct rehydrated lactic bacteria at a concentration of 10 mg·L^{-1}, as recommended by the supplier (Laffort, Bordeaux, France). Fermentation samples were taken every 2 to 5 days for measuring L-malic acid decrease as a proxy of the MLF progress. The MLF ongoing was fitted by a 3 parameters logistic function using the R package (*drm* function of *drc* package), four kinetics parameters were extracted: t_MLF_lag, and t_MLFend, correspond to the time to initiate and to achieve the MLF, respectively. t_MLF50 corresponds to the time for consuming 50% of malic acid, and rmax_MLF is the maximal malic acid consumption rate (g·L^{-1}·h^{-1}). Data fit and MLF parameters are illustrated in the Table S3.

2.7. Enzymatic Assay of Wine

At the end of the alcoholic fermentation, a sample volume of 800 µL was manually transferred in Micronics tubes (Novazine, Lyon, France, ref: MP32033L) and stored at −20 °C. The concentrations of the following organic metabolites were measured: acetic acid, glycerol, malic acid, pyruvate, acetaldehyde and total SO_2 using the respective enzymatic kits: K-ACETGK, K-GCROLGK, K-LMAL-116A, K-PYRUV, K-ACHYD, K-TSULPH, (Megazyme, Bray, Ireland) following the instructions of the manufacturer. Glucose and fructose were assayed by using the enzymatic method described by Stitt et al. [53]. All the enzymatic assays were performed by a robotic platform using the Bordeaux metabolomics facilities (http://metabolome.cgfb.u-bordeaux.fr/). Only the strains able to complete the fermentations (<1.5 g·L^{-1} of residual sugars) were retained.

2.8. Use of Previous Phenotypic Datasets

Two previously published datasets were used for estimating the heritability and the efficiency of this marker assisted selection program. Fermentation phenotypes were measured in the same conditions and in the same grape musts allowing to compare the data. Malic Acid Consumption (*MAC*) was the ratio of malic acid consumed ($[\text{L-malic acid}]_{\text{initial}}$-$[\text{L-malic acid}]_{\text{final}}$)/$[\text{L-malic acid}]_{\text{initial}}$ expressed in %. The *MAC* values of the 35 enological strains and the 94 progenies of SBxGN hybrid were extracted form Peltier et al. (2018a) [46] and Peltier et al. (2020) [52], respectively. The *MAC* values of M2xF15 offspring were measured in the same conditions described by Peltier et al. (2018b) [48] but are only released in this present work.

2.9. Statistical Analyses

All the statistical and graphical analyses were carried out using R software [54]. Plots were computed using the *ggplot2* package and analyses of variance were carried out using the *car* package. Four linear models were applied: LM1 investigated the effect of QTL numbers on *MAC* according to the formula:

$$y_i = m + EA_i + \epsilon_i, \quad (1)$$

where y is the MAC value for a strain carrying i Enhancers Alleles ($i = 1 \ldots 11$) and ϵ_i the residual, m is the overall mean of the 154 FMGSs progenies phenotyped. The LM2 model

estimated the effects of the following factors must, strain, temperature, nitrogen, and malic acid as well as the first order interaction of all the factors on MAC value according to the formula:

$$y_{ijklm} = m + strain_i + must_j + temp_k + nitro_l + malic_m + inter^2_{ijklm} + \epsilon_{ijkmn}, \quad (2)$$

where y_{ijklm} is the *MAC* value for a strain i (i = 1 ... 6) fermented in a must j (j = 1,2) at the temperature k (k = 1,2) and containing the nitrogen level l (l = 1,2) and the malic acid level m (m = 1,2,3). Levels taken by each factor are given in the Online Resource 2. The term $inter^2_{ijklm}$ represents the first order interactions for each factor and ϵ_{ijklm} the residual, m is the overall mean of the 84 fermentations carried out in triplicate in an equilibrated fractional design. This model was refined with the models LM3 and LM4 allowing the estimation of each factors in the SB16 and M15 must, respectively.

The analysis of variance of models LM2, 3 and 4 allows the estimation of the primary effect of the strain, the grape must, the temperature, the nitrogen and the malic acid concentration as well as their primary interaction effects. The normal distribution of residues as well as the homoscedasticity of the variances were tested by Shapiro test and Levene test (*car* package).

3. Results

3.1. A Basic Breeding Strategy for Improving Malic Acid Consumption

The main goal of this study consists to significantly increase the *S. cerevisiae* consumption of malic acid during alcoholic fermentation. Previously, it has been reported that wine strains were unable to consume more than 50% of the malic acid grape juice in controlled conditions. This overall feature was previously confirmed in our laboratory by measuring the residual malic acid concentration at the end of the alcoholic fermentation for 35 strains in 5 grape juices [52]. Since the initial amount of malic acid varied according to the grape juice, we computed the percentage of malic acid consumed (*MAC*) for comparing strains within multiple environmental conditions. On average, *MAC* ranged between 30.0 and 53.0% within a population of 31 commercial starters plus the four founder strains of this study (SB, GN, M2, F15), that are haploid derivative from commercial starters. Strong differences between grape musts were found (Table S3). The continuous distribution of this trait was illustrated for a red grape juice Merlot 2015 in the Figure 1a. Strains SB and GN had extreme phenotypes, metabolizing between 40.0% and 3.5% of malic acid, respectively. Strains M2 and F15 had an intermediate phenotype, consuming up to 20% of malic acid in the same conditions. This analysis demonstrated that MAC is a quantitative trait with important variation among commercial starters and therefore we decided to took advantage of this natural variation by initiating a selection program with a breeding approach.

We decided to use two previously generated mapping populations as starting material. These populations originated from two hybrids, resulting from the mating of SB with GN (SBxGN) and M2 with F15 (M2xF15) that were sporulated to generate 94 and 96 segregants, respectively. The rational being that meiosis segregation would reveal transgressive phenotypes. SBxGN offspring was phenotyped for MAC in a previous study [48] and the phenotypic characterization of M2xF15 offspring was achieved in the same conditions but the *MAC* values are only released in this present work (Table S3). The fermentation conditions applied (grape juice, fermentation volume, temperature) are the same in all the studies. In both F1-hybrids, the meiotic segregation yields few clones consuming up to 60% of malic acid (Figure 1b,c). Thanks to this first approach, we were able to select two progeny clones GS-28B from SBxGN offspring and FM-8D from M2xF15 one that had high *MAC* value of 58.0%and 45.4%, respectively. These high values correspond to the reported limits of *Saccharomyces cerevisiae* physiology [43,55–57].

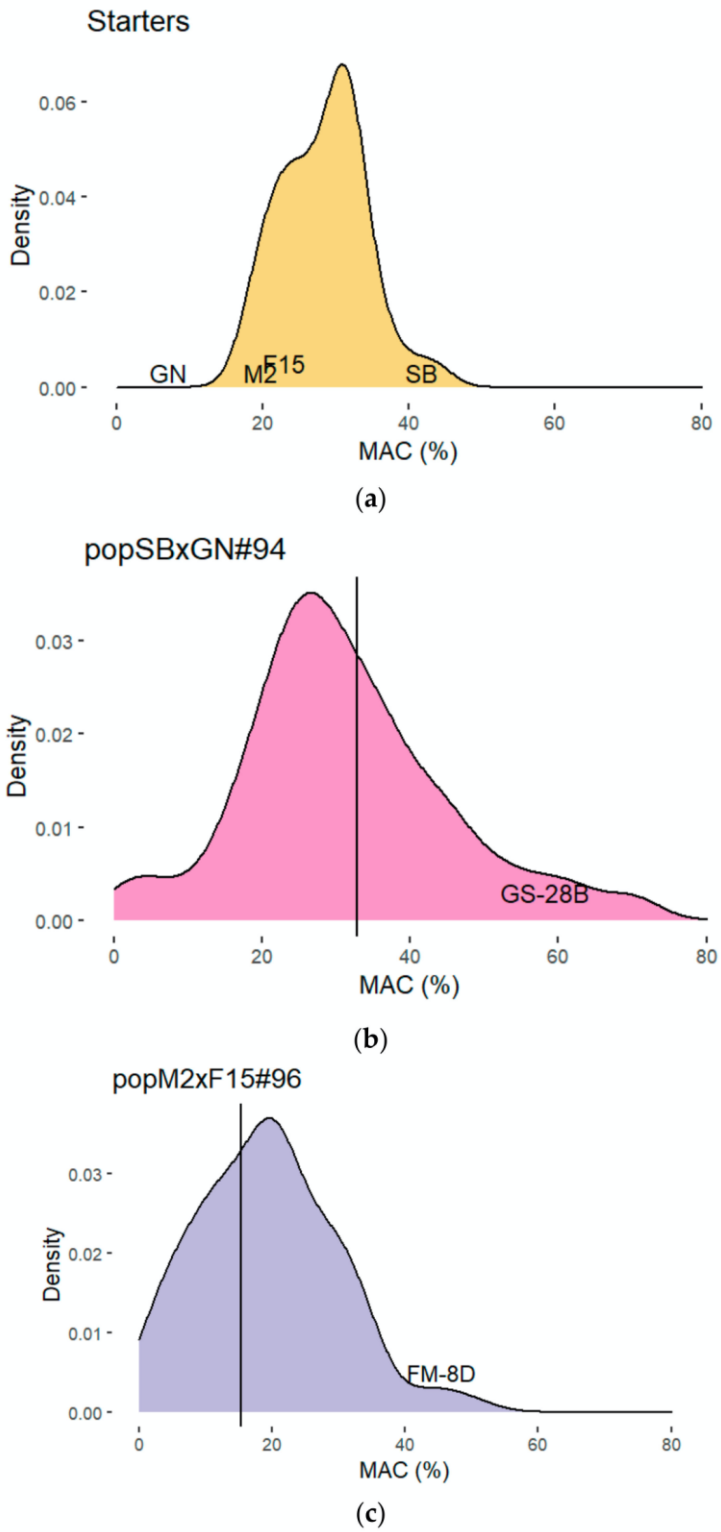


Figure 1. *Cont.*

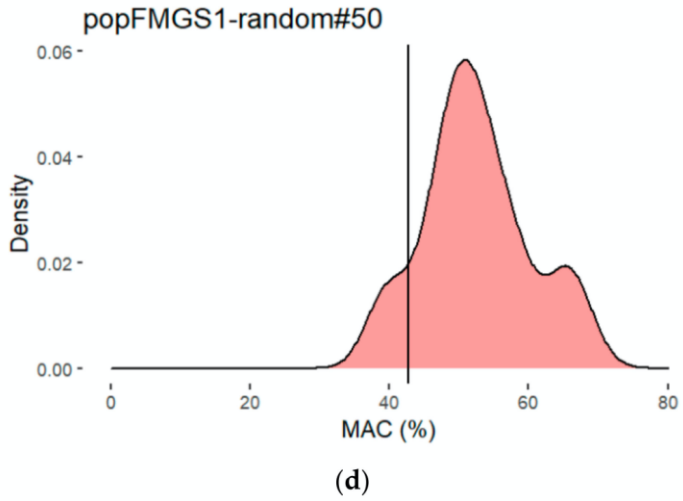


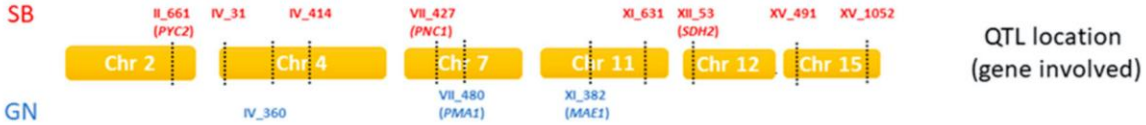
Figure 1. Malic acid consumed (MAC) at the end of alcoholic fermentation in a merlot grape juice. (**a**) Phenotypic distribution of 31 starters and relative position of the 4 founder strains SB, GN, M2, F15 (data previously published by [52]). (**b**) Phenotypic distribution of SBxGN hybrid and 94 related progeny clones including GS-28B (data previously published [46]). (**c**) Phenotypic distribution of M2xF15 hybrid and 96 related progeny clones including FM-8D (this work). All fermentations were carried out in the same conditions. (**d**) Phenotypic distribution of FMGS1 hybrid and 50 related progeny clones. The vertical bar indicated the value of the F1-hybrid of each progeny.

To pursue trait improvement, strains GS-28B and FM-D8 were mated by manually paring spores of both strains. The resulting F1-hybrid (FMGS-1) should have inherited from both genetic backgrounds independent genetic factors controlling MAC that can be recombined through meiosis. Therefore, FMGS-1 was sporulated and a high number of spores were purified by taking advantage of the hydrophobicity properties of spore wall. A spore preparation of 95% was obtained and around 1000 clones were isolated on solid YPD and stored at −80 °C. Fifty randomly picked individuals were fermented in the M15 grape juice and their *MAC* was measured. In this population, the consumption levels of malic acid were on average significantly much higher than those previously observed in SBxGN and M2xF15 progenies as well as in the commercial starters panel (Figure 1d). This result demonstrates that the simple cross of two optimal individuals derived from different background is very efficient for optimizing complex phenotypes. Such strategy is routinely used by microbiologists for improving yeast strains and have been reported and reviewed by many authors [11,14,36,58,59].

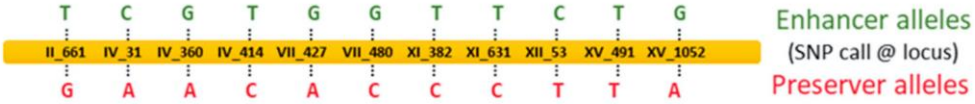
3.2. Use of Marker Assisted Selection (MAS) for Enhancing Malic Acid Consumption

To go further, we used at a large-scale genetic markers for selecting optimal individual prior any phenotypic tests. Indeed, getting genotypic data is generally easier than setting up a phenotypic characterization. For testing the relevance of this strategy, we first defined a set of eleven genetic makers that are nucleotide variations genetically linked to QTL controlling MAC and identified in SBxGN background [46,47]. The Figure 2a,b summarize SNP position and *enhancer* or *preserver* alleles that were define for each QTL. The genotype of the founder strains (SB, GN, M2 and F15) and the parental strains GS-28B and FM-8D is given panel c. We applied this genotypic screening on the ~1000 meiotic spores previously isolated from FMGS-1 and sought for meiotic spore clones having preferably inherited Quantitative Trait Loci (QTL) allele promoting the malic acid consumption. As detailed in the material and method section, each QTL was tracked by using the MASS-array technology able to discriminate in a unique experiment eleven pairs of biallelic Single Nucleotide Polymorphisms (SNP) (Table S1). The inheritance of the 11 loci for 924 FMGS-1 progenies was interrogated. SNP calling was higher than 94% and most of the strains were

fully genotyped. A first segregation analysis confirmed that spore purification was very efficient for removing residual F1-hybrids (fully heterozygous). Indeed, most of the clones (77%) were fully homozygous while a minor fraction (15.5%) harbored both homozygous and heterozygous loci and could be considered as sibling-pair hybrids (F2) (Figure S1 left panel). Only fully homozygous strains with less than one missing genotype were analyzed representing 583 individuals. According to parental genotypes, the segregation of five loci was expected (Figure 2). Indeed, 5 *enhancer* alleles were fixed (IV_360G, IV_414T, VII_480G, XI_382T, and XI_631T), one *preserver* allele was fixed (II_661G), and the five other loci segregated in a mendelian fashion (chi^{2}test $p > 0.05$) (Figure 3a).



(**a**)



(**b**)

Strain	II_661	IV_31	IV_360	IV_414	VII_427	VII_480	XI_382	XI_631	XII_53	XV_491	XV_1052
SB	T	C	A	T	G	C	C	T	C	C	G
GN	G	A	G	C	A	G	T	C	T	T	A
M2	G	A	G	T	A	G	T	T	T	T	A
F15	G	A	G	T	A	G	C	T	T	T	G
GS-28B	G	C	G	T	G	G	T	T	C	C	G
FM-8D	G	A	G	T	A	G	T	T	T	T	A

(**c**)

Figure 2. Quantitative trait loci (QTLs) tracked, and mass-array markers used, dotted lines represent the position of a marker. (**a**) Genetic position of the eleven QTLs mapped modulating malic acid consumption in SBxGN hybrid. The parental allele (GN or SB inheritance) promoting a stronger consumption was indicated for each locus. (**b**) SNP corresponding to the enhancer and preserver allele, the exact position is given in Table S1. (**c**) SNP call in the four founder and in the parental strains GS-28B and FM-8D.

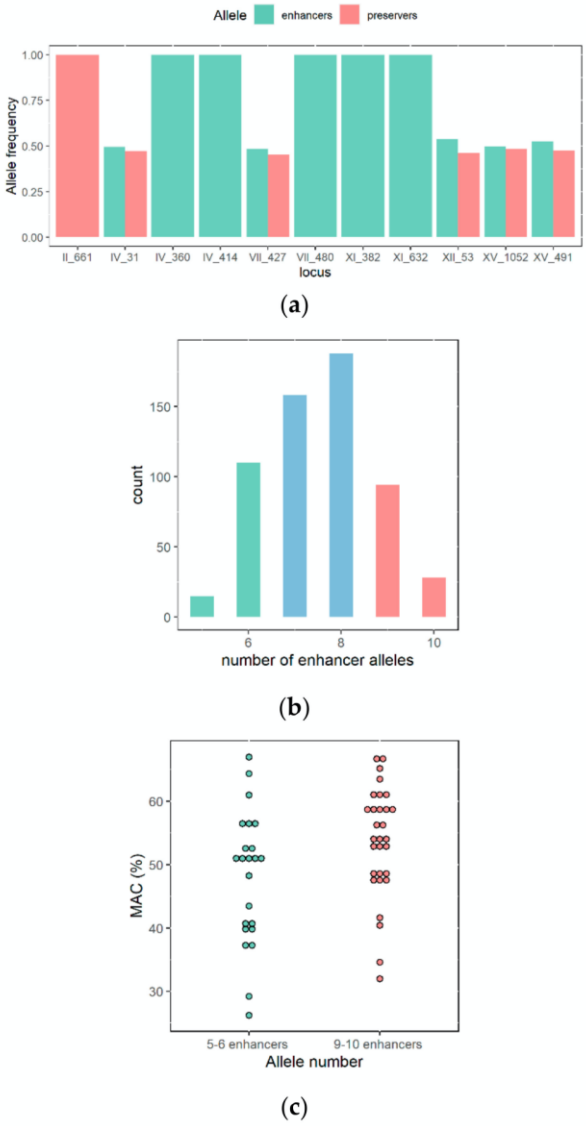


Figure 3. Marker assisted selection of FMGS-1 progeny for five segregating alleles. (**a**) Allele frequency in the progeny, enhancer and preserver alleles are shown in green and red, respectively. (**b**) Distribution of the number enhancer alleles counted in the FMGS-1 progeny (593 fully genotyped progenies). (**c**) Phenotypic difference between progenies having enriched and minimized number of enhancer alleles; values indicated represented the average percentage of Malic acid Consumed (MAC) for each strain (3 replicates).

Next, we investigated the phenotypic impact of markers segregation. The progeny population was split in two groups according to the number of *enhancer* alleles detected. 122 individuals harbor 9 or 10 *enhancer* alleles while 125 individuals harbor only 5 or 6 *enhancer* alleles (Figure 3b). Each group represents nearly 20% of the total population genotyped. In order to test QTLs effect in the FMGS-1 background, 30 and 23 individuals belonging to each group were randomly selected and phenotyped in the Merlot grape juice. Strains enriched for *enhancer* alleles (9 to 10 optimal loci) consumed more malic acid than the control group (Figure 3c). On average, the *enhancer* group consumes 53.7% of malic acid while the control only 48.0% of malic acid (Wilcoxon test $p < 0.05$). Although the phenotypic gain observed (+5.7% of malic acid) was moderated, this demonstrates that MAS allows the blind selection of highly performing individuals without any phenotypic screening.

3.3. Construction and Characterization of a Backcrossed FMGS-2 Population

A possible cause of the moderate efficiency of *MAS* in the FMGS-1 background would be the fact that many QTLs were fixed (i.e., have a homozygous inheritance). Therefore, a second hybrid (FMGS-2) was obtained in order to increase the number of segregating loci. A progeny clone of FMGS-1 (FMGS-1#647) was crossed with GS-41B, one segregant of SBxGN hybrid. FMGS-1#647 has an optimal MAC (65.7%) and harbor 8 out 11 *enhancer* alleles. GS-41B has a moderated *MAC* value (40.4%) but allows the introduction of other enhancer alleles in the resulting hybrid. Indeed, in the FMGS-2 hybrid, all but two QTLs (VII_480 and XII_53) were heterozygous (Figure 4a). From FMGS-2, 1232 spore clones were isolated and genotyped using the same procedure than for FMGS-1 offspring. Mass-array analysis confirmed the Mendelian segregation of 9 out the 11 markers typed (chi^{2}test $p > 0.05$). The genotyping of these clones allows the identification of nearly 12% of residual F1-hybrids and F2-sibling pair hybrids due to a less efficient spore purification. In the further analysis, 797 fully homozygous meiotic segregants were used. In order to test *MAS* efficiency in this new background, the same approach than for FMGS-1 was used. Fifty randomly selected individuals were phenotyped for characterizing the overall performance of FMGS-2 progeny. Random progenies of FMGS-1 and 2 have similar average values (52.6 and 48.7) (Table 2 and Figure S2), however the phenotypic variability (variance) in the FMGS-2 offspring is two folds higher (130 vs. 58). This result is likely due to the higher number of QTLs in segregation in FMGS-2 population. Beside this difference, the proportion of clones with optimal malic acid consumption is broadly the same (Table 2).

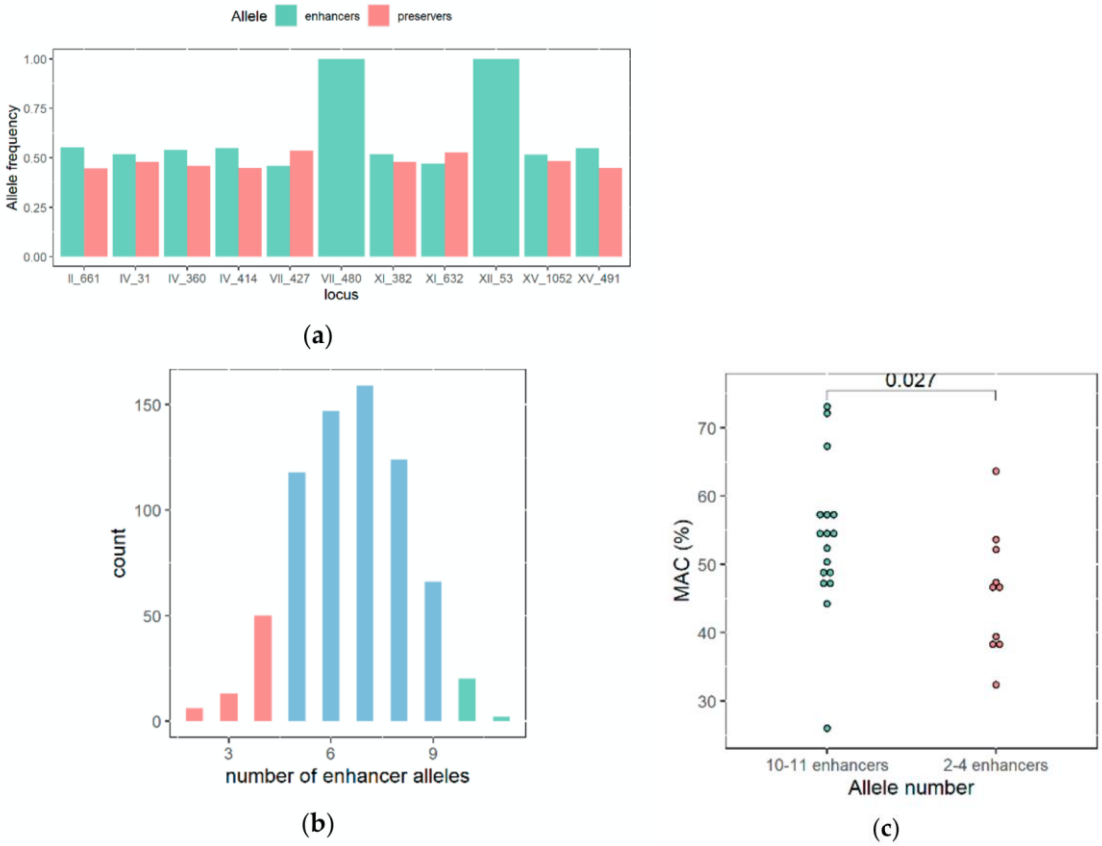


Figure 4. Marker assisted selection of FMGS-2 progeny for nine segregating alleles. (**a**) Allele frequency in the progeny, enhancer and preserver alleles are shown in green and red, respectively. (**b**) Distribution of the number enhancer alleles counted in the FMGS-2 progeny (797 fully genotyped progenies). (**c**) Phenotypic difference between progenies having enriched and minimized number of enhancer alleles; values indicated represented the average percentage of Malic acid Consumed (MAC) for each strain (3 replicates).

Table 2. Biometric comparison of the populations used.

Populations	Number	Mean	Variance	Quantiles			% of Individuals with a MAC Above	
				50%	75%	95%	65%	70%
Starters	31	28.3	33.3	28.2	31.5	36.5	no	no
pop SBxGN	93	31.0	206.6	28.6	36.9	58.5	2.2	2.2
pop M2xF15	94	17.8	138.9	18.6	24.8	34.1	no	no
random FMGS-1	50	52.6	58.6	51.7	57.2	65.7	12.0	no
selected FMGS-1 [a]	30	53.7	77.0	54.1	59.2	65.8	10	no
random FMGS2-1	50	48.7	130.4	49.0	56.6	64.3	4.0	2.0
selected FMGS2-2 [b]	17	53.7	121.2	54.2	57.1	72.3	17.6	11.7

[a] Genetically selected for 9–10 enhancer alleles; [b] genetically selected for 10–11 enhancer alleles; no: not observed.

Similarly, a *MAS* was carried out by selecting individuals according to their genotype. Seventeen individuals that have inherited of 10 and 11 *enhancer* loci were selected among the 797 FMGS-2 progeny genotyped. This *enhancer*-rich group was compared to a small group of 10 strains harboring only 2 to 4 *enhancers* loci. On average, the *enhancer* group consumes +8% of malic acid at the end of the alcoholic fermentation (53.7% vs. 45.8%, Wilcoxon test $\alpha = 0.05$) (Figure 4c). This also demonstrates that, in the FMGS-2 background, *MAS* is a convenient way for selecting extreme individuals in a small group of strains. Indeed, within the 17 genetically selected strains, two strains consumed more than 70% of malic acid which represents 11% of the population (Table 2); this proportion is significantly higher than those expected in the random population (hyper geometric test $\alpha = 0.05$).

3.4. Study of QTL Penetrance in FMGS-1 and FMGS-2 Populations

Although a significant difference was found between *enhancers* and *preservers* groups (Figures 3c and 4c), many individuals with a nearly optimal genotype reach a moderate MAC value. Encompassing the random and marker-selected strains of each FMGS populations, a total of 154 strains were phenotypically and genetically characterized (Table S4). This set of strains was used for estimating the cumulative impact *enhancer* allele on the malic acid consumption. A linear model confirms that, the more the number of *enhancer* alleles, the stronger the percentage of malic acid consumed (Figure 5a). Although, this correlation is very significative (Spearman test, rho = 0.23, *p*-value < 0.0001); a great residual variation within extreme groups is still observed. Indeed, the number of QTLs explained only 4.2% of the total variance observed in the linear model (LM1 see methods). This result illustrates the low penetrance level of the QTL pool used, which is a frequent case in *MAS* programs [40].

The effect of each QTL on malic acid consumption was tested and compared to their effect observed in the SBxGN background that was used for their identification. When a significative difference was observed (one-way ANOVA $\alpha = 0.05$), the part of variance explained was indicated (Table 3). Since all the loci did not segregate in both progenies this test was in some cases not applied.

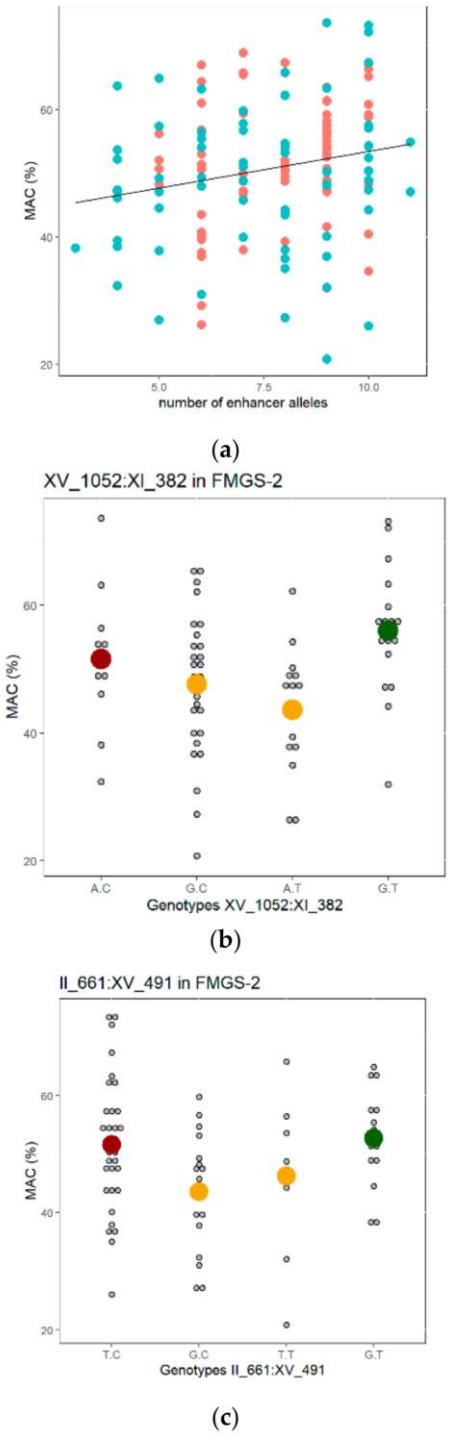


Figure 5. Overall QTL effect and pair wise interactions. (**a**) Linear relationship between the number of enhancer alleles and the MAC value in the 154 FMGS' progenies tested; the red and blue dots represent the FMGS-1 and FMGS-2 progenies, respectively. (**b**,**c**) Genetic interactions between QTLs observed in the FMGS-2 population (72 individuals). The red, orange, and green dots are the average values observed for the bad, intermediate, and good combinations expected.

Table 3. QTL penetrance in various populations [a].

Population	II_661	IV_31	IV_360	IV_414	VII_427	VII_480	XI_382	XI_631	XII_53	XV_491	XV_1052
Gene validated	*PYC2*				*PNC1*	*PMA1*	*MAE1*		*SDH2*	*PTC5*	
SBxGN (n = 94)	3.87	4.22	ns	ns	ns	8.20	2.97	ns	4.45	7.61	ns
FMGSs (n = 154)	-	7.14	-	-	ns	-	-	-	-	ns	4.92
FMGS-1 (n = 82)	-	9.31	-	-	ns	-	-	-	ns	ns	9.74
FMGS-2 (n = 72)	ns	6.41	ns	ns	ns	-	ns	3.10	-	ns	ns

[a] Part of variance explained in %, one-way analysis of variance, p-value <0.05. ns: not significative, -: not relevant.

Among the eleven QTLs used, only six have a significative effect in the SBxGN population (Anova $\alpha = 0.05$). This result is explained by the fact that those QTLs were detected in three different environments [46] which is not the case in the present analysis of variance. Surprisingly, although the effect of the causative genes *PNC1* has been molecularly validated [46], the inheritance of the marker *VII_427* did not impact *MAC* in the population. When the two FMGSs populations were pooled, two of the four segregating QTLs had a significative effect (*IV_31* and *XV_1052*). The locus *IV_31* has a complete penetrance since is significative in the four populations. The locus *XV_1052* has a more contrasted effect; it accounted for 9.7% of total variance observed in FMGS-1 progeny but did not significantly impact the malic acid consumption in FMGS-2 or SBxGN populations. Finally, the locus *XI_631* has a significative effect only in the FMGS2 population (Table 3). These results show that most of the QTLs tracked have an incomplete penetrance in the FMGS hybrids. This result can be explained by at least three causes: (i) the limited number of progenies tested (less than 100), (ii) the epistatic relations withing QTLs, (iii) the presence of modifier loci. We tested possible pair-wise interactions within the nine segregating QTLs in the FMGS-2 population (Table S5). Interestingly, two significative interactions were found (*XV_1052* vs. *XI_382*) and (*II_661* vs. *XV_491*). In the first case, the inheritance of the allele XI_382^C impairs the enhancer effect of the allele XV_1052^G (Figure 5b). This explains why XV_1052^G allele has a much higher penetrance in the FMGS-1 progeny where the XI_382^T allele is fixed (Figure 2). Concerning the *II_661* and *XV_491* interaction, the best combination expected II_661^G/XV_491^T has a similar effect than the worst one II_661^T/XV_491^C (Figure 5c); suggesting a possible metabolic trade-off between their associated genes *PYC2* and *PTC5*.

3.5. Phenotypic Characterization of High Malic Acid Consuming Strains

Four malic consumer strains belonging to both FMGS' populations (FMGS_215, FMGS_889, FMGS2_107, FMGS2_265) were selected in order to be phenotypically characterized in various environmental conditions. These strains were compared to SB and GN which represent the extreme limits of the starter population (Figure 1a). Small-volume alcoholic fermentations were carried out in two grape musts (M15 and SB16) adjusted with different concentrations of malic acid and nitrogen and incubated at two different temperatures (see methods). The objective was to assess the impact of several basic environmental factors in enology that could affect *MAC*. The part of variance of each factor as well as their first-order interactions were estimated by applying a fractional experimental design (see methods). The effect of each factor is represented in the Figure 6a and summarized in Table S6.

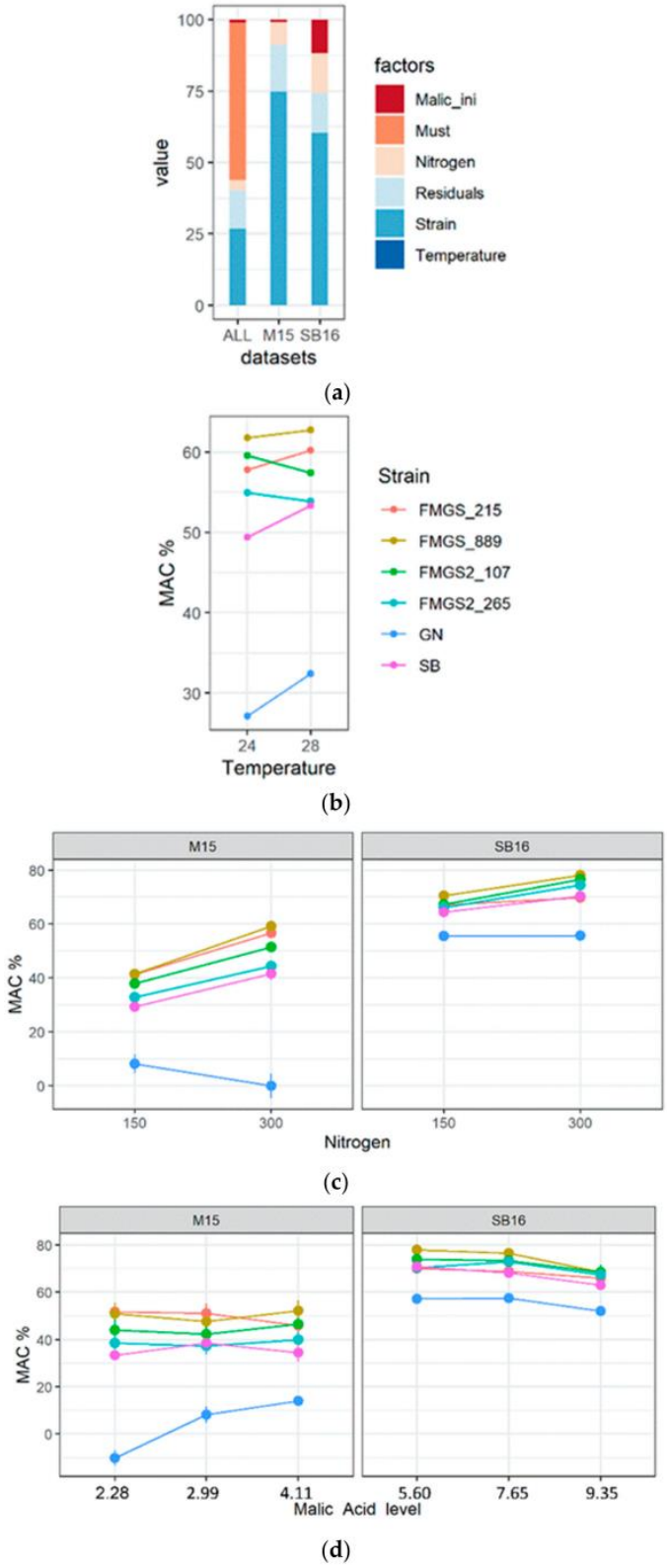


Figure 6. *Cont*.

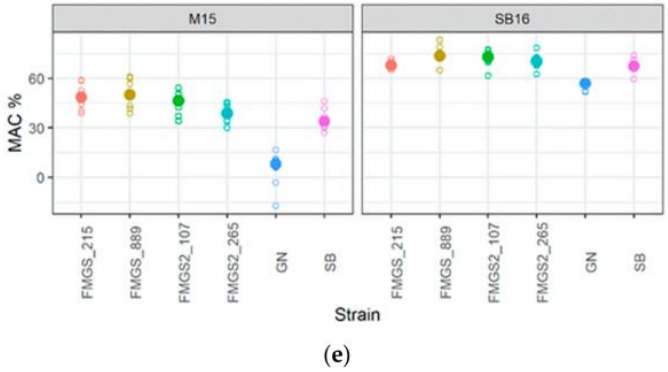


Figure 6. Biotic and abiotic factors affecting the variation of malic acid consumption. (**a**) Bar graphs indicate the part of variance explained by the different factors considered by the linear model applied: malic acid concentration, must origin, nitrogen content, strain origin, and temperature. (**b**) Temperature effect according to the strains used; (**c**) nitrogen effect according to the strains used; (**d**) initial malic acid concentration effect according to the strains used; (**e**) strain effect in both grape juices. For panels (**b–e**) the full dots represent the average values.

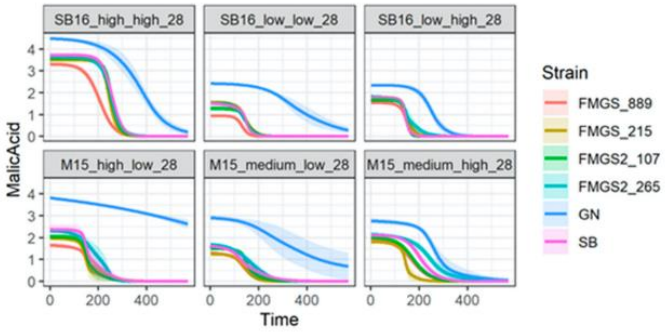
All conditions combined; *MAC* varies mainly with the nature of the must (55.2% of total variance explained). Indeed, yeast strains consumed in average 32.5% more malic acid in white must compared to red must (Figure 6a). In contrast, temperature had a low impact on MAC and can be considered as poorly significant in the present study (Figure 6b). The important must-effect observed is likely due to the highest malic acid concentrations found in white must (SB16) compared to those of the red one (M15). This difference strongly impacts the pH of the matrix and may affect the diffusion of malic acid through cell membrane since this acid is protonated below pH = 3.4 [60].

In order to dissociate this matrix effect, a second set of ANOVA was carried out by splitting the dataset according to the must. The new models shed light on the effect of nitrogen and malic acid concentrations that were partially hidden by the must effect. Nitrogen concentrations impacted malic acid consumption in both musts (Figure 6c). In our experiment, malic acid consumed was 10% higher in low nitrogen condition (150 mg/L N). However, this trend was not observed for the strain GN that consumes more malic acid (+4%) in rich nitrogen condition (300 mg/L N). The impact of grape juice concentration of malic acid on *MAC* was also strongly contrasted. As shown on the Figure 6d, the malic acid level accounts for 11.8% of the total variance explained in the white wine but only 0.9% of the total variance in the red one. In the white grape juice, the *MAC* decreases (−8%) for the highest malic acid concentration (9.39 g/L) while this trait is steadier in red grape juice. Again, the response of GN differs from the malic consumer strains. Indeed, in red must, the impact of initial malic acid was stronger, with an increased MAC with higher initial malic acid concentration.

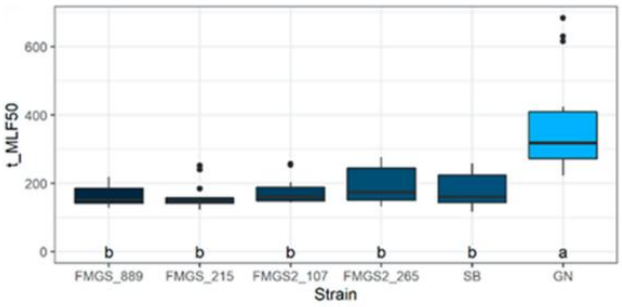
Finally, this experiment demonstrates that in both musts, the factor "strain" has the major impact explaining 74.8% and 60.5% of "red" and "white" models, respectively (Figure 6e). As expected, all the strains selected by genetic markers consumed significantly more malic acid than the control GN. The strongest consumer is the strain FMGS_889 that metabolized 32% and 11% more malic acid than the strains GN and SB, respectively (Wilcoxon test $p < 0.01$). Although minor variations were attributed to nitrogen and malic acid content, those strains steady consume malic acid which is not the case of the strain GN. The founder strain SB belongs to the malic consuming group but has always a lower MAC than the four individuals selected. As a direct consequence, the resulting pH of wine at the end of the alcoholic fermentation was drastically impacted (Figure S2). Indeed, in the most discriminating conditions, the pH difference measured in produced wine between extreme strains (GN and FMGS_889) was of 0.42 units (M15_high_initial_24), demonstrating the strong impact of *S cerevisiae* strains in the modulation of wine pH.

3.6. Ongoing of Malolactic Fermentation

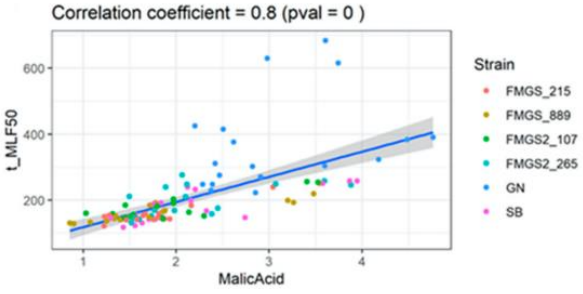
During the winemaking process, most of the red wines and some white wines underwent the malolactic fermentation (MLF) due to the development of the lactic bacteria *Oenococcus oeni* [44]. The malic acid is one key energetic substrate of the MLF and its content in wine at the end of the alcoholic fermentation has an obvious impact on the process. It has been reported that L-malic acid concentration delays the MLF duration but does not drastically affect the *O. oeni* fermentation rate [61,62]. In addition, low pH has a negative impact on MLF initiation and efficiency [44]. Since strains selected clearly impacted malic acid concentration (Figure 6e) and wine pH (Figure S2), their influence on MLF was evaluated by inoculating the different wines produced with a malolactic starter (see methods). MLF ongoing was monitored by measuring the L-malic acid degradation by an enzymatic assay. MLF kinetics were fitted by a logistic function and four kinetics parameters were extracted (*t_MLF50*, *t_MLFend*, *t_MLF_lag*, *rmax_MFL*) (Figure S3). For the malic consuming yeast strains, the MLFs were completed between 10 and 15 days. In contrast, MLFs carried out in wines fermented by GN were much slower and were sometimes not achieved (Figure 7a).



(a)

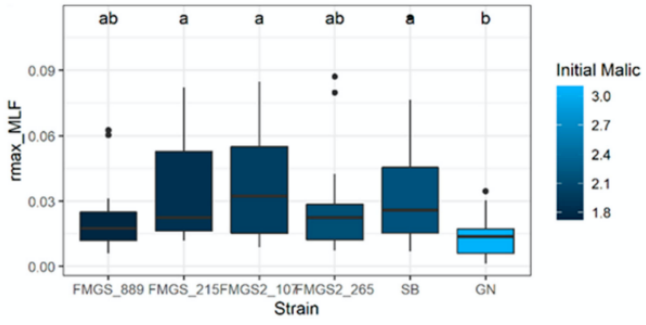


(b)



(c)

Figure 7. *Cont.*



(**d**)

Figure 7. Malolactic fermentations with high malic consumer strains. (**a**) Malic acid concentration (g/L) according to the time (h) for each stain used in the 6 inoculated wines. (**b**) Time (h) to achieve 50% of the MLF according to the strains used. (**c**) Correlation between the time at 50% of the MLF (t_MLF50) and the malic acid concentration (g/L). (**d**) Maximum fermentation rate (g/L/h) according to the strains used.

A correlation analysis performed on the 36 wines (6 strains and 6 conditions) clearly demonstrated a positive correlation between the initial malic acid content and the time for consuming 50% of malic acid (t_MLF50) (Figure 7b). Indeed, the amount of malic acid in wines fermented with GN was 1.1 $g{\cdot}L^{-1}$ higher, on average, than those from other strains (Figure 7c). GN was different from the other strains (Kruskal–Wallis test, $\alpha = 0.05$) and MLFs of wines fermented with GN required twice more time for consuming 50% of total malic acid. In contrast, the maximum fermentation rate (*rmax_MLF*) was quite similar between the strains. Indeed, strains FMGS_889 and GN have similar *rmax_MLF* but opposed *t_MLF50* values (Figure 7d). Thus, the time for completing MLF (*t_MLFend*) proved to be faster (less than 300 h) when malic acid concentration was below 2 $g{\cdot}L^{-1}$. In addition, a low initial concentration in malic acid does not seem to impair the initiation of MLF since the lag time of MLF (*t_MLFlag*) is quite similar in all the conditions tested. Altogether, these results suggested that malic consuming strains are compatible with sequential inoculation of lactic acid bacteria and should facilitate the MLF by increasing wine pH and reducing the malic acid concentration to consume.

4. Discussion

4.1. Assesment of a Wide MAS Program for Improving a Complex Trait

Marker assisted selection (MAS) constitutes the applied side of quantitative genetics and has been widely used for improving plant varieties and animal races [32,63,64]. For the last two decades, *Saccharomyces cerevisiae* has been raised as a perfect model for addressing fundamental questions in quantitative genetics [65–68]. Furthermore, the budding yeast is also the prime industrial microorganism involved in bioethanol and fermented foods production. Although methods for identifying QTLs are perfectly controlled [31], their use for improving yeast strains for industrial applications is poorly documented. In the present study, we aimed to increase the malic acid consumption of wine starters in order to propose new strains with outlier features. To achieve this program, we get a new F1-hybrid (FMGS-1) resulting from the cross of two optimal unrelated strains (GS-28b and FM-8d). Then, we implemented for the first time a wide MAS program using 11 QTLs controlling the same trait.

First, a robust protocol was developed for isolating and genotyping a wide number of meiotic progenies derived from prototrophic and homothallic yeast. Although these features complexify breeding operations [14], the purification of FMGSs hybrids yielded more than 80% of spore clones (doubled haploids) or sibling-pair hybrids (F2) (Figure S1). The use of robotic-assisted DNA extraction and mass array genotyping allow a SNP call average of 94% and most of the strains were unambiguously genotyped. This technique

allows the parallel genotyping of up to 40 markers which would strongly decrease the genotyping cost per SNP.

Second, we demonstrated that individuals carrying a high proportion of *enhancer* alleles consumes statistically more malic acid than those carrying a high proportion of *preserver* alleles in both FMGS progenies (Figures 3c and 4c). This result confirms that a blind preselection of strains can be achieved on the basis of their genotype. Nevertheless, the overall effect of this pool of alleles is limited regards to the general cross effect observed. Indeed, many FMGS-1 and FMGS-2 progenies reach high *MAC* value whatever their genotype. This result indicates that the simple selection of optimal outbred parental strains is very efficient for optimizing quantitative phenotypes. Indeed, the average malic acid consumed by randomly selected FMGS-1 progenies (50 strains) is statistically similar to average value of the FMGS-1 *enhancer* group (52.6% vs. 53.7%). This is explained by the fact that five *enhancer* alleles are homozygous (fixed) in FMGS-1 hybrid and only five QTLs are in segregation (Figure 2a) reducing the proportion of non-optimal genotypes. Thus, 36% of randomly selected segregants (18) carry at least 8 *enhancer* alleles of the 11 possible one. These strains include FMGS-647, FMGS-215 and FMGS-889 that are among the top malic consumers. A second blind selection was then carried out in the FMGS-2 progeny. Since 10 of 11 QTL segregated, the difference of MAC between *enhancer* and *preserver* groups was larger (8%). A tight significative difference was found between random and *enhancer* enriched groups (48.7% vs. 53.7%, respectively, Wilcoxon test < 0.1). In addition, the proportion of optimal clones consuming more than 65 or 70% is higher than in random population (Table 2). This highest proportion supports the idea that MAS would be efficient for the blind selection of many optimal individuals and should be used as a prescreening step. Since many other quantitative traits segregate independently in FMGS's offspring, MAS may allow the selection of a collection of top-degrading malic acid strains to be used in downstream selection programs focusing on other traits (aromatic, fermentation performances, temperature adaptation . . .).

4.2. Possible Causes of Incomplete QTLs Penetrance

Although it was successfully applied in many organisms, the efficiency of MAS is strongly depending on the number of marker used, the type of breeding design, the complexity, and the number of traits to improve [32,40,64,69]. Indeed, when QTLs detected in a specific background are introduced in an outbred individual, their expressivities are much lower than those observed in the initial QTL population [40]. This general trend was also observed here since only three QTLs of the eleven used has a significant effect of FMGSs hybrids (Table 2). This incomplete penetrance can be explained by several causes. First, the 11 QTLs used were detected in a multiple environmental design which increase the detection power of QTLs [46,47]. Thus, it is not surprising that minor QTLs showing GxE does not raise the significant threshold imposed in the subset of 94 SBxGN progenies. Second, possible alleles controlling *MAC%* in the M2xF15 background were not considered at all. Since 50% of FMGS-1 genome is constituted by M2 or F15 alleles, QTLs governing the phenotypic variability of M2xF15 background are likely influencing the *MAC%* of FMGS's progeny. Third, the lack of penetrance observed for the marker VII_427 related to the causative gene *PNC1* would result to the complex genetic structure of the chromosome VII that contains many causative genes including *PMA1* a major contributor of MAC% [43]. Finally, some QTLs seems to show epistatic interactions (Figure 5b,c) that modulate their expressivity in a non-predicable manner.

4.3. Outlier Strains for Specific Enological Applications. Lowering the Acidity of Rich Malic Wines and Shortening the MLF of Red Wines

From an enological point of view, this study provides new strains for managing atypical vinification conditions. Indeed, *S. cerevisiae* strains consuming more than 50% of the total malic acid are quite rare and have been scarcely described [55,57,70,71]. Such strains would be useful to better managing wine acidity and may have two distinct enological applications. First, in very acidic grape juice (cold climate white grapes), the excess

malic acid is a concern since the taste of related wines is quite sour [72]. Therefore, the biotechnological reduction of malic acid would contribute to provide smoother wines (less aggressive) from a tasty point of view. In the Sauvignon blanc grape juice containing 9.39 g/L of L-malic acid, the difference in final L-malic acid concentration reached 1.3 g/L (Figure 7a) representing a pH shift close to 0.2 pH unit (Figure S2) with a possible impact on the acidity perception. Second, such strains would contribute to facilitate malolactic fermentation by increasing wine pH and reducing L-malic concentration. Indeed, we demonstrated that none of the FMGSs strains selected has a negative impact MLF ongoing inoculated with a commercial starter (Lactoenos® B7 Direct, Laffort oenology, FRANCE). In contrast, the malic acid reduction and the pH increase promote the MLF by reducing its duration. Therefore, whatever the residual amount of L-malic acid at the end of the alcoholic fermentation, the MLF is initiated readily without any significative impact on the consumption rate of bacteria.

By measuring the *MAC%* in several musts and conditions (temperature, nitrogen composition and L-malic concentration) we demonstrated that the high consumption properties of FMGS's strains are quite robust to environmental changes. Temperature did not impact this trait and the main factor is the nature of grape juice (Figure 6a). Although the compositions of the two grape juices are not chemically defined, a possible cause of this discrepancy should be the pH of the media that affects the charge and the transport of malic acid [70]. However, in a specific must, L-malic acid concentration has moderated effect (Figure 6c). Beside the nature of the must, the impact of nitrogen seems to be the most important factor with strain specific interactions that has been previously reported [57]. Indeed, malic-consumer strains achieved a stronger MAC in nitrogen rich conditions which is not the case of GN. The GN's behavior was previously reported for other wine yeast strains [57]. This suggest that the high malic-consumer strains selected would have a particular response to nitrogen level.

Although significantly improved, the consumption of the total amount of malic acid by *S. cerevisiae* seems to be illusive. Indeed, the strain FMGS_889 consumed 84% of malic acid in SB16 and 67% in M15. In both cases, the final concentration of malic acid fall to 0.9 g/L constituting a kind of physiological threshold. This limit is likely due to the absence of an active malic acid transporter able to pump in this organic acid through the cell membrane [73]. A further improvement of malic acid consuming ability would require the enhancement of malic acid uptake. Beside the expression of recombined proteins [41], this new skill might be acquired by activating quiescent transmembrane transporter by mutagenesis as achieved for xylulose import [74].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7040304/s1, Table S1: SNPs detected by mass array, Table S2: Composition of grape must, pH and nitrogen sources added, Table S3: *MAC* measured in 31 starters and the four founder strains [52], Figure S1: Purification rate of progeny clones of FMGS-1 and FMGS-2 hybrids, Table S4: FMGS-1 and FMGS-2 genotypes and phenotypes, Table S5: Part of variance of pair-wise interactions in FMGS-2 population, Table S6: Analysis of variance of environmental and strain effect in selected strains, Figure S2: pH of wines at the end of the alcoholic fermentation, Figure S3: MFL parameters.

Author Contributions: Conceptualization, P.M., C.V. and E.P.; investigation, M.B., M.M., C.V. and E.P.; data Analysis, E.P., C.V. and P.M.; writing P.M. and C.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Aquitaine Region, grant number SESAM.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Strains used have been deposited on the WDCM-791 (CRB ISVV, Bordeaux).

Acknowledgments: Mass array assay was performed at the Genome Transcriptome Facility of Bordeaux (grants from the Conseil Régional d'Aquitaine no 20030304002FA and 20040305003FA, from the European Union FEDER no 2003227 and from Investissements d'Avenir ANR-10-EQPX-

16-01), with the help of Erwan Guichoux". Enzymatic assays were performed at the metabolomic Facility of Bordeaux (grants from PHENOME-ANR-INBS-0012) with the help of Yves Gibon.

Conflicts of Interest: All the authors belong to the Biolaffort company; the funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Sicard, D.; Legras, J.L. Bread, beer and wine: Yeast domestication in the Saccharomyces sensu stricto complex. *C. R. Biol.* **2011**, *334*, 229–236. [CrossRef] [PubMed]
2. Rainieri, S.; Pretorius, I.I.S. Selection and improvement of wine yeasts. *Ann. Microbiol.* **2000**, *50*, 15–31.
3. Marullo, P.; Dubourdieu, D. Yeast selection for wine flavour modulation. In *Managing Wine Quality Vol 2*; Reynolds, A.G., Ed.; Woodhead Publishing Limited: Cambridge, UK, 2010; pp. 296–320. ISBN 9781845697983.
4. Gammacurta, M.; Marchand, S.; Albertin, W.; Moine, V.; De Revel, G. Impact of yeast strain on ester levels and fruity aroma persistence during aging of bordeaux red wines. *J. Agric. Food Chem.* **2014**, *62*, 5378–5389. [CrossRef] [PubMed]
5. Marchal, A.; Marullo, P.; Durand, C.; Moine, V.; Dubourdieu, D. Fermentative Conditions Modulating Sweetness in Dry Wines: Genetics and Environmental Factors Influencing the Expression Level of the Saccharomyces cerevisiae HSP12 Gene. *J. Agric. Food Chem.* **2015**, *63*, 304–311. [CrossRef] [PubMed]
6. Murat, M.-L.; Masneuf, I.; Darriet, P.; Lavigne, V.; Tominaga, T.; Dubourdieu, D. Effect of Saccharomyces cerevisiae Yeast Strains on the Liberation of Volatile Thiols in Sauvignon blanc Wine. *Am. J. Enol. Vitic.* **2001**, *52*, 136–139.
7. Julien, A.; Roustan, J.L.; Dulau, L.; Sablayrolles, J.M. Comparison of nitrogen and oxygen demands of enological yeasts: Technological consequences. *Am. J. Enol. Vitic.* **2000**, *51*, 215–222.
8. Berthels, N.J.; Otero, R.R.C.; Bauer, F.F.; Thevelein, J.M.; Pretorius, I.S. Discrepancy in glucose and fructose utilisation during fermentation by Saccharomyces cerevisiae wine yeast strains. *FEMS Yeast Res.* **2004**, *4*, 683–689. [CrossRef] [PubMed]
9. Marullo, P.; Mansour, C.; Dufour, M.; Albertin, W.; Sicard, D.; Bely, M.; Dubourdieu, D. Genetic improvement of thermo-tolerance in wine Saccharomyces cerevisiae strains by a backcross approach. *FEMS Yeast Res.* **2009**, *9*, 1148–1160. [CrossRef]
10. Esteve-Zarzoso, B.; Gostıncar, A.; Bobet, R.; Uruburn, F.; Querol, A. Selection and molecular characterization of wine yeasts isolated from the "El Penedès" area (Spain). *Food Microbiol.* **2000**, *17*, 553–562. [CrossRef]
11. Romano, P.; Soli, M.G.; Suzzi, G. Improvement of a wine Saccharomyces cerevisiae strain by a breeding program. *Appl. Environ. Microbiol.* **1985**, *50*, 1064–1067. [CrossRef]
12. Marullo, P.; Bely, M.; Masneuf-Pomarède, I.; Pons, M.; Aigle, M.; Dubourdieu, D.; Masneuf-Pomarede, I.; Pons, M.; Aigle, M.; Dubourdieu, D. Breeding strategies for combining fermentative qualities and reducing off-flavor production in a wine yeast model. *FEMS Yeast Res.* **2006**, *6*, 268–279. [CrossRef] [PubMed]
13. De Vero, L.; Solieri, L.; Giudici, P. Evolution-based strategy to generate non-genetically modified organisms Saccharomyces cerevisiae strains impaired in sulfate assimilation pathway. *Lett. Appl. Microbiol.* **2011**, *53*, 572–575. [CrossRef]
14. Steensels, J.; Snoek, T.; Meersman, E.; Nicolino, M.P.; Voordeckers, K.; Verstrepen, K.J. Improving industrial yeast strains: Exploiting natural and artificial diversity. *FEMS Microbiol. Rev.* **2014**, *38*, 1–49. [CrossRef] [PubMed]
15. Marullo, P.; Durrens, P.; Peltier, E.; Bernard, M.; Mansour, C.; Dubourdieu, D. Natural allelic variations of Saccharomyces cerevisiae impact stuck fermentation due to the combined effect of ethanol and temperature; a QTL-mapping study. *BMC Genom.* **2019**, *20*, 680. [CrossRef] [PubMed]
16. Marullo, P.; Aigle, M.; Bely, M.; Masneuf-Pomarède, I.; Durrens, P.; Dubourdieu, D.; Yvert, G. Single QTL mapping and nucleotide-level resolution of a physiologic trait in wine Saccharomyces cerevisiae strains. *FEMS Yeast Res.* **2007**, *7*, 941–952. [CrossRef]
17. Zimmer, A.; Durand, C.; Loira, N.; Durrens, P.; Sherman, D.J.; Marullo, P. QTL dissection of lag phase in wine fermentation reveals a new translocation responsible for Saccharomyces cerevisiae adaptation to sulfite. *PLoS ONE* **2014**, *9*, e86298. [CrossRef]
18. Martí-Raga, M.; Peltier, E.; Mas, A.; Beltran, G.; Marullo, P. Genetic Causes of Phenotypic Adaptation to the Second Fermentation of Sparkling Wines in Saccharomyces cerevisiae. *G3 Genes Genomes Genet.* **2017**, *7*, 399–412. [CrossRef]
19. Ambroset, C.; Petit, M.; Brion, C.; Sanchez, I.; Delobel, P.; Guerin, C.; Chiapello, H.; Nicolas, P.; Bigey, F.; Dequin, S.; et al. Deciphering the Molecular Basis of Wine Yeast Fermentation Traits Using a Combined Genetic and Genomic Approach. *G3 Genes Genomes Genetics* **2011**, *1*, 263–281. [CrossRef]
20. Steyer, D.; Ambroset, C.; Brion, C.; Claudel, P.; Delobel, P.; Sanchez, I.; Erny, C.; Blondin, B.; Karst, F.; Legras, J.-L. QTL mapping of the production of wine aroma compounds by yeast. *BMC Genom.* **2012**, *13*, 573. [CrossRef] [PubMed]
21. Cubillos, F.A.; Parts, L.; Salinas, F.; Bergström, A.; Scovacricchi, E.; Zia, A.; Illingworth, C.J.R.; Mustonen, V.; Ibstedt, S.; Warringer, J.; et al. High-resolution mapping of complex traits with a four-parent advanced intercross yeast population. *Genetics* **2013**, *195*, 1141–1155. [CrossRef] [PubMed]
22. Jara, M.; Cubillos, F.A.; García, V.; Salinas, F.; Aguilera, O.; Liti, G.; Martínez, C. Mapping genetic variants underlying differences in the central nitrogen metabolism in fermenter yeasts. *PLoS ONE* **2014**, *9*, e86533. [CrossRef]
23. Salinas, F.; Cubillos, F.A.; Soto, D.; Garcia, V.; Bergström, A.; Warringer, J.; Ganga, M.A.; Louis, E.J.; Liti, G.; Martinez, C. The genetic basis of natural variation in oenological traits in Saccharomyces cerevisiae. *PLoS ONE* **2012**, *7*, e49640. [CrossRef]

24. Brice, C.; Sanchez, I.; Bigey, F.; Legras, J.-L.; Blondin, B. A genetic approach of wine yeast fermentation capacity in nitrogen-starvation reveals the key role of nitrogen signaling. *BMC Genom.* **2014**, *15*, 495. [CrossRef] [PubMed]
25. Roncoroni, M.; Santiago, M.; Hooks, D.O.; Moroney, S.; Harsch, M.J.; Lee, S.A.; Richards, K.D.; Nicolau, L.; Gardner, R.C. The yeast IRC7 gene encodes a b-lyase responsible for production of the varietal thiol 4-mercapto-4-methylpentan-2-one in wine. *Food Microbiol.* **2011**, *28*, 926–935. [CrossRef] [PubMed]
26. Huang, C.; Roncoroni, M.; Gardner, R.C. MET2 affects production of hydrogen sulfide during wine fermentation. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 7125–7135. [CrossRef] [PubMed]
27. Noble, J.; Sanchez, I.; Blondin, B. Identification of new Saccharomyces cerevisiae variants of the MET2 and SKP2 genes controlling the sulfur assimilation pathway and the production of undesirable sulfur compounds during alcoholic fermentation. *Microb. Cell Fact.* **2015**, *14*, 68. [CrossRef] [PubMed]
28. Cordente, A.G.; Heinrich, A.; Pretorius, I.S.; Swiegers, J.H. Isolation of sulfite reductase variants of a commercial wine yeast with significantly reduced hydrogen sulfide production. *FEMS Yeast Res.* **2009**, *9*, 446–459. [CrossRef]
29. Linderholm, A.L.; Findleton, C.L.; Kumar, G.; Hong, Y.; Bisson, L.F. Identification of genes affecting hydrogen sulfide formation in Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **2008**, *74*, 1418–1427. [CrossRef]
30. Linderholm, A.; Dietzel, K.; Hirst, M.; Bisson, L.F. Identification of MET10-932 and Characterization as an Allele Reducing Hydrogen Sulfide Formation in Wine Strains of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **2010**, *76*, 7699–7707. [CrossRef]
31. Peltier, E.; Friedrich, A.; Schacherer, J.; Marullo, P. Quantitative Trait Nucleotides Impacting the Technological Performances of Industrial Saccharomyces cerevisiae Strains. *Front. Genet.* **2019**, *10*, 683. [CrossRef]
32. Xu, Y.; Crouch, J.H. Marker-Assisted Selection in Plant Breeding: From Publications to Practice. *Crop Sci.* **2008**, *48*, 391–407. [CrossRef]
33. Dekkers, J.C.M. Marker-assisted selection for commercial crossbred performance1. *J. Anim. Sci.* **2007**, *85*, 2104–2114. [CrossRef]
34. Yao, F.-J.; Lu, L.-X.; Wang, P.; Fang, M.; Zhang, Y.-M.; Chen, Y.; Zhang, W.-T.; Kong, X.-H.; Lu, J.; Honda, Y. Development of a Molecular Marker for Fruiting Body Pattern in Auricularia auricula-judae. *Mycobiology* **2018**, *46*, 72–78. [CrossRef] [PubMed]
35. Dufour, M.; Zimmer, A.; Thibon, C.; Marullo, P. Enhancement of volatile thiol release of Saccharomyces cerevisiae strains using molecular breeding. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 5893–5905. [CrossRef] [PubMed]
36. Marullo, P.; Yvert, G.; Bely, M.; Aigle, M.; Dubourdieu, D. Efficient use of DNA molecular markers to construct industrial yeast strains. *FEMS Yeast Res.* **2007**, *7*, 1295–1306. [CrossRef] [PubMed]
37. Javelot, C.; Girard, P.; Colonna-Ceccaldi, B.; Vladescu, B. Introduction of terpene-producing ability in a wine strain of Saccharomyces cerevisiae. *J. Biotechnol.* **1991**, *21*, 239–251. [CrossRef]
38. Blondin, B.; Noble, J.; Sanchez, I. Méthode de Contrôle de la Production de Sulfites, D'hydrogène Sulfureux et D'acétaldéhyde par des Levures. European Patent EP2807247B1.
39. Mackay, T.F.C. The Genetic Architecture of Quantitative Traits. *Annu. Rev. Genet.* **2001**, *35*, 303–339. [CrossRef]
40. Lecomte, L.; Duffé, P.; Buret, M.; Servin, B.; Hospital, F.; Causse, M. Marker-assisted introgression of five QTLs controlling fruit quality traits into three tomato lines revealed interactions between QTLs and genetic backgrounds. *Theor. Appl. Genet.* **2004**, *109*, 658–668. [CrossRef] [PubMed]
41. Volschenk, H.; Viljoen, M.; Grobler, J.; Petzold, B.; Bauer, F.; Subden, R.E.; Young, R.A.; Lonvaud, A.; Denayrolles, M.; van Vuuren, H.J. Engineering pathways for malate degradation in Saccharomyces cerevisiae. *Nat. Biotechnol.* **1997**, *15*, 253–257. [CrossRef]
42. Redzepovic, S.; Orlic, S.; Majdak, A.; Kozina, B.; Volschenk, H.; Viljoen-Bloom, M. Differential malic acid degradation by selected strains of Saccharomyces during alcoholic fermentation. *Int. J. Food Microbiol.* **2003**, *83*, 49–61. [CrossRef]
43. Delcourt, F.; Taillandier, P.; Vidal, F.; Strehaiano, P. Influence of pH, malic acid and glucose concentrations on malic acid consumption by Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 321–324. [CrossRef]
44. Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A. *Handbook of Enology, Vol. 1: The Microbiology of Wine and Vinifications*, 2nd ed.; Wiley: Hoboken, NJ, USA, 2006; ISBN 9780470010341.
45. Volschenk, H.; van Vuuren, H.J.J.; Viljoen–Bloom, M. Malo-ethanolic fermentation in Saccharomyces and Schizosaccharomyces. *Curr. Genet.* **2003**, *43*, 379–391. [CrossRef] [PubMed]
46. Peltier, E.; Vion, C.; Abo Saada, O.; Friedrich, A.; Schacherer, J.; Marullo, P. Flor yeast rewire the central carbon metabolism during wine alcoholic fermentation. *bioRxiv* **2021**. [CrossRef]
47. Peltier, E.; Marullo, P.; Coulon, J. Levures Œnologiques Permettant le Contrôle de la Teneur en Acide des Vins et Méthodes de Sélection de Telles Levures. Patent FR 33390, 2020.
48. Peltier, E.; Sharma, V.; Raga, M.M.; Roncoroni, M.; Bernard, M.; Gibon, Y.; Marullo, P.; Jiranek, V.; Gibon, Y.; Marullo, P. Dissection of the molecular bases of genotype x environment interactions: A study of phenotypic plasticity of Saccharomyces cerevisiae in grape juices. *BMC Genom.* **2018**, *19*, 772. [CrossRef]
49. Chernova, M.; Albertin, W.; Durrens, P.; Guichoux, E.; Sherman, D.J.; Masneuf-Pomarede, I.; Marullo, P. Many interspecific chromosomal introgressions are highly prevalent in Holarctic Saccharomyces uvarum strains found in human-related fermentations. *Yeast* **2018**, *35*, 141–156. [CrossRef]
50. Gabriel, S.; Ziaugra, L.; Tabbaa, D. SNP genotyping using the sequenom massARRAY iPLEX Platform. *Curr. Protoc. Hum. Genet.* **2009**, *60*, 2.12. 1–2.12. 18. [CrossRef] [PubMed]

51. Den Dunnen, J.T.; Antonarakis, S.E. Mutation nomenclature extensions and suggestions to describe complex mutations: A discussion. *Hum. Mutat.* **2000**, *15*, 7–12. [CrossRef]
52. Peltier, E.; Bernard, M.; Trujillo, M.; Prodhomme, D.D.; Barbe, J.-C.; Gibon, Y.; Marullo, P. Wine yeast phenomics: A standardized fermentation method for assessing quantitative traits of Saccharomyces cerevisiae strains in enological conditions. *PLoS ONE* **2018**, *13*, e0190094. [CrossRef]
53. Stitt, M.; McC, L.R.; Gerhardt, R.; Heldt, H.W. Metabolite Levels in Specific Cells and Subcellular Compartements of Plant Leaves. *Methods Enzymol.* **1989**, *174*, 518–552.
54. R Development Core Team, R. R: A Language and Environment for Statistical Computing. *R Found. Stat. Comput.* **2011**, *1*, 409.
55. Taillandier, P.; Gilis, M.; Strehaiano, P.; Taillandier, P.; Gilis, M.; Strehaiano, P. Deacidification by Schizosaccharomyces: Interactions with Saccharomyces. *J. Biotechnol.* **1995**, *40*, 199–205. [CrossRef]
56. Yéramian, N.; Chaya, C.; Suárez Lepe, J.A. L-(-)-malic acid production by *Saccharomyces* spp. during the alcoholic fermentation of wine (1). *J. Agric. Food Chem.* **2007**, *55*, 912–919. [CrossRef] [PubMed]
57. Vilanova, M.; Ugliano, M.; Varela, C.; Siebert, T.; Pretorius, I.S.; Henschke, P.A. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by Saccharomyces cerevisiae wine yeasts. *Appl. Microbiol. Biotechnol.* **2007**, *77*, 145–157. [CrossRef]
58. Mortimer, R.K.; Romano, P.; Suzzi, G.; Polsinellif, M. Genome Renewal: A New Phenomenon Revealed From a Genetic Study of 43 Strains of Saccharomyces cerevisiae Derived From Natural Fermentation of Grape Musts. *YEAST* **1994**, *10*, 1543–1552. [CrossRef] [PubMed]
59. Giudici, P.; Solieri, L.; Pulvirenti, A.M.; Cassanelli, S. Strategies and perspectives for genetic improvement of wine yeasts. *Appl. Microbiol. Biotechnol.* **2005**, *66*, 622–628. [CrossRef] [PubMed]
60. Salmon, J. l-Malic-acid permeation in resting cells of anaerobically grown Saccharomyces cerevisiae. *Biochim. Biophys. Acta Biomembr.* **1987**, *901*, 30–34. [CrossRef]
61. Vailiant, H.; Formisyn, P.; Gerbaux, V. Malolactic fermentation of wine: Study of the influence of some physico-chemical factors by experimental design assays. *J. Appl. Bacteriol.* **1995**, *79*, 640–650. [CrossRef]
62. Versari, A.; Parpinello, G.P.; Cattaneo, M. Leuconostoc oenos and malolactic fermentation in wine: A review-Journal of Industrial Microbiology and Biotechnology. *J. Ind. Microbiol. Biotechnol.* **1999**, *23*, 447–455. [CrossRef]
63. Hospital, F.; Charcosset, A. Marker-assisted introgression of quantitative trait loci. *Genetics* **1997**, *147*, 1469–1485. [CrossRef]
64. Bouchez, A.A.; Hospital, F.F.; Causse, M.; Gallais, A.A.; Charcosset, A. Marker-assisted introgression of favorable alleles at quantitative trait loci between maize elite lines. *Genetics* **2002**, *162*, 1945–1959. [CrossRef]
65. Bloom, J.S.; Kotenko, I.; Sadhu, M.J.; Treusch, S.; Albert, F.W.; Kruglyak, L. Genetic interactions contribute less than additive effects to quantitative trait variation in yeast. *Nat. Commun.* **2015**, *6*, 8712. [CrossRef]
66. Steinmetz, L.M.; Sinha, H.; Richards, D.R.; Spiegelman, J.I.; Oefner, P.J.; McCusker, J.H.; Davis, R.W. Dissecting the architecture of a quantitative trait locus in yeast. *Nature* **2002**, *416*, 326–330. [CrossRef]
67. Sinha, H.; David, L.; Pascon, R.C.; Clauder-Münster, S.; Krishnakumar, S.; Nguyen, M.; Shi, G.; Dean, J.; Davis, R.W.; Oefner, P.J.; et al. Sequential Elimination of Major-Effect Contributors Identifies Additional Quantitative Trait Loci Conditioning High-Temperature Growth in Yeast. *Genetics* **2008**, *180*, 1661–1670. [CrossRef] [PubMed]
68. Liti, G.; Louis, E.J. Advances in Quantitative Trait Analysis in Yeast. *PLoS Genet.* **2012**, *8*, e1002912. [CrossRef] [PubMed]
69. Bonnett, D.G.; Rebetzke, G.J.; Spielmeyer, W. Strategies for efficient implementation of molecular markers in wheat breeding. *Mol. Breed.* **2005**, *15*, 75–85. [CrossRef]
70. Fatichenti, F.; Farris, G.A.; Deiana, P.; Ceccarelli, S. Malic acid production and consumption by selected of Saccharomyces cerevisiae under anaerobic and aerobic conditions. *Appl. Microbiol. Biotechnol.* **1984**, *19*, 427–429. [CrossRef]
71. Ramon-Portugal, F.; Seiller, I.; Taillandier, P.; Favarel, J.L.; Nepveu, F.; Strehaiano, P. Kinetics of Production and Consumption of Organic Acids during Alcoholic Fermentation by Saccharomyces cerevisae. *Food Technol. Biotechnol.* **1999**, *37*, 235–240.
72. Kunkee, R.E. Some roles of malic acid in the malolactic fermentation in wine making. *FEMS Microbiol. Lett.* **1991**, *88*, 55–71. [CrossRef]
73. Volschenk, H.; van Vuuren, H.J.J.; Viljoen-Bloom, M. Malic Acid in Wine: Origin, Function and Metabolism during Vinification. *S. Afr. J. Enol. Vitic.* **2006**, *27*. [CrossRef]
74. Jeppsson, H.; Yu, S.; Hahn-Hägerdal, B. Xylulose and glucose fermentation by Saccharomyces cerevisiae in chemostat culture. *Appl. Environ. Microbiol.* **1996**, *62*, 1705–1709. [CrossRef]

2. Préservation de l'acidité des vins par l'utilisation de souches acidifiantes

2.1. Contexte et problématique de l'étude

Le changement climatique impacte fortement la composition chimique des moûts de raisin. Comme évoqué dans la revue bibliographique, la hausse des températures entraine notamment une diminution de la concentration en acide malique dans les baies de raisin, poussant les vinificateurs à adopter de nouvelles stratégies pour préserver la fraîcheur de leurs vins. L'objectif de ce deuxième volet était de développer de nouvelles souches de *S. cerevisiae* produisant de l'acide malique pendant la fermentation alcoolique. Ce type de souche pourrait être intégré à des itinéraires de vinification en rouge associés à des cépages précoces, atteignant tôt leur maturité et étant de ce fait plus sensibles aux hausses des températures. C'est le cas pour les cépages traditionnellement utilisés dans le sud de la France tels que le Grenache et le Syrah mais également dans les vignobles espagnols (Tempranillo, Grenache, Monastrel), argentins (Malbec, Syrah, Cabernet sauvignon), chilien (Cabernet sauvignon, Merlot) ou encore australiens (Syrah, Cabernet sauvignon, Merlot). Ces vignobles de l'hémisphère sud sont déjà largement concernés par les problématiques liées au changement climatique. Pour cela, un programme de croisement basé sur une sélection phénotypique a été mis en place afin de préserver l'acidité des vins. La fréquence des allèles préservant l'acidité des vins dans de telles souches a été étudiée à postériori permettant de valider l'impact de certaines formes alléliques dans plusieurs fonds génétiques. Les principaux résultats obtenus au cours de ce travail d'amélioration génétique sont présentés dans l'article 2 intitulé « *New malic acid producer strains of Saccharomyces cerevisiae for preserving wine acidity during alcoholic fermentation* » paru en décembre 2022 dans *Food Microbiology*

2.2. Principaux résultats

Cette sous-partie fait l'objet d'un article scientifique. Le design des expériences a été réalisé par Philippe Marullo (PM) et moi-même. Les expériences de sporulation, croisement et isolation des clones, extraction d'ADN et génotypage ont été réalisées par Valentine Fautré (stagiaire de Master 2 encadrée par PM), Margaux Bernard (MB) et Maitena Muro (MM). J'ai pour ma part participé aux expériences de phénotypage pas fermentation en petit volume, dosages enzymatiques, micro-vinifications, mise en place des séances d'analyse sensorielle avec l'aide Bruce Richard pendant son stage de Master 2. J'ai également pris en charge le traitement statistique des données avec l'aide de PM. Enfin, l'article a été rédigé par PM et moi-même et revu par Sophie Tempère pour la partie analyse sensorielle.

Food Microbiology 112 (2023) 104209



Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm





New malic acid producer strains of *Saccharomyces cerevisiae* for preserving wine acidity during alcoholic fermentation

Charlotte Vion [a,b,1], Maitena Muro [a,b,1], Margaux Bernard [a,b], Bruce Richard [b], Fautre Valentine [b], Nadine Yeramian [c], Isabelle Masneuf-Pomarède [b], Sophie Tempère [b], Philippe Marullo [a,b,*]

[a] Biolaffort, Bordeaux, France
[b] UMR 1366 Œnologie, Université de Bordeaux, INRAE, Bordeaux INP, BSA, ISVV, France
[c] Microbiology Division, Department of Biotechnology and Food Science, Faculty of Science-University of Burgos, France

ARTICLE INFO

keywords:
pH
Malic acid
Breeding
Acidity perception
Wine yeast

ABSTRACT

In the context of climate change, the chemical composition of wines is characterized by a massive drop of malic acid concentration in grape berries. Then wine professionals have to find out physical and/or microbiological solutions to manage wine acidity. The aim of this study is to develop wine *Saccharomyces cerevisiae* strains able to produce significant amount of malic acid during the alcoholic fermentation. By applying a large phenotypic survey in small scale fermentations, the production level of malic acid in seven grape juices confirmed the importance of the grape juice in the production of malic acid during the alcoholic fermentation. Beside the grape juice effect, our results demonstrated that extreme individuals able to produce up to 3 g/L of malic acid can be selected by crossing together appropriate parental strains. A multivariate analysis of the dataset generated illustrate that the initial the amount of malic acid produced by yeast is a determining exogenous factor for controlling the final pH of wine. Interestingly most of the acidifying strains selected are particularly enriched in alleles that have been previously reported for increasing the level of malic acid at the end of the alcoholic fermentation. A small set of acidifying strains were compared with strains able to consume a large amount of malic acid previously selected. The total acidity of resulting wines was statistically different and a panelist of 28 judges was able to discriminate the two groups of strains during a free sorting task analysis.

1. Introduction

Climate change is a direct consequence of the global warming and accentuates the greenhouse effect with a drastic impact for many agricultural productions including vine (IPCC 2020; van Leeuwen and Darriet 2016). It is well documented that temperature increase modifies vine phenology (van Leeuwen and Darriet 2016) and affects grape juice composition by increasing sugar concentration (Coombe and Kliewer, 1987; Poudel et al., 2009; Nistor et al., 2018), and decreasing titratable acidity (Arrizabalaga et al., 2018; Coombe and Kliewer, 1987; Mira de Orduña, 2010; Nistor et al., 2018; Poudel et al., 2009; van Leeuwen and Darriet, 2016) due to a drastic drop of malic acid concentrations. In turn, the resulting wines have a greater alcohol content (Mira de Orduña 2010; van Leeuwen and Darriet 2016) and are also affected in their aroma composition and their sensorial properties (Bureau et al., 2000).

These combined modifications constitute a great challenge for the wine industry that have to adapt winemaking processes and enological products for maintaining wine quality and stability despite the drastic changes of grape juice composition. Concerning the correction of acidity drop, the addition of chemical products (tartaric or malic acids) is efficient but represents an expensive solution. In addition, the context of a new green deal promotes microbiological and biotechnological solutions as a good alternative for wine acidification.

Alcoholic and malolactic fermentations modify the equilibrium of organic acids by the metabolic activity of wine microorganisms as reported by many authors (Redzepovic et al., 2003; Volschenk et al., 2006; Su et al., 2014). However, wine microorganisms mostly reduce the wine acidity by consuming malic acid through malo-alcoholic (Volschenk

* Corresponding author. UMR 1366 Œnologie, Université de Bordeaux, INRAE, Bordeaux INP, BSA, ISVV, 210 chemin de Leysotte, 33882, Villenave d'Ornon, France.
E-mail address: philippe.marullo@u-bordeaux.fr (P. Marullo).
[1] Authors contributes to the same level to this publication.

https://doi.org/10.1016/j.fm.2022.104209
Received 30 August 2022; Received in revised form 7 December 2022; Accepted 14 December 2022
Available online 14 December 2022
0740-0020/© 2022 Elsevier Ltd. All rights reserved.

et al., 1997) and malolactic fermentation (Lonvaud-Funel, 1994). Quite recently, the species *Lachancea thermotolerans* has been proposed for correcting wine acidity due to its ability to produce significant amount of L-lactic acid during the alcoholic fermentation (Hranilovic et al., 2018, 2021). This non-conventional yeast species can be mixed with *Saccharomyces cerevisiae* starters and constitutes a promising solution for managing wine acidity. However, the development of mixed culture in winemaking conditions requires an accurate management of alcoholic fermentation which is sometime not compatible with a routine application in the cellar.

Beside L. *thermotolerans,* studies reporting a microbiological acidification of wine matrix without an elevation of acetic acid are quite rare. Within the *Saccharomyces* genus the species *Saccharomyces uvarum* has been described for its acidification properties (Castellari et al., 1994; Coloretti et al., 2002) that was related to the psychrophilic property of this species. This feature is mostly shared by hybrids between *S. cerevisiae* and *S. uvarum* (Origone et al., 2018) that have been proposed as a solution for coping both acidity drop and high sugar levels in grape juices (da Silva et al., 2015; Origone et al., 2018). Concerning *S. cerevisiae*, former studies described the contribution of strains on malic acid production.

Malic acid plays a central role in yeast metabolism being a key molecule of glyoxylate shunt (Regev-rudzki et al., 2009) and tricarboxylic acid cycle as extensively reviewed by (Saayman and Viljoen-Bloom, 2006). Schwartz and Radler deeply investigated the physicochemical factors influencing the production of malic acid by *S. cerevisiae* reporting production levels of nearly 1 g/L in enological compatible conditions (Schwartz and Radler, 1988). A large screening of natural isolate reported that few strains isolated in Spain or in China are able to produce up to 1 g/L of malic acid (Yéramian et al., 2007) and to increase titratable acidity (Chen et al., 2022). However, this interesting feature has not been deeply exploited.

Recently, the identification of QTLs (Quantitative Trait Loci) influencing malic acid metabolism of *S. cerevisiae* during the alcoholic fermentation (Peltier et al., 2021) paves new avenues for the control of acidity using the main species used in alcoholic fermentation. Recently, we demonstrated that such QTLs can be efficiently used for enhancing the consumption of malic acid during the alcoholic fermentation. Starting from two F1 hybrids obtained from 4 enological strains we drastically increased the percentage of malic acid consumed by enological strain in two rounds of selection (Vion et al., 2021). This strategy could be useful for shortening malolactic fermentation by reducing the amount of malic acid to be degraded by lactic bacteria. However, the use of high malic consumer strains reduces wine acidity which is not suitable in the context of global climate change.

