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Par

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Pilotage des fermentations en culture mixte

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Titre : Pilotage des fermentations en culture mixte

Mots clés : vin, levures, interactions, multiparamétrique, cytométrie en flux, automatisation

Résumé :

Les contraintes actuelles et futures liées au changement climatique et à l'évolution de la demande des consommateurs de vin conduisent la profession à envisager l'adoption de nouvelles pratiques pour répondre aux défis techniques posés par ce contexte : nécessité de garder un taux d'alcool constant malgré des moûts de plus en plus sucrés, recherche de profils aromatiques plus variés, tout en conservant une reproductibilité acceptable.
La conduite de fermentations avec adjonction de levures non-*Saccharomyces* à l'espèce *Saccharomyces cerevisiae* classiquement utilisée pour conduire la fermentation alcoolique de façon maîtrisée, paraît être une alternative intéressante pour atteindre ces objectifs. Néanmoins, les nombreuses interactions existant entre ces différentes levures restent encore mal comprises, ce qui complexifie la maîtrise de ces cocultures.
Un état de l'art a été effectué sur les interactions entre levures *Saccharomyces* et non-*Saccharomyces* et leurs conséquences sur les caractéristiques du vin obtenu, avec un focus sur les méthodologies utilisées pour étudier ces interactions. Cette étude bibliographique a permis de mettre en évidence une grande diversité de résultats selon les études considérées, et donc le possible impact des conditions de fermentation sur les interactions entre levures.
Les travaux se sont donc orientés sur l'étude de l'impact de certaines conditions de fermentation sur les interactions entre *Saccharomyces cerevisiae* et *Lachancea thermotolerans*, une levure ayant des intérêts technologiques (acidification, composés d'arômes, bioprotection). Cette étude est effectuée via une approche globale multicritère (dynamique des populations, déroulement de la fermentation, production de composés d'arômes) s'appuyant sur un plan d'expériences.
Les résultats montrent de multiples impacts des paramètres de fermentation, différents selon les variables étudiées. Les vitesses de croissance des levures sont impactées par la température, le ratio d'ensemencement et par une probable compétition pour l'azote. La persistance de *Lachancea thermotolerans* est modulée par trois facteurs en interaction entre eux : la température, le ratio d'ensemencement et l'oxygénation. La durée de fermentation est fortement impactée par la température. La production d'éthanol est influencée à la fois par le ratio d'ensemencement et l'oxygénation alors que la production d'acide lactique n'est impactée que par le ratio. Pour modifier la production de composés d'arômes, la température, le ratio d'ensemencement et parfois la concentration en azote constituent des leviers importants. Ces résultats illustrent la complexité de la maîtrise d'une fermentation en culture mixte, et la nécessité de trouver le meilleur compromis paramétrique permettant d'atteindre les objectifs techniques ciblés.
Afin de contrôler ces cocultures, la maîtrise des dynamiques de population constitue donc un point clé puisqu'elles impactent à la fois le déroulé de la fermentation, les interactions et la qualité du vin. Un second axe de travail a donc été d'évaluer la possibilité d'utiliser une méthode de suivi en continu des populations microbiennes, donnant des résultats précis et avec le moins de délai possible. La faisabilité d'une automatisation de la cytométrie en flux dans le domaine œnologique a été validée : cette technologie permet de suivre en continu des populations bactériennes ou levuriennes, dans diverses situations et notamment dans le cadre de fermentations en culture mixte.
Les deux volets de la thèse (impact des conditions de fermentation sur les interactions et automatisation de la cytométrie en flux) sont complémentaires et permettent d'entrevoir un pilotage en temps réel des conditions de fermentation en culture mixte avec des applications en recherche, en contrôle qualité voire en cuverie.

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Title: Monitoring of mixed culture fermentations

Keywords: wine, yeasts, interactions, multiparametric, flow cytometry, automation

Abstract:

Current and future constraints linked to climate change and evolution of wine consumer demand are prompting the winemaking industry to consider adopting new practices to address the technical challenges resulting from this context. These challenges include the need to maintain a constant alcohol level despite increased sugar contents in the must, and to seek a wider diversity of aromatic profiles, while maintaining acceptable reproducibility.
Fermentations with addition of non-*Saccharomyces* yeasts to the *Saccharomyces cerevisiae* species traditionally used to conduct alcoholic fermentation seem to be an interesting alternative to achieve these objectives. However, the numerous interactions between these different yeasts are still poorly understood, which complicates the control of these cocultures.
A state-of-the-art review of the interactions occurring between *Saccharomyces* and non-*Saccharomyces* yeasts and their consequences on the characteristics of the resulting wine was conducted, with a specific focus on the methodologies used to study these interactions. This review pointed out the existence of a great diversity of results depending on the studies considered, and thus to a possible impact of fermentation conditions on the interactions between yeasts.
This study therefore focused on studying the impact of selected fermentation conditions on interactions between *Saccharomyces cerevisiae* and *Lachancea thermotolerans*, a yeast with technological interests (acidification, aroma compounds, bioprotection). This study was carried out using a multi-criteria approach (population dynamics, fermentation progress, production of aroma compounds) based on an experimental design.
The results show multiple impacts of the fermentation parameters, which differ depending on the variables studied. Yeast growth rates were affected by temperature, inoculation ratio and by a probable competition for nitrogen. The persistence of *Lachancea thermotolerans* was modulated by three interacting factors: temperature, inoculation ratio and oxygenation. The duration of fermentation is strongly affected by temperature. Ethanol production is influenced by both inoculation ratio and oxygenation, whereas lactic acid production is only affected by the ratio. To modify the production of aroma compounds, temperature, inoculation ratio and in some cases nitrogen concentration constitute significant levers. These results illustrate the complexity of controlling fermentation in mixed cultures, and the necessity to find the best parametric compromise to achieve the targeted technical objectives.
In order to control these cocultures, the control of population dynamics is therefore a key point, as they impact both the fermentation process, microbial interactions and wine quality. A second line of work therefore aimed to evaluate the possibility of using a method of continuous monitoring of microbial populations, giving precise results with the least possible delay. The feasibility of automating flow cytometry in the oenological field was validated: this technology makes it possible to continuously monitor bacterial or yeast populations in various situations, particularly in the context of mixed culture fermentations.
The two parts of the thesis (impact of fermentation conditions on interactions and automation of flow cytometry) are complementary, and allow to envision real-time control of fermentation conditions in mixed cultures, with future applications in research, quality control and even in the cellar.

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Lexique

AFC	Analyse Factorielle des Correspondances
AUC, AUC_{Lt}, AUC_{Sc}	Area Under Curve, aire sous la courbe de croissance des microorganismes, AUC pour *Lt* et pour *Sc*
GC-MS	Gas Chromatography - Mass Spectrometry
DNO	Diplôme National d'Œnologue
L. thermotolerans	*Lachancea thermotolerans*
Lt	*Lachancea thermotolerans*
MS300/MS150	Moût synthétique contenant 300 ou 150 $mg \cdot L^{-1}$ d'azote assimilable.
$N_{max\ Sc}$, $N_{max\ Lt}$	Population maximale de *Sc*, de *Lt*
$\mu_{max\ Sc}$, $\mu_{max\ Lt}$	Vitesse de croissance maximale de *Sc*, de *Lt*
N.S.	Levures non-*Saccharomyces*
S. cerevisiae	*Saccharomyces cerevisiae*
Sc	*Saccharomyces cerevisiae*
T. delbrueckii	*Torulaspora delbrueckii*
Vin synthétique	Produit issu de la fermentation d'un moût synthétique
YPD	Yeast Extract-Peptone-Dextrose
WL	Wallerstein Laboratory Nutrient Agar

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Introduction

Le vin demeure aujourd'hui un produit occupant une place centrale dans la gastronomie mondiale et tout particulièrement française. Son procédé de fabrication, s'il est né il y a plusieurs millénaires d'une nécessité de conservation du raisin (McGovern *et al.*, 2017), s'est peu à peu complexifié à mesure que le vin évoluait au-delà de sa nature de produit de subsistance pour devenir un produit élaboré, à la portée de plus en plus gastronomique et culturelle. Cette transformation du raisin implique de multiples étapes techniques successives, qui s'étendent de la récolte des grappes à la mise en bouteille finale. Tout au long de ce procédé, la flore microbienne est omniprésente, soit en tant qu'alliée lorsqu'elle apporte saveurs et complexité au vin obtenu (fermentation malolactique, élevage sur lies), soit en tant que menace pour la qualité organoleptique du vin (*Brettanomyces bruxellensis,* bactéries acétiques). Mais les microorganismes constituent avant tout l'outil central au cœur de ce procédé d'élaboration, via les levures qui transforment, lors de la fermentation alcoolique, le moût de raisin fraîchement pressé en vin.

L'étape clé du procédé de fabrication du vin est en effet cette fermentation alcoolique, au cours de laquelle les sucres présents dans le moût (glucose et fructose) sont convertis par des levures en éthanol et dioxyde de carbone. Cette biotransformation, d'une durée de 10 à 15 jours, s'accompagne de la production d'autres métabolites qui contribuent à la qualité organoleptique du vin : glycérol, acides, composés d'arômes (alcools, esters, aldéhydes, cétones, thiols, terpènes…). Ces composés volatils proviennent directement du raisin, mais aussi et surtout de la libération d'arômes variétaux à partir de précurseurs et de la synthèse d'arômes fermentaires, via l'équipement enzymatique des levures. Le vin final est composé à 85-90 % d'eau, avec 10-15 % d'éthanol, 0.4-1.0 % de glycérol, 0.5-1.5 % d'acides organiques et 1 % de composés d'arômes (Swiegers & Pretorius, 2005), un équilibre précis entre tous ces composés définissant la qualité organoleptique du vin.

En vinification traditionnelle, la fermentation alcoolique démarre de manière spontanée et est effectuée par la flore microbienne issue des baies de raisin, mais aussi par les microorganismes présents dans l'environnement de vinification et sur les équipements utilisés. Ce consortium microbien, constitué essentiellement de levures non-*Saccharomyces*, contribue à l'obtention d'un vin avec une richesse aromatique importante, caractéristique généralement recherchée par le consommateur. La levure *Saccharomyces cerevisiae*, initialement présente en très faible quantité dans le moût, se développe plus rapidement que les autres microorganismes, et devient par conséquent au cours du processus de fermentation la principale levure présente dans le milieu (Fleet, 2003). Ces dynamiques de population particulières sont nécessaires pour la fermentation complète des sucres du moût, les levures non-*Saccharomyces* n'étant pas ou peu capables de se développer à des niveaux élevés d'éthanol (Borren & Tian, 2020). En revanche, la qualité sensorielle est difficilement reproductible et contrôlable, puisqu'elle dépend en grande partie de la flore naturelle présente sur les baies. De plus, en fonction des dynamiques de population du consortium, des déroulements non souhaités de la fermentation peuvent avoir lieu : fermentations languissantes voire stoppées si *Saccharomyces cerevisiae* ne s'implante pas correctement dans le milieu, voire développement de microorganismes d'altération affectant la qualité finale du vin obtenu (arômes indésirables, acidification) (Mas & Portillo, 2022).

Afin de mieux maîtriser la fermentation et la production des métabolites impactant la qualité sensorielle, la profession pratique l'ensemencement du moût avec des levures connues ("starter" de fermentation à

base de levures sèches actives). Ce starter permet d'assurer un démarrage rapide de la fermentation et la complétion de celle-ci, ainsi que d'éviter le développement de microorganismes indésirables. En revanche, pour des questions de reproductibilité, ces starters ne sont généralement constitués que d'une seule souche de levure *Saccharomyces cerevisiae*.

Or, la profession doit actuellement répondre à de nouvelles attentes du consommateur qui recherche des vins moins riches en alcool (à la fois par intérêt sensoriel et pour des préoccupations de santé) et des caractéristiques sensorielles différentes : plus de rondeur, plus de notes fruitées ou encore une diversité aromatique plus importante (Goold *et al.*, 2017). La demande du consommateur s'oriente également de plus en plus vers des vins qui, de la vigne au procédé de fabrication, s'inscrivent dans une démarche de durabilité (Pomarici & Vecchio, 2019) : diminution des intrants, procédés écologiques et durables, diminution des additifs notamment pour lutter contre l'oxydation et le développement microbien. L'évolution du climat engendre également de nouvelles problématiques, puisque l'augmentation de température conduit à des raisins contenant plus de sucres et moins d'acide malique, d'où des vins plus concentrés en alcool et moins acides à procédé identique, ce qui risque de déséquilibrer leurs caractéristiques sensorielles (Querol *et al.*, 2018).

Afin de répondre à ces problématiques, la maîtrise d'une fermentation mixte alliant *Saccharomyces cerevisiae* à des levures non-*Saccharomyces* apparaît comme une alternative intéressante et fait donc actuellement l'objet de nombreux travaux de recherche (Englezos *et al.*, 2022; Romani *et al.*, 2020; Roudil *et al.*, 2020). En effet, ces levures comportent pour certaines des atouts techniques importants : modulation du profil aromatique des vins (Englezos *et al.*, 2022; Padilla *et al.*, 2016), faible rendement en éthanol ou acidification du moût (permettant de compenser la sucrosité supérieure liée au réchauffement climatique) (Vicente *et al.*, 2022), ou encore bioprotection pour limiter le développement de flores d'altération (*Brettanomyces bruxellensis* par exemple) (Simonin *et al.*, 2018). Néanmoins, ces fermentations mixtes s'avèrent complexes à maîtriser du fait de l'existence de nombreuses interactions entre les levures non-*Saccharomyces* et *S. cerevisiae*. Ces interactions impactent non seulement les dynamiques de population, mais aussi la biotransformation du moût et donc la qualité du vin. Ces interactions multiples et complexes rendent difficiles la prévision du déroulement de la fermentation et la reproductibilité de telles cocultures (Comitini *et al.*, 2021; Englezos *et al.*, 2022).

L'objectif final de ces travaux de thèse est de maîtriser et contrôler ces fermentations en culture mixte. Leur maîtrise passe nécessairement par leur compréhension et demande donc d'approfondir nos connaissances fondamentales sur leur fonctionnement et notamment les interactions qui s'y déroulent. L'application de cette maîtrise à des cas concrets de contrôle, en laboratoire puis en cave, nécessite également d'identifier des métriques à surveiller ainsi que des leviers d'action. Ceux-ci sont essentiels, à la fois pour prévoir le déroulement de ces fermentations et pour les orienter, soit de manière anticipée, soit en temps réel. Le développement de nouvelles technologies et méthodologies d'analyse peut en outre permettre d'améliorer la précision et la justesse de mesure des métriques identifiées, et par conséquent affiner l'usage subséquent des leviers d'action. La problématique est donc double : d'une part, améliorer la compréhension des interactions à l'œuvre, afin d'identifier les facteurs impactant le plus le déroulement des fermentations en culture mixte (et donc les plus susceptibles d'être utilisables comme leviers d'action pour contrôler celles-ci) et, d'autre part, évaluer la possibilité de suivre plus précisément et de façon automatisée les populations de levure, afin de pouvoir agir sur elles à l'avenir en modulant les facteurs les plus impactants.

Devant la complexité des interactions impliquées dans les fermentations mixtes, une attention particulière doit être apportée à la méthodologie mise en œuvre lors de la réalisation d'expérimentations visant à mieux comprendre ces interactions.

La démarche a donc été la suivante. Dans un premier temps, un état de l'art a été réalisé, focalisé sur les interactions entre levures non-*Saccharomyces* et *S. cerevisiae*, les méthodologies utilisées pour les étudier, et les impacts de ces interactions sur la modification du milieu. Cette analyse de l'existant a fait l'objet, dans le cadre de ces travaux de thèse, d'une revue bibliographique centrée sur la partie méthodologie (Bordet *et al.*, 2020). Elle a notamment permis de mettre en évidence les résultats parfois contradictoires des nombreux travaux de recherche menés sur les fermentations mixtes, qui pourraient être expliqués par des modes opératoires et des milieux utilisés différents selon les équipes. Ces études sont, de plus, souvent monofactorielles, ce qui limite les comparaisons possibles entre les paramètres de fermentation étudiés.

Afin d'étudier les interactions en fermentation mixte, il apparaît donc nécessaire dans un second temps d'aborder la problématique avec une méthodologie qui permettra à la fois de standardiser les conditions d'étude et d'avoir une approche globale via une étude plurifactorielle. La deuxième partie de ce manuscrit est donc dédiée à ces travaux, qui se sont focalisés sur les fermentations mixtes avec le couple *Saccharomyces cerevisiae* / *Lachancea thermotolerans* qui présente des intérêts technologiques particuliers. Après une première étape de définition des paramètres de fermentation à étudier, un plan expérimental multifactoriel a été mis en place et a permis d'évaluer de façon simultanée l'impact de différents paramètres de fermentation (ratio d'ensemencement, composition en azote du milieu de culture, oxygénation du moût, température de fermentation) sur la fermentation mixte, à la fois sur l'aspect dynamique des populations et sur celui de la composition du milieu. Les résultats ont fait l'objet d'un article (Joran *et al.*, 2022). Ces travaux ont ensuite été poursuivis à la fois par un deuxième plan expérimental complémentaire qui a permis de consolider les résultats du premier et de mettre en évidence des interactions entre facteurs, et par une approche sensorielle des caractéristiques des vins synthétiques obtenus. Ces études montrent l'importance de certains paramètres de fermentation, notamment le ratio d'ensemencement et la température, sur les interactions et les conséquences sur le produit.

En outre, si l'on souhaite maîtriser la conduite d'une fermentation mixte, et à l'avenir agir en modulant les paramètres de fermentation, il apparaît nécessaire d'améliorer les procédés d'analyse existants afin d'assurer un meilleur suivi des populations levuriennes. En effet, le suivi actuel des populations microbienne, que ce soit par méthode culturale ou par cytométrie en flux, reste encore aujourd'hui limité dans sa fréquence et son amplitude de mesure par la nécessité pour les chercheurs d'être présents et d'effectuer eux-mêmes prélèvements, dilutions, marquages, et lancement de la mesure. Cette limitation de la fréquence par l'aspect chronophage de l'analyse de populations microbiennes constitue un obstacle important à l'obtention de courbes de croissance détaillées, et par conséquent à la mesure des métriques en découlant (phase de latence, vitesse maximale de croissance), pourtant essentielles pour l'étude des interactions et la maîtrise des dynamiques de population en culture mixte. C'est pourquoi la troisième partie de ce manuscrit est dédiée à une étude de faisabilité sur l'utilisation d'un système automatisé de cytométrie en flux pour contrôler les populations microbiennes, et ce dans différents contextes œnologiques. Ces travaux ont fait l'objet d'une publication soumise dans le journal OENO One.

1. Les cultures mixtes, un lieu d'interactions complexe à étudier et à contrôler : état de l'art

1.1. Une grande diversité de non-*Saccharomyces* d'intérêt œnologique

La microflore naturelle du raisin est dépendante de différents facteurs (variété, conditions climatiques, pratiques culturales, état des grains), mais est essentiellement constituée de levures non-*Saccharomyces* appartenant à une grande diversité d'espèces (Querol *et al.*, 2018). Longtemps considérées comme des microorganismes indésirables, pouvant être à l'origine de défauts des vins, les levures non-*Saccharomyces* se révèlent être des acteurs clés pour l'obtention de vins de qualité. L'intérêt œnologique de certaines a en effet été étudié et démontré au fil du temps et des études réalisées sur le sujet. Elles appartiennent aux genres suivants : *Torulaspora*, *Candida, Hanseniaspora*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Lachancea* et *Pichia* (Jolly *et al.*, 2014). Leur prévalence sur les grains de raisin est variable : *Hanseniaspora spp*. et *Starmerella bacillaris* sont présentes en grande quantité, *Metschnikowia pulcherrima* et *Wickerhamomyces anomalus* sont assez abondantes, alors que d'autres sont présentes en faible quantité (*Lachancea thermotolerans*, *Torulaspora delbrueckii*, *Pichia kluyveri*, *Schizosaccharomyces pombe*). Ces levures présentent également des tolérances à l'éthanol variées (3 à 14 % v/v) qui laissent présager des capacités de persistance différentes au cours de la fermentation alcoolique (Borren & Tian, 2020).

Différents intérêts technologiques de ces levures non-*Saccharomyces* ont été mis en évidence dans la littérature, en premier lieu en culture simple, mais aussi en coculture avec *S. cerevisiae*.
Tout d'abord, la possibilité d'obtenir un vin moins concentré en éthanol est un point clé motivant les recherches, puisque la maîtrise de la production d'alcool au cours de la fermentation permettrait d'atténuer l'impact du climat (augmentation du titre alcoométrique de 1 % (v/v) tous les 10 ans), mais aussi de répondre à la demande du consommateur (moins d'alcool pour des raisons de santé ou pour des raisons sensorielles, l'alcool en trop forte quantité masquant la perception des notes fruitées et déséquilibrant le ressenti sensoriel) (Ciani *et al.*, 2016; Gonzalez *et al.*, 2021).
La concentration en éthanol inférieure obtenue lors d'une fermentation mixte avec des levures non-*Saccharomyces* par rapport à une culture pure de *S. cerevisiae* peut être liée à plusieurs phénomènes : un rendement plus faible en éthanol, une efficacité de fermentation plus faible, ainsi qu'un métabolisme respiro-fermentaire plus important chez les levures non-*Saccharomyces* (Romani *et al.*, 2020). En effet, certaines levures non-*Saccharomyces* ont un rendement intrinsèque en éthanol plus faible que *S. cerevisiae*, pouvant être expliqué par l'implication de voies métaboliques différentes conduisant à des composés secondaires autres que l'éthanol (Ciani *et al.*, 2016; Gobbi *et al.*, 2014). Dans des conditions classiques de fermentation, *S. cerevisiae* transforme 60 % des sucres en éthanol, 30 % en dioxyde de carbone et le reste en biomasse et métabolites divers (Gonzalez *et al.*, 2021). Le rendement sucre / éthanol de *S. cerevisiae* est globalement stable quelles que soient les conditions, et supérieur à celui des levures non-*Saccharomyces* (Ciani *et al.*, 2016; Contreras *et al.*, 2015). Pour ces dernières, une variabilité du rendement est observée selon l'espèce mais également selon la souche (Gobbi *et al.*, 2014).
En outre, *S. cerevisiae* a la particularité, dans des milieux riches en sucres comme le moût de raisin, d'utiliser la fermentation comme principale voie métabolique (avec production d'éthanol), même en présence d'oxygène (effet Crabtree). Cet effet est moins important voire inexistant chez les autres

levures qui, en conditions aérobies, dégradent les sucres par oxydation via la respiration (Ciani *et al.*, 2016; Gonzalez *et al.*, 2013; Williams *et al.*, 2015). L'équilibre entre métabolisme respiratoire et fermentaire et les conséquences sur la production d'autres métabolites que l'éthanol dépendent chez les non-*Saccharomyces* de l'espèce, de la souche et des conditions environnementales (Ciani *et al.*, 2016). L'implication plus importante de la respiration et la capacité à produire d'autres composés que l'éthanol sont mises à profit dans des fermentations mixtes non-*Saccharomyces* / *S. cerevisiae* pour obtenir une réduction de l'éthanol de 1 à 4 % (v/v) (Ciani *et al.*, 2016; J. Varela & Varela, 2019).
Cette diminution de la production d'éthanol peut également résulter d'un métabolisme du carbone différent, avec une déviation vers la production de glycérol à la place de l'éthanol. À concentration élevée, ce composé peut avoir un impact sur le caractère doux, sucré du vin mais assez peu sur sa viscosité (Goold *et al.*, 2017). Une augmentation de la production de glycérol est souvent observée dans des fermentations mixtes en présence de levures non-*Saccharomyces* par rapport à une fermentation avec *S. cerevisiae* seule (Borren & Tian, 2020; Ciani & Comitini, 2015).