In the present study, we applied a phenotype driven breeding program aiming to select *S. cerevisiae* strains able to preserve the wine acidity. Starting from the parental strains used by Vion et al. (2021) new strains able to produce up to 3.2 g/L of malic acid at the end of the alcoholic fermentation were obtained. These high malic producer strains are statistically enriched in QTL's alleles that preserve wine acidity constituting a new genetic resource for managing acidity in wine. A preliminary comparison of malic acid producer and consumer strains was carried out, the resulting wines can be clearly discriminated on the basis of the acidity perception in a *Sauvignon blanc* matrix.

2. Methods

2.1. Yeast strains, conservation, and propagation methods used

Yeast (*S. cerevisiae*) were propagated on YPD 2% (1% peptone, 1% yeast extract, 2% glucose) at 28 °C in both liquid and plate cultures (2% agar). Long term storage at −80 °C was achieved by adding one volume of glycerol to YPD 2% overnight cultures. All the strains are homozygous for the *HO* locus and are therefore diploids. The main yeast strains used were described in Table 1 and are derived from the two F1-hybrids (M2 × F15 and SB × GN) used in previous QTL mapping studies (Huang et al., 2014; Peltier et al., 2018b). Meiotic spore clones of SB × GN (GS-56 B, GS-13C, GS-8B) and of M2 × F15 (FM-8B, FM-1C, FM-3C) were used as parental candidates for achieving a breeding program aiming to increase the production of malic acid during the alcoholic fermentation. These strains have been previously characterized for their ability to produce small amounts of malic acid (Vion et al., 2021). A first F1-hybrid was obtained by crossing the strains GS-8b and FM-1C. A second hybridization cycle was achieved using the monosporic clones AC1-13, AC1-46, AC1-191, AC1-217. Finally, three *S. cerevisiae* strains C1-4, M2-9, and M6-7 previously described to produce mild amount of malic acid during the alcoholic fermentation (Yéramian et al., 2007) were used as control.

Table 1
S. cerevisiae strains used.

Strain	Description	Reference
ZF15	Zymaflore F15	Laffort, FRANCE
ZF10	Zymaflore Fx10	Laffort, FRANCE
M2	parental strain, a meiotic spore clone from Enoferm M2 (Lallemand, Canada)	Huang et al. (2014)
F15	parental strain, a meiotic spore clone from Zymaflore F15 (Laffort, France)	Huang et al. (2014)
GN	parental strain, a meiotic spore clone from Zymaflore VL1 (Laffort, France)	(Peltier et al., 2018b)
SB	parental strain, a meiotic spore clone from Zymaflore BO213 (Laffort, France)	(Peltier et al., 2018b)
M2xF15	F1-hybrid (M2 × F15)	Huang et al. (2014)
SBxGN	F1-hybrid (SB × GN)	(Peltier et al., 2018b)
C4-2	Yeast strain isolated from Cordoba region, mild malic acid producer	Yéramian et al. (2007)
M2-9	Yeast strain isolated from Madrid region, mild malic acid producer	Yéramian et al. (2007)
M6-7	Yeast strain isolated from Madrid region, mild malic acid producer	Yéramian et al. (2007)
GS-56 B	meiotic clone of SB × GN, mild malic acid producer	Vion et al. (2021)
GS-13C	meiotic clone of SB × GN, mild malic acid producer	Vion et al. (2021)
GS-8B	meiotic clone of SB × GN, mild malic acid producer	Vion et al. (2021)
FM-8B	meiotic clone of M2 × F15, mild malic acid producer	Vion et al. (2021)
FM-1C	meiotic clone of M2 × F15, mild malic acid producer	Vion et al. (2021)
FM-3C	meiotic clone of M2 × F15, mild malic acid producer	Vion et al. (2021)
AC1	F1-hybrid (GS-8b x FM-1C)	this work
AC1-191	meiotic clone of AC1, malic acid producer strain	this work
AC1-13	meiotic clone of AC1, high malic acid producer	this work
AC1-217	meiotic clone of AC1, high malic acid producer	this work
AC1-46	meiotic clone of AC1, high malic acid producer	this work
AC2	F1-hybrid (AC1-191 × AC1-46)	this work
AC3	F1-hybrid (AC1-13 × AC1-217)	this work
AC2-1	meiotic clone of AC2, high malic acid producer	this work
AC2-467	meiotic clone of AC2, high malic acid producer	this work
AC3-22	meiotic clone of AC3, high malic acid producer	this work
FMGS_889	meiotic clone of FMGS, high malic consumer	Vion et al. (2021)
FMGS_71	meiotic clone of FMGS, high malic consumer	Vion et al. (2021)
FMGS_263	meiotic clone of FMGS, high malic consumer	Vion et al. (2021)
FMGS2_107	meiotic clone of FMGS2, high malic consumer	Vion et al. (2021)
FMGS2_714	meiotic clone of FMGS2, high malic consumer	Vion et al. (2021)

2.2. Spore mating and isolation

F1-hybrids (AC1, AC2 and AC3) were obtained by spore to spore mating on YPD 2% using the method previously described (Marullo

et al., 2009). After few hours, zygotes (trilobed shaped) were observed and manually isolated; their genotype was confirmed by a SNP genotyping (see below). Each F1-hybrids was sporulated by cultivating 10^8 cells in 5-ml of potassium acetate 1% (ACK) during 3 day at 24 °C. After a digestion by cytohelicase (2 mg/L) (Sigma, France) spores were isolated by micromanipulation or by spore purification according to the procedure previously described (Vion et al., 2021). As the yeast strains of this study are homozygous for the *HO* gene, colonies derived form a spore are considered as diploids and fully homozygous.

2.3. Cell culture and DNA extraction in microplates

Strains were cultivated in 96-Well microplate containing 200 μL of YPD coated with gaz-permeable sheets allowing CO_2 production. Genomic DNA was extract by using a Li–Ac SDS protocol previously adapted for microplate handling (Chernova et al., 2018). Broadly, 5×10^6 cells (200 μL of overnight cultures) were centrifuged and incubated with 50 μL of 200 mM LiAc/1% SDS at 70 °C for 5 min. Genomic DNA was extracted by mixing cell lysates with 150 μL of pure ethanol and vortexed for 15 s. After a briefly spin (2 min, 4000 rpm) the pellet was washed with 70% ethanol and was the solubilized in 200 μL of milliQ water at 60 °C for 5 min.

2.4. Mass ARRAY genotyping of selected clones

According to a recent publication (Peltier et al., 2021), QTLs that possibly enhance the malic acid production in the SB × GN background were used. These QTLs have the same localization than those used by Vion et al. for enhancing the consumption of malic acid (Vion et al., 2021). For optimizing the genotyping procedure, the allelic form tracked in the present study were specific to a malic consumption feature. In addition, for most QTLs a pair of SNPs narrowing the region was used in order to increase the quality of the genotyping. The genotypes of each strain founder (M2, F15, SB, GN) and of the parental strains of hybrids AC1, AC2, and AC3 are given in Table S1. The genotyping of these SNP was carried out by using Mass ARRAY technology, which allows large multiplex SNP detection (Gabriel et al., 2009). Less than 15 ng of DNA were used for genotyping the strain. Primers were design using the tool MassARRAY® Assay Design (version 4.0.0.2) and amplified fragment of less than 120 bases having a mass difference between 4545.0 and 8633.7 Da. The genotype of selected progenies of hybrids AC1 (37), AC2 (28) and AC3 (29) was carried out in the same multiplex. All the spore clones genotyped have a fully homozygous genotype consistent with their meiotic progeny nature (Table S2).

2.5. Alcoholic fermentation assays

2.5.1. Grape juices

The grape juices Marullo et al., 2019 and Marullo et al., 2019 (M13 and M18) and Chernova et al., 2018, 2014, 2015, 2017 and 2019 (CS13, CS14, CS15, CS27 and CS19) used were provided by Vignobles Ducourt (Ladaux, France) and stored at −20 °C. Red grape juices have been thermically treated by the cellar and only the liquid phase was used. Grape juices were sterilized by membrane filtration and their nitrogen content were adjusted to 200 mg/L N in order to provide a sufficient nitrogen nutrition. The composition of grape juice in terms of fermenting sugars, nitrogen sources and malic acid content as well their pH are listed in Table S3 for original and corrected musts.

2.5.2. Small scale fermentation monitoring

Small-volume alcoholic fermentations were implemented in screwed vials fermentations according to the general procedure described in (Peltier et al., 2018a). Briefly, 20 ml-screwed vials (Fisher Scientific, Hampton, New Hampshire, USA) were filled with 12.0 ml of filtered grape must were tightly closed with screw cap-magnetic (Fisher Scientific, Hampton, New Hampshire, USA) perforated by hypodermic needles for allowing the CO_2 release. Vessel was inoculated by 2×10^6 viable cell. mL^{-1} precultured in liquid YPD for 24 h. Cellular concentration and viability was estimated by flow cytometry using a Cytoflex apparatus (Beckmann Coulter, Indianapolis, USA). Fermentation took place at 24 °C and was each vial shaken at 175 rpm by an orbital shaker (SSL1, Stuart, Vernon Hills, Illinois, USA). The fermentation kinetics was estimated by monitoring manually (2–3 times per day) the weight loss caused by CO_2 release using a precision balance with automatic weight recording (Mettler Toledo, Greifensee, Switzerland). The amount of CO_2 released according to time was modeled by local polynomial regression fit allowing the estimation of parameters related to fermentation (Peltier et al., 2018a): the maximal amount of CO_2 released (CO_2max in $g.L^{-1}$), the lag phase (h), the time to release 35 and 80 g/L of CO_2 after subtracting the lag phase (*t35* and *t80* in h). The CO_2max value was divided by the amount of sugar consumed for computing the *yield* of the fermentation.

2.6. Enzymatic assay of wine

At the end of the alcoholic fermentation, a sample volume of 800 μL was manually transferred in Micronics tubes (Novazine, Lyon, France, ref: MP32033L) and stored at −20 °C. The concentrations of the following organic metabolites were measured: acetic acid, glycerol, malic acid, pyruvate, acetaldehyde and total SO_2 using the respective enzymatic kits: K-ACETGK, K-GCROLGK, K-LMAL-116 A, K-PYRUV, K-ACHYD, K-TSULPH, K–SUCCI (Megazyme, Bray, Ireland) following the instructions of the manufacturer. Glucose and fructose were assayed by using the enzymatic method previously described. All the enzymatic assays were performed by a robotic platform using the Bordeaux metabolomics facilities (http://metabolome. cgfb. u-bordeaux.fr/). The Malic Acid Consumption (MAC%) is the ratio of malic acid consumed and was computed according to the following formula:

$$MAC\% = \frac{\left([\mathrm{L-malic\ acid}]_{initial} - [\mathrm{L-malic\ acid}]_{final}\right)}{[\mathrm{L-malic\ acid}]_{initial}} \times 100$$

2.7. Sensory analysis

2.7.1. Wines preparation

Twelves strains were used for assessing the sensorial impact of malic acid production/consumption on a Sauvignon Blanc wine. The wine was produced at the laboratory scale from a Sauvignon blanc grape juice harvested in 2020 in the Bordeaux area (Vignobles Ducourt, Ladaux, FRANCE) containing 35 mg/L of total SO_2. The composition of this grape juice is given in Table S3. Fermentations were carried out in 1.2 L bioreactors initially incubated at 18 °C. At the fifth day of fermentation, 30 mg N/L of nitrogen (Ammonium sulfate) were added for enhancing the fermentation kinetics. At the seventh day, the temperature was rise up 22 °C for ensuring a rapid achievement of the fermentation. Bioreactors were shaken daily for 60 s with a magnetic stirrer in order to resuspend yeast cells and the CO_2 production rate was followed by manual weighting. At the end of the fermentation (between 11 and 13 days), wines were settled for a week at 4 °C and yeast lees were resuspended each 48 h for preserving wines from oxidation. A week before tasting wines were sulfited (50 mg/L) and transferred in tapped bottles and stored at 12 °C.

2.7.2. Sensory analysis conditions

Sample assessments were undertaken in a temperature-controlled room, using covered, white ISO glasses (ISO 3591:1977) containing about 25 ml of liquid, coded with random three-digit numbers. Sessions lasted approximately 15 min. Judges were selected on the basis of availability and interest. A total of 28 volunteers belonging to the Laffort and ISVV staff and showed different levels of expertise in sensory analysis but representing different domains or expertise of the wine

industry. The ages of the panelists ranged from 25 to 50 years old, and the panel was constituted by 55% of women.

2.7.3. Preliminary control of acidity perception by the panel

A preliminary session was carried out using a commercial *Sauvignon blanc* wine (Marullo et al., 2019, Languedoc Roussillon) with spiked concentrations of malic acid. The aim of this preliminary session was to control that all the judges correctly perceive malic acid contribution in a Sauvignon blanc wine. The panelists performed a ranking test (ISO 8587:2007), sorting four samples according to their acidity from least to most intense. The sums of ranks for increasing concentrations of malic acid (1.0, 1.7, 2.4 and 3.0 g/L) were 43, 60, 66, and 81, respectively. A Page test (Conover, 1999) assuming the increasing concentration of malic acid in wine confirmed that judges have a correct perception of acidity.

2.7.4. Free sorting task

Two days after the control session a descriptive sorting task (Chollet et al., 2014) was used to characterize the olfactory and gustatory differences between 12 Sauvignon Blanc wines produce in the laboratory. The protocol of the sorting focused on both a full evaluation (olfactory and gustatory). The panelists (n = 28) were asked to constitute groups of wines according to their acidity. Judges must constitute at least two groups of wines and each group must contain at least two wines.

2.8. Use of former datasets for figures

Two previously published datasets were used for illustrating the efficiency of this breeding programs. In such dataset phenotypes were measured in a Marullo et al., 2019 grape juice in the same conditions and in the same grape juices allowing to compare the data. Malic acid consumption of 35 enological strains and 193 progeny clones of M2 × F15 or SB × GN hybrids were extracted form (Emilien Peltier, Bernard, et al., 2018) and (Vion et al., 2021). These distributions were presented in Fig. S1.

2.9. Statistical analyses

All the statistical and graphical analyses were done using R software (R Core Team, 2018). Median differences were assayed using the non-parametric Kruskal test (*agricolae package*). When required, multiple comparisons were achieved using a post-hoc test using the criterium Fisher's least significant difference with Benjamini-Hochberg corrected p values (alpha = 0.05).

The effect of grape juice and strains on MAC% was estimated by a two-way analysis of variance (*car* package) using the data collected for the strains SB, GN, AC2 and AC3 in the grape juices CS14 and CS15. The linear model used is expressed in the following formula

$$MAC\% = \mu + Strain_i * Must_j + \varepsilon$$

Where μ is the average value observed in the dataset, *Strain* is the nature of the strain taking four levels *i*, *Must* is the nature of the grape juice taking two levels *j* and ε is the residual error of the model. The analysis of variance was carried out after checking the normal distribution of the residues of the model (Shapiro test) and the homoscedasticity of the variance (Levene test).

To evaluate the contribution of different metabolites on final pH of wine a multiple linear regression model was carried out. The initial model computed is expressed in the following formula.

$$pH_{wine} \sim pH_{must} + [malic\ acid]_{wine} + [acetic\ acid]_{wine} + [succinic\ acid]_{wine} + [residual\ sugar]_{wine} + \varepsilon$$

Where ε represents the error of the model. A subsequent analysis of variance of the model was applied in order to estimate which variables have a significative impact on the total variance observed.

Page test was carried out for controlling the correct perception of acid malic in a spiked Sauvignon blanc. The L value obtained for 28 judges was 685 with four wines presented. The threshold L′ value was equal to 4.15 allowing to reject the null hypothesis. L' is computed assuming the normal distribution of the samples according to the following formula:

$$L' = \frac{12 \times L - 3 \times J \times P(P+1)^2}{P(P+1) \times \sqrt{J \times (P-1)}}$$

where *J* is the number of judges (n = 28) and *P* is the number of wines presented (n = 4). Multidimensional scaling was assed using the R package *smacof* (de Leeuw and Mair, 2009) Dissimilarities between samples were analyzed using the cooccurrence of wines belonging to the same group as proposed by (Cox and Cox, 2001).

3. Results

3.1. Phenotypic characterization of parental candidates

The aim of the present work was to increase the production level of malic acid by *S. cerevisiae* strains in order to prevent de drastic drop of acidity caused by climatic change.

Six monosporic clones (GS-56 B, GS-13C, GS-8B, FM-8B, FM-1C, FM-3C) constitutes the starting genetic material of this study. They were obtained from the F1-hybrids M2 × F15 and SB × GN. Their MAC% in a Merlot grape juice of 2015 was previously reported (Vion et al., 2021). These strains outcompeted a panel of 35 commercial starters and some of them had a weak production of malic acid at the end of the alcoholic fermentation (Fig. S1). In order to better characterized such strains, the amount of malic acid at the end of the alcoholic fermentation of seven red grape juices characterized by a wide range of initial malic acid content (1.45–4.40 g/L) was measured. The fermentation characteristics of such strains were compared to some reference ones including two commercial starters (Zymaflore Fx10 and Zymaflore F15), three malic acid producer strains (C1-4, M2-9 and M6-7) (Yéramian et al., 2007) and the strain FMGS_889 which is a high malic acid consumer strains selected by (Vion et al., 2021). Fermentations were carried out in duplicate, and traits collected are given in Table S4.

In order to compare all the conditions, the MAC% was computed as described in material and methods. Average MAC% values measured in the seven grape juices was significantly different according to the yeast strain (Kruskal test, pval = 3.7×10^{-9}). A Post-hoc test using the criterium Fisher's LSD revealed that FM-8B and FMGS-889 were statistically different to two commercial Fx10 and F15 (Fig. 1A). However, most of the acidifier strains cannot be statistically differentiated due to a strong variability of MAC% observed within the grape juice fermented. This significative effect of grape juice is confirmed by Kruskal test, p = 9.3×10^{-14} and subsequent post hoc analysis (Fig. 1B). The impact of grape juice was clearly correlated to the initial concentration of malic acid (Spearman's correlation test: rho = 0.60, pval$<2.2 \times 10^{-16}$). Interestingly, for low initial malic acid concentrations (1.41 g/L in M-2018 and 2.02 in CS-2014) average MAC% reaches negative values (malic acid production) suggesting that such matrices are relevant for the selection of acid malic producing strains.

In order to better characterize the parental candidates, we narrowed our analysis on these two grape juices. Table 2 provides the average phenotypic values measured for 13 quantitative traits in M-2018 and CS-2014 with the attribution of statistical groups (Kruskal test with *post hoc* group attribution. The average amount of malic acid at the end of the alcoholic fermentation ranged between 1 g/L and 2.6 g/L for the extreme strains FMGS-889 and GS-8B. Significative differences between strains were also observed for other organic acids such as acetate but were not correlated to the production of malic acid (Fig. S2).

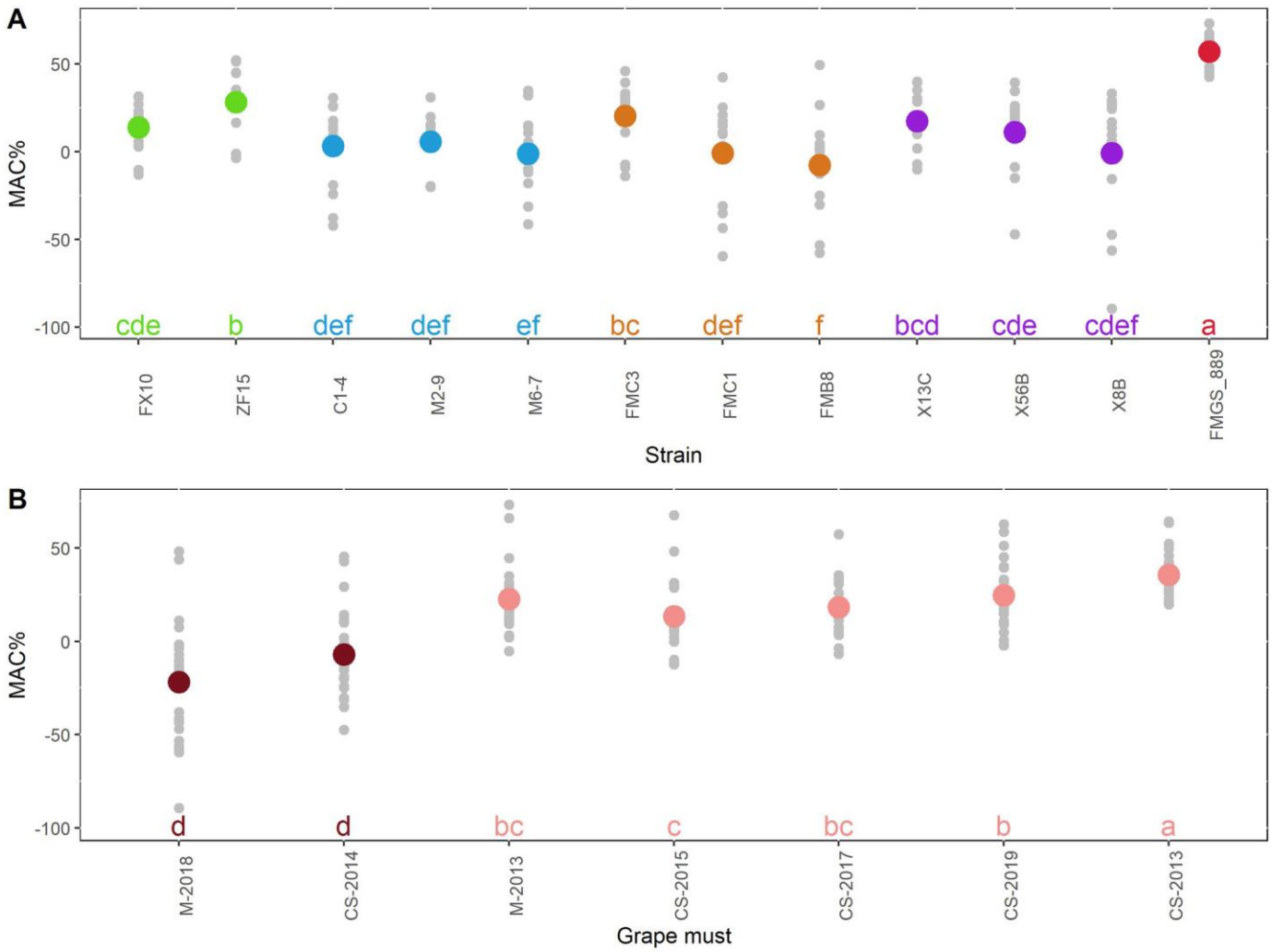


Fig. 1. Phenotypic characterization of acidifying strains in seven red grape juices.
Panel A. The dot plot indicates the distribution of MAC% value measured for 12 strains in seven grape juices (2 replicates per grape juice). Strains were colored according to their origin. Green = industrial starters; light blue = malic producer strains isolated in Spain, orange M2xF15 progenies, purple = SBxGN progenies, red = malic consumer strain. The letters below the dot plot indicate post hoc significative differences between strains (Fisher's least significant difference). Groups that do not share a common letters can be considered as statistically different (Kruskal test, alpha = 0.05).
Panel B. The dot plot indicates the distribution of MAC% value measured for 12 strains in seven grape juice (2 replicates per grape juice). Grape juices were sorted according to their initial pH values. Negative MAC% values (indicating a malic acid production) were obtained in only two grape must colored in burgundy color. The letters below the dot plot indicate post hoc significative differences between strains (Fisher's least significant difference). Groups that do not share a common letters can be considered as statistically different (Kruskal test, alpha = 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Phenotypic optimization of malic acid production by a breeding program

The two best malic acid producer strains GS-8B and FM-1C were crossed by pairing their spores using a micromanipulator and the F1-hybrid AC1 was isolated. Forty-one progeny clones of the AC1 hybrid were recovered and analyzed for their MAC% in the CS14 grape juice that positively promotes malic acid production (Fig. 1B). The AC1 hybrid showed a negative MAC% value similar to those of parental strains (−74.2%) (Fig. 2A). In this grape juice this MAC% value represented a final malic acid production of 1.5 g/L. The hybrid AC1 was sporulated and 41 progeny clones were isolated. All of them reached negative MAC% ranging between −23.7% and −146.8%. Four strains AC1-13, AC1-191, AC1-46 and AC1-217 were selected for achieving a second round of phenotypic optimization. The hybrid AC2 (AC1-191 × AC1-46) and AC3 (AC1-13 × AC1-217) were isolated by using the micromanipulator. The MAC% of these new F1-hybrids was clearly improved respect to AC1 with values of −130.7% and −149% for AC2 and AC3, respectively. To our knowledge, such levels of malic acid production (up to 3.0 g/L) were never reported in an enological context and constitute a technological rupture compared to the phenotypic variability of industrial starters that was illustrated by the four founder strains of this study (SB, GN, M2 and F15) (Fig. 2A).

The phenotypic segregation of MAC% of AC2 and AC3 hybrids was measured for a small set of progenies (AC2 = 14, AC3 = 19) and compared to the 41 AC1 progenies (Fig. 2B). Both populations showed an average MAC% significantly lower than the AC1 population (Wilcoxon test, alpha = 0.05). The phenotypic variance of each progeny was statistically similar (Variance F-test, alpha = 0.05). For AC1 progeny, 70% of clones had a MAC% value higher than the F1-hybrid while for AC2 and AC3 the distribution was mostly centered around the respective F1-hybrid value. This second segregation step allowed the selection of strains with a −160% lower MAC% representing a production of malic acid of 3.2 g/L.

Table 2
Average phenotypic values of initial acidifying strains.

Strain	MAC%	pH wine	Acetic acid (g.L-1)	Malic acid (g.L-1)	Glycerol (g.L-1)	SO2 (mg.L-1)	Succinic acid (g.L-1)	CO_2 max (g/L)	lp (h)	V50-80 (g.h-1.L-1)	t35 g (h)	t50 g (h)	t80 g (h)
GS-8B	−52.1 f	3.6	0.10 d	2.6 a	9.2 a	8.6	1.1abc	107.4	8.0	0.91 de	38.5 bc	55.3 abc	109.8 ab
FM-1C	−42.2 f	3.6	0.20 abcd	2.5 ab	8.9 ab	7.2	1.5a	110.1	6.5	0.93 de	38.8 bc	55.3 bc	106.8 ab
FM-8B	−41.5 f	3.6	0.20abcd	2.4 ab	8.2 abcd	6.1	1.1abc	110.8	7.3	1.13 bc	38.0 bc	53.0 bcd	96.0 bc
C1-4	−30.7 ef	3.7	0.13 bcd	2.3 abc	6.5 d	6.8	1.1abc	110.0	6.8	1.05 cd	34.8 cd	50.3 cde	96.8 b
M6-7	−24.1 def	3.7	0.18 bcd	2.1 abc	6.8 cd	4.8	1.4ab	110.9	6.3	0.81 e	45.3 a	68.0 a	132.8 a
GS-56 B	−14.9 bcde	3.7	0.14 abc	2.0 bcd	7.3 bcd	8.9	0.8c	110.4	7.0	0.85 e	41.8 ab	59.0 ab	116.5 a
M2-9	−13.0 cde	3.7	0.23 ab	2.0 abcd	7.3 abcd	7.5	1.0abc	110.2	6.3	0.85 e	40.8 ab	58.8 abc	116.5 a
FM-C3	−4.7 bcd	3.7	0.27 cd	1.8 cd	8.7 abc	10.9	1.4ab	111.3	7.0	1.32 ab	32.5 d	45.5 def	80.8 cd
GS-13C	−1.3 abc	3.7	0.12 a	1.7 cde	6.1 d	5.2	0.9bc	112.0	7.3	0.95 cde	39.3 bc	57.0 abc	108.5 ab
Zymaflore FX10	0.6 abc	3.8	0.31 abc	1.7 cde	7.3abcd	8.6	0.8bc	111.8	8.8	1.48 a	29.3 d	41.0 f	73.8 d
Zymaflore F15	5.7 ab	3.8	0.23 a	1.6 de	6.1 abcd	5.7	1.3abc	112.0	8.0	1.46 a	31.3 d	44.3 ef	77.0 d
FMGS-889	45.0 a	4.0	0.19 abcd	1.0 e	8.0 abcd	8.8	1.2abc	113.7	9.0	1.55 a	32.0 d	45.3 def	77.8 d

In order to confirm these findings, MAC% was assayed in a second grape juice (CS15) using additional progenies of AC2 (n = 26) and AC3 (n = 22). In this grape juice, AC2 and AC3 hybrids as well as the control strains GN and SB produced less malic acid since their MAC% was significantly higher than in CS14 grape juice. This effect was quantified by two-way ANOVA (Fig. S3) underlining the impact of the grape juice discussed above. Despite this environmental effect a similar phenotypic segregation was observed for both hybrids and some progenies produced more malic acid than their respective hybrids (Fig. 2C).

3.3. Multivariate analysis of acidic strains behavior

The large dataset of 484 fermentations characterized by 15 quantitative variables was generated (Table S5) offering the opportunity to investigate traits linked to the strong production of malic acid of acidic strains. This represents the phenotypic values of 127 strains phenotyped in CS14 and/or in CS15 at least in triplicate. The phenotypic variability of this dataset was figured out in a PCA analysis (Fig. 3A). The first two components represent 60% of the total inertia and clearly discriminate the hybrid background and the grape juice origin. Correlation circles indicates that the content of malic acid drives the inertia in the same direction that t50 and t80 but was mostly decoupled to succinic acid, glycerol, CO_2, and acetic acid production (Fig. 3B). Correlation analysis (Fig. S4) confirmed the strong positive correlation between kinetics parameters and malic acid content suggesting that acidifying strains have some difficulties to ferment efficiently.

This was also corroborated by the strong correlation between malic acid production and residual sugars (rho = 0.45, pvalue = 2×10^{-26}) that are highlighted by the 123 stuck fermentations (open symbol) shown on Fig. 3A. The distribution of malic acid produced in completed and stuck fermentations was figured out Fig. 5. Stuck fermentations mostly occurred in the CS15 grape juice which is characterized by a lower CO_2 yield than observed in CS14 (0.427 vs 0.456 g/g, Wilcoxon test, alpha = 0.05). In CS15, the production level of succinic acid also significantly higher than observed in CS14 (1.61 vs 0.52 g/L, Wilcoxon test, alpha = 0.05) contributing to separate the two grape juices on the PCA. As expected, the pH value of the resulting wine was negatively correlated with malic acid content in both grape juice (rho = −0.40 and −0.69 for CS14 and CS15, respectively, alpha = 0.001) (Fig. 3C). This demonstrates that the selection of strains controlling malic acid concentration was quite efficient for modulating the pH. On average, the dynamic range of final pH of wine was 3.41–3.82 for CS14 and 3.52–4.01 for CS15.

Since other organic acids than malate would influence the wine acidity, we applied a linear model for predicting the quantitative factors statistically linked to the final pH. Five quantitative parameters were used for building the model: the initial pH of the matrix, the organic acids produced by yeast (malic, acetic, succinic) as well as the residual sugar in the grape juice were interrogated. Except succinic acid, all the parameters significantly contributed to the multiple linear regression of the final pH according to the following equation.

$$pH = 0.982 - 0.0058\ x\ [Malic\ acid]p + 0.1287\ x\ [Acetic\ acid]p + 0.678\ x\ pHi - 0.0013\ x\ [residual\ sugar]$$

The adjusted R square of the model is 0.392 (pvalue $< 2 \times 10^{-16}$), suggesting that a large part of the explaining factors was not identified as illustrated Fig. 3D. A variance analysis of this linear model indicated that an important part of final pH variation was explained by initial pH of grape juice and the malic acid produced by yeast (25 and 13%, respectively). In contrast, acetic acid, and residual sugars accounted for a very minor part of the model.

3.4. Malic acid producer strains are enriched in ACIDIC alleles

In a previous study (Vion et al., 2021), we applied a marker assisted selection using eleven QTLs related to MAC% and mapped on the SB × GN genome (Peltier et al., 2021). The penetrance of such QTLs was only partial due to complex genetic interactions. Since SB and GN genome constitute 50% or the AC's hybrids we hypothesized that the most malic acid producer strains should be enriched in alleles conferring low MAC% values. The QTLs linked to MAC% were given in Table S1 and were tracked with 19 mass array markers suitable to interrogate all the genotypes in the same assay. Most of the QTLs were narrowed by a pair of markers in order to increase the robustness of genotyping.

Fig. 4A shows the relative position of such markers on yeast chromosomes and specify the contribution of SB and GN alleles respect to the MAC%. Alleles promoting malic consumption were named DEMALIC

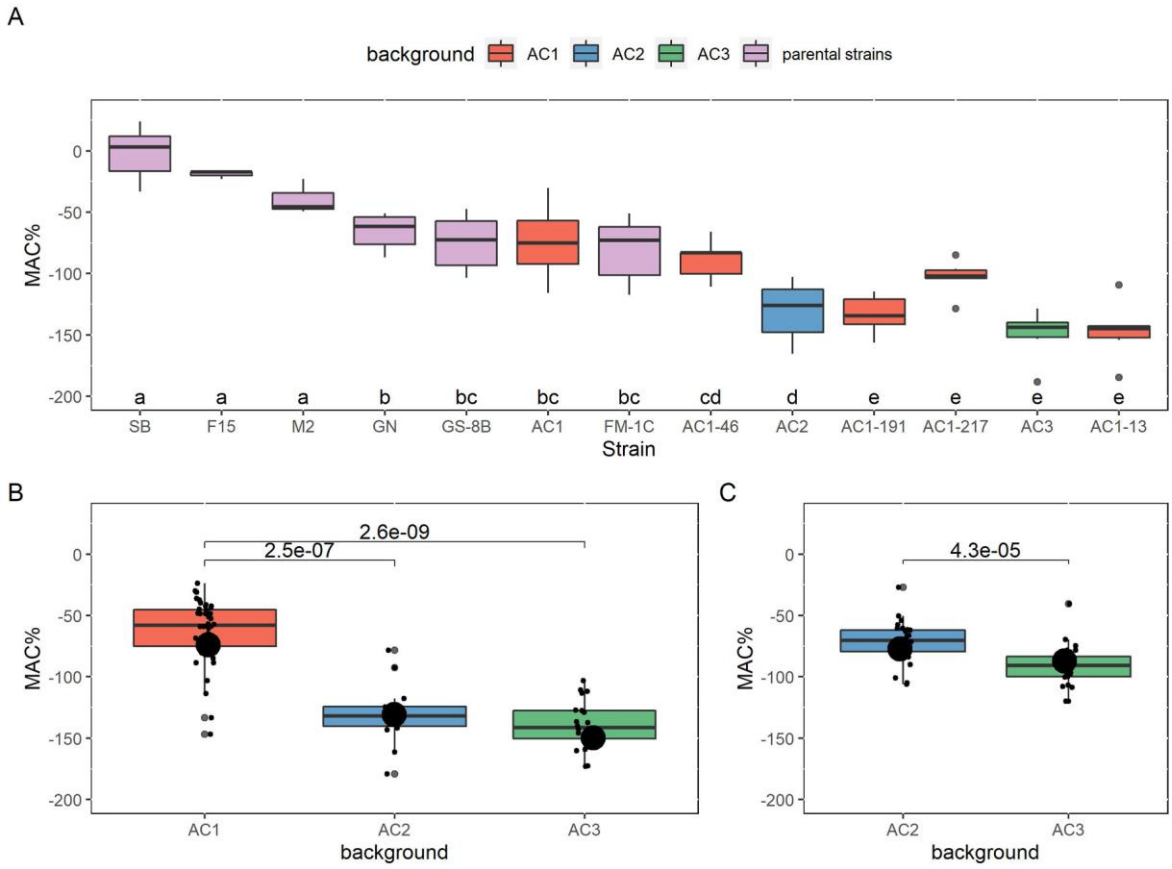


Fig. 2. Breeding program for enhancing malic acid production

Panel A. Box plot indicates the optimization of MAC% values by a phenotype driven breeding program. The purple color indicates the MAC% distribution of founder strains GN, SB, M2 and F15 and parental strains selected GS-8B and FM-1C. The resulting F1 hybrid AC1 and its four optimal progeny clones are colored in red. The second-generation hybrids AC2 and AC3 are respectively colored in red and green; each F1-hybrid is narrowed by its respective parents. The letters below the box plots indicate post hoc significative differences between strains (Fisher's least significant difference). Groups that do not share a common letters can be considered as statistically different (Kruskal test, alpha = 0.05).

Panel B. Distribution of MAC% values in AC1 AC2 and AC3 progenies. Data presented are the average values of triplicates and were measured in the CS_14 grape juice. The large black dot indicates the average of values per background. The p value above the box plot indicates a statistical difference of MAC% according to the hybrid background (Kruskal test).

Panel C. Distribution of MAC% values in AC2 and AC3 progenies. Data presented are the average values of triplicates and were measured in the CS_15 grape juice. The large black dot indicates the average of values per background. The p value above the box plot indicates a statistical difference of MAC% according to the hybrid background (Kruskal test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

while alleles promoting malic acid production were named ACIDIC. Fig. 4B indicated the SNP call of both DEMALIC and ACIDIC alleles that are represented by green and red, respectively. Among the 19 ACIDIC alleles, 14 are derived from the parental strain GN encompassing nine of the 11 QTLs tracked. The allele inheritances for the four parental strains and the hybrids AC1 to AC3 are also detailed in Fig. 4C. Homozygous alleles are represented by green or red shaded area and can be considered as fixed in the respective hybrid's progeny. For instance, the acidic allele of the QTLs XI_382 corresponding the gene *MAE1* is present in all the AC's hybrids of this study. Conversely, for the loci VII_425 and VI_426, the GN allele was lost indicating that the acidifying copy of *PNC1* is not present in the progeny analyzed (Fig. 4C). Among the 118 progeny strains phenotyped in this work, 94 were randomly genotyped for their inheritance for these 19 markers using a mass array strategy (see methods). The genotype of each strain is given in Table S2.

As shown in Fig. 5A, the number of ACIDIC alleles was negatively correlated with MAC% values. However, this number was quite different according to the progeny genotyped. Indeed, the number of segregating markers was different according to the hybrid. In the AC1 progeny the number of ACIDIC alleles ranged between 2 and 13 while AC2 and AC3 progenies had fixed a greater number of ACIDIC alleles (between 10 and 14) (Fig. 4C). Two QTLs (IV_28 and VII_480) segregated in all the progenies. Their effect on MAC% was confirmed in each AC's hybrid as illustrated in Fig. 5 B and C. Interestingly, this effect was much higher in the AC2 and AC3 backgrounds where numerous other acidic alleles were present. This result illustrated modifications of QTL penetrance according to the genetic background.

3.5. *Malic acid consumer and producer strains drastically impact the acidity perception of wine*

The organoleptic impact of strains able to modulate the amount of malic acid at the end of the alcoholic fermentation was evaluated by the sensory analysis of a Sauvignon blanc wine. In order to have a representative image of the technological potential of such strains, five high malic acid producers and five high malic acid consumers were selected. The producer strains are progeny clones of hybrids AC1, AC2 and AC3 while the consumer strains are progeny clones of FMGS and FMGS2 hybrids previously described by Vion et al., (2021). Two commercial starters (BO213 and RB4, Laffort, France) were added to complete this panel. Those commercial starters showed the highest difference in MAC % among 32 commercial starters tested (Peltier et al., 2018a). Each

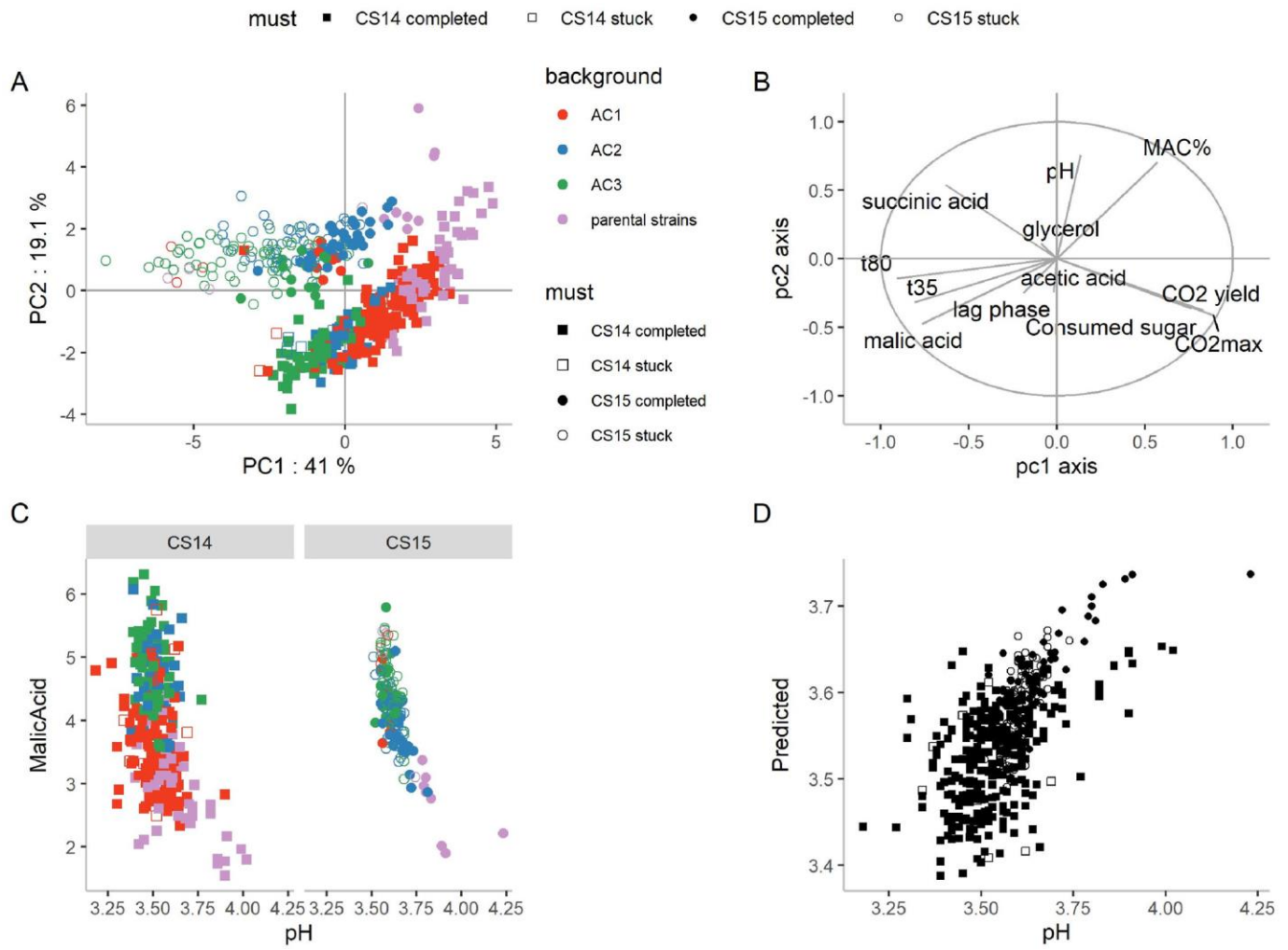


Fig. 3. Multivariate analysis

Panel A. The phenotypic behavior of 127 strains for 12 quantitative traits was figured out in the first two dimension of a Principal Component Analysis (encompassing 60.1% of the total inertia). A total of 484 fermentations were carried out in two grape juices (CS_14 and CS15). The color of the dots indicated the yeast background (AC1, AC2, AC3 and control strains) while the shape indicated the grape must fermented. Open and full symbols indicates if the fermentations were stuck or completed, respectively.

Panel B. Circle of correlation of the 12 variables used for the PCA.

Panel C. Correlation between the final malic acid content and the final pH of the wine in both CS_14 and CS_15 grape juices, the dot key is the same than for panel A.

Panel D. Real and predicted values of the final pH according to following linear model regression $pH = 0.0631 - 0.0058\,x\,[Malic\ acid] + 0.1282\,x\,[Acetic\ acid] + 0.9495\,x\,pHi - 0.0014\,x\,[residual\ sugar]$. Open and full symbols indicates if the fermentations were stuck or complete, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

strain was fermented in a single bioreactor of 1.2 L according to standard enological procedure, control of temperature, nitrogen management and preservation to oxidation (see methods). The final composition of wines is presented in Table 3.

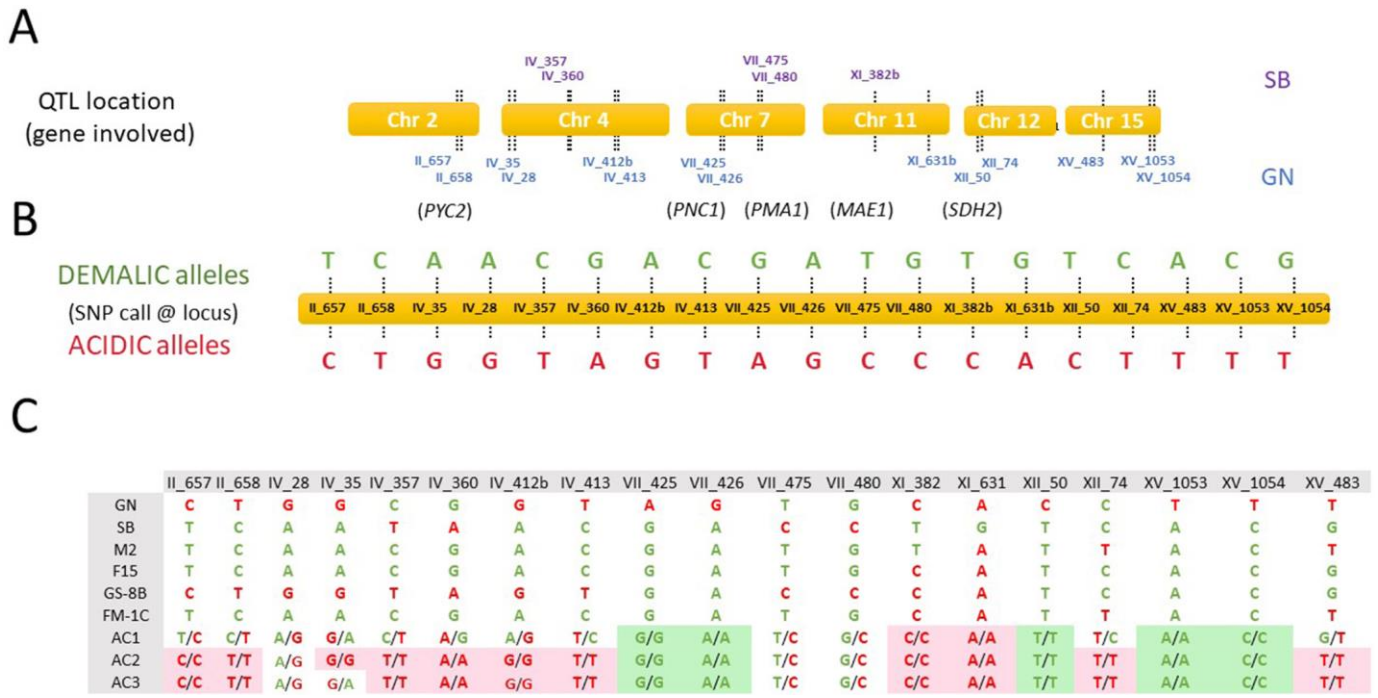
As expected, high producer and consumer strains showed significative differences in malic acid content with a direct impact on pH and total acidity (Fig. 6A) which is a good indicator of the overall acidity of wines (Plane et al., 1980). Other traits such as residual sugar, acetic acid, glycerol, or ethanol production were not significantly different between groups underlining a relative variability of strains belonging to the same group.

The resulting wines were presented to a panel of 28 tasters previously trained to appreciate the acidity in a Sauvignon Blanc wine. Dissimilarities between samples were analyzed using non-metric multidimensional scaling (MDS) allowing to figure out the perceptive proximities between wines on a map. The average stress value of the analysis was 0.051 which is significantly lower than explained by chance for 28 tasters and 12 wines (FDR of 5% 0.064). The two-dimensional MDS configuration reveals (Fig. 6B) that the wines were grouped according to the type of the strain and discriminates more specifically the malic acid consumers and producers. Such approach demonstrated that wines had different acid profiles that were clearly perceived by the tasters.

4. Discussion

4.1. The nature of the grape juice and yeast strains are determining factors to modulate the malic acid level and final pH of wines

Our findings shed light on the impact of the grape juice on the production of malic acid by *S. cerevisiae* strains corroborating previous studies (Delcourt et al., 1995; Vilanova et al., 2007). As previously suggested, the MAC% parameter is useful for comparing the behavior of various strains in different grape juices (Vion et al., 2021). By measuring this trait for 12 *S. cerevisiae* strains in seven grape juices we observed a strong effect of the grape juice origin (Fig. 1B). This variability is partially related to the initial content of malic acid in the grape juice.



	II_657	II_658	IV_28	IV_35	IV_357	IV_360	IV_412b	IV_413	VII_425	VII_426	VII_475	VII_480	XI_382	XI_631	XII_50	XII_74	XV_1053	XV_1054	XV_483
GN	C	T	G	G	C	G	G	T	A	G	T	G	C	A	C	C	T	T	T
SB	T	C	A	A	T	A	A	C	G	A	C	C	T	G	T	C	A	C	G
M2	T	C	A	A	C	G	A	C	G	A	T	G	T	A	T	T	A	C	T
F15	T	C	A	A	C	G	A	C	G	A	T	G	C	A	T	C	A	C	G
GS-8B	C	T	G	G	T	A	G	T	G	A	C	C	C	A	T	C	A	C	G
FM-1C	T	C	A	A	C	G	A	C	G	A	T	G	C	A	T	T	A	C	T
AC1	T/C	C/T	A/G	G/A	C/T	A/G	A/G	T/C	G/G	A/A	T/C	G/C	C/C	A/A	T/T	T/C	A/A	C/C	G/T
AC2	C/C	T/T	A/G	G/G	T/T	A/A	G/G	T/T	G/G	A/A	T/C	G/C	C/C	A/A	T/T	T/T	A/A	C/C	T/T
AC3	C/C	T/T	A/G	G/A	T/T	A/A	G/G	T/T	G/G	A/A	T/C	G/C	C/C	A/A	T/T	T/T	A/A	C/C	T/T

Fig. 4. QTLs genotyped by Mass Array in AC's hybrids
Panel A. Schematic localization of QTLs tracked on the genomic map of SBxGN hybrid. Alleles conferring a malic acid production were colored in purple and blue for SB and GN, respectively.
Panel B. SNP call detected by mass array genotyping for each locus tracked in this study. The DEMALIC alleles (in green) enhance the consumption of malic acid while the ACIDIC alleles enhance the production of malic acid (in red).
Panel C. Genotypes of the four founders of the study (GN, SB, M2 and F15), the two parental strains GS-8B, FM-1C and the three hybrids AC1, AC2 and AC3. For each locus, the green and red colored areas indicated if the DEMALIC or ACIDIC allele is fixed in the hybrid background. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The lowest the initial malic acid concentration, the highest the malic acid production by yeast. Since there is an obvious relation between malic acid concentration and pH of grape juice, it is not clear if the amount of malic acid of grape itself determines its production by yeast. Indeed, previous studies reported that malic acid production would be related to the pH of the medium with a similar concentration of malic acid (Schwartz and Radler, 1988). Malic acid is a diacid with two *pKa* of 3.46 and 5.10, respectively. Since the pH of grape juice ranges between 3.2 and 4.0, malic acid is mostly found in its undissociated and mono-dissociated form (H_2M and HM). Once it enters into the cell, malic acid mostly takes its deprotonated configuration (M) since the intracellular pH of the yeast is around 5–6. A proton efflux ensured by active pumps maintains the intracellular pH between 5 and 6. Malic acid entrance in the cell has been described as happening by facilitated diffusion (Salmon, 1987) as *S. cerevisiae* does not possess any known diacid transporter. In such case, only the protonated configuration of malic acid (H_2M) can enter into the cell which represent only 50% of the total malic acid available at pH = 3.46. Since low pH values enhance the H_2M/HM ratio, more di-protonated form is consumed triggering a higher deacidification of the media. This explains why high MAC% values are generally reached in more acid grape juices. In contrast, mechanisms triggering the expulsion of malic acid outside the cell much are less documented. Salmon (1987) provides a preliminary evidence of a malic acid efflux dependent on glucose in resting cells suggesting the existence of an active transporter. However such transporter was never characterized (Casal et al., 2008) despite several efforts. In addition, other abiotic factors such as nitrogen composition, sugar content or vitamins concentrations would also impact the production and consumption of malic acid during the alcoholic fermentation (Delcourt et al., 1995; Vilanova et al., 2007; Schwartz and Radler, 1988).

Beside the composition of grape juice, our findings demonstrate that yeast strain has a definitive impact on wine malic acid content. Although the range of MAC% in the population of commercial starters is not very large (0–45%), the selection of strains metabolically biased toward production or consumption of malic acid allows the modification of wine pH in a surprising manner. Indeed, the dynamic range of pH for the same initial grape juice was higher than 0.4 units which is highly relevant in an enological context. Indeed, such range of pH variation drastically affect the antimicrobial activity of SO_2 (Divol et al., 2012), as well as the anthocyanins stability with a great influence on the color of red wines (Ribérau-Gayon et al., 2006). The composition of 484 wines obtained during the breeding program allows the investigation of the main factors influencing the final pH of wine by a multiple linear regression approach. The main two contributors detected by the model and confirmed by an analysis of variance were the pH of grape juice and the malic acid produced by yeast. Although significative, the quite low adjusted R^2 (0.392) of the model suggested that many other compounds present in wines and/or produced by yeast should influence the final pH of wines. Among them potassium salt and tartaric acid concentrations could likely modulate the variability observed. The role of yeast strains regarding these factors remains to be explored.

4.2. *Phenotypic characterization of wine yeast strains according to their malic acid production level*

The phenotypic characterization of progeny clones derived from three newly made F1-hybrids illustrates the efficiency of a breeding program for enhancing a complex trait. This result has been likely obtained because we crossed together extreme strains having distinct genetic origins. As previously documented for other quantitative traits of enological interest the phenotypic variability of the parental strains of this study (FM-1C and GS-8B) are mostly determined by distinct allelic

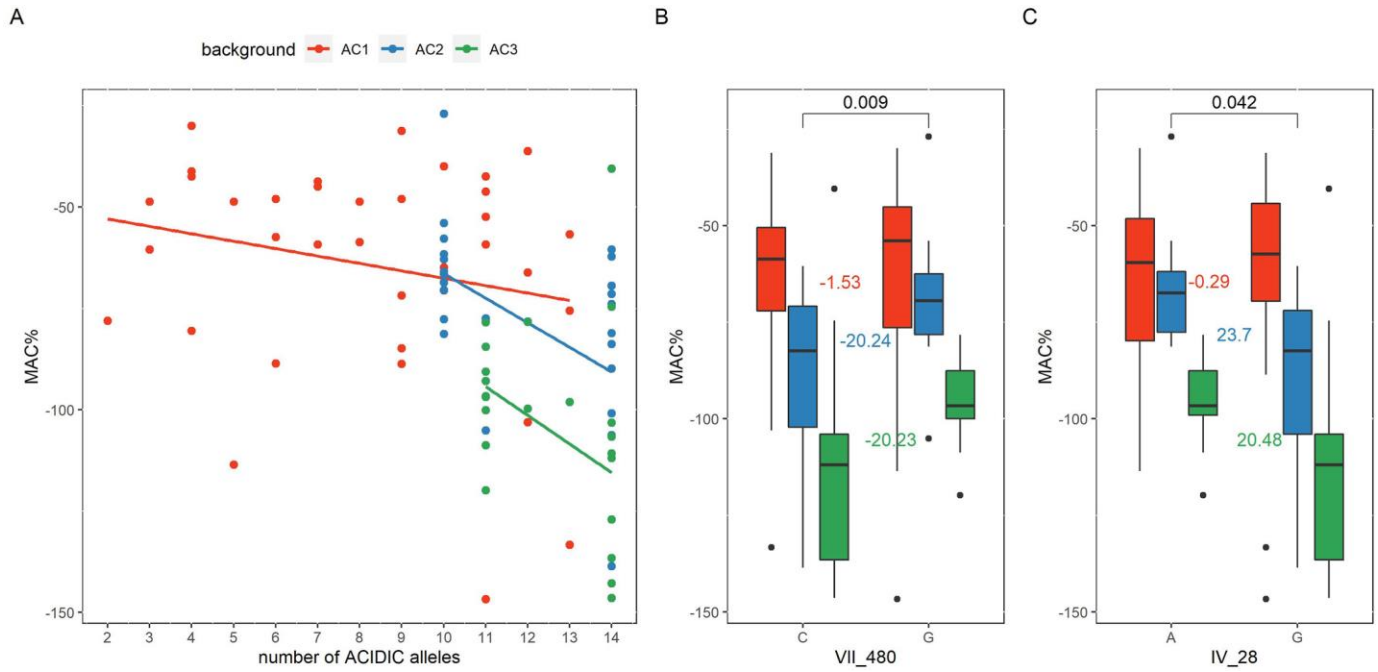


Fig. 5. QTL effect

Panel A. Linear regression of the MAC% according to the number of ACIDIC alleles carried by the progeny clones of the hybrids AC1, AC2 and AC3 that are colored in red, blue and green respectively.

Panel B. The boxplot shows the MAC% values of strains carrying the DEMALIC (C) or ACIDIC (G) allele of the locus VII_480 according to the hybrid background. Colored numbers indicated the MAC% differences between the genotype (same key than panel A). The p value above the box plot indicates a statistical difference of MAC% according to the genotype in indicated (Wilcoxon test).

Panel C. The boxplot shows the MAC% values of strains carrying the DEMALIC (A) or ACIDIC (G) allele of the locus IV_28 according to the hybrid background. Colored numbers indicated the MAC% differences between the genotype (same key than panel A). The p value above the box plot indicates a statistical difference of MAC% according to the genotype in indicated (Wilcoxon test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Main characteristics of wine evaluated by sensory analysis.

Strain	Group	Ethanol (%, v/v)	Total acidity (g/L H_2SO_4)	pH	Malic acid (g/L)	acetic acid (g/L)	Glycerol (g/L)	Residual sugar (g/L)
AC1_191	high producer	12.27	4.23	3.29	3.71	0.13	8.50	<2
AC2-1 A	high producer	12.25	4.40	3.27	3.90	0.12	8.56	<2
AC3-22	high producer	12.39	3.71	3.34	3.34	0.29	6.75	<2
AC1-217	high producer	12.22	4.29	3.29	3.57	0.26	8.81	<2
AC2-467	high producer	12.16	4.74	3.24	4.01	0.11	8.81	<2
FMGS2-714	high consumer	12.34	3.51	3.4	2.30	0.74	8.63	<2
FMGS-889	high consumer	12.42	3.02	3.44	2.00	0.27	8.37	<2
FMGS-71	high consumer	12.28	3.22	3.42	2.23	0.24	8.63	<2
FMGS2-107	high consumer	12.28	3.21	3.44	2.04	0.49	8.66	<2
FMGS-263	high consumer	11.57	3.94	3.35	3.38	0.36	6.79	>5
BO213	starter	12.40	3.68	3.33	3.36	0.29	6.69	<2
RB4	starter	12.36	3.61	3.37	2.98	0.40	7.22	<2

pools (Peltier et al., 2018b). Therefore, their comparable MAC% (−75%) results from the combination of different alleles controlling different metabolic targets. Consequently, merging such allelic pools in a new hybrid background (AC1) allows the emergence of a wide genetic and phenotypic variability with some strains showing high transgressive values. Interestingly, the distribution of MAC% in AC1 progeny (Fig. 2B) suggested that the inheritance of this trait is mostly non additive since only few progenies produce more malic acid than their F1-hybrid. However, once alleles controlling a high malic acid production have been fixed by crossing tail distribution progenies, it was possible to get many strains with very high acidifying properties. Thus, nearly 50% of the AC2 and AC3 progenies showed very low MAC% values offering the possibility to select a palette of malic acid producer strains that could segregates for many other traits not related to the malic acid production.

For instance, the multivariate phenotypic analysis of 127 strains presented in Fig. 3 showed that the three main secondary metabolites produced by yeast (acetic acid malic acid and glycerol) are not statistically correlated each togethers (Fig. S4). In contrast, a weak positive correlation (rho = 0.37) was found between malic and succinic acids suggesting than the production of both acids might be partially coupled which is consistent with their metabolic relationships (Camarasa et al., 2003). This analysis also suggested a possible phenotypic trade-off between fermentation completion and malic acid production. In fact, the most acidifying strains have the slowest fermentation kinetics (high values of t35 and t80) and some of them did not complete the alcoholic fermentation (residual sugars >2 g/L). Therefore, the production of high concentrations of malic acid during the alcoholic fermentation could result in a loss of fitness for *S. cerevisiae* explaining why most of the

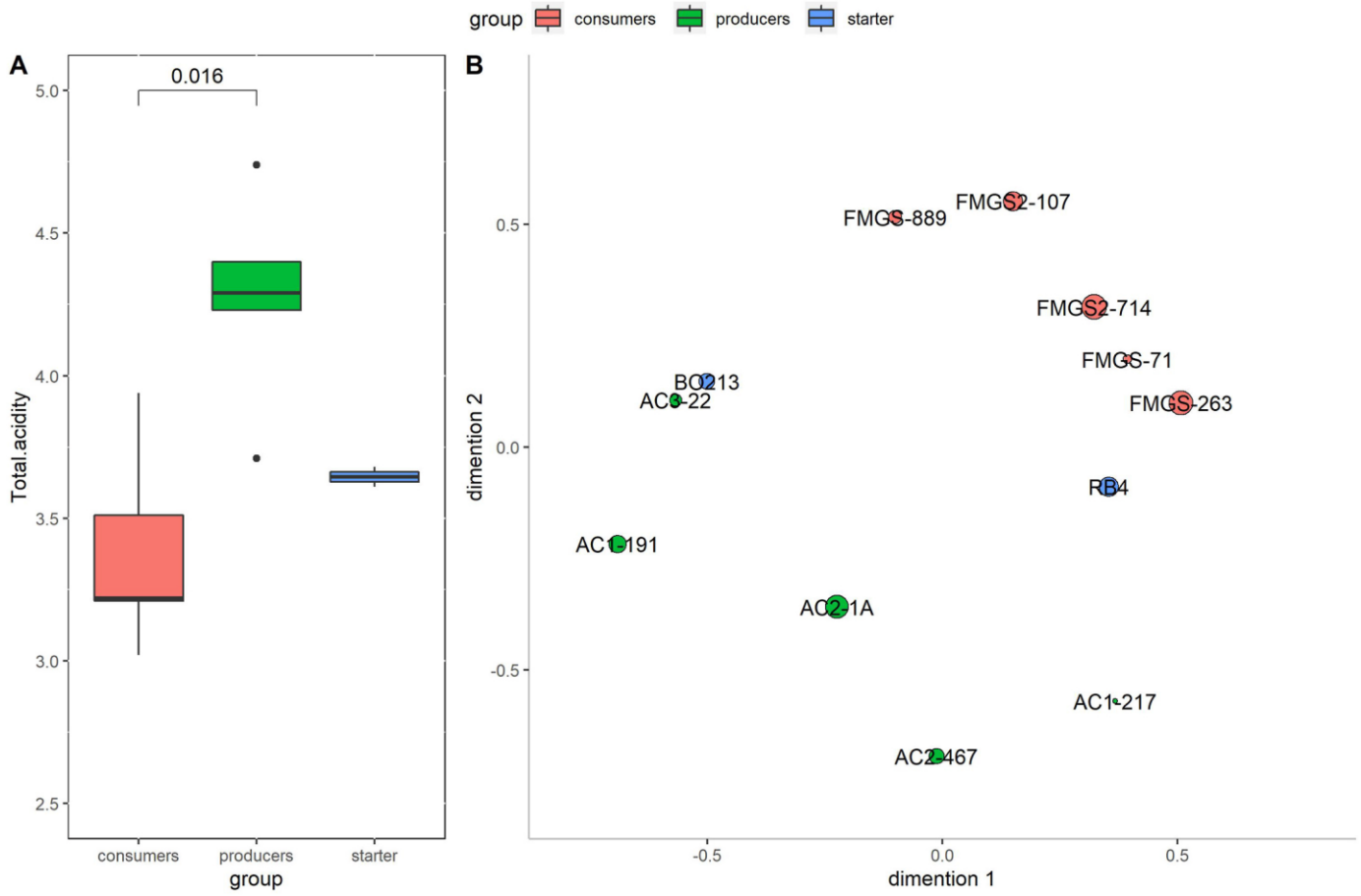


Fig. 6. Acidity perception estimated by sensory analysis
Panel A. The boxplot figures out the distribution total acidity for each groups of strains. The significative difference between malic acid consumers and producers is confirmed by a Wilcoxon test (alpha<0.05).
Panel B. The relative position of the wines tasted are figured out on the first two dimensions of a MDS analysis. Wines are represented by points that are positioned such that the distances between the pairs of points reflect distances between the pairs of wines. The average stress value of the analysis was 0.051 which is significantly lower than explained by chance for 28 tasters and 12 wines (FDR of 5% 0.064). The area of each point is proportional to the individual stress indicating the confidence of the projection. The colors indicate the group of the strain, starter, malic acid consumers or producers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

commercial starters empirically selected are malic acid consumers. Complementary studies with more phenotypes and more conditions will be necessary for confirming these preliminary conclusions.

4.3. Despite a partial penetrance QTLs previously mapped are statistically linked to the MAC% of malic producer strains

From a genetic point of view, the hybrids AC1, AC2, and AC3 and their relative progenies can be considered as mosaic strains derived from two parental strains (FM-1C and GS-8B). Such parental strains provided a large set of allelic variations enhancing the production of malic acid during the alcoholic fermentation that have been inherited from the four founder strains M2, GN, F15 and SB that well represent the genetic diversity of commercial starter population (Peltier et al., 2018b). A fraction of this allelic pool has been previously mapped by a QTL analysis defining ACIDIC and DEMALIC alleles that modulate the production or consumption of malic acid, respectively. In order to evaluate what is the contribution of such alleles in the AC-hybrids, we randomly genotyped 94 progenies to find out statistical link between the inheritance of these alleles and the phenotype of the strains. The small number of progenies genotyped, and the high number of QTL tracked does not allow to test individually the effect of each QTL. However, some relations between genotype and phenotype have been elucidated. First, we found a negative correlation between the number of ACIDIC alleles and the MAC % whatever the background indicating that higher is the number of ACIDIC alleles stronger is the production of malic acid. Second, the global effect of two QTLs (VII_480 and IV_34) that played an important role in the MAC% determinism (Vion et al., 2021) was confirmed in new genetic backgrounds. Interestingly, such QTLs have a stronger effect in the AC2 and AC3 background which have already fixed many ACIDIC alleles respectively to AC1. This finding illustrates that major QTLs may have different level of penetrance due to the presence of other segregating alleles commonly called “modifiers” as review by several authors (Hou et al., 2016; Peltier et al., 2019; Yadav et al., 2016). The fixation of these buffering alleles in the AC2 and AC3 backgrounds allows a better expressivity of the QLTs VII_480 and VI_28 as shown in Fig. 5 B and C.

4.4. Selection of a new type of S. cerevisiae strains for coping the drastic drop of acidity in wines

Despite numerous stuck fermentations of high malic acid producers, this study allows to isolate eight strains that completed the alcoholic fermentation and produced more than 3 g/L of malic acid (Fig. S5). This selection proposes a set of strains with a very contrasted phenotype respectively to those previously selected by Vion et al., (2021) enhancing considerably the technological palette of resulting wines by

modulating the wine acidity during the alcoholic fermentation. This original feature is particularly relevant in an enological context for coping with drastic drops of acidity level observed in grape juices. In order to test the enological relevance of such strains, five malic producer strains were compared to five malic consumers strains as well as two commercial starters. Small scale vinifications were carried out on a Sauvignon blanc matrix that was evaluated by a sensory panel using a sorting task approach. After a preliminary test of the aptitude of the panel to discriminate wine acidity, the twelves wines were evaluated and the strains showing contrasted malic acid metabolism were well separated. This very preliminary sensory characterization agrees with the chemical analyses of wines tasted that statistically differs in their total acidity, their pHs and their malic acid content.

4.5. Concluding remarks

The climate change has drastic consequences of the acidity of grape juice and constitutes a pregnant problem for wine making. The use of selected microorganisms during the alcoholic fermentation is a promising solution to moderate this drop of acidity and preserve wine quality. In order to propose a reliable alternative to the use of non-conventional yeast species such as *L. thermotolerans* we applied a selection program aiming to obtained *S. cerevisiae* strains able to produce up to 3 g/L of malic acid during the alcoholic fermentation. This result has been obtained by applying a classical breeding strategy narrowing the ability of strain to produce malic acid in different grape juices with a significant impact on wine pH and titratable acidity. This result has been obtained by the accumulation of acidifying alleles of several QTLs that have been previously identified. The large number of alcoholic fermentation achieved allowed the discrimination of some biotic and abiotic factors that control the final pH of wine in *S. cerevisiae* pure cultures. Beside a strong effect of the grape juice origin, the production of malic acid by yeast strongly impacted wine pH which was not the case of succinic or acetic acid production. However, high concentrations of malic acid reached had negative consequences of fermentation kinetics due to the strong acidification of the fermenting matrix. From a sensory view point we demonstrated that acidifying strains could be clearly discriminated from the high malic acid consuming ones. This work demonstrated that breeding programs based on QTLs detection is an efficient lever for selecting new starters able to manage wine acidity.

Author contributions

Conceptualization CV and PM; Formal analysis CV and PM; Funding acquisition IM, PM; Investigation CV, MM, MB, VF, BR; Methodology MM, ST; Resources NY, IM, PM; Software CV, PM; Supervision PM; Writing - original draft CV, NY, PM; Writing - review & editing ST, CV PM.

Conflict of interest

PM, CV, MB, and MM reports a relationship with Biolaffort company that includes full time employment.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marullo Philippe reports financial support was provided by Biolaffort. Vion Charlotte reports financial support was provided by Biolaffort. Maitena Muro reports financial support was provided by Biolaffort. Margaux Bernanrd reports financial support was provided by Biolaffort.

Acknowledgments

This work was funded by the grant MEGAVIP from the Regional Concil of Aquitaine. Enzymatic assays were performed at the metabolomic Facility of Bordeaux with the help of Yves Gibon.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104209.

References

Arrizabalaga, M., Morales, F., Oyarzun, M., Delrot, S., Gomès, E., Irigoyen, J.J., Hilbert, G., Pascual, I., 2018. Tempranillo clones differ in the response of berry sugar and anthocyanin accumulation to elevated temperature. Plant Sci. 267, 74–83. https://doi.org/10.1016/j.plantsci.2017.11.009.

Bureau, S.M., Razungles, A.J., Baumes, R.L., 2000. The aroma of Muscat of Frontignan grapes: effect of the light environment of vine or bunch on volatiles and glycoconjugates. J. Sci. Food Agric. https://doi.org/10.1002/1097-0010.

Camarasa, C., Grivet, J.P., Dequin, S., 2003. Investigation by 13C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways of succinate formation in Saccharomyces cerevisiae during anaerobic fermentation. Microbiology (Road Town, V. I. (Br.)) 149, 2669–2678. https://doi.org/10.1099/mic.0.26007-0.

Casal, M., Paiva, S., Queirós, O., Soares-Silva, I., 2008. Transport of carboxylic acids in yeasts. FEMS Microbiol. Rev. 32, 974–994. https://doi.org/10.1111/j.1574-6976.2008.00128.x.

Castellari, L., Ferruzzi, M., Magrini, A., Giudici, P., Passarelli, P., Zambonelli, C., 1994. Unbalanced wine fermentation by cryotolerant vs. non-cryotolerant Saccharomyces strains. Vitis. ISSN 00427500.

Chen, Y., Jiang, J., Song, Yaoyao, Zang, X., Wang, G., Pei, Y., Song, Yuyang, Qin, Y., Liu, Y., 2022. Yeast diversity during spontaneous fermentations and oenological characterisation of indigenous Saccharomyces cerevisiae for potential as wine starter cultures. Microorganisms 10, 1455.

Chernova, M., Albertin, W., Durrens, P., Guichoux, E., Sherman, D.J., Masneuf-Pomarede, I., Marullo, P., 2018. Many interspecific chromosomal introgressions are highly prevalent in Holarctic Saccharomyces uvarum strains found in human-related fermentations. Yeast 35, 141–156. https://doi.org/10.1002/yea.3248.

Chollet, S., Valentin, D., Abdi, H., 2014. Free sorting task. In: Tomasco, P., Ares, G. (Eds.), Novel Techniques in Sensory Characterization and Consumer Profiling, ISBN 9781138034273, pp. 207–227. Boca Raton.

Coloretti, F., Zambonelli, C., Castellari, L., Tini, V., Rainieri, S., 2002. The effect of DL-malic acid on the metabolism of L-malic acid during wine alcoholic fermentation. Food Technol. Biotechnol. 40, 317–320. ISSN 1330-9862.

Conover, 1999. Counts and categories, 1999. In: Conover (Ed.), Practical Nonparametric Statistics. Wiley, pp. 165–195. Wiley, pp. 380–383.

Coombe, B.G., 1987. Influence of temperature on composition and quality of grapes. Acta hortic. In: Kliewer, W.M. (Ed.), Symposium on Grapevine Canopy and Vigor Management. ISHS, California Univ., Davis, CA (USA). Dept. of Viticulture and Enology).- Wageningen (Netherlands), ISBN 90-6605-442-5, pp. 23–35, 1987.

Cox, T., Cox, M., 2001. Multidimensional Scaling, second ed., ISBN 978-3-540-33037-0 New York.

da Silva, T., Albertin, W., Dillmann, C., Bely, M., la Guerche, S., Giraud, C., Huet, S., Sicard, D., Masneuf-Pomarede, I., De Vienne, D., Marullo, P., 2015. Hybridization within Saccharomyces genus results in homoeostasis and phenotypic novelty in winemaking conditions. PLoS One 10, e0123834. https://doi.org/10.1371/journal.pone.0123834.

de Leeuw, J., Mair, P., 2009. Multidimensional scaling using majorization: SMACOF in R. J. Stat. Software 31, 1–30. https://doi.org/10.18637/jss.v031.i03.

Delcourt, F., Taillandier, P., Vidal, F., Strehaiano, P., 1995. Influence of pH, malic acid and glucose concentrations on malic acid consumption by Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol. 43, 321–324. https://doi.org/10.1007/BF00172832.

Divol, B., Du Toit, M., Duckitt, E., 2012. Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. Appl. Microbiol. Biotechnol. 95, 601–613. https://doi.org/10.1007/s00253-012-4186-x.

Gabriel, S., Ziaugra, L., Tabbaa, D., 2009. SNP genotyping using the sequenom massARRAY iPLEX Platform. Curr. Protoc. Hum. Genet. https://doi.org/10.1002/0471142905.hg0212s60.

Hou, J., Sigwalt, A., Fournier, T., Pflieger, D., Peter, J., de Montigny, J., Dunham, M.J., Schacherer, J., 2016. The hidden complexity of mendelian traits across natural yeast populations. Cell Rep. 16, 1106–1114. https://doi.org/10.1016/j.celrep.2016.06.048.

Hranilovic, A., Albertin, W., Capone, D.L., Gallo, A., Grbin, P.R., Danner, L., Bastian, S.E. P., Masneuf-Pomarede, I., Coulon, J., Bely, M., Jiranek, V., 2021. Impact of Lachancea thermotolerans on chemical composition and sensory profiles of Merlot wines. Food Chem. 349, 129015 https://doi.org/10.1016/j.foodchem.2021.129015.

Hranilovic, A., Gambetta, J.M., Schmidtke, L., Boss, P.K., Grbin, P.R., Masneuf-Pomarede, I., Bely, M., Albertin, W., Jiranek, V., 2018. Oenological traits of Lachancea thermotolerans show signs of domestication and allopatric differentiation. Sci. Rep. 8, 14812 https://doi.org/10.1038/s41598-018-33105-7.

Huang, C., Roncoroni, M., Gardner, R.C., 2014. MET2 affects production of hydrogen sulfide during wine fermentation. Appl. Microbiol. Biotechnol. 98, 7125–7135. https://doi.org/10.1007/s00253-014-5789-1.

IPCC, 2020. Climate change and land, international encyclopedia of geography: people, the earth, environment and technology. https://doi.org/10.1002/9781118786352.wbieg0538.

Lonvaud-Funel, A., 1994. La Desacidification Biologique des Vins; Etat de la question, perspectives d'avenir. J. Int. des Sci. la Vigne du Vin 28, 161–170. https://doi.org/10.20870/oeno-one.1994.28.2.1149.

Marullo, P., Durrens, P., Peltier, E., Bernard, M., Mansour, C., Dubourdieu, D., 2019. Natural allelic variations of Saccharomyces cerevisiae impact stuck fermentation due to the combined effect of ethanol and temperature; a QTL-mapping study. BMC Genom. 20, 680. https://doi.org/10.1186/s12864-019-5959-8.

Marullo, P., Mansour, C., Dufour, M., Albertin, W., Sicard, D., Bely, M., Dubourdieu, D., 2009. Genetic improvement of thermo-tolerance in wine Saccharomyces cerevisiae strains by a backcross approach. FEMS Yeast Res. 9, 1148–1160. https://doi.org/10.1111/j.1567-1364.2009.00550.x.

Mira de Orduña, R., 2010. Climate change associated effects on grape and wine quality and production. Food Res. Int. 43, 1844–1855. https://doi.org/10.1016/j.foodres.2010.05.001.

Nistor, E., Dobrei, A.G., Dobrei, A., Camen, D., 2018. Growing season climate variability and its influence on sauvignon blanc and pinot gris berries and wine quality: study case in Romania (2005-2015). S. Afr. J. Enol. Vitic. 39, 196–207. https://doi.org/10.21548/39-2-2730.

Origone, A.C., Rodríguez, M.E., Oteiza, J.M., Querol, A., Lopes, C.A., 2018. Saccharomyces cerevisiae × Saccharomyces uvarum hybrids generated under different conditions share similar winemaking features. Yeast 35, 157–171. https://doi.org/10.1002/yea.3295.

Peltier, E., Bernard, M., Trujillo, M., Prodhomme, D.D., Barbe, J.-C., Gibon, Y., Marullo, P., 2018a. Wine yeast phenomics: a standardized fermentation method for assessing quantitative traits of Saccharomyces cerevisiae strains in enological conditions. PLoS One 13, 191353. https://doi.org/10.1101/191353.

Peltier, E., Friedrich, A., Schacherer, J., Marullo, P., 2019. Quantitative trait nucleotides impacting the technological performances of industrial Saccharomyces cerevisiae strains. Front. Genet. 10, 683. https://doi.org/10.3389/fgene.2019.00683.

Peltier, E., Sharma, V., Raga, M.M., Roncoroni, M., Bernard, M., Gibon, Yves, Marullo, P., Jiranek, V., Gibon, Y., Marullo, P., 2018b. Dissection of the molecular bases of genotype x environment interactions: a study of phenotypic plasticity of Saccharomyces cerevisiae in grape juices. BMC Genom. 19, 772. https://doi.org/10.1186/s12864-018-5145-4.

Peltier, E., Vion, C., Abou Saada, O., Friedrich, A., Schacherer, J., Marullo, P., 2021. Flor yeasts rewire the central carbon metabolism during wine alcoholic fermentation. Front. Fungal Biol. 2 https://doi.org/10.3389/ffunb.2021.733513.

Plane, R., Mattick, L., Weirs, L., 1980. An acidity index for the taste of wines. Am. J. Enol. Vitic. 31, 258–265. ISSN : 0002-9254.

Poudel, P.R., Mochioka, R., Beppu, K., Kataoka, I., 2009. Influence of temperature on berry composition of interspecific hybrid wine grape "Kadainou R-1" (Vitis ficifolia var. ganebu × V. vinifera 'Muscat of Alexandria'). J. Japanese Soc. Hortic. Sci. 78, 169–174. https://doi.org/10.2503/jjshs1.78.169.

R Core Team, 2018. R: A Language and Environmentfor Statistical Computing. R Found. Stat. Comput., Vienna, Austria https//www.R-project.org/.

Redzepovic, S., Orlic, S., Majdak, A., Kozina, B., Volschenk, H., Viljoen-Bloom, M., 2003. Differential malic acid degradation by selected strains of Saccharomyces during alcoholic fermentation. Int. J. Food Microbiol. 83, 49–61. https://doi.org/10.1016/S0168-1605(02)00320-3.

Regev-rudzki, N., Battat, E., Goldberg, I., Pines, O., 2009. Dual localization of fumarase is dependent on the integrity of the glyoxylate shunt. Mol. Microbiol. 72, 297–306. https://doi.org/10.1111/j.1365-2958.2009.06659.x.

Ribérau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, D., 2006. Handbook of Enology Volume 2 the Chemistry of Wine Stabilization and Treatments, ISBN 978-0-470-01037-2.

Saayman, M., Viljoen-Bloom, M., 2006. The biochemistry of malic acid metabolism by wine yeasts – a review. S. Afr. J. Enol. Vitic. 27, 113–122. https://doi.org/10.21548/27-2-1612.

Salmon, J., 1987. l-Malic-acid permeation in resting cells of anaerobically grown Saccharomyces cerevisiae. Biochim. Biophys. Acta Biomembr. 901, 30–34. https://doi.org/10.1016/0005-2736(87)90253-7.

Schwartz, H., Radler, F., 1988. Formation of l(-)malate by Saccharomyces cerevisiae during fermentation. Appl. Microbiol. Biotechnol. 27, 553–560. https://doi.org/10.1007/BF00451631.

Su, J., Wang, T., Wang, Y., Li, Y.Y., Li, H., 2014. The use of lactic acid-producing, malic acid-producing, or malic acid-degrading yeast strains for acidity adjustment in the wine industry. Appl. Microbiol. Biotechnol. 98, 2395–2413. https://doi.org/10.1007/s00253-014-5508-y.

van Leeuwen, C., Darriet, P., 2016. The impact of climate change on viticulture and wine quality. J. Wine Econ. 11, 150–167. https://doi.org/10.1017/jwe.2015.21.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by Saccharomyces cerevisiae wine yeasts. Appl. Microbiol. Biotechnol. 77, 145–157. https://doi.org/10.1007/s00253-007-1145-z.

Vion, C., Peltier, E., Bernard, M., Muro, M., Marullo, P., 2021. Marker Assisted Selection of malic-consuming Saccharomyces cerevisiae strains for winemaking. Efficiency and limits of a QTL's driven breeding program. J. Fungi 17–24. https://doi.org/10.20944/preprints202103.0132.v1.

Volschenk, H., van Vuuren, H.J.J., Viljoen-Bloom, M., 2006. Malic acid in wine: origin, function and metabolism during vinification. S. Afr. J. Enol. Vitic. 27 https://doi.org/10.21548/27-2-1613.

Volschenk, H., Viljoen, M., Grobler, J., Bauer, F., Lonvaud-Funel, A., Denayrolles, M., Subden, R.E., Van Vuuren, H.J.J., 1997. Malolactic fermentation in grape musts by a genetically engineered strain of Saccharomyces cerevisiae. Am. J. Enol. Vitic. 48, 0002-9254.

Yadav, A., Dhole, K., Sinha, H., 2016. Genetic regulation of phenotypic plasticity and canalisation in yeast growth. PLoS One 11, e0162326. https://doi.org/10.1371/journal.pone.0162326.

Yéramian, N., Chaya, C., Suárez Lepe, J.A., 2007. L-(-)-malic acid production by Saccharomyces spp. during the alcoholic fermentation of wine. J. Agric. Food Chem. 55, 912–919. https://doi.org/10.1021/jf061990w.

3. Etude de la réponse phénotypique des souches démaliquantes et acidifiantes

Les deux premiers articles de cette thèse ont permis de valider une méthode de construction de souches consommant ou produisant de l'acide malique. Dans la suite de ce manuscrit les premières seront qualifiées d'acidifiantes (*acidic* en anglais) et les secondes de démaliquantes (*demalic* en anglais).
Ce troisième volet reprend le travail expérimental réalisé par Bruce Richard (étudiant en Master 2 de microbiologie que j'ai encadré au cours de la période de janvier à juin 2022).

3.1. Contexte et problématique

Comme décrit dans les parties 1 et 2 de ce chapitre, l'utilisation de souches de levures sélectionnées pour moduler l'acidité des vins rencontre un intérêt grandissant au sein de la filière viti-vinicole. D'une part, des souches de levures ayant la capacité de consommer jusqu'à 80% de l'acide malique du moût de raisin lors de la fermentation alcoolique ont été sélectionnées (Vion *et al.*, 2021). D'autre part, des souches préservant l'acidité des vins grâce à une production d'acide malique allant jusqu'à +3 g/L dans certaines conditions ont été sélectionnées (Vion *et al.*, 2022). Ces deux types de souches permettent une gestion fine de l'acidité des vins au moyen d'intrants de nature biologique.