Les cultures mixtes entre *S. cerevisiae* et les levures non-*Saccharomyces* ont montré également leur intérêt pour moduler la production d'acides organiques. Elles permettent le plus souvent d'augmenter l'acidité totale via la production d'acides organiques spécifiques, comme les acides lactique, succinique, pyruvique (Vicente *et al.*, 2022), qui ont un impact généralement positif au niveau sensoriel et permettent une meilleure conservation, tout en limitant la production d'acide acétique, qui a un impact sensoriel généralement négatif (Ciani & Comitini, 2015).
Les levures non-*Saccharomyces* produisent des enzymes impliquées dans la formation de composés d'arômes et pouvant être différentes de celles produites par *S. cerevisiae*. Cet équipement enzymatique peut donc permettre, à travers l'usage de ces levures, de moduler le profil aromatique des vins (Borren & Tian, 2020; Padilla *et al.*, 2016). Ces enzymes extracellulaires sont très diverses : alcool déshydrogénase transformant les acides aminés en alcools supérieurs, estérases conduisant à la production d'esters, décarboxylases permettant la formation d'acides gras volatils, glycosidase conduisant aux terpènes (Borren & Tian, 2020). Les non-*Saccharomyces* sont donc susceptibles de produire des composés d'arômes variés et en particulier des alcools supérieurs à note fruitée, des esters à note fruitée ou florale, des terpènes à note fleurie, mais aussi des acides gras à odeur moins agréable (rance, vinaigre) (Borren & Tian, 2020; Mateo & Maicas, 2016). Les levures non-*Saccharomyces* sont également capables, grâce à leur équipement enzymatique (et notamment la β-glucosidase), de libérer des arômes liés à des sucres et présents dans le moût : la libération de terpènes, de thiols volatils ainsi réalisée est particulièrement importante pour le profil aromatique du vin (Mateo & Maicas, 2016; C. Varela, 2016; Zott *et al.*, 2011). En outre, les non-*Saccharomyces* sont susceptibles d'augmenter la concentration en polysaccharides du vin, notamment en libérant des mannoprotéines. Ces polysaccharides ont une influence positive sur la qualité organoleptique du vin en augmentant sa viscosité (Domizio *et al.*, 2014; Mateo & Maicas, 2016).
Certaines levures non-*Saccharomyces* ont également la faculté de contribuer à l'intensité et à la stabilité de la couleur du vin rouge. Cette contribution passe par l'acidification du milieu (notamment par *Lachancea thermotolerans)*, et plus particulièrement par la production d'acide pyruvique qui permet la formation de composés colorés stables. La production de cet acide a notamment été identifiée chez *Schizosaccharomyces pombe* et *Starmerella bacillaris* (Benito *et al.*, 2019; S. Benito, 2020; Englezos *et al.*, 2018; Morata, Escott, *et al.*, 2019; Romani *et al.*, 2020).
Les levures non-*Saccharomyces* ont donc de multiples utilités pour améliorer la qualité organoleptique globale du vin : diminution de l'éthanol, obtention de profils aromatiques différents, ou encore participation à un équilibre acidité / arôme. Néanmoins, certains auteurs relatent la production d'arômes indésirables, impactant négativement le profil sensoriel du vin, lorsque les levures non-*Saccharomyces*

étudiées persistent au cours de la fermentation dans certaines conditions, comme une oxygénation trop importante (Tronchoni *et al.*, 2018). Cette possibilité d'un impact négatif sur la sensorialité du vin souligne l'importance de maîtriser les fermentations impliquant des non-*Saccharomyces*.

Par ailleurs, un autre atout de certaines levures non-*Saccharomyces* est leur capacité de bioprotection. Ainsi *Lachancea thermotolerans* est par exemple capable, via la production d'acide lactique, de composés organiques volatils comme le phényléthanol, ou d'autres composés toxiques, de diminuer la croissance voire de réduire la population de certaines levures ou champignons filamenteux (Gianvito *et al.*, 2022). En réduisant les risques de développement de flores d'altération (*Brettanomyces bruxellensis* notamment) et de production de mycotoxines, l'utilisation de non-*Saccharomyces* peut donc à l'avenir conduire à des vins de meilleure qualité microbiologique sans recourir à l'utilisation de SO_2, ou du moins en réduire la quantité nécessaire, comme cela a été montré avec *Torulaspora delbrueckii* (Simonin *et al.*, 2018) ou testé avec *Lachancea thermotolerans* (Nally *et al.*, 2018; Rubio-Bretón *et al.*, 2018).

Les levures non-*Saccharomyces* présentent donc une grande diversité d'intérêts technologiques : réduction du taux d'éthanol final, amélioration des qualités organoleptiques du vin obtenu et bio-protection contre les flores d'altération. Cependant, leur tolérance à l'éthanol, généralement bien plus faible que celle de *S. cerevisiae*, empêche leur utilisation en culture pure pour la fermentation alcoolique du vin. Afin de bénéficier à la fois des avantages potentiels des non-*Saccharomyces* listés ci-dessus et de la capacité fermentaire élevée de *Saccharomyces*, il apparaît donc essentiel de procéder à des fermentations en culture mixte associant *S. cerevisiae* à une (ou plusieurs) non-*Saccharomyces*.

Cependant, la mise en place de ces cultures mixtes soulève des problématiques techniques spécifiques, notamment en termes de fiabilité et de reproductibilité. En effet, la gestion de plusieurs levures différentes dans une même culture demande non seulement d'anticiper leurs éventuels besoins spécifiques en termes de nutriments (Roca-Mesa *et al.*, 2020), mais également de prendre en compte d'éventuelles interactions entre les levures choisies. Or, des interactions entre *S. cerevisiae* et les non-*Saccharomyces* avec un intérêt œnologique potentiel ont déjà été mises en évidence lors de fermentations en culture mixte (Zilelidou & Nisiotou, 2021). Ces interactions compliquent naturellement les dynamiques de population à l'œuvre et par conséquent la maîtrise de celles-ci. La simple présence de ces interactions au sein des cultures mixtes, mais aussi leur diversité et leur variabilité, rendent donc essentielle leur compréhension afin de pouvoir anticiper et moduler les dynamiques de population au sein d'une fermentation en culture mixte (Bagheri *et al.*, 2020).

1.2. Interactions entre *Saccharomyces cerevisiae* et levures non-*Saccharomyces*

Les interactions entre *S. cerevisiae* (*Sc*) et les levures non-*Saccharomyces* (*N.S.*), comme toutes les interactions microbiennes, peuvent être classées dans différentes catégories selon l'impact qu'elles produisent sur les deux espèces concernées (Figure 1). Ainsi, différents phénomènes peuvent être observés au cours d'une culture mixte : compétition, mutualisme, commensalisme ou amensalisme (Zilelidou & Nisiotou, 2021). Les cas de parasitisme (non représenté sur la figure 1) sont, dans les systèmes fermentaires, difficilement distinguables des cas d'amensalisme, l'impact positif pour la levure "parasite" étant souvent difficile à identifier comme une résultante directe de l'impact négatif qu'elle exerce sur la levure "hôte" (Winters *et al.*, 2019).

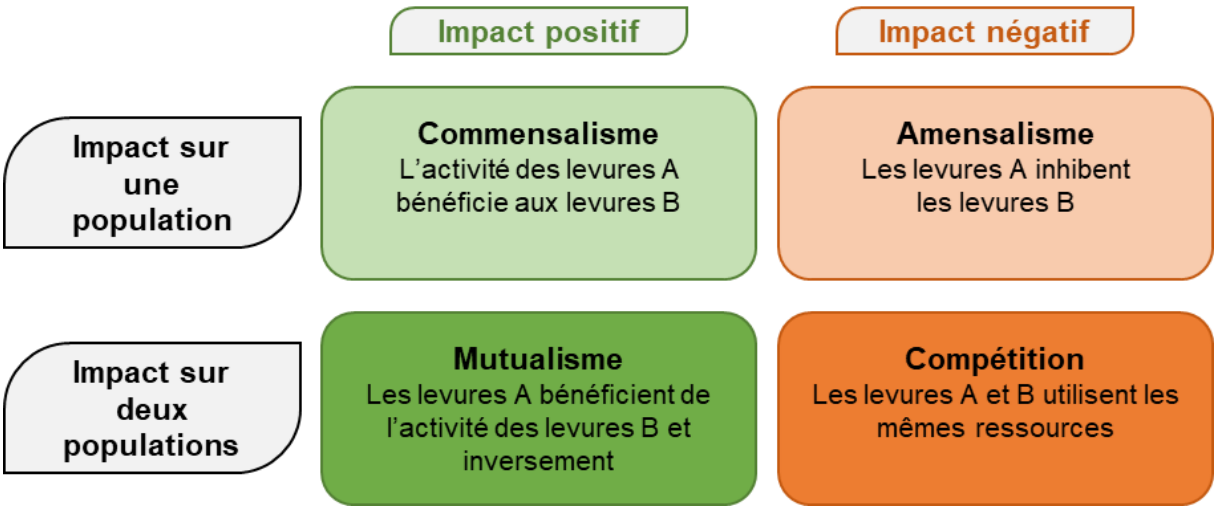

Figure 1. Types d'interactions entre levures au cours d'une fermentation alcoolique

1.2.1. Mise en évidence de l'existence d'interactions *Saccharomyces* / non-*Saccharomyces*

Les interactions entre *S. cerevisiae* et les différentes non-*Saccharomyces* utilisées en œnologie ont pu être mises en évidence dans les études existantes à travers leurs impacts sur différents aspects de la fermentation.

En premier lieu, les interactions entre *Saccharomyces* et non-*Saccharomyces* en contexte fermentaire sont observables directement via l'étude des dynamiques de population au cours de la fermentation alcoolique.
En effet, avant même de s'intéresser au cas des starters microbiens, l'étude des fermentations spontanées obtenues à partir de moûts de raisins pressés et non-ensemencés manuellement par une ou plusieurs souches exogènes permet de montrer que des dynamiques de population complexes sont à l'œuvre et dépendent des interactions existant entre les levures présentes. Toutes les études réalisées sur ce sujet observent une présence importante des non-*Saccharomyces* en début de fermentation, puis celles-ci sont peu à peu supplantées par *S. cerevisiae* au fur et à mesure que le processus de

fermentation progresse (Bagheri *et al.*, 2020; Harlé *et al.*, 2018; Mateo & Maicas, 2016; Sgouros *et al.*, 2018; Zott *et al.*, 2008, 2011).
Ces résultats, et avec eux l'hypothèse de l'existence d'interactions entre levures dans ces conditions fermentaires, sont confirmés par d'autres études effectuées en conditions plus standardisées, qui impliquent cette fois des cultures mixtes avec ensemencement de consortiums plus simples (Zilelidou & Nisiotou, 2021). Des différences concrètes entre les cinétiques de croissance des levures étudiées en culture pure ou en culture mixte ont également pu être observées, allant d'une phase de latence modifiée (Brou *et al.*, 2018) à un phénomène de mort cellulaire (Englezos *et al.*, 2019), en passant par une inhibition de la croissance de l'une des levures (Comitini *et al.*, 2011).

Outre leur impact sur les dynamiques de population, les interactions entre levures au cours de la fermentation peuvent également être révélées par l'étude de leurs métabolismes.
Ainsi, de multiples études ont pu mettre en évidence une production de métabolites différente en culture mixte *Sc* / *N.S.* par comparaison aux cultures pures correspondantes (Gustafsson *et al.*, 2016; Petitgonnet *et al.*, 2019; Roullier-Gall *et al.*, 2022; Sadoudi *et al.*, 2012, 2017). Cette modification métabolique se traduit souvent par une inhibition, par une des deux levures, de la production de certains métabolites chez l'autre levure étudiée. Plus encore, certaines études ont pu aller jusqu'à mettre en évidence l'inhibition de l'expression de certains gènes chez *S. cerevisiae* lorsque cultivée en présence de *Starmerella bombicola* (Milanovic *et al.*, 2012) ou *Metschnikowia pulcherrima* (Mencher *et al.*, 2021). À l'inverse, des effets de synergie peuvent parfois être observés entre deux souches, avec par exemple une production de glycérol ou de certains composés d'arômes plus importante lors d'une coculture que dans les cultures pures des deux espèces (Gobbi *et al.*, 2013; Morales *et al.*, 2019; Shekhawat *et al.*, 2017).

Il est à noter que l'existence et le type d'interactions observées varient selon l'étude, parfois au sein d'une même étude étudiant plusieurs couples *Sc* / *N.S.*, et semblent donc, au vu de l'état de l'art, à la fois espèce et souche-dépendants (Bagheri *et al.*, 2017, 2018; Mateo & Maicas, 2016). Il apparaît donc important de s'intéresser aux mécanismes qui sous-tendent ces interactions afin de mieux comprendre comment elles impactent à la fois le développement des levures œnologiques et leur métabolisme.