L'objectif de cette partie est d'étudier le comportement et la robustesse phénotypique de ces souches dans différentes matrices œnologiques présentant des concentrations variables en sucres et acide malique. Selon différentes études, la composition initiale du moût peut impacter le niveau de production et/ou de consommation d'acide malique par *S. cerevisiae* (Delcourt *et al.*, 1995; Vilanova *et al.*, 2007; Vion *et al.*, 2022, 2021). Cet effet de la matrice est partiellement expliqué par la teneur initiale en acide malique. D'autres facteurs abiotiques tels que les teneurs en azote assimilable ou en sucres fermentescibles peuvent moduler la concentration en acide malique à la fin de la fermentation alcoolique. Afin de caractériser l'étendue de la réponse phénotypique de souches extrêmes à ces variations environnementales, dix souches appartenant au groupe *demalic* et dix souches appartenant au groupe *acidic* ont été choisies pour fermenter douze matrices œnologiques représentées par différents cépages rouges et blancs, présentant des niveaux de sucres et d'acide malique variables.

3.2. Matériels et méthodes

3.2.1. Sélection des souches de S. cerevisiae

Les vingt souches de l'étude ont été sélectionnées en se basant sur deux critères principaux : leur capacité à consommer ou produire de l'acide malique (valeurs de *MAC%* les plus extrêmes) et leurs capacités fermentaires (cinétique franche, achèvement de la fermentation, acidité volatile faible). Quatre souches parentales SB, GN, M2 et F15 ont également été utilisées comme témoins dans l'étude. Ces souches sont les progéniteurs utilisés pour la construction des souches acidifiantes et démaliquantes. L'ensemble des souches utilisées est présenté dans le tableau 1.

Tableau 1. Souches utilisées

Souche	Type	Référence
FMGS_136	*Demalic*	(Vion *et al*., 2021)
FMGS_215	*Demalic*	(Vion *et al*., 2021)
FMGS_482	*Demalic*	(Vion *et al*., 2021)
FMGS_71	*Demalic*	(Vion *et al*., 2021)
FMGS_744	*Demalic*	(Vion *et al*., 2021)
FMGS_889	*Demalic*	(Vion *et al*., 2021)
FMGS2_107	*Demalic*	(Vion *et al*., 2021)
FMGS2_714	*Demalic*	(Vion *et al*., 2021)
FMGS4_263	*Demalic*	(Vion *et al*., 2021)
AC1_191	*Acidic*	(Vion *et al*., 2022)
AC1_197	*Acidic*	(Vion *et al*., 2022)
AC1_203	*Acidic*	(Vion *et al*., 2022)
AC1_217	*Acidic*	(Vion *et al*., 2022)
AC2_1A	*Acidic*	(Vion *et al*., 2022)
AC2_467	*Acidic*	(Vion *et al*., 2022)
AC3_1C	*Acidic*	(Vion *et al*., 2022)
AC3_3A	*Acidic*	(Vion *et al*., 2022)
AC3_22	*Acidic*	(Vion *et al*., 2022)
AC4_2B	*Acidic*	(Vion *et al*., 2022)
SB	Parent	(Peltier *et al*., 2018b)
GN	Parent	(Peltier *et al*., 2018b)
M2	Parent	(Huang *et al.*, 2014)
F15	Parent	(Huang *et al.*, 2014)

3.2.2. Fermentations alcooliques

3.2.2.1. Moûts utilisés

Deux moûts de Merlot 2021 et Sauvignon blanc 2016 ont été utilisés tels quels ou supplémentés en acide malique et/ou sucres fermentescibles (glucose + fructose) pour obtenir un total de huit matrices fermentaires. Quatre autres moûts représentant des conditions contrastées ont été utilisés : Merlot 2013, Merlot 2017, Cabernet sauvignon 2013 et Sauvignon blanc 2014. Les principales informations d'intérêt œnologique de ces matrices sont indiquées dans le tableau 2. L'ensemble des moûts utilisés a été fourni par

les vignobles Ducourt (Ladaux, France) et stockés à -20°C. Les moûts ont été débourbés avant inoculation.

Tableau 2. Caractéristiques œnologiques des 12 matrices de l'étude

Cépage	Type	Millésime	Sucres réducteurs initiaux (g/L)	Acide malique initial (g/L)	SO_2 total (mg/L)	pH	Sucres réducteurs finaux (g/L)	Acide malique final (g/L)	Nom abrégé
Cabernet sauvignon	rouge	2013	184	7,02	22	3,4	184	7,02	CS13
Sauvignon	blanc	2014	194	7,79	34	3,14	194	7,79	SB14
Merlot	rouge	2013	235	3,25	15	3,59	235	3,25	M13
Merlot	rouge	2017	238	1,99	0	3,73	238	1,99	M17
Merlot	rouge	2021	202,4	2,9	16	3,67	202,4	2,9	M21
Merlot	rouge	2021	202,4	2,9	16	3,47	202,4	6	M21A
Merlot	rouge	2021	202,4	2,9	16	3,49	240	2,9	M21S
Merlot	rouge	2021	202,4	2,9	16	3,42	240	6	M21AS
Sauvignon	blanc	2016	201	3,88	22	3,15	201	7,79	SB16
Sauvignon	blanc	2016	201	3,88	22	2,95	201	3,88	SB16A
Sauvignon	blanc	2016	201	3,88	22	3,23	230	7,79	SB16S
Sauvignon	blanc	2016	201	3,88	22	2,96	230	3,88	SB16AS

3.2.2.2. Déroulement des fermentations alcooliques

Des fermentations en petit volume (12 mL) ont été réalisées selon la méthode développée par Peltier *et al.*, (2018a). Brièvement, des flacons de 20 mL avec bouchon à vis ont été remplis avec 12 mL de jus de raisin. Les bouchons sont dotés d'un septum imperméable à l'air perforé d'une aiguille hypodermique permettant le dégagement de CO_2. Les flacons ont été inoculés à $2x10^6$ cellules vivantes/mL provenant d'une préculture en milieu moût/H_2O (1:1) de 24h. La concentration cellulaire et la viabilité ont été estimées par cytométrie en flux (Cytoflex, Beckmann Coulter, Indianapolis, USA). Les fermentations ont été réalisées à 24°C sous agitation orbitale (175 rpm). Les cinétiques de fermentation ont été estimées par une mesure manuelle quotidienne de la perte de poids causée par le dégagement de CO_2. Une balance de précision équipée d'un logiciel d'enregistrement automatique du poids (Mettler Toledo, Greifensee, Suisse) a été utilisée. La concentration de CO_2 dégagée en fonction du temps a été modélisée par une régression polynomiale permettant l'estimation des paramètres liés à la fermentation (Peltier *et al.*, 2018a) : la concentration maximale de CO_2 dégagé (CO_2max en g/L), la phase de latence (h), le temps nécessaire pour atteindre 35 et 80 g/L de CO_2 dégagé (après soustraction de la phase de latence) (T35, T80 en h).

3.2.3. Dosages enzymatiques

A la fin des fermentations, un échantillon de 800 µL a été manuellement transféré dans des tubes Micronics (Novazine, Lyon, France, ref. : MP32033L) et stocké à -20°C. Les concentrations des métabolites suivants ont été mesurées : acide acétique, glycérol, acide malique, acide succinique et SO_2 total en utilisant les kits enzymatiques K-ACETGK, K-GCROLGK, K-LMAL-116A, K-SUCC, K-TSULPH (Megazyme, Bray, Irlande). Les sucres résiduels (glucose et fructose) ont été dosés en utilisant la méthode enzymatique décrite par Stitt *et al*., (1989). Tous les dosages enzymatiques ont été réalisés à la plateforme robotisée Bordeaux métabolome (http://metabolome.cgfb.u-bordeaux.fr/).

3.2.4. Analyses statistiques

Les analyses statistiques et graphiques ont été réalisées à l'aide du logiciel R (R Development Core Team, 2016). Les graphiques ont été générés en utilisant le package *ggplot2*.

3.2.4.1. Analyses multivariées

La variabilité phénotypique des différents types de souches a été visualisée par une Analyse en Composante Principale (ACP) en utilisant le package *ade4*. Afin de déterminer les facteurs les plus discriminants, une Analyse en Composante Principale Discriminante (DACP) a été appliquée en sélectionnant les composantes principales permettant de capturer 90% de l'inertie cumulée en utilisant le package *adegenet*.

3.2.4.2. Analyses de variance et modèle de surface de réponse

Des analyses de variance (ANOVA) ont été réalisées en utilisant le package *car*. Les modèles de réponse de surface ont été générés à l'aide du package *rsm.* Un modèle linéaire (LM) a été appliqué à chaque type de souche (acidifiante ou démaliquante) selon la formule suivante :

(1) $Comp_{ij} = \text{Sucre initial}_i + \text{pH initial}_j$

Comp représente la valeur du paramètre d'intérêt (*MAC%*, glycérol, acide succinique, pH, glycérol, acide acétique, rendement œnologique ou T80), pour toutes les variables, pour une valeur de sucre initiale *i* (comprise entre 180 à 240) et pH initial *j* (comprise entre 2,9 à 3,7). Les interactions de ces deux facteurs ne sont pas prises en compte dans le modèle de surface de réponse.

3.3. Résultats

Afin de mieux caractériser les phénotypes liés à des comportements métaboliques opposés, la réponse phénotypique de 24 souches de *S. cerevisiae* présentant un métabolisme extrême de l'acide malique a été étudiée dans 12 jus de raisin. Pour cela, des fermentations à petite échelle ont été réalisées en triplicata. Le

jeu de données complet représente 864 fermentations (Figure 4) qui ont été caractérisées pour 15 traits quantitatifs mesurés en routine comme décrit par Peltier *et al.*, (2018a).

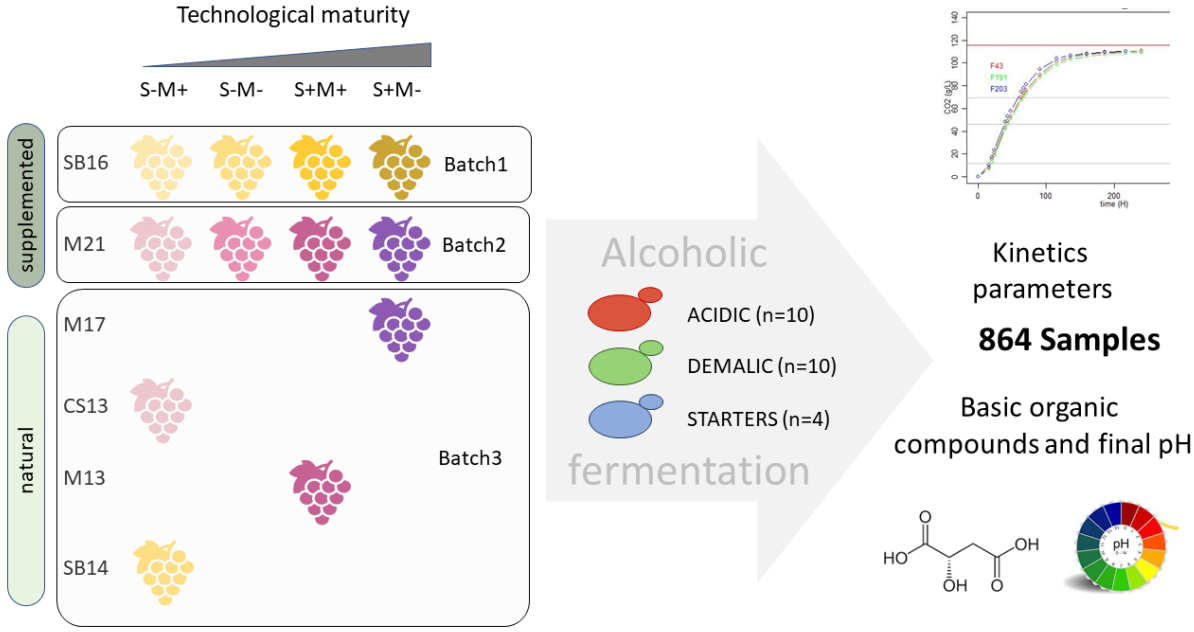


Figure 4. Plan d'expérience présentant, de gauche à droite, les matrices utilisées, les types de souches ayant réalisé la fermentation alcoolique et les différents paramètres mesurés en fin de fermentation.

3.3.1. Caractéristiques fermentaires de souches acidifiantes et démaliquantes

Six jus de raisin naturels ont été utilisés pour définir le paysage phénotypique des différentes souches de *S. cerevisiae*. Deux d'entre eux (M21 et SB16) ont été supplémentés en acide malique et/ou sucres fermentescibles afin de modifier leur densité et acidité. Comme schématisé dans la Figure 5, les milieux utilisés sont caractérisés par une large plage de concentrations en acide malique (1,9 – 7,9 g/L) et en sucres initiaux (180 – 249 g/L) qui correspondent aux différents équilibres sucre/acidité retrouvés dans les conditions de vinification modernes. Les principaux paramètres de ces 12 matrices œnologiques sont récapitulés dans le tableau 2.

L'origine des 24 souches est présentée dans le tableau 1. Les dix souches acidifiantes ont été sélectionnées parmi les descendants de 3 hybrides F1, (AC1, AC2 et AC3), capables de produire de l'acide malique pendant la fermentation alcoolique (Vion *et al.*, 2022). Les dix souches démaliquantes ont été sélectionnées parmi les descendants de 3 hybrides F1 (FMGS1, FMGS2 et FMGS4) (Vion *et al.*, 2021). Quatre souches supplémentaires (SB, GN, BO213 et RB4), référencées comme *starters*, représentent des souches commerciales avec des caractéristiques phénotypiques contrastées. Sur la base de précédents résultats, GN et RB4 sont considérées comme légèrement acidifiantes et SB et BO213 comme légèrement démaliquantes (Peltier *et al.*, 2018b).

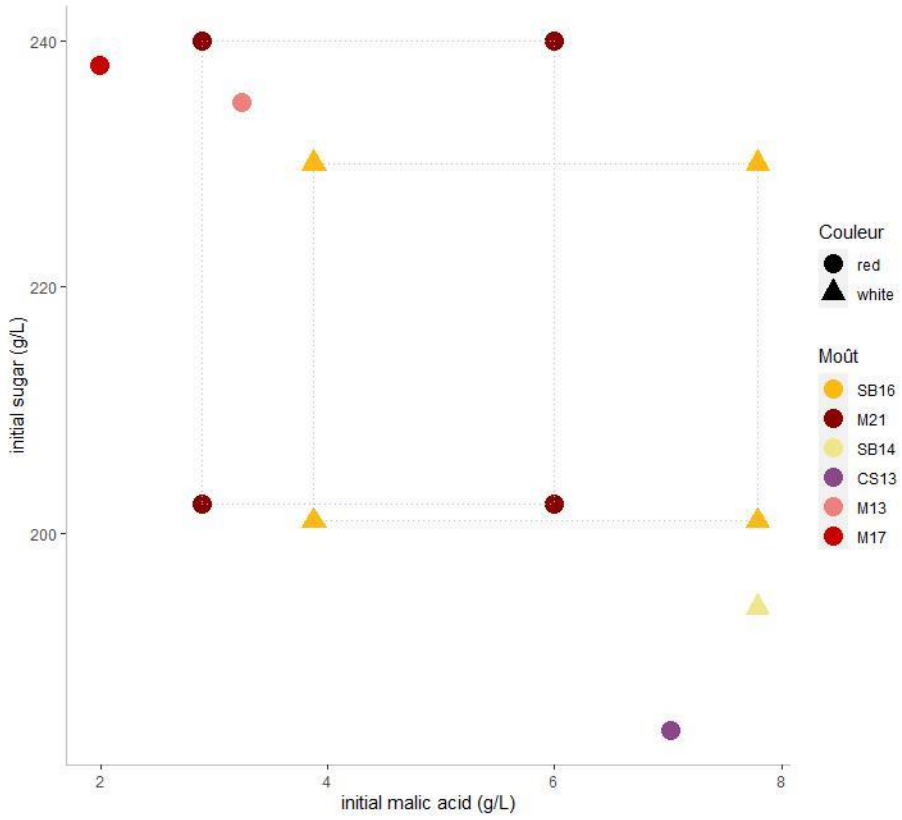


Figure 5: Positionnement des six jus de raisin naturels et des six jus supplémentés en fonction de leur concentration initiale en sucres (g/L) et en acide malique (g/L).

La variabilité des souches dans son ensemble est présentée dans l'Analyse en Composante Principale (Figure 6). Les souches appartenant aux groupes *acidic* et *demalic* sont clairement séparées par la seconde composante expliquant 18,8 % de l'inertie totale. Les souches acidifiantes sont enrichies en acide malique et ont un pH plus faible que les souches démaliquantes. Le T35 et T80 sont également corrélés à l'axe PC1, indiquant que le temps de fermentation des souches acidifiantes est plus long que celui des souches démaliquantes.

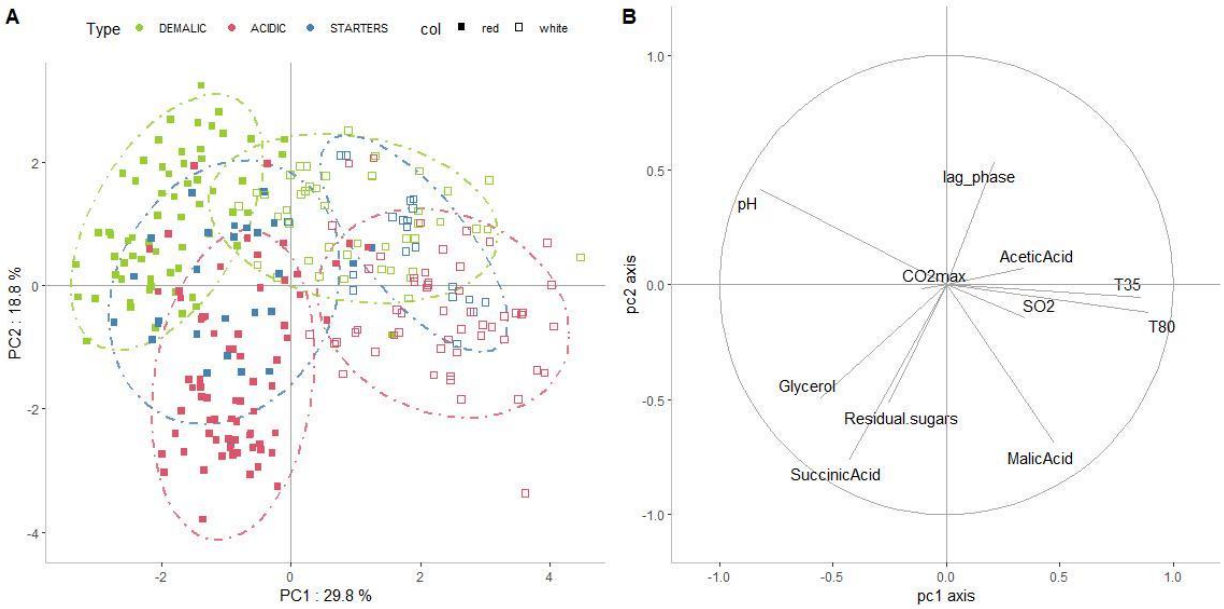


Figure 6: Analyse en composante principale (ACP) des trois groupes dans les douze matrices. **Panel A.** Les deux premiers axes de l'ACP effectuée sur les 3 types de souches dans les 12 matrices. Les axes 1 et 2 expliquent 29,8 et 18,8 % de la variance respectivement. Chaque point représente la moyenne de trois réplicas biologiques pour une souche dans un milieu. Chaque souche est colorée selon son groupe d'appartenance. **Panel B.** Cercle de corrélation indiquant les corrélations des variables étudiées pour les axes 1 et 2. Les moûts rouges et blancs sont séparés par la première composante expliquant 29,8 % de l'inertie totale des paramètres de cinétique de fermentation. Les vins rouges présentent davantage de sucres résiduels, glycérol et acide succinique, et les fermentations en blanc sont caractérisées par une phase de latence plus longue et des T35 et T80 plus importants.

Les jeux de données issus des fermentations en rouge et en blanc ont été analysés séparément par Analyse en Composante Principale Discriminante (DACP) afin d'identifier les métabolites ou paramètres cinétiques discriminant le mieux les types de souches démaliquantes et acidifiantes. Le paramètre *MAC%* a été intégré à l'analyse à la place de la concentration en acide malique afin de s'affranchir de la concentration initiale des moûts et d'évaluer uniquement l'effet des souches.

En rouge, le *MAC%*, et T80 sont les paramètres les plus discriminants, suivis du T35. La variance cumulée expliquée par ces trois paramètres est de 98 %. A lui seul, le *MAC%* explique 68 % de cette discrimination. En blanc, le T35, le CO_2max et le T80 sont les paramètres les plus discriminants et représentent 67 %, 14 % et 10 % de la variance, respectivement. Le *MAC%* arrive en quatrième position et n'explique que 4 % de l'inertie totale.

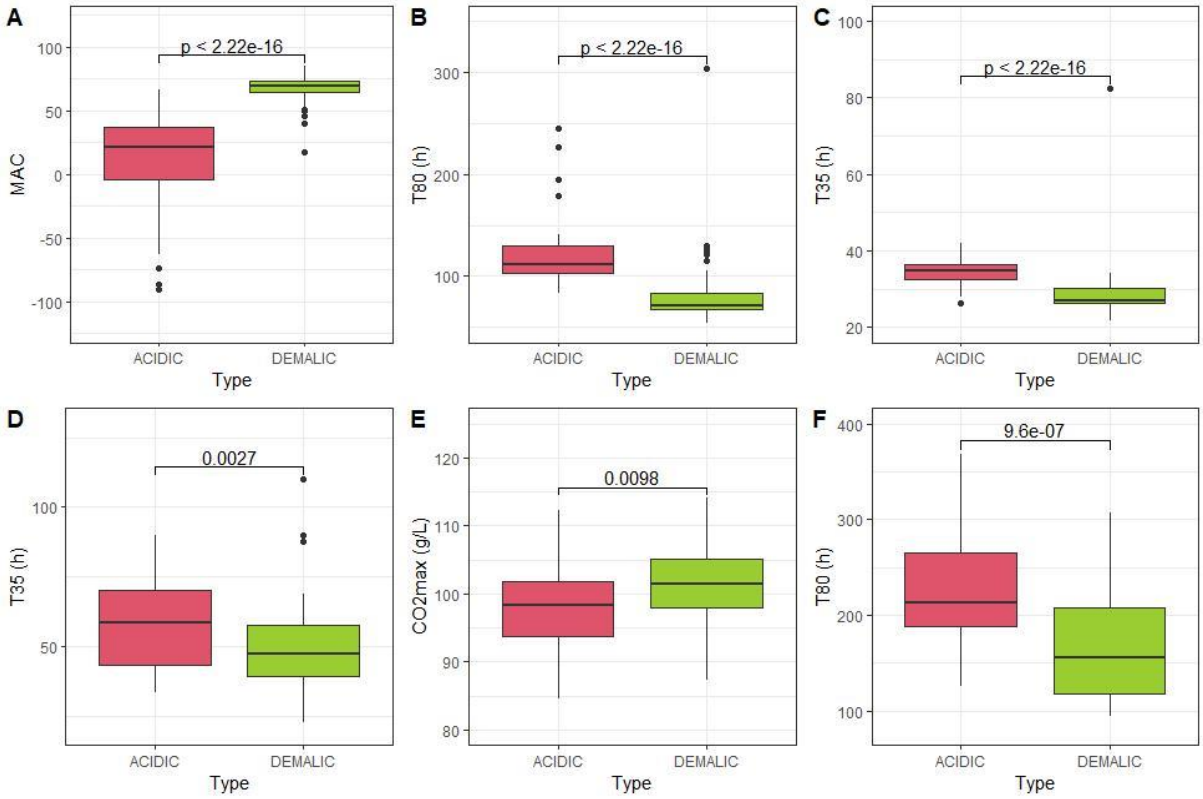


Figure 7. Principaux paramètres impliqués dans la ségrégation des deux types de souches. Les **panels A, B et C** représentent les paramètres les plus discriminants sur l'ensemble des matrices rouges. Les **panels D, E et F** représentent les paramètres les plus discriminants sur l'ensemble des matrices blanches. La valeur de la p-value d'un test de Wilcox entre les deux types de souches est affichée sur les graphiques.

Cette analyse discriminante permet de rendre compte des traits phénotypiques qui différencient le plus les deux groupes de souches. Pour la fermentation des jus de raisin rouges, le *MAC%* est le principal facteur de discrimination suivi par des paramètres cinétiques (Figure 7 A-C). Les souches acidifiantes présentent dans leur ensemble une fermentation plus lente (en moyenne 49 h de plus pour atteindre 80 % de la fermentation). Cependant très peu de fermenteurs n'achèvent pas la fermentation (21/131 acidifiantes et 4/112 démaliquantes) et il n'existe aucune différence significative pour les sucres résiduels entre les deux groupes. Pour la fermentation des jus de raisin blancs, le moût SB16 s'est révélé être une matrice difficile à fermenter, notamment à cause de sa très faible teneur en azote (76 mg N/L) qui n'a pas été supplémentée. Cet oubli a fortement impacté le temps de fermentation de l'ensemble des souches et plus particulièrement celui du groupe des acidifiantes qui présente des vitesses de fermentations plus lentes. Par conséquent, la teneur en sucres résiduels du groupe acidifiant est supérieure à celui du groupe démaliquant avec un nombre conséquent d'arrêts pour certaines souches (respectivement 16 % et 3,6 % des souches acidifiantes et démaliquantes). Ce biais expérimental explique que les paramètres cinétiques et le CO_2max soient les principaux facteurs discriminant de l'analyse DAPC.

3.3.2. La sélection de souches ayant des métabolismes de l'acide malique opposés montre des normes de réaction non parallèles pour de nombreux traits

La fermentation de nombreuses matrices par les deux types de souches de ce travail offre la possibilité de modéliser un champ de réponse phénotypique à l'aide de modèles de surface de réponse. Ces modèles nécessitent de définir des variables explicatives liées à la matrice fermentée permettant de prédire la valeur prise un trait fermentaire en fin de fermentation alcoolique. Dans le cas de notre étude nous avons défini deux variables explicatives qui ont une pertinence œnologique, le pH initial du moût et sa teneur en acide sucre. Le pH initial a été préféré à la teneur en acide malique car il tient compte également de la présence d'autres acides ce qui fait de lui un paramètre plus intégratif. Afin de gommer un peu les différences liées aux arrêts prématurés de fermentation dans les matrices SB16, les fermentations présentant une teneur en sucres résiduels de plus de 10 g/L ont été écartées soit 29 fermentations sur 288. Ces modalités faussent le calcul de certains paramètres tels que le rendement œnologique et risquent de biaiser le modèle de surface de réponse. Pour chaque variable réponse, deux modèles linéaires ont été réalisés, un pour les souches acidifiantes et un pour les souches démaliquantes. Ces modèles permettent de définir des normes de réaction des deux groupes. L'ensemble des normes de réaction calculées est présenté dans le tableau 3. Elles permettent de définir si le pH initial et/ou la teneur en sucres impacte significativement le phénotype des deux groupes de souches dans les 12 matrices fermentées. Si les modèles sont statistiquement différents, alors les souches démaliquantes et acidifiantes ne présentent pas les mêmes normes de réaction. C'est notamment le cas pour le *MAC%*, le pH, l'acide succinique et le glycérol.

Afin de représenter graphiquement cette différence de réponse phénotypique, des modèles de surface de réponse ont été calculés. Un modèle de surface de réponse permet de prédire dans un certain périmètre la valeur d'un phénotype en fonction des variables explicatives du modèle linéaire. La figure 8 montre que les souches acidifiantes et démaliquantes ont des normes de réaction très différentes pour certains phénotypes.

Tableau 3. Part de variance expliquée par le pH initial, sucre initial ou l'interaction pH initial : Sucre initial sur les différents composés et paramètres cinétiques en fonction du type de souche. Les valeurs en bleu gras représentent les valeurs significatives (p-value < 0,05). La colonne p-value indique si les modèles *ACIDIC* et *DEMALIC* sont statistiquement différents et si les normes de réaction peuvent être considérés comme parallèles (NS : non significatif, p-value > 0,05, * : 0,01< p-value < 0,05, ** : 0,001 < p-value < 0,01, *** p-value < 0,001.

	ACIDIC			**DEMALIC**		
	pH initial	Sucre initial	pH : Sucre	pH initial	Sucre initial	pH : Sucre
MAC%	**15,43**	**6,89**	**11,69**	0,19	0,61	**24,17**
Acide succinique	**18,19**	**2,47**	**4,61**	6,15	1,56	4,96
pH final	**42,05**	0,02	**1,15**	**57,07**	**0,56**	**4,10**
Glycérol	**22,25**	**2,66**	0,00	**10,39**	**5,94**	0,00
Acide acétique	**11,55**	**7,54**	**3,35**	**5,46**	**12,29**	**4,22**
Rendement œnologique	**7,88**	**3,92**	0,14	**3,80**	**6,73**	0,03
T80	**60,39**	0,26	**1,32**	**51,58**	0,15	0,54

Les souches démaliquantes ont tendance à consommer toujours la même proportion d'acide malique (en moyenne 65 %) quelle que soit la quantité de sucre initial. Cette consommation est sensiblement moindre lorsque le pH initial est plus basique. Cette élévation du pH initial peut s'expliquer notamment par une quantité d'acide malique initial plus faible, ce qui influence sa consommation (Vion *et al.*, 2021). Les souches acidifiantes, en revanche, sont plus sensibles à la composition des moûts. En effet, les matrices présentant une forte concentration en sucres fermentescibles et un pH de départ élevé semblent induire une production plus forte d'acide malique (Figure 8A). C'est le cas notamment des moûts M17 (238 g/L de sucres et 1,99 g/L d'acide malique) et M21S (240 g/L de sucres et 2,9 g/L d'acide malique) pour lesquels la production d'acide malique par les souches acidifiantes atteint + 2 g/L en M17 et + 1,5 g/L en M21S (Figure supplémentaire 1). De manière intéressante, des moûts avec les mêmes propriétés technologiques (teneur en acide malique en sucres) peuvent générer des MAC% assez différents. Cependant, il a été montré que la teneur en azote assimilable peut influencer le MAC% (Vion *et al.*, 2021) et d'autres facteurs abiotiques non mesurés ici peuvent avoir un effet.

Concernant l'acide succinique, il est produit en plus grande quantité par les souches acidifiantes, et plus particulièrement lorsque la concentration initiale en sucres est forte et le pH est élevé (Figure 8B). Cette production plus importante d'acide succinique par les souches *acidic* est corrélée à une production d'acide malique par ces mêmes souches dans les mêmes conditions. Ce phénomène a déjà été observé dans d'autres matrices (Vion *et al.*, 2022).

Le pH final des vins diffère également en fonction du type de souches avec des surfaces de réponses

non parallèles (panel C) qui diffèrent de celle du *MAC%*. Ce phénomène s'explique probablement par l'effet tampon qu'exerce la teneur en acide malique. A cause de la forte modulation de cet acide entre les deux groupes de souches, cet effet tampon est modifié. Cet effet est détaillé dans la partie discussion.

Enfin, la production de glycérol est impactée par le type de souche utilisé. Les souches acidifiantes produisent plus de glycérol que les souches démaliquantes, là encore avec une différence plus marquée entre les deux types de souches dans des conditions de forte teneur en sucre et pH élevé (panel D).

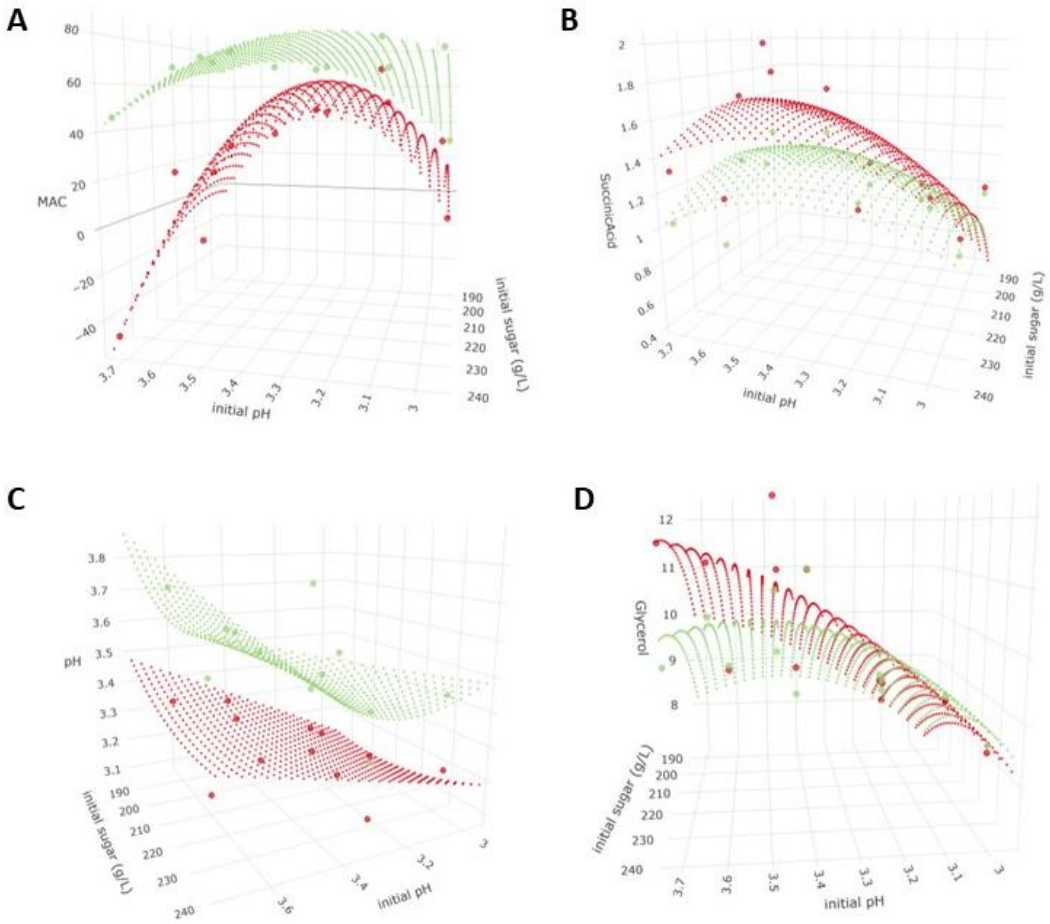


Figure 8. Normes de réaction du MAC% (**Panel A**), acide succinique (g/L) (**Panel B**), pH (**Panel C**) et glycérol (g/L) (**Panel D**) pour les souches démaliquantes et acidifiantes en fonction de la concentration en sucre initial et du pH initial. Les souches démaliquantes sont représentées en vert et les acidifiantes en rouge.

Pour d'autres paramètres de cette étude (acide acétique, rendement œnologique et T80), des différences significatives sont observées entres les deux types de souches (figure supplémentaire 2). Les souches démaliquantes produisent plus d'acide acétique (environ 0,1 g/L) quelle que soit la composition du milieu de départ, avec une légère atténuation de cette différence pour les faibles concentrations en sucres fermentescibles (inférieur à 200 g/L) (Figure S2 Panel A). Le rendement œnologique est légèrement plus fort chez les souches acidifiantes. Ces dernières ont besoin en moyenne de 0,5 g de sucres fermentescibles

supplémentaire pour produire 1° d'éthanol, quelle que soit la composition de la matrice de départ (Panel B). Cette propriété intéressante conduit à une production finale en CO_2 moindre, très probablement corrélé à la teneur en éthanol. Par ailleurs, dans les valeurs les plus extrêmes de sucres et de pH initial, l'ensemble des souches nécessite une plus grande concentration de glucose pour produire une même concentration d'éthanol, ce qui correspond à une réalité œnologique bien connue. Enfin, le temps pour atteindre 80 % de la réaction (T80) est plus long chez les souches acidifiantes (Panel C).

3.4. Discussion

3.4.1. Effet pléiotropique de la sélection génétique sur le phénotype des souches

Les sélections génétiques réalisées dans ce chapitre suggèrent que les souches de *S. cerevisiae* présentant des *MAC%* opposés sont sujettes à des compromis phénotypiques (*phenotypic trade-off*) directs ou indirects. Etant donné que ces souches partagent les mêmes ancêtres, le pedigree global des souches est assez similaire et le génotypage par des marqueurs ne permet pas de différencier les 20 souches de cette étude (résultats non montrés). Cependant, l'introduction de plusieurs QTLs par des programmes de croisement semble avoir eu des impacts phénotypiques qui dépassent la simple modulation du *MAC%*. Pour certains traits (sucres résiduels, SO_2 final) ces deux groupes ne peuvent pas être différenciés, si on exclut les problèmes liés à la matrice SB16. Pour d'autres phénotypes, la sélection appliquée impacte les cinétiques de fermentation, le rendement œnologique et la production d'acidité volatile (Figure S2). De manière intéressante et nouvelle, il a été démontré que les normes de réaction de ces deux types de souches ne sont pas toujours parallèles. C'est en particulier le cas pour le pH final et la production de certains composés organiques tels que l'acide succinique et le glycérol. Dans le cas du glycérol, ce *trade-off* phénotypique pourrait être lié à l'effet génétique du gène *PNC1* qui contrôle à la fois la teneur en acide malique et la production de glycérol (Peltier *et al.*, 2021). Cependant la pénétrance de ce QTL est complexe et incomplète (Vion *et al.*, 2021) et l'effet de ce gène doit être validé par des études génétiques complémentaires.

L'impact de la nature des souches de levures fermentaires sur le pH des vins a été précédemment illustré sur deux moûts de raisins. Dans cette présente étude, nous confirmons le fort impact de souches *demalic* et *acidic* sur le pH des vins et mettons en évidence que la sélection génétique du MAC% entraîne des conséquences pléiotropiques sur les caractéristiques fermentaires des levures.

3.4.2. Les souches démaliquantes sont plus robustes aux variations de maturité technologique que les souches acidifiantes

L'évaluation du *MAC%* en fonction des matrices a permis de montrer que les souches démaliquantes sont robustes à la maturité technologique des moûts (sucre et acidité), (Figure supplémentaire 1). Sur les 10 souches étudiées, ce groupe consomme en moyenne 65 % de l'acide malique initial de manière assez invariante. En revanche, les souches acidifiantes sont plus sensibles à la composition du moût. Dans les jus

de raisin rouges peu concentrés en acide malique et avec une forte teneur en sucres fermentescibles (M17 et M21S) ces souches produisent de l'acide malique (*MAC%* négatif). Les souches les plus acidifiantes présentent un *MAC* de -100 % en M17, ce qui correspond à une production nette de 2 g/L.

Dans les jus de raisin blancs et les rouges ayant une maturité technologique faible, les souches acidifiantes préservent l'acidité de départ ou limitent à minima la consommation de l'acide malique du jus de raisin (*MAC* < 50 %).

La surproduction d'acide malique par les souches acidifiantes dans les matrices dites « chaudes » présente un intérêt certain pour pallier le manque de fraîcheur de ces matrices. Ce type de jus de raisin est de plus en plus fréquent dans les vignobles des régions chaudes (Rogiers *et al.*, 2022; Van Leeuwen *et al.*, 2019) et tend à devenir la norme du fait du changement climatique. L'utilisation de levains œnologiques capables de produire de l'acide malique lors de la fermentation alcoolique s'avère donc être un outil prometteur pour gérer le manque d'acidité de ce type de vins.

D'une manière générale, les souches acidifiantes présentent des capacités fermentaires plus faibles avec des temps de fermentation plus longs. Cette caractéristique est probablement une cause indirecte liée aux conditions fermentaires induites par l'abaissement du pH de la matrice fermentée. En effet l'impact du pH sur les cinétiques fermentaires a été particulièrement bien documenté en œnologie (Arroyo-López *et al.*, 2009; Ribéreau-Gayon *et al.*, 2006). Des expériences complémentaires visant à suivre les cinétiques fermentaires de telles souches dans un milieu à pH constant (ajout de base et acide en bioréacteur, contrôlé par ordinateur pour respecter un pH consigne) pourraient permettre d'établir le lien entre le pH du milieu et les cinétiques fermentaires. Il est également possible que certains QTLs impliqués dans la production d'acide malique soient directement responsables de la vitesse plus lente de telles souches. En effet le gène *PMA1* qui code la principale ATP-ase membranaire de la levure est directement associé au contrôle du *MAC%* (Vion *et al.*, 2021). Ce gène a un effet pléiotropique bien documenté et sa forme allélique acidifiante impacte négativement les cinétiques fermentaires des levures (Martí-Raga *et al.*, 2017; Peltier *et al.*, 2021, 2018b). Un autre aspect technologique intéressant des souches acidifiantes est leur rendement œnologique plus fort. Ce trait robuste à l'environnement (Figure S2) entraîne une légère diminution de l'éthanol qui peut être un atout dans le contexte de la vinification de matrices « chaudes », qui produisent des vins trop alcooleux.

3.4.3. Les souches démaliquantes impactent plus fortement le pH des vins

D'un point de vue technologique, le pH du vin est un paramètre très important qui influence à la fois la stabilité chimique et microbiologique des vins (Comuzzo and Battistutta, 2019). La figure 9 illustre les corrélations entre les valeurs finales de pH et d'acide malique pour les trois groupes de souches étudiées : acidifiantes, démaliquantes et *starters*. Pour les trois groupes, les corrélations sont fortement significatives.

L'impact des souches démaliquantes sur le pH est plus important que celui des autres groupes comme cela est illustré par la pente des droites de régression linéaire. Ce phénomène s'explique par l'effet tampon de l'acide malique dans le milieu. Plus cet acide faible est présent, moins l'ajout de base ou d'acide modifie le pH du milieu. La surconsommation de l'acide malique par ce groupe réduit l'effet tampon de la matrice fermentée et entraîne des variations de pH plus importantes. A l'inverse, la production d'acide malique par les souches acidifiantes renforce cet effet tampon, ce qui explique que les souches acidifiantes aient la capacité de mieux préserver le pH final des vins après la fermentation alcoolique.

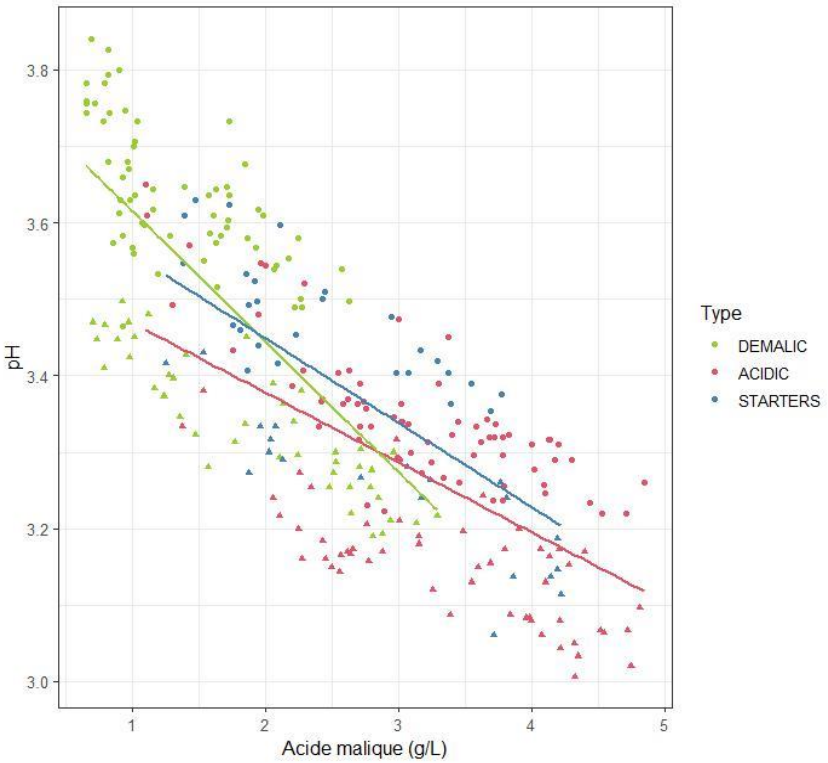


Figure 9. Valeurs de pH en fonction de l'acide malique (g/L) pour les trois types de souches, une analyse de corrélation réalisée pour les trois types de souches montre un effet très significatif (méthode de Spearman). Les lignes indiquent la régression linéaire du pH en fonction de la concentration en acide malique.

Données supplémentaires

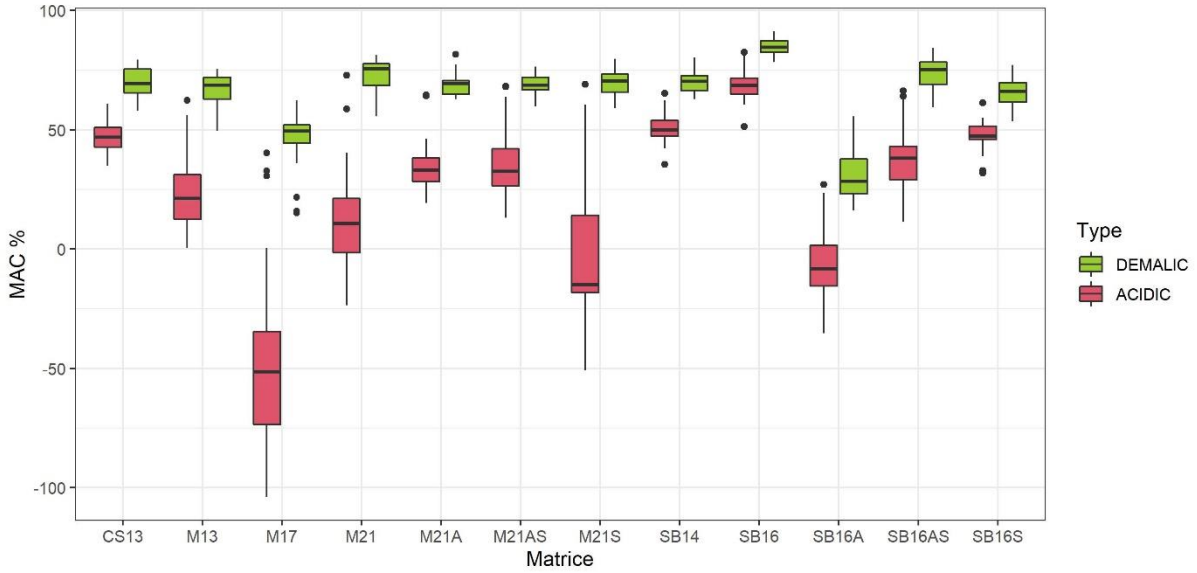


Figure S1. Représentation du *MAC%* en fonction des différentes matrices œnologiques. Les souches *demalic* sont représentés par les boxplots verts et les souches acidic par les boxplots rouges

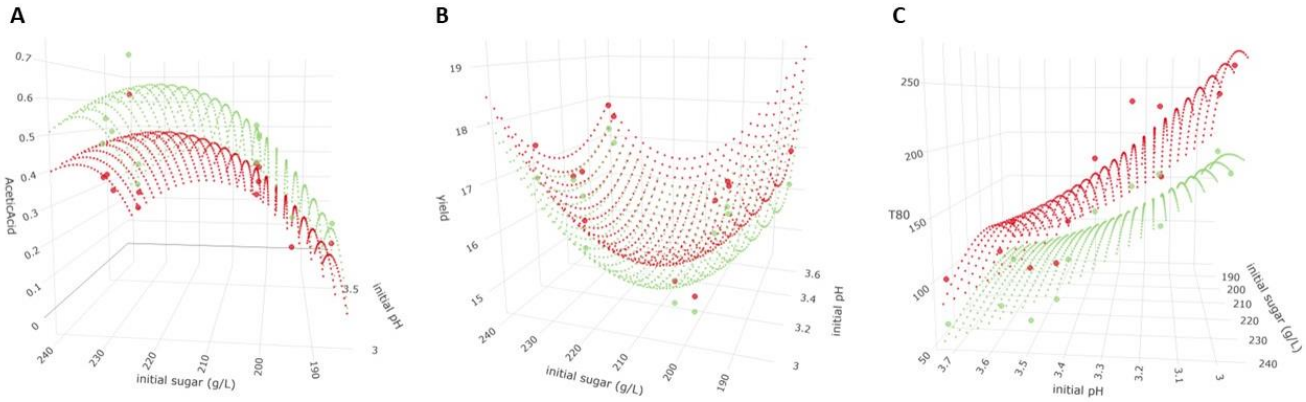


Figure S2. Normes de réaction de l'acide acétique (A), du rendement œnologique (g sucre / ° éthanol produit) (B) et du T80 (h) (C) pour les souches *demal* et *acidic* en fonction de la concentration en sucre initial (g/L) et du pH initial.

4. Evaluation sensorielle de l'impact de nouvelles souches extrêmes sur l'acidité perçue dans les vins

4.1. Problématique

L'objectif de cette partie est de valider si les vins produits avec des souches acidifiantes et démaliquantes présentent un profil organoleptique perçu comme différent par un panel de dégustateurs. Pour cela, deux nano-vinifications ont été réalisées à partir de moûts blancs et rouges avec cinq souches appartenant au groupe *acidic*, cinq souches appartenant au groupe *demalic*. A ces dix souches, deux souches industrielles représentant les bornes extrêmes d'un panel de 34 souches commerciales ont été ajoutées comme témoins (Peltier *et al.*, 2018a). Dans les cas des vinifications en blanc, l'utilisation des différentes souches module le niveau d'acide malique final. Lors de la vinification des vins rouges, la fermentation alcoolique est suivie d'une FML qui transforme l'acide malique en acide lactique. Dans ce cas, l'utilisation de levures modulant l'acide malique impacte indirectement la concentration finale du vin en acide lactique. L'analyse sensorielle des vins ainsi produits à partir d'une même matrice de départ permet d'établir si les différences de concentration en acide malique ou lactique sont perçues lors de la dégustation. L'analyse des douze vins blancs a été brièvement décrite dans l'article 2, elle sera reprise ici en incluant la démarche sensorielle générale mise en œuvre.

Une troisième nano-vinification a été réalisée sur un moût blanc avec trois souches appartenant au groupe des levures acidifiantes et trois souches au groupe des levures démaliquantes. Une troisième modalité a été générée en ajoutant aux vins produits avec les souches démaliquantes, une quantité d'acide malique correspondant à la différence entre les groupes acidifiants et démaliquants. Cette troisième vinification permet d'obtenir un groupe de vins à faible concentration en acide malique et deux groupes ayant une même concentration en acide malique. Le but est d'affiner l'analyse sensorielle des vins en évaluant la capacité d'un panel à distinguer les deux concentrations en acide malique mais également déterminer si les deux groupes avec la même concentration en acide malique sont perçus comme différents ou non.

4.2. Matériels et méthodes

4.2.1. Nano-vinifications et analyses effectuées

Les nano-vinifications ont été réalisées au laboratoire dans des bioréacteurs de 1,2 L. Les caractéristiques des moûts utilisés pour les trois expériences sont détaillées dans le tableau 4. Le dégagement de CO_2 a été suivi indirectement pas des pesées quotidiennes tout au long des fermentations. Les souches de *S. cerevisiae* utilisées sont référencées dans le tableau 5.

Dans le cas des deux fermentations sur Sauvignon blanc, les bioréacteurs ont été inoculés à $1x10^6$ cellules vivables/mL et placés à 18°C pendant 5 jours. Au cinquième jour, 30 mg N/L d'azote (sulfate d'ammonium) ont été ajoutés pour accélérer les cinétiques de croissance. Les bioréacteurs ont ensuite été

placés à 22°C au septième jour pour terminer rapidement les fermentations.

Pour la fermentation en rouge les bioréacteurs ont été inoculés à $1x10^6$ cellules vivables/mL et placés à 24 °C. A la fin des fermentations, les bioréacteurs sont placés 5 jours à 4°C. Les vins ont ensuite été soutirés pour éliminer les lies puis inoculés avec *Oenococcus oeni* (souche LACTOENOS® B7 Direct, Laffort, France) à 1 g/hL. Le suivi des FML a été effectué par dosage des acides lactique et malique quite à un prélèvement quotidien de 100 µL à la seringue (Kits Megazyme, K-LMAL-116A et K-LATE). A la fin des fermentations, différentes analyses œnologiques ont été réalisées : titre alcoolémique volumique (TAV), acidité totale (g/L H_2SO_4), concentration en acide malique (ou lactique) et pH. Une semaine avant les dégustations, les vins ont été sulfités (50 mg/L) afin de les préserver de l'oxydation et stockés à 12°C.

Tableau 4. Caractéristiques des moûts utilisés pour les nano-vinifications

	Nano-vinification n°1	**Nano-vinification n°2**	**Nano-vinification n°3**
Cépage	Sauvignon blanc	Cabernet sauvignon	Sauvignon blanc
Millésime	2020	2017	2022
Sucres réducteurs (g/L)	194	210	217
Acide malique (g/L)	4,49	3,95	4,00[a]
pH	3,31	3,57	3,38

[a] Le Sauvignon blanc 2022 a été supplémenté en acide malique. Sa concentration originale était 2,91 g/L

Tableau 5. Souches utilisées pour les nano-vinifications

Souche	**Type**	**Référence**
AC1_191	*Acidic*	(Vion *et al.*, 2022)
AC1_217	*Acidic*	(Vion *et al.*, 2022)
AC2_1A	*Acidic*	(Vion *et al.*, 2022)
AC2_467	*Acidic*	(Vion *et al.*, 2022)
AC3_22	*Acidic*	(Vion *et al.*, 2022)
FMGS_71	*Demal*	(Vion *et al.*, 2021)
FMGS_889	*Demal*	(Vion *et al.*, 2021)
FMGS2_107	*Demal*	(Vion *et al.*, 2021)
FMGS2_714	*Demal*	(Vion *et al.*, 2021)
FMGS4_263	*Demal*	(Vion *et al.*, 2021)
BO213	Industrielle	(Peltier *et al.*, 2018a)
RB4	Industrielle	(Peltier *et al.*, 2018a)

4.2.2. Test sensoriel : la tâche de tri

L'évaluation sensorielle des vins a été réalisée sous forme de tâches de tri avec les conseils de Sophie Tempère, Maître de conférences dans l'unité de recherche. Cette épreuve est une méthode qualitative qui consiste à regrouper des échantillons en fonction de leur similarité. Elle permet d'identifier s'il existe des propriétés sensorielles différentes entre des échantillons. Les vins sont présentés aux dégustateurs dans un ordre aléatoire et ces derniers doivent former des groupes présentant des similarités sensorielles communes (Barbe *et al*., 2021). Une tâche de tri peut être libre (processus holistique) ou dirigée (Chollet *et al.*, 2014). Pour une tâche de tri dirigée, un ou plusieurs critères d'évaluation sont donnés au panel pour former les groupes, à l'inverse de la tâche de tri libre qui ne présente pas de contrainte. Dans le cadre de notre étude, les dégustateurs ont été amenés à effectuer des tâches de tri libres puis dirigées, sur les mêmes séries d'échantillons. Lors des tâches de tri dirigées, il a été demandé aux dégustateurs de se baser uniquement sur la sensation d'acidité en bouche pour former les groupes. Il était impératif de former au minimum deux groupes et chacun des groupes devait contenir au moins deux vins. Les données ont été collectées sous forme de matrices de similarité et traitées par analyse statistique « *Multidimensional Scaling* » (MDS) (Faye *et al.*, 2011). Ce type de traitement des données permet de visualiser dans un espace à 2 dimensions le positionnement de chaque échantillon par rapport aux autres selon le résultat de la matrice de similarité (Cox, 2001).

4.2.3. Panels

Trois panels ont été constitués afin de participer aux tâches de tri sur les différentes matrices. Les participants sont tous des membres de l'unité de recherche Œnologie ou de la société Laffort et ont une implication différente dans la filière et une expérience de l'analyse sensorielle allant de sujet initié à sujet qualifié. Le panel était adapté à la réalisation d'une tâche de tri (Faye *et al.*, 2011) et chaque dégustateur a été familiarisé avec la procédure en amont du test.

Le premier panel est composé de 28 personnes âgées de 22 à 60 ans et constitué de 55 % de femmes. Ce panel a participé à l'évaluation sensorielle de 12 vins blancs.

Le second panel est composé de 27 personnes âgées de 22 à 60 ans et constitué de 63 % de femmes. Ce panel a participé à l'évaluation sensorielle de 12 vins rouges.

Le troisième panel est composé de 31 personnes âgées de 22 à 47 ans et constitué de 58 % de femmes. Ce panel a participé à l'évaluation sensorielle de 9 vins blancs.

4.2.4. Environnement de l'analyse sensorielle et matériels utilisés

Les dégustations ont eu lieu dans la salle d'analyse sensorielle de l'ISVV (Villenave d'Ornon) (NF EN ISO 8589 Mai 2010) et dans la salle de dégustation de la société Laffort (Floirac). La température des salles était contrôlée et les verres utilisés respectaient la norme ISO 3591 : 1977. Chaque verre était identifié à l'aide d'un numéro à 3 chiffres et l'ordre de présentation des échantillons randomisé entre les dégustateurs.

4.3. Résultats

Pour les nano-vinifications n°1 et 2, une première tâche de tri libre a été effectuée par les panels. En effet, les souches utilisées ont été sélectionnées en se basant principalement sur un trait phénotypique : le *MAC%*. Cependant, les programmes de croisements peuvent mener à la sélection d'autres caractères portés par les populations choisies. Si ces derniers ont un impact sensoriel, ils peuvent être décrits lors de la tâche de tri libre. Lors de cette tâche de tri, les dégustateurs avaient la possibilité de donner un ou plusieurs descripteurs, s'ils en identifiaient, justifiant le choix des groupes réalisés.

Un second exercice a ensuite été donné au panel. Il consistait à classer 4 vins ayant une concentration croissante en acide malique (ou lactique pour le second panel). L'objectif de cet exercice était d'initier tous les participants à l'impact de l'acide organique étudié sur la sensation en bouche. Après cet exercice, une tache de tri dirigée avec pour consigne de se baser uniquement sur la sensation d'acidité en bouche a été effectuée par les panels, sur les mêmes vins que ceux évalués lors de la tâche de tri libre.

4.3.1. Analyses physico-chimique des vins

L'analyse préalable des vins blanc et rouge a montré des différences significatives de pH, acidité totale et concentration en acide malique (ou lactique) entre les groupes de souches démaliquantes et acidifiantes (figure 10, tests Wilcox, p-value < 0.05). Ces différences significatives prouvent l'efficacité de la sélection de telles souches pour moduler l'acidité des vins.

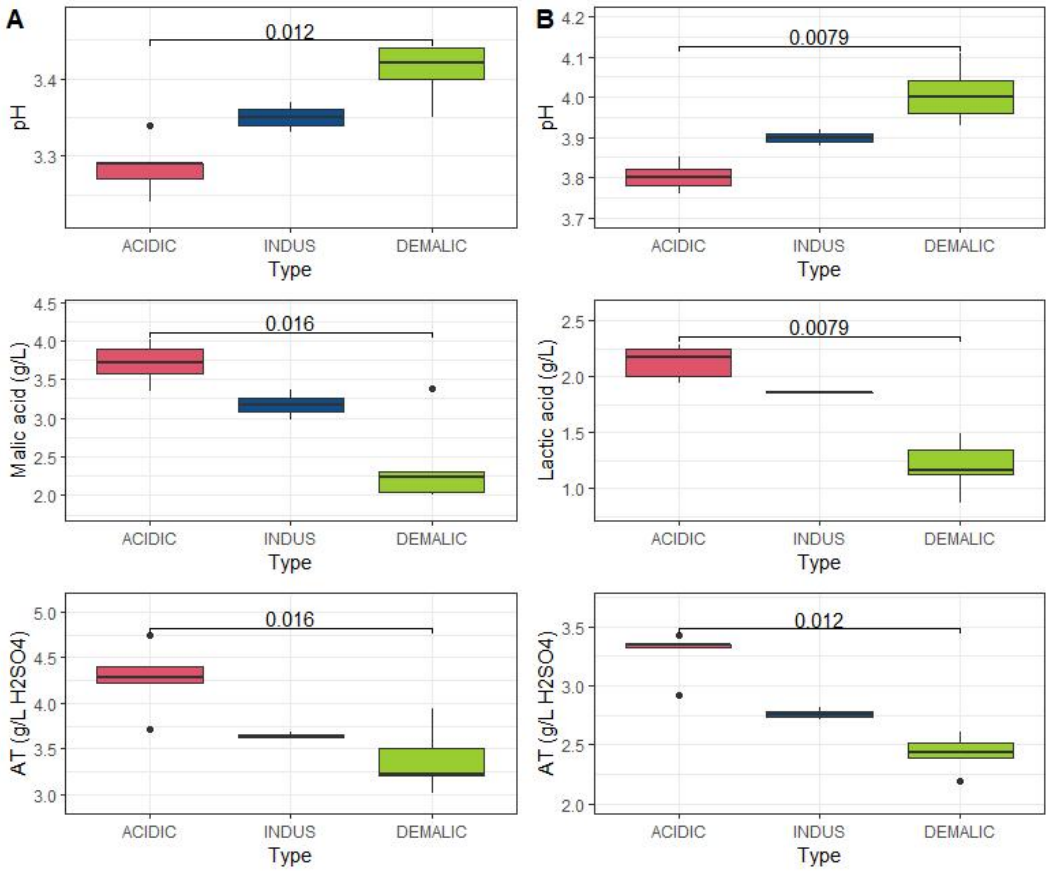


Figure 10. Analyses des vins blancs (**Panel A**) et rouges (**Panel B**). La p-value du test de Wilcox entre les groupes *acidic* et *demalic* est affichée sur les graphiques.

4.3.2. Analyses sensorielles

L'impact de ces différences physico-chimiques a ensuite été évalué au niveau sensoriel. Les réponses de chacun des dégustateurs ont permis de construire des matrices de similarité entre les vins. Plus deux vins ont été placés dans un même groupe par les dégustateurs, plus la valeur de la matrice entre ces deux vins est proche de 1. A l'inverse, si deux vins ont été très rarement placés dans le même groupe, la valeur de la matrice entre ces deux vins sera proche de 0.

La représentation sur deux dimensions des vins blancs montre que les vins ont été groupés en fonction du type de souche avec laquelle ils ont été vinifiés et discrimine particulièrement les groupes démaliquant et acidifiant (figure 11A). Les deux souches commerciales ont été placées de façon plus aléatoire entre les deux groupes, montrant une indécision du panel à percevoir leur impact sur la sensation d'acidité. Pour les vins rouges (figure 11B), la distinction entre les groupes de souche est moins nette mais reste significative (test de Kruskal-Wallis, p-value = 0.025 < 0.05 sur la dimension 1). La dégustation en rouge est rendue plus difficile du fait du caractère astringent des vins, amenant une fatigue sensorielle plus rapide du panel.

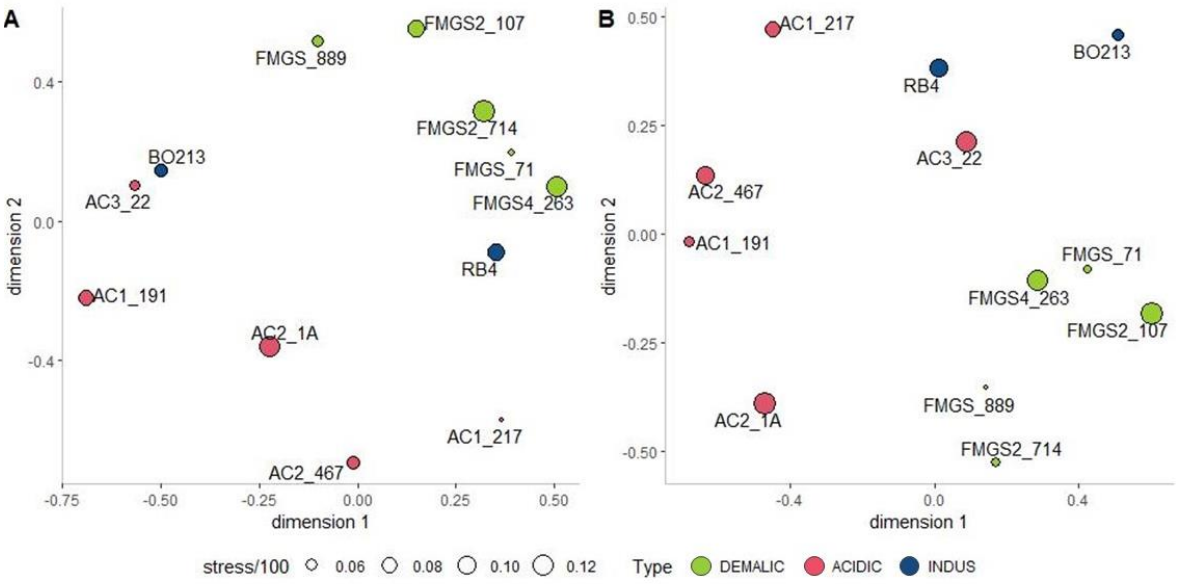


Figure 11: Position relative des vins blancs (**Panel A**) et rouges (**Panel B**) évalués lors d'une tâche de tri dirigée sur les deux premières dimensions d'une analyse MDS. Les vins sont représentés par des points qui sont positionnés de sorte que la distance entre deux points reflète la distance entre deux vins. L'aire de chaque point est proportionnelle au stress, indiquant la confiance de projection.

Pour les deux vinifications, les groupes de vins formés lors de la tâche de tri libre étaient sensiblement les mêmes que ceux formés lors de la tâche de tri dirigée (données non présentées). Les souches sélectionnées semblent donc impacter principalement la sensation d'acidité en bouche et aucune autre caractéristique ne se démarque.

Cette approche démontre que les vins produits avec des souches démaliquantes ou acidifiantes ont des profils d'acidité différents qui sont clairement perçus par les dégustateurs.

4.3.3. Effet de la concentration en acide malique vs effet des souches utilisées

Une troisième nano-vinification en Sauvignon blanc avait pour objectif de confirmer la distinction entre la perception acide des vins blancs produits à partir de souches acidifiantes et démaliquantes. Cette troisième modalité a permis d'ajouter un contrôle en corrigeant la teneur en acide malique des vins produits avec les levures démaliquantes. Cette teneur est ramenée au niveau observé chez les souches acidifiantes. Ainsi, ce dernier groupe de vins possède la même concentration en acide malique que le groupe *acidic* et présente un pH légèrement différent (figure 12A, test de Student, p-value < 0,05). L'ajout d'acide malique en fin de fermentation n'a donc pas complètement compensé les écarts d'acidité entre ces vins.

Pour cette étude, une seule tâche de tri dirigée a été effectuée par le panel en précisant que le tri devait être fait sur la base de la perception d'acidité en bouche. L'analyse MDS montre une séparation des trois groupes de souches (Figure 12B). Un test de Kruskal-Wallis effectué sur la dimension 1 permet de montrer

que les trois groupes sont significativement distincts (p-value = 0,027 < 0,05).

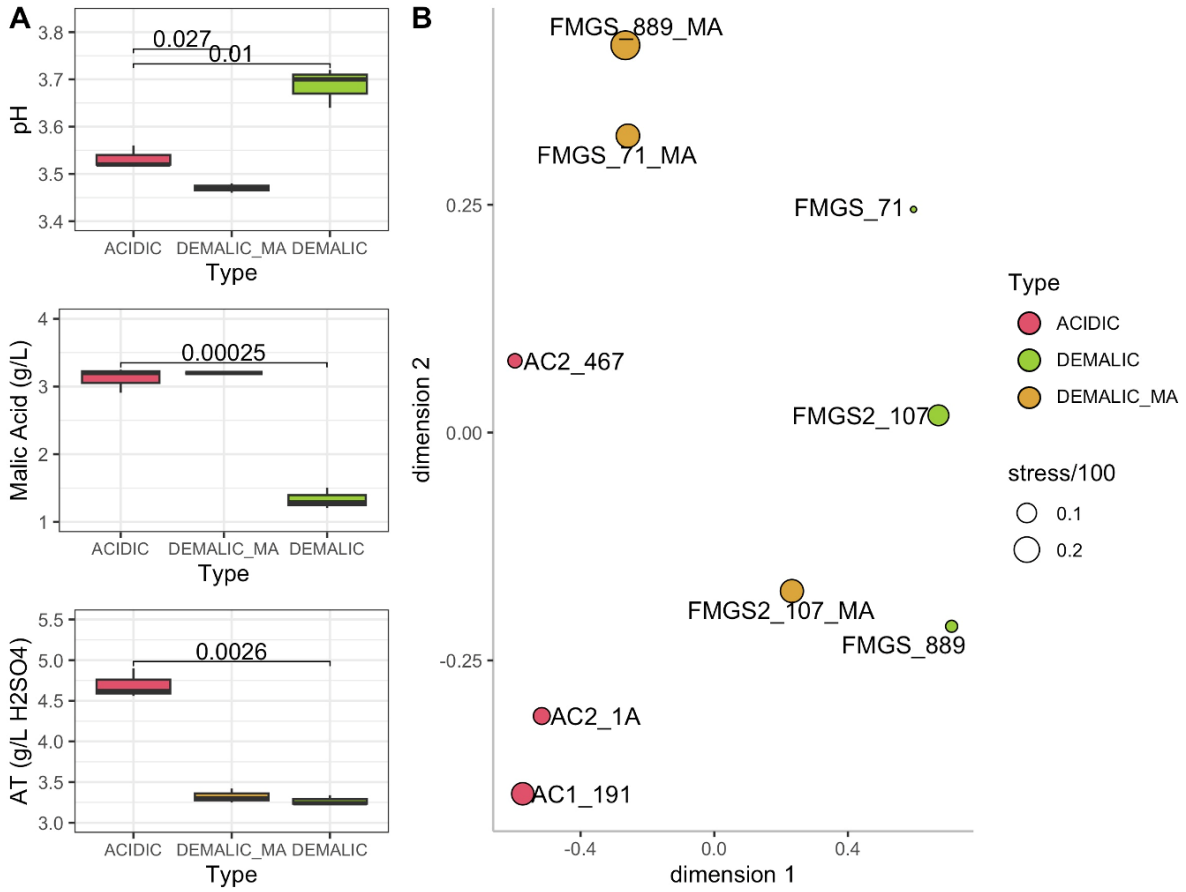


Figure 12. Résultats de la troisième nano-vinification. **Panel A.** Valeurs de pH, concentration en acide malique et acidité totale pour les trois groupes de souche. Le groupe noté *demalic_ma* correspond aux vins produits avec les souches démaliquantes et supplémentés en acide malique en fin de fermentation alcoolique. La p-value du test de Student est affichée pour la comparaison entre les groupes *acidic* et *demalic* et entre le groupe *acidic* et *demalic_ma* pour le pH. **Panel B.** Position relative des vins évalués lors d'une tâche de tri dirigée sur les deux premières dimensions d'une analyse MDS. Les vins sont représentés par des points qui sont positionnés de sorte que la distance entre deux points reflète la distance entre deux vins. L'aire de chaque point est proportionnelle au stress, indiquant la confiance de projection.

L'ajout d'acide malique au groupe de levures démaliquantes permet un décalage vers la droite de ces souches sur l'axe 1 de la MDS. Ce décalage a lieu vers le nuage de souches acidifiantes et signifie de manière indirecte que l'image sensorielle acide est augmentée. Cette expérience valide bien que l'ajout de 2 g/L d'acide malique a un effet sur la perception d'acidité pour les souches démaliquantes. Cependant la correction d'acidité ne permet pas de confondre parfaitement les souches *acidic* et *demalic_ma*. A concentration égale en acide malique, (3,2 g/L) les vins *acidic* et *demalic_ma* présentent un pH légèrement différent (3,47 et 3,53). Cette variation de pH de 0,06 unités est faible et n'explique pas à elle seule cette différenciation. La distinction de perception entre ces deux groupes pourrait être également liée à la

production d'autres acides organiques. En effet, le groupe de souches démaliquantes a produit environ deux fois plus d'acide acétique que le groupe des souches acidifiantes (0,42 g/L vs 0,19 g/L), ce qui a probablement été perçu par les dégustateurs. La production d'acide succinique était également supérieure de 0,2 g/L en moyenne au sein du groupe des acidifiantes ce qui a pu renforcer la perception acide des vins produits avec ces souches. De plus, les souches utilisées pour la FA étant différentes, d'autres éléments de différenciation ont pu être indirectement perçus par les dégustateurs malgré une dégustation orientée sur l'acidité.

5. Evaluation au chai de deux souches acidifiantes

Le potentiel œnologique de deux souches extrêmes a été évalué lors d'essais préliminaires au chai. Pour cela, des vinifications ont été conduites en moûts rouge et blanc sur deux souches de *S. cerevisiae* sélectionnées au laboratoire. Ce type d'essai permet d'évaluer, dans un contexte œnologique, les capacités fermentaires et acidifiantes des souches afin d'évaluer leur potentiel commercial. La souche acidifiante AC1_191 a été choisie car elle a terminé la fermentation alcoolique dans l'ensemble des modalités de la partie 3 de cette étude. Cependant d'autres essais menés au laboratoire ont indiqués qu'elle présente quelques faiblesses fermentaires (Vion *et al.*, 2022). De ce fait, une autre souche a été construite (« BABY »). Cette souche est plus robuste au niveau fermentaire mais produit légèrement moins d'acide malique. Ces deux levures acidifiantes ont été comparées à une souche témoin commerciale (Zymaflore®F15 en rouge, Zymaflore®X5 en blanc) présentant en moyenne des *MAC%* de 30%.

5.1. Description de l'essai et analyse des moûts

Les vinifications ont été conduites sur les vendanges de l'années 2022, au chai expérimental WEC (Wine Experimental Cellar) de la société Biolaffort. Le tableau 6 résume les conditions des essais et la composition physico-chimique des moûts utilisés.

Les levains liquides ont été préparés au laboratoire dans des bioréacteurs de 400 mL en régime aérobique par Margaux Bernard, technicienne de l'équipe. Avant ensemencement, la viabilité a été déterminée par cytométrie en flux. Pour les vinifications en rouge, la fermentation alcoolique a été suivie d'une fermentation malolactique avec ensemencement d'une souche d'*Oenococcus oeni* (Lactoenos® B7 Direct).

Tableau 6. Détails des essais et composition des moûts.

Moût	**Merlot**	**Sauvignon Blanc**
Origine	Château Cantegrill 2022	
TAP (rendement théorique)	14,3 % VOL (16,83 g/%/vol)	12 % vol (16,83 g/%/vol)
pH	3,53	3,41
AT (g/L H_2SO_4)	2,28	2,96
Acide L-malique (g/L)	1,12	1,67
SO_2 total (mg/L)	61	20
SO_2 libre (mg/L)	17	<LQ
Azote assimilable (mg N/L)	122	158
Volume de chaque modalité	1 hL (120 kg de vendange)	1,6 hL
Contenant	Cuves inox 2 hL	
Lieu de vinification	WEC	
Modalités	Zymaflore®F15 (LSA)	Zymaflore®X5 (LSA)
	AC1_191 (levain liquide)	
	BABY (levain liquide)	
Sulfitage du moût	4 g/hL	5 g/hL
Niveau de levurage	20 g/hL (LSA) ou 2.10^6 cell/mL (levain liquide)	
Utilisation d'un produit de réhydratation/autre	20 g/hL Superstart®Rouge, même avec les levains liquides + Lafase HE Grand Cru 3 g/hL	20 g/hL Superstart®Blanc, même avec les levains liquides
Nutrition azotée	DAP 30 g/hL au 1/3 de FA	23 g/hL au 1/3 de FA

5.2. Résultats des essais

L'ensemble des vins présentés dans cet essai ne présentent pas de traces de sucres résiduels au bout de 10 jours de fermentation. La levure AC1_191 présente cependant une cinétique fermentaire plus lente, notamment en fin de fermentation alcoolique (données non montrées).

Tableau 7. Paramètres œnologiques des vins en fin de fermentation alcoolique. Les valeurs mise en avant en bleu gras sont celles qui diffèrent de la souche témoins pour chaque vinification.

	Merlot			Sauvignon Blanc		
	F15	**AC1_191**	**BABY**	**X5**	**AC1_191**	**BABY**
TAV (%vol)	13,62	**13,32**	**13,39**	12,72	12,71	12,8
Glu + Fru (g/L)	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
AT (g/L H_2SO_4)	4,71	**6,03**	**5,92**	3,88	**4,26**	3,85
pH	3,48	**3,39**	**3,41**	3,31	**3,24**	3,3
AV (g/L H_2SO_4)	0,22	< LQ	0,14	0,48	**<LQ**	**0,15**
Acide L-malique (g/L)	1,19	**2,23**	**2,15**	1,46	**2,06**	**1,76**
Acide L-lactique (g/L)	<LQ	< LQ	< LQ	< LQ	< LQ	< LQ
Glycérol (g/L)	9,68	9,8	9,77	5,84	**7,16**	**7,42**
SO_2 total (mg/L)	< LQ	< LQ	< LQ	17	15	< LQ

En rouge, les souches AC1_191 et BABY produisent 1,04 et 0,96 g/L d'acide malique supplémentaire par rapport à la souche commerciale témoin. Elles impactent le pH de 0.08 unités en moyenne et augmentent l'acidité totale de 1,26 g/L (exprimée en g/L H_2SO_4). Leur impact est également retrouvé sur le TAV qui est abaissé de 0,3 % vol avec AC1_191 et 0.23 % vol avec BABY.

En blanc, la souche AC1_191 semble avoir plus d'effet que la souche BABY. Elle produit 0,6 g/L d'acide malique supplémentaire par rapport à la souche commerciale témoins contre 0,3 g/L pour BABY. Cette dernière n'impacte pas le pH final du vin par rapport à la souche commerciale.

Après la fermentation alcoolique, les vins rouges sont ensemencés avec une souche commerciale d'*Oenococcus oeni* afin de réaliser la FML. Les paramètres œnologiques de ces vins post-FML en lien avec l'acidité sont présentés dans le tableau 8. Il en ressort que les deux levures acidifiantes permettent d'augmenter d'un facteur deux la teneur en acide lactique des vins. Cette différence de production d'acide lactique impacte sensiblement le pH et l'AT des vins.

Tableau 8. Paramètres chimiques des vins rouges post-FML en lien avec l'acidité

	F15	AC1_191	BABY
AT (g/L H_2SO_4)	4,28	**5,08**	**5,02**
pH	3,59	**3,49**	**3,52**
AV (g/L H_2SO_4)	0,29	0,18	0,2
Acide L-malique (g/L)	< LQ	< LQ	< LQ
Acide L-lactique (g/L)	0,63	**1,21**	**1,2**

Les FML ont été suivies par dosage de l'acide malique régulier après ensemencement. De manière triviale, plus la concentration initiale en acide malique est importante, plus l'achèvement de la FML

nécessite du temps. Cependant l'intégralité de l'acide malique a été transformé en acide lactique dans les trois vins et les cinétiques de dégradation sont parallèles (Figure 13).

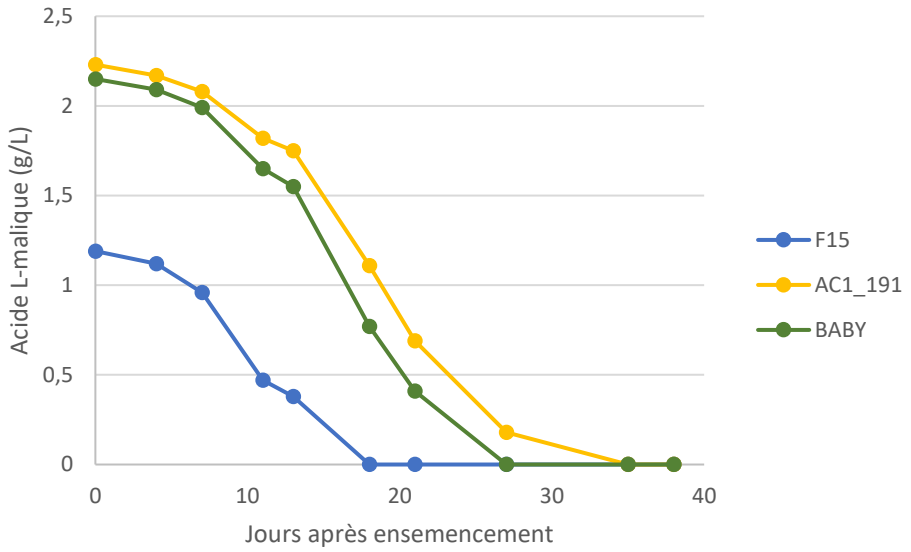


Figure 13. Evolution de la concentration en acide malique (g/L) après encensement de *Oenococcus oeni*.

Cet essai permet de conclure positivement sur la pertinence des deux souches acidifiantes AC1_191 et BABY pour la vinification d'un moût rouge présentant une faible acidité initiale. Ces souches ont non seulement un pouvoir acidifiant, mais également la capacité de réduire légèrement le degré d'alcool par rapport à un témoin commercial. Ces résultats à l'échelle du chai corroborent ceux observés dans la partie 3 de ce chapitre, à l'échelle du laboratoire.

Conclusion du chapitre 1

Dans ce premier chapitre, la sélection de souches de levures de *S. cerevisiae* pour leur capacité à moduler le niveau d'acide malique en fermentation alcoolique, ainsi que la caractérisation de ces panels de souches au niveau phénotypique et sensoriel ont été étudiés. Les principaux éléments à retenir sont les suivants :

- La sélection assistée par marqueurs génétiques a permis de faire émerger des souches capables de consommer jusqu'à 80% de l'acide malique présent dans les jus de raisin. Ces souches, issues de croisements, sont enrichies en allèles induisant une consommation d'acide malique.
- Des souches acidifiantes ont été sélectionnées. Elles peuvent produire jusqu'à 3 g/L d'acide malique supplémentaire à l'issue de la fermentation alcoolique.
- Les souches démaliquantes sont robustes à leur environnement : elles consomment des quantités stables d'acide malique, quel que soit la concentration initiale. En revanche, les souches acidifiantes sont plus impactées par le milieu : plus la maturité technologique est élevée, plus la production d'acide malique par ces souches est importante.
- Des différentes de *MAC%* ont cependant été observées dans des moûts à concentrations en acide malique initial similaires. Ce résultat suggère que d'autres facteurs abiotiques non mesurés ici ont un impact sur la modulation de cet acide organique par les souches.
- La sensation acide en bouche des vins produits par ces deux types de souches est perçue différemment à la dégustation, en blanc et en rouge.
- Un premier essai au chai de deux souches acidifiantes sur les vendanges 2022 a permis de montrer la pertinence de l'utilisation de ces souches sur des matrices rouges « chaudes ».

Chapitre 2 : Etude des variations métaboliques inter- et intraspécifiques de souches du genre *Saccharomyces*

Chapitre 2 : Etude des variations métaboliques inter- et intraspécifiques de souches du genre *Saccharomyces*

1. Contexte

L'objectif de cette deuxième partie était d'explorer la variabilité phénotypique de souches de levures avec un focus spécifique sur leur capacité à consommer ou à produire de l'acide malique au cours de la vinification. Dans ce contexte, des données métaboliques ont été collectées sur un large panel de souches suspectées de dégrader ou préserver l'acide malique. Cette exploration a nécessité le développement de protocoles expérimentaux nouveaux pour l'unité de recherche, afin de caractériser de manière plus exhaustive les matrices fermentées. Jusqu'à la réalisation de ce travail, mon équipe avait mis en place des tests enzymatiques permettant d'étudier en routine un nombre relativement limité de composés en fin de fermentation alcoolique. L'avantage des tests enzymatiques est de permettre un dosage standardisé offrant un large niveau de parallélisation, dans de volumes faibles (inférieur au millilitre) ce qui est compatible avec les approches de nano-fermentations réalisées au laboratoire (Peltier *et al.*, 2018a). Cependant, une petite dizaine de composés seulement sont quantifiables et chaque composé nécessite un dosage spécifique.

Nous avons entrepris de développer une méthode plus exhaustive en conservant un faible volume d'analyse et un niveau de parallélisation satisfaisant. Pour ce faire, nous avons utilisé la spectroscopie par RMN du proton en collaborant avec nos collègues Tristan Richard et Grégory de Costa de l'axe MIB de l'unité de recherche. En ce qui concerne l'analyse des vins, notre protocole a été adapté d'une méthode déjà en place au sein de l'unité de recherche (Gougeon *et al*., 2018; Le Mao *et al*., 2021). Cette technique quantitative est un outil puissant permettant d'accéder à un plus grand nombre de composés appartenant à des familles chimiques différentes (Le Mao *et al.*, 2023). La mise en application de ce protocole a permis de caractériser le niveau de production de 15 métabolites du vin par un panel d'une cinquantaine de souches de levures dans plusieurs matrices (Article 3).