1.2.2. Mécanismes d'interactions

De nombreux mécanismes différents ont été mis en évidence dans la littérature comme étant à l'œuvre lors des interactions *Saccharomyces* / non-*Saccharomyces* pendant une fermentation alcoolique. Ces mécanismes sont de types très variés, allant d'interactions très indirectes (avantage adaptatif, compétition pour les nutriments) à des interactions plus directes comme le *quorum sensing* ou le contact cellulaire (Zilelidou & Nisiotou, 2021).

Adaptation à l'environnement

S. cerevisiae est mieux adaptée que les levures non-*Saccharomyces* à la survie en milieu stressant que constitue le moût de raisin (forte concentration en sucre en début de fermentation puis milieu épuisé en nutriments, acidité, faible quantité d'oxygène, présence de SO_2 et présence d'éthanol en fin de fermentation). La plus forte sensibilité des non-*Saccharomyces* à ces conditions particulières se traduit par une diminution plus rapide de leur population que celle de *S. cerevisiae*, qui devient la population dominante (Alonso-del-Real *et al.*, 2017; Ciani & Comitini, 2015). Parmi les paramètres

environnementaux observés comme pouvant exercer une pression de sélection sur le consortium microbien, on peut ainsi trouver le taux d'éthanol (Pina *et al.*, 2004), la disponibilité de l'oxygène (Holm Hansen *et al.*, 2001), ou encore la température (Goddard, 2008).

Néanmoins, la résistance au milieu stressant varie ici aussi selon l'espèce étudiée : les levures *Lachancea thermotolerans*, *Lachancea fermentati* et *Schizosaccharomyces pombe* sont notamment citées comme faisant partie des espèces les plus résistantes à la pression osmotique, à la présence d'éthanol et à l'acidité (García *et al.*, 2021).

Compétition pour les nutriments

Lors d'une culture avec plusieurs types de levures, une compétition pour les nutriments peut s'instaurer du fait de vitesses de consommation et d'efficacité de transformation différentes selon les espèces en pr*ése*nce, entraînant alors un développement plus important de l'une des souches. Cette compétition peut être augmentée du fait de réactions induites par la présence d'autres microorganismes : sécrétion de molécules qui vont empêcher l'assimilation de nutriments par les autres, augmentation de la vitesse de consommation de certains substrats (sucres, composés azotés…) (Gianvito *et al.*, 2022; Harlé *et al.*, 2018). En effet, des analyses transcriptomiques permettent de montrer qu'une culture mixte entraîne des modifications de l'expression des gènes des deux espèces en présence, avec une réponse spécifique de chacune d'elles (modification du métabolisme du carbone, de l'azote, de l'absorption d'ions métalliques, du stérol) (Shekhawat *et al.*, 2019). La protéine Hsp12 semble jouer un rôle dans la communication intercellulaire et servir de signal pour activer les réponses au stress induit par la coculture. Le gène codant pour cette protéine a été observé comme surexprimé dans les premières heures d'une coculture entre *S. cerevisiae* et *T. delbrueckii,* et cela dans les deux levures, avec comme conséquence une stimulation de l'activité métabolique (glycolyse, métabolisme de l'azote) et une accélération de la consommation des substrats. La réponse de *T. delbrueckii* intervenant quelques heures plus tard que celle de *S. cerevisiae*, un phénomène de compétition pour les substrats pourra avoir un effet négatif sur sa croissance (Curiel *et al.*, 2017; Tempère *et al.*, 2018; Tronchoni *et al.*, 2017). Ces phénomènes de compétition pour les nutriments peuvent avoir des conséquences concrètes sur le déroulé des fermentations. Ainsi, la diminution de la disponibilité en azote du fait d'une consommation rapide en début de fermentation par des levures non-*Saccharomyces*, peut entraîner des fermentations languissantes ou stoppées, voire compromettre la croissance de *S. cerevisiae* si le ratio d'ensemencement *S. cerevisiae* / non-*Saccharomyces* est en faveur des non-*Saccharomyces* (Medina *et al.*, 2012). En effet, *S. cerevisiae* n'a plus suffisamment de sources azotées pour conduire la fermentation (celle-ci nécessitant plus d'azote que d'autres voies métaboliques comme l'oxydation, comme suggéré par une étude génomique (Backhus *et al.*, 2001)). L'ajout d'azote et de vitamines permet d'améliorer la fermentation, montrant également une compétition entre non-*Saccharomyces* et *S. cerevisiae* pour les vitamines ou mettant en évidence un effet de synergie entre azote et vitamines pour la croissance des levures (Medina *et al.*, 2012).

Si les levures sont en général en compétition pour des nutriments, avec un effet négatif sur la croissance d'une espèce, la situation peut également s'inverser au cours de la fermentation et se rapprocher d'une situation de commensalisme. Ainsi, en se développant en début de fermentation, les non-*Saccharomyces* consomment rapidement les acides aminés et vitamines, qui ne sont plus disponibles pour *S. cerevisiae.* Mais leur activité protéolytique peut également permettre d'enrichir le milieu en azote, disponible pour *S. cerevisiae* (Ciani & Comitini, 2015).

Composés toxiques

Au cours de la fermentation, différents métabolites sont produits par le consortium de levures (*Saccharomyces* et non-*Saccharomyces*) présent au sein du moût. Ces métabolites peuvent inhiber la croissance, voire entraîner la mort de certaines espèces de levures. Parmi ces métabolites se trouvent les composés contribuant à la qualité sensorielle du vin (alcools, acides) mais aussi des molécules avec un rôle toxique particulier (comme les peptides antimicrobiens).
Le principal composé toxique produit est bien sûr l'éthanol, vis-à-vis duquel les levures non-*Saccharomyces* semblent plus sensibles comme évoqué plus haut. Des effets toxiques sont observés dès 3 % (Borren & Tian, 2020). L'effet Crabtree, en inhibant le métabolisme respiratoire favorise les voies métaboliques fermentaires, et permet donc à *S. cerevisiae* d'utiliser en condition aérobie comme anaérobie les sucres pour produire de l'éthanol et du dioxyde de carbone. Ces composés inhibent la croissance des non-*Saccharomyces*, et confèrent ainsi à *S. cerevisiae* un avantage adaptatif sur celles-ci.
Les alcools supérieurs cycliques, les acides gras à chaîne moyenne et les acides organiques (par exemple, l'acide acétique) peuvent également avoir un effet toxique ou inhibiteur sur les levures (Ciani & Comitini, 2015). Les composés volatils tels que le phényléthanol peuvent également jouer un rôle dans les interactions entre levures (Gianvito *et al.*, 2022).
Un autre type de mécanisme lié à des composés toxiques est la production par une souche de peptides antimicrobiens, qui entraînent la mort des autres levures présentes dans le milieu, via l'endommagement de la membrane et/ou la modification du pH intracellulaire, mais avec des effets souche-dépendants (Branco *et al.*, 2014, 2015, 2018; Branco, Francisco, *et al.*, 2017; Branco, Kemsawasd, *et al.*, 2017). *S. cerevisiae* est connue pour inhiber via ce mécanisme certaines non-*Saccharomyces* (Albergaria *et al.*, 2010; Albergaria & Arneborg, 2016; Pereznevado *et al.*, 2006), mais certaines non-*Saccharomyces* sont également capables de synthétiser des peptides avec un impact sur d'autres levures et notamment la levure indésirable *Brettanomyces bruxellensis* (Ciani & Comitini, 2015; Escott *et al.*, 2017). La production de protéines dites "killer", létales pour des microorganismes qui y sont sensibles, est également rapportée, à la fois chez *S. cerevisiae* et les non-*Saccharomyces* (Gianvito *et al.*, 2022).

Quorum sensing

Une communication intercellulaire via des molécules signal a été mise en évidence dans des cultures mixtes de levures. Ce phénomène, appelé *quorum sensing*, se traduit par la production, par une cellule, de métabolites dans le milieu, qui, à partir d'une certaine concentration ("quorum"), se lient à des récepteurs d'autres cellules, entraînant une modification de l'expression des gènes chez celles-ci (Avbelj *et al.*, 2016). Ces molécules signal, produites en très faible concentration (µM) sont connues pour avoir un rôle dans la coordination du comportement d'une population de levures en réponse à l'environnement, comme le passage à une forme filamenteuse en cas de forte densité cellulaire chez *Candida*. Mais ce système de communication entre cellules, dépendant de la densité cellulaire, paraît également impliqué dans les interactions entre levures au cours de la fermentation alcoolique (Johansen & Jespersen, 2017; Zupan *et al.*, 2013).

Différentes molécules avec un rôle signal ont été identifiées comme pouvant être produites, pendant leur phase de croissance par *S. cerevisiae* et les levures non-*Saccharomyces* mais cette aptitude et les composés signal dépendent de l'espèce. Ces molécules proviennent souvent de la transformation des acides aminés aromatiques : tryptophol, mélatonine issus du tryptophane, tyrosol issu de la tyrosine, 2-

phényl-éthanol de la phénylalanine… (Johansen & Jespersen, 2017; Valera *et al.*, 2019; Zupan *et al.*, 2013). La production des alcools aromatiques avec un rôle signal est impactée par les conditions du milieu (concentration en azote, densité cellulaire, présence d'oxygène, d'éthanol) (Avbelj *et al.*, 2016).
L'ajout de tryptophol ou de mélatonine à des moûts synthétiques permet de confirmer le rôle de ces molécules sur la régulation de la croissance des levures : des changements dans les dynamiques de populations (phase de latence, vitesse de croissance, dominance) sont observés, avec des effets variables selon la concentration en composé bioactif (tryptophol, mélatonine), le type de culture (*Sc* seule, mélange de *N.S.*, ou mixte *Sc* / *N.S.*), les espèces de levures étudiées, et la concentration en azote du milieu (Valera *et al.*, 2019).
Cependant, la classification ou non comme *quorum sensing* des mécanismes d'interactions entre levures faisant appel à des molécules signal est complexe, et les critères de classification font l'objet de discussions au sein de la communauté scientifique (Winters *et al.*, 2019).

Contact cellulaire

Dès 2003, Nissen *et al.* (Nissen *et al.*, 2003) mettent en évidence que l'arrêt de croissance observé lors d'une culture mixte de *S. cerevisiae* avec certaines levures non-*Saccharomyces* n'est ni lié à une limitation par les nutriments, ni à la présence de composés inhibant la croissance, mais à un autre type d'interactions : le contact cellulaire. La croissance des non-*Saccharomyces* est inhibée en présence de grandes concentrations de levures *S. cerevisiae* viables et serait liée à un mécanisme de contact entre les deux levures. La séparation physique de deux populations de levures (membrane semi-perméable, réacteur cloisonné) permet en effet de limiter la décroissance des non-*Saccharomyces* observée en culture mixte (Englezos *et al.*, 2019; Kemsawasd *et al.*, 2015; Nissen *et al.*, 2003; Petitgonnet *et al.*, 2019; Renault *et al.*, 2013).
Il faut noter que dans certains cas, un effet inhibiteur sur la croissance de *S. cerevisiae* via un mécanisme lié à un contact physique avec des levures non-*Saccharomyces* a pu également être observé (Hu *et al.*, 2022; Luyt *et al.*, 2021).

Le contact cellulaire impacte non seulement la croissance des levures mais aussi leur métabolisme : augmentation de la consommation de nutriments, induction de la synthèse d'acides aminés nécessaires à la croissance, suppression de certaines voies métaboliques (Ehrlich), modification des métabolites produits et notamment des composés d'arômes (Hu *et al.*, 2022; Luyt *et al.*, 2021; Petitgonnet *et al.*, 2019).

D'un point de vue mécanistique, les interactions par contact cellulaire semblent étroitement liées à la fois à la densité cellulaire de la culture et aux mécanismes d'agrégation et de floculation présents chez les levures étudiées.
Tout d'abord, il semble en effet consensuel dans la littérature que leur impact varie significativement en fonction de la densité cellulaire de la culture étudiée. Ainsi, de multiples études ont montré que l'effet inhibiteur de *S. cerevisiae* sur diverses non-*Saccharomyces* par contact cellulaire ne prend place que lorsque la densité cellulaire est suffisamment élevée (Nissen *et al.*, 2003; Pereznevado *et al.*, 2006; Perrone *et al.*, 2013; Renault *et al.*, 2013).