De plus, une méthode d'extraction des métabolites intracellulaires des levures a été mise au point, permettant de quantifier de manière reproductible une vingtaine de composés intracellulaires sur un panel de 17 souches de levures. Les résultats obtenus dans cette seconde expérience sont présentés dans l'article 4.

2. Etat de l'art sur l'analyse métabolomique par RMN ^{1}H

2.1. Principe de la RMN du proton

La spectroscopie Résonnance Magnétique Nucléaire (RMN) est une technique d'analyse utilisée en chimie, biochimie et physique pour l'identification et la caractérisation de composés moléculaires. Le

principe de base de la RMN est de faire interagir les noyaux atomiques avec un champ magnétique extérieur. Dans le cas de la RMN du proton, la résonnance des noyaux d'hydrogène présents dans les molécules est étudiée. Les protons (^{1}H) présentent une propriété physique appelée spin nucléaire, tout comme d'autres noyaux : ^{13}C,^{15}N, ^{17}O, ^{19}F, ^{31}P. Lorsque ces noyaux sont placés dans un champ magnétique extérieur, leur spin peut être aligné soit dans le sens du champ magnétique (état bas en énergie), soit dans le sens opposé (état haut en énergie) (Figure 14).

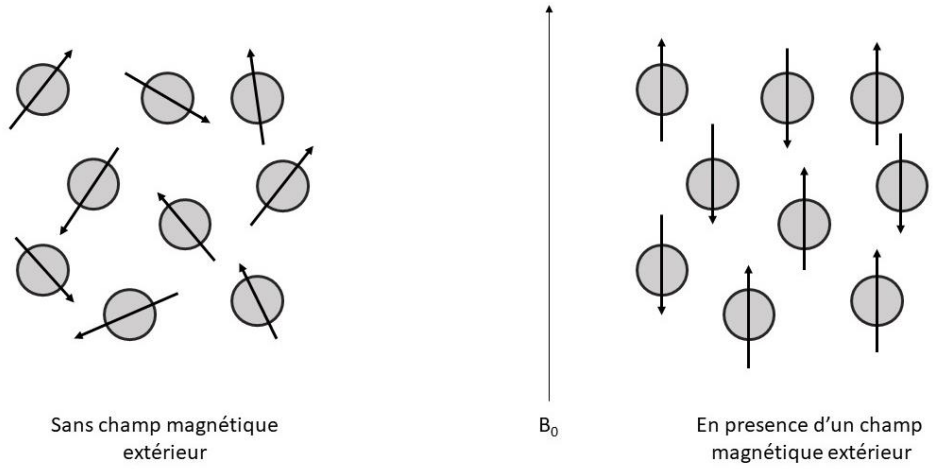


Figure 14. Alignement des protons dans le champ magnétique externe B_0.

Lorsque l'on applique une onde radiofréquence (RF) à la molécule, cela va perturber l'alignement des spins des protons dans le champ magnétique, les protons vont passer de l'état bas en énergie à l'état haut en énergie. Ce phénomène est appelé résonnance. Lorsque l'onde radiofréquence est arrêtée, il n'y a plus d'excitation et les protons retournent à leur état initial. Ce phénomène est appelé relaxation. Ces deux phénomènes induisent une variation de l'aimantation macroscopique. Cette variation peut être détectée à l'aide d'une antenne réceptrice (la sonde RMN) par induction électromagnétique. Le signal recueilli est appelé FID (*Free Induction Decay*) ou encore signal de précession libre, en français. La mesure du signal en RMN est donc liée à un dispositif d'émission/réception. La séquence de base pour la spectroscopie RMN du proton est présentée en Figure 15. Elle est constituée d'une impulsion RF suivi du recueil de la FID. Le signal mesuré est complexe : il contient plusieurs fréquences en fonction des noyaux étudiés et de leur environnement. Le traitement du signal par transformée de Fourier permet d'obtenir le spectre RMN à partir du FID.

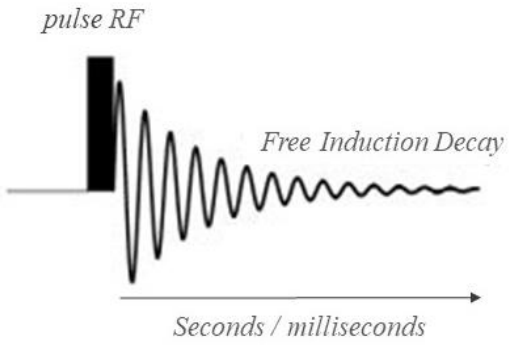


Figure 15. Séquence de base pour la spectroscopie RMN. Après l'impulsion RF, le signal est détecté pendant la durée du FID

Dans les analyses métabolomiques par RMN, le principal noyau observé est le noyau d'hydrogène, ^{1}H, du fait de sa présence dans la quasi-totalité des composés d'intérêt et de sa forte abondance isotopique naturelle (99,98 %) (Hatzakis, 2019). Chaque proton d'une molécule génère un pic sur le spectre RMN à une fréquence qui dépend de son environnement chimique. Cette fréquence est appelée déplacement chimique (δ) et s'exprime en parties par million (ppm). La présence de liaisons chimiques (et donc de protons voisins) induit la formation de multiplets. Cette multiplicité du signal est caractérisée par une constante de couplage, notée *J*, qui exprime l'écart entre les pics d'un multiplet en Hz. L'aire du pic est proportionnelle au nombre de spins qui résonnent à cette fréquence au sein de l'échantillon. La mesure de cette aire permet de déterminer la concentration moléculaire d'un métabolite.

2.2. Applications de la métabolomique par RMN en science des aliments

L'analyse chimique exhaustive d'une matrice associée à un traitement statistique des données par des approches multivariées est la base de la métabolomique. La métabolomique est, par définition, l'identification et la quantification de l'ensemble des métabolites d'une cellule, d'un organite, d'un tissu, d'un organe, ou d'un organisme à un temps donné et dans des conditions données. Les techniques majeures actuellement utilisées pour l'analyse métabolomique sont la spectrométrie de masse et la RMN (Verpoorte *et al.*, 2008).

La RMN est utilisée dans l'analyse des aliments depuis plusieurs décennies, avec un essor important depuis la fin des années 80. Cela est dû notamment à une préoccupation croissante des consommateurs pour la sécurité des aliments et leur origine. La RMN du proton est utilisée pour l'authentification et la classification (Marcone *et al.*, 2013), le contrôle qualité (Minoja and Napoli, 2014), l'évaluation de molécules sensorielles (Malmendal *et al.*, 2011) et la compréhension des mécanismes moléculaires et interactions des composés alimentaires (Fernandes *et al.*, 2015). Le développement des techniques de suppression des solvants ainsi que les avancées technologiques dans le traitement des données ont permis l'émergence des applications de la RMN du proton à la science des aliments qui était auparavant considérée

comme trop coûteuse, et pas assez résolutive (Hatzakis, 2019). Ainsi, la RMN peut être appliquée à l'étude des boissons fermentées telles que le cidre (Cusano *et al.*, 2020) ou le vin *(Gougeon et al., 2018; Le Mao et al.*, 2021). Dans le cas de Cusano *et al.* (2020), la RMN a été utilisée pour établir le profil métabolique de jus de pommes fermentés avec différentes espèces de levures. Les aliments et boissons constituent des matrices complexes, contenant des centaines voire des milliers de composés. Des analyses multivariées sont donc très souvent nécessaires pour appréhender simultanément l'ensemble des variables (Hatzakis, 2019).

3. Etude des variations métabolomiques chez des populations de levures du genre *Saccharomyces* : analyse des vins par RMN du proton

Cette sous-partie a fait l'objet d'un article scientifique soumis dans le journal *Food Microbiology*. La conceptualisation des expériences a été réalisée par Philippe Marullo (PM) et moi-même. Le génotypage microsatellite a été réalisé par Maïtena Muro. J'ai réalisé les fermentations alcooliques au laboratoire et préparé puis analysé les échantillons en RMN avec l'aide d'Inès Le Mao et Grégory Da Costa. J'ai participé à l'identification des molécules à partir des spectres RMN avec l'aide d'Inès Le Mao et Grégory Da Costa, puis traité les données générées avec le logiciel R sous la supervision de PM. Enfin, j'ai participé à l'écriture du manuscrit avec PM et Tristan Richard a relu et complété le document final.

Targeted ^{1}-H-NMR wine analyses revealed specific metabolomic signatures of yeast populations belonging to the *Saccharomyces* genus.

Vion Charlotte[1,2], Le Mao Inès[2], Nadine Yeramian[3], Muro Maïtena[1,2], Da Costa Gregory[2], Richard Tristan[2], Marullo Philippe[1,2]

[1]Biolaffort, Bordeaux, FRANCE

[2] UMR 1366 Œnologie, Université de Bordeaux, INRAE, Bordeaux INP, BSA, ISVV

[3]Microbiology Division, Department of Biotechnology and Food Science, Faculty of Science-University of Burgos, Spain

Abstract

This study aimed to explore the non-volatile metabolomic variability of large panel of strains (44) belonging to the *Saccharomyces cerevisiae* and *Saccharomyces uvarum* species in the context of the wine alcoholic fermentation. For the *S. cerevisiae* strains *flor*, *fruit* and *wine* strains isolated from different anthropic niches were compared. This phenotypic survey was achieved with a special focus on acidity management by using natural grape juices showing opposite level of total acidity. A ^{1}H NMR spectroscopy assay was developed for quantifying fifteen wine metabolites that showed important quantitative variability within the strains. Thanks to the robustness of the assay and the low amount of sample required this tool is relevant for the analysis of the metabolomic profile of numerous wines. The *S. cerevisiae* and *S. uvarum* species displayed significant differences for malic, succinic, and pyruvic acids, as well as for glycerol and 2,3-butanediol production. As expected, *S. uvarum* also showed weaker fermentation fitness, but showed interesting acidifying properties. In addition, the three groups of *S. cerevisiae* strains showed different metabolic profiles that were mostly due to their production and consumption of organic acids. More specifically, *flor* yeast consumed more malic acid and produce more acetic acid than the other *S. cerevisiae* strains which was never reported before. These features might be linked to the ability of *flor* yeasts to shift their metabolism during wine oxidation.

Key words

^{1}H-NMR, fermenting yeast, wine metabolome, malic acid, flor and wine yeast strain.

Introduction

Metabolic activities of microorganisms strongly impact the chemical composition of fermented products and modify their nutritional and organoleptic properties [1,2]. Thus, the quantification of chemical compounds in fermented goods is a critical step to understand the role of microbes and to control their development. In this context, wine alcoholic fermentation has been particularly well investigated, and many wine compounds were identified using Gaz or Liquid Chromatography coupled with Mass and NMR spectroscopies [3–6]. As widely reviewed [7–9], the yeast metabolism impacts the chemical composition of resulting wines with direct enological consequences. Although they broadly share the same metabolic pathways, yeast species and strains show a high variability in their metabolites production. Thus, yeast species involved in grape juice fermentation are characterized by specific metabolic signatures for primary metabolites [10,11], fermentative esters [12–14], and off-flavor compounds [15]. In mixed cultures, metabolic profiling is also useful for characterizing microbial interactions [16–18] that may impact wine complexity [19]. At the intra specific level, analytical chemistry methods are also decisive for driving yeast selection aiming to better control ethanol content [20], wine acidity [10,21], volatile thiols content [22] or fermentative esters production [13].

The natural genetic variability of the species *Saccharomyces cerevisiae* [23] constitutes an important source of metabolomic variability that has been deciphered for volatile [24,25] and non-volatile compounds [26,27] by applying quantitative genetic. These studies required the use of analytical methods such as HPLC [28], enzymatic assays [26], targeted GC-MS [24] allowing the quantification, for large sample sizes of wine metabolites belonging to different chemical family. Alternatively, untargeted metabolomics approaches may be applied for quantifying hundreds of metabolic features able to discriminate the biochemical signature of few *Saccharomyces cerevisiae* strains during the alcoholic fermentation [29][5]. However, the exact identification of such discriminating compounds remains a chemical challenge [30].

In this context, the exploration of extracellular metabolomic variability of yeast strains during the alcoholic fermentation for many classes of compounds constitute a challenging task. A performing and versatile analytical technique to quantify yeast metabolites is the proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy [31,32]. The main ^{1}H NMR spectroscopy assets are the simplicity of sample preparation and high reproducibility of the quantification. It is also possible to investigate a large range of metabolites belonging to different chemical families that are displayed on the same spectrum. This non-destructive technique also gives the opportunity to carry out several analyses on the same sample. NMR spectroscopy is quantitative since the signal intensity is directly proportional to the metabolite concentration and the number of nuclei in the molecule [33,34]. Despite its high potentiality to study fermented products, ^{1}H NMR investigations are still scarce and face two major challenges [31]. First, the sensitivity of the technique is lower than mass spectrometry which explains that the latter is often preferred for non-targeted analyses.

This issue can be addressed by applying a high number of scans during the analysis, but this will extend the time required for the analysis. Secondly, peak overlaps from multiple metabolites pose major challenges. 2D NMR or supplementation with pure compounds of interest can be carried out to address this issue [35].

In the present study, we explored the metabolomic variability of different wine yeasts with a special focus on the management of wine acidity by malic acid. Indeed, the level of this organic acid tends to drastically decrease in grape juices due to climate change with important enological consequences [36]. As previously reported by several authors, strains and species of the *Saccharomyces* genus may modulate malic acid concentrations of wines [37–43]. Recently, pools of alleles belonging to *flor* or *wine* yeast populations were partially linked to the metabolic variability of malic acid [44] suggesting a possible effect of the yeast ecological origin. In this context, we reevaluated the natural variability of several *Saccharomyces* strains presenting different level of malic acid production or consumption during wine fermentation. In order to have a wide overview of possible metabolic trade-off related to malic acid metabolism, we developed new protocols aiming to quantify extracellular yeast metabolites using ^{1}H-NMR based metabolomics, adapting methods already used for describing wine composition [45][46].

1. Materials and Methods

1.1. Yeast strains used and culture methods

The forty-four strains of *S. cerevisiae* and *S. uvarum* isolated in different enological niches are listed in Table 1. *S. cerevisiae* strains were propagated on YPD 2 % (1 % peptone, 1 % yeast extract, 2 % glucose) at 30°C in both liquid and plate cultures (2 % agar). *S. uvarum* strains were propagated on YPD 6 % to avoid sporulation as described by [47]. Long term storage at -80°C was achieved by adding one volume of glycerol to YPD overnight cultures.

Table 1: Yeast strain used

Strain	Species	Ecological niche	Substrate/origin	Geographical area	Collection	Reference
FMGS1_889	*S. cerevisiae*	Control	Extreme malic acid consuming strain	Miscelaneous (Breeding)	UMR Oenology (ISVV)	[21]
FMGS3_191	*S. cerevisiae*	Control	Extreme malic acid producing strain	Miscelaneous (Breeding)	UMR Oenology (ISVV)	[70]
14.280 (SV2)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	[71]
23.10 (S34V)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	[71]
34.220 (SV3)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	[71]
36.2J (S4V)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	[71]
8.1J (S6V)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	[71]
DBVPG4695	*S. cerevisiae*	Flor	Vino Santo	Lungarotti winery, Italy	1002 genomes	[23]
CBS4079	*S. cerevisiae*	Flor	Velum	Spain	1002 genomes	[23]
CBS4092	*S. cerevisiae*	Flor	Velum	Spain	1002 genomes	[23]
CBS4093a	*S. cerevisiae*	Flor	Velum	Spain	1002 genomes	[23]
Y-1301	*S. cerevisiae*	Flor	Wine	Unknown	NRRL collection	
YB-210	*S. cerevisiae*	Fruit	Spoiled banana	Costa Rica	NRRL collection	
Y-747	*S. cerevisiae*	Fruit	Cider	Illinois, USA	NRRL collection	
YB-1191	*S. cerevisiae*	Fruit	Citrus juice	Louisiana, USA	NRRL collection	
Y-6678	*S. cerevisiae*	Fruit	Olives	Spain	NRRL collection	
Y-2230	*S. cerevisiae*	Fruit	Fruit juice	The Netherlands	NRRL collection	
YB-360	*S. cerevisiae*	Fruit	Applesauce	Unknown	NRRL collection	
YB-2541	*S. cerevisiae*	Fruit	Benzolated cider at 18C	Unknown	NRRL collection	
Y-641	*S. cerevisiae*	Fruit	Cider	Unknown	NRRL collection	
Y-35	*S. cerevisiae*	Fruit	Fruit (Ilex aquifolium)	Unknown	NRRL collection	
Y-6275	*S. cerevisiae*	Fruit	Orange concentrate	Unknown	NRRL collection	
Y-129	*S. cerevisiae*	Fruit	Orange juice	Unknown	NRRL collection	
YB-4081	*S. cerevisiae*	Fruit	Ripe goyave	Unknown	NRRL collection	
YB-2573	*S. cerevisiae*	Fruit	Sauerkraut	Unknown	NRRL collection	
YB-369	*S. cerevisiae*	Fruit	Sauerkraut	Unknown	NRRL collection	
Y-964	*S. cerevisiae*	Fruit	Sour figs	Unknown	NRRL collection	
Y-767	*S. cerevisiae*	Fruit	Tomato product (B-117)	Unknown	NRRL collection	
RC4-15	*S. cerevisiae*	Uvarum	Wine	Alsace, France	UMR Oenology (ISVV)	[49]
BR6-2	*S. uvarum*	Uvarum	Cider	Britany/Normandy, France	UMR Oenology (ISVV)	[49]
CBS 377	*S. uvarum*	Uvarum	Fruit juice	Germany	UMR Oenology (ISVV)	[49]
P3	*S. uvarum*	Uvarum	Wine	Sancerre, France	UMR Oenology (ISVV)	[49]
CBS 425	*S. uvarum*	Uvarum	Cider	Switzerland	UMR Oenology (ISVV)	[49]

Strain	Species	Ecological niche	Substrate/origin	Geographical area	Collection	Reference
GN	*S. cerevisiae*	Wine	meiotic spore clone from Zymaflore VL1	Bordeaux, France	UMR Oenology (ISVV)	[43]
C1-4	*S. cerevisiae*	Wine	Wine	Cordoba, Spain	UMR Oenology (ISVV)	[40]
C4-2	*S. cerevisiae*	Wine	Wine	Cordoba, Spain	UMR Oenology (ISVV)	[40]
C9-10	*S. cerevisiae*	Wine	Wine	Cordoba, Spain	UMR Oenology (ISVV)	[40]
SB	*S. cerevisiae*	Wine	meiotic spore clone from Actiflore BO213	France	UMR Oenology (ISVV)	[43]
F15msp	*S. cerevisiae*	Wine	meiotic spore clone from Zymaflore F15	France	UMR Oenology (ISVV)	[72]
M10-7	*S. cerevisiae*	Wine	Wine	Madrid, Spain	UMR Oenology (ISVV)	[40]
M2-2	*S. cerevisiae*	Wine	Wine	Madrid, Spain	UMR Oenology (ISVV)	[40]
M2-9	*S. cerevisiae*	Wine	Wine	Madrid, Spain	UMR Oenology (ISVV)	[40]
M2msp	*S. cerevisiae*	Wine	meiotic spore clone from Enoferm M2 (Lallemand)	Unknown	UMR Oenology (ISVV)	[72]
BO213	*S. cerevisiae*	Wine	Wine starter	France	Laffort	[26]

1.1.1. Grape juices

Two grape juices, Sauvignon blanc 2019 (SB19) and Grenache 2021 (GR21) were collected in different geographic areas and were stored at -20°C. The GR21 is a grape juice with particularly low malic acid content. The SB19 was supplemented with L-malic acid in order to artificially increase its acidity. The final composition of these grape juices in fermenting sugar, malic acid content and pH is listed in Table 2.

Table 2: Composition of the different grape juices used in the experiments.

Composition	SB19 [a]	GR21
L-malic acid (g/L)	5.31	0.52
pH	3.20	3.52
Fermentable sugars (g/L)	202.4	240.0
Assimilable nitrogen (mg N/L)	124	106
Total SO_2 (mg/L)	53	32

[a] The SB19 has been supplemented to increase its malic acid content and decrease the pH. Its original composition was a malic acid concentration at 3.73 g/L and a pH of 3.38.

1.1.2. Alcoholic fermentation monitoring

Small-volume alcoholic fermentations were implemented in screwed vials fermentations according to the general procedure described in [48]. Rapidly, 20 mL-screwed vials (Thermo Fisher Scientific, Bordeaux, France) were filled with 12 mL of grape juice and were tightly closed with screw cap-magnetic (Agilent Technologies, hdsp cap 18 mm PTFE/il 100 pk, Les Ulis, France) perforated with hypodermic needles (G26-0.45 x 13 mm, Terumo, Shibuya, Tokyo, Japan) for allowing CO_2 release. Vessel was inoculated by 2.10^6 viable cell.mL^{-1} precultured in liquid media 50 % filtered must, 50 % sterile H_2O for 24h. Cellular concentration and viability was estimated by flow cytometry using a CytoFlex (Beckman Coulter, Villepinte, France). Fermentation took place at 24°C in shaken vials by using an orbital shaker (SSL1, Stuart, Vernon Hills, IL, USA) at 175 rpm. Fermentation kinetics were estimated by monitoring manually (1-2 times per day) the weight loss caused by CO_2 release using a precision balance with automatic weight recording (LabX system, Mettler Toledo, Viroflay, France). The amount of CO_2 released according to time was modeled by local polynomial regression fit [48]. This model allows the estimation of the time necessary to reach the maximum CO_2 produced, the lag phase, the speed between 15 % and 50 % of the fermentation (V15_50), the speed between 50 % and 80 % (V50_80), the time to reach 80 % of CO_2 produced (T80) and the maximal theoretic CO_2 produced (CO_2max). Final pH was monitored using Five Easy Plus pH-meter (Mettler Toledo, Viroflay France) with a micro probe LE422 (Mettler Toledo, Viroflay France).

1.2. Microsatellite genotyping

The genomic DNA of the 39 *S. cerevisiae* strains was quickly extracted in 96-wells microplate format using a customized LiAc-SDS protocol [49]. Fourteen polymorphic microsatellite loci (*SCAAT3*, *C3*, *C5*, *SCYOR267C*, *C8*, *C11*, *SCAAT2*, *YKL172*, *C9*, *C4*, *SCAAT5*, *SCAAT1*, *C6*, *SCAAT5*, *SCAAT1*, *C6*, *YPL009*) were used for estimating the genetic relationships within those strains using PCR conditions previously described [50]. Two multiplex PCRs allowing genotyping of seven loci were carried out in a final volume of 12.5 µL containing 6.25 µL of Qiagen Multiplex PCR master mix and 1 µL of DNA template. 1.94 µL of each mix was added in the mixture using the concentrations indicated. Both reactions were run with the following program: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 57°C for 2 min, 72°C for 1 min, and a final extension at 60°C for 30 min. The size of PCR products was analyzed by the MWG company (Ebersberg, Germany) using 0.2 µL of 600 LIZ (GeneScan) as a standard marker. Chromatograms were analyzed with the GeneMarker (V2.4.0, Demo) program.

1.3. Metabolites analysis by ^{1}H NMR

1.3.1. Wine samples preparation

Samples were collected during the alcoholic fermentation. Sixty µL of centrifuged wine sample was diluted ten times in phosphate buffer prepared in D_2O (pD 4.2, 0.1 M final). Sixty µL of solution of calcium formate (FCa) 1 mM and trimethylsilylpropanoic acid (TSP) 1.25 mM in D_2O was added to the preparation. The FCa is used as an internal standard and the TSP to set the spectrum at 0 ppm [46].

1.3.2. NMR spectra acquisition

Spectra were recorded on a 600 MHz Avance III NMR spectrometer (Brucker, Wissembourg, France) operating at 600.25 MHz, equipped with a TXI 5 mm probe with z gradient coils. The measurement was performed at 293 K using TopSpin 4.0.8 software (Brucker, Wissembourg, France). The sequence used was a *noeysygpps1d*. The acquisition parameters were set as follows: 64 free induction decays (FIDs) were collected into a time domain of 64k data points, with a spectral width of 16 ppm, an acquisition time of 3.40 s and a relaxation delay (RD) of 5 s per scan. The 90° pulse calibration was carried out for each sample automatically, and the shimming was set manually in *gs* mode for each spectrum in order to obtain the finest possible line width (lower than 1 Hz). Water suppression was achieved during the RD using a shape pulse with a multiple-band selective solvent suppression (20 Hz centered on each water signal), with a power level for presentation of 50.37 dB and a shaped pulse for presaturation of 34.83 dB. The FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor prior to the Fourier transformation. Manual phase followed by automatic baseline corrections were applied to the resulting spectrum, which was aligned to zero using the TSP signal.

Wine metabolite identification was performed by comparison with literature data [51], and addition of pure standards. Individual compounds were quantified by targeted analysis, by the global spectral deconvolution method (GSD) [52], using the simple mixture analysis (SMA) plugin of MestReNova 12.0 software (Mestrelab Research, Santiago de Compostela, Spain), as previously described [46].

1.4. Statistical analyses

All the statistical and graphical analyses were carried out using R software [53] and plots were generated using the base or *ggplot2* packages.

1.4.1. Phylogenic analysis

The microsatellite dataset was manipulated using the *adegenet* package implemented in R and the genetic distance within the strains was estimated using the Bruvo's distance using the *poppr* package [73]. The phylogram was built by Neighbor Joining (*ape* package) [74]. In order to evaluate a possible genetic structuration between *fruit*, *wine* and *flor* yeasts, a Discriminant Analysis of the seventh Principal

Components was applied using the *adegenet* package omitting the two control strains (FMGS_889 and AC1_191). The pairwise *Fst* between populations was estimated using the *genet.dist* function.

1.4.2. Multivariate analyses

Spearman correlation tests between traits were computed using the *corr* function (*psych* package) corrected for multiple tests using the Benjamini-Hochberg method ($\alpha = 0.001$). Results were displayed with the *corrplot* function (*corrplot* package). The multivariate phenotypic variability of yeast strains was visualized by a Principal Component Analysis (PCA) using the *ade4* package. In order to highlight the most discriminating variables of PCA, Discriminant Analysis of Principal Component (DAPC) (*adegenet* package) was applied by selecting principal components allowing to capture up to 60% of the cumulated inertia.

1.4.3. Analysis of variance

Analyses of Variance (ANOVA) were carried out using the *car* package. The phenotypic values measured in the grape juices SB19 and GR21 were analyzed using the linear model (LM1) in order to estimate the effect of the following factors: media, population, strain in population as well as the first order interaction of all the factors according to the formula (1).

(1) $y_{ijk} = media_i + population_j + strain(population)_{jk} + inter^2_{ijk} + \varepsilon_{ijkl}$

where *y* is value of all the variables for a media *i* (*i*=1,2) in which *j* (*j*=1,2,3,4) groups of yeast strains fermented. Each population is composed of *k* strains. The factor strain is nested in the factor population and *k* varies between 1:5 and 1:16 according to the number of strains per population. The term $inter^2_{ijk}$ represents the first order interaction of each factor and ε_{ijkl} the residual. The analysis of variance of model LM1 allows the estimation of the primary effect of the media, population, and strain in population on several quantitative variables, as well as their primary interaction effect. The normal distribution of residues as well as the homoscedasticity of variances were tested by Shapiro test and Levene test (*car package*), respectively. When necessary, non-parametric comparison of samples were carried out using the Wilcoxon-Mann-Whitney or Kruskal test with corrected p values (Benjamini-Hochberg method, $\alpha = 0.05$).

2. Results

2.1. Genetic characterization of a panel of *Saccharomyces* strains

The forty-two strains used were classified according to their origin and were denominated thereafter “flor” (n=10), “wine” (n=11), “fruit” (n=16), and “uvarum” (n=5) (Table 1). *Flor* and *wine* yeast populations (*S. cerevisiae*) share the same ecological niches but have been clearly separated by molecular phylogenetic studies [54,55]. As mentioned in the introduction, several genes related to central metabolism and proton homeostasis have been linked to the ability to consume, or not, malic acid during the alcoholic fermentation [44]. For most of them, alleles of the *flor* origin were related to a stronger consumption of malic acid

suggesting a possible metabolic adaptation of this population to malic acid. However, phenotypic differences of malic acid consumption between *flor* and *wine* yeasts were never reported to our knowledge. To enlarge the genetic diversity of this metabolic survey, several *S. cerevisiae* strains (16) related to the fermentation of fruits (agrums, apple) and acidic substrates (sauerkraut or tomato juice) were included in the panel. Finally, five *S. uvarum* strains presenting different genetic origin [42] were integrated to this panel since this psychrophilic species of the *Saccharomyces* genus is a strong producer of malic acid [56]. In addition, two *S. cerevisiae* strains AC1_191 and FMGS_889 were included to this panel as "control" for a total of 44 strains. These last two strains have been recently selected for their extreme ability to consume or produce malic acid [21,57] and have a mixed inheritance between *flor* and *wine* origin. The Bruvo's genetic distance between the 37 *Saccharomyces cerevisiae* strains used was computed by using 14 microsatellites markers as previously described [26]. The genetic distances range is between 0.11 and 0.96, indicating that all the strains are unique as illustrated by the genetic tree shown in Figure 1A with some confusions between *flor* and *wine* populations. A discriminant analysis of the ten first Principal Components (60 % of the cumulative variance) allows the separation of the three populations (Figure 1B) with a probability of assignation of 0.90, 1.00 and 0.93 for *Flor*, *Wine,* and *Fruit* populations respectively. The pairwise Fst between *flor* and *wine* strains was 0.169 indicating a clear separation between the two populations. The five strains of *S. uvarum* are also unique and represent the variability of holoarctic *S. uvarum* as reported in a former study [42].

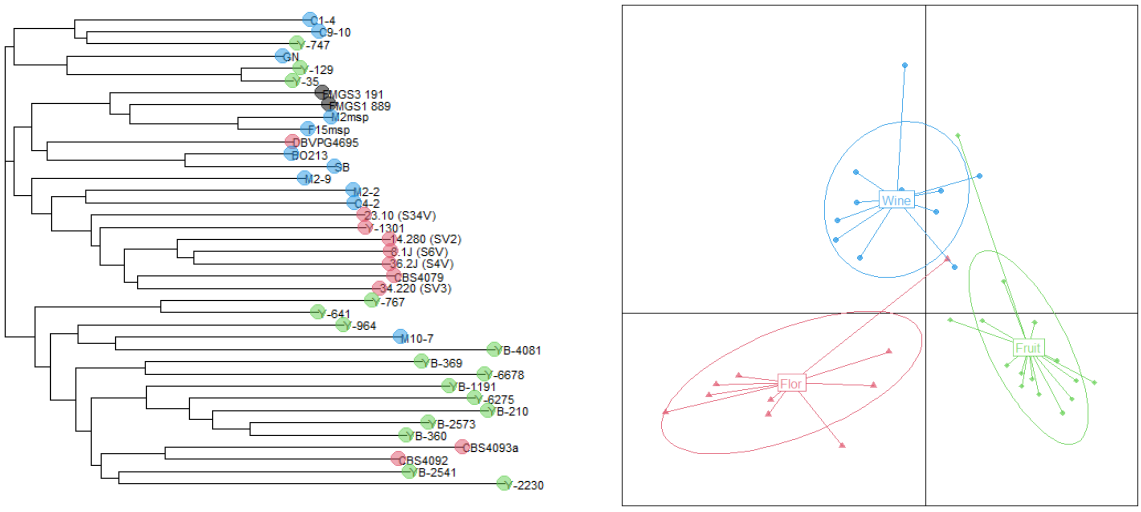


Figure 1: (A) Genetic relationships between the 37 S. cerevisiae strains of the study (Bruvo's distance) computed from the genotyping of 14 variable microsatellites, the colors red, green, and blue indicated the Flor, Fruit, and Wine origin, respectively. the two control strains are indicated by a black dot (b) Discriminant Analysis of Principal Components computed with microsatellite data. The three main populations are figured out by the same colors.

2.2. Assessment of wine metabolites variability of fermenting yeasts by targeted ^{1}H-NMR analysis.

2.2.1. Targeted ^{1}H-NMR analysis of yeast

A targeted ^{1}H-NMR metabolomics approach was applied for quantifying metabolites of fermenting yeasts belonging to the *Saccharomyces* genus. The main wine metabolites produced and consumed by yeasts during the alcoholic fermentation were quantified from sixty microliters of wine stored at -80°C. Samples were simply thawed and diluted in an appropriate buffer before analysis as detailed in methods. The typical ^{1}H-NMR spectrum after water suppression is presented in Figure 2. The signals at 0.00 ppm and 8.28 ppm correspond to TSP and FCa respectively; other signals correspond to wine constituents. The ^{1}H-NMR spectra were dominated by ethanol, and glycerol, followed by organic acids. Nitrogen compounds such as amino acids were not detected in all the samples since they depend on the initial grape juice composition and the yeast strain used [58]. The Table 3 shows the chemical shifts and the coupling constants used for identification and quantification of 15 metabolites including six organic acids (acetic acid, citric acid, malic acid, pyruvic acid, succinic acid, and tartaric acid), three alcohols (ethanol, 2,3-butanediol, glycerol), two reducing sugars (fructose and glucose), one ester (ethyl acetate), one aldehyde (ethanal), and two amino acids (tyrosine and arginine). The concentration range and the average coefficient of variation (CV) of each metabolite measured in one red (GR21) and one white grape juice (SB19) is also indicated, as well as the average concentration in both grape juices.

Table 3: Chemical shifts and coupling constants used for compounds identification. The signals chosen for quantification are in bold. Variation coefficients and average concentration are displayed for all compounds in both media.

Compound	δ1 H (Multiplicity, J in Hz, Assignment)	Average CV SB19	Average concentration SB19 (g/L)	Average CV GR21	Average concentration GR21 (g/L)
acetic acid	**2.08** (s, CH3)	21.06	0.2786	20.27	0.3166
arginine	**1.68** (m, CH3)	8.67	0.0031	43.09	0.0074
2,3-butanediol	**1.13** (d, 6.2, 2CH3),	15.84	0.4027	17.77	0.5089
citric acid	**2.67** (d, 15.6, CH2),	7.49	0.4265	49.74	0.0953
ethanal	**2.23** (d, 3.0, CH3), 9.79 (q, 2.85, CH)	60.44	0.0023	34.15	0.0141
ethanol	**1.17** (t, 7.2, CH3), 3.65 (q, CH2)	7.89	59.6569	4.82	104.2476
ethyl acetate	1.26 (t, 7.2, CH3), **2.03** (s, CH3), 4.12 (q, CH2)	8.48	0.7078	5.97	1.1418
fructose	3.77 (m, CHCHCH2), 3.87 (dd, 9.9, 3.4, CH), 3.97 (m, CH), **4.01** (dd, 12.8, 1.0 CH2), 4.09 (m, 2CH)	37.38	1.3929	49.99	1.8364
glucose	**4.65** (d, 7.9, CH), **5.23** (d, 3.6, CH)	25.47	0.2182	32.71	0.1882
glycerol	**3.55** (dd, 11.8 and 6.5, CH2), 3.77 (m, CH)	5.50	10.1624	9.01	9.0169
malic acid	2.59 (dd, 16.3 and 7.0, CH), **2.79** (dd, 16.3 and 4.5, CH), 4.36 (dd, CH)	5.44	6.9769	12.28	1.2349
pyruvic acid	**2.38** (s, CH3)	18.14	0.0542	21.83	0.0341
succinic acid	**2.54** (s, 2CH2)	10.81	1.8568	15.24	1.4810
tartaric acid	**4.39** (s, 2CH)	40.52	0.5950	15.31	1.1052
tyrosine	**6.86** (m, 8.4, 2CH), 7.17 (m, 8.6, 2CH)	16.34	0.0470	15.05	0.0517

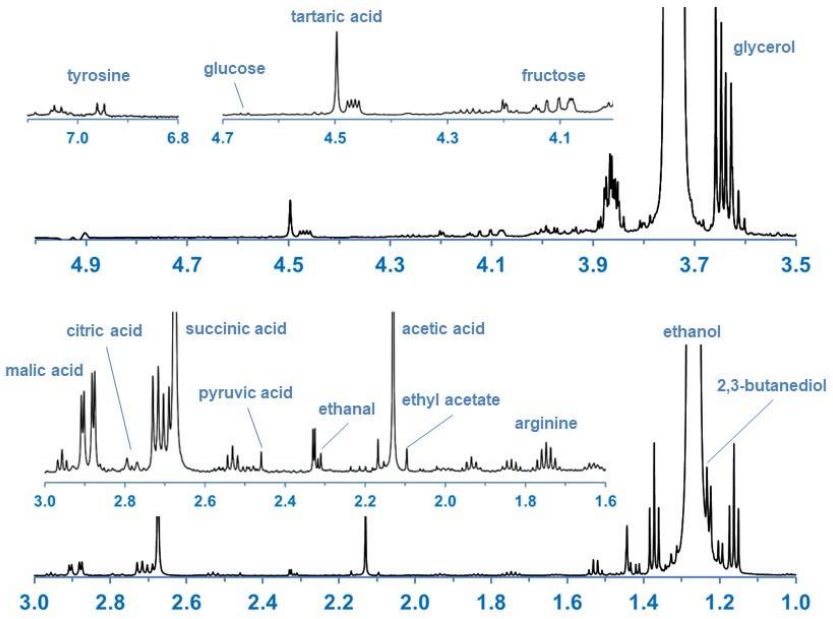


Figure 2: Annotated typical [1]H-NMR spectrum of wine metabolites after water suppression (NOESYGPPR1D). Identified constituents are listed in Table 3.

In both grape juices, most compounds had a CV lower than 30 % and in the SB19, six compounds had a CV under 10 % which shows the good repeatability and reliability of the analysis. Some compounds (residual sugars, ethanal, arginine and tartaric acid) were not accurately quantified due to their low concentrations and their instability to sample concentration (see discussion). The quantification of four key metabolites (acetic acid, malic acid, succinic acid, and glycerol) was compared to enzymatic assay methods commonly used in enology [43]. As expected, strong correlations between enzymatic and [1]H-NMR quantifications were found for all the metabolites (Spearman correlation analysis). The average CV of both methods were similar in the two grape juices, demonstrating that [1]H-NMR assay was also very reproducible for such metabolites (Figure S1 panels A and B). For acetic acid, malic acid, and succinic acid, the high correlations coefficient (Rho > 0.81) indicated a good level of agreement between the two methods. Glycerol concentrations were also correlated despite a weaker Spearman's coefficient (rho = 0.57, p-value = 2.79×10^{-21}). Linear regressions suggested that quantification by [1]H-NMR of malic acid and succinic acid was slightly overestimated respect to enzymatic assays. This was also the case of glycerol for which concentrations estimated by [1]H-NMR were 2-folds higher than those estimated by enzymatic assay (Figure S1, panel C). As discussed below these results underlined possible deviation in one of the methods.

2.2.2. Multivariate analysis of wine metabolites

Fermentations were monitored daily by following weight loss, and six kinetic parameters (lag phase, V15_50, V50_80, T35, T80 and CO_2max) were extracted as previously reported [48]. The fifteen end-point metabolites were quantified for a panel of 44 strains of different origins (Table 1) in two grape juices (SB19 an GR21) fermented in triplicate. A Principal Component Analysis (PCA) was applied to average values in order to capture the overall variability of the 15 wine metabolites quantified by ^{1}H-NMR (Figure 3). The final concentrations of wine metabolites are mostly structured by the grape juice nature and are clearly separated by the first component (40.3 % of inertia). Indeed, the SB19 was enriched in malic acid and citric acid compared to the GR21. In contrast, GR21 displayed a higher sugar content resulting in a higher production of ethanol, ethyl-acetate and ethanal. Beside this grape juice effect, *S. uvarum* and *S. cerevisiae* species are partially separated by the second component which captured 16.9 % of the total inertia. This axis is mostly correlated with glycerol and succinic acid content which are overproduced by *S. uvarum* strains. In addition, the presence of reducing sugars at the end of the alcoholic fermentation was detected for some S. *uvarum* and *fruit* strains which is also strongly related to the axis 2. In contrast, the quantification of the 15 metabolites did not allow a clear separation of the three *S. cerevisiae* populations in both matrices.

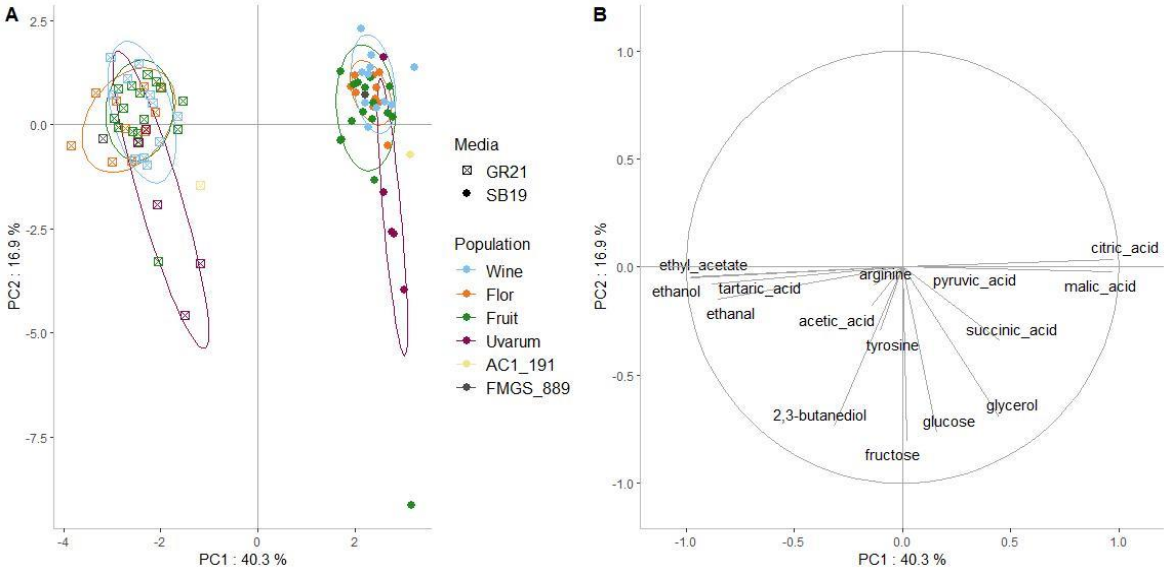


Figure 3: Principal Component Analysis (PCA) (A) The two first axis of the PCA performed for the 44 strains in the two grape juices. Axes 1 and 2 explain 40.3 and 16.9 % of total variation respectively. Each point represents the average of three biological replicates of a single strain. Each strain is colored according to its population. (B) Correlation circle indicating the correlation of the variables for axes 1 and 2.

In order to identify metabolic signatures able to better discriminate yeast populations, the two grape juice datasets were analyzed separately using a Discriminant Analysis of Principal Component (DAPC). In the SB19, malic acid is the most discriminating compound followed by glycerol and succinic acid. The

cumulated variability explained by these three metabolites on main linear discriminant axis is 98.3 %. As shown in Figure 4A, wines fermented by the *S. uvarum* species are significantly more concentrated in malic acid, glycerol, and succinic acid (Kruskal test, alpha < 0.05) than *S. cerevisiae*. These two species were also well discriminated in GR21 grape juice by three compounds: malic acid, succinic acid, and acetic acid which represent 64 %, 31 %, and 2 % of the discriminating inertia, respectively (Figure 4B). Beside this major species effect, significant differences of minor magnitudes were also observed within *S. cerevisiae* populations with a special emphasis for *wine* and *flor* populations that were always significantly different. In GR21 grape juice, the four populations were better separated than in the SB19 grape juice. Indeed, in GR21 each population was statistically different for malic acid content (Kruskal-Wallis' test, p-value < 0.05) while in SB19, only *S. uvarum* produced significantly more malic acid than the three other groups. This result could be linked to the initial characteristics of GR21 that contained a very low amount of malic acid.

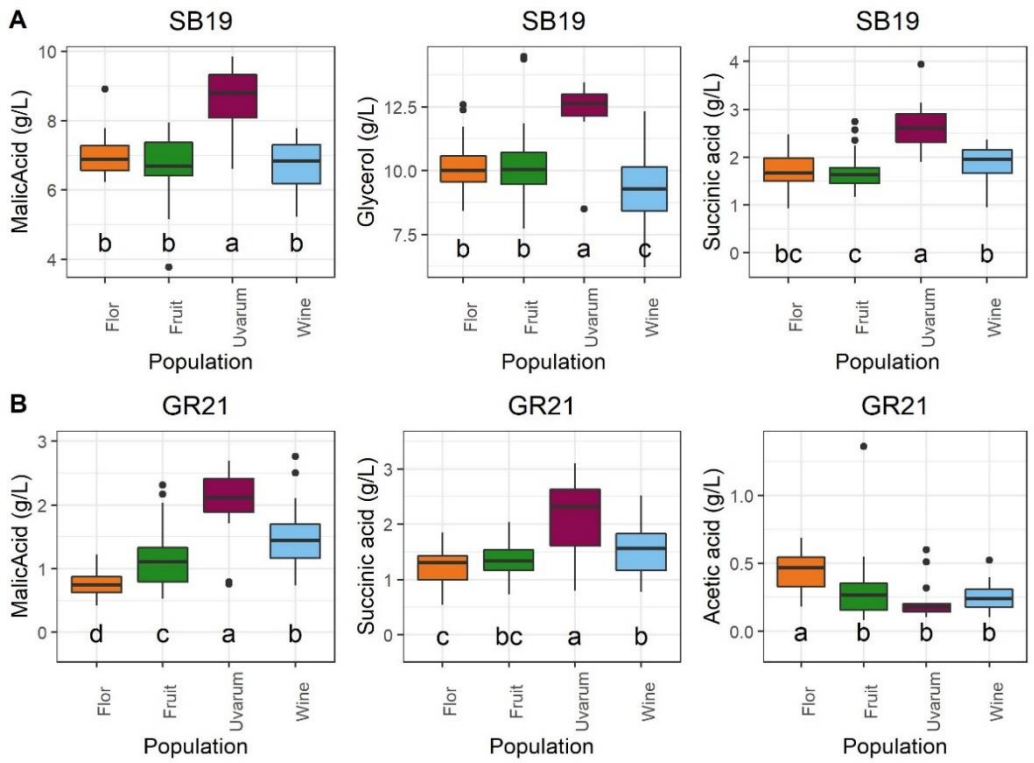


Figure 4: Main compounds involved in populations separation. (**A**) Concentrations (g/L) of malic acid, glycerol, and succinic acid, in SB19 for the 4 populations. (**B**) Concentrations (g/L) of malic acid, succinic acid, and acetic acid in GR21 for the four yeast populations. A Kruskal test has been applied to the compounds in both media. Different letters indicate a significant difference between the populations ($\alpha < 0.05$).

2.2.3. Contributions of genetic and environmental effect on the phenotypic variability of Saccharomyces strains

To deeply investigate factors influencing kinetic and metabolic traits, a nested analysis of variance was applied, aiming to estimate the impact of environment (grape juice) and genetic factors and their possible interactions. The genetic contribution effect was decomposed in population effect and strain within population effect as detailed by the linear model 1 (see methods). The contribution of each factor on 21 quantitative traits is summarized in Figure 5. The variability of ethanol, citric acid, malic acid, ethyl acetate, tartaric acid, and ethanal was mainly due to the grape juice effect. Indeed, ethanol and ethanal are directly linked to the initial sugars concentration that differs between red and white grape juices (SB19 = 202.4 g/L, GR21 = 240 g/L). Ethyl acetate is a carboxylate ester which is a secondary metabolite produced during alcoholic fermentation, derived from ethanol [59]. Even though its production can vary according to the strain, the very different ethanol content between the two wines led to significantly different amount of ethyl acetate. Tartaric acid is almost two times more concentrated in GR21 than SB19 (1.10 g/L vs 0.59 g/L) and the average amount of citric acid is about 4 times more important in SB19 than in GR21 (0.426 g/L vs 0.095 g/L respectively). Finally, malic acid varied greatly with the environment as the two grape juices were selected for their extreme acidities.

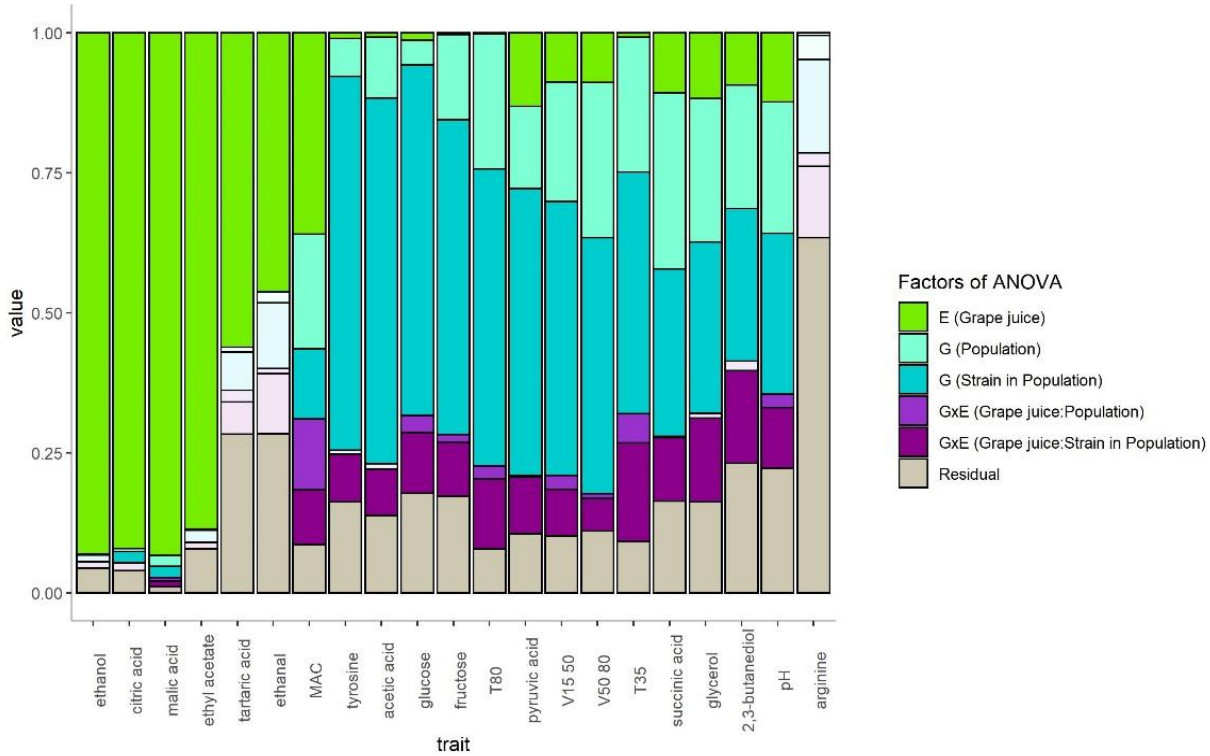


Figure 5: Bar graphs indicating the part of variance explained by the different factors of the ANOVA. The letters E and G represent the environmental (grape juice) and the genetic factors, respectively. The nested ANOVA applied allows to evaluate the effect of the population and the effect of strain within each population (strain). GxE represents the interaction between genetic and environment and was decomposed in two nested factors grape juice: population and grape juice: strain in population. Gray tons indicate non significative effect of the factors.

To better estimate the genetic contribution on malic acid content, we used the variable *MAC* (Malic Acid Consumed) that represents the ratio of malic acid consumed expressed in percentage (Figure 6A). *MAC* variability was evenly influenced by G (33 % var), E (36 % var) and GxE interactions (22 % var). The *MAC* values of the two control strains AC1_191 [57] and FMGS_889 [21] highlighted their great impact on acidity management since they produced and consumed significantly more than any other *S. cerevisiae* strains of this study. Quantitatively, the differences of malic acid concentrations between wines made by these extreme strains were 2.51 and 5.19 g/L for GR21 and SB19, respectively. Intuitively, the *MAC* is strongly correlated to the final pH (Figure 6B) since wine pH depends on the concentration of grape juice organic acids and especially the amount of malic acid. The Figure 6C shows the correlation of *MAC* with other metabolites in both grape juices. Overall, malic producer strains such as *S. uvarum* and AC1_191 produced more succinic acid than other.

For other metabolites (acetic acid, 2,3-butanediol, fructose, glycerol, pyruvic acid, succinic acid, and tyrosine) as well as kinetics parameters (V15_50, V50_80, T35 and T80), the genetic effect is greater than the grape juice's one. The decomposition of genetic effect in population and strain contribution indicated that within the same population, the metabolic variability of strains is generally stronger than the metabolic variability observed between groups (Figure 5). This is well illustrated by acetic acid variability that is more explained by the strain within populations (65 %) than by population effect itself (11 %). This can be explained for instance by some *flor* (CBS4079) and *fruit* (Y-2230, Y-6678) strains that overproduced acetic acid compared to other strains from their own populations (Figure S2). Finally, arginine was the unique metabolite that was neither impacted by grape juice or yeast strain, likely due to its low concentration level at the end of the fermentation.

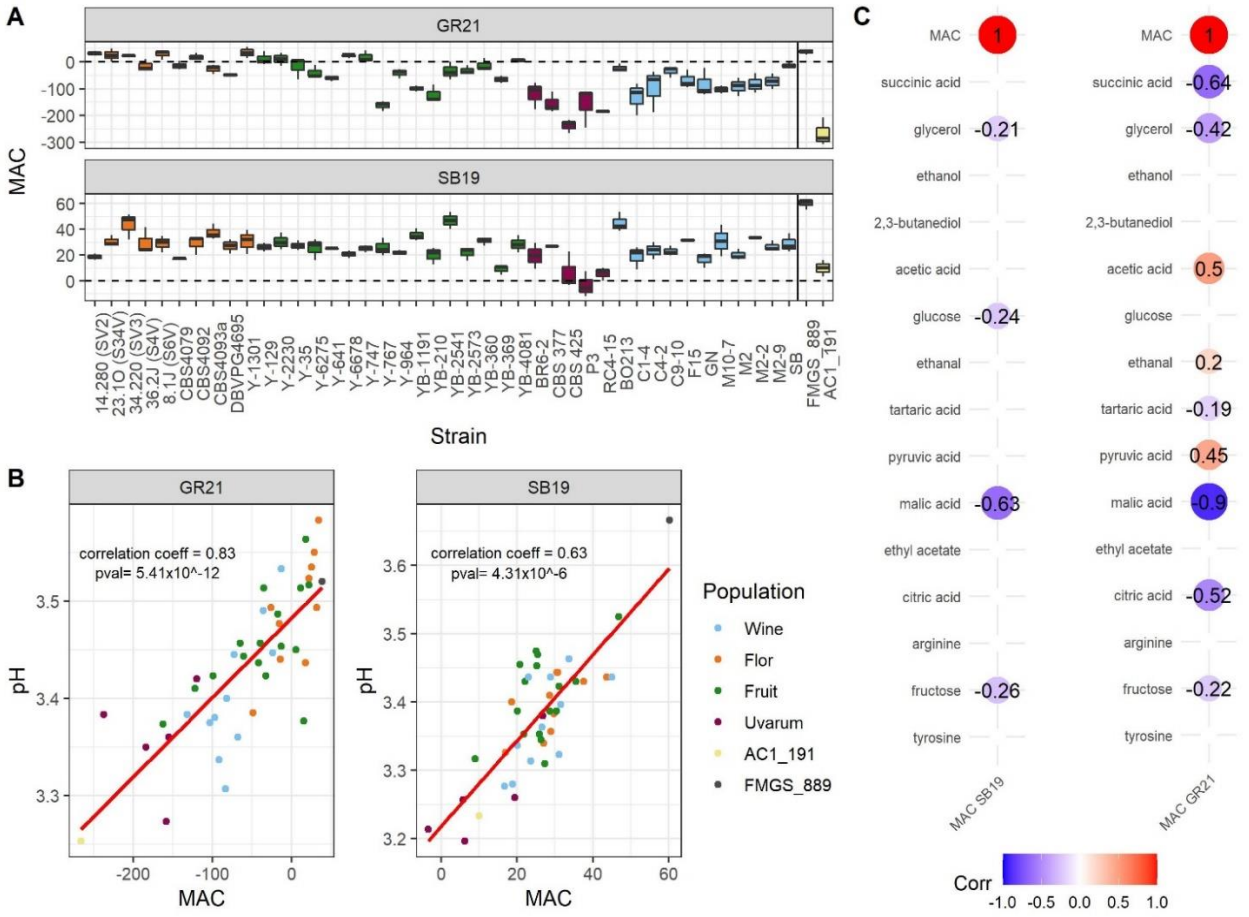


Figure 6: (**A**) MAC values measured for all strains in the two grape juices (3 replicates per strain). Strains were colored according to their population. The dashed line represents the 0% which corresponds to no malic acid consumed or produced. Positive values correspond to a consumption of malic acid and negative values to a production. (**B**) Correlation of MAC and pH for the two grape juices. The red line represents the linear regression line, correlation coefficients and p-values are written on the graphs. The dots represent all the tested strains (3 replicates per strains). They are colored according to their genetic group. (**C**) Correlation matrix between MAC and all other metabolites in the two grape juices. The value displayed corresponds to the correlation coefficient. Red values correspond to a negative correlation while blue values correspond to a positive correlation, only significative correlations ($\alpha < 0.05$) were indicated.

3. Discussion

3.1. ^{1}H NMR as an effective tool for analyzing wine microorganism metabolites.

Alcoholic beverages are complex matrices composed of several hundred volatile and non-volatile molecules that participate to the overall quality of the product. Therefore, analytical chemistry efforts aiming to characterize such complex matrices must be done for understanding and quantifying the role of microorganisms that participate to their elaboration. From a methodological viewpoint, trade-offs exist between the number of biological samples to analyze, the number of compounds assayed, the volume of sample required for their quantification, and the cost of the analysis. Generally, most of the microbiology

studies lay emphasis on the number of biological sample and conditions analyzed in order to better understand the complex interactions existing between microbiological diversity and environmental conditions. This wide phenotypic characterization requires a reduction of fermentation volumes that allows a more efficient parallelization of culture conditions [43]. However, this strategy generally reduced the number of metabolites investigated by reducing the available sample volume. Thus, it is necessary to use high throughput methods such as enzymatic assay [43] or HPLC analyses [28]. Enzymatic assays are cost effective and can be easily robotized, however they require a specific assay per compounds with a quite limited number of metabolite available. HPLC analyses are more expensive and require the use of specific methods for quantifying organic acid and sugars [60], or nitrogen compounds [61].

NMR metabolomics is particularly amenable to detect compounds that are less tractable by liquid chromatography such as sugars, organic acids, alcohols polyols, and other highly polar compounds [33]. Those classes of compounds are well represented in wine reinforcing the interest of this technique. In this study, we used an analytical method able to quantify from a small volume (less than 100 µL) using quantitative ^{1}H-NMR spectroscopy. The advantages of NMR are the simplicity of sample preparation, measurement rapidity and the possibility to detect compounds belonging to different chemical families on one spectrum, in a single experiment. Previous studies were focused on the characterization of commercial wines and analysis of the cultivar, geographic origin, or vintage [45,46,64]. Our study focused on the characterization of wines fermented in the laboratory with different yeasts strains. The ^{1}H-NMR method allowed the identification and quantification of 15 wine soluble metabolites of 264 biological samples requiring only 60 µL of wine for each experiment. This original approach allows studying the origin of strains and its impact on metabolites production from a same matrix.

The NMR analytical method was cross assessed by comparing the quantifications obtained by enzymatic assays for three organic acids (malate, succinate, and acetate) and for glycerol. Data obtained were accurately correlated for these metabolites and both methods had low coefficients of variation. However, linear regression applied indicated that NMR method could overestimate both malic and succinic acids respect to enzymatic assay (Figure S1). These problems of linearity could be corrected by the introduction of correction factors to obtain absolute quantification [62] but does not impair the relative comparison of the strains that is the scope of this study. The quantification of glycerol seems more problematic even if the coefficient of variation of repetition is quite low (5.5% in SB19 and 9.01% in GR21). As shown Figure S1, the ^{1}H-NMR method over-estimated glycerol content possibly due to the deconvolution of the double doublet at 3.55 ppm of ^{1}H-NMR data. Indeed, this region of the spectrum is very dense and dominated by ethanol signal at 3.65 ppm. An overlap might have led to uncertainty of measurement.

In the present study, ethanol was quantified on all spectra as it is a compound of major interest in

phenotypic analysis. However, the high intensity of the ethanol signal masks the signal of other compounds such as acids present in lower quantity (γ-aminobutyric acid, galacturonic acid, glucuronic acid, sorbic acid…). Therefore, the sensitivity of this method could be optimized in order to identify a higher number of compounds by suppressing the ethanol signal which is dominant after water using a selective pulse. Another solution would consist of introducing a lyophilization step to suppress the water and ethanol signals. This would improve the signal-to-noise ratio and better observe non-volatile compounds close to the signals of water and ethanol. These extra steps would increase the time of preparation and analysis but would allow the identification and accurate quantification of a higher number of other compounds.

Interestingly, the method applied is also useful for quantifying compounds that are not easily quantified by classical analyses such as ethyl acetate and ethanal. Ethyl acetate can be of interest as it is the major ester in several distilled spirits such as whiskey, rum and cachaça [63,64]. Ethanal, is found in wines in various concentrations and can be formed by yeasts or be the indication of a contamination by acetic acid bacteria, lactic acid bacteria or be the result of auto-oxidation of ethanol and phenolic compounds [65]. The monitoring of its concentration in wines during fermentation and aging could be useful as its presence in small concentration gives a pleasant fruity aroma but at high concentrations it gives a pungent irritating odor. Finally, ^{1}H-NMR analytical method is also applicable to musts and ongoing fermentations. The present study focused on the analysis of wines, but samples taken earlier during alcoholic fermentation could allow the assay of nitrogen compounds which are more abundant in musts and early stages of fermentation. On some grape juices' spectra, nitrogen compounds such as leucine, isoleucine, valine, threonine, arginine, proline, and choline were clearly identified (personal communication) in the 0.9 – 3.3 ppm region of the spectrum. However, those compounds are not found in finished fermentations' spectra, except for arginine which is initially present in high concentration in grape juices and is lately consumed by yeasts [58].

3.2. Wine metabolome is partially impacted by the population origin of the fermenting strains.

The metabolomic characterization of two grape juices fermented by a large panel of yeast strains was achieved. Those strains provided by microorganism collections are associated with acidic fermented matrices such as wine, cider, fruit juices, and sauerkraut. As confirmed by microsatellite analysis, the four groups of strains collected in different ecological or human associated niches are discriminated by population genetics tools. The three *S. cerevisiae* groups belong to distinct populations (*flor*, *wine*, and *fruit*) the last group being constituted by *S. uvarum* strains isolated in cider and wine environments. This last species has been reported to have acidifying properties and to produce large quantities of malic acid [42].

The GR21 appeared to be more discriminant than SB19 as the Kruskal tests performed on all metabolites were more often significant for population differences in GR21 than SB19. Initially, the GR21 had a low content in malic acid compared to SB19 which enhanced the ability of some strains to produce more malic acid. As malic acid has a buffer effect, an addition of malic acid to the medium reinforced this buffer effect.

On the contrary, in SB19 most strains consumed malic acid, limiting the buffer effect. The correlation between MAC and pH is strong in both grape juices (Figure 6B) as malic acid is the organic acid influencing the most the pH. Tartaric acid is also a strong influencer of the acidity, but it is not modulated by yeasts during alcoholic fermentation. Indeed, the figure 5 shows that this organic acid is only impacted by the grape juice and there is no significant effect of the strains or populations.

The main insight of this study is that metabolome variations are partially structured by the origin of the strain. First, the phenotypic discrepancies between the two main species involved in the alcoholic fermentation (*S. uvarum* and *S. cerevisiae*) were confirmed. Previous studies reported that *S. uvarum* produced higher concentrations of malic acid [42,11], succinic acid [66,67] and glycerol [11,67] that *S. cerevisiae*. In addition, this species is characterized by weaker fermentation performances than *S. cerevisiae* [11,67] and by the production of specific fermentative aromas [66,68]. Da Silva *et al.* (2015) reported that *S. uvarum* produced more acetic acid, but this finding was not supported in our study except for one strain (RC4-15) which produced 0.6 g/L of acetic acid in GR21 while the 4 other *S. uvarum* strains produced about 0.2 g/L in the same grape juice (figure S2). Using ^{1}H-NMR-based metabolomics, fifteen non-volatile metabolites were quantified. The results obtained confirmed the differences reported for glycerol, malic acid, and succinic acid content (Figure 4). In addition, this species reveals to produce more 2,3-butanediol (in average 1.5 times more than *S. cerevisiae* strains). The amount of pyruvic acid was in average 2 times less important for *S. uvarum* than *S. cerevisiae* strains, and residual fructose was the indication of some stuck fermentations by *S. uvarum* (~10 g/L of fructose remaining).

Second, *wine* and *flor* yeasts can be distinguished by handful of compounds, mostly belonging to organic acid family (cf. figure 4 and 5). This metabolic signature could be the consequence of a differential management of central carbon metabolism as previously proposed by [44]. This might be explained by the fact that *flor* yeasts are adapted to shift their metabolism to an oxidative metabolism when sugar and nitrogen are depleted [69]. In the context of a shift diauxic during the velum formation the aptitude to consume malic acid as a secondary carbon source may constitute a selective advantage. Moreover, a correlation was found between malic acid production and succinic acid production in GR21 (no production occurred in SB19). This observation was previously reported by [70]. It is consistent with their metabolic relationships and raise the question of the metabolic pathways taken by produced and consumed malic acid. This correlation could explain a significative difference of succinic acid content between *wine* and *flor* yeasts as they already differ for malic acid consumption.

Finally, the two control strains displayed extreme differences for malic acid content, demonstrating the efficiency of the selection they came from [21,70]. They frame the natural variability of all the other strains regarding the MAC values (Figure 6A). Only some strains of *S. uvarum* can produce as much malic acid as AC1_191. On the PCA (Figure 3), the strain AC1_191 is close to *S. uvarum* group in terms of acidity

management. Indeed, this strain was selected for its ability to produce important amounts of malic acid and happens to also produce important amount of succinic acid. The extreme strain AC1_191 appears to be a good strain to cope with low amounts of malic acid in musts in a context of climate change. It is able to produce malic and, secondarily, succinic acid which decrease the pH of the final wine [65]. Finally, it was able to complete the fermentation unlike some *S. uvarum* and produced low amounts of acetic acid (Figure S3) which makes it suitable for winemaking. this strain could be used for vinification itineraries aiming to conserve the freshness of white wines. The FMGS_889 is also a strain of enological interest for lowering the acidity of rich malic wines and shortening the malo-lactic fermentation of red wines [21].

Supplementary data

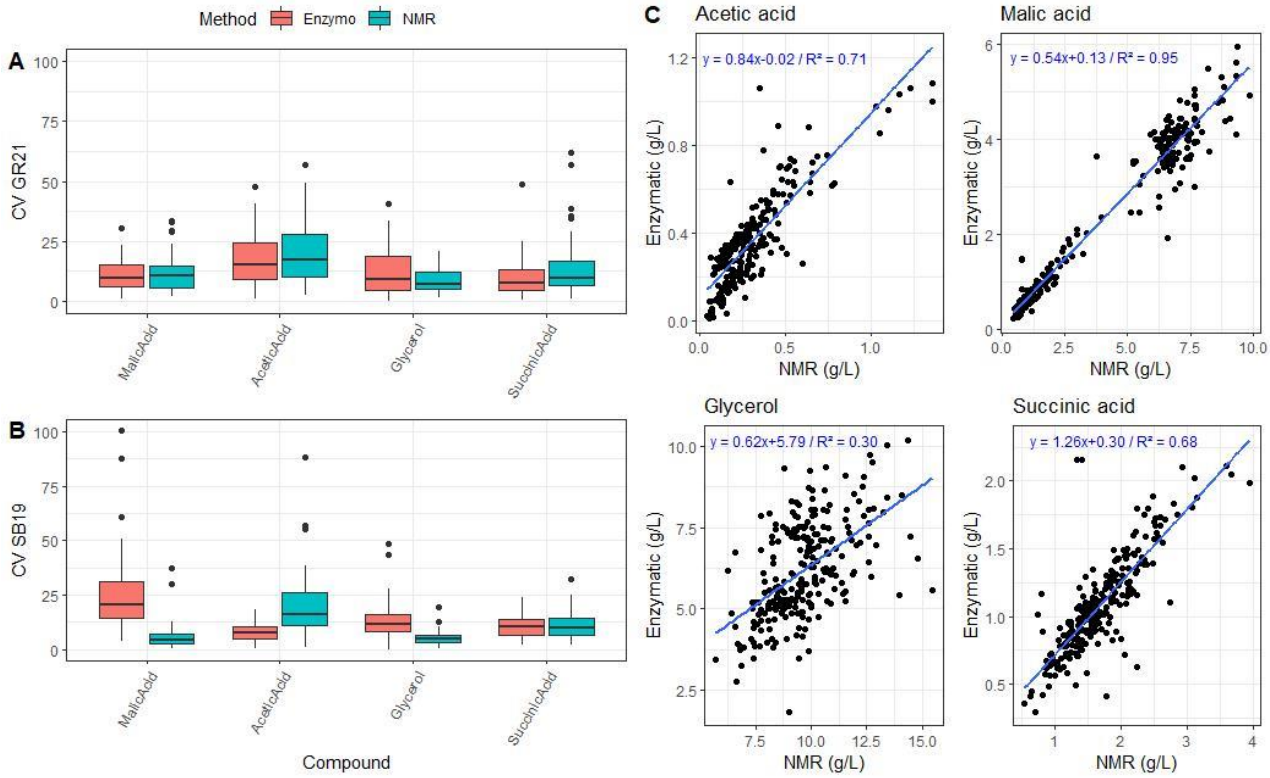


Figure S1: Correlation between enzymatic assay and NMR analysis for acetic acid, glycerol, malic acid, and succinic acid. The Spearman correlation coefficients are 0.81, 0.82, 0.94 and 0.57 for acetic acid, succinic acid, malic acid, and glycerol, respectively. The concentrations on both axes are expressed in g/L. There is an overestimation of the concentration of malic acid, succinic acid, and glycerol with the NMR method.

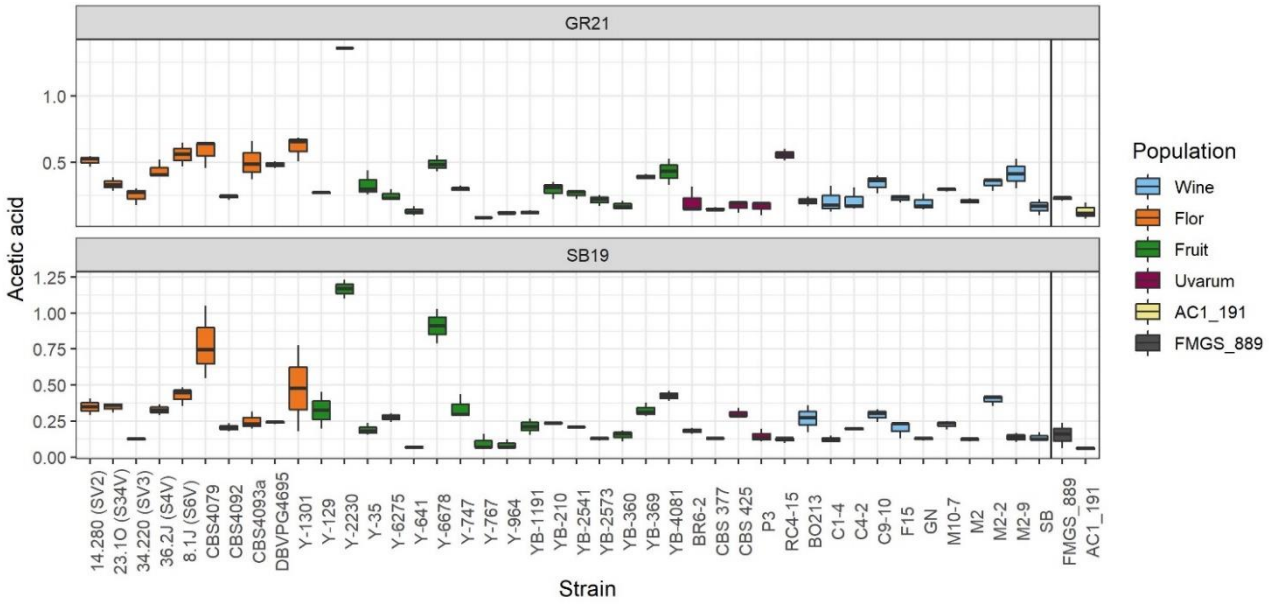


Figure S2: Concentration in acetic acid (g/L) at the end of the fermentation for all strains in both grape juices. The boxplots are colored according to their population.

Fundings

- « This study received financial support from the French government in the framework of the IdEX Bordeaux University "Investments for the Future" program / GPR Bordeaux Plant Sciences ». PM received a financial support from Aquitaine lander: program MEGAVIP, 2020-2023

Aknowledgements

This work was supported by the Bordeaux Metabolome Facility and MetaboHUB (ANR-11-INBS-0010 project).

Authors warmly thanks Prof Hervé Alexandre, Prof Joseph Schacherer and Dr Jean-Luc Legras (CIRM) for providing some of the *flor* yeast strains used in this work.

References

1. Tamang JP, Watanabe K, Holzapfel WH. Review: Diversity of microorganisms in global fermented foods and beverages. Front Microbiol. 2016;7. doi:10.3389/fmicb.2016.00377

2. Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP, *et al.* Food fermentations: Microorganisms with technological beneficial use. Int J Food Microbiol. 2012;154: 87–97. doi:10.1016/j.ijfoodmicro.2011.12.030

3. Ebeler SE, Thorngate JH. Wine chemistry and flavor: Looking into the crystal glass. J Agric Food Chem. 2009;57: 8098–8108. doi:10.1021/jf9000555

4. Alañón ME, Pérez-Coello MS, Marina ML. Wine science in the metabolomics era. TrAC Trends Anal Chem. 2015;74: 1–20. doi:https://doi.org/10.1016/j.trac.2015.05.006

5. Pinu FR. Grape and Wine Metabolomics to Develop New Insights Using Untargeted and Targeted Approaches. Fermentation. 2018. doi:10.3390/fermentation4040092

6. Lorrain B, Ky I, Pechamat L, Teissedre P-L. Evolution of Analysis of Polyhenols from Grapes, Wines, and Extracts. Molecules. 2013. pp. 1076–1100. doi:10.3390/molecules18011076

7. Fleet GH. Wine yeasts for the future. FEMS Yeast Res. 2008;8: 979–995. doi:10.1111/j.1567-1364.2008.00427.x

8. Ribéreau-Gayon, P., Dubourdien, D., Donéche B& AL. Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition. 2006.

9. Swiegers JH, Bartowsky EJ, Henschke PA, Pretorius IS. Yeast and bacterial modulation of wine aroma and flavour. Aust J Grape Wine Res. 2005;11: 139–173. doi:10.1111/j.1755-0238.2005.tb00285.x

10. Hranilovic A, Gambetta JM, Schmidtke L, Boss PK, Grbin PR, Masneuf-Pomarede I, *et al*. Oenological traits of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation. Sci Rep. 2018;8: 1–13. doi:10.1038/s41598-018-33105-7

11. Masneuf-Pomarède I, Bely M, Marullo P, Lonvaud-Funel A, Dubourdieu D. Reassessment of phenotypic traits for *Saccharomyces bayanus var. uvarum* wine yeast strains. Int J Food Microbiol. 2010;139: 79–86. doi:10.1016/j.ijfoodmicro.2010.01.038

12. Da Silva T, Albertin W, Dillmann C, Bely M, La Guerche S, Giraud C, *et al.* Hybridization within *Saccharomyces* genus results in homoeostasis and phenotypic novelty in winemaking conditions. PLoS One. 2015;10: 1–24. doi:10.1371/journal.pone.0123834

13. Viana F, Gil J V, Genovés S, Vallés S, Manzanares P. Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol. 2008;25: 778–785. doi:https://doi.org/10.1016/j.fm.2008.04.015

14. Soles RM, Ough CS, Kunkee RE. Ester Concentration Differences in Wine Fermented by Various Species and Strains of Yeasts. Am J Enol Vitic. 1982;33: 94–98.

15. Chatonnet P, Dubourdie D, Boidron J, Pons M. The origin of ethylphenols in wines. J Sci Food Agric. 1992;60: 165–178. doi:https://doi.org/10.1002/jsfa.2740600205

16. Petitgonnet C, Klein GL, Roullier-Gall C, Schmitt-Kopplin P, Quintanilla-Casas B, Vichi S, *et al.* Influence

of cell-cell contact between *L. thermotolerans* and *S. cerevisiae* on yeast interactions and the exo-metabolome. Food Microbiol. 2019;83: 122–133. doi:10.1016/j.fm.2019.05.005

17. Howell KS, Cozzolino D, Bartowsky EJ, Fleet GH, Henschke P a. Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. FEMS Yeast Res. 2006;6: 91–101. doi:10.1111/j.1567-1364.2005.00010.x

18. Renault P, Coulon J, Moine V, Thibon C, Bely M. Enhanced 3-Sulfanylhexan-1-ol Production in Sequential Mixed Fermentation with *Torulaspora delbrueckii/Saccharomyces cerevisiae* Reveals a Situation of Synergistic Interaction between Two Industrial Strains. Frontiers in Microbiology. 2016. p. 293.

19. Tempère S, Marchal A, Barbe J-C, Bely M, Masneuf-Pomarede I, Marullo P, *et al*. The complexity of wine: clarifying the role of microorganisms. Appl Microbiol Biotechnol. 2018;102: 3995–4007. doi:10.1007/s00253-018-8914-8

20. Tilloy V, Ortiz-Julien A, Dequin S. Reduction of ethanol yield and improvement of glycerol formation by adaptive evolution of the wine yeast *Saccharomyces cerevisiae* under hyperosmotic conditions. Appl Environ Microbiol. 2014;80: 2623–32. doi:10.1128/AEM.03710-13

21. Vion C, Peltier E, Bernard M, Muro M, Marullo P. Marker assisted selection of malic-consuming *Saccharomyces cerevisiae* strains for winemaking. Efficiency and limits of a qtl's driven breeding program. J Fungi. 2021;7. doi:10.3390/jof7040304

22. Dufour M, Zimmer A, Thibon C, Marullo P. Enhancement of volatile thiol release of *Saccharomyces cerevisiae* strains using molecular breeding. Appl Microbiol Biotechnol. 2013;97: 5893–5905. doi:10.1007/s00253-013-4739-7

23. Peter J, De Chiara M, Friedrich A, Yue J-X, Pflieger D, Bergström A, *et al.* Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates Species-wide genetic and phenotypic diversity. Nature. 2018;556: 339–347.

24. Steyer D, Ambroset C, Brion C, Claudel P, Delobel P, Sanchez I, *et al*. QTL mapping of the production of wine aroma compounds by yeast. BMC Genomics. 2012;13: 573. doi:10.1186/1471-2164-13-573

25. Eder M, Sanchez I, Brice C, Camarasa C, Legras JL, Dequin S. QTL mapping of volatile compound production in *Saccharomyces cerevisiae* during alcoholic fermentation. BMC Genomics. 2018;19: 1–19. doi:10.1186/s12864-018-4562-8

26. Peltier E, Sharma V, Martí Raga M, Roncoroni M, Bernard M, Jiranek V, *et al.* Dissection of the molecular bases of genotype x environment interactions: A study of phenotypic plasticity of *Saccharomyces cerevisiae* in grape juices. BMC Genomics. 2018;19: 1–20. doi:10.1186/s12864-018-5145-4

27. Cubillos FA, Brice C, Molinet J, Tisné S, Abarca V, Tapia SM, *et al*. Identification of Nitrogen Consumption Genetic Variants in Yeast Through QTL Mapping and Bulk Segregant RNA-Seq Analyses. G3 Genes, Genomes, Genet. 2017;7: 1693–1705. doi:10.1534/g3.117.042127

28. Bony M, Bidart F, Camarasa C, Ansanay V, Dulau L, Barre P, *et al*. Metabolic analysis of *S. cerevisiae* strains engineered for malolactic fermentation. FEBS Lett. 1997;410: 452–456. doi:10.1016/S0014-5793(97)00637-6

29. Bordet F, Roullier-Gall C, Ballester J, Vichi S, Quintanilla-Casas B, Gougeon RD, *et al*. Different wines from different yeasts? "*Saccharomyces cerevisiae* intraspecies differentiation by metabolomic signature and sensory patterns in wine." Microorganisms. 2021;9: 1–17. doi:10.3390/microorganisms9112327

30. Reed LK, Baer CF, Edison AS. Considerations when choosing a genetic model organism for metabolomics studies. Curr Opin Chem Biol. 2017;36: 7–14. doi:10.1016/j.cbpa.2016.12.005

31. Cusano E, Cagliani LR, Consonni R, Simonato B, Zapparoli G. NMR-based metabolic profiling of different yeast fermented apple juices. Lwt. 2020;118. doi:10.1016/j.lwt.2019.108771

32. Avenoza A, Busto JH, Canal N, Peregrina JM. Time course of the evolution of malic and lactic acids in the alcoholic and malolactic fermentation of grape must by quantitative 1H NMR (qHNMR) spectroscopy. J Agric Food Chem. 2006;54: 4715–4720. doi:10.1021/jf060778p

33. Emwas AH, Roy R, McKay RT, Tenori L, Saccenti E, Nagana Gowda GA, *et al*. NMR Spectroscopy for Metabolomics Research. Metabolites. 2019;9. doi:10.3390/metabo9070123

34. Puig-Castellví F, Alfonso I, Piña B, Tauler R. 1H NMR metabolomic study of auxotrophic starvation in yeast using Multivariate Curve Resolution-Alternating Least Squares for Pathway Analysis. Sci Rep. 2016;6: 30982. doi:10.1038/srep30982

35. Vignoli A, Ghini V, Meoni G, Licari C, Takis PG, Tenori L, *et al*. High-Throughput Metabolomics by 1D NMR. Angew Chemie - Int Ed. 2019;58: 968–994. doi:10.1002/anie.201804736

36. Dequin S, Escudier J-L, Bely M, Noble J, Albertin W, Masneuf-Pomarède I, *et al.* How to adapt winemaking practices to modified grape composition under climate change conditions. OENO One. 2017;51: 205. doi:10.20870/oeno-one.2016.0.0.1584

37. Volschenk H, van Vuuren HJJ, Viljoen-Bloom M. Malic Acid in Wine: Origin, Function and Metabolism during Vinification. South African J Enol Vitic. 2006;27. doi:10.21548/27-2-1613

38. Van Leeuwen C, Destrac-Irvine A. Modified grape composition under climate change conditions requires adaptations in the vineyard. Oeno One. 2017;51: 147–154. doi:10.20870/oeno-one.2016.0.0.1647

39. Bigard A, Berhe DT, Maoddi E, Sire Y, Boursiquot JM, Ojeda H, *et al*. Vitis vinifera L. fruit diversity to breed varieties anticipating climate changes. Front Plant Sci. 2018;9: 0–16. doi:10.3389/fpls.2018.00455

40. Yéramian N, Chaya C, Suárez Lepe JA. L-(-)-malic acid production by *Saccharomyces* spp. during the alcoholic fermentation of wine. J Agric Food Chem. 2007;55: 912–919. doi:10.1021/jf061990w

41. Gao C, Fleet GH. Degradation of malic and tartaric acids by high density cell suspensions of wine yeasts. Food Microbiol. 1995.

42. Coloretti F, Zambonelli C, Castellari L, Tini V, Rainieri S. The effect of DL-malic acid on the metabolism of L-malic acid during wine alcoholic fermentation. Food Technol Biotechnol. 2002;40: 317–320.

43. Peltier E, Bernard M, Trujillo M, Barbe J-C, Prodhomme D, Gibon Y, *et al*. Wine yeast phenomics: A standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. PLoS One. 2018; 1–23. doi:10.1371/journal.pone.0190094

44. Peltier E, Vion C, Abou Saada O, Friedrich A, Schacherer J, Marullo P. Flor Yeasts Rewire the Central Carbon Metabolism During Wine Alcoholic Fermentation. Front Fungal Biol. 2021;2. doi:10.3389/ffunb.2021.733513

45. Le Mao I, Martin-Pernier J, Bautista C, Lacampagne S, Richard T, Da Costa G. 1H-Nmr Metabolomics as a Tool for Winemaking Monitoring. Molecules. 2021;26: 1–13. doi:10.3390/molecules26226771

46. Gougeon L, da Costa G, Guyon F, Richard T. 1H NMR metabolomics applied to Bordeaux red wines. Food Chem. 2019;301: 125257. doi:10.1016/j.foodchem.2019.125257

47. Albertin W, Da Silva T, Rigoulet M, Masneuf-Pomarede I, De Vienne D, Sicard D, *et al.* The Mitochondrial Genome Impacts Respiration but Not Fermentation in Interspecific *Saccharomyces* Hybrids. PLoS One. 2013.