Des expérimentations ont également mis en évidence le rôle central des mécanismes d'adhésion et de floculation dans la modulation des interactions levuriennes par contact cellulaire (Rossouw *et al.*, 2015; Shekhawat *et al.*, 2019). En effet, la délétion ou surexpression de certains gènes codant pour des protéines pariétales liées aux mécanismes d'adhésion chez *S. cerevisiae* (gènes *FLO*) modifient ainsi

significativement les impacts observables des interactions sur la croissance de diverses non-*Saccharomyces*, et par conséquent les dynamiques de population des cultures mixtes correspondantes (Rossouw *et al.*, 2018). Cette dernière étude évoque ainsi la possibilité d'agir sur ces propriétés de floculation et d'agrégation pour impacter les interactions par contact cellulaire et donc de les utiliser comme levier d'action potentiel pour contrôler les dynamiques de population en culture mixte. Une autre étude a permis de mettre en évidence des modifications métaboliques à la fois chez *S. cerevisiae* et *L. thermotolerans* lors de cocultures, concomitantes à des réponses transcriptomiques liées à la structure pariétale ainsi qu'aux phénomènes d'adhésion, ce qui laisse penser à un lien potentiel avec le contact cellulaire (Shekhawat *et al.*, 2019).

Mécanismes multiples

Néanmoins, tous ces mécanismes d'interactions entre levures ne sont pas complètement indépendants. La forte densité cellulaire à l'origine des interactions par contact cellulaire peut également favoriser les interactions via des composés inhibiteurs, en augmentant leur concentration et facilitant donc leur action. Elle peut également être à l'origine du *quorum sensing*, la liaison des molécules signal à leurs récepteurs n'intervenant que lorsqu'une certaine concentration de cellules et donc de composés signal est atteinte ("quorum") (Avbelj *et al.*, 2016).
Les interactions peuvent combiner plusieurs mécanismes comme le contact cellulaire et l'implication de peptides antimicrobiens (Kemsawasd *et al.*, 2015). Les peptides peuvent en effet s'accumuler au niveau de la membrane de la paroi et provoquer la mort des non-*Saccharomyces* par contact cellulaire (Branco, Kemsawasd, *et al.*, 2017). D'autres auteurs suggèrent également que le contact cellulaire pourrait entraîner la production de composés toxiques (comme les acides gras à chaîne courte ou moyenne) ou de composés volatils (comme le phényléthanol) pouvant avoir un rôle signal (Luyt *et al.*, 2021). La production de ces derniers peut en outre être très influencée par les paramètres environnementaux (Dzialo *et al.*, 2017).
Le contact cellulaire peut également avoir comme conséquence une accélération de la consommation de nutriments et entraîner ou accentuer un phénomène de compétition entre souches, nuisant au développement de l'une d'elle, comme observé entre différentes espèces de *Saccharomyces* (Alonso-del-Real *et al.*, 2019).

Devant la complexité de ces interactions ainsi que la diversité des mécanismes qui les sous-tendent, il est nécessaire pour les étudier de faire appel à de multiples techniques d'analyses et des méthodologies variées. La partie suivante de cet état de l'art s'intéresse à ces méthodologies.

1.3. Méthodologies d'étude des interactions entre levures

La synthèse de l'état de l'art sur les méthodologies d'étude des interactions entre levures a donné lieu à une publication dans le journal *Microorganisms* sous la forme d'une revue bibliographique (Bordet *et al.*, 2020).

Ces travaux de synthèse méthodologique sont en effet rapidement apparus nécessaires, à la fois pour donner un meilleur cadre d'interprétation aux résultats parfois contradictoires ou peu reproductibles observés dans la littérature, ainsi que pour dresser un état des lieux des méthodologies utilisables et pertinentes pouvant par la suite aider à mieux appréhender la complexité des interactions entre levures discutée plus haut.

Cette revue s'attache donc dans un premier temps à dresser l'état des lieux des méthodologies existantes et éprouvées, ainsi que des méthodologies en émergence, notamment autour des études transcriptomiques et métabolomiques. Ensuite, ces travaux font la synthèse des résultats obtenus par ces nouvelles méthodologies, concernant l'impact des interactions levure / levure sur la composition chimique du vin, à la fois en composés volatils et non-volatils. Enfin, la revue s'intéresse aux résultats récents reliant les interactions levuriennes pendant la fermentation au profil sensoriel réel obtenu sur le vin fini.

microorganisms

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Review

Yeast–Yeast Interactions: Mechanisms, Methodologies and Impact on Composition

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Abstract: During the winemaking process, alcoholic fermentation is carried out by a consortium of yeasts in which interactions occurs. The consequences of these interactions on the wine matrix have been widely described for several years with the aim of controlling the winemaking process as well as possible. In this review, we highlight the wide diversity of methodologies used to study these interactions, and their underlying mechanisms and consequences on the final wine composition and characteristics. The wide variety of matrix parameters, yeast couples, and culture conditions have led to contradictions between the results of the different studies considered. More recent aspects of modifications in the composition of the matrix are addressed through different approaches that have not been synthesized recently. Non-volatile and volatile metabolomics, as well as sensory analysis approaches are developed in this paper. The description of the matrix composition modification does not appear sufficient to explain interaction mechanisms, making it vital to take an integrated approach to draw definite conclusions on them.

Keywords: yeast–yeast interactions; wine; mixed culture; methodologies; fermentation conditions; omics; sensory

1. Introduction

The transformation of grape must into wine is a complex process involving various microorganisms: yeasts, molds, and bacteria. The main step, alcoholic fermentation, is performed by yeasts. In natural fermentation, microflora comes from grape berries but also from winery equipment and surroundings. Yeast biodiversity on grape berries is governed by various biotic and abiotic factors such as grape variety, climatic conditions and viticultural practices [1–3]. Yeasts present on grapes are mainly from non-*Saccharomyces* genera (essentially *Hanseniaspora*, *Candida*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Cryptococcus* and *Rhodotorula* [3]) while *Saccharomyces* genera are very rare. However, although non-*Saccharomyces* yeasts initiate fermentation and develop during the first hours, their population declines rapidly in favor of *Saccharomyces cerevisiae* (*S. cerevisiae*), which becomes the dominant species until the end of alcoholic fermentation. The evolution of yeast populations during fermentation seems to be linked to several modifications that make the medium more selective. The establishment of nutrient depletion, anaerobic conditions, increased acidity, the production of sulfur dioxide, and increasing levels of ethanol (up to 15%v/v) results in a drop in yeast diversity [2]. This modification of

the matrix environment allows the survival of *S. cerevisiae* because of its overall better resistance to stress compared to non-*Saccharomyces* species [4].

Producers have used wine starters for many decades to ensure proper fermentation initiation and the quality and reproducibility of wine. Indeed, starter yeasts allow efficient fermentation management that limits contaminations and avoids deviations due to interrupted or sluggish fermentations [5]. These starter yeasts are selected for their specific metabolic properties: resistance to various stresses, fermentation capacity, or the presence of enzymatic activities [6]. The ability of *S. cerevisiae* to grow in a selective medium as described above, to carry out efficient and quick alcoholic fermentations, make this species a tool of choice as an oenological starter [5].

However, in recent years, non-*Saccharomyces* yeasts have been used for wine production since several yeast species have shown high oenological potential [7,8]. Indeed, yeasts like *Saccharomyces* non-*cerevisiae* [9,10], non-*Saccharomyces* [6,7,11–16], and even natural hybrids [17–21] are of interest, because their different metabolisms compared to *S. cerevisiae* brings diversity to quantitative and qualitative composition of final wine (for example, ethanol content, organic acids, aroma production) [3,22,23]. Nevertheless, all these studies show that the utilization of these yeasts, in combination with *S. cerevisiae*, as wine starters, is still a challenge, since the results are unpredictable and lack of reproducibility. The conduct of fermentations by managing the simultaneous or successive implantation of different strains to obtain the desired impacts on wine has not yet been mastered. It is therefore necessary to understand the phenomena involved in the evolution of the yeast ecosystem during alcoholic fermentation, to control these mixed cocultures more efficiently. In recent years, numerous researchers have furthered research in understanding interaction mechanisms between microorganisms, since these interactions impact not only the population dynamics but also the metabolism of each strain, with consequences on the compounds produced, and eventually on final wine quality.

Authors have shown the existence of different interaction mechanisms between yeasts: competition for nutrients, the production of inhibitory or toxic compounds, the modification of metabolism by a quorum-sensing answer or induced by cell-contact. But all these results highlight that yeast interactions during wine alcoholic fermentation are very complex because of the variations according to yeasts (species, strain), medium composition, and abiotic conditions (oxygen, temperature). These fickle results (as described in a recent review from Conacher et al. (2019) [24]) seem to be linked to the sheer diversity of the methodologies employed. Each team often works with different strains including commercial and indigenous strains, different types of culture media, various matrixes (synthetic must or musts from different grape varieties) and also different culture modalities Each of these factors can impact the population and fermentation dynamics, by tipping the balance of a fragile equilibrium between species one way or another. Despite the great need for an overview of these methodological differences, so far none is available, although it would lead to better comparisons of results, and provide a synthesis of standard protocols for newcomers in the field.

The objective of this review is to highlight recent scientific developments concerning yeast–yeast interactions. First, the different methodologies employed will be discussed, including recent contributions from transcriptomic and metabolomic approaches. The impact of interactions on volatile and non-volatile composition will then be considered. Finally, the consequences of interactions on wine sensory characteristics will be discussed in-depth.

2. Methodologies

2.1. Parameters: Inoculation and Culture Conditions

Numerous studies have been performed on mixed cultures between *S. cerevisiae* and non-*Saccharomyces* to understand how yeast interactions can impact wine quality. Authors have monitored population dynamics, fermentation parameters, metabolite production, especially aroma compound production, and highlighted interaction mechanisms. But contradictory results can be found in this field, as shown in Table 1 and Table S1, which include the conditions and results of experiments for several couples of yeasts, those most studied to improve wine quality.

Table 1. Diversity of methodologies and results in *S. cerevisiae/Lachancea thermotolerans* interaction experiments.

Species		*S. cerevisiae/Lachancea thermotolerans*																				
Matrix		Synthetic Medium										Grape Juice/Must (Unspecified Color)										
		SGJ	WYPD		SGJ	WYPD		SGJ				Grape Juice		Grape Must					Grape Must			
Sugar Initial Concentration (g/L)		210 (Glc)	200 (Glc)		210 (Glc)	200 (Glc)		200 (Glc 100 Fru 100)				162		160					231			
YAN/PAN (mg/L)																			PAN 154/NH3 22			
Temperature (°C)		25	25		25	25		25				25		20					25			
Oxygen		+/-	+/-	+	+/-	+		-	+	++	+++	+/-	+	+					-			
Inoculation delay between two strains (h)		0	0		0	0		0				0	0	0	24	48	72		0			
Ratio Sc/NS		1:1	1:1		1:1	1:1		1:10				1:1	1:1	1:1					1:1	1:100	1:10,000	
Sc population	Max	2	1	1	2	1	1	3	3	3	3	2	2	1	5	7	7		2	4	15	
	Dominance	+	=		+	+ 1	+ 1	+				+	+	+ 1	+ 2	+ 7	+ 12		+ 1	+ 10	+ 15	
	Decrease	-	2.5	2.5	-	-	-	-	3	3	3	6	-	-	-	-	-		-	-	-	
NS population	Max	2	1	1	2	1	1	1	1	1	1	2	2	3	2	5	3		1	3	3	
	Decrease	3	2.5	2.5	3-6	1	1	-	-	-	-	3	4	3	6	6	10		3	10	15	
	Detectable End	Low 6			Low 6-10	No 6	Low 6/Yes					Low 4	Low 6	No 7	No 8	Low 8 No 30	Low 30		No 15	No 22	No 22	
Fermentation completion		10			10	6	5					10	10	+	+	+	+		+			
Ethanol								=	- -	- -	- -			- -	- -	- -	=		- -			
Glycerol								++	- -	- -	- -			=	++	++	++		++			
Organic acids	TA													++	++	++	++		++			
	VA													- -	- -	- -	- -		- -			
	LA													++	++	++	++					
	AA																					
Impacting Factors								OX/SPE				OX		DEL					RAT/SPE			
Interaction Mechanisms		No TOX/No COMP/CCC	No TOX/No COMP/No QS/CCC		COMP	TOX/CCC		COMP				No TOX/COMP										
Reference		[25]	[26]		[27]	[28]		[29]				[30]		[31]					[32]			