48. Peltier E, Bernard M, Trujillo M, Prodhomme DD, Barbe J-C, Gibon Y, *et al*. Wine yeast phenomics: a standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. Schacherer J, editor. PLoS One. 2018;13: 191353. doi:10.1101/191353

49. Albertin W, Chernova M, Durrens P, Guichoux E, Sherman DJ, Masneuf-Pomarede I, *et al*. Many interspecific chromosomal introgressions are highly prevalent in Holarctic *Saccharomyces uvarum* strains found in human-related fermentations. Yeast. 2017;35: 141–156. doi:10.1002/yea.3248

50. Börlin M, Venet P, Claisse O, Salin F, Legras JL, Masneuf-Pomarede I. Cellar-associated *Saccharomyces cerevisiae* population structure revealed high-level diversity and perennial persistence at Sauternes wine estates. Appl Environ Microbiol. 2016;82: 2909–2918. doi:10.1128/AEM.03627-15

51. Airoldi C, Tripodi F, Guzzi C, Nicastro R, Coccetti P. NMR analysis of budding yeast metabolomics: A rapid method for sample preparation. Mol Biosyst. 2015;11: 379–383. doi:10.1039/c4mb00452c

52. Cobas C, Seoane F, Domínguez S, Sykora S. A new approach to improving automated analysis of proton NMR spectra through Global Spectral Deconvolution (GSD). Spectrosc Eur. 2011;23: 26–30.

53. R Development Core Team. R: A language and environment for statistical computing. 2016;2: 1–12.

54. Peter J, De Chiara M, Friedrich A, Yue J-X, Pflieger D, Bergström A, *et al.* Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. Nature. 2018;556: 339–344. doi:10.1038/s41586-018-0030-5

55. Coi AL, Bigey F, Mallet S, Marsit S, Zara G, Gladieux P, *et al.* Genomic signatures of adaptation to wine biological aging conditions in biofilm-forming flor yeasts. Mol Ecol. 2017. doi:10.1111/mec.14053

56. Chernova M, Albertin W, Durrens P, Guichoux E, Sherman DJ, Masneuf-Pomarede I, *et al*. Many interspecific chromosomal introgressions are highly prevalent in Holarctic *Saccharomyces uvarum* strains found in human-related fermentations. Yeast. 2018;35: 141–156. doi:10.1002/yea.3248

57. Vion C, Muro M, Bernard, Margaux, Richard B, Fautre V, Yeramian N, *et al.* New malic acid producer strains of *Saccharomyces cerevisiae* for preserving wine acidity during alcoholic fermentation. Food Microbiol. 2022;112. doi:10.1016/j.fm.2022.104209

58. Crépin L, Nidelet T, Sanchez I, Dequin S, Camarasa C. Sequential use of nitrogen compounds by *Saccharomyces cerevisiae* during wine fermentation: A model based on kinetic and regulation characteristics of nitrogen permeases. Appl Environ Microbiol. 2012;78: 8102–8111. doi:10.1128/AEM.02294-12

59. Park YC, Shaffer CEH, Bennett GN. Microbial formation of esters. Appl Microbiol Biotechnol. 2009;85: 13–25. doi:10.1007/s00253-009-2170-x

60. Calull M, Marcé RM, Borrull F. Determination of carboxylic acids, sugars, glycerol and ethanol in wine and grape must by ion-exchange high-performance liquid chromatography with refractive index detection. J Chromatogr A. 1992;590: 215–222. doi:10.1016/0021-9673(92)85384-6

61. Lehtonen P. Determination of Amines and Amino Acids in Wine — A Review. Am J Enol Vitic. 1996;47: 127 LP – 133. doi:10.5344/ajev.1996.47.2.127

62. Godelmann R, Kost C, Patz CD, Ristow R, Wachter H. Quantitation of compounds in wine using 1H NMR spectroscopy: Description of the method and collaborative study. J AOAC Int. 2016;99: 1295–1304.

doi:10.5740/jaoacint.15-0318

63. Nascimento ESP, Cardoso DR, Franco DW. Quantitative ester analysis in cachaça and distilled spirits by Gas Chromatography-Mass Spectrometry (GC-MS). J Agric Food Chem. 2008;56: 5488–5493. doi:10.1021/jf800551d

64. Alcarde AR, Souza LM, Bortoletto AM. Formation of volatile and maturation-related congeners during the aging of sugarcane spirit in oak barrels. J Inst Brew. 2014;120: 529–536. doi:10.1002/jib.165

65. Liu SQ, Pilone GJ. An overview of formation and roles of acetaldehyde in winemaking with emphasis on microbiological implications. Int J Food Sci Technol. 2000;35: 49–61. doi:10.1046/j.1365-2621.2000.00341.x

66. Pérez D, Jaehde I, Guillamón JM, Heras JM, Querol A. Screening of *Saccharomyces* strains for the capacity to produce desirable fermentative compounds under the influence of different nitrogen sources in synthetic wine fermentations. Food Microbiol. 2021;97. doi:10.1016/j.fm.2021.103763

67. Minebois R, Pérez-Torrado R, Querol A. A time course metabolism comparison among *Saccharomyces cerevisiae, S. uvarum and S. kudriavzevii* species in wine fermentation. Food Microbiol. 2020;90: 103484. doi:10.1016/j.fm.2020.103484

68. Pérez D, Denat M, Heras JM, Guillamón JM, Ferreira V, Querol A. Effect of non-wine *Saccharomyces* yeasts and bottle aging on the release and generation of aromas in semi-synthetic Tempranillo wines. Int J Food Microbiol. 2022;365. doi:10.1016/j.ijfoodmicro.2022.109554

69. Legras JL, Moreno-Garcia J, Zara S, Zara G, Garcia-Martinez T, Mauricio JC, *et al.* Flor yeast: New perspectives beyond wine aging. Front Microbiol. 2016;7: 1–11. doi:10.3389/fmicb.2016.00503

70. Vion C, Muro M, Bernard, Margaux, Richard B, Fautre V, Yeramian N, *et al.* New malic acid producer strains of *Saccharomyces cerevisiae* for preserving wine acidity during alcoholic fermentation. Food Microbiol. 2022.

71. David-Vaizant V, Alexandre H. Flor yeast diversity and dynamics in biologically aged wines. Front Microbiol. 2018;9: 1–16. doi:10.3389/fmicb.2018.02235

72. Huang C, Roncoroni M, Gardner RC. MET2 affects production of hydrogen sulfide during wine fermentation. Appl Microbiol Biotechnol. 2014;98: 7125–7135. doi:10.1007/s00253-014-5789-1

73. Kamvar ZN, Tabima JF, Grünwald NJ. Poppr: a R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ. 2014;2: e281. doi:10.7717/peerj.281

74. Paradis E, Claude J, Strimmer K. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics. 2004;20: 289–290. doi:10.1093/bioinformatics/btg412

4. Comparaison métabolique de deux populations de *Saccharomyces cerevisiae* au niveau intracellulaire : développement d'une méthode d'extraction des métabolites et analyse par RMN du proton

4.1. Contexte de l'étude

Dans la section précédente, nous avons pu observer des différences phénotypiques entre des souches appartenant à différentes populations. Les levures de voile et de vin ont notamment montré des différences significatives pour la production et consommation de certains acides organiques (acides malique et succinique). De ces observations découle la question de l'existence de signatures métaboliques différentes entre ces deux populations. Afin d'investiguer plus précisément ces différences métaboliques, nous avons décidé d'étudier les métabolites intracellulaires d'un pool de 15 souches de *S. cerevisiae* appartenant aux deux populations. Pour cela, une méthode d'extraction des composés intracellulaires a été mise au point au laboratoire à partir d'un petit volume de culture. L'identification et la quantification des métabolites ont été réalisées par RMN du proton, en collaboration avec l'axe MIB de notre unité de recherche. Cette équipe dispose d'une méthode de dosage des principaux composés du vin par RMN ^{1}H qui a été adaptée (préparation des échantillons, durée d'analyse, température d'analyse et choix d'un étalon interne) afin de convenir à des métabolites intracellulaires de levures. Le développement de la méthode d'extraction des composés intracellulaires chez *S. cerevisiae* ainsi que les expériences préliminaires visant à choisir la meilleure séquence d'analyse de ces composés en RMN du proton ont fait l'objet du stage de Master 2 de Mathilde Brambati. Lors de son stage, Mathilde a déterminé les meilleures conditions pour l'extraction des métabolites en optimisant différents paramètres (volume de culture, solvant utilisé, homogénéité de l'échantillon) ainsi que certains paramètres d'analyses par RMN du proton (choix de l'étalon interne pour la quantification, nombre de scan et délais de relaxation à appliquer).

4.2. Principaux résultats

Cette sous-partie a fait l'objet d'un article scientifique. La conception des expériences a été effectuée par PM et moi-même. La mise au point de l'extraction des métabolites intracellulaires a été réalisée par Mathilde Brambati dans le cadre de son stage de Master 2. J'ai réalisé les extractions et préparé les échantillons pour l'analyse RMN. Pour ces analyses, j'ai bénéficié de l'aide d'Inès Le Mao et Grégory Da Costa. J'ai effectué le traitement statistique des données et rédigé le document avec l'aide de PM. Tristan Richard a relu et complété le document.

Endo metabolomic profiling of flor and wine yeasts by ^{1}H-NMR approach.

Vion Charlotte[1,2], Brambati Mathilde[1,2], Da Costa Grégory[2], Richard Tristan[2], Marullo Philippe[1,2]

[1]Biolaffort, Bordeaux FRANCE

[2] UMR 1366 Œnologie, Université de Bordeaux, INRAe, Bordeaux INP, BSA, ISVV

Abstract

This study explored the intracellular metabolic variations between 17 strains of *S. cerevisiae* belonging to two different genetic populations: *flor* and *wine* yeasts, in the context of alcoholic fermentation. These two populations are closely related as they share the same ecological niche but display distinct genetic characteristics. A protocol was developed for intracellular metabolites extraction and ^{1}H-NMR analysis. With this methodology, we were able to identify and quantify 21 intracellular metabolites at two different fermentation steps: exponential and stationary phases. With this work, we evidenced significant differences of abundance of intracellular metabolites which are strain- and time- dependent revealing complex interactions. Moreover, those differences of abundance appeared to be correlated with life-history traits such as average cell size and specific glycolytic flux revealing unsuspected phenotypic trade-offs between metabolite load and fermentation activity.

Key words

Phenotypic trade-offs, *flor* and *wine* yeast, metabolite load

1. Introduction

Genetic and enzymatic regulations of metabolic pathways are decisive for the ecological and evolutionary success of living organisms. A well-studied case of such metabolic control is the Crabtree effect that governs the catabolism of glucose by yeast in a sugar-rich environment (Pronk, Steensma, & van Dijken, 1996). The fitness benefit provided by Crabtree effect is well illustrated by the alcoholic fermentation performed by the budding yeast *Saccharomyces cerevisiae* that generally outcompetes other yeast species present in grape juice (Ribéreau-Gayon, P., Dubourdien, D., Donéche, 2006). In such conditions, *S. cerevisiae* preferentially produces alcohol from sugar by repressing the use of oxidative respiration even in presence of oxygen (Compagno, Merico, Sulo, & Pis, 2007; Piškur, Rozpedowska, Polakova, Merico, & Compagno, 2006). This metabolic strategy promotes the fast development of its biomass (Pfeiffer, Schuster, & Bonhoeffer, 2001) coupled with ethanol production (Goddard, 2008) and temperature increase (Balsa-canto, Alonso-del-real, & Querol, 2020). Interestingly, ethanol-producing yeasts such as *Saccharomyces* and *Brettanomyces* genera can also efficiently catabolize ethanol at the end of the alcoholic fermentation process (Compagno, Piskur, & Hagman, 2013). This metabolic transition (diauxic shift) has been intensively investigated by molecular approaches (Zampar *et al*., 2013). The ability of yeast to switch from ethanol production (fermentation) to ethanol consumption (respiration) is based on the presence of parallel enzymatic reactions including alcohol dehydrogenase enzymes (Thomson *et al.*, 2005) that arose after the whole genome duplication. This fast production and consumption of ethanol is referred as the ‘‘make-accumulate-consume’’ strategy (Compagno *et al.*, 2013; Piškur *et al*., 2006) and might be considered a specialization hallmark of *S. cerevisiae* to sweet fruits. However, according to other authors this species might be a generalist able to survive in a wide range of environments (Goddard & Greig, 2015).

Resource-utilization strategies are intimately linked to metabolic trade-off and are subjected to natural selection. For example, individuals of the same species may take different paths of metabolic adaptation in a changing environment as recently demonstrated (Ekkers *et al*., 2022).

The winemaking process constitutes an anthropic niche in which at least two specific populations of the same species may coexist. As demonstrated by several phylogenomic studies, *flor* (velum) yeast and *wine* yeast are two closely related groups of yeasts with distinct genetic characteristics (Coi *et al.*, 2017; Eldarov *et al*., 2018; Peltier, Sharma, *et al.*, 2018). These divergent populations are characterized by genomic signatures in many genes involved in the biofilm formation (*FLO11*), metal transport (*FRE-FIT region*, *ZFR1*), sugar uptake (*HXT3*), and signaling pathways (*RAG2*, *SFL1*) (Coi *et al.*, 2017; David-Vaizant & Alexandre, 2018; Eldarov *et al.*, 2018; Fidalgo, Barrales, Ibeas, & Jimenez, 2006). Interestingly, *flor* and *wine* yeast populations allowed the emergence of a specific “Champagne” cluster of *wine* yeasts that result from their hybridization. Several quantitative genetics studies demonstrated that the segregation

of *flor* and *wine* alleles in a F1-hybrid progeny clearly impact the phenotypic properties of yeast during the alcoholic fermentation. Indeed flor-specific alleles were reported to control asparagine uptake (*ASP1*) (Marullo *et al.*, 2007), fermentation kinetics and pH resistance (*PMA1*) (Martí-Raga, Peltier, Mas, Beltran, & Marullo, 2017) glycerol production and malic acid consumption (*PNC1, SDH2, MAE1, PYK2, MSB2, PMA1*) (Peltier *et al.*, 2021). Beside these genetics evidence, proteomics and transcriptomic analyses depicted the transition of metabolic pathways occurring during the velum formation such as stress resistance, oxidative carbon metabolism, nutrient uptake, protein maintenance, DNA reparation, and cell wall biogenesis (Moreno-García, García-Martínez, Millán, Mauricio, & Moreno, 2015; Moreno-García, García-Martínez, Moreno, & Mauricio, 2015).

However, despite the numerous studies focusing on flor yeast specificities, comparative physiological studies between flor and wine yeast are surprisingly rare (Esteve-Zarzoso, Peris-Torán, García-Maiquez, Uruburu, & Querol, 2001). Recently, an ^{1}H-NMR-based metabolomic analysis revealed that the final concentration of malic and succinic acids found may be significantly different between *flor* and *wine* yeast populations (Vion *et al.*, 2023). These organic acids play a central role in the TCA cycles and would be markers of divergent metabolic strategies between *flor* and *wine* groups. In order to better characterized these possible metabolic specificities, we implemented our ^{1}H-NMR-based analysis to quantify the intracellular content of the most abundant metabolites of yeast. This method was applied for comparing for the first time the endo-metabolome of a restricted number of *flor* and *wine* strains.

2. Materials and Methods

2.1. Yeast strains used and culture methods

Seventeen strains of *S. cerevisiae* were used and are listed in Table 1. Seven of them belong to the *flor* group while seven others are well characterized *wine* yeast. Three other strains SB, FMGS_889 and AC1_191 have a mixed genome related to *flor* and *wine* yeasts.

Table 1: Yeast strain used.

Strain	*Species*	*Ecological niche*	*Substrate/origin*	*Geographical area*	*Collection*	*Reference*
FMGS_889	*S. cerevisiae*	Control	Extreme malic acid consuming strain	Miscelaneous (Breeding)	ISVV	(Vion, Peltier, Bernard, Muro, & Marullo, 2021)
AC1_191	*S. cerevisiae*	Control	Extreme malic acid producing strain	Miscelaneous (Breeding)	ISVV	(Vion *et al.,* 2022)
23.10 (S34V)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	(David-Vaizant & Alexandre, 2018)
34.220 (SV3)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	(David-Vaizant & Alexandre, 2018)
36.2J (S4V)	*S. cerevisiae*	Flor	Velum	Dijon, France	Prof. Hervé Alexandre	(David-Vaizant & Alexandre, 2018)
CLIB2D	*S. cerevisiae*	Flor	Velum	Sadinia, Italy	CLIB	(Coi *et al.,* 2017)
DBVPG4695	*S. cerevisiae e*	Flor	Vino Santo	Lungarotti winery, Italy	1002 genomes	(Peter *et al*., 2018)
CBS4092	*S. cerevisiae*	Flor	Velum	Spain	1002 genomes	(Peter *et al*., 2018)
Y-1301	*S. cerevisiae*	Flor	Wine	Unknown	NRRL collection	(Vion *et al*., 2023)
GN	*S. cerevisiae*	Wine	meiotic spore clone from Zymaflore VL1	Bordeaux, France	UMR Oenology (ISVV)	(Peltier, Bernard, Trujillo, Barbe, *et al*., 2018)
SB	*S. cerevisiae*	Wine	meiotic spore clone from Actiflore BO213	France	UMR Oenology (ISVV)	(Peltier, Bernard, Trujillo, Barbe*, et al*., 2018)
F15msp	*S. cerevisiae*	Wine	meiotic spore clone from Zymaflore F15	France	UMR Oenology (ISVV)	(Huang, Roncoroni, & Gardner, 2014)
M2msp	*S. cerevisiae*	Wine	meiotic spore clone from Enoferm M2 (Lallemand, Canada)	Unknown	UMR Oenology (ISVV)	(Huang *et al*., 2014)
VL3	*S. cerevisiae*	Wine	Wine starter	Bordeaux, France	Laffort	
Fx10	*S. cerevisiae*	Wine	Wine starter	Bordeaux, France	Laffort	
X5	*S. cerevisiae*	Wine	Wine starter	Bordeaux, France	Laffort	
RB4	*S. cerevisiae*	Wine	Wine starter	Beaujolais	Laffort	(Peltier, Bernard, Trujillo, Barbe*, et al*., 2018)

S. cerevisiae strains were propagated on YPD 2 % (1 % peptone, 1% yeast extract, 2 % glucose) at 30°C in both liquid and plate cultures (2 % agar). Long term storage at -80°C was achieved by adding one volume of glycerol to YPD overnight cultures.

2.2. Alcoholic fermentation assays

2.2.1. Grape juices

The grape juice used for the alcoholic fermentation was a Sauvignon Blanc 2021 (SB21). It was collected in the Bordeaux area and stored at -20°C. Its final composition in fermenting sugar, malic acid content and pH is listed in Table S1.

2.2.2. Alcoholic fermentation monitoring

Small-volume alcoholic fermentations were implemented in screwed vials fermentations according to the general procedure described in (Peltier, Bernard, Trujillo, Prodhomme, *et al.*, 2018). Rapidly, 20 mL-screwed vials (Thermo Fisher Scientific, Bordeaux, France) were filled with 12 mL of grape must and were tightly closed with screw cap-magnetic (Agilent Technologies, hdsp cap 18 mm PTFE/il 100 pk, Les Ulis, France) perforated with hypodermic needles (G26-0.45 x 13 mm, Terumo, Shibuya, Tokyo, Japan) for allowing CO_2 release. Vessel was inoculated by 2.10^6 viable cell.mL^{-1} precultured in liquid media 50 % filtered must, 50 % sterile H_2O for 24h. Cellular concentration and viability was estimated by flow cytometry (see below). Fermentation took place at 24°C in shaken vials by using an orbital shaker (SSL1, Stuart, Vernon Hills, IL, USA) at 175 rpm. Fermentation kinetics were estimated by monitoring manually (1-2 times per day) the weight loss caused by CO_2 release using a precision balance with automatic weight recording (LabX system, Mettler Toledo, Viroflay, France). The amount of CO_2 released according to time was modeled by local polynomial regression fit (Peltier, Bernard, Trujillo, Prodhomme, *et al.*, 2018) allowing the estimation of the time necessary to reach the maximum CO_2 produced, the lag phase, the speed between 15 % and 80 % of the fermentation (V15_50), the speed between 50 % and 80% (V50_80), the time to reach 80 % of CO_2 produced (T80) and the maximal theoretic CO_2 produced (CO_2max). Final pH was monitored using Five Easy Plus pH-meter (Mettler Toledo, Viroflay France) with a micro sonde LE422 (Mettler Toledo, Viroflay France).

2.3. Biomass collection

Some vials were set aside at 10 % and 50 % of CO_2 produced for the extraction of intracellular metabolites. Five milliliters were taken per vial for quenching and metabolites extraction. For each sample, measurements of cell density, viability and cell size were taken by flow cytometry (Cytoflex, Beckman Coulter apparatus). Enzymatic assays of malic acid, acetic acid, glycerol, succinic acid, and residual sugars were performed on all samples. The figure 1 summarizes the experimental steps from fermentations to NMR analysis.

2.3.1. Flow cytometry analysis

Flow cytometry measures were taken for each biomass sample and for adjust the cellular concentration at the inoculation moment using a CytoFlex (Beckman Coulter, Villepinte, France). 20 µL of homogenized culture with 3 µM propidium iodide (PI) were diluted in 1mL final McIlvaine buffer (sodium phosphate dibasic 0.2 M, citric acid 0.1 M) and was analyzed by flow cytometry (Zimmer *et al.*, 2014). 50000 events were recorded per experiment. The viability of cells was estimated using 488 nm excitation, with a 610/20 nm long bandpass filter to detect PI fluorescence (dead cells). The size of the cells was estimated with the mean of the Forward Scatter parameter (Mean FSC).

2.3.2. Intracellular yeast metabolites extraction and sample preparation

A sample of 5 mL of fermenting grape juice was taken from the vials and quickly quenched in cold pure methanol with a (1:5) ratio sample/methanol in 35 mL Teflon centrifuge tubes with screw closure (Nalgene, Thermo Fisher Scientific) to avoid metabolites leaks during centrifugation (Villas-Bôas, Højer-Pedersen, Åkesson, Smedsgaard, & Nielsen, 2005). Tubes were centrifuged at 4000 g for 5 min to remove the medium. The pellet was resuspended in 1 mL of cold pure methanol and placed at -80°C with agitation for 30 min. Tubes were centrifuged at 10000 g for 5 min and the supernatant was stored in a 1.8 mL microtube. The pellet was washed again with 0.5 mL of cold pure methanol, placed at -80°C with agitation for 30 min. Then tubes were centrifuged at 10000 g for 5 min and the supernatant was collected in the same microtube as before. Methanol was evaporated with a SpeedVac vacuum (Radid Vap, Labconco, Kansas City, USA) at 32°C, 25 % agitation for 4 hours. The microtubes were stored at -80°C until NMR analysis.

2.3.3. Metabolites analysis by 1H-NMR

2.3.3.1. Samples preparation

Intracellular metabolites were resuspended in 660 µL of phosphate buffer (Na_2HPO_4, 0.1 M, pH 7.0) in 90 % H_2O, 10 % D_2O with 1,3-dimethylamylamine (DMMA) 1 mM as internal standard to achieve a quantitative experiment and TSP to set the spectrum at 0 ppm. The resulting solution was placed into the NMR tube. Since most of the intracellular compounds quantified can be found in the extracellular media, the efficiency of the extraction was verified by adding 5 g/L of galactose in the samples before methanol quenching. This sugar is not present in grape juices or in *S. cerevisiae* metabolome, therefore its detection would be the evidence of an extracellular contamination. Less than 0.1 % of galactose added was found in supplemented samples, which proved the efficiency of the extraction method applied (data not shown).

2.3.3.2. NMR spectra acquisition

Spectra were recorded on a 600 MHz Avance III NMR spectrometer (Brucker, Wissembourg, France) operating at 600.25 MHz, equipped with a TXI 5 mm probe with z gradient coils. The measurement

of intracellular compounds was performed at 303 K using TopSpin 4.0.8 software (Brucker, Wissembourg, France) and the number of scans was set at 256 resulting in an analysis time of 35 minutes per sample. The acquisition parameters were set as follows: the Free Induction Decay (FID) was collected into a Time Domain (TD) of 64K data points, with a Spectral Width (SW) of 16 ppm, an acquisition time (AQ) of 3.40 s and a relaxation delay of 5 s per scan. Then 90° pulse calibration was carried out for each sample automatically, and the shimming was set manually in *gs* mode for each spectrum in order to obtain the finest possible line width (lower than 1 Hz). Water suppression was achieved during the Relaxation Delay (RD) using a shape pulse with a multiple-band selective solvent suppression (20 Hz centered on each water signal), with a power level for presentation of 50.37 dB and a shaped pulse for presaturation of 34.83 dB. The FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor prior to the Fourier transformation. Manual phase followed by automatic baseline corrections were applied to the resulting spectrum, which was aligned to zero using the TSP signal.

Intracellular compounds were identified based on previous studies (Puig-Castellví, Alfonso, Piña, & Tauler, 2015), the use of available databases as YMDB (Jewison *et al.*, 2012) and dosed additions made it possible to identify some other compounds. Compounds were quantified by targeted analysis, by the global spectral deconvolution method (GSD) (Cobas, Seoane, Domínguez, & Sykora, 2011), using the simple mixture analysis (SMA) plugin of MestReNova 12.0 software (Mestrelab Research, Santiago de Compostela, Spain).

2.4. Enzymatic assay

For each sample taken during the alcoholic fermentation and at the end, a volume of 800 µL was manually transferred in Micronics tubes (Novazine, Lyon, France, ref: MP32033L) and stored at -20°C. The concentrations of the following organic metabolites was measured: acetic acid, glycerol, malic acid and succinic acid using the respective enzymatic kits : K-ACETGK, K-GCROLGK, K-MAL-116A, K-SUCC (Megazyme, Bray, Ireland) following the instructions of the manufacturer. Glucose and fructose were assayed by using the enzymatic method described by (Stitt, McC., Gerhardt, & Heldt, 1989). All the enzymatic assays were performed by a robotic platform using Bordeaux metabolomics facilities (http://metabolome.cgfb.u-bordeaux.fr/).

2.5. Statistical analyses

All the statistical and graphical analyses were carried out using R software (R Development Core Team, 2016) and plots were generated using the base or *ggplot2* packages.

2.5.1. Multivariate analyses

Spearman correlation tests between traits were computed using the *corr* function (*psych* package) corrected for multiple tests using the Benjamini-Hochberg method (α=0.001). Results were displayed with the *corrplot* function (*corrplot* package). The multivariate phenotypic variability of yeast strains was visualized by a Principal Component Analysis (PCA) using the *ade4* package. In order to highlight the most discriminating variables of PCA, Discriminant Analysis of Principal Component (DAPC) (*adegenet* package) were applied by selecting principal components allowing to capture up to 60% of the cumulated inertia. The heatmap was generated with the *ComplexHeatmap* package. Data were scaled and centered to compare variables which did not have the same order of magnitude.

2.5.2. Analysis of variance

Analyses of Variance (ANOVA) were carried out using the *car* package. The phenotypic variability of the 21 intracellular metabolites was estimated by a linear model (LM1) according to the formula (1):

(1) $y_{ijk} = time_i + group_j + strain(group)_{jk} + inter^2_{ijk} + \varepsilon_{ijkl}$

where y is value of all the variables for a fermentation time *i* (*i*=1,2), *j* (*j*=1,2) groups of yeasts strains fermented a grape juice. Each group is composed of *k* strains. The factor strain is nested in the factor group and *k* varies between 1:7 and 1:8 according to the number of strains per group. The term $inter^2_{ijk}$ represents the first order interaction of each factor and ε_{ijkl} the residual. When necessary, non-parametric comparison of samples were carried out using the Wilcoxon-Mann-Whitney or Kruskal test with corrected p values (Benjamini-Hochberg method α=0.05).

3. Results and discussion

3.1. Experimental design

In this study an ^{1}H-NMR method was developed to quantify the main intracellular metabolites of fermenting yeasts during the alcoholic fermentation. We focused our effort on the characterization of *flor* and *wine* yeasts that constitute two distinct *S. cerevisiae* populations (Coi *et al*., 2017; Peltier, Sharma, *et al*., 2018) and share similar ecological niches. As recently demonstrated, wines made by these two groups may have statistical differences for several organic compounds including malic, acetic, and succinic acids (Vion *et al*., 2023). The genetic material used is listed in (Table 1). Seven *flor* strains were compared to eight *wine* strains. In addition, the two control strains (FMGS_889 and AC1_191) were included in the experiment; these strains have been selected for their opposed malic acid metabolism as previously reported (Vion *et al*., 2022, 2021). As detailed in methods, yeast cells were collected during the alcoholic fermentation and immediately quenched using cold methanol. Samples were taken at two fermentation moments: growth phase (10 % of CO_2 produced) and stationary phase (50 % of CO_2 produced). Just before sampling, the concentration and the cell size of yeasts were estimated by flow cytometry. To ensure the

reliability of quantification five biological repetitions were carried out per modality. In addition, a small volume of fermenting juice was frozen for enzymatic metabolic analyses. The experimental design of this work is shown Figure 1.

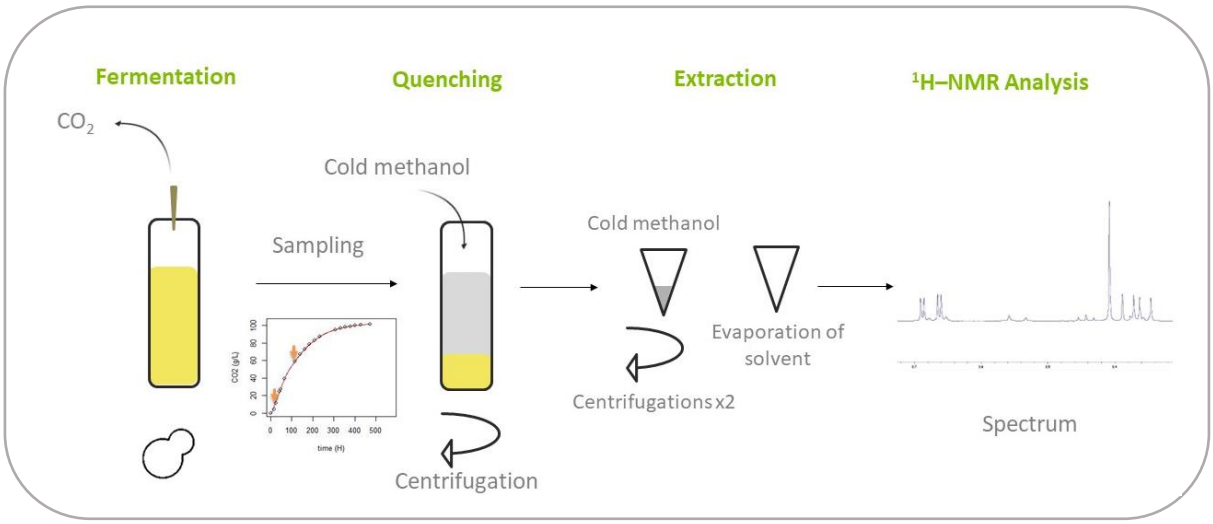


Figure 1: Schematic representation of the experimental steps from fermentation to ^{1}H-NMR analysis

3.2. Fermentation and yeast growth monitoring

The fermentation kinetics of the different strains are shown in Figure 2, panel A. The quantification of cell growth and viability by flow cytometry at two different time courses of the alcoholic fermentation allowed a brief comparison of *flor* and *wine* populations. The average viability of fermenting yeast in the growth and the stationary phases are 92 % and 81 %, respectively. These traits were not statistically different between *flor* and *wine* populations. In contrast, the cellular concentration reached during the stationary phase is significantly different between *flor* and *wine* yeasts with average values of 6.15×10^{7} and 8.6×10^{7} (Wilcoxon test, $\alpha = 0.05$) (Figure 2B). The FSC parameter of the flow cytometer was used as a proxy for determining the cell size of both populations. Interestingly, the evolution of cell size between the *flor* and *wine* groups was statistically different. *Wine* yeasts are smaller than *flor* yeasts during the growth phase, but they became larger during the stationary phase (Figure 2C) as confirmed by a strong effect of interaction between group and sampling time (2-way ANOVA 21.8% of the variance explained, p value = 0.009).

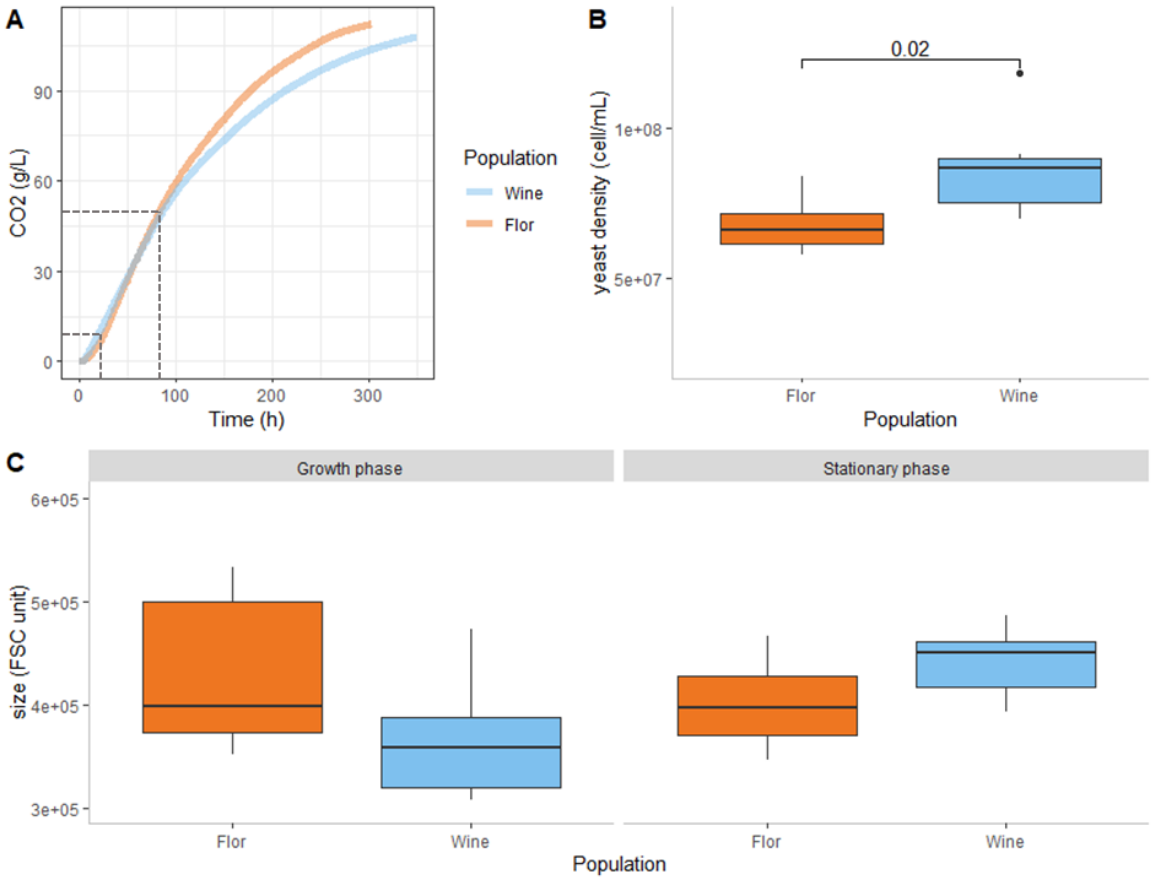


Figure 2: Biomass analysis of yeast cells at the sampling time. **Panel A.** The time course CO_2 release of the *flor* and *wine* strains. Dot lines represent the two sampling points (exponential and stationary phases, 10 and 50 g/L released CO_2 respectively). **Panel B.** The cellular concentrations at stationary phase (cell/ml) of flor and wine yeast were compared (Wilcoxon test, α=0.05). **Panel C.** The average yeast size (FSC unit) of *flor* and *wine* yeast did not evolve in the same way according to the sampling point (ANOVA test, interaction effect α=0.01).

3.3. Development and optimization of a methodology to extract intracellular metabolites from *S. cerevisiae* and analysis by ^{1}H-NMR.

The typical ^{1}H-NMR spectrum after water suppression is presented in Figure 3. The signals at 0.00 ppm and 1.26 ppm correspond to TSP and DMMA respectively; other signals correspond to intracellular metabolites. The ^{1}H-NMR spectra were dominated by sugar content and organic acids followed by some amino acids. The chemical shifts and coupling constant used for identification and absolute quantification of 21 organic compounds are listed in Table 2. The abundance of each compound was normalized by the number of cells per milliliter present in the sample. For both sampling times, the average abundance and CV of each compound and phase are indicated in the Table 2. The average abundance of each strain at each sampling point is provided in the additional Figure S1.

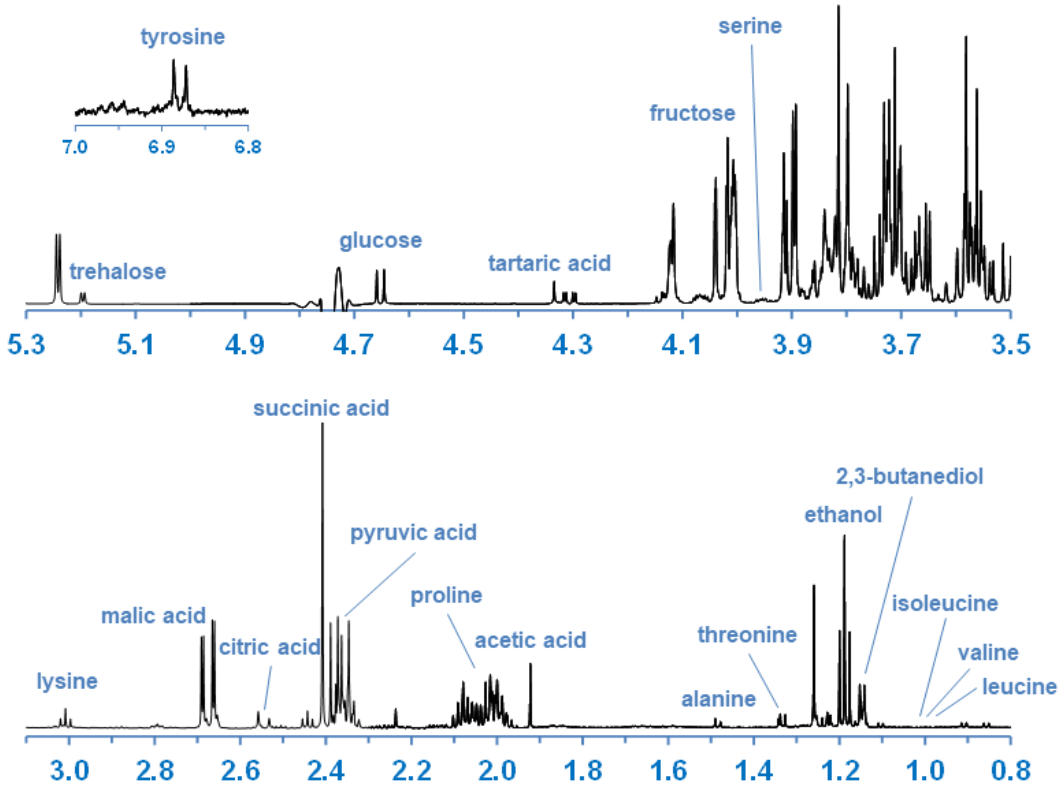


Figure 3: Annotated typical ^{1}H-NMR spectrum of intracellular yeasts' metabolites after water suppression (*noesyggpr1d*).

Table 2: Chemical shifts and coupling constants used for compounds identification. The signal chosen for quantification is in bold. Abondance (normalized by the cell number) and Coefficients of Variation (CV) of intracellular compounds are indicated for both sampling time.

Compound	Δ1 H (Multiplicity, J in Hz, Assignment)	CV growth phase (10 % FA)	Abundance (mmol/10^7 cells)	CV stationary phase (50% FA)	Abundance (mmol/10^7 cells)
acetic acid	**1.90** (s, CH3)	38.25	$1.12x10^{-04}$	33.85	$1.76x10^{-04}$
alanine	**1.50** (d, 7.2, CH3)	75.92	$8.23x10^{-05}$	38.20	$1.34x10^{-04}$
2,3-butanediol	**1.13** (d, 6.2, 2CH3), 3.61 (m, 2CH)	32.73	$4.97x10^{-05}$	31.43	$2.88x10^{-04}$
citric acid	**2.54** (d, 15.6, CH2), 2.66 (d, 15.6, CH2)	27.97	$5.45x10^{-04}$	26.40	$5.24x10^{-04}$
ethanol	**1.17** (t, 7.2, CH3)	25.32	$8.72x10^{-04}$	38.75	$1.63x10^{-03}$
fructose	3.97 (m, CH), **4.01** (dd, 12.8, 1.0 CH2), 4.09 (m, 2CH)	22.72	$7.73x10^{-02}$	23.59	$5.11x10^{-02}$
glucose	**4.65** (d, 7.9, CH), **5.23** (d, 3.6, CH)	22.36	$6.4x10^{-02}$	24.08	$2.69x10^{-02}$
glycerol	3.55 (dd, 11.8 and 6.5, CH2), **3.64** (dd, CH2)	25.70	$3.44x10^{-03}$	24.61	$5.39x10^{-03}$
isoleucine	0.93 (t, 7.4, CH3), **1,01** (d, 7.0, CH3), 1.24 (m, CH2), 1.45 (m, CH2), 1.97 (m, CH), 3.66 (d, 3.9, CH)	97.20	$1.12x10^{-08}$	54.58	$1.83x10^{-06}$
leucine	**0.96** (t, 2CH3), 1.70 (m, CH3)	63.17	$5.02x10^{-06}$	129.30	$6.75x10^{-06}$
lysine	2,12 (dtd, CH), **3,01** (t, 7.05, CH2)	33.74	$1.95x10^{-04}$	23.79	$2.71x10^{-04}$
malic acid	2.36 (dd, 16.3 and 7.0, CH), **2.67** (dd, 16.3 and 4.5, CH), 4.30 (dd, CH)	24.19	$6.02x10^{-03}$	23.05	$5.34x10^{-03}$
proline	**2.02** (m, CH2), 2.06 (m, CH)	29.40	$8.38x10^{-04}$	44.33	$1.13x10^{-03}$
pyruvic acid	**2.36** (s, CH3)	45.39	$8.82x10^{-05}$	29.57	$1.44x10^{-04}$
serine	**3.96** (m, CH2)	27.09	$2.37x10^{-03}$	23.69	$1.64x10^{-03}$
succinic acid	**2.56** (s, 2CH2)	24.42	$2.70x10^{-04}$	26.71	$9.71x10^{-04}$
tartaric acid	**4.47** (s, 2CH)	16.35	$1.25x10^{-03}$	22.51	$1.11x10^{-03}$
threonine	**1.32** (d, 6.7, CH3), 2.58 (d, 4.9, CH), 4.24 (m, CH)	25.15	$1.01x10^{-04}$	35.18	$2.71x10^{-04}$
trehalose	**5.2** (d, 3.8, CH2)	47.50	$3.47x10^{-03}$	35.91	$7.71x10^{-03}$
tyrosine	3.02 (dd, CH2), 3.17 (dd, CH2), 3.92 (dd, CH), **6.88** (d, 8.4, 2CH), 7.17 (m, 8.6, 2CH)	27.39	$1.30x10^{-06}$	32.40	$4.10x10^{-05}$
valine	**0.99** (d, 7.3, CH3), 1.04 (d, 7.3, CH3), 2.28 (m, CH)	40.77	$1.04x10^{-05}$	69.01	$1.46x10^{-05}$

These analytical results demonstrated that the experimental procedure developed can efficiently extract and quantified intracellular metabolites of fermenting *S. cerevisiae*. The extraction methodology was adapted from previous intracellular metabolite extraction protocols. According to (Villas-Bôas *et al.*, 2005), cold methanol acts as a non-selective solvent and quenching agent allowing the efficient extraction of different chemical families' intracellular metabolites. The addition of galactose in the fermentation medium and its absence in the intracellular extracts confirms the efficiency of our protocol (data not shown). Extracted metabolites were then identified and quantified by ^{1}H-NMR using the procedures applied in a previous study (Vion *et al.*, 2023).

In order to compare biological samples in the same physiological state, yeast cells were collected according to their amount of CO_2 released (10 and 50 g/L) during the alcoholic fermentation. These sampling points corresponded to the growth and stationary phases as confirmed by flow cytometry analyses (Figure 2). The time monitoring of these metabolites may provide information about the evolution of the physiological status of the cells. For example, trehalose is a typical quiescence marker and a key storage carbohydrate (Gray *et al*., 2004). The abundance of this compound is 2.2 folds higher during the stationary phase in our experiment (Table 3) (test de Wilcox, p-value = 1.14e-08 < 0.05). The detection of this accumulation confirmed the overall physiological changes occurring in fermenting yeasts.

Interestingly, a similar approach was applied for characterizing genetic and environmental factors modulating the intracellular content of *S cerevisiae* in synthetic media cultures (Airoldi, Tripodi, Guzzi, Nicastro, & Coccetti, 2015). Compared to this former study, we quantified additional intracellular compounds such as ethanol, 2,3-butanediol, and proline. The detection of additional metabolites would require a higher number of scans and more concentrated biological materials. The analytical parameters are already of 256 scans for an analysis time of 35 minutes per sample. In addition, the evolution of these intracellular compounds was achieved for a largest number of strains (17) compared to Airoldi *et al.* (only 2). By increasing the number of strains, we aim to explore the intraspecific variability of intracellular metabolite abundance that has been poorly investigated so far.

3.4. Wine and flor yeast showed life history traits and metabolic trade-offs.

The relative abundance of the 21 intracellular metabolites quantified by ^{1}H-NMR was represented by a heatmap according to populations and sampling times (Figure 4). Interestingly, the sum of the abundance of each metabolite quantified is statistically different between *flor* and *wine* populations during the growth phase. Strains belonging to the *wine* population displayed a lower pool of intracellular metabolites than strains belonging to the *flor* group (Wilcoxon test, p-value = 0.05, Panel A). In contrast, during the stationary phase, this sum of abundance is not significantly different between the two populations. This surprising finding could reflect a real difference of metabolite concentration inside the cell or could be due to biases in abundance normalization. As routinely done, we normalized NMR intensity by the number

of viable cells per milliliter. We alternatively normalized data by the total volume of fermenting yeast that is the product of the cell volume and the number of cells. In both cases, the cumulated metabolite load is significantly higher for the *flor* group revealing unsuspected variations between *S. cerevisiae* populations during the growth phase of the alcoholic fermentation.

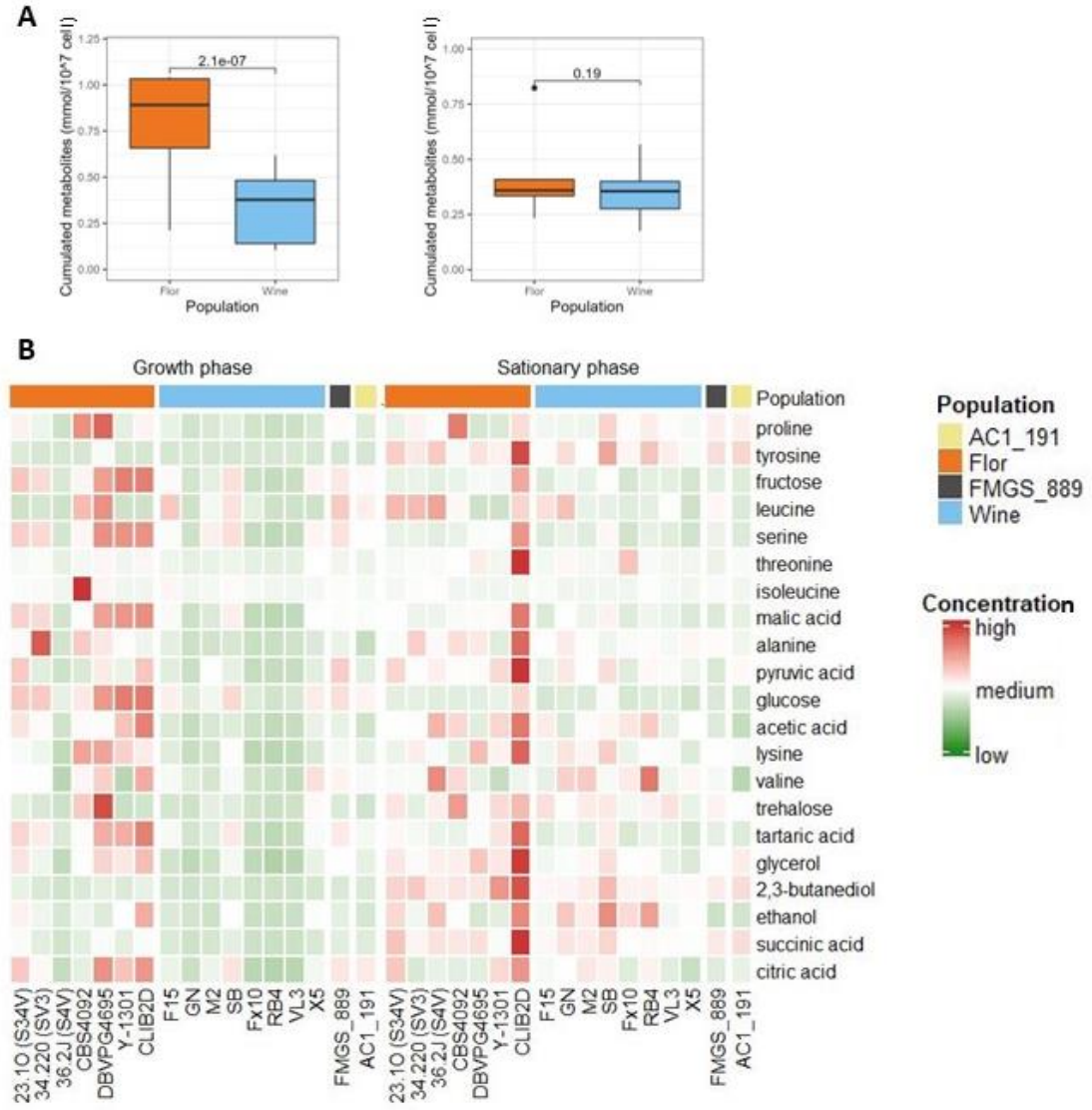


Figure 4: Panel A. Boxplots representing the distribution of the sum of the abundance of each metabolite quantified (cumulated abundance). A Wilcoxon test was applied between the flor and wine populations and the resulting p-values of the test were annotated on the plot. **Panel B**. Heatmap of the intracellular metabolites' concentration per strain. Both sampling times are represented (growth phase, and stationary phase). Strains are clustered and colored by population. Concentration values are normalized and expressed as low, medium, or high concentration of metabolites. The control strains AC1_191 and FMGS_889 were also added to the heatmap.

Previous studies reported strong correlations between the specific glycolytic flux, the number of fermenting cells and their average size among yeast strains of different origins. These phenotypic trade-offs were reported in different sugar levels concentrations (Spor *et al*., 2009; Spor, Wang, Dillmann, de Vienne, & Sicard, 2008) and during different alcoholic fermentation conditions (Albertin *et al*., 2011). The

correlation between these life-history traits defined two styles of growth strategies named “ants” and “grasshoppers”. This trade-off reflects the energetic impossibility to make at the same time big and numerous cells in a nutritional limited resource context. *Wine* yeasts have been reported to belong to the “ants” group being small, numerous, and fast fermenting at their populational level (Albertin *et al*., 2011).

The quantification of the overall metabolites content of fermenting cells provides the opportunity to test the impact of this new metabolic parameter. First, we confirmed the negative relationship between the cell size and the maximum biotic capacity previously reported by (Spor *et al.,* 2008) (Figure 5A). In addition, a weak yet highly significant positive correlation (rho = 0.49, p-value = 0.0002) was found between the overall metabolite load and the cell size during the growth phase (Figure 5B). Broadly, biggest cells are those that contain the highest intracellular metabolite specific concentration. This trend was only observed during the growth phase when the variation of the cell size between the strains is particularly high (Figure S2). Finally, we tested the correlation between the overall metabolite load and the specific glycolytic flux measured between 15 and 50 % of CO_2 released (Figure 5C). There is a strong and very significative positive correlation between the specific glycolytic flux and the overall metabolite concentration observed in both populations. Strains with a high metabolic load have a higher specific CO_2 production rate and could be considered as “grasshopper”.

Beside the broad differences found between *flor* and *wine* groups, important strain-specific variations were found. Interestingly, the strain SB had the highest relative abundance of intracellular metabolites within the *wine* population and was the biggest size of the *wine* yeasts in growth phase. This might be explained by its mosaic origin between *flor* and *wine* yeasts (Peltier *et al.,* 2021). In contrast, the *flor* yeast 36.2J (S4V) exhibited a low relative abundance of intracellular metabolites, like most strains of the *wine* group. Other yeast strains displayed distinctive metabolic profiles, such as the *flor* yeast CLIB-2D which had a much higher content of intracellular metabolites in the stationary phase compared to all other strains. However, its average cell size is not particularly high during this phase (Figure S1). This important strain variability suggested that the cumulated load of metabolites is a complex parameter that remains to be investigated.

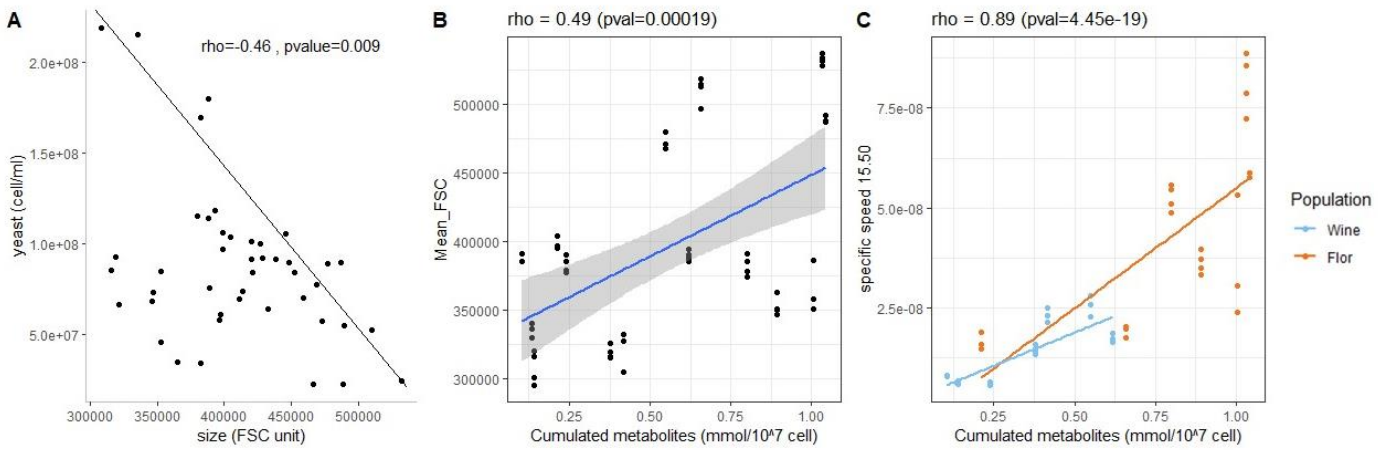


Figure 5: Each dot represents a biomass sample that was quantified by flow cytometry before to be submitted to metabolite extraction. The mean FSC parameter is a proxy of the cell size. **Panel A.** Correlation between the cell size and the cumulated concentration of intracellular metabolites during the growth phase. **Panel B.** Correlation between the cell size and the total cellular concentration during the growth phase. **Panel C. Correlation** between the cumulated concentration of intracellular metabolites and the specific glycolytic flux (per cell) during the growth phase.

3.5. The variability of metabolites abundance is mostly characterized by complex genetic × sampling time interactions.

To deeply investigate the differences of metabolites content between populations and sampling phases, a nested analysis of variance was applied. This analysis estimated the effects of the fermentation phase (Time), genetic factors and their possible interactions. The genetic contribution effect was decomposed in population and strain within population effects as detailed by the linear model 2 (see methods). The contribution of each factor on 17/21 quantitative traits is summarized in Figure 6. A Levene test ($\alpha = 0.05$) was performed to test the variance homogeneity and the four traits were excluded from the analysis.

Some compounds such as 2,3-butanediol, tyrosine and succinic acid are greatly impacted by the sampling moment which explained almost 50% of the variance for 2,3- butanediol and tyrosine. Indeed, intracellular 2,3-butanediol and tyrosine globally increased four times between growth and stationary phases while succinic acid is 3 times more concentrated inside the cells at stationary phase. The accumulation of 2,3-butanediol by yeast cells during stationary phase is figured out (figure 7A). This compound is the end metabolite of the acetoin degradation a minor by-product of alcoholic fermentation. Beside these few metabolites, most of the compounds showed a quite steady intracellular concentration between the two phases. This phenomenon highlighted that yeast cells are able to maintain a relative intracellular homeostasis of their metabolites during the alcoholic fermentation process. This observation also implies that the concentrations measured were not contaminated by the extracellular medium. For example, extracellular

fructose drastically decreased between growth and stationary phase (> 20 g/L), but its intracellular concentration is quite similar according to the sampling phases (figure 7B). Interestingly, most of the metabolic compounds (14/17) assayed are strongly impacted by the genetic component which represented more than 25% of the total variance. Although the population origin was always significative, the decomposition of the population and strain variances indicated a strong strain variability. In addition, an important effect of genetic per time (GxT) interaction was observed for several compounds. This effect ranged between 8 and 31 % of the total variance and highlighted the difficulty to establish clear pattern of intracellular metabolic content between flor and wine populations as illustrated for fructose, glycerol, and malic acid. Figure 7 B, C, and D, respectively.

Population genomic studies reveals that the species *S. cerevisiae* is constituted by several distinct populations that are characterized by specific genetic and metabolic features. Wine and flor yeast constitutes two distinct groups of strains (Coi *et al.*, 2017). Although they share the same ecological niche (wine environment), they developed alternative metabolic strategies for surviving. The first produces ethanol from grape juice while the second consumes ethanol from wine by forming a velum (Legras *et al.,* 2016). Surprisingly few studies compared the phenotypic properties of wine and flor yeast during the alcoholic fermentation (Esteve-Zarzoso, Peris-Torán, García-Maiquez, Uruburu, & Querol, 2001). Recently, we identified unsuspected differences in the extracellular composition of wines produced by flor and wine strains. Among others, the content of malic acid at the end of the alcoholic fermentation well discriminated the two groups, the flor group being a stronger consumer of this organic acid (Vion *et al.,* 2023). Since flor and wine yeast have evolved under different energetic metabolism (fermentation or respiration) we speculated that they might carry intracellular metabolic hallmarks of these very different selective pressures. The previous section highlighted that the cumulated content of the intracellular metabolites is significantly different between these two groups during the first stages of the alcoholic fermentation. Taken individually, none of the metabolites are able to clearly discriminate those groups. Among the most impacting metabolites identified by our nested ANOVA, glucose, fructose, malic acid, citric acid, glycerol has a strong population effect accounting for more than 20 % of the total variance explained. However, the strain variability effect within groups is always stronger impairing to define if those metabolic compounds are group specific.

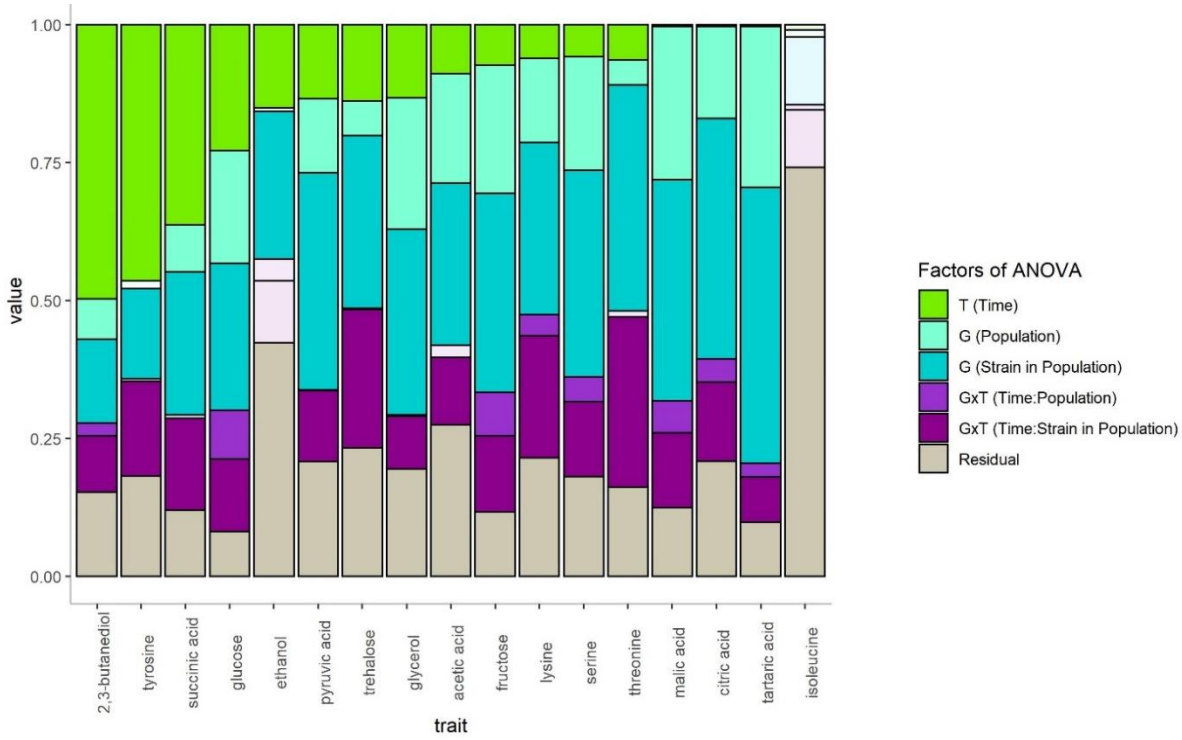


Figure 6: Bar graphs indicating the part of variance explained by the different factors of the ANOVA. The letters T and G represent the time of fermentation and the genetic factors, respectively. The nested ANOVA applied allows to evaluate the effect of the genetic group (population) and the effect of strain within each population (Strain). GxT represents the interaction between genetic and time and was decomposed in two nested factors Time:Population and Time:Strain in Population. Gray tons indicate non significative effect of the factors.

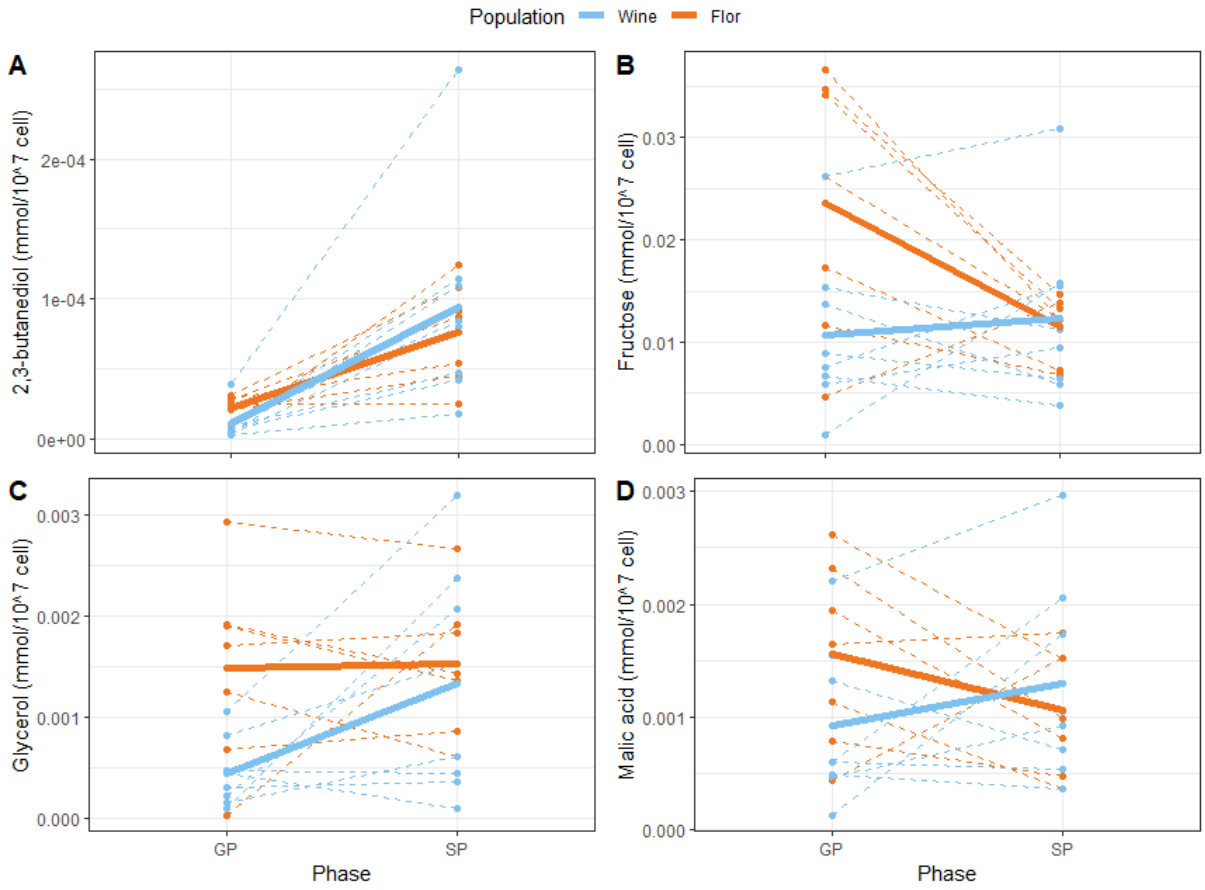


Figure 7: Panel A. Evolution of intracellular malic acid content between growth phase (GP) and stationary phase (SP) for the 15 strains. Strains are colored according to their population origin. The bold line represents the average value of the evolution for each population. **Panel B.** Evolution of intracellular 2,3-butanediol content between GP and SP. **Panel C.** Evolution of intracellular fructose between GP and SP. **Panel D.** Evolution of intracellular glycerol between GP and SP.

3.6. Comparison of intracellular metabolites abundance of two strains showing opposed malic acid metabolism

The intracellular metabolic profile of the two control strains (AC1_191 and FMGS_889) were compared in this last section. These strains were selected for their strong discrepancy regarding their malic acid metabolism which is illustrated in the Figure 8A. From the stationary phase sampling point, the extracellular malic acid concentration between the two strains is significantly different (1.23 g/L of difference) and reached 2.4 g/L at the end of the alcoholic fermentation. These differences are much higher than those observed between *flor* and *wine* groups that only differs by 0.15 g/L at the end of the alcoholic fermentation (Figure 8 panel B). The NMR quantification method developed provided the opportunity to investigate which intracellular compounds could reflect differences in malic acid metabolism observed at the extracellular level. Surprisingly, the different organic acids quantified showed very similar concentrations between the two strains. Thus, whatever the dynamic of malic acid consumption and/or production, the intracellular concentrations of malic acid as well as other organic acids (succinic, citric, and

pyruvic) are quite steady (Figure 9A). In contrast, the intracellular concentrations of valine, alanine, and threonine measured at the stationary phase displayed a statistical difference between the two strains (Figure 9B). These amino acids are indirectly derived from pyruvic and alpha keto-glutaric acid and might resulted from a difference of malic acid assimilation. However additional experiments are necessary for establishing a metabolic link between these compounds and the malic consumption differences observed between AC1_191 and FMGS_889.

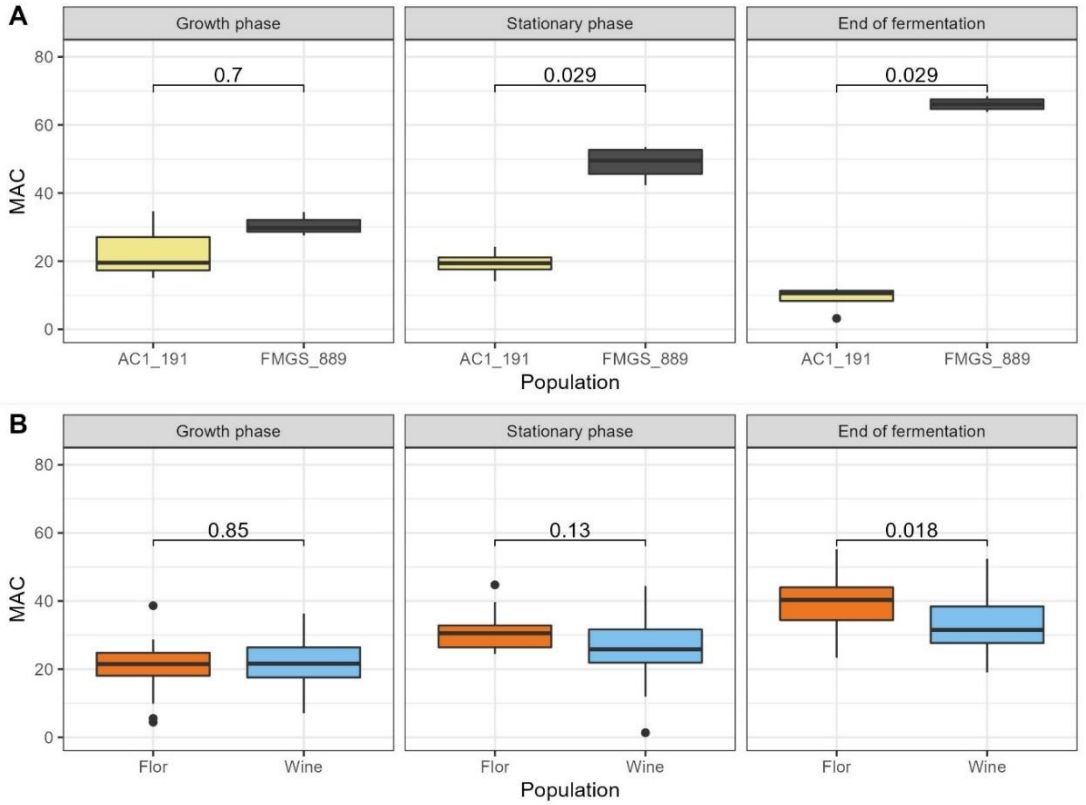


Figure 8: extracellular malic acid (g/L) according to the fermentation phase. **Panel A.** A Wilcox test has been performed between AC1_191 and FMGS_889 strains and the resulting p-value is displayed on the graphs. **Panel B.** A Wilcox test has been performed between flor and wine populations and the resulting p-value is displayed on the graphs.

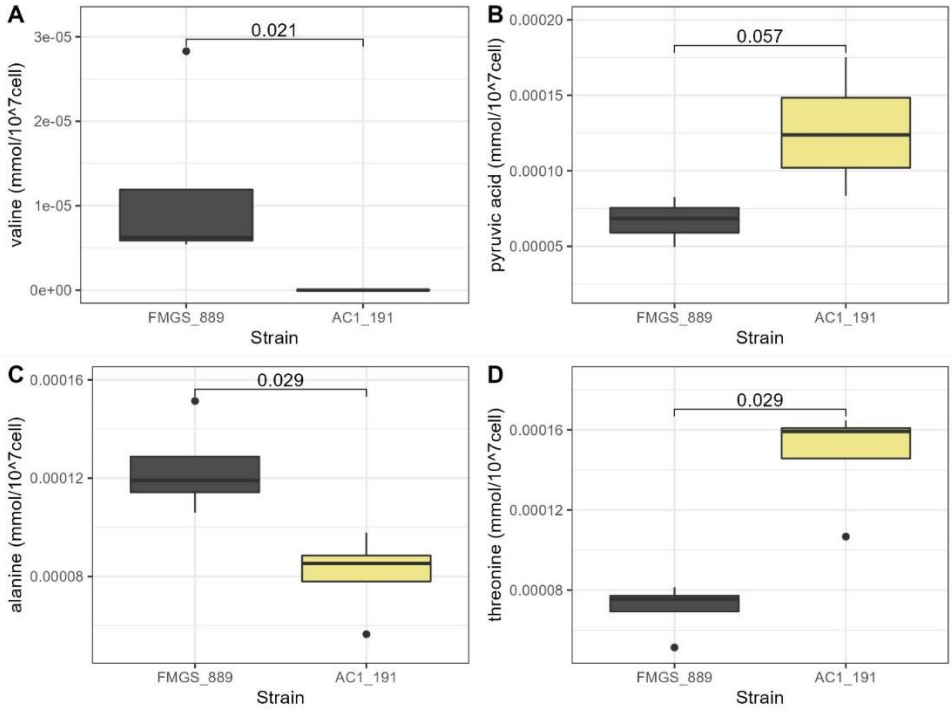


Figure 9: Concentrations of intracellular valine (**Panel A**), pyruvic acid (**Panel B**), alanine (**Panel C**) and threonine (**Panel D**) for the two control strains in stationary phase. A Wilcox test has been performed between the two strains and the p-values are displayed on the plots.

Conclusion

In this work we developed a protocol for quantifying by ^{1}H-NMR the intracellular concentration of yeast biomass during the alcoholic fermentation of natural grape juice. This protocol was applied to study the phenotypic variability of the metabolic content of several *Saccharomyces cerevisiae* strains belonging to two distinct genetic populations the *flor* and the *wine* yeasts. The precise quantification of 21 metabolites at two time series provided new results to the scientific community. First, the method applied allow to detect significative differences between strains demonstrating that the intracellular amount of the main metabolites is variable. The cumulated concentration of these metabolites is significantly different between two group of strains at least during the growth phase. This difference of abundance is also correlated with life history traits such as the average size of the cell. In addition, we demonstrated that intracellular metabolic variability is governed by the sampling time and the yeast strain with complex interactions that impair to draw simple physiological conclusions. For instance, strains with a very different metabolic production of malic acid are very similar for most of their intracellular metabolites. Despite these complex differences the method and the first results obtained reinforce the interest of this analytic tool for studying the role of intracellular metabolite concentration in the physiology of the fermenting yeast *S. cerevisiae*.

Supplementary data

Table S1: Composition of the grape juice used in the experiments.

	SB21
L-malic acid (g/L)	4.20
pH	3.30
Fermentable sugars (g/L)	186.1
Assimilable nitrogen (mg N/L)	141
Total SO_2 (mg/L)	34

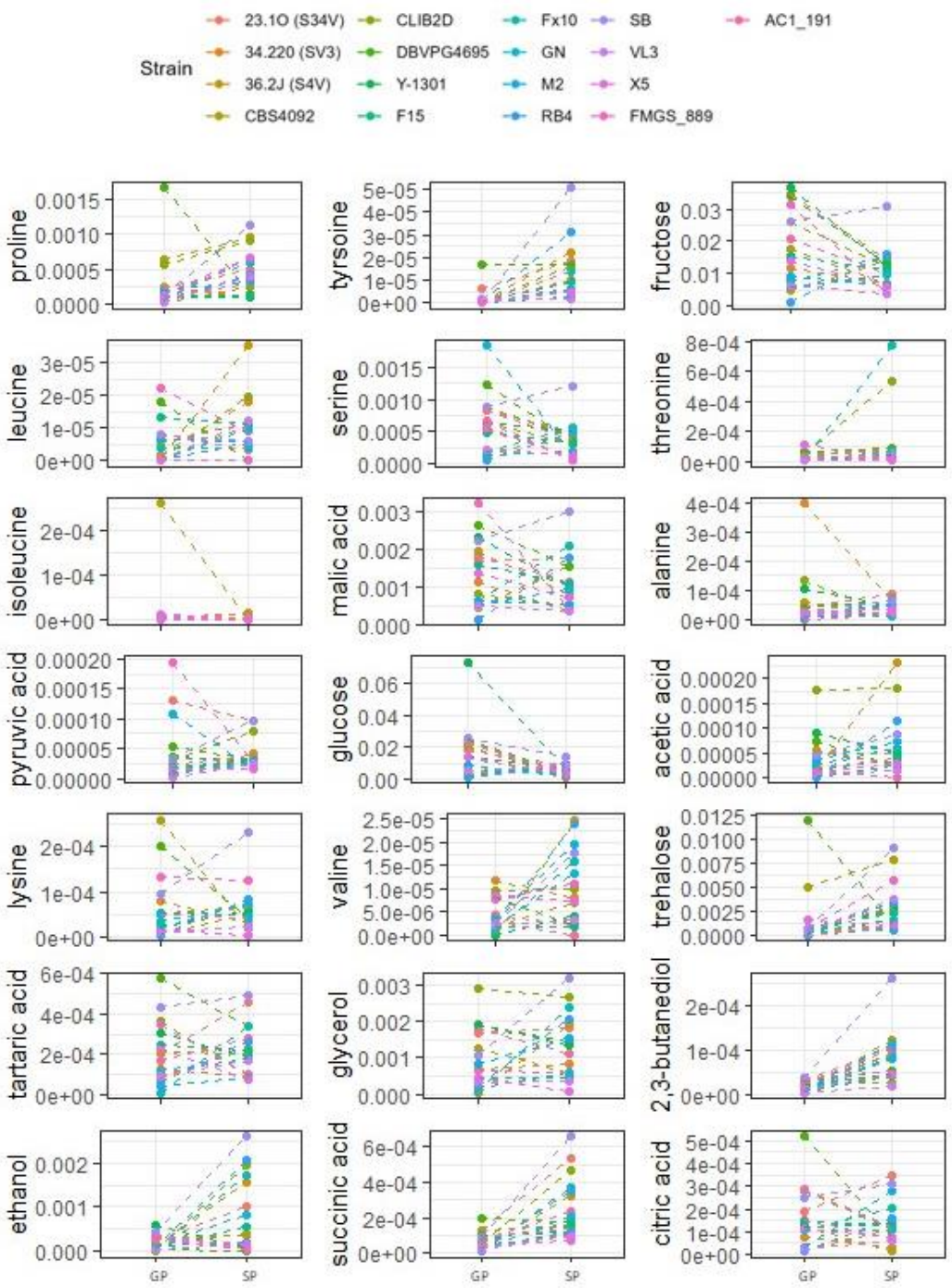


Figure S1: Evolution of intracellular content of the 21 metabolites between growth phase (GP) and stationary phase (SP) for the 17 strains. All concentrations are expressed in mmol/10^7cell.

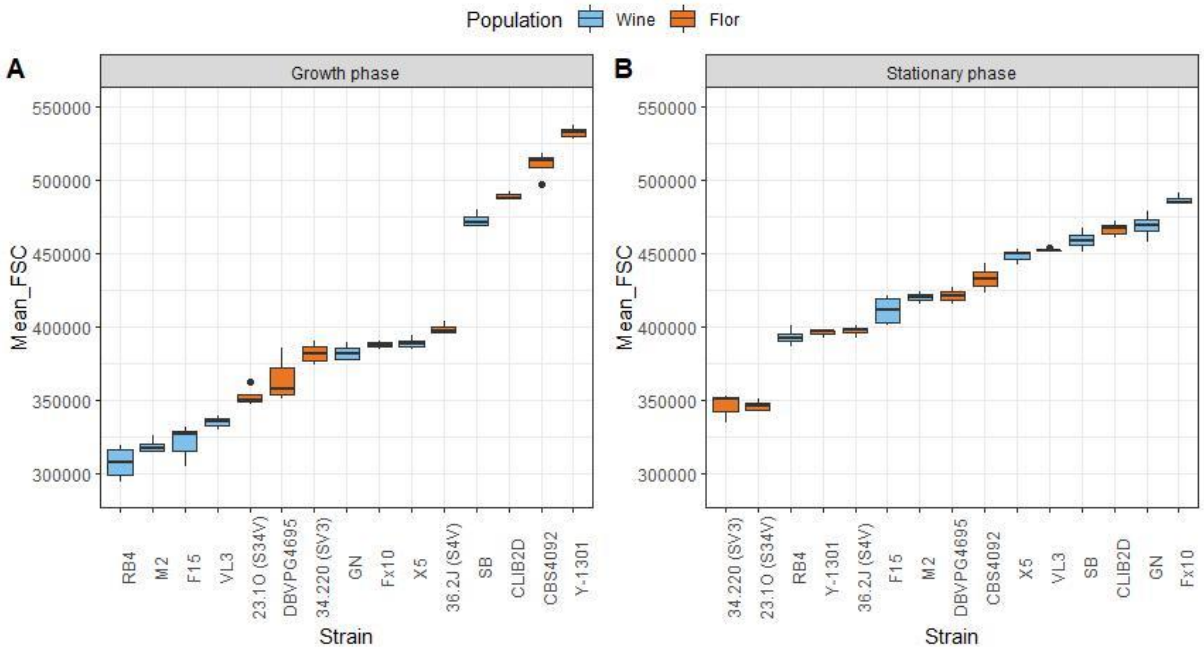


Figure S2. Each box plot represents the mean FSC parameter considered as a proxy of the cell size for the 15 strains belonging to the *flor* and *wine* groups. The quantification of cell size was shown for the growth and stationary phase on panel A and B, respectively.

Fundings

This study received (1) financial support from the French government in the framework of the IdEX Bordeaux University "Investments for the Future" program / GPR Bordeaux Plant Sciences (2) Region Aquitaine funding Program MEGAVIP

Acknowledgements

This work was supported by the Bordeaux Metabolome Facility and MetaboHUB (ANR-11-INBS-0010 project)

Authors warmly thanks Prof. Hervé Alexandre, Prof. Joseph Schacherer, and Dr. Jean-Luc Legras (CIRM) for providing some of the *flor* yeast strains used in this work. We also thanks Dr. Warren Albertin for her suggestions and revision of the manuscript.

Bibliography

Airoldi, C., Tripodi, F., Guzzi, C., Nicastro, R., & Coccetti, P. (2015). NMR analysis of budding yeast metabolomics: A rapid method for sample preparation. *Molecular BioSystems*, *11*(2), 379–383. https://doi.org/10.1039/c4mb00452c

Albertin, W., Marullo, P., Aigle, M., Dillmann, C., de Vienne, D., Bely, M., & Sicard, D. (2011). Population size drives industrial *Saccharomyces cerevisiae* alcoholic fermentation and is under genetic control. *Applied and Environmental Microbiology*, *77*(8), 2772–2784. https://doi.org/10.1128/AEM.02547-10

Alexandre, H. (2013). Flor yeasts of *Saccharomyces cerevisiae*-Their ecology, genetics and metabolism. *International Journal of Food Microbiology*, *167*(2), 269–275. https://doi.org/10.1016/j.ijfoodmicro.2013.08.021

Balsa-canto, E., Alonso-del-real, J., & Querol, A. (2020). *Temperature Shapes Ecological Dynamics in Mixed Culture Fermentations Driven by Two Species of the* Saccharomyces *Genus*. *8*(August), 1–12. https://doi.org/10.3389/fbioe.2020.00915

Cobas, C., Seoane, F., Domínguez, S., & Sykora, S. (2011). A new approach to improving automated analysis of proton NMR spectra through Global Spectral Deconvolution (GSD). *Spectroscopy Europe*, *23*(1), 26–30.

Coi, A. L., Bigey, F., Mallet, S., Marsit, S., Zara, G., Gladieux, P., … Legras, J. L. (2017). Genomic signatures of adaptation to wine biological ageing conditions in biofilm-forming flor yeasts. *Molecular Ecology*, *26*(7), 2150–2166. https://doi.org/10.1111/mec.14053

Compagno, C., Merico, A., Sulo, P., & Pis, J. (2007). *Fermentative lifestyle in yeasts belonging to the Saccharomyces complex*. *274*, 976–989. https://doi.org/10.1111/j.1742-4658.2007.05645.x

Compagno, C., Piskur, J., & Hagman, A. (2013). *Yeast '" Make-Accumulate-Consume "' Life Strategy Evolved as a Multi-Step Process That Predates the Whole Genome Duplication*. *8*(7). https://doi.org/10.1371/journal.pone.0068734

David-Vaizant, V., & Alexandre, H. (2018). Flor yeast diversity and dynamics in biologically aged wines. *Frontiers in Microbiology*, *9*(SEP), 1–16. https://doi.org/10.3389/fmicb.2018.02235

Ekkers, D. M., Tusso, S., Moreno-Gamez, S., Rillo, M. C., Kuipers, O. P., & Van Doorn, G. S. (2022). Trade-Offs Predicted by Metabolic Network Structure Give Rise to Evolutionary Specialization and Phenotypic Diversification. *Molecular Biology and Evolution*, *39*(6), 974–984. https://doi.org/10.1093/molbev/msac124

Eldarov, M. A., Beletsky, A. V., Tanashchuk, T. N., Kishkovskaya, S. A., Ravin, N. V., & Mardanov, A. V. (2018). Whole-genome analysis of three yeast strains used for production of sherry-like wines revealed genetic traits specific to flor yeasts. *Frontiers in Microbiology*, *9*(MAY), 1–13. https://doi.org/10.3389/fmicb.2018.00965

Esteve-Zarzoso, B., Peris-Torán, M. J., García-Maiquez, E., Uruburu, F., & Querol, A. (2001). Yeast Population Dynamics during the Fermentation and Biological Aging of Sherry Wines. *Applied and Environmental Microbiology*, *67*(5), 2056–2061. https://doi.org/10.1128/AEM.67.5.2056-2061.2001

Fidalgo, M., Barrales, R. R., Ibeas, J. I., & Jimenez, J. (2006). Adaptive evolution by mutations in the FLO11 gene. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(30), 11228–11233. https://doi.org/10.1073/pnas.0601713103

Goddard, M. R. (2008). Quantifying The Complexities Of *Saccharomyces cerecisiae's* Ecosystem Engineering Via Fermetnation. *Ecology*, *89*(8), 2077–2082.

Goddard, M. R., & Greig, D. (2015). *Saccharomyces cerevisiae*: a nomadic yeast with no niche? *FEMS Yeast Research*, *15*(3), fov009. https://doi.org/10.1093/femsyr/fov009

Gray, J. V., Petsko, G. A., Johnston, G. C., Ringe, D., Singer, R. A., & Werner-Washburne, M. (2004). "Sleeping Beauty": Quiescence in *Saccharomyces cerevisiae* . *Microbiology and Molecular Biology Reviews*, *68*(2), 187–206. https://doi.org/10.1128/mmbr.68.2.187-206.2004

Huang, C., Roncoroni, M., & Gardner, R. C. (2014). MET2 affects production of hydrogen sulfide during wine fermentation. *Applied Microbiology and Biotechnology*, *98*(16), 7125–7135. https://doi.org/10.1007/s00253-014-5789-1

Jewison, T., Knox, C., Neveu, V., Djoumbou, Y., Guo, A. C., Lee, J., … Wishart, D. S. (2012). YMDB: The yeast metabolome database. *Nucleic Acids Research*, *40*(D1), 815–820. https://doi.org/10.1093/nar/gkr916

Legras, J. L., Moreno-Garcia, J., Zara, S., Zara, G., Garcia-Martinez, T., Mauricio, J. C., … Budroni, M. (2016). Flor yeast: New perspectives beyond wine aging. *Frontiers in Microbiology*, *7*(APR), 1–11. https://doi.org/10.3389/fmicb.2016.00503

Martí-Raga, M., Peltier, E., Mas, A., Beltran, G., & Marullo, P. (2017). Genetic Causes of Phenotypic Adaptation to the Second Fermentation of Sparkling Wines in *Saccharomyces cerevisiae*. *G3: Genes|Genomes|Genetics*, g3.116.037283. https://doi.org/10.1534/g3.116.037283

Marullo, P., Aigle, M., Bely, M., Masneuf-Pomarède, I., Durrens, P., Dubourdieu, D., & Yvert, G. (2007). Single QTL mapping and nucleotide-level resolution of a physiologic trait in wine *Saccharomyces cerevisiae* strains. *FEMS Yeast Research*, *7*(6), 941–952. https://doi.org/10.1111/j.1567-1364.2007.00252.x

Moreno-García, J., García-Martínez, T., Millán, M. C., Mauricio, J. C., & Moreno, J. (2015). Proteins involved in wine aroma compounds metabolism by a *Saccharomyces cerevisiae* flor-velum yeast strain grown in two conditions. *Food Microbiology*, *51*, 1–9. https://doi.org/10.1016/j.fm.2015.04.005

Moreno-García, J., García-Martínez, T., Moreno, J., & Mauricio, J. C. (2015). Proteins involved in flor yeast carbon metabolism under biofilm formation conditions. *Food Microbiology*, *46*, 25–33. https://doi.org/https://doi.org/10.1016/j.fm.2014.07.001

Peltier, E., Bernard, M., Trujillo, M., Barbe, J.-C., Prodhomme, D., Gibon, Y., & Marullo, P. (2018). Wine yeast phenomics: A standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. *PLoS ONE*, 1–23. https://doi.org/10.1371/journal.pone.0190094

Peltier, E., Bernard, M., Trujillo, M., Prodhomme, D. D., Barbe, J.-C., Gibon, Y., & Marullo, P. (2018). Wine yeast phenomics: a standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. *PLOS ONE*, *13*(1), 191353. https://doi.org/10.1101/191353

Peltier, E., Sharma, V., Martí Raga, M., Roncoroni, M., Bernard, M., Jiranek, V., … Marullo, P. (2018). Dissection of the molecular bases of genotype x environment interactions: A study of phenotypic plasticity of *Saccharomyces cerevisiae* in grape juices. *BMC Genomics*, *19*(1), 1–20. https://doi.org/10.1186/s12864-018-5145-4

Peltier, E., Vion, C., Abou Saada, O., Friedrich, A., Schacherer, J., & Marullo, P. (2021). Flor Yeasts Rewire the Central Carbon Metabolism During Wine Alcoholic Fermentation. *Frontiers in Fungal Biology*, *2*(October). https://doi.org/10.3389/ffunb.2021.733513

Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergström, A., … Schacherer, J. (2018). Genome

evolution across 1,011 *Saccharomyces cerevisiae* isolates Species-wide genetic and phenotypic diversity. *Nature*, *556*, 339–347.

Pfeiffer, T., Schuster, S., & Bonhoeffer, S. (2001). Cooperation and competition in the evolution of ATP-producing pathways. *Science*, *292*(5516), 504–507. https://doi.org/10.1126/science.1058079

Piškur, J., Rozpedowska, E., Polakova, S., Merico, A., & Compagno, C. (2006). How did *Saccharomyces* evolve to become a good brewer? *Trends in Genetics*, *22*(4), 183–186. https://doi.org/10.1016/j.tig.2006.02.002

Pronk, J. T., Steensma, H. Y., & van Dijken, J. P. (1996). Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast*, *12*, 1607–1633. https://doi.org/10.1038/167653b0

Puig-Castellví, F., Alfonso, I., Piña, B., & Tauler, R. (2015). A quantitative 1H NMR approach for evaluating the metabolic response of *Saccharomyces cerevisiae* to mild heat stress. *Metabolomics*, *11*(6), 1612–1625. https://doi.org/10.1007/s11306-015-0812-9

R Development Core Team. (2016). *R: A language and environment for statistical computing. 2*, 1–12.

Ribéreau-Gayon, P., Dubourdien, D., Donéche, B. & A. L. (2006). *Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition* (Vol. 1).

Spor, A., Nidelet, T., Simon, J., Bourgais, A., De Vienne, D., & Sicard, D. (2009). Niche-driven evolution of metabolic and life-history strategies in natural and domesticated populations of *Saccharomyces cerevisiae*. *BMC Evolutionary Biology*, *9*(1), 1–12. https://doi.org/10.1186/1471-2148-9-296

Spor, A., Wang, S., Dillmann, C., de Vienne, D., & Sicard, D. (2008). "Ant" and "grasshopper" life-history strategies in *Saccharomyces cerevisiae*. *PLoS ONE*, *3*(2). https://doi.org/10.1371/journal.pone.0001579

Stitt, M., McC., L. R., Gerhardt, R., & Heldt, H. W. (1989). Metabolite Levels in Specific Cells and Subcellular Compartements of Plant Leaves. *Methods in Enzymology*, *174*(1984), 518–552.

Thomson, J. M., Gaucher, E. A., Burgan, M. F., De Kee, D. W., Li, T., Aris, J. P., & Benner, S. A. (2005). Resurrecting ancestral alcohol dehydrogenases from yeast. *Nature Genetics*, *37*(6), 630–635. https://doi.org/10.1038/ng1553

Villas-Bôas, S. G., Højer-Pedersen, J., Åkesson, M., Smedsgaard, J., & Nielsen, J. (2005). Global metabolite analysis of yeast: evaluation of sample preparation methods. *Yeast*, *22*(14), 1155–1169. https://doi.org/10.1002/yea.1308

Vion, C., Le Mao, I., Yeramian, N., Da Costa, G., Richard, T., & Marullo, P. (2023). Targeted 1H-NMR wine analyses revealed specific metabolome signatures of yeast populations belonging to the *Saccharomyces* genus. *Submitted*.

Vion, C., Muro, M., Bernard, Margaux, Richard, B., Fautre, V., Yeramian, N., … Marullo, P. (2022). New malic acid producer strains of *Saccharomyces* cerevisiae for preserving wine acidity during alcoholic fermentation. *Food Microbiology*, *112*(xxxx). https://doi.org/10.1016/j.fm.2022.104209

Vion, C., Peltier, E., Bernard, M., Muro, M., & Marullo, P. (2021). Marker assisted selection of malic-consuming *Saccharomyces cerevisiae* strains for winemaking. Efficiency and limits of a qtl's driven breeding program. *Journal of Fungi*, *7*(4). https://doi.org/10.3390/jof7040304

Zampar, G. G., Kümmel, A., Ewald, J., Jol, S., Niebel, B., Picotti, P., … Heinemann, M. (2013). Temporal system-

level organization of the switch from glycolytic to gluconeogenic operation in yeast. *Molecular Systems Biology*, *9*(1), 651. https://doi.org/10.1038/msb.2013.11

Zimmer, A., Durand, C., Loira, N., Durrens, P., Sherman, D. J., & Marullo, P. (2014). QTL dissection of lag phase in wine fermentation reveals a new translocation responsible for *Saccharomyces cerevisiae* adaptation to sulfite. *PLoS ONE*, *9*(1), 37–39. https://doi.org/10.1371/journal.pone.0086298

Conclusion du chapitre 2

Dans ce chapitre, la RMN du proton a été utilisée comme outil pour étudier la diversité métabolique de souches de levures du genre *Saccharomyces*. La variabilité métabolique d'un large éventail de souches (~50) du genre *Saccharomyces* a été évaluée. Ces souches ont été sélectionnées sur la base de leur lieu d'isolement correspondant à des matrices acides (vin, cidre, jus de fruits, choucroute). De plus, deux populations génétiquement distinctes mais partageant une même niche écologique (levures de vin et de voile) ont également été comparées au niveau intracellulaire. Enfin une comparaison de deux souches extrêmes acidifiante et démaliquante, sélectionnées dans le premier chapitre de cette thèse, a été réalisée afin de tenter d'expliquer les différences extracellulaires perçues au niveau intracellulaire. Les principaux éléments à retenir de cette partie sont les suivants :

- Au niveau extracellulaire, une méthode de dosage des composés du vin par RMN déjà disponible au laboratoire a été adaptée à nos échantillons. Plus de 400 échantillons issus des fermentations réalisées au laboratoire ont été analysés par RMN du proton. Ces analyses ont permis de quantifier 15 composés dont certains sont peu regardés avec les méthodes de dosages enzymatiques classiques.
- Certains métabolites tel que le glycérol, l'acide malique ou encore l'acide succinique ont été identifiés comme différentiellement produits ou consommés entre les groupes de souches. Les souches se différencient à deux niveaux distincts. Au niveau de l'espèce *S. cerevisiae* et *S. uvarum* présentent des divergences métabolomiques très nettes. Au niveau de groupe génétique appartenant à la même espèce les levures de voile (*flor*) et de vinifications présentent quelques métabolites distinctifs. Une forte variabilité intra-groupe a également été observée pour d'autres composés tels que le tréhalose ou l'acide acétique.
- Une méthode simple d'extraction des composés intracellulaires de la levure a été mise au point au laboratoire à partir de cultures en petit volume. Au total, 350 extractions ont été effectuées et analysées par RMN du proton. Ces analyses ont permis de quantifier 21 composés intracellulaires de *S. cerevisiae*.
- Au niveau intracellulaire, des différences d'abondance de métabolites entre les souches et à différentes étapes de la fermentation alcoolique ont été mise en évidence. Une tendance à une surabondance de l'ensemble des métabolites étudiés a été observée chez les levures de voile en phase de croissance. Ce phénomène est très corrélé à la vitesse spécifique de croissance des levures de voile ainsi qu'à la taille des cellules.
- La comparaison de deux levures extrêmes acidifiante et démaliquante au niveau intracellulaire a montré qu'il n'y a pas de différence significative au niveau du pool de métabolites étudiés. Les

différences de concentrations en acides organiques extracellulaires, notamment l'acide malique, sont donc probablement liées à l'existence de flux d'entrée et de sortie de cet acide. La question de l'implication d'un transporteur est donc soulevée par ces résultats.

Chapitre 3 : Etude des voies métaboliques empruntées pas l'acide malique lors de la fermentation alcoolique

Chapitre 3 : Etude des voies métaboliques empruntées par l'acide malique lors de la fermentation alcoolique

1. Contexte et objectifs

Les différences métaboliques observées entre les différentes souches au niveau extracellulaire ont soulevé la question des voies métaboliques empruntées lors de la consommation et production de ces différents composés. Ce troisième chapitre s'intéresse aux flux d'assimilation de cet acide organique au cours de la fermentation alcoolique qui demeurent encore méconnues. Pour cela, des fermentations avec de l'acide malique marqué avec du ^{13}C, comme seconde source de carbone, ont été réalisées. L'objectif était d'effectuer une filiation isotopique afin de déterminer les composés ayant intégré du ^{13}C et ainsi retracer les routes métaboliques empruntées par l'acide malique consommé. Des mesures temporelles ont également été réalisées afin de dépasser le simple bilan global et mieux comprendre les phénomènes de consommation et relargage observés chez les souches de *S. cerevisiae*.

Le travail mené dans ce chapitre a été effectué en partenariat avec l'unité Science Pour l'Œnologie (SPO) de Montpellier, avec l'équipe FLAM (« *Fermentation alcoolique : Levures, Arômes, Métabolisme* »). Leur expertise sur le fonctionnement du métabolisme des levures en œnologie a été une aide précieuse pour la mise au point des expériences et l'interprétation des résultats. Les expériences de fermentations avec l'acide malique marqué ont été réalisées à l'unité de recherche UMR Œnologie de l'Université de Bordeaux. Les extractions des métabolites ainsi que les analyses par RMN ont également été réalisés à l'unité de recherche, en collaboration avec l'axe MIB. Cette étude a été l'opportunité d'effectuer des expériences de RMN du proton et RMN 2D ((^{13}C-^{1}H)-HSQC) permettant d'accéder à des informations sur les métabolites marqués au niveau extra- et intracellulaire. Enfin les analyses par GC-MS et LC-MSMS ont été effectués à l'unité SPO et à la Faculté des Sciences de Montpellier respectivement.

2. Principaux résultats

Cette partie a fait l'objet d'un article scientifique.

La conceptualisation des expériences a été réalisée par Philippe Marullo (PM), Carole Camarasa (CC), Audrey Bloem (AB), Tristan Richard (TR), et moi-même. J'ai réalisé les expériences de fermentation en milieu synthétique contenant l'acide malique marqué au sein de l'unité de recherche Œnologie de l'ISVV. Après avoir extrait les métabolites intracellulaires et préparé les échantillons extracellulaires, j'ai passé l'ensemble de ces échantillons en RMN ^{1}H et RMN 2D avec l'aide de Grégory Da Costa. J'ai été accueillie

au sein de l'unité de Recherche SPO pendant 1 mois pour effectuer les expériences de détermination d'enrichissement isotopique des acides aminés en GC-MS. J'ai préparé les échantillons et ces derniers ont été passés sur la masse par le personnel de l'unité de recherche SPO. Les échantillons de surnageant ont été passés en LC-MS/MS à la plateforme de métabolomique de la Faculté des Sciences de Montpellier par Gilles Valette. J'ai traité l'ensemble des résultats recueillis par les différentes méthodes et participé au traitement statistique de ces derniers sous la supervision de PM. J'ai rédigé l'article avec PM et CC et AB ont apporté leurs corrections et relecture au document ainsi que Tristan Richard pour la partie méthodologie concernant la RMN.

Investigation of malic acid metabolic pathways during alcoholic fermentation using GC-MS, LC-MS, and NMR derived ^{13}C-labeled data

Vion Charlotte[1,2], Bloem Audrey[3], Valette Gilles[4], Da Costa Grégory[2], Richard Tristan[2], Camarasa Carole[3], Marullo Philippe[1,2]

[1]Biolaffort, Bordeaux, FRANCE

[2] UMR 1366 Œnologie, Université de Bordeaux, INRAE, Bordeaux INP, BSA, ISVV

[3] UMR SPO, INRA, SupAgroM, Université de Montpellier

[4] LPM, Université de Montpellier

Abstract

Malic acid has a strong impact on wine pH and the contribution of fermenting yeasts to modulate its concentration has been intensively investigated in the past. Recent advances in yeast genetics have shed light on the unexpected property of some strains to produce large amounts of malic acid ("acidic strains") while most of the wine starters consume it during the alcoholic fermentation. Being a key metabolite of the central carbohydrate metabolism, malic acid participates to TCA and glyoxylate cycles as well as neoglucogenesis. Although present at important concentrations in grape juice, the metabolic fate of malic acid has been poorly investigated. In this work, we used ^{13}C-labeled malic acid to understand the main routes of its consumption and its *de novo* production. Two strains selected for their opposed malic acid metabolism were compared by combining several analytical chemistry techniques. The isotopic enrichment of intracellular amino acids was measured by GC-MS, the relative quantification of intracellular and extracellular labeled compounds was achieved by 2D-NMR, and the absolute quantification of labeled and unlabeled extracellular organic acids was achieved by LC-MS/MS. Although, both strains consume most of the malic acid provided, the "acidic strain" produces *de novo* malic acid during the second part of the alcoholic fermentation. In addition, ^{13}C-filiation analyses provided evidence that most of the TCA is fed by glycolytic pyruvate and/or by cytosolic acetyl-CoA. Our results also confirmed that malic acid may be a secondary source of TCA cycle during alcoholic fermentation especially in high malic acid consuming strains that has an efficient malo-ethanolic fermentation. Finally, ^{13}C-labeled compounds belonging to amino acids, alcoholic fermentation and neoglucogenesis pathways were identified, highlighting the pleiotropic position of malic acid in both catabolic and anabolic routes.

Key words

^{13}C-labeling, malic acid, central carbon metabolism

1. Introduction

Central carbon metabolism encompasses enzymatic pathways that convert carbohydrate nutrients into energy, building blocks, and byproducts. The budding yeast *Saccharomyces cerevisiae* preferentially metabolizes reducing sugars such as glucose and fructose by the glycolytic pathway that are oxidized in pyruvate providing to the cell 2 molecules of ATP [1]. When oxygen is available, and reducing sugars are present in limited concentrations, the pyruvate produced by the glycolysis is fully oxidized in CO_2 and H_2O through the TCA cycle. This pathway requires the transport of pyruvate into the mitochondrial matrix by the mitochondrial pyruvate carrier (MPC) expressed during growth on nonfermentable carbon sources [2]. Pyruvate is then oxidized in acetyl-CoA by the pyruvate dehydrogenase complex [3]. The TCA cycle also requires another essential metabolite, oxaloacetate, that is produced in the cytoplasm by pyruvate carboxylases Pyc1p/Pyc2p [4]. When it is fully functional, the TCA cycle is coupled with oxidative phosphorylation and provides to the cell 36 molecules of ATP [5]. Under anaerobic conditions, or in high sugar concentrations, pyruvate is mostly transformed in ethanol and CO_2 through the alcoholic fermentation [6]. This metabolic regulation is due to two distinct causes. First, *S. cerevisiae* is a Crabtree positive organism that mostly uses fermentation rather than respiration in high sugar conditions [7]. Second, as oxygen plays the role of final electron acceptor of the respiratory chain, the regeneration of redox co factors of the TCA cycle cannot be achieved. This inactivation of the respiratory chain does not impair the enzymatic reactions between organic acids that play a key role in the biosynthesis of several amino acids [8]. In such conditions, the TCA cycle is split into two branches, oxidative and reductive [9]. The reductive branch has been described as producing oxaloacetate and malate from pyruvate, while the oxidative branch produces citrate, isocitrate and α-ketoglutarate [10]. Succinate can be synthetized through 2 pathways : the oxidative branch of the TCA cycle via Sdh2p or by the reduction of fumarate via Osm1p/Fdr1p [11].

During the alcoholic fermentation, the feeding of TCA cycle by pyruvate has not been totally elucidated. Theoretically, a fraction of pyruvate may enter in the mitochondrial matrix, however its conversion into acetyl-CoA is debated since the E3 subunit of this complex is highly repressed by high sugar concentration and hypoxia [12,13]. As acetyl-CoA is essential for fatty acid metabolism [14], it has been proposed that its synthesis could be achieved by a cytosolic route the "pyruvate dehydrogenase by-pass". This route is mostly due to the acetate formation by Ald6p as previously demonstrated [12]. The so formed acetyl-CoA could be imported into the mitochondria and be condensed with oxaloacetate by the citrate synthase.

During the wine alcoholic fermentation, important amounts (g/L) of malic acid are found in grape must (usually between 2-6.5 g/L) [15,16]. Malic acid can enter the cell by simple diffusion and be utilized by *S. cerevisiae* as reported by several studies [17–22]. This external organic acid source may provide a mitochondrial pool of pyruvate through the action of the malate enzyme that is encoded by MAE1 in the mitochondria [15]. However, the metabolic fate of this compound during the alcoholic fermentation was

never precisely monitored.

To unravel the fate of consumed compounds in the metabolic pathways, labeling of a carbon source turns out to be a powerful tool. ^{13}C labeling is a technique used to investigate metabolic pathways and monitor compounds evolution overtime in microorganisms. The common approach is to grow a microorganism in a medium containing a labeled carbon source. As the microorganisms consume the labeled substrate, the ^{13}C atoms are incorporated into the metabolic pathways and ultimately into the end products of the metabolism. The details of these pathways can provide important insight for the physiology and biochemistry of microorganisms [23]. *S. cerevisiae* is a well-studied yeast and ^{13}C-labeling studies have been used extensively to investigate its metabolism. For example, ^{13}C-labeling studies have been used to investigate the pathways of glycolysis [24,25], explore the TCA cycle pathways during alcoholic fermentation [10], or investigate amino acids metabolism [26]. It is usually coupled with analytical methods such as NMR [27], GC-MS [24,28] or LC-MS [25]. In most cases, labeled compounds used are glucose [24,28–30], aspartate [10], glutamate [10], or acetate [27,28]. In this work, the metabolic use of labeled malic acid was achieved in the context of wine alcoholic fermentation using two distinct yeast strains of *S. cerevisiae* which were selected for their extreme phenotype regarding malic acid modulation. Time series samples were made to understand the metabolic routes of exogenous malic acid. Targeted LC-MS/MS was used to quantify extracellular organic acids and determine the number of labeled carbons per acid. 2D-NMR was used to relatively quantify intracellular and extracellular labeled compounds between the two strains. Finally, targeted GC-MS was used to evaluate the isotopic enrichment of proteinogenic amino acids. Altogether these analytical chemistry tools suggest that malic acid might be a secondary source in the TCA cycle feeding during alcoholic fermentation, especially in the *demalic* strain.

2. Materials and Methods

2.1 Yeast strains used and culture methods.

The two *S. cerevisiae* strains used have been chosen for their contrasted ability to consume or produce malic acid during alcoholic fermentation. As previously described, the strain FMGS_889 is able to consume more than 70% of malic acid in grape juice [31], while the strain AC1_191 may produce significant amount of this organic acid [32]. Yeast strains were propagated on YPD 2 % (1 % peptone, 1% yeast extract, 2 % glucose) at 30°C in both liquid and plate cultures (2 % agar). Long term storage at -80°C was achieved by adding one volume of glycerol to YPD overnight cultures.

2.2. Alcoholic fermentation assays

2.2.1. Synthetic medium

Model synthetic medium simulated a standard grape juice and contained the following components

(expressed in g/L): glucose (115), fructose (115), tartaric acid (3), citric acid (0.3), malic acid (2). Both 4-carbons ^{13}C labeled and regular malic acid were used that were purchased at Eurisotop and Sigma, respectively. Nitrogen sources were adjusted to 280 mg total N/L as $(NH_4)_2SO_4$ (0.39) and amino acids solution (eq. 187 mg N/L). Vitamins (mg/L): meso-inositol (300), biotin (0.04), thiamin (1), pyridoxine (1), nicotinic acid (1), pantothenic acid (1), *p*-amino benzoic acid (1). Mineral salts (mg/L): $MgSO_4$ (200), KH_2PO_4 (2000), $MnSO_4 \cdot H_2O$ (4), $ZnSO_4 \cdot 7H_2O$ (4), $CuSO_4 \cdot 5H_2O$ (1), KI (1), $CoCl_2 \cdot 6H_2O$ (0.4), $(NH_4)_6Mo_7O_{24} \cdot 4H2O$ (1), H_3BO_3 (1). pH was adjusted to 3.6 using KOH pellets. Before yeast inoculation, the medium was sterilized by filtration (nitrate cellulose membrane, 0.45 µm, Millipore, France). The malic acid concentration was set to 2 g/L in order to allow the malic consuming strain to degrade malic.

2.2.2. Alcoholic fermentation monitoring

Small-volume alcoholic fermentations were implemented in screwed vials fermentations according to the general procedure described in [33]. Rapidly, 20 mL-screwed vials (Thermo Fisher Scientific, Bordeaux, France) were filled with 12 mL of synthetic medium and were tightly closed with screw cap-magnetic (Agilent Technologies, hdsp cap 18 mm PTFE/il 100 pk, Les Ulis, France) perforated with hypodermic needles (G26-0.45 x 13 mm, Terumo, Shibuya, Tokyo, Japan) for allowing CO_2 release. Vessel was inoculated by 2.10^6 viable cell.mL^{-1} precultured in liquid media 50 % synthetic medium 50% sterile H_2O for 24h. Fermentation took place at 24°C in shaken vials by using an orbital shaker (SSL1, Stuart, Vernon Hills, IL, USA) at 175 rpm. Fermentation kinetics were estimated by monitoring manually (1-2 times per day) the weight loss caused by CO_2 release using a precision balance with automatic weight recording (LabX system, Mettler Toledo, Viroflay, France). The amount of CO_2 released according to time was modeled by local polynomial regression fit [33] allowing the estimation of the time necessary to reach the maximum CO_2 produced. Some vials were set aside at 10 %, 40 % 60% and 100 % of CO_2 produced for analysis.

2.3. Flow cytometry analysis

Cellular concentration and viability were estimated by flow cytometry using a CytoFlex apparatus (Beckman Coulter Inc, Brea, California USA). 20 µL of homogenized culture with 3 µM propidium iodide (PI) were diluted in 1 mL final McIlvaine buffer (sodium phosphate dibasic 0.2 M, citric acid 0.1 M, pH = 4.5) and was analyzed by flow cytometry [34]. 50000 events were recorded per experiment. The viability of cells was estimated using 488 nm excitation, with a 610/20 nm long bandpass filter to detect PI fluorescence (dead cells).

2.4. Enzymatic assay of extracellular metabolites

For all samples, a volume of 800 µL was manually transferred in Micronics tubes (Novazine, Lyon, France, ref: MP32033L) and stored at -20°C. The concentrations of the following organic metabolites were

measured: acetic acid, glycerol, and malic acid using the respective enzymatic kits: K-ACETGK, K-GCROLGK, K-LMAL-116A, (Megazyme, Bray, Ireland) following the instructions of the manufacturer. Glucose and fructose were assayed by using the enzymatic method described by [35]. All the enzymatic assays were performed by a robotic platform using the Bordeaux metabolomics facilities (http://metabolome.cgfb.u-bordeaux.fr).

2.5. NMR analysis of intra and extra-cellular metabolites

2.5.1. wine samples preparation for NMR analysis

Wine samples were prepared according to [36]. Briefly, 60 µL of wine was diluted ten times in deuterated phosphate buffer (pD 4.2, 0.1 M final). Sixty µL of solution of calcium formate (FCa) 1 mM and trimethylsilylpropanoic acid (TSP) 1.25 mM in D_2O was added to the preparation. The FCa is used as an internal standard and the TSP to set the spectrum at 0 ppm [36,37].

2.5.2. Intra cellular yeast metabolites extraction and sample preparation for NMR analysis

The extraction of intracellular metabolites was carried out according to our previous work [38]. A sample of 5 mL of fermenting medium was taken from the vials and quickly quenched in cold pure methanol with a (1:5) ratio sample/methanol in 35 mL Teflon centrifuge tubes with screw closure (Nalgene, Thermo Fisher Scientific) to avoid metabolites leaks during centrifugation. Tubes were centrifuged at 4000 g for 5 min to remove the medium. The pellet was resuspended in 1 mL of cold pure methanol and placed at -80°C with agitation for 30 min. Tubes were centrifuged at 10000 g for 5 min and the supernatant was stored in a 1.8 mL microtube. This protocol was repeated twice. Methanol was evaporated with a SpeedVac vacuum (Radid Vap, Labconco, Kansas City, USA) at 32°C, 25 % agitation for 4 hours. The microtubes were stored at -80°C until NMR analysis. Intracellular metabolites were resuspended in 660µL of phosphate buffer (Na_2HPO_4, 0.1 M, pH 7.0) in 90 % H_2O, 10 % D_2O with 1,3-dimethylamylamine (DMMA) 1 mM as internal standard to achieve a quantitative experiment and TSP to set the spectrum at 0 ppm. The resulting solution was placed into the NMR tube.

2.5.3. NMR spectra acquisition

NMR spectra were recorded on a Bruker Avance 600 NMR spectrometer (600 MHz for ^{1}H and 151 MHz for ^{13}C experiments) equipped with a z-axis pulsed field gradient 5 mm TXI probe. Experiments were conducted at 303 K and 293 K for intracellular and extracellular samples respectively. The following experiments were conducted : ^{1}H-NOESY and 2D-(^{1}H-^{13}C)-HSQC.

1D-NOESY experiments were conducted using the parameters previously described [36]: noesygpps1d sequence, 64 scans, 5 s relaxation delay, 16 ppm spectral width, 64k data points (128k after zero-filling). Pulse calibration was carried out for each sample. An exponential apodization of 0.3 Hz was

applied. 2D-HSQC spectra were recorded using the hsqcetgpsisp.2 using the following parameters: 2 s relaxation delay, 180 ppm in f1 and 14 ppm in f2, 300 data points in f1 and 2048 data points in f2, 88 and 44 scans for intracellular and extracellular samples, respectively. All spectra were zero-filled. 2D experiments were carried out for the 48 samples (labeled and unlabeled fermentations of the 2 strains, sampled at 4 times and carried out in triplicates).

2.5.4. NMR spectra analysis

Metabolite identification was performed by comparison with literature data and databases, and addition of pure standards [39]. For 1D spectra, Quantification of individual metabolites was performed by spectral deconvolution using the simple mixture analysis (SMA) plugin of MestReNova 12.0 software (Mestrelab Research, Santiago de Compostela, Spain) [40]. For 2D spectra, Relative quantification of individual metabolites was obtained by segmentation of the spectra. Each segment formed by a single resonance for each metabolite. Segments of spectra with unlabeled compounds were subtracted to the equivalent spectra (same strain and fermentation step) containing labeled compounds. This manipulation was done to remove the 1% of natural abundance of 13C molecules.

2.6. Measurement of the isotopic enrichment of intracellular amino acids

2.6.1. Biomass hydrolysis

The determination of the isotopic enrichment of proteinogenic amino acids was performed using 1-2 mg dried biomass pre-hydrolyzed in 1200 µL of 6 M HCl for 16h at 105 °C in tightly close glass tubes in a dry heat oven. The sample, after addition of 1200 µL of distilled water, was centrifuged at 3000 g for 5 min to remove cellular debris. The supernatant was distributed in 6 fractions of 400 µL, which were further dried at 105°C. These fractions were then utilized for amino acid derivatization using 3 different agents. The ECF method targets 13 amino acids : alanine, glycine, valine, leucine, isoleucine, threonine, serine, proline, aspartate, glutamate, phenylalanine, lysine, and histidine. The DMF/DMA method targets 6 amino acids : alanine, glycine, valine, aspartate, glutamate, and phenylalanine. Finally, arginine is the only amino acid targeted by the BSTFA method.

The ethyl-chloroformate (ECF) derivatization procedure was described in [41]. The dried hydrolysate was dissolved in 200 µL of 20 mM HCl and 133 µL of a pyridine-ethanol mixture (1:4). The amino acids in this mixture were extracted in 500 µL of dichloromethane. For analysis, the upper organic phase was collected after 4 min of centrifugation at 10000 g and injected directly into the GC-MS system.

The N,N-dimethylformamide dimethyl acetal (DMF/DMA) derivatization procedure *was described* in [41]. The dried hydrolysate was dissolved in 50 µL of methanol and 200 µL of acetonitrile, and the mixture was derivatized by adding 300 µL of DMF/DMA. The extract was directly injected in the GC-MS system.

The N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization procedure was described in

[42]. The dried hydrolysate was suspended in 200 µL of acetonitrile. 200 µL of BSTFA was added in hermitically closed glass tubes which were incubated at 135°C for 4h. The extract was directly injected in the GC-MS system.

2.6.2. GC-MS analysis.

Samples were then analyzed with a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a CTC Combi PAL Autosampler AOC-5000 (Schimadzu, Columbia, SC, USA) and coupled to an HP 5973 mass spectrometer. The instrument was controlled, and the data were analyzed using HP G1701DA ChemStation software. The gas chromatograph was fitted with a 30 m x 0.25 mm DB-17 ms columns with a film thickness of 0.15 µm (Agilent Technologies) and helium as the carrier gas. Compounds obtained from derivatization were separated using the analytical conditions described by [43]. The MS was operated in selected ion monitoring (SIM) mode with positive ion electron impact at 70 eV using the characteristic ions of amino acid fragment reported in [43]. For each amino acid fragment, the outcome of the analysis was a cluster of intensities corresponding to its different mass isotopomers. These data were subsequently processed using the *IsoCor* software developed by [44] to correct for natural labeling and to assess the isotopic enrichment of amino acids, which is defined as the fraction of labeled compound with respect to its total amount in proteins (expressed in percentage).

2.7. Quantification of labeled extracellular organic acids by LC-MS/MS

2.7.1. Materials and reagents

Malic acid, oxaloacetic acid, citric acid, succinic acid, sodium pyruvic acid, glyoxylic acid, α-ketoglutaric acid, hippuric acid (internal standard), and ammonium acetate were purchased from Sigma Aldrich (St Louis, MO, USA). Sodium pyruvic acid ^{13}C was purchased from Eurisotop (Saint-Aubin, France). ULC/MS grade acetonitrile and formic acid were purchased from Biosolve (Dieuze, France).

2.7.2. Optimizing Multiple Reaction Mode (MRM) Transitions of organic acids

Standard organic acids were dissolved in 100 % ethanol to obtain a concentration of 10 g/L. Each compound diluted to obtain a concentration of 100 ng/mL in water. Ten µL of each solution were infused directly into the MS system to obtain optimum conditions for MRM transition in negative mode (Table 1).

2.7.3. Instrumentation and conditions LC-MS/MS analysis

UHPLC analysis

The chromatographic apparatus consisted of Nexera X2 UHPLC system (Shimadzu, Marne la Vallée, France) equipped with a binary pump, solvent degasser, and thermostated column compartment. An Atlantis Premier BEH C18 AX (2.1 x 100 mm ; 1.7 µm ; Waters) was used for the sample separation. The

mobile phase comprised (A) 0.1 % aqueous formic acid and (B) a mixture of 60 % 100 mM ammonium acetate solution and 40% acetonitrile, all acidified with 0.1% formic acid at a flow rate of 0.6 mL/min. The gradient program used was as follows: Injection under 100 % eluent A for up to 30 seconds, then linear increase over 2 minutes to pass to 100% eluent B, followed by a plateau for 3 minutes and a return to initial conditions in 10 seconds. The oven temperature was set to 40°C.

MS/MS detection

MS/MS experiments were carried out using the Shimadzu UHPLC system described above coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer using the multiple reaction monitoring (MRM) technique operating in negative ion mode. All transitions and the dwell times were optimized by LabsSolution Main (Shimadzu). The interface settings were as follows. Nebulizing-gas flow rate: 2.5 L/min; Heating gas flow : 10 L/min, Interface temperature : 300°C, drying-gas flow rate: 10.0 L/min; DL temperature: 250 °C; heat block temperature: 400 °C; interface voltage : 4.0 kV. The data analysis was performed by LabSolutions Insight LCMS (Shimadzu).

Table 1: MRM parameters for the ESI-MS/MS detection of unlabeled and labeled organic acids

Compounds	RT min	Precursor Ion	Product ions	Dwell time	Q1 Prebias *	CE (eV) **	Q3 Prebias *
Malic acid	1.93	133.1	**115.15**/73.1/71.5	6	15.0	15.0	11.0
Oxaloacetic acid	3.21	131.15	**43.05**/40.95	11	27.0	15.0	10.0
Citrate	2.87	191.2	**111.15**/87.05/85.0	6	23.0	13.0	10.0
α-ketoglutaric acid	2.85	145.2	**101**/72.95/57.05	6	17.0	12.0	14.0
Succinate	1.97	117.1	**73.10**	25	25.0	14.0	10.0
Pyruvic acid	2.79	87.4	**43.10**	25.0	19.0	11.0	14.0
Glyoxylic acid	1.91	73	**44.9**	25.0	29.0	11.0	15.0
Hyppuric acid (IS)	2.74	117.9	**134.20**	6.0	20.0	15.0	13.0
Lactic acid	1.47	83.3	**43.10**	11	18.0	12.0	15.0
Malic acid (4C13)	1.93	137.1	**119.15**/74.95/73.95	6	15.0	15.0	10.0
Oxaloacteic acid (4C13)	3.21	135.15	**45.05**	11	27.0	15.0	10.0
Citrate (4C13)	2.87	195.20	**115.0**/114/90/88	6	23.0	13.0	10.0
Citrate (6C13)	2.87	197.20	**116.0**/90.0/89.0	6	23.0	13.0	10.0
α-ketoglutaric acid (3C13)	2.85	148.2	**103.15**/104.15/75/0/59	6	17.0	12.0	14.0
α-ketoglutaric acid (5C13)	2.85	150.20	**105.0**/75/60.05	6.	17.0	12.0	14.0
Succinate (4C13)	1.97	121.0	**76.15**	11	25.0	13.0	10.0
Pyruvic acid (3C13)	2.79	90.2	**45.0**	10	19.0	12.0	11.0
Glyoxylic acid (2C13)	1.91	75	**45.90**	25	29.0	11.0	15.0
Lactic acid (3C13)	1.47	92.3	**45.0**	11	18.0	1.0	15.0

*Q1/Q3 Prebiasis: tension if quadripoles 1 and 3 respectively (in Volt)
**CE : Collision Energy
*** Oxaloacetic and glyoxylic acid were not detected, neither labeled or unlabeled

Method Quantification

Calibration curves standards were constructed over a range of 10-200 ng/mL (10; 15; 25; 40; 60; 80; 100; 200 ng/mL). Quality control (QC) samples at two concentrations (QC30 et QC150) were used to validate the analytical run. Analyte to IS ratios were plotted against the corresponding concentrations to obtain calibration curves (linear correlation coefficient, slope) to quantify analytes in samples. Labeled analytes were quantified on the basis of the response of unlabeled analytes.

2.8. Statistical analyses

All statistical and graphical analyses were carried out using R software [45] and plots were generated using the base or *ggplot2* packages. The differences in the metabolic compounds kinetics during the fermentation were estimated by estimating the statistical effect of the sampling point, the strain, and their possible interaction using a 2-way paired ANOVA with interaction (α=0.05). the homoscedasticity and the normality of the residues of the linear model were controlled by using a Leven and a Shapiro test, respectively.

3. Results and discussion

3.1. Rational of the study and analytical chemistry analyses

In the present work, the incorporation of ^{13}C-labeled malic acid was investigated, aiming to identify both the catabolic and anabolic routes related to this organic acid during alcoholic fermentation. The two *S. cerevisiae* strains used frame the natural variability of malic acid modulation of the *S. cerevisiae* species during the alcoholic fermentation [32,36]. FMGS_889 is a strong consumer [31], while AC1_191 may produce relatively high amount of this compound in grape juices containing low amount of initial malic acid [32]. Thus, these two strains would reflect contrasted metabolic regulations in the context of the alcoholic fermentation. Thereafter, the strong consumer strain will be referred as "demalic" strain while the producer strain will be referred as "acidic" strain. In order to study the dynamics of malic acid uptake and to trace the metabolic compounds derived from its assimilation, we applied a time series sampling during the alcoholic fermentation. Wine samples were taken a 10 %, 40 %, 60 % and 100 % of CO_2 produced. These sampling points occurred at different times since strains did not have the same fermentation kinetics (Figure 1). Both strains achieved the alcoholic fermentation, and the composition of the main extracellular compounds determined by enzymatic assays as well as the cellular concentration and viability are given in the table S1.

At each sampling point, the biomass was collected and characterized by flow cytometry. Then, cell pellets were collected in order to extract the intracellular metabolites using a cold methanol protocol previously developed [38]. Both extra and intracellular samples were submitted to different analytical chemistry analyses using 2D-NMR, GC-MS and LC-MSMS approaches (Figure 1).

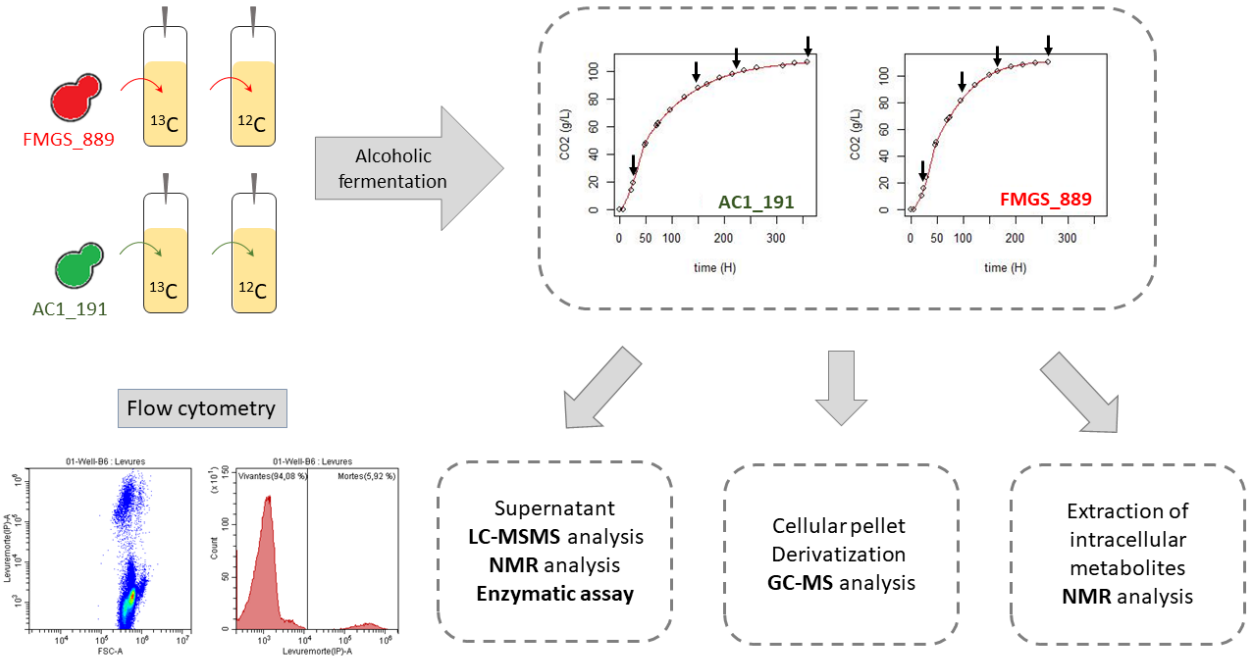


Figure 1: Workflow of the experiments carried out: the two strains fermented with ^{13}C-malic acid or ^{12}C-malic acid, samples were taken at 10 %, 40 %, 60 %, and 100 % of CO_2 produced for both, flow cytometry analysis allowed to determine the cellular density and viability of each sample. Analytical chemistry experiments were carried out for each sample. Black arrows on the kinetics represent the samples taken for analysis.

Wine samples fermented with ^{13}C-malic acid were analyzed by LC-MSMS to determine the absolute quantification of six extracellular organic compounds: malic, succinic, citric, α-ketoglutaric, lactic, and pyruvic acids. They were also analyzed by 2D NMR to determine the relative concentration of seven labeled compounds between the two strains: malic acid, succinic acid, citric acid, glucose, ethyl acetate, phenethyl alcohol and isoamyl alcohol, and by ^{1}H-NMR to determine the relative concentration of the same compounds regardless of their labelling. Finally, enzymatic assays were carried out to determine the concentration of malic acid, acetic acid, glycerol, and residual sugars, regardless of their labeling. The same wines fermented with ^{12}C-malic acid were used as control. For NMR analysis, spectra recorded with unlabeled compounds were subtracted to spectra recorded with ^{13}C-labeled compounds to remove the ~1 % of natural abundance of ^{13}C contained in the samples. Intracellular composition of ^{13}C-labeled samples was investigated by GC-MS to assess the isotopic enrichment of proteinogenic amino acids. 2D-NMR analyses were also conducted on intracellular compounds to determine the relative concentration of 11 intracellular

labeled compounds: acetic acid, alanine, citric acid, ethanol, fumaric acid, glucose, malic acid, proline, pyruvic acid, succinic acid, and trehalose. ^{1}H-NMR spectra were recorded to quantify those same compounds for both strains regardless of their labeling. As for extracellular 2D-NMR analysis, the spectra obtained with unlabeled compounds were subtracted to those obtained with labeled compounds to remove the 1 % of natural abundance of the ^{13}C.

3.2. Consumption and *de novo* synthesis of malic acid during the alcoholic fermentation.

The evolution of extracellular malic acid during the alcoholic fermentation was quantified by LC-MS/MS analysis that allowed the distinction between labeled and unlabeled molecules. Since the synthetic medium fermented contained only ^{13}C-labelled malic acid, the assimilation and the *de novo* production of this compound was precisely quantified for the first time in the context of the alcoholic fermentation. As expected, the overall evolution of malic acid concentration differs between the two strains (Figure 2A). Although the method slightly underestimated the amount of malic acid (the initial concentration expected is 2000 mg/L), the malic acid concentrations measured are well correlated with those routinely quantified by enzymatic assay (rho = 0.606, p-value = 0.001). At 10 % of the alcoholic fermentation, the concentration of malic acid was slightly reduced for both strains. After this point, a clear distinction can be made between strains with a strong decrease for FMGS_889 and a slight increase for AC1_191. At the end of the alcoholic fermentation, AC1_191 displayed two times more malic acid than FMGS_889 in the medium with a quantitative discrepancy of 1 g/L.

Interestingly, those differences are not only caused by a simple consumption and production but result from the combination of both mechanisms. The profile of extracellular ^{13}C-labeled malic acid (Figure 2B) showed that both strains assimilate malic acid during the whole fermentation, but with a different efficiency. Simultaneously to the consumption, a production of unlabeled malic acid has been observed, corresponding to the de novo formation of malic acid from sugars (Figure 2C). The *acidic* strain AC1_191 increased its production through the fermentation to reach almost 1 g/L of *de novo* malic acid produced while FMGS_889 produced up to 250 mg/L of malic acid between 10 % and 60 % of the fermentation. Consequently, the discrepancy of *de novo* malic acid produced is mostly due to a strong production rate of AC1_191 during the second part of the alcoholic fermentation. These results shed light on the existence of anabolic and catabolic routes of malic acid that are activated simultaneously during the alcoholic fermentation.

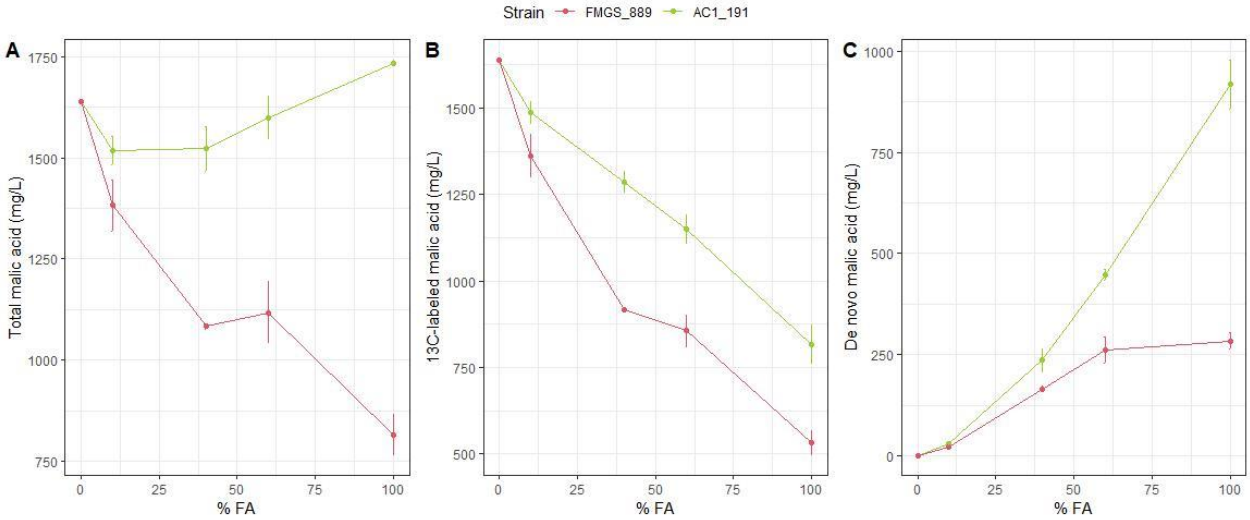


Figure 2: Evolution of the concentration (mg/L) of total malic acid (**A**), ^{13}C-labeled malic acid (**B**) and de novo produced malic acid (**C**) by the two strains FMGS_889 and AC1_191 during alcoholic fermentation.

The quantification of both forms of extracellular malic acid allows to make some hypotheses concerning the transport flux of this organic acid. The amount of labeled malic acid is a proxy of malate import while the amount of unlabeled malic acid is a proxy of malate export. This import (F_{imp}) and export (F_{exp}) flux of malate at the different moments of the fermentation were figured out in Figure 3. Fluxes were expressed in mmol of malate per g of CO_2 for 10^7 cells. The two strains showed different input and output malic acid flux. Indeed, the import of malic acid was two times more efficient for the *demalic* strain until the mid-term of the alcoholic fermentation. However, in the later stage both strains raised a similar specific rate. The export of malate was also quite different according to the strain, malate efflux was broadly similar until the mid-term of the alcoholic fermentation, then the *acidic* strain showed a strong elevation of malate efflux. This additional malate efflux occurred in non-optimal osmotic conditions for the AC1_191 strain. Indeed, the extracellular malic acid concentration was twice higher for this strain while intracellular concentration of malic acid was quite similar between those strains [38]. This additional osmotic gap suggested that the export of malic acid by AC1_191 could be due to an active transport that might be more efficient for this strain.

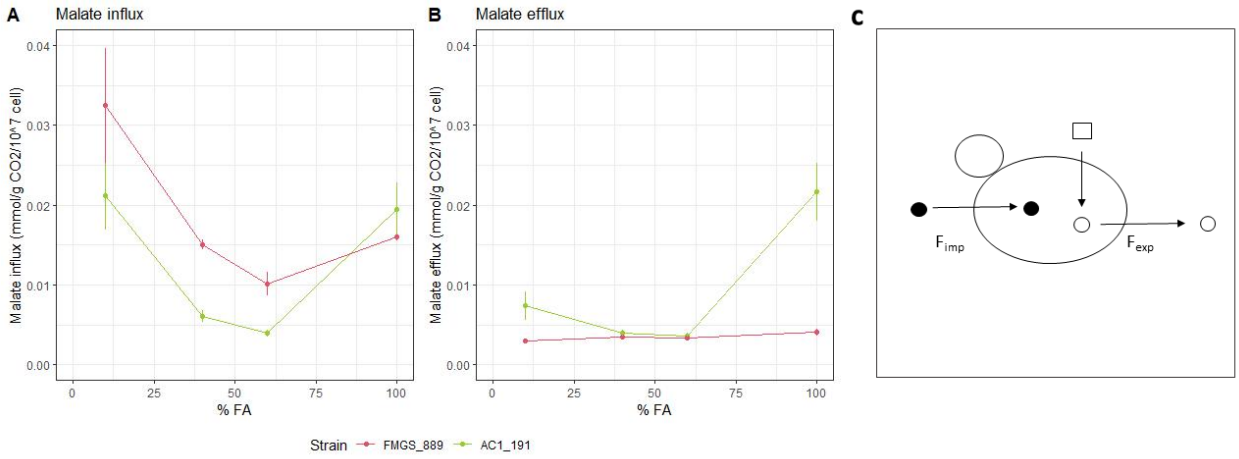


Figure 3: Panels A and B. Malate influx and efflux for both strains during the alcoholic fermentation. **Panel C.** Schematic representation of the import and export flux of malate through the cell. Black dots represent le labeled malic acid, white dots the unlabeled malic acid and the square represents the glucose which is unlabeled.

3.3. Most part of succinic acid produced could be derived from glyoxylate shunt.

As demonstrated in the previous section, malic acid production and uptake can be discriminated by assaying the unlabeled and ^{13}C-labeled molecules. The high proportion of malic acid consumed by yeast (even for the *acidic* strain) addresses the question of the metabolic fate of this organic acid that is quantitatively, the second source of assimilable carbon after fructose and glucose. By using LC-MS/MS analysis, we quantified both the labeled and unlabeled isotopes of pyruvic, lactic, succinic, citric, and α-ketoglutaric acids present in the fermented medium. These extracellular concentrations represent a metabolic overflow and not the actual intracellular metabolic concentration. However, variations of their concentrations may provide important clues for understanding their metabolic origin. No ^{13}C-labeled molecules were found for pyruvic acid and lactic acid. In contrast, succinic, citric, and α-ketoglutaric acids had ^{13}C isotopes, suggesting that these organic acids are directly or indirectly derived from external malic acid which is the sole source of ^{13}C isotope in our experiment. The kinetics of those labeled compounds and the associated statistical analyses (ANOVA) were presented in the supplementary figure S1 and Table S2, respectively.

Both strains consumed a high amount of malic acid reaching a final uptake of 6.5 and 8.5 mmol for AC1_191 and FMGS_889, respectively (Figure 4A). Surprisingly, the concentration of ^{13}C-labelled metabolites directly connected to malate catabolism are quite low. The Figure 4B illustrates the evolution of ^{13}C-labeled succinate which was detected in few amounts until 60 % of the reaction. At this stage, 4.25 and 6.5 mmol of malate have been assimilated for AC1_191 and FMGS_889, respectively. However, only 0.1 mmol have been converted in succinate. During the later step of the alcoholic fermentation, a production

peak of labelled succinate was detected for the FMGS_889 strain. This overproduction of 0.3 mmol is in phase with the additional 2 mmol of malate consumed by FMGS_889 respect to AC1_191. This first analysis indicated that newly consumed malic acid is not directly converted into succinate. In contrast, higher amounts of unlabeled succinate were quantified by the same method (Figure 4C). This production reaches a plateau at the mid-term of the fermentation corresponding to 4 and 2.5 mmol for AC1_191 and FMGS_889, respectively. That is to say that succinate is mostly derived from label-free molecules and results from *de novo* synthesis rather than labeled malate conversion.

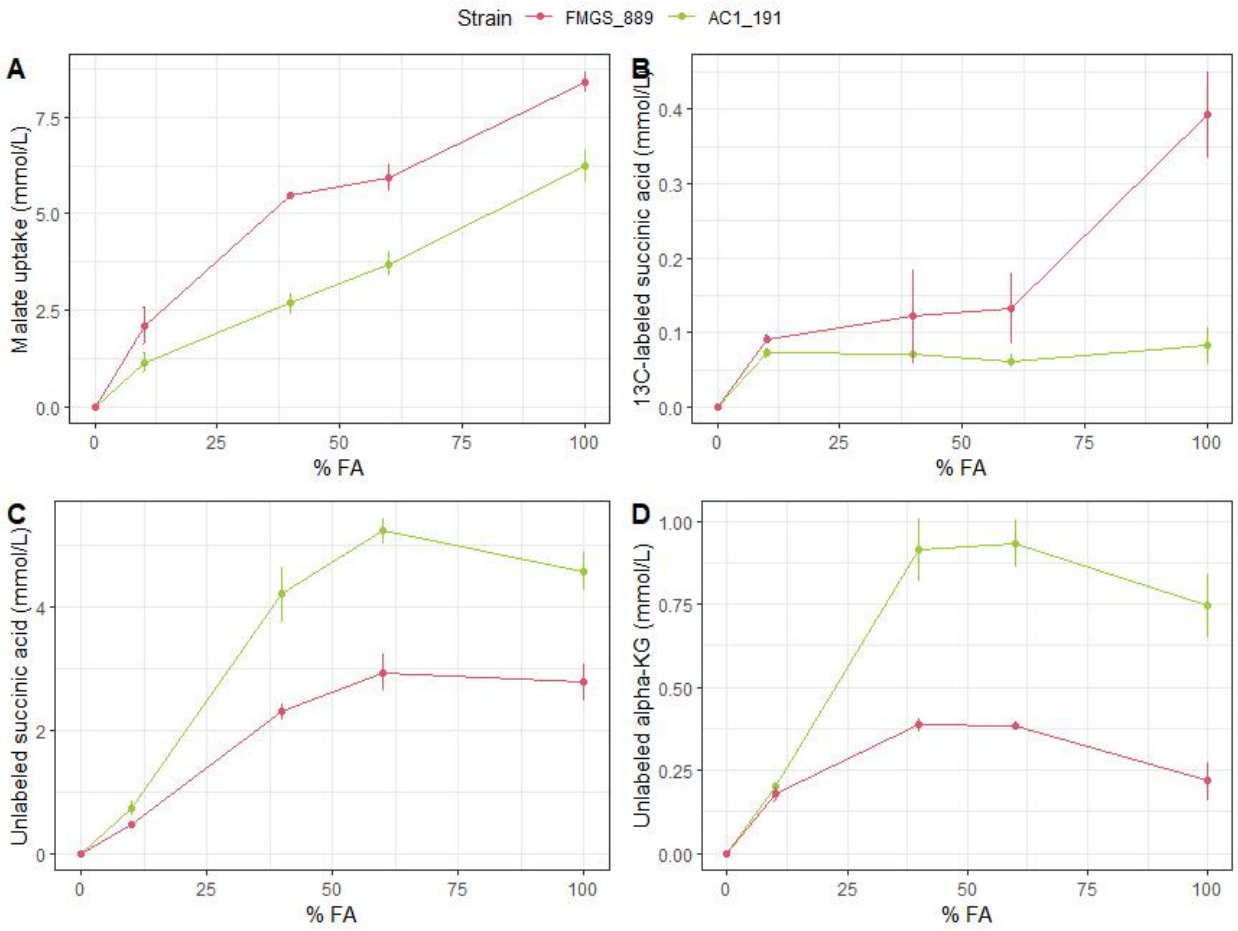


Figure 4: Panel A. Malate uptake for both strains during the alcoholic fermentation (mmol/L). **Panel B.** Amount of 13C labeled succinic acid for both strains during the alcoholic fermentation (mmol/L). **Panel C.** Amount of unlabeled succinic acid for both strains during the alcoholic fermentation (mmol/L). **Panel D**. Amount of unlabeled α-ketoglutarate for both strains during the alcoholic fermentation (mmol/L).

Succinic acid can be produced by three metabolic pathways, the reductive branch of the TCA that is mitochondrial (figure 5, red route) and its parallel cytosolic reduction from malic acid (blue route). Alternatively, succinate can result from the glyoxylate shunt, from the condensation of oxaloacetate and

acetyl-CoA that provide citrate and isocitrate (yellow route).

The cytosolic malate reduction pathway is not consistent with the very few amounts of labeled succinate detected (about 2 %), our findings suggested that succinate might be mostly derived from the glyoxylate shunt. Interestingly, the *demalic* strain FMGS_889 showed a higher fraction of labeled succinate (0.3 mmol), corresponding to 15 % of its total production occurring in the later fermentation part. This labeled succinate can be derived directly from labeled malic acid through the direct reduction of malate into fumarate. At this stage the mitochondrial or cytosolic origin of this reduction cannot be explained. This specific trait clearly differentiates the *demalic* and *acidic* strains.

By following the evolution of unlabeled α-ketoglutaric we confirmed that the glyoxylate shunt could be the main route of succinate production (Figure 4D). Indeed, the α-ketoglutaric acid is derived from the reduction of D-*threo*-isocitrate throughout the glyoxylate shunt (yellow route). For both strains, the production kinetics of unlabeled α-ketoglutaric acid followed those observed for unlabeled succinic acid, suggesting that both acids were produced by the same pathway. The AC1_191 strain is a stronger producer of both succinic and α-ketoglutaric acids. In a less extensive way, unlabeled citric acid follows a similar pattern with a strain discrepancy at the 40% of the alcoholic fermentation. However, the presence of this acid in the medium complexifies the analysis of its production (Fig S1, panel C).

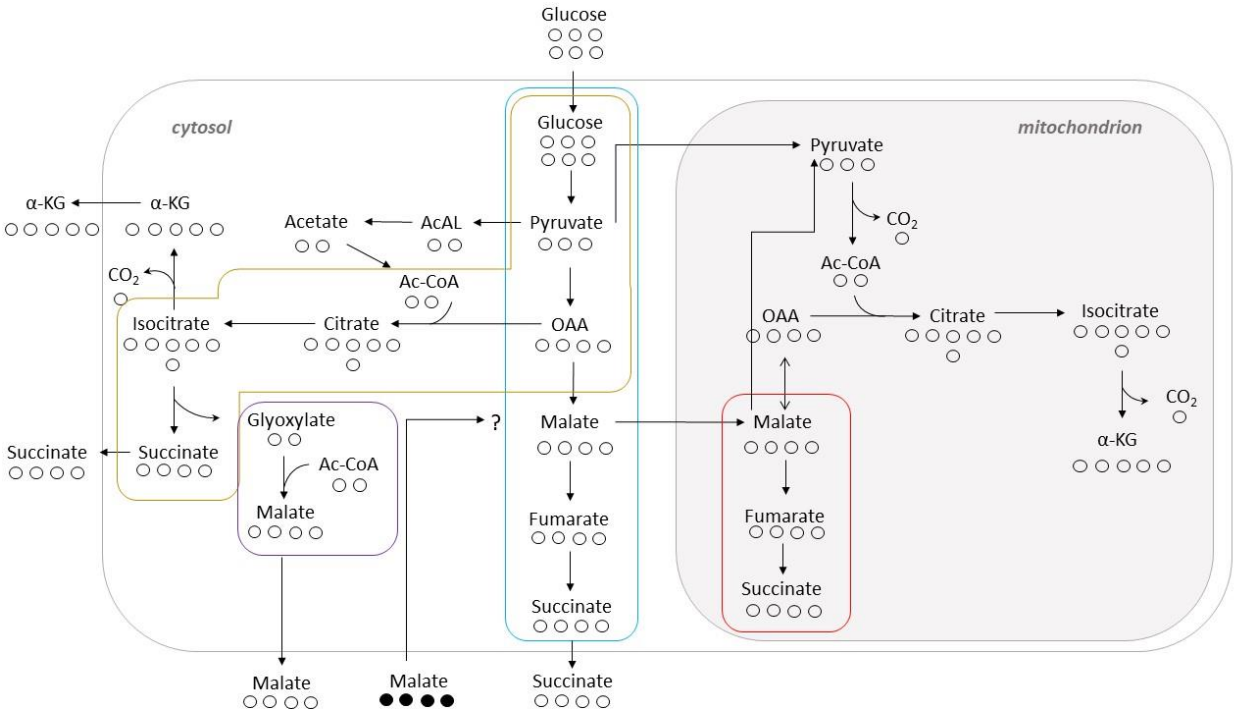


Figure 5: ^{13}C filiation from labeled malate through the TCA oxidative pathway and glyoxylate shunt during alcoholic fermentation. Black dots are for labeled carbons and white dots for unlabeled carbons. The carbons are organized according to the position of the carbons on the molecule. AcAL stands for acetaldehyde, Ac-CoA for acetyl-CoA, OAA for oxaloacetate and α-KG for α-ketoglutarate.

3.4. Undirect evidence of the glyoxylate shunt as the main source of de novo malate synthesis until mid-alcoholic fermentation.

Both strains produced unlabeled malate from sugar (figure 2C). According to biochemical knowledges, this *de novo* malate synthesis may have two main origins. (1) The conversion of pyruvate to oxaloacetate which is directly converted in malate by the cytosolic malate dehydrogenase activity (Mdh2p) (Figure 5, blue route). (2) The condensation of oxaloacetate with cytosolic acetyl-CoA providing citrate and isocitrate. The isocitrate is then involved in the glyoxylate shunt producing succinate and glyoxylate (yellow route). Then, malate synthase Mls1p condensates glyoxylate and cytosolic acetyl-CoA to produce *de novo* malic acid (Figure 5, purple box). Due to the large amount of malate produced the mitochondrial origin (red route) of malate synthesis was not considered here.

According to the hypothesis developed in the previous paragraph, succinate production is mostly derived from the glyoxylate shunt. Following this pathway, the glyoxylic acid produced must be converted in malate by the malate synthase. The analysis of the time course of the *de novo* production succinate and malate suggested a more complex origin. Indeed, *de novo* succinate and malate productions are not perfectly correlated depending on the strain.

For the high malate consuming strain FMGS_889, succinate and malate are perfectly coupled suggesting that glyoxylate shunt was the main biosynthetic route for both acids. This feature is not observed for the strain AC1_191. For this *acidic* strain, succinate is produced until 60% of the fermentation (Figure 4C) while malate production occurred during all the alcoholic fermentation, with a strong increase after 60 % (figure 2C). This suggested that, at this stage, the malate biosynthesis is not coupled to the glyoxylate pathway anymore, otherwise succinate should be also produced. The alternative *de novo* malate production pathway is the cytosolic reduction of oxaloacetate in malate by the malate dehydrogenase Mdh2p. This enzyme has been described to be inactivated in presence of high glucose concentration [46,47]. However, Minard *and al*. reported that a truncated version of *MDH2* without 12 residues from the amino terminus of the polypeptide is not subject to phosphorylation following an addition of glucose [47] suggesting that intraspecific variability for this regulation is possible. In addition, during the later step of the alcoholic fermentation the catabolic repression of glucose is lower and the Mdh2p might be more active. Thus, malic acid can be produced at this stage from oxaloacetate for the AC1_191 strain.

These findings suggest that the production of de novo malic acid by yeasts occurs in the cytosol, by the action of Mdh2p and Mls1p enzymes, with a different temporality. Mls1p generates malate until 60% of the alcoholic fermentation then Mdh2p does. However, the origin of malate from Mdh2p addresses several questions that remain to be clarified. Why are fumarate and succinate not produced from cytosolic malate ? Why is this route taken only by the high producing strain AC1_191? Why does the FMGS_889 strain

produce succinate from the labeled malate but not from the unlabeled one?

3.5. ^{13}C isotopic filiation of α-ketoglutaric acid revealed that malic acid is assimilated differentially by *acidic* and *demalic* strains.

In the present section we interrogated the becoming of labeled malate. To decipher this point we analyzed the ratio of the two distinct ^{13}C isotopes of α-ketoglutaric acid. This acid was mostly detected with its 3 carbon-labeled form. However, a minor fraction of 5-carbon-labeled isotope was also detected. The strains FMGS_889 and AC1_191 showed significative differences in their α-ketoglutaric acid isotopic ratio with 14% and less than 1%, respectively (Figure 6A). This isotopic ratio was conserved during all the alcoholic fermentation (data not shown).

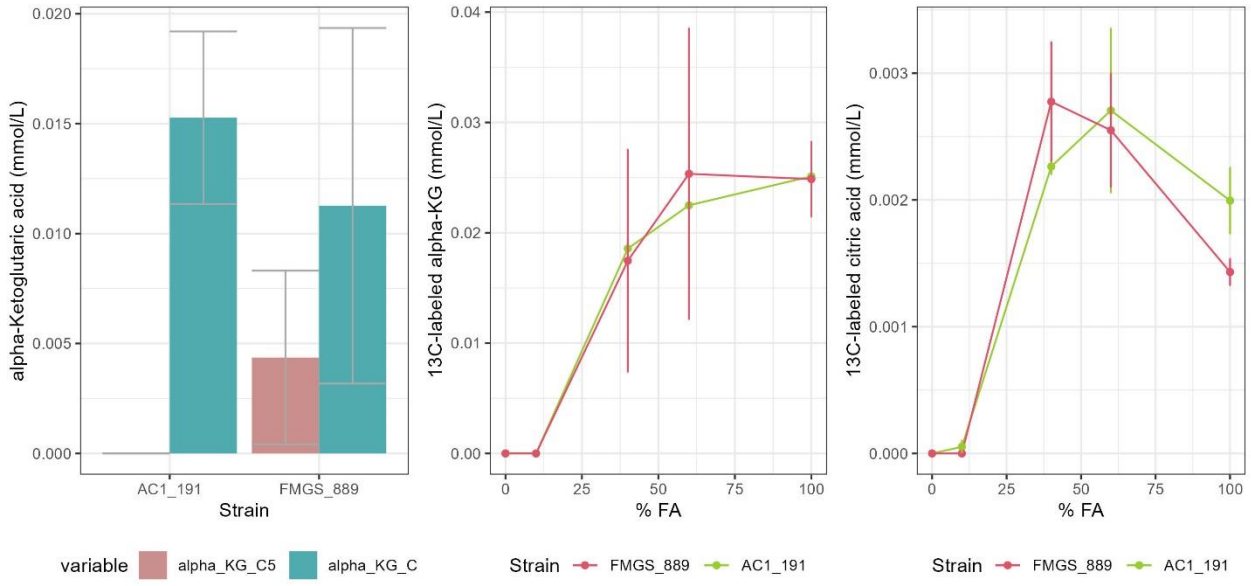


Figure 6A: Panel A. Proportion α-ketoglutaric acid labeled on 5 carbons and 3 carbons for both strains. The mean of each compound at the 4 sampling times is represented in mmol/L. Panel B. Amount of total labeled alpha-ketoglutaric acid during the alcoholic fermentation. Panel C. Amount of labeled citric acid during the alcoholic fermentation

The 5-carbon-labeled α-ketoglutarate is necessarily the result of the decarboxylation of 6-carbon-labeled isocitrate which comes from 6-carbon-labeled citrate. The 6-carbon-labeled citrate must be produced exclusively from 4-carbon-labeled malate. This can be explained by the entry of labeled malate into the mitochondrion. Then, in the mitochondrial matrix, malate can be oxidized by the malate dehydrogenase Mdh1p or be decarboxylated into pyruvate, by the malic enzyme Mae1p. This ^{13}C-labeled pyruvate would give in turn ^{13}C-labeled acetyl-CoA that may be condensed with ^{13}C-labeled oxaloacetate to produce 6 carbon labeled citrate. In our experiment, the detection of 5-carbon-labeled α-ketoglutarate is the indirect proof of the input of labeled malate into the TCA cycle by the combined action of Mdh1p and Mae1p. labeled pyruvate by the enzyme Mae1p. This malic acid input is much stronger for the *demalic* strain

FMGS_889 since up to 14 % of the α-ketoglutarate quantified followed this pathway. The very low amount of extracellular citrate detected, and the low fraction of C6-labeled citrate explain the non-detection of this last compound.

The origin of the 3-carbon-labeled α-ketoglutarate is more complex to decipher and may have both cytosolic and mitochondrial origin.

.

LIN

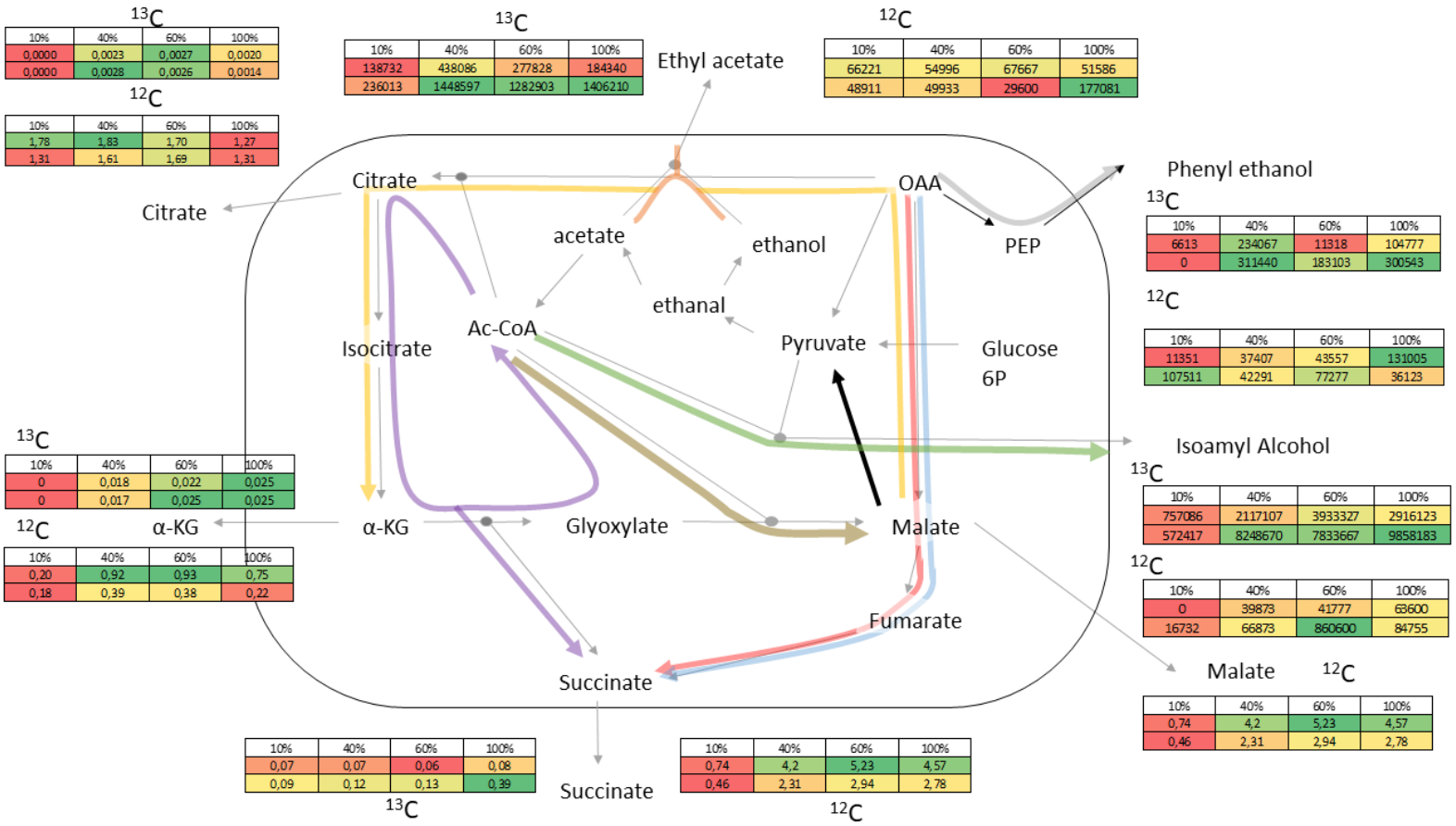


Figure 7: Schematic representation of the different pathways for organic acid production and derided. The blue and red lines represent the cytosolic and mitochondrial routes of oxaloacetate conversion to succinate. The yellow line represents the pathway taken by malate until α-ketoglutarate formation. This route is either cytosolic or mitochondrial. The purple line represents the glyoxylate cycle with succinate formation. This pathway is cytosolic. The brown line represents the cytosolic pathway of malate production via glyoxylate. The concentrations of labeled (13C) and unlabeled (12C) compounds are displayed in tables next to the name of the extracellular compounds. The concentration is expressed in mmol at the different fermentation steps of the AF. The top line is for the *acidic* strains and the bottom line the *demalic* strain

3.6. 2D NMR approach captured additional labeled molecules that are markers of the pathways taken by ^{13}C labeled malic acid.

In order to capture a greater number of ^{13}C-labeled wine metabolites, the same samples were analyzed using (^{1}H-^{13}C)-NMR approaches. The ^{1}H-NOESY applied allows the quantification of the total amount of several wine metabolites as previously described. The (^{1}H-^{13}C)-HSQC method applied allows the relative comparison of signal intensity between samples for the same molecule. However, the relative fraction of labelled and unlabeled compounds was not possible with such analysis. Beside malic, citric, and succinic acids this method detected three additional metabolites: isoamyl alcohol, ethyl acetate and phenyl ethanol. The relative evolutions of total and labeled molecules during the alcoholic fermentation are shown Figure S2. Broadly, the total concentration of these compounds was similar for both strains. In contrast, the ^{13}C-labeled fraction is much higher for the *demalic* strain FMGS_889. The Figure 7 summarized the differential composition of labeled and unlabeled metabolites found in the medium for the two strains detected by LC-MS/MS and NMR.

These findings suggested that labeled malic acid provides metabolic intermediates that are shared by the glyoxylate shunt and the oxidative branch of the TCA cycle (yellow arrow). Interestingly the amount of ethyl acetate is much stronger for the demalic strain (orange arrow). This compound resulted from the condensation of ^{13}C-acetyl-CoA and ethanol which is an indirect proof of the presence of ^{13}C-pyruvate. This ester can be produced in the mitochondrion by the Eat1p enzyme. The highest amount of ethyl acetate in the *demalic* strain is an additional proof of the mitochondrial assimilation of malic acid by the malic enzyme Mae1p (black arrow). Although, the detection of ^{13}C-labeled higher alcohols (isoamyl and phenyl ethanol) cannot be directly explained by metabolic pathways, these compounds indicated that the malic acid skeleton is dispatched in the whole yeast metabolome in the late stages of the alcoholic fermentation. Phenyl ethanol is expected to be produced from Phospho-Enol Pyruvate (PEP) derived from oxaloacetate. The presence of labeled phenyl ethanol (grey route) is therefore an indirect prof of the cytosolic conversion of malate in oxaloacetate. This finding suggested that this key anaplerotic route is partially active during the alcoholic fermentation. No difference was found among the strains of this study.

3.7. Isotopic enrichment of proteinogenic amino acids

Organic acids are the starting point of the biosynthesis of several amino acids that constitute the primary building blocks of yeast proteins. As previously demonstrated, the measurement of isotopic enrichment of amino acids is very informative for deciphering the biosynthetic pathways of secondary metabolites [42] as well as to study environmental factors influencing their production [41]. In this section, the isotopic enrichment of 14 amino acids: alanine, arginine, aspartate, glutamate, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, and valine, was quantified by GC-MS

analysis. The isotopic enrichment has been calculated for each sampling points and for both strains. A two-way paired analysis of variance was applied for estimating the effect of the yeast strain as well as the effect of sampling point. The part of variance explained by each factor is indicated in the Table 2.

Neither the strain nor the fermentation progress influences the isotopic enrichment of proline, lysine, arginine, phenylalanine, serine, histidine, and glycine. The average fraction of ^{13}C labeled isotopes for these amino acids is 1.41 % for both strains suggesting that consumed malate did not participate to the biosynthesis of those amino acids. In contrast, all the other amino acids assayed were enriched in ^{13}C labeled isotopes with an average isotopic ratio of $^{13}C/^{12}C$ of 5.88%. Interestingly, all these amino acids can be produced in the mitochondrion. This is the case of branched and aliphatic amino acids (alanine, leucine, valine, and isoleucine) [49], they are derived from ^{13}C- pyruvate and ^{13}C-oxaloacetate. Aspartate and threonine are also derived from ^{13}C-oxaloacetate but can be produced both from mitochondrial and cytosolic routes. Finally, glutamate is also derived from α-ketoglutarate by the action of the mitochondiral NADP-Glutamate dehydrogenase (Gdh3p). Altogether, amino acids connected to the mitochondrial pool of pyruvate and oxaloacetate showed an isotopic enrichment of 8.45%, 4.17%, respectively. In contrast, other amino acids derived from α-ketoglutarate (arginine, lysine, and proline) are produced in the cytosol and are not particularly affected by the input of labeled malic acid (average isotopic enrichment of 2.07%).

Interestingly, a shift of incorporation of nearly 3% was observed for the *demalic* strain FMGS_889 between 10 and 40 % of the fermentation. This trend was observed for all the amino acids derived from pyruvate and oxaloacetate and in a less extend for glutamate. In contrast, the *acidic* strain AC1_191 showed a similar isotopic ratio throughout all the alcoholic fermentation. The Figure 8 illustrated this discrepancy for valine. After 40 % of the fermentation, the growth phase is over and there is no more formation of proteinogenic amino acids. Therefore, the percentage of isotopic enrichment does not evolve, implying that consumed malic acid is not directed to amino acids production anymore.

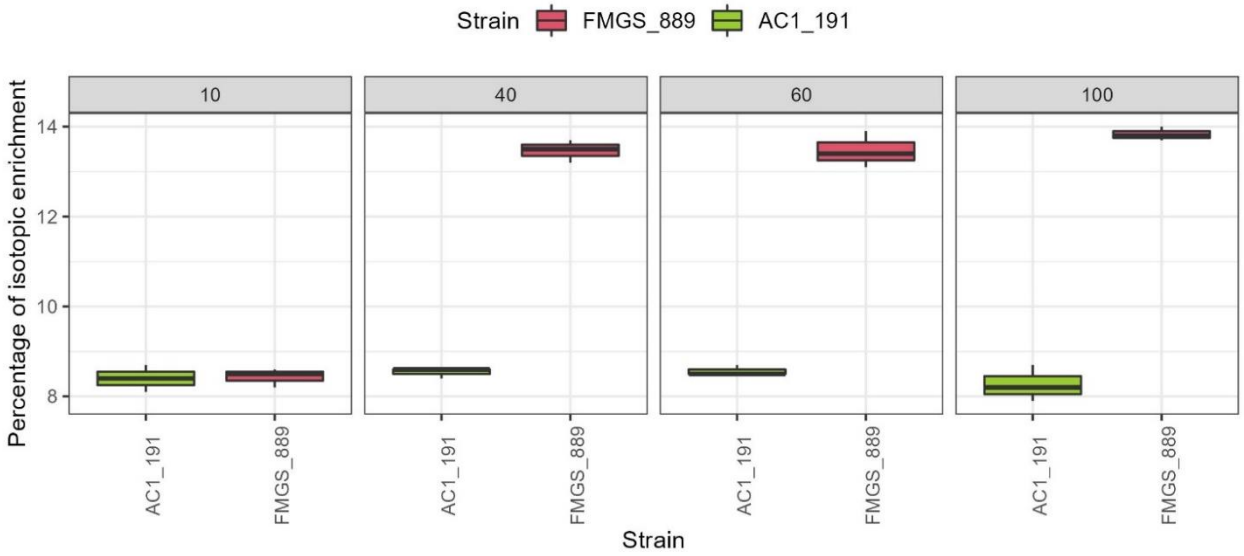


Figure 8: Percentage of isotopic enrichment of the valine for the two strains at the four sampling times (10 %, 40 %, 60 %, and 100 %).