Table 1. *Cont.*

Species		*Saccharomyces cerevisiae/Lachancea thermotolerans*									
Matrix		Red Grape Juice/Must									
		Sangiovese - Cabernet Sauvignon 1:1		Sangiovese		Tempranillo	Tempranillo	Shiraz	Tempranillo	Tempranillo	Pinot Grigio
Sugar Initial Concentration (g/L)		254		222		249	226	280 (Glc 140 Fru 140)	245	226	236
YAN/PAN (mg/L)		PAN 160/NH_3 36				PAN 167	PAN 241	YAN 170 (supp)	YAN 181	PAN 333	YAN 235
Temperature (°C)		20	30	25		20	25	25		25	22
Oxygen		++		+		+	-	++	+	+	+
Inoculation delay between two strains (h)		0		0	48	96	96	96	72	72	48
Ratio Sc/NS		1:10		1:10	1:10	10:1	1:1	1:1	1:1	1:1	1:1
Sc population	Max	4	1	4	7	6	8				
	Dominance	-									
	Decrease	-	-	4	7	-	-	-		-	-
NS population	Max	4	4	2	2	4	4				
	Decrease	18	4	4	4	4	4	+	+	5	3
	Detectable End		No 7	Low 10	Low 10	No 8	Low 10	No 7	No 6		Low 5
Fermentation completion		24	13			12	10	17			14
Ethanol		--	=	--	--	--	-	=	--	--	--
Glycerol		++	+	++	++	++	++		+	=	++
Organic acids	TA	++	++	++	++						
	VA	+	--	++	++			++	= or ++		
	LA	++	+	++	++		++			++	+/-
	AA					--	--			--	++
Impacting Factors		TEMP/DEL/GRA/REAC						SPE	SPE		SPE/NS
Interaction Mechanisms											
Reference		[33]				[34]	[35]	[36]	[37]	[13]	[14]

Table 1. *Cont.*

Species		*Saccharomyces cerevisiae/Lachancea thermotolerans*																			
Matrix		White Grape Juice/Must																			
		Grape Must			Riesling	Emir				Airen		Sauvignon Blanc	Airen + Catechin Addition	Chardonnay		Riesling	Pedro Ximenez/Sun-Dried			Grape Must
Sugar Initial Concentration (g/L)		224			237	205				245		217	234	218		225	487			212
YAN/PAN (mg/L)		PAN 154/NH3 22			PAN 147					PAN 177		YAN 170 (supp)				YAN 250 (supp)				251
Temperature (°C)		25			20	18				25		15	25			20	22			20
Oxygen		-				+				+		+	-	-	++	+	+			+
Inoculation delay between two strains (h)		0	24	48	48	0	24	48	72	0	96	96	168	0		48	0			24
Ratio Sc/NS		1:10	1:10	1:10	10:1	1:1				1:2.5	1:2	1:1	1:1.3	1:10		1:1	1:5	1:20	1:50	1:1
Sc population	Max	2	1	2	4	4				4	8			4	2	4				3
	Dominance	+ 11	-	-		+											-	-	-	+
	Decrease	-	-	-	-	6	6			-	-			6	6	14				-
NS population	Max	1	2	2	2						4			0		0	6	6	6	1
	Decrease	11			2	4	6			+	4	+	+ or -	4	6	4	-	-	-	5
	Detectable End	Low 18			No 15	Low 7	Yes			No 6	No 8	No 14		No 6	No 8	No 6				
Fermentation completion		+	-	-	+	7	9			10	14	19		10	12	+	-	-	-	16
Ethanol		- -	- -	- -	-	=	- -			-	- -	=	=	=	- -	=	- -	- -	- -	- -
Glycerol		+	++	++	++					-	++			=	=	++	- -	- -	- -	
Organic acids	TA	++	++	++		++	++						- -			=				
	VA	- -	- -	- -		++	+++					- -	- -							
	LA	++	++	++	+					++	+++		++			++				
	AA				=					=	- -	-		=	=	=	- -	- -	- -	
Impacting Factors		TEMP/DEL/GRA/REAC			SPE	DEL						SPE	MED	OX/SPE		DEL	RAT			
Interaction Mechanisms														COMP						CCC/COMP
Reference		[33]			[38]	[39]				[40]		[41]	[42]	[43]		[44]	[45]			[46]

YAN = yeast assimilable nitrogen/**PAN** = primary amino nitrogen. **Sc** = *S. cerevisiae*/**NS** = Non-*Saccharomyces*. **TA** = total acidity/**VA** = volatile acidity/**LA** = lactic acid/**AA** = acetic acid. **SGJ** = Synthetic Grape Juice/**Glc** = Glucose/**Fru** = Fructose/**WYPD** = Yeast Peptone Dextrose medium modified for wine fermentation/**Supp** = with supplementation. **Oxygen**: - = anaerobia, +/- semi-anaerobia, low oxygenation, + = semi-anaerobia, ++ aerobia, +++ aerobia, with higher oxygenation. **Population columns**: **Max =** maximal population reached by day x/**Dominance**: + = dominance of *S. cerevisiae*, = similar populations, "x" = dominance obtained after x days/**Decrease** = decrease since day x/**Low** = low population since day x/**No** = population not detectable since day x/**Yes** = population still detectable at day x. **Fermentation completion**: x = reached at day x, +/- = reached/not reached during experimentation. **Ethanol, glycerol and organic acids** are compared to Sc pure culture: +++ very high increase, ++ high increase, + increase, +/- slight decrease, = no change, - decrease, - - high decrease. **Impacting factors**: inoculation delay (**DEL**), inoculation ratio between *S. cerevisiae* and non-*Saccharomyces* yeast (**RAT**), yeast species (**SPE**), yeast strain *S. cerevisiae* (**SC**) or non-*Saccharomyces* (**NS**), medium composition (**MED**), grape nature (**GRA**), temperature (**TEMP**), oxygenation (**OX**), type of reactor (lab, pilot, industrial) (**REAC**). **Interaction mechanisms**: involvement of quorum sensing mechanisms (**QS**), toxic compounds (including ethanol, antimicrobial peptides) (**TOX**), competition for nutrient (including oxygen) (**COMP**), cell-cell contact mechanisms (CCC)/**No** = mechanism involvement has been ruled out by the study.

Variability in population dynamics results can be observed depending on the various studies. The *S. cerevisiae* population is not affected in most experiments by the presence of another yeast, even if some exceptions exist [12,29,39,46,47]. On the other hand, the presence of *S. cerevisiae* usually negatively impacts non-*Saccharomyces* growth and early decline and even early death are often observed, but some authors have observed the stability of non-*Saccharomyces* yeasts during a longer period [33,45]. Fermentation kinetics can also be different. Mixed cultures with non-*Saccharomyces* yeasts can lead either to complete fermentations (within different timeframes) [48,49], or to incomplete fermentation [12,33]. The production of metabolites such as glycerol, acids, and aroma compounds is also variable [31,33].

Yeasts are often inoculated at a cell count of 10^6 cells/mL since this corresponds to the conditions occurring in natural fermentation [50], in which there is dominance of non-*Saccharomyces* populations at the early stage, but inoculation density can vary between 5.10^4 [26] and 2.10^7 cells/mL [29,51].

The first hypothesis to explain this diversity of results is medium composition, which is known to impact yeast physiology, metabolism, and yeast interactions. Table 1 and Table S1 show that numerous authors choose to use real grape juice or must to approach winemaking conditions. But natural grape must is not standardized and its composition varies depending, for example, on the year, harvest time, and grape variety. Englezos et al. (2016) [12] and Nisiotou et al. (2018) [49] both conducted mixed fermentation with *Starmerella bacillaris* (*S. bacillaris*) in similar conditions (temperature and inoculation) but obtained different results. Indeed, contradictory results are reported in terms of non-*Saccharomyces* persistence and fermentation completion reflecting the influence of the matrix composition on yeast interactions. However, other differences (must sterilization, yeast strain) in methodology can also explain these discrepancies.

Initial sugar concentration can impact yeast growth but also the capacity of yeasts to interact with other yeasts. The ability to take up glucose varies with glucose concentration with a species-dependent effect. Outside the 160-190 g/L range, non-*Saccharomyces* yeasts are less able to take up glucose and become less able to compete with *S. cerevisiae* in mixed cultures [27]. In addition, initial sugar concentration and the amount of sugar metabolized by *S. cerevisiae* could have an impact on toxic compound production and further on population dynamics in mixed cultures: a delay in *Hanseniaspora guillermondii* (*H. guillermondii*) death can be observed when the initial sugar concentration is 100 g/L, compared to a standard medium with 200 g/L [52]. These effects are not always verified since *Lachancea thermotolerans* (*L. thermotolerans*) can survive until the end of fermentation with 200 g/L of initial sugars [39] but not with 160 g/L of initial sugars [31], although other factors can be involved (total population, oxygenation, strain). At high sugar concentrations (up to 200-300 g/L), yeasts can delay their growth and have a lower growth rate, with a possible effect on population dynamics [53]. However, *L. thermotolerans* can be used in the mixed fermentation of sun-dried grapes with a very high sugar content and become the dominant strain if inoculated at a high ratio [45].

Other medium components are also important to manage since *S. cerevisiae* and non-*Saccharomyces* yeasts have different needs and do not metabolize nutrients in the same way. A key point is nitrogen source quality since ammonium and amino acids may be assimilated or not, with various rates according to the strain and culture conditions like temperature [54–57]. In addition, competition for nutrients can occur between strains and limiting nutrients concentrations can increase their interactions. For example, in sequential mixed cultures, an insufficient initial amount of assimilable nitrogen can be entirely consumed by non-*Saccharomyces* yeast before the inoculation of *S. cerevisiae*, resulting in incomplete fermentation [51]. Moreover, nitrogen availability has an impact on the ability of yeast to compete with other strains. When nitrogen is limited, indigenous *S. cerevisiae* strains are more competitive toward commercial *S. cerevisiae* and can co-dominate fermentation; they have higher nitrogen demand and can quickly remove nitrogen from the medium, which is then no longer available for commercial strains [58]. In addition, as the production of aromatic alcohols (as tyrosol, tryptophol), known as quorum sensing molecules, is linked to nitrogen metabolism, the amount of nitrogen can impact this production or the effects of these molecules [59].

All these differences in must composition (sugars, YAN) can cause variabilities in yeast interactions and complicate understanding of the mechanisms involved. Considering this must variability, some authors choose to standardize their fermentation medium by supplementing must with sugar or nitrogen sources. However, referring to Table 1 and Table S1, it can be seen that the target level is not always the same, making comparisons between studies difficult. Moreover, Englezos et al. (2018) [60] observed that even with standardized sugar and YAN composition, grape variety still has a significant impact on non-*Saccharomyces* persistence during the fermentation process, hinting at the impossibility of effectively standardizing real must.

An alternative to these limits would be to use a synthetic medium with a fixed composition that can simulate natural must. Some researchers have chosen to use a quite simple medium such as a classical laboratory medium supplemented with sugars [26,28], while others have used compositions more similar to natural must, called "synthetic must" or "synthetic juice" [61]. Here, several key choices subsist, as some nutrients can impact yeast dynamics: the proportion of fructose in sugars [62,63], vitamins, and growth factors [64]. Authors have usually used this type of media to allow for effective standardization while studying the impact of a specific factor on interactions. For example, Wang et al. in 2016 [65] used synthetic must to show strain dependence in interactions between multiple *Saccharomyces*/ non-*Saccharomyces* couples. Shekhawat et al. (2017) [29] also used synthetic grape juice to study the impact of must oxygenation on mixed cultures of *L. thermotolerans*/*S. cerevisiae*.

The inoculation procedure is also an important parameter. Authors have conducted mixed fermentations with the simultaneous or sequential inoculation of yeasts, with various times between both inoculations. The addition of *S. cerevisiae* after non-*Saccharomyces* can delay their death and increase their influence on wine characteristics. *L. thermotolerans* can be present until the end of fermentation, when *S. cerevisiae* is added 24, 48, or 72 h afterwards [33,39], but its population decreases drastically when a longer delay is used (4 days) [35,41]. However, some authors have observed a decrease with inoculation performed after 24 h [46], 48 h [14,31,44], or 72 h [37,66] indicating that other factors (medium composition, temperature, oxygenation) can interact. Non-*Saccharomyces* can also, in sequential culture, become the dominant strain and negatively impact *S. cerevisiae* growth, as was shown for *L. thermotolerans* [39], and *S. bacillaris* [12,47,49,60,67–69].