Table 2: Isotopic enrichment (%) of amino acids per strain and percentage of CO_2 produced. The last two columns represent the part of variance explained by the factors $\%CO_2$ and Strain. The bold blue values are the one with a significative effect

Amino acid	Ions	Method	Origin	FMGS_889 10	FMGS_889 40	FMGS_889 60	FMGS_889 100	AC1_191 10	AC1_191 40	AC1_191 60	AC1_191 100	% CO_2 effect	Strain effect
Alanine	Ala158 Ala99	ECF - DMF/DMA	Pyruvate	6.89 ± 0.34 %	8.25 ± 0.66 %	8.15 ± 0.73 %	8.30 ± 0.70 %	7.25 ± 0.27 %	7.08 ± 0.24 %	7.07 ± 0.22 %	6.75 ± 0.45 %	5.03	**46.96**
Leucine	Leu158	ECF	Pyruvate	6.33 ± 0.06 %	10.80 ± 0.10 %	10.93 ± 0.31 %	11.30 ± 0.10 %	6.03 ± 0.23 %	6.30 ± 0.10 %	6.07 ± 0.15 %	6.17 ± 0.29 %	**12.57**	**62.00**
Valine	Val127 Val143 Val144 Val186	ECF - DMF/DMA	Pyruvate	7.99 ± 0.55 %	12.83 ± 0.77 %	13.12 ± 0.63 %	13.33 ± 0.81 %	8.12 ± 0.69 %	8.02 ± 0.61 %	7.91 ± 0.62 %	7.84 ± 0.66 %	**11.21**	**59.70**
Aspartate	Asp115 Asp188 Asp216	ECF - DMF/DMA	OAA	3.00 ± 0.48 %	5.51 ± 0.49 %	6.00 ± 0.78 %	6.81 ± 0.85 %	3.26 ± 0.50 %	3.34 ± 0.40 %	3.39 ± 0.29 %	3.71 ± 0.43 %	**27.25**	**45.48**
Isoleucine	Ile158	ECF	OAA	3.53 ± 0.06 %	6.40 ± 0.10 %	6.77 ± 0.40 %	7.43 ± 0.31 %	3.77 ± 0.25 %	3.83 ± 0.06 %	3.80 ± 0.10 %	4.00 ± 0.10 %	**21.23**	**51.02**
Threonine	Thr146 Thr175	ECF	OAA	1.62 ± 0.15 %	5.18 ± 1.85 %	4.87 ± 0.89 %	5.27 ± 0.57 %	1.92 ± 0.16 %	2.05 ± 0.18 %	2.28 ± 0.10 %	2.42 ± 0.82 %	**22.69**	**41.36**
Arginine	Arg174	BSTFA	alpha-KG	3.75 ± 0.07 %	3.55 ± 0.78 %	2.1 ± 0.00 %	3.27 ± 1.27 %	3.60 ± 0.00 %	3.43 ± 0.00 %	3.85 ± 0.64 %	3.23 ± 0.91 %	0.69	0.01
Glutamate	Glu230 Glu202	ECF - DMF/DMA	alpha-KG	0.93 ± 0.18 %	4.20 ± 0.18 %	4.52 ± 0.40 %	5.27 ± 0.29 %	1.25 ± 0.14 %	2.42 ± 0.26 %	2.65 ± 0.44 %	2.78 ± 0.35 %	**46.99**	**25.01**
Lysine	Lys156	ECF	alpha-KG	1.47 ± 0.06 %	1.83 ± 0.21 %	1.83 ± 0.31 %	2.17 ± 0.06 %	1.77 ± 0.06 %	1.77 ± 0.06 %	1.57 ± 0.15 %	1.77 ± 0.21 %	**23.62**	5.29
Proline	Pro142	ECF	alpha-KG	0.80 ± 0.10 %	0.53 ± 0.12 %	0.63 ± 0.21 %	0.77 ± 0.06 %	1.00 ± 0.10 %	0.83 ± 0.06 %	0.57 ± 0.06 %	0.77 ± 0.15 %	6.76	9.83
Phenylalanine	Phe143 Phe192	ECF - DMF/DMA	Glycolysis	1.32 ± 0.12 %	1.40 ± 0.15 %	1.32 ± 0.12 %	1.30 ± 0.13 %	1.33 ± 0.12 %	1.30 ± 0.11 %	1.30 ± 0.15 %	1.30 ± 0.09 %	6.22	6.29
Serine	Ser132 Ser175	ECF	Glycolysis	0.30 ± 0.23 %	0.45 ± 0.50 %	0.55 ± 0.44 %	0.37 ± 0.15 %	0.30 ± 0.17 %	0.20 ± 0.20 %	0.15 ± 0.10 %	0.32 ± 0.11 %	8.90	0.75
Histidine	His327	ECF	Pentose Phosphate	0.10 ± 0.10 %	0.17 ± 0.15 %	0.20 ± 0.14 %	0.23 ± 0.06 %	0.40 ± 0.30 %	0.10 ± 0.00 %	0.00 ± 0.00 %	0.10 ± 0.10 %	0.04	10.13
Glycine	Gly102 Gly144 Gly85	ECF - DMF/DMA	Glyoxylate / OAA / Glycolysis	1.83 ± 1.21 %	1.88 ± 1.13 %	1.85 ± 1.14 %	1.95 ± 1.29 %	1.88 ± 1.23 %	1.83 ± 1.15 %	1.75 ± 1.05 %	1.72 ± 1.13 %	1.93	14.98

3.8. Relative quantification of intracellular metabolites using a 2D NMR approach

In this last section, we quantified intracellular metabolites of fermenting yeast using the extraction procedure previously developed in the laboratory [38]. Intracellular content was normalized by the number of viable cells per milliliter determined by flow cytometry. The intracellular samples were analyzed by NMR spectroscopy using a (^{1}H-^{13}C)-HSQC method. A representative NMR spectrum is shown in figure 9, allowing the identification of eleven compounds: ethanol, alanine, acetic acid, pyruvic acid, proline, succinic acid, citric acid, malic acid, glucose, trehalose, and fumaric acid. The method applied did not allow neither an absolute quantification nor the estimation of the isotopic enrichment. However, the evolution of intracellular compounds concentration over the fermentation can be analyzed as well as possible differences between the two strains.

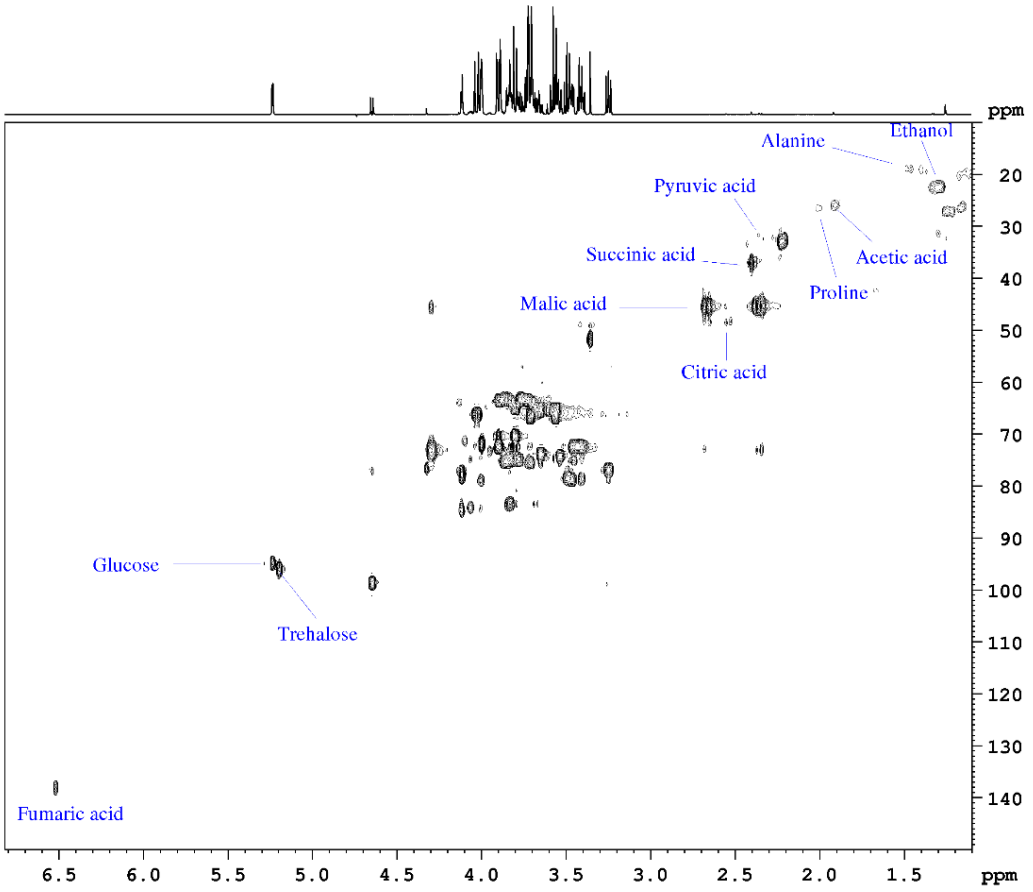


Figure 9: Representative spectrum : Contour plot of a (^{1}H-^{13}C)-HSQC NMR spectrum (region shown δ_H =1-7 ppm, δ_C = 10-150 ppm) for an intracellular sample.

A two-way analysis of variance demonstrated that most of the intracellular ^{13}C-labeled metabolites are significantly accumulated inside the cell during the alcoholic fermentation (Table 3). This is the case for acetic acid, fumaric acid, proline, pyruvic acid, and succinic acid (Figure 10). Beside compounds found in the TCA and glyoxylate cycles, other ^{13}C-labeled metabolites belonging to peripheral metabolic pathways such as alcoholic fermentation (ethanol), acetic acid production (acetate),

amino acid production (proline and alanine), and anabolic routes (trehalose) were observed. These results confirm that incorporated labeled malic acid is gradually distributed to several metabolic pathways through the fermentation ongoing. The evolution of those compounds is statistically similar for both strains, except for acetic acid for which the deacidifying strain FMGS_889 accumulated more labeled acetate at the later stage of the alcoholic fermentation. These similar patterns contrasted with the strong differences found at the extracellular level, indicating that fermenting yeast keep similar levels of intracellular compounds despite the strong difference observed at the extracellular level.

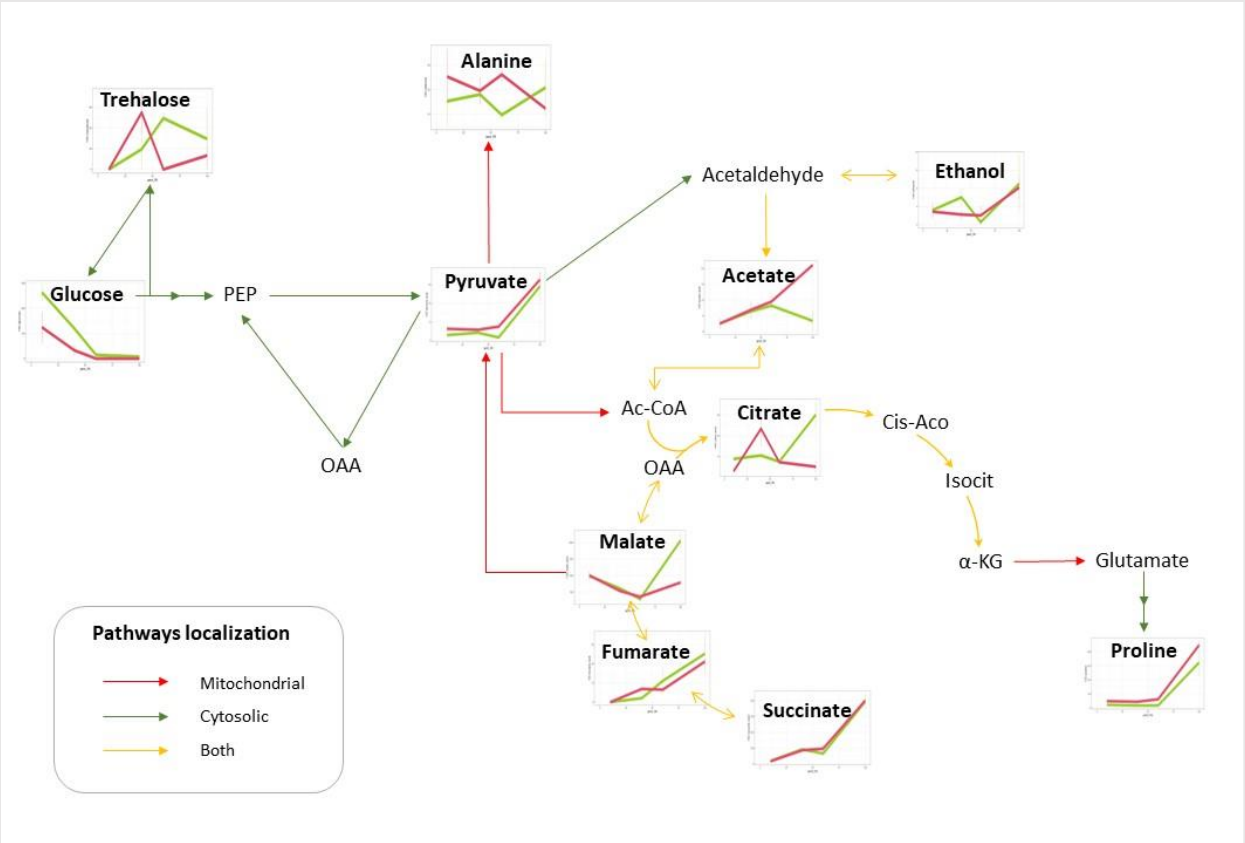


Figure 10 : Relative concentration of intracellular ^{13}C-labeled compounds overtime for the two strains. The red curve is for the deacidifying strain FMGS_889 and the green one for the acidifying strain AC1_191. The compounds have been placed on metabolic pathways and the color of the arrow indicates the compartmentation of the pathway.

Table 3: Part of variance explained by the factors "Strain","% CO_2"and the interaction effect of Strain and CO_2 for the intracellular labeled compounds. The bold, blue numbers are the one with a significative effect (p-value < 0.05)

	^{13}C-labeled		
	CO_2	Strain	CO_2 : Strain
Acetic acid	**31.38**	**16.21**	**26.83**
Alanine	1.23	3.68	6.49
Citric acid	10.70	10.10	**20.00**
Ethanol	14.65	1.50	0.02
Fumaric acid	**63.97**	0.26	1.05
Glucose	**54.11**	**10.97**	**7.37**
Malic acid	8.79	11.49	**20.00**
Proline	**63.81**	2.31	1.02
Pyruvic acid	**61.18**	1.39	0.01
Succinic acid	**82.18**	0.01	0.01
Trehalose	4.93	2.60	3.63

Conclusion

This work aimed to better understand the metabolic pathways involved in malic acid metabolism during alcoholic fermentation by two strains of *Saccharomyces cerevisiae* selected for their opposite behavior regarding malic acid modulation. Several analytical tools were combined to analyze data derived from fermentations with ^{13}C-labeled malic acid as secondary carbon source. The combination of GC-MS, LC-MSMS and NMR allowed the collection of complementary metabolic data. First, their analysis shed light on two distinct pathways for malic acid consumption and malic acid *de novo* production. A difference in the utilization of the Mae1p pathway between the two strains was observed. For the *demalic* strain the mitochondrial assimilation of malate by the malo ethanolic fermentation. This result is supported by the existence of 5-carbon labeled α-ketoglutarate. In contrast the *acidic* strain produced de novo malic acid via the cytosolic enzyme Mdh2p. This hypothesis is based on the fact that at the end of alcoholic fermentation malic and succinic acids production are decoupled. Overall, malic acid consumption fed several pathways such as amino acid biosynthesis and the anaplerotic route converting malate into oxaloacetate. Malic acid seems to have an essential role in the metabolism of the two strains studied. However, the difference observed between the strains at the end of the fermentation raise the question of the link between malic acid production and loss of fitness observed in the *acidic* strain.

Supplementary data

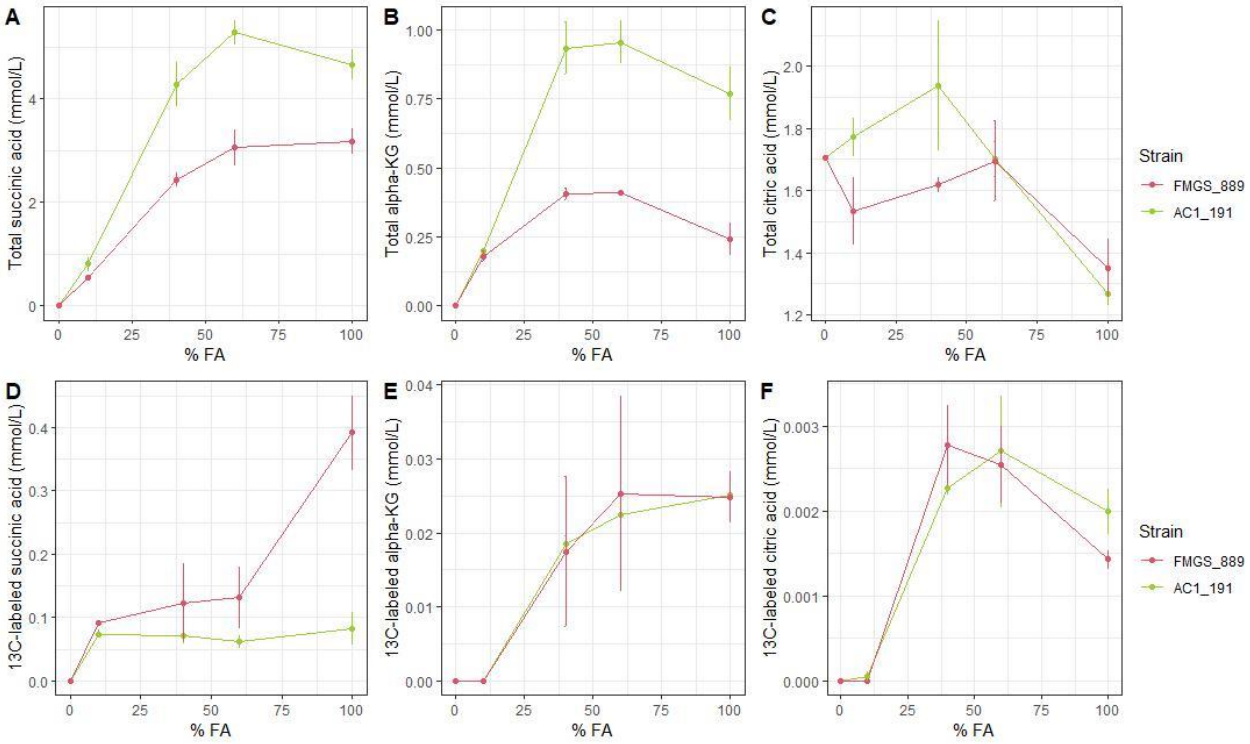


Figure S1: Evolution of the concentration of total succinic (**A**), α-ketoglutaric (**B**), and citric acids (**C**) and ^{13}C-labeled succinic (**D**), α-ketoglutaric (α-KG) (**E**), and citric (**F**) acids. Concentrations are expressed in mmol/L and time in percentage of CO_2 produced.

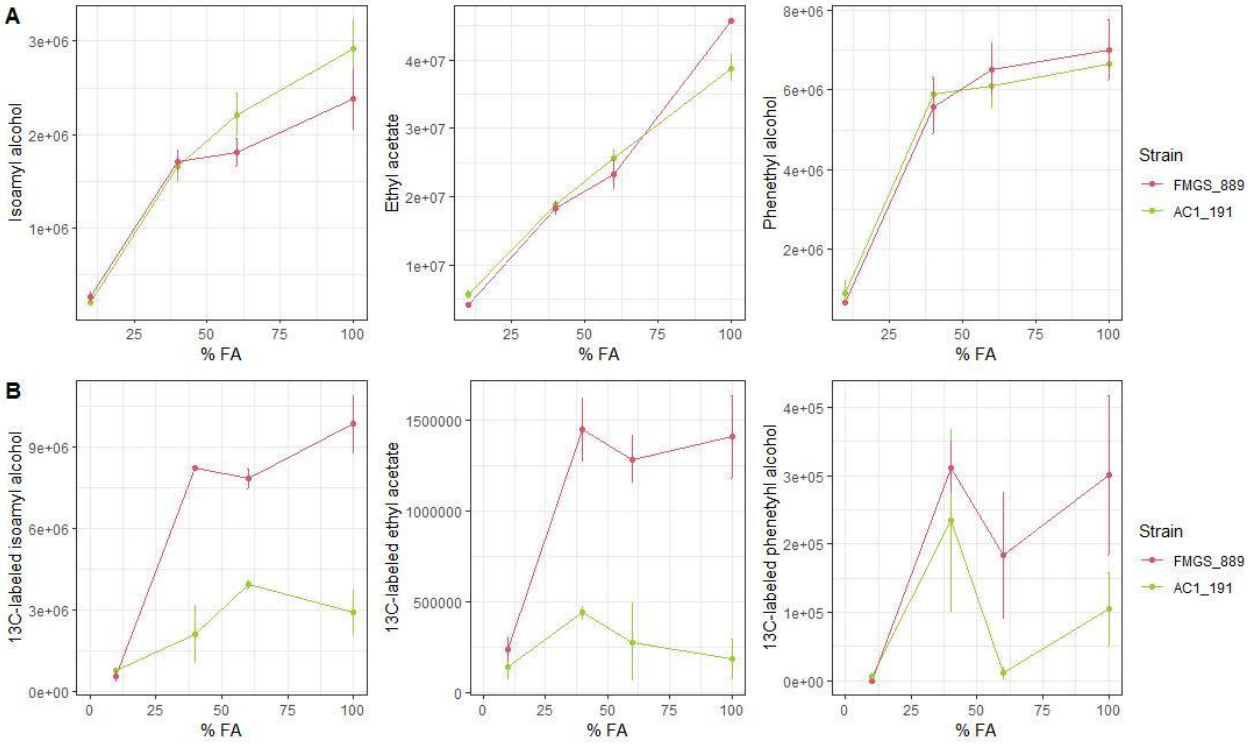


Figure S2: Concentration of total isoamyl alcohol (**A**), ethyl acetate (**B**), phenethyl alcohol (**C**) and 13C-labeled isoamyl alcohol (**D**), ethyl acetate (**E**), and phenethyl alcohol (**F**). Concentrations are relative and expressed as the area of the peak used for identification.

Table S1: Organic compounds assayed by enzymatic analysis and cellular concentration and viability determined by flow cytometry

Percentage FA (%)	Strain	Malic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Viability (%)	cell/mL (x 10^7)
10	FMGS_889	2.72 ± 0.36	0.38 ± 0.03	1.53 ± 0.39	91.8 ± 1.49	5.96 ± 1.04
10	AC1_191	2.79 ± 0.15	0.38 ± 0.04	2.16 ± 0.26	94.58 ± 0.90	6.64 ± 1.68
40	FMGS_889	2.05 ± 0.40	0.52 ± 0.08	3.78 ± 0.34	71.91 ± 3.48	11.5 ± 0.75
40	AC1_191	2.01 ± 0.35	0.34 ± 0.07	4.30 ± 0.33	93.91 ± 1.20	10. 7 ± 0.64
60	FMGS_889	1.79 ± 0.34	0.46 ± 0.04	3.78 ± 0.42	78.88 ± 2.74	11.2 ± 0.91
60	AC1_191	2.42 ± 0.62	0.27 ± 0.11	5.05 ± 0.22	91.50 ± 0.82	14.5 ± 2.02
100	FMGS_889	1.03 ± 0.09	0.40 ± 0.05	4.23 ± 0.89	48.03 ± 4.98	10.6 ± 0.62
100	AC1_191	2.25 ± 0.16	0.30 ± 0.07	4.92 ± 0.49	37.20 ± 10.37	9.27 ± 0.98

Table S2: Part of variance explained by the factors "Strain" and "% CO2" for the 3 organic acids (total + labeled) referenced in the figure 3. The bold, blue numbers are the one with a significative effect (p-value < 0.05)

	Succinic acid		α-ketoglutaric acid		Citric acid	
	Total	**^{13}C-labeled**	**Total**	**^{13}C-labeled**	**Total**	**^{13}C-labeled**
	Fig S1 A	*Fig S1 D*	*Fig S1 B*	*Fig S1 E*	*Fig S1 C*	*Fig S1 F*
Strain	**14.18**	**20.35**	**35.73**	0.02	3.91	0.19
% CO2	**54.30**	**29.84**	**16.21**	**47. 07**	**27.82**	**33.35**
Residuals	31.52	49.81	48.06	52.91	68.27	66.46

References

1. Faria-Oliveira F, Puga S, Ferreira C. Yeast: World's Finest Chef. Food Ind. 2013;11: 13. Available: https://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics
2. Herzig S, Raemy E, Montessuit S, Veuthey JL, Zamboni N, Westermann B, *et al*. Identification and functional expression of the mitochondrial pyruvate carrier. Science (80-). 2012;336: 93–96. doi:10.1126/science.1218530
3. KRESZE G -B, RONFT H. Pyruvate Dehydrogenase Complex from Baker's Yeast: Purification and Some Kinetic and Regulatory Properites. Eur J Biochem. 1981;119: 573–579. doi:10.1111/j.1432-1033.1981.tb05646.x
4. Walker E, Val DL, Rohde M, Wallace JC, Devenish J. Yeast Pyruvate Carboxylase: Identification of Two Genes Encoding Isoenzymes. Biochem Phys Res Commun. 1991;176: 1210–1217.
5. Kozlowski TT, Pallardy SG. Enzymes, Energetics, and Respiration. Physiology of Woody Plants (Second Edition). 1997. pp. 134–158.
6. A. Rosa C, Péter G. The Yeast Handbook : Biodiversity and Ecophysiology of Yeasts. Biodiversity and Ecophysiology of Yeasts. 2006. doi:10.1007/3-540-30985-3_4
7. Piškur J, Rozpedowska E, Polakova S, Merico A, Compagno C. How did *Saccharomyces* evolve to become a good brewer? Trends Genet. 2006;22: 183–186. doi:10.1016/j.tig.2006.02.002
8. Costenoble R, Picotti P, Reiter L, Stallmach R, Heinemann M, Sauer U, *et al*. Comprehensive quantitative analysis of central carbon and amino-acid metabolism in *Saccharomyces cerevisiae* under multiple conditions by targeted proteomics. Mol Syst Biol. 2011;7: 1–13. doi:10.1038/msb.2010.122
9. Gombert AK, Dos Santos MM, Christensen B, Nielsen J. Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression. J Bacteriol. 2001;183: 1441–1451. doi:10.1128/JB.183.4.1441-1451.2001
10. Camarasa C, Grivet JP, Dequin S. Investigation by 13C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways of succinate formation in *Saccharomyces cerevisiae* during anaerobic fermentation. Microbiology. 2003;149: 2669–2678. doi:10.1099/mic.0.26007-0
11. Arikawa Y, Kuroyanagi T, Shimosaka M, Muratsubaki H, Enomoto K, Kodaira R, *et al*. Effect of gene disruptions of the TCA cycle on production of succinic acid in *Saccharomyces cerevisiae*. J Biosci Bioeng. 1999;87: 28–36. doi:https://doi.org/10.1016/S1389-1723(99)80004-8
12. Pronk, Jack T; Steensma, H. Yde; Van Dijken JP. Pyruvate Metabolism in *Saccharomyces cerevisiae*. Yeast. 1996;12: 1607–1633.
13. SB B, Zaman Z, LP C, AJ B, IW D. Positive regulation of the LPD1 gene of *Saccharomyces cerevisiae* by the HAP2/HAP3/HAP4 activation system. Mol Gen Genet. 1992;231: 296–303.
14. Flikweert MT, Van der Zanden L, Janssen WMTM, Steensma HY, Van Dijken JP, Pronk JT. Pyruvate Decarboxylase : An Indispensable Enzyme for Growth of *Saccharomyces cerevisiae* on Glucose. Yeast. 1996;12: 247–257. doi:10.1016/s0021-9258(19)76974-9
15. Volschenk H, van Vuuren HJJ, Viljoen-Bloom M. Malic Acid in Wine: Origin, Function and Metabolism during Vinification. South African J Enol Vitic. 2006;27. doi:10.21548/27-2-1613
16. Chidi BS, Bauer FF, Rossouw D. Organic Acid Metabolism and the Impact of Fermentation Practices on Wine Acidity : A Review. South African J Enol Vitic. 2018;39.
17. Volschenk H, van Vuuren HJJ, Viljoen-Bloom M. Malo-ethanolic fermentation in *Saccharomyces* and *Schizosaccharomyces*. Curr Genet. 2003;43: 379–391. doi:10.1007/s00294-003-0411-6
18. Delcourt F, Taillandier P, Vidal F, Strehaiano P. Influence of pH, malic acid and glucose concentrations on malic acid consumption by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. 1995;43: 321–324. doi:10.1007/BF00172832
19. Peltier E, Vion C, Abou Saada O, Friedrich A, Schacherer J, Marullo P. Flor Yeasts Rewire the Central Carbon Metabolism During Wine Alcoholic Fermentation. Front Fungal Biol. 2021;2. doi:10.3389/ffunb.2021.733513
20. Redzepovic S, Orlic S, Majdak A, Kozina B, Volschenk H, Viljoen-Bloom M. Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. Int J Food Microbiol. 2003;83: 49–61. doi:10.1016/S0168-1605(02)00320-3
21. Ramon-Portugal F, Seiller I, Taillandier P, Favarel JL, Nepveu F, Strehaiano P. Kinetics of Production and Consumption of Organic Acids during Alcoholic Fermentation by *Saccharomyces cerevisae*. Food Technol Biotechnol. 1999;37: 235–240.
22. Salmon JM. l-Malic-acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*. BBA - Biomembr. 1987;901: 30–34. doi:10.1016/0005-2736(87)90253-7
23. Tang JKH, You L, Blankenship RE, Tang YJ. Recent advances in mapping environmental microbial

metabolisms through 13C isotopic fingerprints. J R Soc Interface. 2012;9: 2767–2780. doi:10.1098/rsif.2012.0396

24. Kleijn RJ, Geertman JMA, Nfor BK, Ras C, Schipper D, Pronk JT, *et al*. Metabolic flux analysis of a glycerol-overproducing *Saccharomyces cerevisiae* strain based on GC-MS, LC-MS and NMR-derived 13C-labelling data. FEMS Yeast Res. 2007;7: 216–231. doi:10.1111/j.1567-1364.2006.00180.x
25. Van Winden WA, Van Dam JC, Ras C, Kleijn RJ, Vinke JL, Van Gulik WM, *et al*. Metabolic-flux analysis of *Saccharomyces cerevisiae* CEN.PK113-7D based on mass isotopomer measurements of 13C-labeled primary metabolites. FEMS Yeast Res. 2005;5: 559–568. doi:10.1016/j.femsyr.2004.10.007
26. Hazelwood LA, Daran JM, Van Maris AJA, Pronk JT, Dickinson JR. The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol. 2008;74: 2259–2266. doi:10.1128/AEM.02625-07
27. den Hollander JA, Behar KL, Shulman RG. 13C NMR study of transamination during acetate utilization by *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A. 1981;78: 2693–2697. doi:10.1073/pnas.78.5.2693
28. Dos Santos MM, Gombert AK, Christensen B, Olsson L, Nielsen J. Identification of in vivo enzyme activities in the cometabolism of glucose and acetate by *Saccharomyces cerevisiae* by using 13C-labeled substrates. Eukaryot Cell. 2003;2: 599–608. doi:10.1128/EC.2.3.599-608.2003
29. Zelle RM, De Hulster E, Van Winden WA, De Waard P, Dijkema C, Winkler AA, *et al*. Malic acid production by *Saccharomyces cerevisiae*: Engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. Appl Environ Microbiol. 2008;74: 2766–2777. doi:10.1128/AEM.02591-07
30. Den Hollander JA, Brown TR, Ugurbil K, Shulman RG. 13C nuclear magnetic resonance studies of anaerobic glycolysis in suspensions of yeast cells. Proc Natl Acad Sci U S A. 1979;76: 6096–6100. doi:10.1073/pnas.76.12.6096
31. Vion C, Peltier E, Bernard M, Muro M, Marullo P. Marker assisted selection of malic-consuming *Saccharomyces cerevisiae* strains for winemaking. Efficiency and limits of a qtl's driven breeding program. J Fungi. 2021;7. doi:10.3390/jof7040304
32. Vion C, Muro M, Bernard, Margaux, Richard B, Fautre V, Yeramian N, *et al.* New malic acid producer strains of *Saccharomyces cerevisiae* for preserving wine acidity during alcoholic fermentation. Food Microbiol. 2022;112. doi:10.1016/j.fm.2022.104209
33. Peltier E, Bernard M, Trujillo M, Barbe J-C, Prodhomme D, Gibon Y, *et al.* Wine yeast phenomics: A standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. PLoS One. 2018; 1–23. doi:10.1371/journal.pone.0190094
34. Zimmer A, Durand C, Loira N, Durrens P, Sherman DJ, Marullo P. QTL dissection of lag phase in wine fermentation reveals a new translocation responsible for *Saccharomyces cerevisiae* adaptation to sulfite. PLoS One. 2014;9: 37–39. doi:10.1371/journal.pone.0086298
35. Stitt M, McC. LR, Gerhardt R, Heldt HW. Metabolite Levels in Specific Cells and Subcellular Compartements of Plant Leaves. Methods Enzymol. 1989;174: 518–552.
36. Vion C, Le Mao I, Yeramian N, Da Costa G, Richard T, Marullo P. Targeted 1H-NMR wine analyses revealed specific metabolome signatures of yeast populations belonging to the *Saccharomyces* genus. submitted. 2023.
37. Gougeon L, Da Costa G, Le Mao I, Ma W, Teissedre PL, Guyon F, *et al.* Wine Analysis and Authenticity Using 1H-NMR Metabolomics Data: Application to Chinese Wines. Food Anal Methods. 2018;11: 3425–3434. doi:10.1007/s12161-018-1310-2
38. Vion C, Brambati M, Da Costa G, Richard T, Marullo P. Endo metabolomic profiling of flor and wine yeasts by ^{1}H-NMR approach. submitted. 2023.
39. Airoldi C, Tripodi F, Guzzi C, Nicastro R, Coccetti P. NMR analysis of budding yeast metabolomics: A rapid method for sample preparation. Mol Biosyst. 2015;11: 379–383. doi:10.1039/c4mb00452c
40. Gougeon L, da Costa G, Guyon F, Richard T. 1H NMR metabolomics applied to Bordeaux red wines. Food Chem. 2019;301: 125257. doi:10.1016/j.foodchem.2019.125257
41. Rollero S, Mouret JR, Bloem A, Sanchez I, Ortiz-Julien A, Sablayrolles JM, *et al*. Quantitative 13C-isotope labelling-based analysis to elucidate the influence of environmental parameters on the production of fermentative aromas during wine fermentation. Microb Biotechnol. 2017;10: 1649–1662. doi:10.1111/1751-7915.12749
42. Bloem A, Rollero S, Seguinot P, Crépin L, Perez M, Picou C, *et al.* Workflow based on the combination of isotopic tracer experiments to investigate microbial metabolism of multiple nutrient sources. J Vis Exp. 2018;2018: 1–17. doi:10.3791/56393
43. Crépin L, Truong NM, Bloem A, Sanchez I, Dequin S, Camarasa C. Management of multiple nitrogen sources during wine fermentation by *Saccharomyces cerevisiae*. Appl Environ Microbiol. 2017;83. doi:10.1128/AEM.02617-16
44. Millard P, Letisse F, Sokol S, Portais JC. IsoCor: Correcting MS data in isotope labeling experiments.

Bioinformatics. 2012;28: 1294–1296. doi:10.1093/bioinformatics/bts127
45. R Development Core Team. R: A language and environment for statistical computing. 2016;2: 1–12.
46. Minard KI, McAlister-Henn L. Glucose-induced degradation of the MDH2 isozyme of malate dehydrogenase in yeast. J Biol Chem. 1992;267: 17458–17464.
47. Minard KI, McElister-Henn L. Glucose-Induced Phosphorylation of the MDH2 Isoenzyme of Malate Dehydrogenase in *Saccharomyces cerevisiae*. Arch Biochem Biophys. 1994; 302–309.
48. Ovádi J, Srere PA. Macromolecular Compartmentation and Channeling. Int Rev Cytol. 1999;192: 255–280. doi:10.1016/s0074-7696(08)60529-x
49. Ljungdahl PO, Daignan-Fornier B. Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. Genetics. 2012;190: 885–929. doi:10.1534/genetics.111.133306

Conclusion du chapitre 3

Ce chapitre était dédié à acquérir une meilleure compréhension du métabolisme de l'acide malique chez la levure *S. cerevisiae* dans un contexte de fermentation alcoolique. Plusieurs techniques analytiques ont été combinées afin de recueillir un maximum d'information sur les voies métaboliques. Ce travail collaboratif avec l'unité SPO (Montpellier), et l'axe MIB de notre unité de recherche a permis de générer des données originales sur le marquage de différents métabolites retrouvés au niveau extra- et intracellulaire. Les principales informations à retenir sont les suivantes :

- L'acide malique est consommé et produit aussi bien par des souches acidifiantes que démaliquantes. Les différences phénotypiques observées entre ces souches résultent de l'efficacité de ces voies de consommation et de production.
- La consommation de l'acide malique lors de la phase de croissance sert en partie à la biosynthèse des acides aminés. L'acide malique consommé est également dispatché dans diverses voies métaboliques et mène à la production de nombreux composés organiques.
- La production d'acide malique *de novo* vient, selon nos hypothèses, majoritairement de la condensation du glyoxylate et de l'acétyl-CoA cytosolique par l'enzyme Mls1p. En fin de fermentation alcoolique, cette voie s'atténue. Les souches acidifiantes mettent en œuvre une voie alternative liée à la transformation de l'oxaloacétate en malate par l'enzyme cytosolique Mdh2p.
- L'acide succinique produit par les souches de *S. cerevisiae* est souvent corrélé à la production d'acide malique (voir les précédents chapitres de cette thèse). Cette production passe majoritairement par le cycle du glyoxylate et plus précisément par la réduction de l'isocitrate en succinate. Notre étude démontre également une corrélation entre la production de succinate et d'α-kétoglutarate sur les deux souches étudiées. Cette voie n'est plus active en fin de fermentation.
- Ces travaux posent la question du transport et de l'export de l'acide malique hors de la cellule. Ce dernier sort contre le gradient de concentration puisque cet acide est déjà présent dans le milieu. De plus, les différences d'efficacité de consommation et production de cet acide organique entre les deux souches étudiées interroge sur l'existence d'un transport actif de l'acide malique. De plus amples investigations seraient nécessaires pour élucider cette question.

Chapitre 4 : Utilisation de la métabolomique non ciblée pour la discrimination de différents groupes de souches

Chapitre 4 : Utilisation de la métabolomique non ciblée pour la discrimination de différents groupes de souches

1. Contexte et problématique

Ce dernier chapitre propose un travail préliminaire utilisant la métabolomique non ciblée pour caractériser des vins produits avec des souches de *S. cerevisiae* présentant des phénotypes variés. La métabolomique non ciblée est un outil puissant et polyvalent qui permet d'analyser l'ensemble des métabolites présents à l'état dissous dans le vin, qu'ils proviennent des moûts ou de l'action des microorganismes lors de la vinification. En utilisant la métabolomique non ciblée, il est donc possible de comparer les profils métaboliques de différents vins afin de déterminer des différences significatives entre eux. Cette approche permet également d'identifier des métabolites spécifiques qui peuvent être des marqueurs du cépage ou des microorganismes utilisés.

Dans notre cas, cette méthode a été utilisée pour discriminer des vins produits avec des souches démaliquantes et acidifiantes. Pour réaliser ces expérimentations, une collaboration avec nos collègues de l'axe QIV de notre unité de recherche a été conçue. Les vins produits avec les souches démaliquantes et acidifiantes, présentés dans le chapitre 1.3 de cette thèse, ont été analysés en chromatographie liquide couplée à la spectrométrie de masse à haute résolution afin d'établir si certaines molécules permettent de discriminer les vins en fonction de la levure utilisée. Des outils de traitements statistiques multivariés ont été développés pour traiter les jeux de données générés par ces analyses.

2. Principaux résultats

Cette partie a fait l'objet d'un article scientifique.

La conception de l'étude a été effectuée par Philippe Marullo (PM), Axel Marchal (AM), Marie Le Scanff (MLS) et moi-même. Les expériences de fermentations, pool des vins générés et préparation des échantillons pour les analyses en LC-MSMS ont été réalisés par Bruce Richard dans le cadre de son stage de Master 2 et moi-même pour le dernier batch ayant été analysé après la fin de son stage. Les analyses par LC-MSMS ont été effectuées par Tom Estier et MLS. PM a pris en charge le développement d'une méthode de traitement des données automatisée adaptée au large jeu de données généré par la LC-MSMS. Les expériences de phénotypage en milieu synthétique faisant intervenir le composé X ont été réalisées par Faustine Rose dans le cadre de son stage de Master 2. L'analyse des données ainsi que leur interprétation ont été réalisées par PM, MSL et moi-même. Enfin j'ai participé à la rédaction de l'article avec PM et MLS. L'article sous sa forme définitive sera revu par MLS, AM, PM et moi-même.

Untargeted LC-HRMS metabolomics reveals phenotypic specificities of extreme *S. cerevisiae* strains selected for their acidification properties.

Vion Charlotte*[1,2], Le Scanff Marie*[2], Estier Tom[2], Rose Faustine[1,2], Richard Bruce[1,2], Marchal Axel[2], Marullo Philippe[1,2]

[1]Biolaffort, Bordeaux FRANCE

[2] UMR 1366 Œnologie, Université de Bordeaux, INRAe, Bordeaux INP, BSA, ISVV

[*]The authors contributed equally to this work

Abstract

Yeasts strains used for alcoholic fermentation display an important variability on several quantitative traits of enological interest. One of these traits is the ability of *S. cerevisiae* strains to modulate acidity during alcoholic fermentation by consuming or producing malic acid. In this study, we used a panel of malic consuming and malic producing strains that fermented in twelve oenological matrices and characterized them with untargeted metabolomics methods. We used LC-HRMS and multivariate analysis in an attempt to discriminate wines produced with the different groups of strains. This work shed light on a handful of compounds discriminating the two groups of strains. A focus was made on one of these compounds to precisely investigate its effect on malic acid production.

Key words

LC-HRMS, *acidic* and *demalic* strains,

1. Introduction

Untargeted metabolomics is a booming technology that has been proposed as a powerful strategy to globally characterize the chemical composition of fermented products, for a review see Gao *et al.* (2021). Defined as the whole set of small molecules of a biological system (Nicholson 1999), metabolomics is the most recent and complex "omics" method located at final level in the flow of information of life organism before the phenotype (Vignoli *et al.*, 2019). Untargeted metabolomics can be achieved using mass or NMR spectroscopy. Mass spectrometry approaches are by far the most high-throughput methods for non-targeted analyses since they enable the access to a large number of metabolic features (Farrés, Piña, and Tauler 2015; Verpoorte *et al*. 2008; Bordet *et al*. 2021). Gas Chromatography coupled to Mass spectrometry (GC-MS) provides high sensitivity and takes advantage of well-established spectral databases. However, this approach required complex pre-treatments and applied only to volatile compounds unless derivatization procedures are used to modify the polarity of metabolites and facilitate their volatility (Fiehn, 2016; Kim *et al*., 2013; Marshall and Powers, 2017). Liquid Chromatography coupled to Mass Spectrometry (LC-MS) does not require volatile inputs. This feature reduces background signal noise and provides higher quality data allowing a more extensive metabolite detection (Aydoğan, 2020; Gao *et a*l., 2021). This technique is widely used for profiling soluble metabolites belonging to a wide range of concentration and chemical families such as flavonoids, saponins, peptides, nucleotides, organic acids, alkaloids, phospholipids, and polyamines. The recent advances in mass spectrometry instrumentation led to the generalization of tandem mass spectrometry equipment with one of the analyzers allowing High Resolution Mass Spectrometry (HRMS). Such spectrometers can produce thousands of MS^2 spectra per sample, with mass measurement accurate enough o associate empirical formulas to the detected ions (Marshall and Hendrickson, 2008). In the last decade, several efforts have been done to exploit the huge flow of data generated by such instruments. Suites of bioinformatic tools offers the possibility to interrogate databases (Dührkop *et al.,* 2019; Reed *et al.,* 2017) using user-friendly interfaces. Tools such as *CSI:FingerID* are based on the prediction of a molecular fingerprint of a query compound from its fragmentation tree and spectrum (Dührkop *et al*., 2015). When the MS^2 mass spectra are not resolved, the systematic annotation of compounds can be achieved by using class assignment and ontology prediction using tools such as CANOPUS (Dührkop *et al.*, 2021).

Microorganisms such as fungi and bacteria are associated to the production of fermented foods (breads, pickled vegetables, sausages, cheeses and yogurts, amino acid/peptide sauces) (Ashaolu, 2019; Navarrete-Bolaños, 2012; Tamang *et al.*, 2016) or alcoholic beverages (wine, beer, sake) (Sicard and Legras, 2011). Beside the transformation of carbohydrates and nutrient sources for their development, microorganism activities consume and release in the medium a wide panel of secondary metabolites that confer to the fermented goods their nutritional qualities, their texture, and their organoleptic properties. The impact of fermenting microorganisms on foods and beverages quality has been investigated in several matrices including dairy (Mozzi *et al*., 2013), bakery (Nissen *et al.,* 2020), traditional fermented

foods and beverages (Babini *et al.*, 2017). These studies allow to better understand the positive contribution of strains and species by identifying metabolic markers that positively contribute to the quality of the fermented product.

Recently we demonstrated that the level of malic acid at the end of the alcoholic fermentation can be efficiently controlled by applying genetic selection programs assisted by molecular markers (Vion *et al.*, 2022, 2021). Briefly, some strains hereafter named "*acidic*" does not consume and often produce malic acid during the alcoholic fermentation while others, named "*demalic*", consume a large part (>70%) of this organic acid with direct consequences on wine pH. The basic fermentation properties of such *acidic* and *demalic* strains were compared in various enological matrices underlining the existence of metabolic trade-offs between the two groups (Chapter 1.4 of this thesis). Indeed, *acidic* strains were described as slower fermenters than *demalic* strains which might be indirectly linked to the fact that they acidify the medium during the alcoholic fermentation. They are also more sensitive to the medium composition. For example, they produce more malic acid when its initial concentration is low, while *demalic* strains consume a steady amount of malic acid regardless of the initial amount (Chapter 1.4 of this thesis). In the present study, we stepped up this characterization by applying an untargeted metabolomic approach.

Here, we applied non-targeted metabolomics for characterizing molecular signatures of wines fermented by different *Saccharomyces cerevisiae* strains. The metabolomic profile of 12 wines fermented with yeast strains having a strong impact of wine acidity were analyzed using LC-HRMS instruments. The 216 samples analyzed were submitted to the bioinformatic pipeline SIRIUS that was coupled to multivariate statistical analysis tools. Some metabolites were statistically linked to the capability of strain to produce malic acid. These preliminary results pave new avenues for understanding the metabolic control of acidity management of the *S. cerevisiae* species in the context of the alcoholic fermentation of grape juice.

2. Methods

2.1. Origin and preparation of the wine sample analyzed.

The 216 wine samples analyzed in this study were obtained by achieving laboratory scale fermentations following the procedure detailed in a previous publication (Chapter 1.3. of this thesis). Briefly, twelve different wines were derived from six natural white and red grape juices collected in the Bordeaux area (Vignoble Ducourt, Ladeaux). Two red grape juices M13 and M17 resulted from thermo-treated Merlot grapes collected in 2013 and 2017 respectively. One red grape juice CS13 resulted from a thermo-treated Cabernet sauvignon. One white grape juice SB14 was a Sauvignon blanc harvested in 2014. These four grape juices had different levels of sugar acidity balance that were summarized in the chapter 1.3. of this thesis. Two others grape juice SB16 (Sauvignon blanc 2016) and M21 (Merlot 2021) were supplemented with malic acid and/or fermentable sugars, in order to mimic the differences of

technological maturity met in enology. The condition enriched in malic acid (M+) was obtained by adding 3.9 and 3.1 g/L of L-malic acid in the M21 and SB16 grape juices respectively. The conditions enriched in sugar was obtained by adding 40 and 30 g/L of an equimolar mix of D-glucose and D-fructose in the respective grape juices. The combination of this conditions (S-M-, S-M+, S+M- and S+M+) lead to a modification of the acidity and density of M21 and SB16 matrices. Thus, twelves different matrices with complementary levels of acidity and sugar concentrations were fermented.

At the end of fermentation, pool of wines fermented with the different group of strains were prepared. According to the analytical results of wines obtained in the chapter 1, we selected five *acidic* strains able to produce low-pH wines, and five *demalic* strains able to produce high-pH wines. The phenotypic differences of *acidic* and *demalic* strains have been widely characterized and resulted from several genetic specificities (Peltier *et al.*, 2021; Vion *et al.*, 2022, 2021). For the twelve matrices ,the samples preparation went as follows. 200 µL of wine produced by each strain of the *demalic* or *acidic* groups were pooled together for a resulting pool of 1 mL per condition. As three biological replicates were conducted per strain and conditions, six pools were prepared per fermentation condition for a total of 72 pools of wines produced with *acidic* strains and 72 pools of wines produced with *demalic* strains. Wines produced with parental strains were also added to the experiment without pooling, adding 72 samples for a total of 216 samples of wines (Table S1). Samples were stocked at 4°C until LC-MSMS analysis. Before the injection, sampled were diluted 5 times in H_2O and filtered at 0.22 µm.

2.2. LC-HRMS acquisition

The liquid chromatography apparatus consisted of a Vanquish system (Thermo Fisher Scientific, Les Ulis, France) with binary pumps, an autosampler and a heated column compartment. Mass detection was performed using a Q-Exactive Plus mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe (from Thermo Fisher Scientific, Les Ulis, France). Chromatographic separation was carried out with a Hypersil Gold C18 column (100 × 2.1 mm, 1.9 µm, Thermo Fisher Scientific). The mobile phases were composed of water (Eluent A) and acetonitrile (Eluent B), both acidified with 0.1% of formic acid. The flow rate was set at 600 µL/min and the injection volume was 2 µL. The temperature of the column chamber was fixed at 30 °C in forced air mode. Eluent B varied as follows: 0 min, 2 %; 1 min, 2 %; 3 min, 10 %; 6.5 min, 70 %; 6.8 min, 98 %; 8 min, 98 %; 8.15 min, 2 %; 10 min, 2 %. The mass analyzer was calibrated each week with Pierce®ESI Negative Ion Calibration solutions (Thermo Fisher Scientific). The source parameters were set as follows : sheath gas flow rate 65 arbitrary units (a.u.); auxiliary gas flow rate 20 a.u.; sweep gas flow rate 0 a.u.; HESI probe temperature 360 °C; spray voltage -3.5 kV; capillary temperature 340 °C; S-lens RF level 60 a.u. Mass spectra were acquired in negative ionization with a Full MS-dd-MS^2 mode. Concerning the Full-MS scan, resolution was set at 70,000 FWHM within the range of from 100 to 1800 *m/z*. MS^2 scan were acquired with a Top 5 with normalized collision energy of 10, 30 and 50 eV and the resolution was set as 17,500 FWHM. The value of automatic gain control target was fixed at 1.10^6 ions and at 2.10^5 for

Full-MS and MS^2 respectively. The maximum injection time was set at 200 ms for Full-MS and 50 ms for MS^2.The samples were diluted by 5 with ultrapure water before being filtered at 0.2 µm to be injected. They were separated in three batches to be analyzed. Batch 1 encompassed the wines made from CS13, M13, M17 and SB14 grape juices while batches 2 and 3 encompassed the wine samples made from supplemented SB16 and M21 grape juices, respectively. Each batch contains 72 samples and were randomly disposed on the sampler. A Quality Control (QC) was prepared from an aliquot of each sample and analyzed every 20 samples.

2.3. Raw data treatment and QC filtering procedure

Raw data files (.raw) were compiled with MZmine 2.53 software (Schmid *et al*., 2023) in order to release a mascot generic format file (.mgf) as well as a tabular data set (.csv) encompassing the quantification of all metabolic features with a unique ID number per batch and ionization mode. The mass detection step was realized with a noise level of 1.10^5 for MS^1 filter and 1.10^4 for MS^2. Chromatograms were built with a minimum time span of 0.01 min, a minimum height of 1.10^4 and a *m/z* tolerance of 10 ppm. The "baseline cut off" algorithm was used to perform the chromatogram deconvolution, with a minimum peak height of 1.10^4, a peak duration range from 0.01 to 1 min and a baseline level of 1.10^3. The MS^2 scan pairing was set at *m/z* 0.02 Da. The deisotoped step was carried out using the isotopic peaks grouper algorithm with a *m/z* tolerance of 10 ppm, a retention time tolerance of 0.1 min, a maximum charge of 3 and the representative isotope used was the most intense. Chromatographic peaks were aligned using the join aligner algorithm with a tolerance of 10 ppm for *m/z* and 0.1 min for retention time. The weight for *m/z* was 75 % and 25 % for retention time. Peak finder module was used to perform the gap filling with a tolerance of 10 % for intensity, 10 ppm for *m/z* and 0.2 min for retention time. The peak list was filtered with duplicate peak filter using the mode "new average" with a *m/z* tolerance of 0.001 Da and a retention time tolerance of 0.03 min.

In this study only the fragmented metabolic features (MS^2) that have been considered. The *mgf* file was used for the automatic characterization of metabolic features by the Sirius 4 software suite (Dührkop *et al*., 2019). The tabular data was analyzed using home developed R scripts that are available upon request. First, chromatograms were time-trimmed at 6.8 minutes which correspond to the time of a massive compounds release. Second, data were manipulated using a basic metadata management. A first dataset (X) contains metabolite quantification, a second data set (EXP) contains sample information, and a third dataset (MOL) contains the molecules information generated by SIRIUS and subsequent statistical analyses. Quality control was applied according to the main guideline recommendations (Broadhurst *et al*., 2018). QC samples were displayed on a PCA plot to assess the stability of the analytical system; the first five QC samples places at the beginning of the run for equilibrate the column were omitted for this analysis. Variables were removed from the data set if one of these three conditions was met: (*i*) their reproducibility was inadequate (coefficient of variation CV > 30 % in QC samples), (*ii*) they were observed in < 70 % of QC samples, (*iii*) a linear model indicates a linear correlation

according to the injection order (α=0.05). The Figure S1 illustrated the QC operations applied.

2.4. Identification and classification of molecules

Mascot generic format files of each LC-MS batch were treated with the SIRIUS 4 (Dührkop *et al*., 2019). MS/MS spectra were analyzed with *CSI:FingerID* for molecular structure prediction searching in the Bio Database (Dührkop *et al*., 2015). We limited ionization adducts to $[M - H]^-$, elemental composition to $C_{3–60}$ $H_{4–122}$ $O_{0–20}$ $N_{0–3}$ $S_{0–2}$, P_{0-4} and mass tolerance to 10 ppm. The assignation of molecule to chemical family was achieved with CANOPUS (Dührkop *et al.*, 2021). A home-made R script allows the retrieving of relevant SIRIUS outputs (molecular formula, molecule ID, structures predicted, Tanimoto score, SIRIUS score, CANOPUS class annotation). These information were integrated as metadata and associated to each metabolic feature detected. The script is available upon request.

2.5. Statistical analyses and graphical representations

All the statistical analysis were made with the R program (R Development Core Team, 2016) and graphics were implemented with the *ggplot* package suite. The statistical differences between group of strain were done using non-parametric tests to avoid problem of homoscedasticity and normality. Differences between SB, GN, *acidic* and *demalic* groups were tested using the *kruskal* function (*agricolae*). Since many compounds were tested, the statistical threshold was adjusted using the Benjamini-Holchberg "BH" method. Differences between sugar content, malic acid content and *acidic* vs *demalic* group were tested using a Wilcoxon test with "BH" probability value correction. Class enrichment analysis were carried out by testing the proportion of metabolic features in a selected subset or in the general population (χ^2 test α =0.01). Chemical classes containing less than 8 compounds were discarded from the analysis. Probabilities values were corrected according to the BH method.

Two type of discriminant analyses were carried out. Discriminant analysis of the principal component (DAPC) were implemented using the *adegenet* package (Jombart *et al.*, 2010). The number of PCA axes retained for the analysis were set according to the cumulated part of variance explained (> 80 %). Factors with two and four levels were treated by one and two Linear Discriminant (LD) dimensions, respectively. The selection of most impacting metabolic compounds was carried out according to the following procedure. Metabolites were ranked according to their contribution to the LD retains and those participation to more than 60 % of the cumulated part of variance were selected. Sparse Partial Least Square Discriminant analysis (Lê Cao *et al*., 2011) was implemented using the *mixomics* package. Variables that contribute to separate individuals in the first to principal component were selected using a similar procedure that for DAPC. The most impacting factors retained contribute to 60 % of the part of variance of the two principal axes.

3. Results and Discussion

3.1. Experimental design

In a recent study we demonstrated that yeast strains selected for their aptitude to produce or consume malic acid during the alcoholic fermentation had distinct fermentation behavior in terms of fermentation kinetics (Chapter 1.3 of this thesis), production of organic compounds (Vion *et al.*, 2023) and sensory analysis (Vion *et al*., 2022) (Chapter 1.4 from this thesis). The use of such strains would be particularly relevant for managing the acidity of wines (see the Review in the introduction of this thesis). In order to better understand the physiology and the metabolism of such opposed styles of strains, we applied an untargeted metabolomic approach aiming to identify possible discriminatory metabolites. For this purpose, the metabolome of twelves wines resulting from micro-scale laboratory fermentations were analyzed. As summarized in Figure S2, these twelves fermented matrices were characterized by a wide concentration range in malic acid (1.9 - 7.9 g/L) and initial sugars (180 – 249 g/L) which correspond to different sugar/acidity balances met in winemaking (Figure 1A).

The contribution of the yeast genetic background was tested by comparing four modalities. The first two modalities were represented by biological triplicates of pure cultures made with the strains GN and SB (Figure 1B). These two *S. cerevisiae* strains showed moderate differences in their malic acid metabolism as previously reported (Peltier *et al.*, 2021; Vion *et al.*, 2023, 2022). The two other modalities were represented by pool of wines made with five *acidic* and five *demalic* strains that showed particularly high differences in their malic acid metabolism (Figure 1C). The characteristics of these wine pools were summarized in Table 1. The metabolomic fingerprints of SB, GN, *acidic* and *demalic* strains were then compared using an analytical workflow that include: (*i*) wine sample preparation, (*ii*) LC-HRMS analysis, (*iii*) structure prediction and class annotation using the SIRIUS4 suite, (*iv*) multivariate analysis aiming to select metabolomic signatures linked to *acidic* and *demalic* strains (Figure 1D).

Table 1: Overall characteristics of wine pools

	Ethanol (%Vol)		*Malic acid (g/L)*		*Succinic acid (g/L)*		*Acetic Acid (g/L)*		*Glycerol (g/L)*		*pH*	
	Low-pH [a]	High-pH [b]	Low-pH [a]	High-pH [b]	Low-pH [a]	High-pH [b]	Low-pH [a]	High-pH [b]	Low-pH [a]	High-pH [b]	Low-pH [a]	High-pH [b]
SB16	13.5	13.9	2.62	1.15	1.14	0.89	0.34	0.48	8.98	8.04	3.01	3.26
SB16A	12.7	13.2	4.25	2.49	1.14	0.97	0.35	0.50	8.29	7.89	2.87	3.05
SB16AS	13.1	13.4	2.60	1.03	1.08	0.86	0.32	0.46	8.26	8.05	2.98	3.22
SB16S	14.3	15.0	4.06	2.48	1.22	1.05	0.54	0.66	9.41	8.90	2.91	3.14
M21	12.2	12.6	2.48	0.86	1.86	1.43	0.23	0.48	10.86	10.65	3.37	3.77
M21A	12.2	12.8	3.89	1.83	1.79	1.40	0.23	0.37	10.94	9.57	3.24	3.64
M21AS	14.2	14.0	3.95	1.67	2.22	1.31	0.28	0.55	10.78	11.79	3.24	3.61
M21S	14.2	14.8	3.09	0.91	1.83	1.38	0.23	0.44	12.68	11.45	3.37	3.81
CS13	10.6	11.0	3.64	2.18	1.08	0.91	0.07	0.14	8.52	8.37	3.24	3.53
M13	13.1	13.5	2.43	1.15	1.28	0.92	0.22	0.26	10.15	10.63	3.24	3.53
M17	13.5	14.1	2.85	1.17	1.37	0.98	0.13	0.19	11.40	9.83	3.20	3.58
SB14	11.4	11.8	3.69	2.26	0.91	0.80	0.16	0.22	9.27	8.24	3.01	3.21

[a] Data for low-pH wines are the average values of the strains AC1-191, AC1-227, AC2-1A, AC2-467 and AC3-22 (*acidic* strains)
[b] Data for high-pH wines are the average values of the strains FMGS1-889, FMGS1-71, FMGS2-107, FMGS2-714, FMGS4-263 (*demalic* strain)

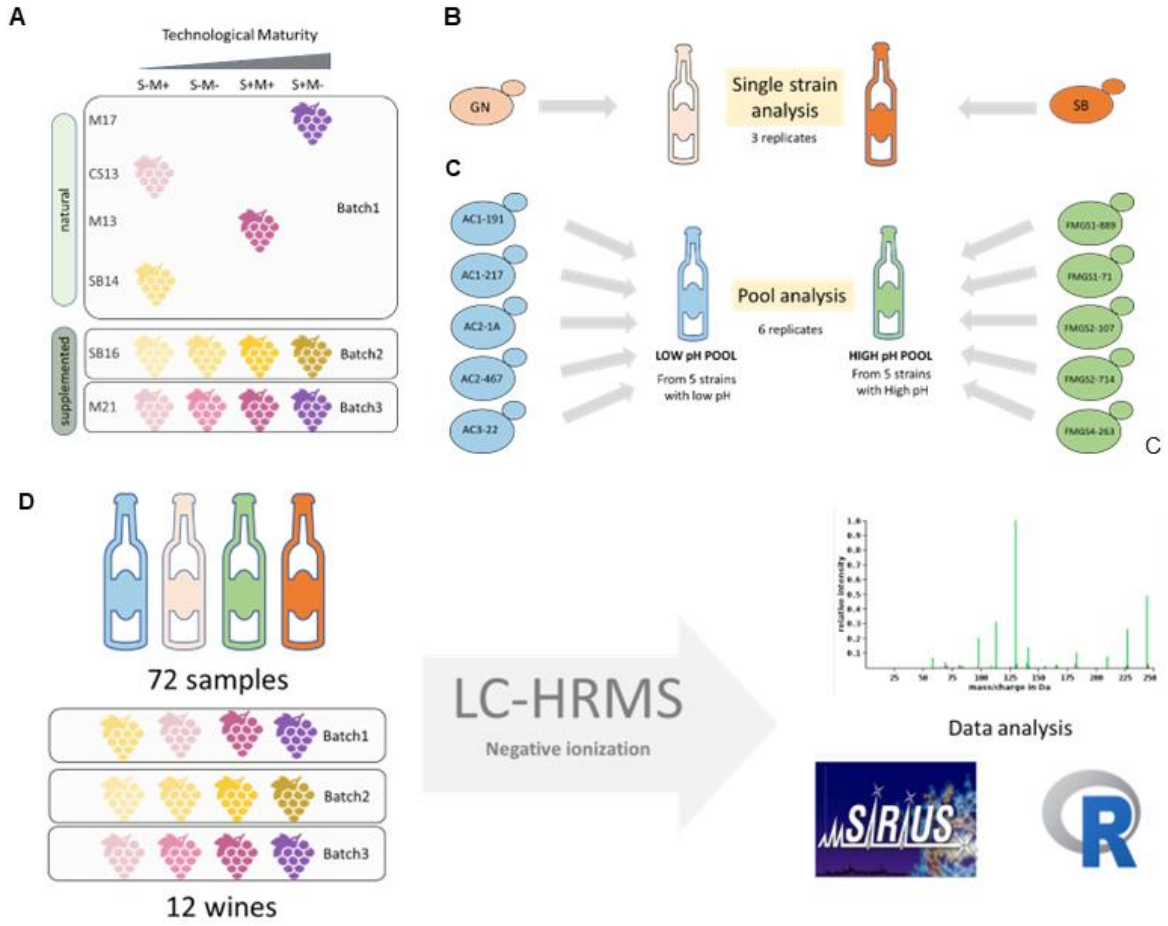


Figure 1: LC-HRMS analysis of twelves wines produced by *acidic* and *demalic* strains. Wines analyzed were previously obtained by laboratory scale nano-vinification (chapter 1.3 of this thesis). **Panel A.** Wines were obtained from six natural grape juices. Four grape juices (M17, CS13, M13, and SB14) with different levels of sugar acidity balance were used as is. Two others SB16 and M21 were supplemented with malic acid or sugars in order to mimic different technological maturity levels met in enology. **Panel B.** The reliability of the method was tested by analyzing the 12 wines produced by the strains SB and GN. Each grape juice x strain combination was independently fermented 3 times. **Panel C.** The impact of *demalic* and *acidic* strains was evaluated by pooling fermentation samples in order to create low-pH and high-pH wines. **Panel D.** In order to adapt this large dataset to the LC-MS capability, samples were split in three distinct batches: *batch 1* encompassed the four unrelated grape juices (M17, CS13, M13, and SB14); *batch 2* encompassed the SB16 with 4 combinations of sugar and malic acid concentrations. *batch 3* encompassed the M21 with 4 combinations of sugar and malic acid concentrations. For each batch, negative ionization was applied for quantifying non-volatile molecules by LC-HRMS technology.

3.2. Set up of a LC-HRMS analysis on non-volatile metabolome workflow: data filtering, automatic annotation of metabolite features and identification of impacting molecules.

The non-targeted metabolomic strategy applied was carried out by using LC-HRMS technology able to analyze thousands metabolic features (Wolfender *et al.*, 2019). Indeed, the qualitative and quantitative description of the samples can be achieved by measuring the exact mass of each metabolite and its intensity. In addition, automated acquisition of MS^2 spectra of some metabolites is useful to generate structural information. In the present study, metabolic compounds were analyzed in order to identify metabolomic signatures of strains having a strong impact on wine acidity. The entire dataset encompassed three independent batches of 72 wines pooled. The MS^2 mass spectra of metabolic features

detected were automatically annotated by using the SIRIUS software suite (Dührkop *et al*. 2019). The procedure for the automatic identification of molecular formula (*SIRIUS*), the attribution of chemical class (*CANOPUS*) and the putative chemical identification by using fragmentation tree of MS^2 mass spectra (*CSI:fingerID*) are detailed in method section. The Table 2 summarizes the number of metabolic features retained by the different steps. In the present work, only metabolic features having an assigned molecular class by the *CANOPUS* algorithm were retained for further analyses leading to the quantification of 877, 540, and 1028 compounds for the baches 1, 2 and 3, respectively. Using MS^2 fragmentation, an additional chemical structure of a subset of metabolite weas proposed by the *CSI:fingerID* algorithm with a Tanimoto index cut-off set at 0.6 (Dührkop *et al.*, 2015). Each dataset was analyzed separately by using univariate, multivariate, and semantic analyses.

Table 2: Number of compounds identified by LC-HRMS.

Dataset	Grape juices	Ionization	total ions [a]	MS^2 [b]	QC passed [c]	Canopus annotation	CSI finder identification [d]
Batch1	CS13, M17, M13, SB14	negative	13.465	2.793	1.268	877	346
Batch2	SB16 (supplemented)	negative	24.501	2.292	728	540	138
Batch3	MR21 (supplemented)	negative	25.011	3.592	1.450	1.028	417

[a] after trimming
[b] only non-missing values
[c] CV <30%. 70 % of QC. LQ=5E5. non-structured
[d] Tanimoto index > 0.6

3.3. Decomposition of the metabolomic variability of four unrelated grape juices

The first batch analyzed encompassed 877 metabolic features commonly detected in four unrelated grape juices (M17, CS13, M13, and SB14). A Principal Component Analysis (PCA) of the dataset illustrated that the most part of the wine variability is related to the nature of the grape juice fermented (Figure 2A). Indeed, the first principal component accounted for 37% of the total inertia and clearly separated wines according to their grape origin. The two Merlot wines being grouped in the right part of the axis 1 while the white wine (SB14) is placed in the left part. The second axis (accounting for 16% of the total inertia) separated the Cabernet sauvignon (CS13) from the other wines. The study of compounds that discriminate the different wines is not the purpose of this work, but this first finding suggested that the method applied would be very efficient for identifying chemical markers associated to the grape juice origin.

The Figure 2B illustrated that non-volatile compounds were also able to well discriminate wines according to the fermenting yeast strains, whatever the grape juice fermented. The projection figured out explained 10.9 % of the total variance and well discriminated the four group of strains. Interestingly, wines made by GN and *acidic* strains are placed of the right part of the axis 4, while wines made by SB

and *demalic* strains are placed on the left side of the plot. The axis 5 is also able to discriminate the parental strains SB and GN.

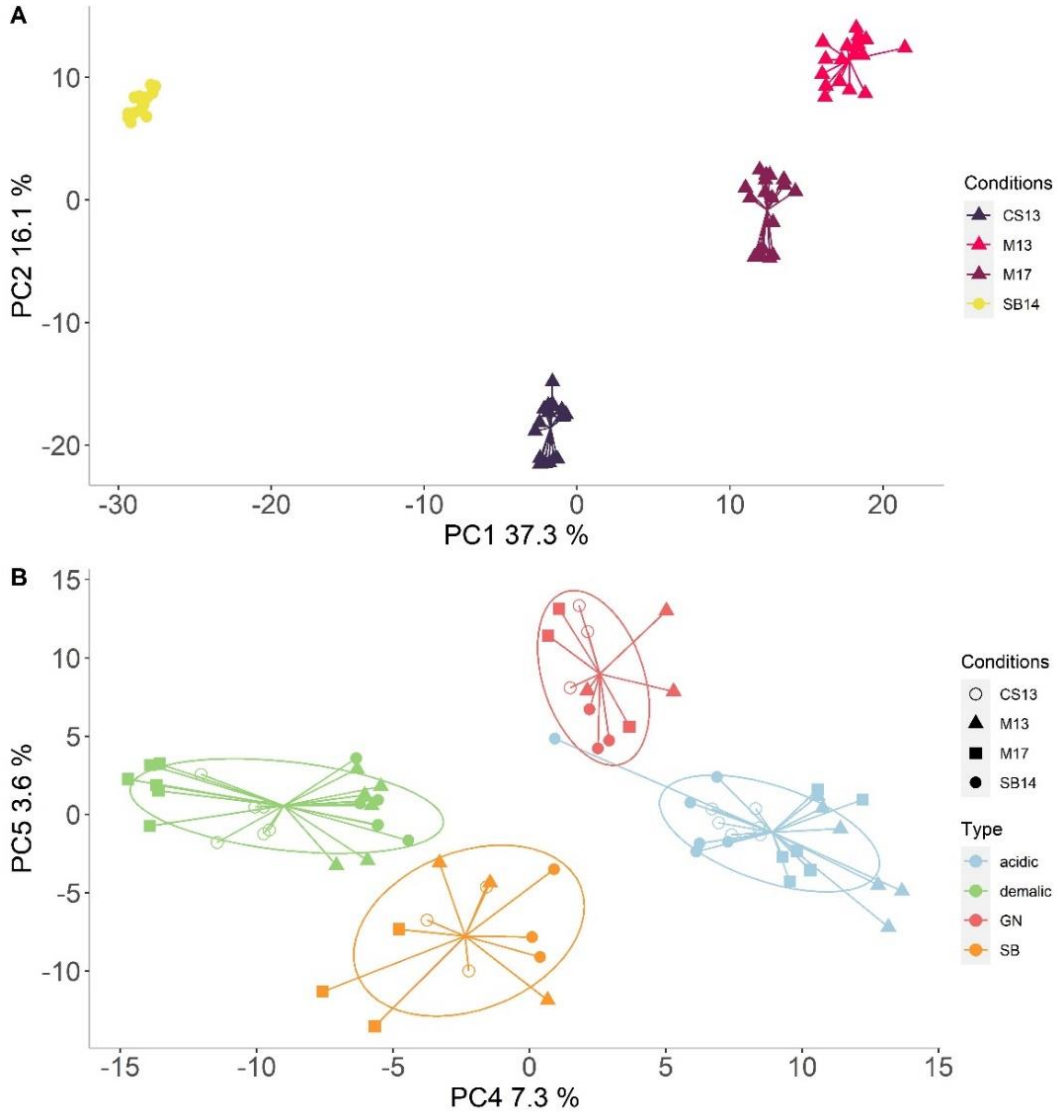


Figure 2: A principal component analysis was applied to the dataset composed of 877 metabolic features detected in 72 samples derived from 4 distinct grape juices M17, CS13, M13, and SB14. **Panel A.** Wines were clustered according to the grape juice origin in the first two components accounting for 53.4% of the total inertia. **Panel B.** The effect of the fermenting yeast is well illustrated by the fourth and the fifth components explaining together 10.9% of the total inertia.

The very high number of compounds structuring the dataset required to applied various univariate and multivariate analyses for narrowing the most relevant compounds discriminating of each group of strain (Sailwal *et al.*, 2020). In order to capture the widest number of compounds, three complementary statistical methods were applied successively. First, basic non-parametric tests (Kruskal test) were coupled with quantitative cut-off (1.5 folds change). The association of these two univariate tests allow the identification of metabolic features that statistically discriminate one of the fourth yeast background or one of the fourth grape juices. Second, a Discriminant Analysis of a Principal Component

Analysis (DAPC) was applied. This method is useful to identify the most relevant quantitative variables able to separate the samples according to the strain or grape juice origin. Third, a sparse Partial Least Square Discriminant Analysis (sPLS-DA) was implemented. This alternative PLS-DA method allows a more effective selection of explicative variables by applying a penalty to highly correlated features (Lê Cao *et al.*, 2011). The Figure 3 illustrates the effect of the metabolic feature #386 identified by the *CSI:fingerID* algorithm as the 2-oxoisoleucine (Figure 3A). This compound is a fatty acyl derived from yeast metabolism that well separates the yeast strains. The Figure 3B compared the average value of this compound for each group of strain. Wines made with the *acidic* and *demalic* strains being those having the highest and lowest concentration of 2-oxoisoleucine, respectively. The Figure 3C shows the overall separation of the four strain groups by the sparse PLS discriminant analysis applied. The panel D illustrates the specific discriminant power of 2-oxoisoleucine that is strongly correlated to the component 1, which globally represent 8 % of the variance explained. The Table 3 summarizes the number of molecules that discriminate the strain and the grape juice effects in this dataset.

Table 3: Identification of compounds impacted by the yeast or grape juice origin in the batch 1.

Dataset (batch 1)	Yeast effect	Grape juice effect
Statistically different [a]	165	700
DAPC [b]	118	285
sPLSDA [c]	210	613
Unique features [d]	246	746
Common and significative features [e]	80	14

[a] features with 1.5 folds change and significative Kruskal test (alpha =0.05, BH adjusted)
[b] features capturing 60% of total inertia LD1 and LD2
[c] features capturing 60% of total inertia of component 1 and 2
[d] captured by Kruskal test or sPLSDA or DAPC
[e] common between sPLSDA and DAPC and statistically different

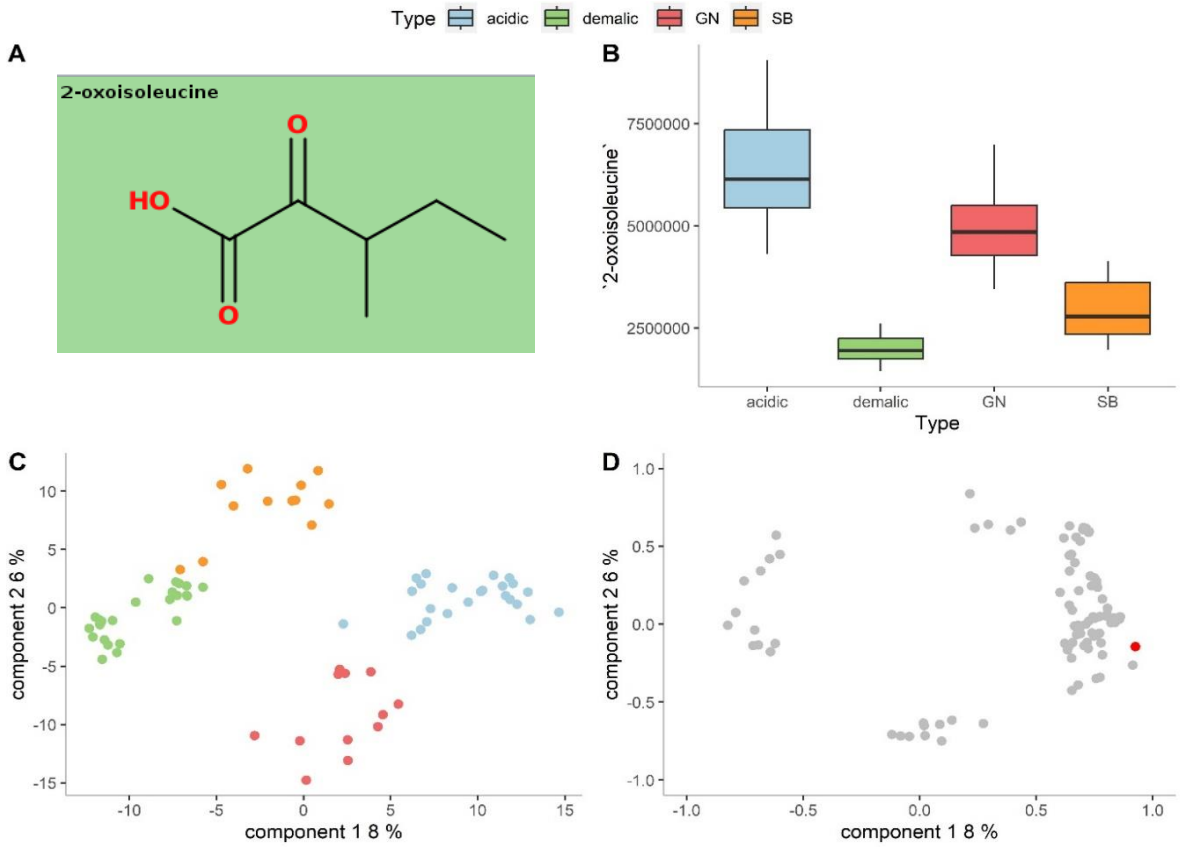


Figure 3: **Panel A.** Structure prediction of the 2-oxoisoleucine by *CSI:fingerID* (Tanimoto index 0.82). **Panel B.** Abundance of 2-oxoisoleucine in the M21 matrix according to the yeast strain. Each boxplot represents the values measured in S-M-, S-M+, S+M- and S+M+ conditions. Each strain is significantly different from the others, Kruskal test with post hoc treatment. **Panel C.** Individual plot of the sPLS Discriminant analysis, each point represents a wine sample and is colored according to the key on the top of the figure. **Panel D.** Variable contribution of the 2-oxoisoleucine (red dot) that is strongly correlated to the first component of the s-PLSDA.

The statistical workflow implemented to identify discriminant metabolites had different success. Concerning the grape juice origin, with univariate analyses (Kruskal test and fold change cut off), most of the compounds reached the 1.5 folds change threshold and were significantly different (700 out of 877). However, the two discriminant analyses applied were not congruent. Indeed, on the 746 metabolites detected by one of the three methods only 14 (1.8%) were commonly detected by the three statistical filters. This result highlights the difficulty to find out discriminatory molecules among a large number of variables (877) with a small number of samples with important intrinsic differences (four types of unrelated wines and only 72 samples). The discriminant analysis of the strain effect was more convergent. On the 239 metabolic features detected by at least one method, 54 of them (22%) were common to the three statistical approaches. This small group of molecules well discriminated the strain whatever the grape juice fermented. They constitute possible candidates able to better discover metabolic specificities between the *acidic* and *demalic* strains.

The recent advances in LC-MS/MS technology allow the easy semi-quantification of thousands

of metabolomics compounds that characterized biological samples of different origins. The massive number of compounds quantified constitute a statistical challenge to flesh out the most informative explanatory variables of such complex matrices. In this work, we applied both univariate and multivariate methods for narrowing such compounds. Univariate analyses were initially applied to identify compounds showing a relevant quantitative discrepancy among the modalities and being statistically different. These basic approaches constitute a necessary step prior to the use of more complex analyses (Lazar *et al*., 2015). However, this approach faces a major drawback: it cannot evaluate the relationship or correlation between the changes occurring within the different groups of metabolites due to their biological origin. To overcome this problem, we applied two types of multivariate analyses (chemometric methods). A non-supervised method was applied to capture the genuine variability of the metabolomic landscape of wines analyzed. The clear separation of samples according to their modalities is a prerequisite for applied additional supervised methods. Two of them were applied DAPC and s-PLSDA. DAPC have been proposed to identify among a large set of genetic markers those that better structure different populations of individuals (Jombart *et al*., 2010). This method can be also applied to any kind of quantitative dataset. s-PLSDA is an improved version of PLS that classifies the variance of data into 2 major components. The component first depends on the variable of interest, whereas the component second depends on uncorrelated data (i.e., orthogonal component) (Lê Cao *et al*., 2011). Both analyses were able to propose a set of discriminating variables characterized by their high VIP (Variable Importance in the Projection). However, those methods were not very congruent and many of the compounds listed are not statistically different between the modalities. This is especially true for metabolites that discriminate the grape juice origin despite the clear differences existing between samples. This result means that contrasted matrices are not only characterized by small set of discriminating molecules. In contrast, the analysis of metabolites able to discriminate the yeast strain was much more congruent, suggesting that the yeast origin can significantly impact the non-volatile wine metabolome.

3.4. Yeast strain, sugar, and malic acid effect on the metabolomic variability of supplemented grape juices

As illustrated in the Figure 2A, the contribution of the four grape juices of different maturity levels and different cultivar has a complex impact on their metabolomic fingerprint. Since the chemical composition of such matrix is unknown, it is impossible to identify abiotic factors governing this variability. In this section, we analyzed two other datasets made of wines obtained from two grape juices (SB16 and M21) that were supplemented with malic acid and/or reducing sugars (Supplementary file S2). These simpler matrices allow the identification of metabolites that were impacted by the addition of malic acid or fermenting sugars. These enological parameters represent the main abiotic factors impacted by the climate change (Van Leeuwen *et al.,* 2019). These additional datasets are also useful

for narrowing metabolic features linked to *acidic* and *demalic* strains.

A large number of metabolic features (540 and 1028) were quantified in the SB16 and M21, respectively (Table 2). The overall variability of these metabolomes is figured out by a Principal Component Analysis (PCA) (Figure 4). Interestingly, in both matrices, the wine composition is mostly structured by the nature of the fermenting yeast. Pool of wines made by *acidic* and *demalic* strains are clearly separated on the first axes of the PCA, representing 20.9 % and 13.9 % of the total inertia for SB16 and M21 wines, respectively. Wines made by the parental strains GN and SB took an intermediate position on the same axis. In both cases, the GN samples were closer to the *acidic* group and SB samples closer to the *demalic* group.

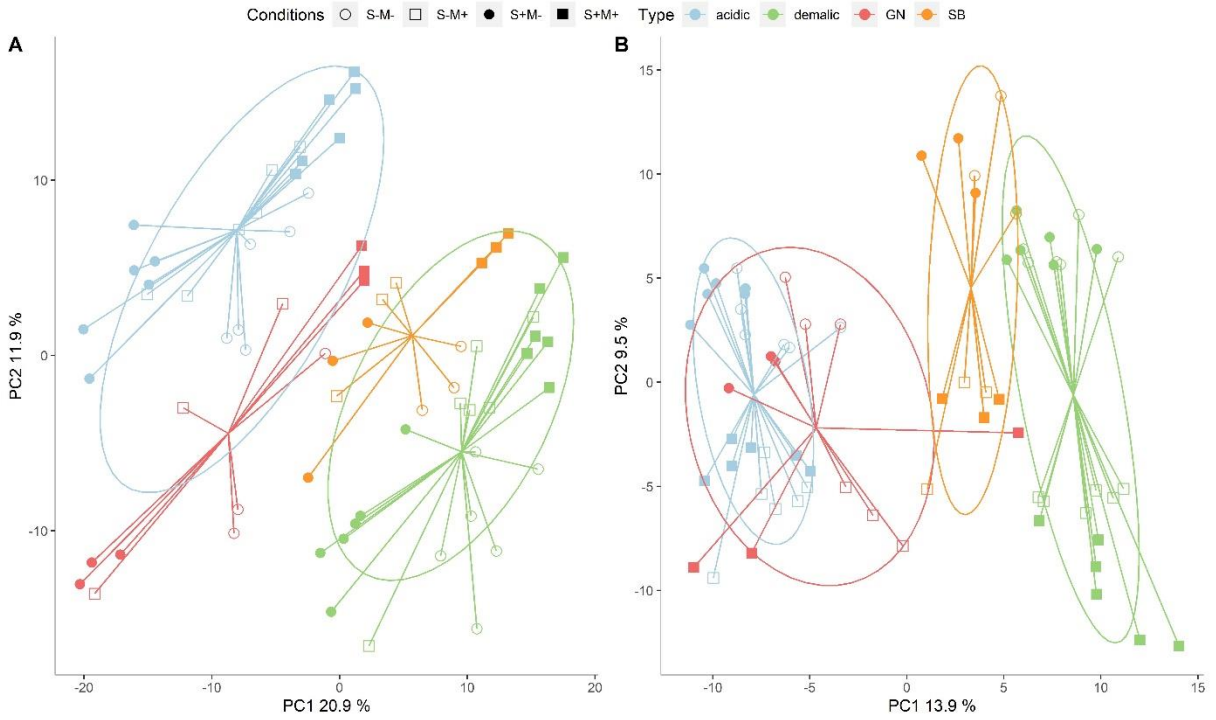


Figure 4: Principal component analyses were applied to the SB16 and M21 datasets that encompassed 540 and 1028 metabolic features, respectively. Each dataset is composed by 72 samples. For both datasets original grape juices were supplemented with malic acid and/or sugars. **Panel A**. SB16 wines were clustered according to the nature of the fermenting yeasts; the first two components accounting for 32.8 % of the total inertia. **Panel B**. M21 wines were clustered according to the nature of the fermenting yeasts, the first two components accounting for 20.4 % of the total inertia.

Table 4: Identification of compounds impacted by the yeast or grape juice origin in the batches 2 and 3.

	SB16 (batch 2)			M21 (batch 3)		
	Yeast effect	Malic acid effect	Sugar effect	Yeast effect	Malic acid effect	Sugar effect
Statistically different [a]	244	20	0	100	19	11
DAPC [b]	125	83	78	99	54	53
sPLSDA [c]	236	437	283	211	239	243
Unique features [d]	306	488	344	225	283	288
Common and significative features [e]	93	2	0	59	0	0

[a] features with 1.5 folds change and significative Kruskal test or Wilcoxon test (alpha =0.05, BH adjusted)
[b] features capturing 60% of total inertia LD1 and LD2 or LD1 in the case of malic and sugar effect
[c] features capturing 60% of total inertia of component 1 and 2
[d] captured by non-parametric tests or by sPLSDA or by DAPC
[e] common between sPLSDA and DAPC and statistically different

In order to identify metabolic features influenced by biotic (strain) and abiotic (sugar and malic acid contents) factors, sPLS-DA, DAPC and non-parametric tests were again applied (Table 4). A large number of metabolites linked to malic acid and sugar addition were proposed by sPLS-DA and DAPC methods. Again, the weak level of congruence between the statistical methods applied suggested that the addition of malic acid or sugar did not clearly impact wine metabolomes. Indeed, only a few numbers of these compounds showed a relevant dynamic range and were statistically validated (Wilcoxon test, alpha = 0.05). Among them, the L-malic acid was identified by the *CSI:finger* algorithm with a Tanimoto score of 1. As expected, the abundance of L-malic acid was significantly higher in wines made with grape juices supplemented with L-malic acid (Figure 5A). The second panel of the figure illustrates the effect of the yeast background: wines made with *acidic* strains being more concentrated in malic acid than the others (Figure 5B).

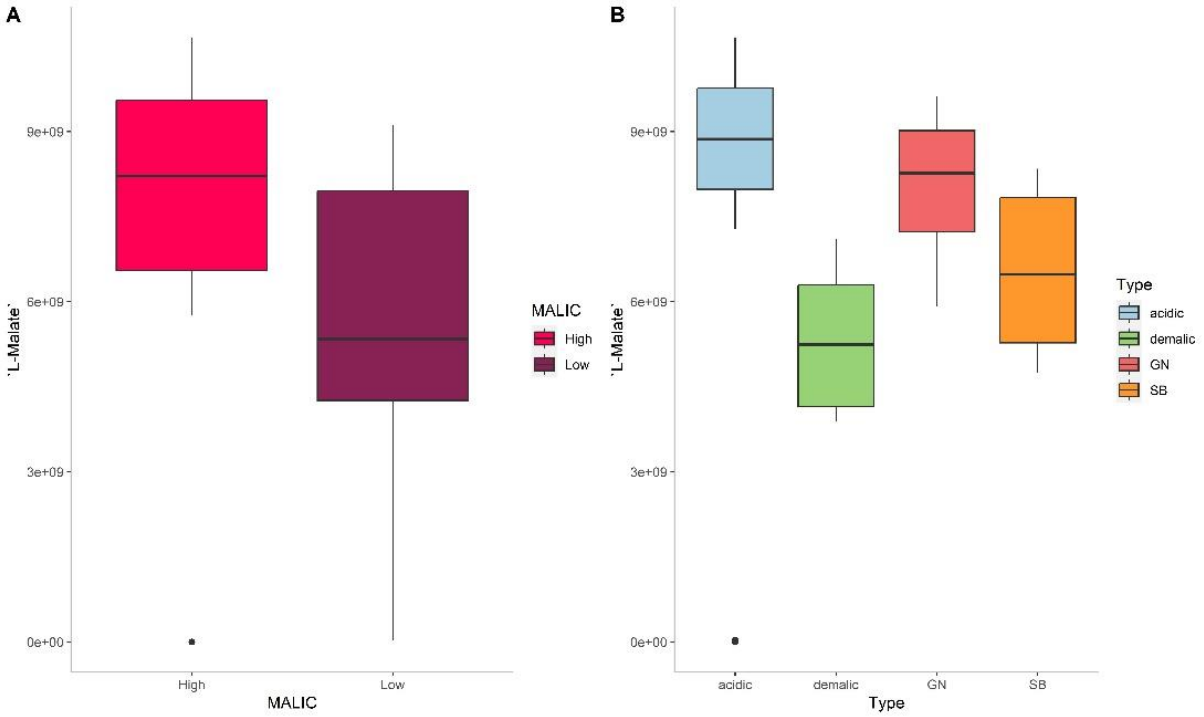


Figure 5: Distribution on the L-malic acid abundance detected in the M21 and SB16 matrices. The abundance was presented represent the area of the peak. **Panel A.** Distribution of abundance according to the low and high malic acid supplementation. **Panel B.** Distribution of abundance according to the fermenting strain.

Interestingly, many metabolic features were statistically different according to the nature of the fermenting yeast. The yeast background impacted 306 and 225 metabolic features for the SB16 and M21 grape juice respectively (Table 4). As demonstrated for the previous batch analyzed, the proportion of compounds that were captured by the three statistical analyses are relatively high (30 and 25%). These captured compounds constitute a robust list of candidates to be explored.

Previous studies reported that the use of selected wine yeasts can drastically modify the wine pH at the end of the alcoholic fermentation (Vion *et al*., 2022, 2021). In the present study, we analyzed wines made with such opposite strains. The difference of wine pH observed between the *acidic* and *demalic* groups (Table 1) may modify indirectly the equilibrium of many protonated molecules and could, in turn, modify the concentration of many wine metabolites. Since a large number of metabolites significantly differ according to the strain inoculated, it is necessary to understand if such differences are due to their intrinsic metabolome or to chemical consequences of their pH management.

By supplementing two independent matrices with malic acid (mean : +3,5 g/L) we have the possibility to discriminate the chemical effect of wine pH to the yeast background effect. As shown in Table 4, a very small number of molecules is significantly different between wines made from low and high malic acid content. Among those molecules, we found the malic acid itself that act as an internal control (Figure 5). This result suggests that most of the variability found between yeast strains is due to intrinsic metabolomic differences that remain to be clarified at this stage.

3.5. Semantic analysis of molecular signatures discriminating *acidic* and *demalic* strains

The analysis of 216 wines by LC-HRMS in three independent batches allowed the identification of 2445 metabolic features with a resolved chemical formula. These metabolic features received a class annotation among the 14 major chemical categories proposed by the CANOPUS algorithm. In the previous sections, multivariate analyses demonstrated that the fermenting yeast has a strong impact on the variability of a subset of 770 metabolites that can be considered as possible discriminant markers of the strain origin (239, 306, and 225 for batches 1, 2, and 3, respectively). These metabolic features are listed in Tables S4. The class assignation of these metabolites was compared to the class distribution of the 2445 metabolic features annotated. The relative proportion for each class indicated that strain-discriminant compounds are significantly enriched in four main chemical classes (χ^2 test, alpha = 0.01). As shown in Figure 6, the proportion of strain-specific metabolic features belonging to pyrimidine nucleotides (12/13), hydroxy acids and derivates (37/70), Coumarins and derivates (21/38) as well as carboxylic acids and derivates (141/366) are higher that the proportion expected by chance (770/2445).

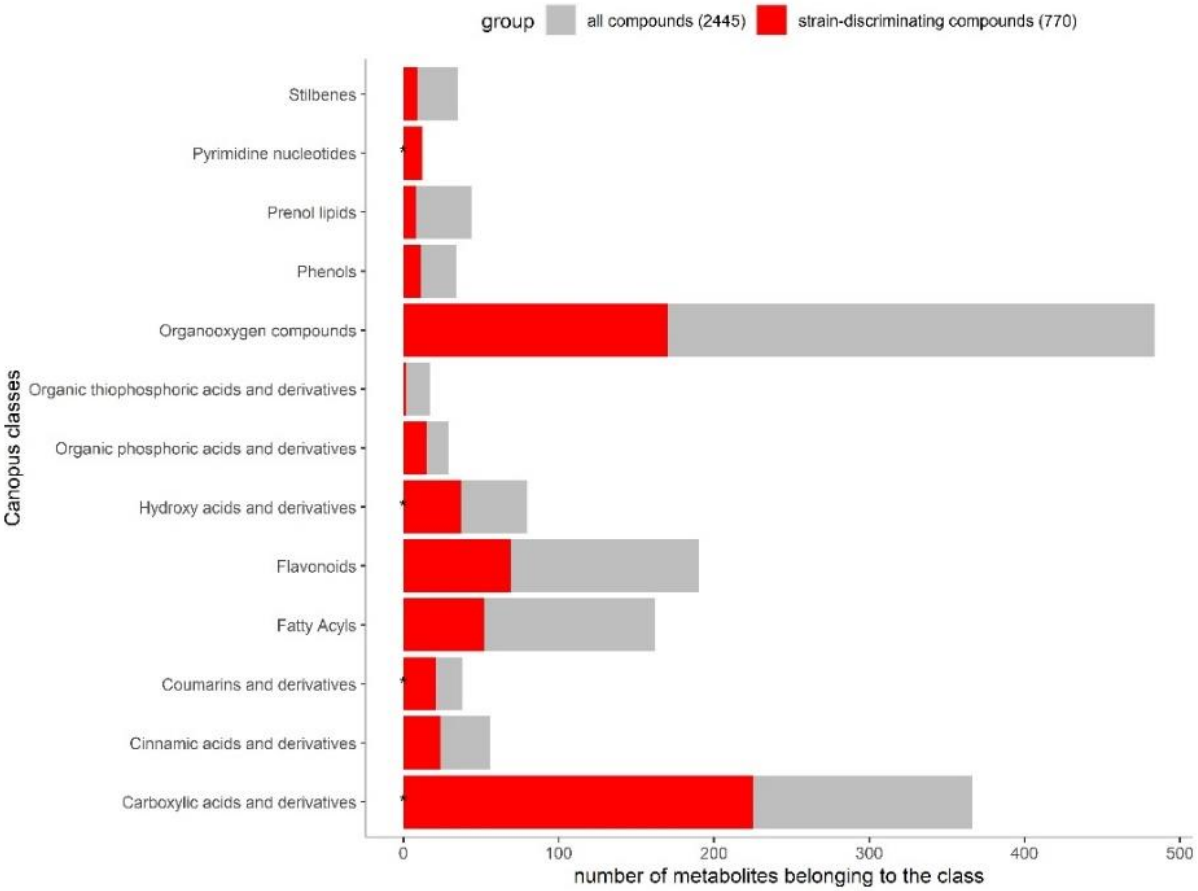


Figure 6: Relative proportions of the 770 molecules able to discriminate the yeast strains among the 14 main chemical classes attributed by CANOPUS analysis. Molecules belonging to four chemical classes were significantly enriched: Carboxylic acids and derivates, Coumarins and derivates, Hydroxy acids and derivates and Pyrimidine nucleotides. The enrichment was test using a $\chi 2$ test, alpha=0.01 using as control the relative proportion found in the 2445 compounds annotated.

In this section, we used the bioinformatic facilities proposed by the SIRIUS software suite to analyze as a whole the semantic annotation of 2445 compounds that were automatically annotated by

CANOPUS analysis. These compounds were detected in one or more LC-HRMS batches and encompassed all the wine metabolic features considered in this study. As a first attempt, we applied a basic semantic analysis based on the class annotation performed by CANOPUS. This analysis demonstrated that some chemical classes are statistically more represented than expected by chance. These preliminary analyses should be completed by other datamining procedures taking into account other annotation features proposed by SIRIUS (KEGG number, size of molecules, molecular network building…).

3.6. Identification of a short list of metabolic compounds discriminating *acidic* and *demalic* strains

The list of the 770 compounds associated to the strain origin was subjected to a last filtering analysis taking into account the compounds' molecular structure. Among these compounds, 545 have a unique molecular formula and correspond to distinct putative molecules according to their mass spectra. 218 of these unique features received a structure annotation with a Tanimoto score higher than 0.6 (*CSI:fingerID* algorithm). In order to narrow specifically compounds that discriminate wines made with *demalic* vs *acidic* strains, we computed their average log2 ratio of abundance and tested a possible significative difference between the two groups. A few numbers of molecules (12) were detected in the three LC-HRMS batches, reinforcing their chance to be key discriminant compound between the two groups (Table 5). The relative effect of these molecules was figured out on a volcano plot (Figure 7A).

Table 5: List of compounds significantly different between *acidic* and *demalic* strains and found in the three LM-HRMS datasets.

Name	m.z	retention time (min)	Formula	KEGGtag	Tanimoto score	CANOPUS class	mean demalic	mean acidic	Wilcoxon test (pvalue)	log2 ratio
5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one	481.099	1.63	C21H22O13	NA	65%	Flavonoids	1,55E+06	2,12E+06	1,49E-07	5,88E-01
b-glucogallin	331.067	1.92	C13H16O10	C01158	77%	Tannins	1,73E+07	1,17E+07	3,48E-08	6,25E-01
(+)-Ampelopsin F	453.134	5.69	C28H22O6	NA	68%	Stilbenes	6,53E+05	1,02E+06	4,77E-10	6,49E-01
Afalanina	206.081	5.15	C11H13NO3	C03519 C056	80%	Carboxylic acids and derivatives	8,84E+06	6,00E+06	8,17E-04	7,11E-01
2-hydroxyoctanedioic Acid	189.076	4.30	C8H14O5	NA	62%	Organooxygen compounds	1,92E+06	3,20E+06	2,32E-09	7,37E-01
coumarate	163.039	3.52	C9H8O3	C06738 C008	66%	Cinnamic acids and derivatives	1,25E+06	2,19E+06	3,24E-06	8,08E-01
2-methylcitrate	205.034	0.86	C7H10O7	C02225	73%	Carboxylic acids and derivatives	7,34E+06	1,02E+07	1,79E-02	9,57E-01
CompX	[redacted]				64%	[redacted]	3,41E+07	1,45E+07	9,33E-11	1,23E+00
2,3-dihydroxy-4-methoxy-4-oxobutanoate	163.023	0.76	C5H8O6	NA	70%	Fatty Acyls	1,12E+06	2,89E+06	1,54E-06	1,36E+00
Quertine	301.035	5.81	C15H10O7	C00389	94%	Flavonoids	3,83E+06	5,92E+06	1,74E-03	1,61E+00
uridylate	323.028	0.55	C9H13N2O9F	C00105	99%	Pyrimidine nucleotides	7,70E+06	6,95E+06	1,68E-04	2,41E+00
glycylisoleucine	187.108	2.70	C8H16N2O3	NA	65%	Carboxylic acids and derivatives	2,00E+05	1,89E+06	3,34E-10	3,34E+00

One of the most impacting compounds was named CompX and was overrepresented in all the wines made by the *demalic* strains respect to those made by the *acidic* ones (Figure 7 panel B). In contrast, wines made with the SB and GN strains are not significantly different for this compound. For confidentiality, we cannot reveal at the moment the identity of this compound.

In this section, we demonstrated that the combination of different datasets is useful for narrowing down the research of impacting compounds. Indeed, the final selection of metabolic compounds that

were found significantly different in 12 matrices increase the chance to study relevant molecular markers. It is noteworthy that the samples analyzed here are constituted by pool of wines made from different strains sharing the same phenotypic specificities. Despite the increased metabolic noise generated by this pool strategy, many discriminating metabolic features emerged from our analysis. This reinforces the idea that real and strong metabolomic differences exist between *acidic* and *demalic* strains. These differences are not due to the wine pH values, but are the consequences of their metabolic activity and specificity.

This paragraph also demonstrate that the SIRIUS software suite was efficient for identifying the chemical structure of 218 molecules through the *CSI:fingerID* algorithm. Although these identifications were not confirmed by additional mass spectra for all the metabolites of Table 5, the annotation proposed may be useful for getting new molecular insights. This is the case of the CompX, which is a promising molecule that discriminate specifically *acidic* and *demalic* strains. The injection of the pure molecule of a mass spectrometer confirmed the identity of the ion detected (same m.z and same retention time, data not shown).

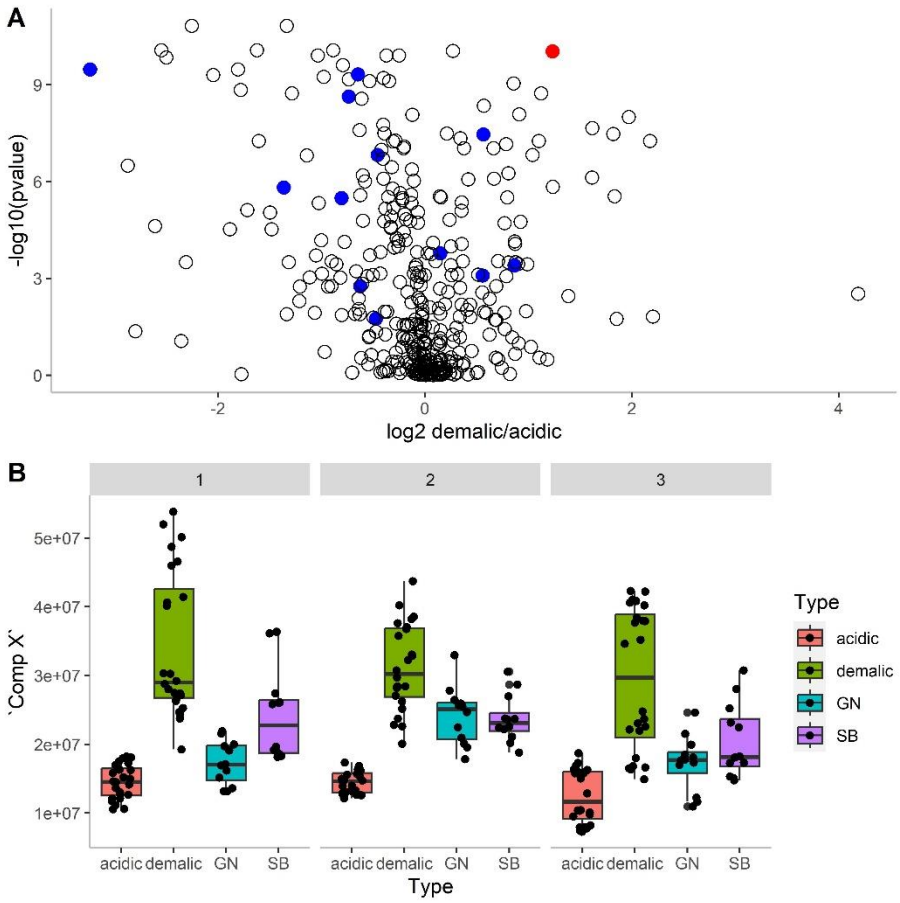


Figure 7: Panel A. Log2 ratio of the abundance of the 218 compounds annotated by CSI:fingerID algorithm (Tanimoto score > 0.6). The colored dots represent significative molecules that have been detected in the three LC-HRMS batches. The red dot represents the CompX, a molecule that is particularly enriched in the wines made with the *demalic* strains. **Panel B.** distribution of the abundance of CompX in the three LC-HRMS batches of this study, each dot represents a wine sample.

3.7. Experimental validation that the CompX is linked to malic acid metabolism

In this section, we focused on the CompX. This compound originates from the grape juice (personal communication) and was differently quantified at the end of alcoholic fermentation between the *acidic* and *demalic* groups of wines. Indeed, wines produced with *demalic* strains were particularly enriched in CompX regarding wines produced with *acidic* strains. To investigate its effect on malic acid modulation, alcoholic fermentations were carried out with two extreme strains, FMGS_889 (*demalic*) and AC1_191 (*acidic*) and two parental strains, SB, and GN. The SB strain is considered as a *demalic* strain and GN as an *acidic* strain, as they were reported as the two most extreme individuals among the industrial strains. The alcoholic fermentations were carried out in synthetic grape juice supplemented with increasing amount of CompX. The kinetics monitoring and enzymatic assays of several compounds were carried out with a well-established methodology at the laboratory (Peltier *et al.*, 2018).

The Figure 8 shows that for the two *acidic* strains, the production of malic acid steady increased with the elevation of CompX concentration with significant differences between extreme concentration (Kruskal test, p-value < 0.05). Interestingly the production of malic acid by the two *demalic* strains was not boosted by the increasing concentration of CompX.

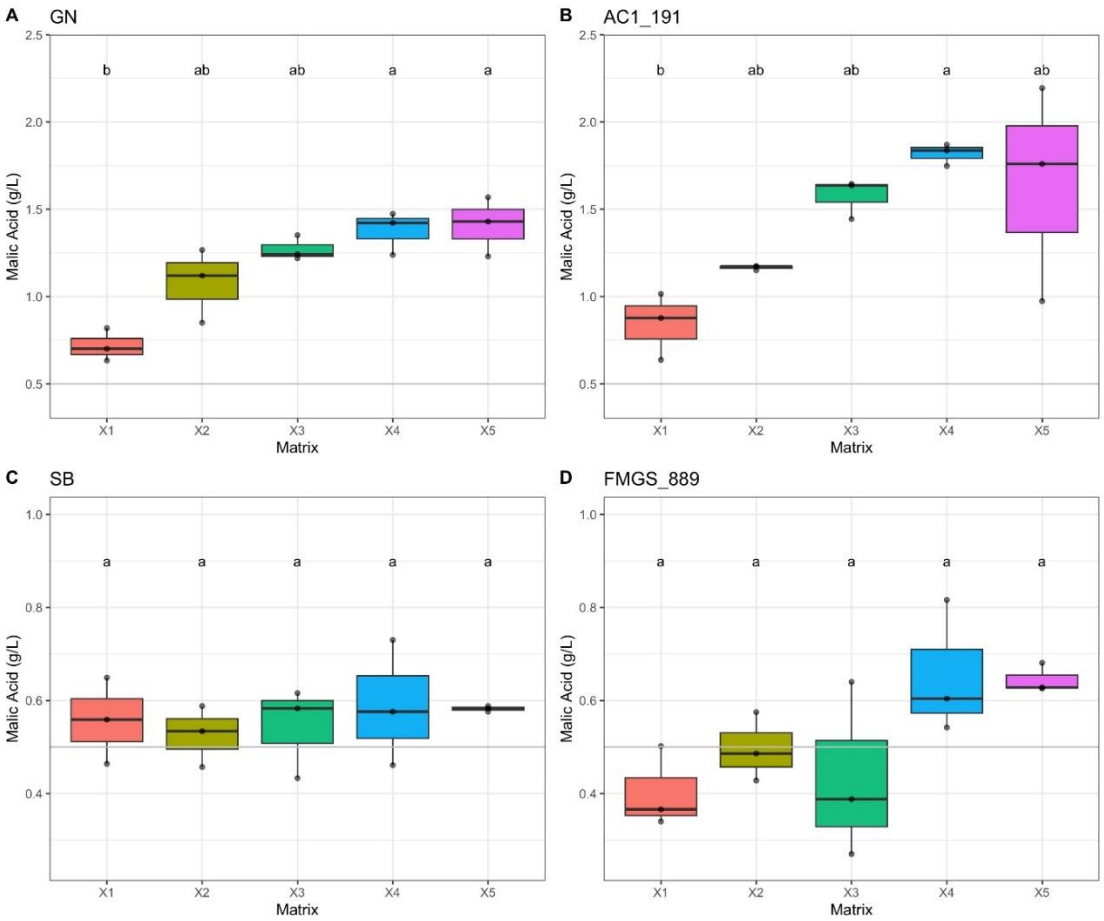


Figure 8: Malic acid concentration per strain as function of the concentration of the CompX. The grey horizontal line shows the initial concentration of malic acid in the medium. Different letters indicate significant differences between groups (Kruskal-Wallis' test, significance level α=0.05)

The table 6 contains the values of concentrations of other compounds (acetic and succinic acids) and some kinetic information for the two groups of strains according to the concentration of CompX. Acetic acid production was not affected by the increase in CompX except when this compound is absent from the medium. Interestingly, succinic acid production, which has been shown to be correlated with malate production in previous studies (Vion *et al.*, 2023, 2022) does not increase for the two *acidic* strains. For these strains the succinic acid production is steady while it increased with the amount of CompX for the two *demalic* strains. Finally, lag phase and T80 were not affected by the CompX concentration, except for the condition without the CompX.

Table 6: Metabolites concentrations and kinetic parameters for *acidic* and *demalic* strains as function of the increasing concentration in the X compound

		X1	X2	X3	X4	X5
Acetic acid (g/L)	**ACIDIC**	0.9	0.1	0.1	0.1	0.1
	DEMALIC	1	0.4	0.2	0.2	0.2
Succinic acid (g/L)	**ACIDIC**	0.4	0.5	0.4	0.4	0.5
	DEMALIC	0.5	0.8	1.3	1.1	1
lag phase (h)	**ACIDIC**	66	9	9	9	9
	DEMALIC	39	11	12	12	12
T80 (h)	**ACIDIC**	370	250	284	303	293
	DEMALIC	269	148	269	280	213

The CompX is differently assimilated by *acidic* and *demalic* strains during alcoholic fermentation. The Figure 7B *showed* that acidic strains consumed a larger amount of this compound compared to *demalic* strains. In addition, the Figure 8 demonstrated that the incorporation of this compound boosted the production of malic acid for a representative member of *acidic* strains (AC1_191). These findings revealed an interesting metabolic dependence that remain to be clarified. In a previous study, we observed that some grape juices with the same pH of amount of initial malic acid led to different levels of malic acid modulation by the same strains (Vion *et al.*, 2022). The amount of CompX in those musts is for the moment unknown but could be an interesting lead to explain the differences of MAC%.

Conclusion

In this study, we used untargeted metabolomic and multivariate analysis to discriminate wines produced with different strains of *S. cerevisiae* and grape juices. The implementation of SIRIUS tools for the LC-HRMS analysis and the development of R code for automatically manage large dataset provide an efficient platform for achieved metabolomic studies. The integration of different univariate and multivariate statistical tools as well as analysis of different wines allow to discriminate both biotic and abiotic factors shaping the metabolomic landscape of 216 wine samples. Our analyses showed that wines produced from different grape juices are difficult to compare due to the complexity of the matrix. However, wines produced from a same grape must with different strains revealed a handful of compounds discriminating the *acidic* and *demalic* groups of strains. The annotation of compounds was carried out with SIRIUS software and 28% of the identified compounds were given a putative name and class. A particular compound found in grape juices structured the two group of strains, revealing differences in its uptake between *acidic* and *demalic* strains. A focus was made on this compound to investigate specifically the consequences of its variation in supplemented synthetic media showing significative differences between *acidic* and *demalic* strains. The increase of its availability in a synthetic grape juice induced a higher production of malic acid for two malic producing strains.

Supplementary files

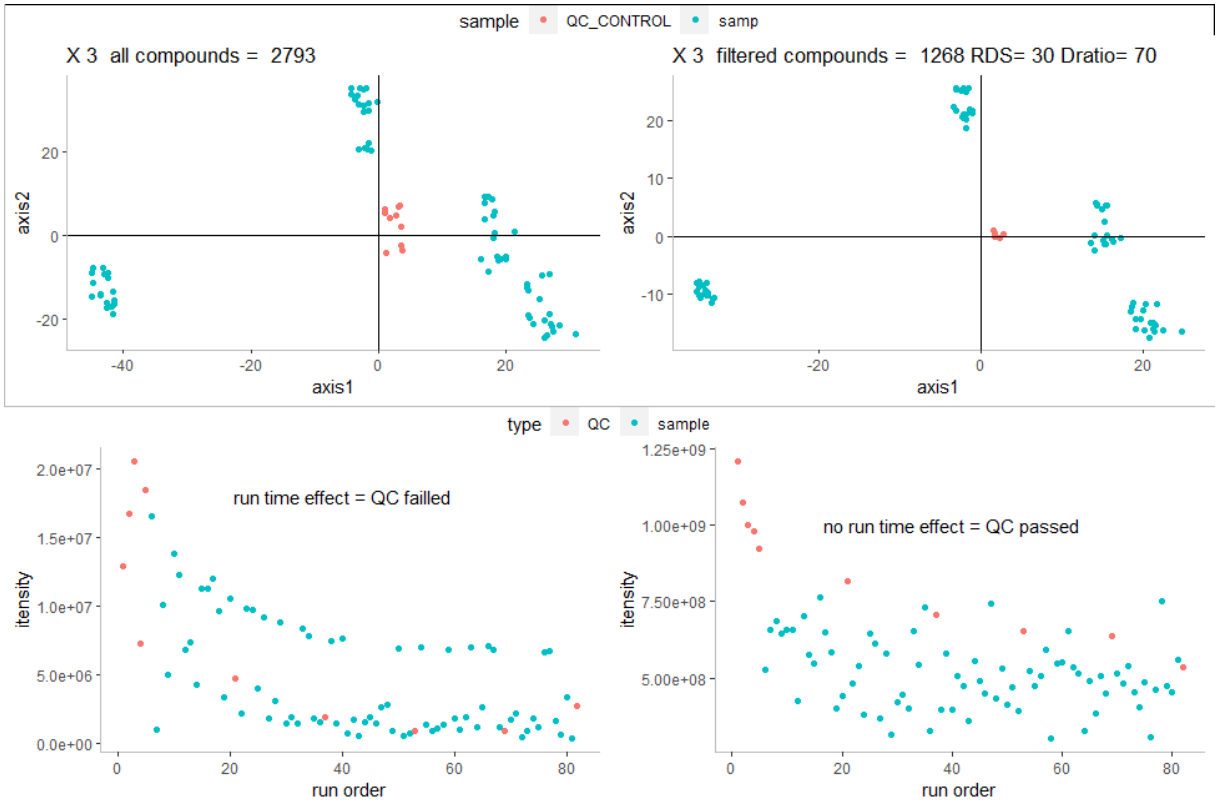


Figure S1: Overview of quality control procedure. **Upper panels.** Samples and QC samples were analyzed by on a PCA plot to assess the stability of the analytical system. Metabolic features with an inadequate reproducibility (relative standard deviation [RSD] > 30 % in QC samples) or present in < 70 % of QC samples were removed. **Lower panels.** A linear model *intensity=f(run order)* was applied to each metabolic features in order to detect any order run effect. Molecules with significant linear regression were discarded ($\alpha = 0.05$).

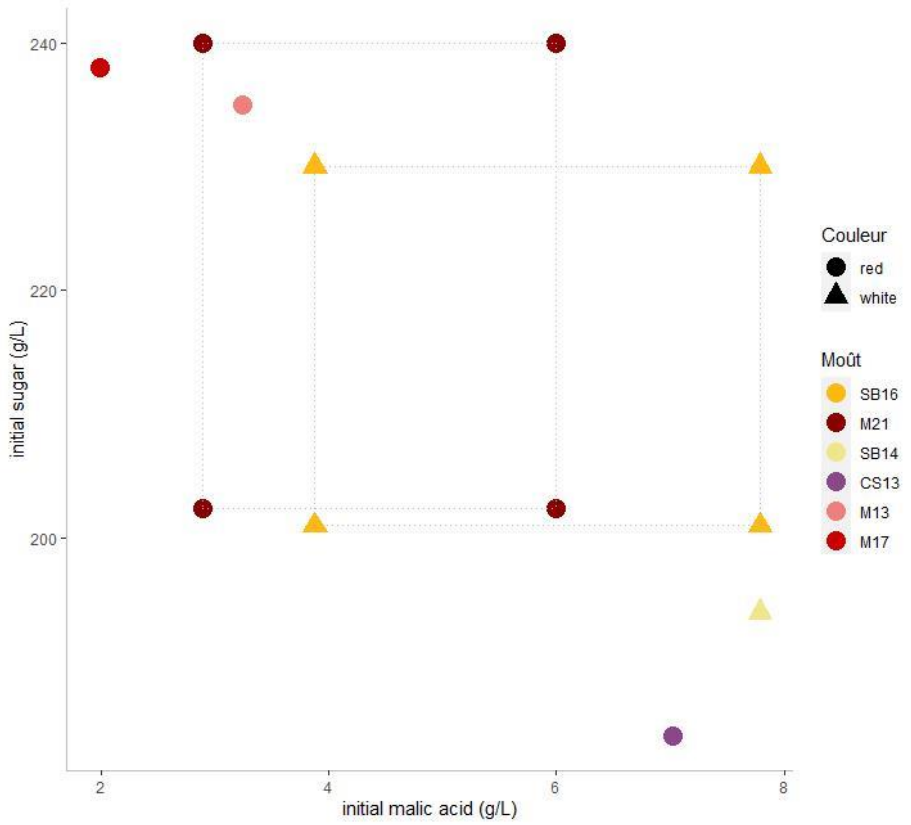


Figure S2: Sugar and malic acid composition of the twelves grape juices used in the experimental design.

File 1 : Pools of wines prepared for the three batches (available online at https://filesender.renater.fr/?s=download&token=09d0622f-be77-47c8-bac0-b3a6358d3fc9)

References

Ashaolu, T.J., 2019. A review on selection of fermentative microorganisms for functional foods and beverages: the production and future perspectives. Int. J. Food Sci. Technol. 54, 2511–2519. https://doi.org/10.1111/ijfs.14181

Aydoğan, C., 2020. Recent advances and applications in LC-HRMS for food and plant natural products: a critical review. Anal. Bioanal. Chem. 412, 1973–1991. https://doi.org/10.1007/s00216-019-02328-6

Babini, E., Tagliazucchi, D., Martini, S., Dei Più, L., Gianotti, A., 2017. LC-ESI-QTOF-MS identification of novel antioxidant peptides obtained by enzymatic and microbial hydrolysis of vegetable proteins. Food Chem. 228, 186–196. https://doi.org/https://doi.org/10.1016/j.foodchem.2017.01.143

Broadhurst, D., Goodacre, R., Reinke, S.N., Kuligowski, J., Wilson, I.D., Lewis, M.R., Dunn, W.B., 2018. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics 14, 1–17. https://doi.org/10.1007/s11306-018-1367-3

Dührkop, K., Fleischauer, M., Ludwig, M., Aksenov, A.A., Melnik, A. V, Meusel, M., Dorrestein, P.C., Rousu, J., Böcker, S., 2019. SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. Nat. Methods 16, 299–302. https://doi.org/10.1038/s41592-019-0344-8

Dührkop, K., Nothias, L.-F., Fleischauer, M., Reher, R., Ludwig, M., Hoffmann, M.A., Petras, D., Gerwick, W.H., Rousu, J., Dorrestein, P.C., Böcker, S., 2021. Systematic classification of unknown metabolites using high-resolution fragmentation mass spectra. Nat. Biotechnol. 39, 462–471. https://doi.org/10.1038/s41587-020-0740-8

Dührkop, K., Shen, H., Meusel, M., Rousu, J., Böcker, S., 2015. Searching molecular structure databases with tandem mass spectra using CSI:FingerID. Proc. Natl. Acad. Sci. U. S. A. 112, 12580–12585. https://doi.org/10.1073/pnas.1509788112

Farrés, M., Piña, B., Tauler, R., 2015. Chemometric evaluation of *Saccharomyces cerevisiae* metabolic profiles using LC–MS. Metabolomics 11, 210–224. https://doi.org/10.1007/s11306-014-0689-z

Fiehn, O., 2016. Metabolomics by Gas Chromatography–Mass Spectrometry: Combined Targeted and Untargeted Profiling. Curr. Protoc. Mol. Biol. 114, 30.4.1-30.4.32. https://doi.org/https://doi.org/10.1002/0471142727.mb3004s114

Gao, Y., Hou, L., Gao, J., Li, D., Tian, Z., Fan, B., Wang, F., Li, S., 2021. Metabolomics Approaches for the Comprehensive Evaluation of Fermented Foods: A Review. Foods 10, 2294. https://doi.org/10.3390/foods10102294

Jombart, T., Devillard, S., Balloux, F., 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet. 11, 94. https://doi.org/10.1186/1471-2156-11-94

Kim, S., Lee, D.Y., Wohlgemuth, G., Park, H.S., Fiehn, O., Kim, K.H., 2013. Evaluation and optimization of metabolome sample preparation methods for *Saccharomyces cerevisiae*. Anal. Chem. 85, 2169–2176. https://doi.org/10.1021/ac302881e

Lazar, A.G., Romanciuc, F., Socaciu, M.A., Socaciu, C., 2015. Bioinformatics Tools for Metabolomic Data Processing and Analysis Using Untargeted Liquid Chromatography Coupled With Mass Spectrometry. Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca, Anim. Sci. Biotechnol. 72, 103–115.

Lê Cao, K.-A., Boitard, S., Besse, P., 2011. Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems. BMC Bioinformatics 12, 253. https://doi.org/10.1186/1471-2105-12-253

Marshall, A.G., Hendrickson, C.L., 2008. High-Resolution Mass Spectrometers. Annu. Rev. Anal. Chem. 1, 579–599. https://doi.org/10.1146/annurev.anchem.1.031207.112945

Marshall, D.D., Powers, R., 2017. Beyond the paradigm: Combining mass spectrometry and nuclear magnetic resonance for metabolomics. Prog. Nucl. Magn. Reson. Spectrosc. 100, 1–16. https://doi.org/10.1016/j.pnmrs.2017.01.001

Mozzi, F., Ortiz, M.E., Bleckwedel, J., De Vuyst, L., Pescuma, M., 2013. Metabolomics as a tool for the comprehensive understanding of fermented and functional foods with lactic acid bacteria. Food Res. Int. 54, 1152–1161. https://doi.org/https://doi.org/10.1016/j.foodres.2012.11.010

Navarrete-Bolaños, J.L., 2012. Improving traditional fermented beverages: How to evolve from spontaneous to directed fermentation. Eng. Life Sci. 12, 410–418. https://doi.org/10.1002/elsc.201100128

Nissen, L., Bordoni, A., Gianotti, A., 2020. Shift of Volatile Organic Compounds (VOCs) in Gluten-Free Hemp-Enriched Sourdough Bread: A Metabolomic Approach. Nutrients. https://doi.org/10.3390/nu12041050

Peltier, E., Bernard, M., Trujillo, M., Barbe, J.-C., Prodhomme, D., Gibon, Y., Marullo, P., 2018. Wine yeast phenomics: A standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. PLoS One 1–23. https://doi.org/10.1371/journal.pone.0190094

Peltier, E., Vion, C., Abou Saada, O., Friedrich, A., Schacherer, J., Marullo, P., 2021. Flor Yeasts Rewire the Central Carbon Metabolism During Wine Alcoholic Fermentation. Front. Fungal Biol. 2. https://doi.org/10.3389/ffunb.2021.733513

R Development Core Team, 2016. R: A language and environment for statistical computing. 2, 1–12.

Reed, L.K., Baer, C.F., Edison, A.S., 2017. Considerations when choosing a genetic model organism for metabolomics studies. Curr. Opin. Chem. Biol. 36, 7–14. https://doi.org/10.1016/j.cbpa.2016.12.005

Sailwal, M., Das, A.J., Gazara, R.K., Dasgupta, D., Bhaskar, T., Hazra, S., Ghosh, D., 2020. Connecting the dots: Advances in modern metabolomics and its application in yeast system. Biotechnol. Adv. 44, 107616. https://doi.org/10.1016/j.biotechadv.2020.107616

Schmid, R., Heuckeroth, S., Korf, A., Smirnov, A., Myers, O., Dyrlund, T.S., Bushuiev, R., Murray, K.J., Hoffmann, N., Lu, M., Sarvepalli, A., Zhang, Z., Fleischauer, M., Dührkop, K., Wesner, M., Hoogstra, S.J., Rudt, E., Mokshyna, O., Brungs, C., Ponomarov, K., Mutabdžija, L., Damiani, T., Pudney, C.J., Earll, M., Helmer, P.O., Fallon, T.R., Schulze, T., Rivas-Ubach, A., Bilbao, A., Richter, H., Nothias, L.-F., Wang, M., Orešič, M., Weng, J.-K., Böcker, S., Jeibmann, A., Hayen, H., Karst, U., Dorrestein, P.C., Petras, D., Du, X., Pluskal, T., 2023. Integrative analysis of multimodal mass spectrometry data in MZmine 3. Nat. Biotechnol. https://doi.org/10.1038/s41587-023-01690-2

Sicard, D., Legras, J.L., 2011. Bread, beer and wine: Yeast domestication in the *Saccharomyces* sensu stricto complex. Comptes Rendus - Biol. 334, 229–236. https://doi.org/10.1016/j.crvi.2010.12.016

Tamang, J.P., Watanabe, K., Holzapfel, W.H., 2016. Review: Diversity of microorganisms in global fermented foods and beverages. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.00377

Van Leeuwen, C., Destrac-Irvine, A., Dubernet, M., Duchêne, E., Gowdy, M., Marguerit, E., Pieri, P., Parker, A., De Rességuier, L., Ollat, N., 2019. An update on the impact of climate change in viticulture and potential adaptations. Agronomy 9, 1–20. https://doi.org/10.3390/agronomy9090514

Verpoorte, R., Choi, Y.H., Mustafa, N.R., Kim, H.K., 2008. Metabolomics: Back to basics. Phytochem. Rev. 7, 525–537. https://doi.org/10.1007/s11101-008-9091-7

Vignoli, A., Ghini, V., Meoni, G., Licari, C., Takis, P.G., Tenori, L., Turano, P., Luchinat, C., 2019. High-Throughput Metabolomics by 1D NMR. Angew. Chemie - Int. Ed. 58, 968–994. https://doi.org/10.1002/anie.201804736

Vion, C., Le Mao, I., Yeramian, N., Da Costa, G., Richard, T., Marullo, P., 2023. Targeted 1H-NMR wine analyses revealed specific metabolome signatures of yeast populations belonging to the *Saccharomyces* genus. submitted.

Vion, C., Muro, M., Bernard, Margaux, Richard, B., Fautre, V., Yeramian, N., Masneuf-pomarède, I., Tempère, S., Marullo, P., 2022. New malic acid producer strains of *Saccharomyces cerevisiae* for preserving wine acidity during alcoholic fermentation. Food Microbiol. 112. https://doi.org/10.1016/j.fm.2022.104209

Vion, C., Peltier, E., Bernard, M., Muro, M., Marullo, P., 2021. Marker assisted selection of malic-consuming *Saccharomyces cerevisiae* strains for winemaking. Efficiency and limits of a qtl's driven breeding program. J. Fungi 7. https://doi.org/10.3390/jof7040304

Wolfender, J.-L., Nuzillard, J.-M., van der Hooft, J.J.J., Renault, J.-H., Bertrand, S., 2019. Accelerating Metabolite Identification in Natural Product Research: Toward an Ideal Combination of Liquid Chromatography–High-Resolution Tandem Mass Spectrometry and NMR Profiling, in Silico Databases, and Chemometrics. Anal. Chem. 91, 704–742. https://doi.org/10.1021/acs.analchem.8b05112

Conclusion du chapitre 4

Ce chapitre final proposait d'aller plus loin dans la caractérisation fine des souches acidifiantes et démaliquantes en utilisant un outil de métabolomique non ciblée. Ce travail collaboratif avec l'axe QIV de notre unité de recherche avait pour ambition de capter un maximum de molécules permettant de discriminer les deux groupes de souches phares de cette thèse. Des analyses statistiques multivariées ont été associées à l'utilisation de la spectrométrie de masse afin de pouvoir traiter de façon automatisée les très larges jeux de données qui en découlent.

Sur le plan purement pratique, de nombreux composés permettant de ségréger les deux groupes ont été trouvés et certains d'entre eux identifiés. L'identification d'un composé en particulier a attiré notre attention et son impact sur la production d'acide malique lors de la fermentation alcoolique a été testé dans un jus de raisin synthétique.

Dans le premier chapitre de cette thèse, nous avions évoqué des différences de *MAC%* pour de mêmes souches fermentant des moûts à pH et/ou concentration en acide initial similaires. D'autres facteurs abiotiques doivent donc avoir un impact. C'est certainement le cas du composé X. Un dosage de ce composé dans les moûts ayant été utilisés dans l'ensemble de cette thèse est à l'étude au laboratoire afin de valider ou non cette hypothèse.

Conclusion générale et perspectives

Conclusion générale et perspectives

Ce travail de thèse a permis d'étudier de façon approfondie la modulation de l'acidité des vins via l'utilisation des souches de *S. cerevisiae* ayant la capacité de consommer ou produire de l'acide malique lors de la fermentation alcoolique. Tout d'abord, des souches ayant une capacité de consommation ou production de l'acide malique extrême par rapport aux souches commerciales et existant naturellement ont été sélectionnées. Ces souches ont ensuite fait l'objet d'une caractérisation phénotypique et sensorielle et un premier essai à l'échelle du chai a été réalisé sur deux d'entre elles. La seconde partie de cette thèse est plus fondamentale et proposait de s'intéresser à la diversité métabolique des souches du genre *Saccharomyces* en lien avec l'acidité puis, plus précisément au métabolisme de l'acide malique lors de la fermentation alcoolique. Pour répondre aux différentes questions posées, plusieurs outils méthodologiques ont été mis en place en plus de ceux déjà disponibles au laboratoire : extraction de composés intracellulaires, dosage des métabolites par RMN ^{1}H au niveau intra-et extracellulaire. D'autres techniques ont également été utilisées grâce à des collaborations avec d'autres laboratoires : LC-MS/MS pour la détection d'acides organiques marqués au ^{13}C et GC-MS pour la détermination de l'enrichissement isotopique des acides aminés protéinogéniques. Enfin une collaboration avec l'équipe QIV de notre unité de recherche nous a permis d'accéder à des outils de métabolomique non ciblée pour une caractérisation encore plus fine des groupes de souches sélectionnées. Les principaux résultats peuvent être décrits selon trois axes.

1. Caractérisation des souches démaliquantes vs acidifiantes

Tout d'abord, la mise en place d'un programme de croisement basé sur la sélection assistée par marqueurs génétiques a permis d'améliorer un trait phénotypique complexe chez *S. cerevisiae* : la capacité de souches à consommer de l'acide malique lors de la fermentation alcoolique. Ces croisements ont permis de générer des souches ayant des valeurs phénotypiques dépassant celles des souches parentales avec des individus extrêmes capables de consommer jusqu'à 80% de l'acide malique des jus de raisin. Ces souches au comportement extrême sont statistiquement enrichies en allèles caractérisés comme induisant une consommation d'acide malique. Cependant, la pénétrance des QTL est incomplète, du fait que ces derniers ne vont pas tous avoir le même impact sur le trait étudié. De plus, certains QTL ne s'expriment pas.

D'un point de vue pratique, il a été montré qu'une surconsommation d'acide malique n'impacte pas négativement la fermentation malolactique. En effet, une teneur plus faible en acide malique n'impacte pas la phase de latence des bactéries lactiques et ces dernières terminent la FML en moins de temps du fait qu'elles ont moins de substrat à consommer.

La sélection de souches au caractère acidifiant s'est montrée particulièrement intéressante pour palier à la chute importante de l'acidité des matrices œnologiques.

La caractérisation fine de ces souches extrêmes visait à bien comprendre l'effet du milieu dans lequel elles sont utilisées. Elle a également permis d'étudier les conséquences de l'acidification sur les vins produits. Il en ressort que les souches démaliquantes sont très robustes à l'environnement tandis que les souches acidifiantes sont davantage impactées par la nature et les propriétés physico-chimiques des moûts. Ces dernières sont particulièrement acidifiantes dans les jus de raisin à forte teneur en sucre et faible teneur en acide malique. Ce type de matrice « chaude » est de plus en plus fréquent du fait des hausses des températures et l'utilisation de souches acidifiantes pourrait permettre de participer à la préservation de la fraîcheur des vins produits. Les défis imposés à la vigne et aux viticulteurs par le changement climatique ne pourront cependant pas être résolus par la seule adaptation du levurage mais plutôt par une combinaison de pratiques : utilisation de cépages plus tardifs et donc moins sensibles aux hausses brutales des température, pratiques viticoles différentes (tressage de la vigne, agroforesterie afin de restaurer la biodiversité), et des techniques de vinification faisant intervenir des levains adaptés à la récolte de l'année.

Enfin, les analyses sensorielles effectuées ont révélé que les vins produits à partir de souches acidifiantes ou démaliquantes se distinguent très bien à la dégustation, en blanc et en rouge même après la réalisation de la FML.

L'effet de la concentration initiale en acide malique dans le jus de raisin n'a pas pu être clairement dissocié de l'effet du pH initial. En effet, ces deux facteurs sont très intriqués puisque la concentration en acide malique est fortement corrélée au pH du moût. De ce fait, il est difficile de distinguer si les levures répondent différemment au pH ou à la concentration d'acide malique en elle-même. Des expériences de fermentation en tamponnant le milieu afin de maintenir un pH constant tout en testant différentes concentrations d'acide malique ont été mises en place au laboratoire mais n'ont pas donné de résultats concluants à ce jour.

2. Métabolisme de l'acide malique

Une partie plus fondamentale de cette thèse s'est intéressée au métabolisme carboné central des levures du genre *Saccharomyces*. Tout d'abord, un paysage phénotypique d'une cinquantaine de souches en lien avec l'acidité a été proposé. Il a permis de démontrer l'existence de métabolites marqueurs de différentes espèces (*S. cerevisiae* vs *S. uvarum*) et populations (levures de voiles vs levures de vin). La question de ces différences entre populations a été poussée au niveau intracellulaire entre les levures de voile et de vin. Il en ressort que les levures de voiles sont plus concentrées en métabolites intracellulaires que les levures de vin en phase de croissance. Cette augmentation de la charge métabolique est corrélée à la vitesse spécifique de croissance des souches sur la première moitié de la fermentation.

Dans un second temps, la question des voies métaboliques de l'acide malique a été étudiée grâce à une expérience de filiation isotopique utilisant de l'acide malique marqué au ^{13}C. Cette expérience a mis en jeu plusieurs collaborateurs scientifiques permettant de générer un large jeu de donnée.

Grâce à cette expérience, deux voies distinctes de consommation et de production de l'acide malique lors de la fermentation alcoolique ont été mises en évidence. Des hypothèses ont également été formulées quant à la voie de synthèse de l'acide malique *de novo*. La voie du cycle de glyoxylate est privilégiée jusqu'à environ 60 % de la fermentation alcoolique. Cette voie est également la source de production de succinate et α-kétoglutarate. En fin de fermentation, la synthèse d'acide malique serait plutôt générée par la conversion d'oxaloacétate en malate par l'enzyme Mdh2p dans le cytosol. A ce stade, les acides succinique et α-kétoglutarique ne s'accumulent plus dans le milieu extracellulaire en fin de fermentation. Enfin, l'acide malique incorporé dans la cellule a plusieurs devenirs. Il sert à la biosynthèse des certains acides aminés lors de la phase de croissance. Ensuite une partie de l'acide malique entre la mitochondrie et est converti en pyruvate par l'action de l'enzyme Mae1p lors de la fermentation malo-éthanolique.

Des perspectives de travail sur le plan fonctionnel sont à l'étude. En effet, la construction de mutants pourrait permettre de valider certaines hypothèses quant aux voies métaboliques empruntées par l'acide malique. Par exemple, un mutant délétère *MAE1* ne serait pas capable de réaliser la fermentation malo-éthanolique. L'intérêt de ce mutant serait de vérifier l'absence d'alpha-kétoglutarate marqué sur 5 carbones et ainsi confirmer le rôle de l'enzyme mitochondriale Mae1p dans la dégradation d'une partie de l'acide malique.

A ce jour, une souche acidifiante possédant l'allèle *MAE1* d'une souche démaliquante a été construite au laboratoire dans le cadre du stage de Master 2 de Faustine Rose. Un essai de fermentation avec la souche acidifiante et son mutant a été réalisé dans trois milieux synthétiques ayant des concentrations croissantes en acide malique. Les premiers résultats montrent une différence significative de concentration finale en acide malique entre la souche et son mutant dans les trois milieux, appuyant l'hypothèse de l'impact de ce gène sur la consommation d'acide malique (Figure 16). D'autres mutants intégrant un ou plusieurs allèles précédemment identifiés comme impactant la concentration en acide malique sont en cours de construction par Margaux Bernard et Faustine Rose. Ils seront ensuite testés en fermentations dans différent milieux.

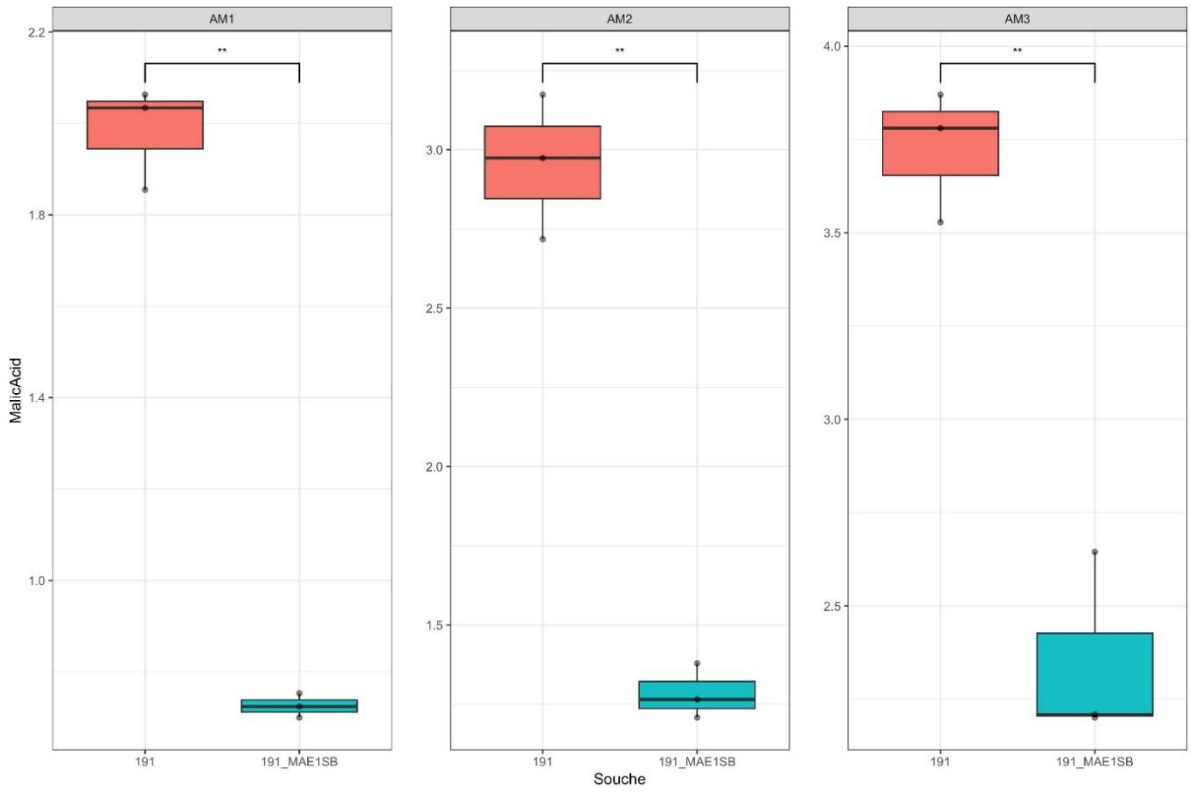


Figure 16: Concentration en acide malique (g/L) par souche (acidifiante en rouge, mutant en bleu) pour trois milieux ayant des concentrations croissantes d'acide malique initial.

La condensation d'oxaloacétate et acetyl-CoA en citrate peut être cytosolique ou mitochondriale. La distinction des voies cytosolique (cycle du glyoxylate) et mitochondriale (cycle du TCA) pour la production de succinate et d'α-kétoglutarate n'a pas été élucidée. Dans notre étude, seul l'α-kétoglutarate marqué sur 5 carbones est un marqueur de la voie mitochondriale. En effet, l'α-kétoglutarate marqué sur 3 carbones peut provenir des deux compartiments.

Cette étude des voies métaboliques a également soulevé la question du transport de l'acide malique dans et hors de la cellule. En effet, l'acide malique est décrit par Salmon (1987) comme entrant par diffusion passive dans la cellule. Cependant la différence d'efficacité de consommation entre les deux souches étudiées pose la question de l'implication d'un transport actif. De plus la sortie de l'acide malique dans le milieu extracellulaire se fait contre un gradient de concentration ce qui nécessite pour la cellule une grande dépense d'énergie. Ces observations suggèrent l'existence d'un transport actif ou facilité, cependant, à notre connaissance, aucune étude suggère l'existence d'un tel transporteur.

Enfin, l'activité enzymatique des principales enzymes d'intérêt pourrait être mesurée chez des souches appartenant aux groupes *acidic* et *demalic*. En effet, des différences d'activité de certaines enzymes clés pourraient permettre d'expliquer en partie des différences de modulation de la concentration en acide malique. Cette étude est l'une des attentes du projet MEGA-VIP et sera réalisée avec l'aide Cécile Miot-Sertier.

3. Techniques de métabolomique employées

D'un point de vue méthodologique, des techniques d'analyses ont été mises en place pour caractériser les métabolites extra- et intracellulaires par RMN du proton. Tout d'abord les principaux métabolites des vins produits ont été dosés par RMN ^{1}H en se basant sur la méthode disponible au laboratoire. Ces analyses ont permis de doser et quantifier 15 composés du vin de façon simultanée sur un échantillon. Ensuite, une méthode simple d'extraction des métabolites intracellulaires des levures a été mise au point. La méthode de dosage des composés extracellulaires a été adaptée pour le dosage de composés intracellulaires. 21 composés ont été identifiés et quantifiés.

La RMN du proton est-elle la technique la plus adaptée pour l'analyse des métabolites du vin et métabolites intracellulaire des levures ?

Des dosages enzymatiques sont effectués en routine au laboratoire. Ces derniers permettent de doser un composé sur un grand nombre d'échantillons grâce à une plateforme robotisée (7 composés sont dosés en routine). L'utilisation de la RMN a permis d'avoir accès à plusieurs composés non regardé avec les dosages enzymatiques traditionnels. Cependant, les échantillons doivent être passés un par un ce qui ne permet pas une analyse haut débit.

Concernant les métabolites intracellulaires, la RMN a permis d'accéder à un nombre important de composés qui ont été quantifiés. Cependant les concentrations de ces composés sont parfois très faibles et les limites de détection peuvent être atteintes. La spectrométrie de masse permet de détecter des concentrations plus faibles.

La RMN 2D (^{1}H-^{13}C)-HSQC a été intégrée dans l'étude des voies métaboliques de l'acide malique (chapitre 3). Avec cette méthode, l'identification de composés marqués appartenant à plusieurs familles chimiques différentes a été possible au niveau extra- et intracellulaire. Cependant, les limites d'utilisation de la RMN 2D en métabolomique résident dans le temps d'analyse des échantillons. En effet, 17h étaient nécessaires par échantillon pour les dosages intracellulaires et 8h par échantillon pour les dosages extracellulaires. Ce temps d'analyse comprend un spectre proton, suivi d'une HSQC et d'un second spectre proton visant à vérifier que l'échantillon ne s'est pas dégradé du fait du long temps d'analyse. Au total, 48 échantillons ont été analysés au niveau extra- et intracellulaire et des concentrations relatives de composés marqués ont été obtenues. Cet outil a permis d'accéder à l'information du marquage de certains métaboliques qui n'étaient pas observés avec les méthodologies de spectrométrie de masse. De plus, cette méthode pourrait être optimisée en utilisant par exemple une sonde à plus forte résolution pour les noyaux de ^{13}C.

L'ensemble de ces techniques de métabolomiques a été utilisé de façon ciblée pour rechercher des métabolites connus dans les matrices fermentées mais leur utilisation en mode « non ciblé » présente

également des avantages. C'est ce qui a été réalisé dans le chapitre 4 de cette thèse, dans lequel la LC-HRMS a été employée comme technique de métabolomique non ciblée. Un ensemble de molécules capables de discriminer des vins produits avec deux groupes de souches (acidifiantes vs démaliquantes) a été identifié L'analyse non ciblée de plusieurs vins et l'application de plusieurs filtres statistiques a révélé l'existence de quelques composés particulièrement intéressants. Ces derniers ont fait l'objet d'une identification à l'aide du logiciel Sirius et d'ajouts dosés pour vérification. Cette analyse ne permet cependant de détecter que des molécules solubles. Des expériences préliminaires ont été réalisées avec une technique comparable (Comprehensive-GC) pour des composés volatiles en collaboration avec le Docteur Alexandre Pons. La figure 17 montre plusieurs composés qui se distinguent entre les deux groupes de souches. Ces derniers restent à être identifiés.

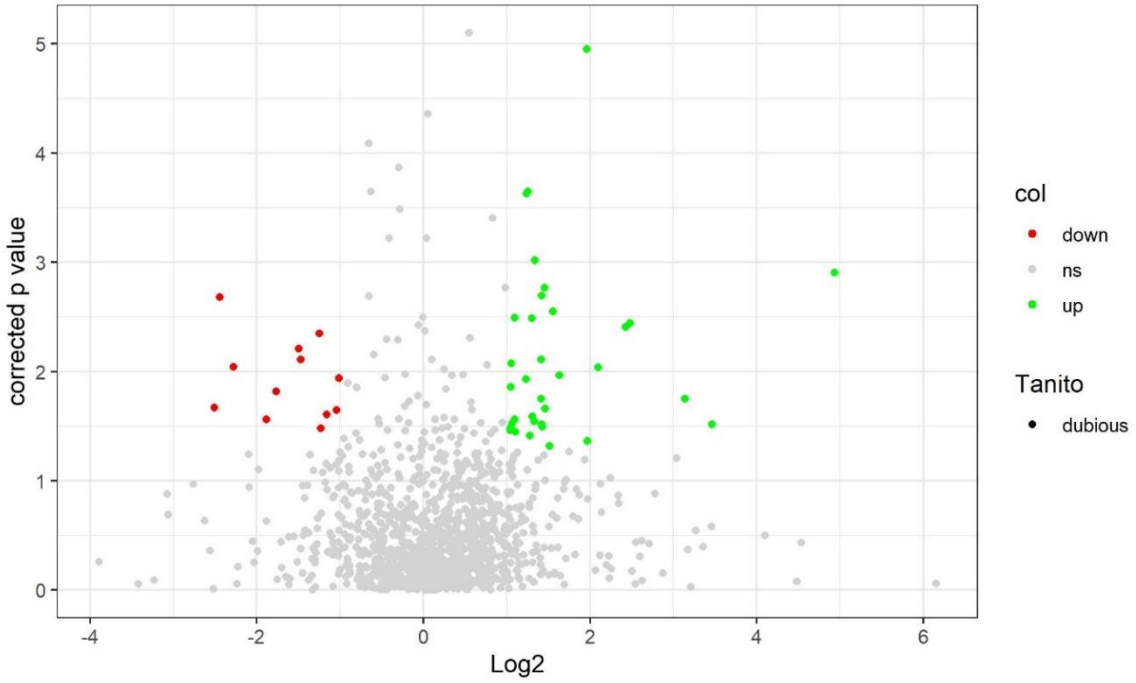


Figure 17: Volcano plot. Les points représentent les molécules détectées. Les composés en rouge sont plus abondants chez les souches démaliquantes et les verts chez les souches acidifiantes

Les composés colorés ici en rouge ont une concentration significativement plus faible chez les souches acidifiantes et les verts une concentration significativement plus forte. L'identification de ces composés devrait permettre de déterminer quelles molécules volatiles sont impactées par l'utilisation de souches acidifiantes ou démaliquantes lors de la fermentation alcoolique. Les composés volatiles ont souvent un impact organoleptique sur les vins produits et des analyses sensorielles pourraient découler de ces expérimentations.

Ce travail basé sur l'utilisation de plusieurs techniques de métabolomique a permis une caractérisation fine et complète de souches de levures aux propriétés technologiques et génétiques différentes

Références bibliographiques

Références bibliographiques

Aa, E., Townsend, J.P., Adams, R.I., Nielsen, K.M., Taylor, J.W., 2006. Population structure and gene evolution in *Saccharomyces cerevisiae*. FEMS Yeast Res. 6, 702–715. https://doi.org/10.1111/j.1567-1364.2006.00059.x

Albertin, W., Marullo, P., Aigle, M., Dillmann, C., de Vienne, D., Bely, M., Sicard, D., 2011. Population size drives industrial *Saccharomyces cerevisiae* alcoholic fermentation and is under genetic control. Appl. Environ. Microbiol. 77, 2772–2784. https://doi.org/10.1128/AEM.02547-10

Arroyo-López, F.N., Orlić, S., Querol, A., Barrio, E., 2009. Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid. Int. J. Food Microbiol. 131, 120–127. https://doi.org/10.1016/j.ijfoodmicro.2009.01.035

Barbe, J.C., Garbay, J., Tempère, S., 2021. The Sensory Space of Wines: From Concept to Evaluation and Description. A Review. Foods 10. https://doi.org/10.3390/foods10061424

Bauer, F.F., Pretorius, I.S., 2000. Yeast Stress Response and Fermentation Efficiency: How to Survive the Making of Wine - A Review. South African J. Enol. Vitic. 21, 27–51. https://doi.org/10.21548/21-1-3557

Camarasa, C., Sanchez, I., Brial, P., Bigey, F., Dequin, S., 2011. Phenotypic Landscape of *Saccharomyces cerevisiae* during Wine Fermentation : Evidence for Origin- Dependent Metabolic Traits. PLoS One 6. https://doi.org/10.1371/journal.pone.0025147

Carpena, M., Fraga-Corral, M., Otero, P., Nogueira, R.A., Garcia-Oliveira, P., Prieto, M.A., Simal-Gandara, J., 2021. Secondary aroma: Influence of wine microorganisms in their aroma profile. Foods 10, 1–26. https://doi.org/10.3390/foods10010051

Chollet, S., Valentin, D., Abdi, H., 2014. Free Sorting Task, in: Varela, P., Ares, G. (Eds.), Novel Techniques in Sensory Characterization and Consumer Profiling.

Coi, A.L., Bigey, F., Mallet, S., Marsit, S., Zara, G., Gladieux, P., Galeote, V., Budroni, M., Dequin, S., Legras, J.L., 2017. Genomic signatures of adaptation to wine biological ageing conditions in biofilm-forming flor yeasts. Mol. Ecol. 26, 2150–2166. https://doi.org/10.1111/mec.14053

Comuzzo, P., Battistutta, F., 2019. Acidification and pH Control in Red Wines, Red Wine Technology. Elsevier Inc. https://doi.org/10.1016/B978-0-12-814399-5.00002-5

Cox, T.F., 2001. Multidimensional scaling used in multivariate statistical process control. J. Appl. Stat. 28, 365–378. https://doi.org/10.1080/02664760120034108

Cusano, E., Cagliani, L.R., Consonni, R., Simonato, B., Zapparoli, G., 2020. NMR-based metabolic profiling of different yeast fermented apple juices. Lwt 118. https://doi.org/10.1016/j.lwt.2019.108771

Delcourt, F., Taillandier, P., Vidal, F., Strehaiano, P., 1995. Influence of pH, malic acid and glucose

concentrations on malic acid consumption by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 43, 321–324. https://doi.org/10.1007/BF00172832

Divol, B., Du Toit, M., Duckitt, E., 2012. Surviving in the presence of sulphur dioxide: Strategies developed by wine yeasts. Appl. Microbiol. Biotechnol. 95, 601–613. https://doi.org/10.1007/s00253-012-4186-x

Faye, P., Courcoux, P., Qannari, E.M., Giboreau, A., 2011. Méthodes de traitement statistiqyes des données issues d"une épreuve de tri libre. Rev. Modul. 1–24.

Fernandes, A., Bras, N., Mateus, N., de Freita, V., 2015. A study of anthocyanin self-association by NMR spectroscopy. New J. Chem 39, 2602–2611. https://doi.org/10.1039/c4nj02339k

Fleet, G.H., Prakitchaiwattana, C., Beh, K.L., Heard, G., 2002. Biodiversity and biotechnology of wine yeasts, in: The Yeast Ecology of Wine Grapes. Research Signpost.

García-Ríos, E., Morard, M., Parts, L., Liti, G., Guillamón, J.M., 2017. The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*. BMC Genomics 18, 1–13. https://doi.org/10.1186/s12864-017-3572-2

Gardner, N., Rodrigue, N., Champagne, C.P., 1993. Combined Effects of Sulfites , Temperature , and Agitation Time on Production of Glycerol in Grape Juice by *Saccharomyces cerevisiaet*. Appl. Environ. Microbiol. 59, 2022–2028.

Giudici, P., Zambonelli, C., 1992. Biometric and Genetic Study on Acetic Acid Production for Breeding of Wine Yeast. Am. J. Enol. Vitic. 43, 370–374.

Gonzalez, R., Morales, P., 2022. Truth in wine yeast. Microb. Biotechnol. 15, 1339–1356. https://doi.org/10.1111/1751-7915.13848

Gougeon, L., Da Costa, G., Le Mao, I., Ma, W., Teissedre, P.L., Guyon, F., Richard, T., 2018. Wine Analysis and Authenticity Using 1H-NMR Metabolomics Data: Application to Chinese Wines. Food Anal. Methods 11, 3425–3434. https://doi.org/10.1007/s12161-018-1310-2

Guillaume, C., Delobel, P., Sablayrolles, J.M., Blondin, B., 2007. Molecular basis of fructose utilization by the wine yeast *Saccharomyces cerevisiae*: A mutated HXT3 allele enhances fructose fermentation. Appl. Environ. Microbiol. 73, 2432–2439. https://doi.org/10.1128/AEM.02269-06

Hatzakis, E., 2019. Nuclear Magnetic Resonance (NMR) Spectroscopy in Food Science : A Comprehensive Review. Compr. Rev. Food Sci. Food Saf. 18, 189–220. https://doi.org/10.1111/1541-4337.12408

Huang, C., Roncoroni, M., Gardner, R.C., 2014. MET2 affects production of hydrogen sulfide during wine fermentation. Appl. Microbiol. Biotechnol. 98, 7125–7135. https://doi.org/10.1007/s00253-014-5789-1

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Amino Acid and Ammonium Utilization by *Saccharomyces cerevisiae* Wine Yeasts From a Chemically Defined Medium. Am. J. Enol. Vitic. 45, 75–83.

Julien, A., Roustan, J.L., Dulau, L., Sablayrolles, J.M., 2000. Comparison of nitrogen and oxygen

demands of enological yeasts: Technological consequences. Am. J. Enol. Vitic. 51, 215–222.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its Importance to Wine Aroma - A review. Underst. Wine Chem. 21, 97–129.

Le Mao, I., Da Costa, G., Richard, T., 2023. 1 H-NMR metabolomics for wine screening and analysis. Oeno One 57, 15–31. https://doi.org/10.20870/oeno-one.2023.57.1.7134

Le Mao, I., Martin-Pernier, J., Bautista, C., Lacampagne, S., Richard, T., Da Costa, G., 2021. 1H-Nmr Metabolomics As a Tool for Winemaking Monitoring. Molecules 26, 1–13. https://doi.org/10.3390/molecules26226771

Legras, J.L., Galeote, V., Bigey, F., Camarasa, C., Marsit, S., Nidelet, T., Sanchez, I., Couloux, A., Guy, J., Franco-Duarte, R., Marcet-Houben, M., Gabaldon, T., Schuller, D., Sampaio, J.P., Dequin, S., 2018. Adaptation of *S. cerevisiae* to fermented food environments reveals remarkable genome plasticity and the footprints of domestication. Mol. Biol. Evol. 35, 1712–1727. https://doi.org/10.1093/molbev/msy066

Lepeltier, P., 2022. Mille Vignes - Penser le vin de demain. Hachette Vins.

Malmendal, A., Amoresano, C., Trotta, R., Lauri, I., Tito, S. De, Novellino, E., Randazzo, A., 2011. NMR Spectrometers as " Magnetic Tongues ": Prediction of Sensory Descriptors in Canned Tomatoes. J. Agric. Food Chem. 59, 10831–10838.

Marcone, M.F., Wang, S., Albabish, W., Nie, S., Somnarain, D., Hill, A., 2013. Diverse food-based applications of nuclear magnetic resonance (NMR) technology. Food Res. Int. 51, 729–747. https://doi.org/10.1016/j.foodres.2012.12.046

Martí-Raga, M., Peltier, E., Mas, A., Beltran, G., Marullo, P., 2017. Genetic Causes of Phenotypic Adaptation to the Second Fermentation of Sparkling Wines in *Saccharomyces cerevisiae*. G3 Genes|Genomes|Genetics 7, 399–412. https://doi.org/10.1534/g3.116.037283

Marullo, P., Bely, M., Masneuf-Pomarede, I., Aigle, M., Dubourdieu, D., 2004. Inheritable nature of enological quantitative traits is demonstrated by meiotic segregation of industrial wine yeast strains. FEMS Yeast Res. 4, 711–719. https://doi.org/10.1016/j.femsyr.2004.01.006

Marullo, P., Bely, M., Masneuf-Pomarède, I., Pons, M., Aigle, M., Dubourdieu, D., Masneuf-Pomarede, I., Pons, M., Aigle, M., Dubourdieu, D., 2006. Breeding strategies for combining fermentative qualities and reducing off-flavor production in a wine yeast model. FEMS Yeast Res. 6, 268–279. https://doi.org/10.1111/j.1567-1364.2006.00034.x

Marullo, P., Mansour, C., Dufour, M., Albertin, W., Sicard, D., Bely, M., Dubourdieu, D., 2009. Genetic improvement of thermo-tolerance in wine *Saccharomyces cerevisiae* strains by a backcross approach. FEMS Yeast Res. 9, 1148–1160. https://doi.org/10.1111/j.1567-1364.2009.00550.x

Mendes-Ferreira, A., Barbosa, C., Falco, V., Leão, C., Mendes-Faia, A., 2009. The production of hydrogen sulphide and other aroma compounds by wine strains of *Saccharomyces cerevisiae* in synthetic media with different nitrogen concentrations. J. Ind. Microbiol. Biotechnol. 36, 571–583. https://doi.org/10.1007/s10295-009-0527-x

Minoja, A.P., Napoli, C., 2014. NMR screening in the quality control of food and nutraceuticals. FRIN. https://doi.org/10.1016/j.foodres.2014.04.056

Mortimer, R., Polsinelli, M., 1999. On the origins of wine yeast. Res. Microbiol. 150, 199–204. https://doi.org/10.1016/S0923-2508(99)80036-9

Murat, M.-L., Masneuf-Pomarede, I., Darriet, P., Lavigne, V., Tominaga, T., Dubourdieu, D., 2001. Effect of *Saccharomyces cerevisiae* Yeast Strains on the Liberation of Volatile Thiols in Sauvignon blanc Wine. Am. J. Enol. Vitic. 52, 136–139.

Peltier, E., Bernard, M., Trujillo, M., Barbe, J.-C., Prodhomme, D., Gibon, Y., Marullo, P., 2018a. Wine yeast phenomics: A standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. PLoS One 1–23. https://doi.org/10.1371/journal.pone.0190094

Peltier, E., Sharma, V., Martí Raga, M., Roncoroni, M., Bernard, M., Jiranek, V., Gibon, Y., Marullo, P., 2018b. Dissection of the molecular bases of genotype x environment interactions: A study of phenotypic plasticity of *Saccharomyces cerevisiae* in grape juices. BMC Genomics 19, 1–20. https://doi.org/10.1186/s12864-018-5145-4

Peltier, E., Vion, C., Abou Saada, O., Friedrich, A., Schacherer, J., Marullo, P., 2021. Flor Yeasts Rewire the Central Carbon Metabolism During Wine Alcoholic Fermentation. Front. Fungal Biol. 2. https://doi.org/10.3389/ffunb.2021.733513

Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergström, A., Sigwalt, A., Barre, B., Freel, K., Llored, A., Cruaud, C., Labadie, K., Aury, J.-M., Istace, B., Lebrigand, K., Barbry, P., Engelen, S., Lemainque, A., Wincker, P., Liti, G., Schacherer, J., 2018. Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates Species-wide genetic and phenotypic diversity. Nature 556, 339–347.

Querol, A., Pérez-Torrado, R., Alonso-del-Real, J., Minebois, R., Stribny, J., Oliveira, B.M., Barrio, E., 2018. New Trends in the Uses of Yeasts in Oenology, in: Advances in Food and Nutrition Research. Elsevier Inc., pp. 177–210. https://doi.org/10.1016/bs.afnr.2018.03.002

R Development Core Team, 2016. R: A language and environment for statistical computing. 2, 1–12.

Remize, F., Cambon, B., Barnavon, L., Dequin, S., 2003. Glycerol formation during wine fermentation is mainly linked to Gpd1p and is only partially controlled by. Yeast 20, 1243–1253. https://doi.org/10.1002/yea.1041

Ribéreau-Gayon, P., Dubourdien, D., Donéche, B.& A.L., 2006. Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B.B., Lonvaud, A.A., Darriet, P., Towey, J., 2006. Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2nd Edition Handbook, Handbook of Enology. https://doi.org/10.1002/9781119588320.ch3

Rogiers, S.Y., Greer, D.H., Liu, Y., Baby, T., Xiao, Z., 2022. Impact of climate change on grape berry

ripening: An assessment of adaptation strategies for the Australian vineyard. Front. Plant Sci. 13, 1–19. https://doi.org/10.3389/fpls.2022.1094633

Romano, P., Soli, M.G., Suzzi, G., 1985. Improvement of a wine *Saccharomyces cerevisiae* strain by a breeding program. Appl. Environ. Microbiol. 50, 1064–1067.

Şener, H., 2018. Effect of Temperature and Duration of Maceration on Colour and Sensory Properties of Red Wine: A Review. South African J. Enol. Vitic. 32, 227–234. https://doi.org/10.21548/39-2-3160

Sicard, D., Legras, J.L., 2011. Bread, beer and wine: Yeast domestication in the *Saccharomyces* sensu stricto complex. Comptes Rendus - Biol. 334, 229–236. https://doi.org/10.1016/j.crvi.2010.12.016

Simonin, S., Alexandre, H., Nikolantonaki, M., Coelho, C., Tourdot-Maréchal, R., 2018. Inoculation of Torulaspora delbrueckii as a bio-protection agent in winemaking. Food Res. Int. 107, 451–461. https://doi.org/10.1016/j.foodres.2018.02.034

Stitt, M., McC., L.R., Gerhardt, R., Heldt, H.W., 1989. Metabolite Levels in Specific Cells and Subcellular Compartements of Plant Leaves. Methods Enzymol. 174, 518–552.

Styger, G., Prior, B., Bauer, F.F., 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 1145–1159. https://doi.org/10.1007/s10295-011-1018-4

Taillandier, P., Ramon Portugal, F., Fuster, A., Strehaiano, P., 2007. Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. Food Microbiol. 24, 95–100. https://doi.org/10.1016/j.fm.2006.04.002

Tomašević, M., Lukić, K., Ćurko, N., Jagatić Korenika, A.M., Preiner, D., Tuščić, V., Jeromel, A., Kovačević Ganić, K., 2023. The Influence of Grape Clone and Yeast Strain on Varietal Thiol Concentrations and Sensory Properties of Graševina Wines. Foods 12, 1–12. https://doi.org/10.3390/foods12050985

Van Leeuwen, C., Destrac-Irvine, A., Dubernet, M., Duchêne, E., Gowdy, M., Marguerit, E., Pieri, P., Parker, A., De Rességuier, L., Ollat, N., 2019. An update on the impact of climate change in viticulture and potential adaptations. Agronomy 9, 1–20. https://doi.org/10.3390/agronomy9090514

Verpoorte, R., Choi, Y.H., Mustafa, N.R., Kim, H.K., 2008. Metabolomics: Back to basics. Phytochem. Rev. 7, 525–537. https://doi.org/10.1007/s11101-008-9091-7

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Appl. Microbiol. Biotechnol. 77, 145–157. https://doi.org/10.1007/s00253-007-1145-z

Vion, C., Muro, M., Bernard, Margaux, Richard, B., Fautre, V., Yeramian, N., Masneuf-pomarède, I., Tempère, S., Marullo, P., 2022. New malic acid producer strains of *Saccharomyces cerevisiae* for preserving wine acidity during alcoholic fermentation. Food Microbiol. 112. https://doi.org/10.1016/j.fm.2022.104209

Vion, C., Peltier, E., Bernard, M., Muro, M., Marullo, P., 2021. Marker assisted selection of malic-consuming *Saccharomyces cerevisiae* strains for winemaking. Efficiency and limits of a qtl's driven breeding program. J. Fungi 7. https://doi.org/10.3390/jof7040304

Yéramian, N., Chaya, C., Suárez Lepe, J.A., 2007. L-(-)-malic acid production by *Saccharomyces* spp. during the alcoholic fermentation of wine. J. Agric. Food Chem. 55, 912–919. https://doi.org/10.1021/jf061990w

Titre : Etude de la modulation de l'acidité des vins par *Saccharomyces cerevisiae* par des approches génétiques et métabolomiques. Rôle de la consommation et de la production de l'acide malique au cours de la fermentation alcoolique

Résumé :

La hausse des températures a de nombreux impacts sur la vigne et les baies de raisin. Parmi eux, une augmentation de la teneur en sucre des baies à maturité et une diminution de leur teneur en acidité. L'adaptation des pratiques viti-vinicoles aux matrices œnologiques de demain passe, entre autres, par l'utilisation de levures de vinification mieux adaptées aux caractéristiques physico-chimiques des moûts. Il existe au sein de l'espèce *Saccharomyces cerevisiae* une grande variabilité génotypique et phénotypique impactant de nombreux traits technologiques. La capacité des levures de vinification à moduler l'acidité lors de la fermentation alcoolique est au centre de ce travail de thèse. Cette propriété technologique a été optimisée grâce à des programmes de croisement assistés à l'aide de marqueurs génétiques liés à des QTLs (*Quantitative Trait Loci*) préalablement identifiés. Des souches fortement consommatrices ou productrices d'acide malique ont été obtenues. Ces souches ont été caractérisées d'un point de vue fermentaire, métabolique et sensoriel dans plusieurs matrices œnologiques afin de les intégrer à différents itinéraires de vinification. Un état des lieux de la modulation de l'acidité d'une cinquantaine de souches du genre *Saccharomyces* a également été entrepris. Ce paysage phénotypique a été réalisé par une approche de métabolomique ciblée par RMN du proton dans différentes matrices œnologiques. Des différences phénotypiques portant sur la teneur finale en acides organiques ont été mises en évidence. Pour un sous-ensemble de souches de *S. cerevisiae* appartenant aux groupes génétiques de vin et de voile, une étude comparative de la concentration en métabolites intracellulaires a été réalisée. Ce travail a nécessité le développement d'une méthode d'extraction et de quantification par spectrométrie RMN du proton. Des *trade-off* métaboliques avec des traits d'histoire de vie ont été mis en évidence entre les deux groupes. Une dernière partie plus fonctionnelle vise à comprendre les modes de production et de consommation de l'acide malique au cours de la vinification. L'ajout dans le milieu fermenté d'acide malique marqué au ^{13}C et l'utilisation de plusieurs approches métabolomiques: LC-MS/MS, GC-MS, RMN-2D, a permis de comprendre les spécificités métaboliques de souches extrêmes. Enfin l'étude métabolomique non ciblée de plusieurs vins obtenus à partir de souches extrêmes a permis de définir des molécules clés capables de moduler l'acidité des vins.

Mots clés :

Contrôle de l'acidité des vins ; Œnologie ; Acide malique ; Sélection génétique ; Métabolomique

Title : Study of the modulation of wine acidity by *Saccharomyces cerevisiae* using genetic and metabolomic approaches. Role of consumption and production of malic acid during alcoholic fermentation.

Abstract:

The increase in temperature has many impacts on grapevines and grape berries. Among them, an increase in the sugar content of ripe grapes and a decrease in their acidity content. The adaptation of winemaking practices to tomorrow's oenological matrices requires, among other things, the use of winemaking yeasts that are better adapted to the physicochemical characteristics of musts. There is a large genotypic and phenotypic variability within the *Saccharomyces cerevisiae* species, which has an impact on many technological traits. The ability of winemaking yeasts to modulate acidity during alcoholic fermentation is the focus of this thesis. This technological property was optimized through breeding programs assisted by genetic markers linked to previously identified QTLs (*Quantitative Trait Loci*). Strains with high malic acid consumption or production were obtained. These strains were characterized from a fermentative, metabolic, and sensory point of view, in several oenological matrices, in order to integrate them into different winemaking itineraries. An overview of the acidity modulation of about 50 strains of the *Saccharomyces* genus was also undertaken. A phenotypic landscape was carried out by a targeted metabolomic approach using ^{1}H NMR in different oenological matrices. Phenotypic differences in the final organic acid content were identified. A comparative study of intracellular metabolite concentration was performed for a subset of *S. cerevisiae* strains belonging to the wine and flor genetic groups. This work required the development of an extraction and quantification method by ^{1}H NMR spectrometry. Metabolic trade-offs with life history traits have been highlighted between the two groups. A last and more functional part aimed at understanding the production and consumption modes of malic acid during the wine making process. The addition of ^{13}C-labelled malic acid to the fermented medium and the use of several metabolomic approaches: LC-MS/MS, GC-MS, 2D-NMR allowed us to understand the metabolic specificities of extreme strains. Finally, the non-targeted metabolomic study of several wines obtained from extreme strains allowed to define key molecules able to modulate the acidity of wines.

Keywords :

Wine acidity control ; Oenology ; Malic acid ; Genetic selection ; Metabolomics

Unité de recherche

[UMR 1366 Œnologie, Université de Bordeaux, INRAe, Bordeaux INP, BSA, ISVV
210 chemin de Leysotte – CS50008 33882 Villenave d'Ornon, France]