Different inoculation modes can also be coupled with different inoculation ratios between the two strains present in mixed culture, which has great importance for population dynamics. In spontaneous fermentation conditions, the yeast population of freshly extracted must is overwhelmingly constituted by non-*Saccharomyces*, with *S. cerevisiae* accounting for less than 1% of the total yeast population [70]. To simulate natural conditions, some researchers have inoculated using a large amount of non-*Saccharomyces* compared to *S. cerevisiae*, with the objective of improving non-*Saccharomyces* persistence during mixed fermentation. Usually, a ratio favoring a specific yeast has a positive impact on the latter's population dynamics; longer persistence, higher population, and dominance, as was shown for *S. bacillaris* [32], *Saccharomyces kudriavzevii* (*S. kudriavzevii*) [9], *H. guilliermondii* [52], *L. thermotolerans* [45]. However, adjusting inoculation ratios is not always enough to obtain the persistence of non-*Saccharomyces* yeasts [48,51]. The initial amount of yeasts can also have an impact since the death of non-*Saccharomyces* yeasts can be linked to the presence of the high cell density of *S. cerevisiae* (*H. guilliermondii* declines when *S. cerevisiae* reaches 10^7 CFU/mL [52]).

The physiological state of yeast can also have an impact on interaction. Branco et al. (2017) [71] showed that *S. cerevisiae* induces the death of non-*Saccharomyces* yeasts in mixed cultures, by different mechanisms, depending on its physiological state when mixed culture begins: cell-contact is involved when *S. cerevisiae* is in stationary phase and not when it is in mid-exponential phase. A potential explanation could be the accumulation of antimicrobial peptides on the cell-surface during *S. cerevisiae* growth, according to these authors.

Other culture parameters such as temperature can also impact population dynamics and ecosystems, since yeasts have different optimal growth temperatures [72]. *S. cerevisiae* is better

adapted to higher temperature and even modifies temperature through heat production during fermentation [73]. The application of low temperature can favor the growth, survival, and even dominance of non-*cerevisiae* species [74–76] and of non-*Saccharomyces* yeasts [77]. Temperature can also increase the competitive ability of non-*Saccharomyces*: *L. thermotolerans* has an inhibitory effect on *S. cerevisiae* growth at 20 °C, while at 30 °C, *S. cerevisiae* competes better and *L. thermotolerans* biomass declines after 4 days [33]. Maturano et al. (2016) [78] even showed that the temperature of cold maceration prior to alcoholic fermentation can positively impact interspecific distribution. Temperature can also impact yeast metabolism. The response of *S. cerevisiae* against the presence of another strain (coculture), by gene expression, is indeed dependent on temperature (transcriptional response higher at 12 °C than at 20 °C) [10]. The variation of fermentation temperature may be involved in the variability of the results obtained by various authors. Differences in fermentation kinetics observed with red and white must (complete at 17 and 24 days respectively) by Whitener et al. and Becker Whitener et al. can be partly explained by differences of temperature (25 and 15 °C) [36,41]. Englezos et al. also explained the different impacts on ethanol content in their works of 2016 and 2018 by temperature differences between protocols [79]. Bagheri et al. recently showed that the population dynamics in a multi-species yeast consortium were affected by temperature, influencing consequently aroma compounds production [80].

One other key point used to explain the differences observed in population dynamics is oxygen availability, induced by different conditions of oxygenation and agitation in various authors' protocols. Oxygen is indeed also known to have impacts on yeast interactions. Non-*Saccharomyces* yeasts are usually less tolerant to low oxygen availability than *S. cerevisiae* [30]: oxygen can increase their survival in mixed culture without affecting *S. cerevisiae*, resulting in a species-dependent variation in population dynamics (persistence) [29,43,67].

On the contrary, removing oxygenation and agitation altogether and allowing fermentations to occur in static conditions makes it possible to get closer to standard vinification conditions. These conditions, besides limiting oxygen intake, also allow the natural sedimentation of yeasts to occur. This sedimentation leads to heterogenous cell distribution, with an increase of local cell density in the sediment and a decrease in the supernatant. Cell density, as shown by Nissen et al. (2003) [26] is a key factor in yeast interactions. Cell density can also favor cell–cell contacts and coaggregation mechanisms which both seem to be involved in the population dynamics and metabolic changes observed in mixed cultures [24].

Studies have also shown that yeast interactions are heavily strain-dependent. Perrone et al. (2013) [81] studied 99 strains of autochthonous *S. cerevisiae* in must and showed that their dominance behavior varies and is expressed only when *S. cerevisiae* senses other yeasts in the same environment. As far as interactions between non-*Saccharomyces* and *S. cerevisiae* are concerned, mechanisms can be influenced by the strain chosen for both species. Wang et al. (2016) [65] observed that culturability loss of non-*Saccharomyces* because of interactions with *S. cerevisiae* is species- and strain-dependent. On the other hand, Englezos et al. (2016, 2019) [12,69] showed that *S. cerevisiae* strain choice also has a key impact on population dynamics (*S. bacillaris* is more or less able to dominate various *S. cerevisiae* strains), sugar consumption, wine composition (ethanol, glycerol, acid production), wine volatile compounds (decrease or increase of aroma production depending on the *S. cerevisiae* strain).

2.2. *Yeast Interactions: Understanding Population Dynamics*

When yeasts are cultivated together in the same medium, as happens in natural must, different interactions occur, with visible and measurable impacts on population dynamics (dominance of one strain, decline or death of others) and cell physiology.

Researchers usually approach population monitoring quantitatively: using the methods described below, they manage to get an overview of general population dynamics. However, a more qualitative approach can supplement this, by giving more information on the physiological state of the yeast cells monitored.

Yeast populations in mixed fermentations are often quantified by traditional methods such as plating using colony morphology, media composition, selective additives, and/or differential growth optima which allow distinguishing between different species [14,60,69,82–84]. Yeast populations can also be discriminated by using a combination of selective and non-selective culture media [26,33,52,65,71,85–87]. The incubation of plates at different temperatures can also be the solution to determine the population of different strains, the main example of this being *S. cerevisiae* growing at 37 °C while non-*cerevisiae* and non-*Saccharomyces* yeasts do not [10,88,89].

These methods are well understood, efficient, and rather precise. Moreover, they allow accurate interspecies discrimination using phenotypic differences, and provide information on the cell cultivability of the populations studied. However, growth time on plates involves a delay in analysis which can prove impractical when monitoring wine fermentation in real time. In addition, these culture-dependent techniques can hardly be used to monitor complex ecosystems, since some strains overcome others in culture medium [90]. They also can overlook microorganisms that grow slowly on artificial media or are present in very small amounts [72]. Moreover, sometimes, no colonies can be observed on plates, since some yeasts are in a viable but non-culturable state (VBNC) as a result of stress induced either by interaction with other yeasts or culture conditions. To confirm this state and evaluate the capacity of yeasts to recover, they are transferred into fresh liquid nutritive medium and incubated for 24-48 h, once or twice: VBNC yeasts after these cultures in ideal conditions can be cultivated again [65,91].

The need for new or adapted analysis methods that shorten the delay in obtaining information has thus emerged. Zupan et al. (2013) [92] presented a quick method to monitor the number of viable yeasts during fermentations, using microscopy and image analysis software; yeasts are observed on a hemocytometer with three settings of the same microscope to count viable, non-viable and total cells.

Flow cytometry is also an interesting technique used to enumerate microbial populations by automating the counting process, and Longin et al. (2017) [93] showed its potentiality for monitoring yeast populations during wine fermentation. As with plating, discriminating between both species studied is essential to monitor populations and highlight yeast interactions. To differentiate various strains, modified strains expressing fluorescent proteins are often used, as in the recent study by Petitgonnet et al. (2019) [46], which makes use of a GFP-modified *S. cerevisiae* to show its capacity to inhibit *L. thermotolerans* by cell–cell contact linked mechanisms. In these cases, it is necessary to verify that these modified strains have the same behavior as wild strains. This allows managing the proportion of *S. cerevisiae* in a mixed culture [94,95]. Another strategy is to use fluorescence in situ hybridization (FISH), like Wang et al. in 2014 [96], who developed specific probes and optimized conditions to monitor *S. cerevisiae* and two non-*Saccharomyces* yeasts in mixed cultures. This method is simple, rapid, and sensitive, but it involves membrane permeabilization and does not give information in real time or on cell viability. Flow cytometry can also be used to obtain extensive information on cell physiological state, as discussed below.

Authors have also used quantitative PCR to monitor yeast populations: from the isolated total DNA, amplifying a gene with species-specific primers gives the proportion of each species, and then extrapolates the population of each species in the total population. This method is rapid and very sensitive [90]. Andorra et al. (2010) [72], Wang et al. (2015) [70] studied the total population present in natural must by different techniques and showed that qPCR can be used to analyze the dynamics during wine fermentation; its advantage over culture-dependent techniques is that it takes into account non culturable yeasts. This qPCR technique was used recently to study the impact of competition between *S. cerevisiae* and other *Saccharomyces* yeasts on growth fitness and to understand the impact of nitrogen on competition between different strains of *S. cerevisiae* [58,76]. Garcia et al. (2017) [97] applied this method to monitor five non-*Saccharomyces* strains in a mixed culture, with satisfying efficiency (good specificity, sensitivity down to 10^3 cells/mL, linearity). Another method, reverse transcription (RT)-qPCR can be used but this methodology underestimates the culturable population in wine due to the decrease of rRNA level in cells facing environmental stress (ethanol, nutrient depletion) [70]. These

RNA/DNA-based methods have several advantages; they save time, they are interesting in the case of microorganisms difficult to cultivate (need for specific medium, VBNC) [70,90] and they provide precise discrimination between strains. However, these methods do not provide any information on cell physiological state or viability, since DNA from dead cells is also detected.

To study the physiological state of yeasts from mixed cultures, authors have used specific staining with different compounds and probes, allowing them to either assess viability, and even to study specific consequences on some aspects of cell physiological state. Coupled with flow cytometry (or epifluorescence microscopy), staining can provide rapid information, but choosing suitable dyes is complex and depends on both the microorganism and medium. Longin et al. (2017) [93] discussed these choices extensively in their review on flow cytometry applied to wine. For example, staining with propidium iodide (PI) can be used to evaluate the viable populations in mixed culture [46], or to study the impact of interaction mechanisms such as anti-microbial peptides on membrane permeability [91,98]. Another marker, fluorescein di-acetate (FDA) or carboxy-FDA (CFDA), can be used as an indicator of cellular vitality since it reflects enzymatic activity (esterase) [93]. This fluorophore is used by Gobert et al. (2017) [55] in cocultures involving *S. bacillaris* and *S. cerevisiae*, to monitor yeast viability during fermentations. Double stainings are often used to help discriminate live cells and assess viability during mixed fermentations [65,93,99].

Fluorophores can also be used to measure intracellular pH (pHi) since this important parameter influences metabolism and can lead to cell membrane disruption if it is modified [93]. Probes such as 5,6-carboxy-2′,7′-dichlorofluorescein diacetate (CDCF) or 5,6-carboxy fluorescein diacetate succimidyl ester (cFDA-SE) (for pH values between 3-4.5 and 4-7 respectively) combined with flow cytometry, epifluorescence microscopy or fluorescence ratio imaging microscopy (FRIM), give information on pHi [91,98], highlighting how exposure to anti-microbial peptides (AMPs) can induce a drop in pHi in non-*Saccharomyces* cells during fermentation.

Based on DNA-techniques, other methods have been developed to show a difference between live and dead cells, by using dyes able to enter cells with compromised membranes and bind to DNA, making them non amplifiable by PCR. Ethidium monoazide bromide (EMA) qPCR can then be used to monitor viable yeasts during must fermentation [70,100]. FISH can also be coupled with live/dead staining such as IP/DAPI [99], to assess the identity and viability of strains in mixed cultures at the same time.

Yeast interactions impact population dynamics and their metabolism with consequences for fermentation kinetics (more or less rapid and complete consumption of substrates (sugars, yeast assimilable nitrogen, oxygen), the production of ethanol) and for the production of other metabolites (differences in quality and content (glycerol, organic acids, aroma compounds)). Authors have usually used the same methods to monitor all these compounds: enzymatic techniques [58,65,84], or high-performance liquid chromatography HPLC [9,54,69,81,101]. Fourier transformed infrared spectroscopy FTIR can also be used since it is very convenient, simple, and rapid, but the results can lack precision. More recently, a new approach is to study more globally the metabolites produced by yeasts [46,84,102] to obtain information on the global metabolism of strains and better evaluate the role of each strain in imprinting its own metabolomic signature on the mixed culture medium.

2.3. *Yeast Interactions: Understanding Mechanisms*

Although most authors have studied population dynamics and metabolite production, some of them have focused on various mechanisms involved in yeast interactions. Interactions can be linked to modifications of medium during fermentation (decrease of nutrients or the production of inhibitory or toxic compounds) or to the direct action of a yeast on another one (with physical cell-contact, through molecules present on the cell surface). To understand these phenomena, authors can employ different strategies often used in parallel studying the impact of specific culture conditions on yeast populations and metabolisms (modification of medium composition, increasing cell contacts,

suppressing cell contacts); highlighting the presence of cell contacts, modification of physiological state induced by interactions.

These different methods are described below. In addition, they are nowadays supplemented by more recent techniques: metabolomic, transcriptomic, and genomic techniques, on which we will focus in part 2 of this review and further.

Nissen et al. (2003) [26] were among the first authors to develop a strategy to understand which mechanisms are involved in yeast interactions in wine. They used modifications of culture conditions (addition of live or dead cells, addition of medium from other cultures, addition of nutrients) to highlight or not different interaction hypotheses.

In mixed culture, when certain yeast populations decline or death is observed, the first possibility is that nutrient competition occurs between both strains. To check this eventuality, authors have studied the addition of fresh culture medium or the replacement of the depleted one [26,68,84] and their impact on population dynamics.

The second hypothesis is the production of specific compounds by a strain impacting the growth of another (whether by toxic properties or other mechanisms like quorum sensing). To include or rule out this possibility, yeasts can be cultivated in pure culture in supernatants obtained from mixed culture (potentially containing toxic or inhibiting compounds (ethanol, fatty acids, peptides)), with supplementation in nitrogen sources to avoid nutrient limitation [52], or without supplementation [25,26,68]. This modus operandi can also be used to test the antimicrobial activity of peptides produced by yeasts in pure or mixed culture: more or less purified supernatants are incubated with different microorganisms [5,91,103]. This strategy makes use of a cell/medium separation technique by centrifugation, that can induce stress in yeasts (as shown by Chlup et al. 2008 [104]) and perhaps impact their metabolism.

To highlight the involvement of physical contact between two different yeast populations, biomass behaviors can be compared between a pure culture and a culture with the addition of another strain in different states and densities. Researchers have observed that the growth of non-*Saccharomyces* yeasts is not impacted by the addition of cellular debris or dead cells of *S. cerevisiae* but is immediately stopped when viable cells are added [25,26]. Thus, they showed that the presence of viable *S. cerevisiae* is necessary to influence the growth of the second yeast. In addition, they observed that a sufficiently high cellular density is required to obtain this impact, indicating the possibility of competition for space or the implication of a cell-contact mechanism. By changing the cell concentration in pure culture fermentation or the ratio of non-*Saccharomyces*/*S. cerevisiae* in mixed cultures, Nissen and Arneborg (2003) [25] showed that early death is not solely a consequence of high cell density or a low ratio but also of different abilities to compete for space.

Therefore, to prove that physical contact is necessary to observe a modification of population dynamics, one strategy is to suppress these contacts artificially by separating both yeast populations by a semipermeable membrane that prevents contacts between the yeasts but allows the exchange of substrates and metabolites between the two compartments. If cell–cell contacts are involved in yeasts interactions, yeast behaviors will be different in these conditions from those in mixed fermentations. Some authors used simple systems (tube or flask with dialysis membrane) with different conditions: without agitation and with a cut-off of 12–14 kDa [9,25,26,46], with agitation and a cut-off of 3.5–5 kDa [28] or 1000 kDa [28,71]. To ensure homogeneity on both sides, some authors measured the concentrations of only a few substrates and metabolites (ethanol for Nissen et al. 2003 [26], glucose and ethanol for Kemsawasd et al. 2015 [28]). Nissen and Arneborg (2003) [25] specified that fouling of the membrane was observed after 4 days of fermentation, with a difference in composition (glucose, ethanol) in both compartments. Kemsawasd et al. (2015) [28] noticed that peptides and proteins were freely transported through a 1000 kDa membrane but that 3.5–5 kDa was slightly permeable to molecules larger than 5 kDa, so that AMPs could be present in both compartments (AMPs derived from GADPH are about 8 kDa according to Branco et al. 2014 [101], AMPs studied by Albergaria et al. 2013 [103] are 2–10 kDa). More recently, Petitgonnet et al. (2019) have evaluated metabolism changes more

globally by using metabolomic techniques and highlighted a notable difference in exo-metabolomes between fermentations with and without physical contact [46]. This aspect will be further developed at a later stage.

To study competition between different strains of *S. cerevisiae*, Perrone et al. (2013) [81] chose to use a partitioned reactor that had already been used in studies of bacteria cocultures (Di Cagno et al. 2009) [105]; a double culture vessel apparatus with compartments separated by a 0.45 µm membrane, and which can be stirred. The membrane allows the transfer of medium compounds and composition homogeneity is verified by HPLC for sugars, alcohol, glycerol, acids; only one difference was observed for sugars but with a value close to instrumental reproducibility. Lopez et al. (2014) [106] and Taillandier et al. (2014) [51] used a reactor composed of two jars interconnected with a hollow fiber membrane (0.1 µm). They regulated the flow between both compartments using alternating pressurization, but they observed fouling after 21 h of culture. The system proposed by Renault et al. (2013) [107] seemed to be more efficient. They designed a new double-compartment reactor; separation was ensured by a 1 µm membrane, a pump circulated the medium between both compartments through a 0.45 µm filter to homogenize it without the transfer of yeasts; it was also equipped with automatic reversal of the pumping direction to avoid clogging. All these systems used different means to separate yeasts while ensuring metabolic homogeneity, but they did not seem to allow the immediate transfer of all the metabolites in both compartments. That is why other authors took a completely different approach to investigate the involvement of cell contact in yeast interactions. Rossouw et al. (2018) [108] used a genetic system (based on *FLO* gene family) to modify cell adhesion properties and show that interspecies contacts impact population dynamics as the mechanism was called cell–cell contact by other authors. They made use of a simple sedimentation rate measurement to assess interspecific coaggregation. This macroscopic approach to aggregation dynamics could then be supplemented by microscopic analysis.

Various microscopy techniques can also be used to study different types of contact between yeasts; cell–cell contact, aggregation, or coaggregation with other cells or solids. Fluorescence microscopy with cell staining was used by Rossouw et al. (2015) [109] to highlight the co-flocculation of *S. cerevisiae* and *Hanseniaspora opuntiae* (*H. opuntiae)* in mixed cultures and to study the involvement of different *FLO* genes on flocculation. Cell staining can also be used with flow cytometry, as shown in Pérez-Torrado et al. (2017) [94], which makes use of sonication to highlight cell aggregation. Caldeira et al. (2019) [110] observed the surface of yeast in mixed cultures by atomic force microscopy and showed the existence of direct cell–cell contact. Scanning electron microscopy (SEM) can be used to highlight the aggregation of yeasts in wine with the same or other yeast species, or with solids (as shown by Govender et al. 2011 [111]).

Epifluorescence microscopy observations can also be used to study how anti-microbial peptides (AMPs) act on yeast. Branco et al. (2017) [86] used chemically synthesized AMPs, with fluorescent labelling, and added them to the medium culture of non-*Saccharomyces* yeasts. They noticed that these AMPs can enter cells and at the same time, cells that internalized these AMPs showed compromised cell membranes (PI-stained).

Other less conventional methods can also be used to study the interaction mechanisms. For example, Branco et al. (2017) [71] used immunologic testing to highlight the involvement of cell contact in AMP activity. After extracting and fractionating surface proteins from *S. cerevisiae*, they analyzed fractions by enzyme-linked immunosorbent assays (ELISA) using a specific antibody against GAPDH-derived AMPs. They showed that these AMPs accumulated on cell surfaces, suggesting a potential link between cell–cell contact mechanisms and these AMPs.

In addition to all the analysis techniques discussed previously, novel omics approaches are nowadays widely used to solve different problems and are used to describe an organism's response to genetic or environmental changes. The impact of cell interactions on yeast metabolism is no exception, and proteomics and transcriptomics in case of yeast–yeast interactions in wine have been used extensively in recent years. Most of the studies performed to characterize the consequences of co-cultures

and interactions between microorganisms on gene expression and protein synthesis have focused on comparisons with *S. cerevisiae*, an organism that has undergone extensive investigation [88,112,113]. The modulation of gene expression has been clearly observed during alcoholic fermentation [9,112,113]. Most of the genes whose expression is modified during co-cultures and interactions are involved in stress response, endocytosis, membrane biogenesis, nutrient uptake, and apoptosis [84,88,89]. Complete metabolic pathways are affected by altered gene expression, as shown by Sadoudi et al. [87], with a change in acetic acid and glycerol metabolism in *S. cerevisiae* in the presence of *Metschnikowia pulcherrima (M. pulcherrima)*. More specifically, in the case of direct cell contact between two populations of distinct species, a change in the expression of *FLO* genes has been described, leading to a modification of population dynamics [109]. We will not develop this aspect in our discussion, as the subject has recently been discussed and detailed by Conacher et al. [24].

3. Environmental Changes Related to Interactions and Sensory Impacts

Proteomics and transcriptomics provide insights into the impact of interactions on wine composition [24,89,112,114] but none of them has so far provided significant progress on the microbial interaction mechanisms involved. Metabolomics is a tool of choice for observing the impact of yeast interactions on the composition of the wine matrix and more interestingly, it can help unravel the as yet unknown mechanisms involved in these interactions. Analytical techniques developed for metabolomics studies allow screening hundreds of metabolites from various metabolic pathways with high-throughput techniques [115] that link the impact of yeast interactions to wine composition [102].

The literature includes various studies in which the specific composition of wine enables distinguishing between wines on the basis of fermentations with different yeast species and strain [116–119] and with single and co-cultures [46,102,120,121].

3.1. Metabolic Profiling

Non-targeted metabolomics studies provide a global vision of the modifications of the matrix. Through this approach, all the products from metabolic pathways affected by interactions with a second microbial population can be studied. Only a few studies aimed at understanding yeast–yeast interactions in wines have been carried out using this non-targeted metabolomic approach. Some studies have used FT-ICR-MS to explore metabolomes in wine [46,102,122]. In 2016, Liu et al. [122] studied fifteen strains of *S. cerevisiae* known to positively or negatively impact malolactic fermentation (MLF) through interaction with lactic acid bacteria. They identified a wide variety of markers such as oligopeptide and sulfur-containing peptide metabolites for each of the yeast phenotypes studied. Later, Petitgonnet et al. [46] highlighted changes in the exo-metabolome of wines from co-culture fermentation, depending on the presence or not of a physical barrier. The originality of this paper resided in the study of the physical separation of the two populations. Indeed, greater diversity of compounds was demonstrated in *L. thermotolerans* alone and contactless *S. cerevisiae/L. thermotolerans* modalities. Biomarkers specific to these modalities were mainly identified as involved in amino acid metabolism and carbon fixation. The general conclusion of the study shows that cell to cell yeast interaction does induce a significant change of diversity and variability in the intensity of metabolic compounds in final wine composition [46]. More recently, Roullier-Gall et al. [102] worked on the non-volatile metabolic fingerprint comparison of three different non-*Saccharomyces* species in single and co-cultures with *S. cerevisiae*. It was pointed out that the metabolite composition of wine from the co-culture did not match the assembly of two wines resulting from single yeast fermentation, an observation already made in previous studies [120,123] involving non-neutral interaction phenomena.

The majority of non-targeted works have focused on the metabolome at the end of alcoholic fermentation and therefore are not able to reveal at what stage of growth and fermentation metabolic changes occur. Fortunately, several papers have focused on the different stages of fermentation, including works from Richter's team in 2015, who conducted alcoholic fermentation on Chardonnay must with *S. cerevisiae* [124]. Significant metabolic changes were identified at each stage of the

fermentation studied. This contribution made it possible to attribute a certain regulation of yeast metabolism during fermentation to the efficiency of the glycolytic pathway, probably due to a reduced activity of several enzymes or to glucose transport. In 2018, Peng et al. [121] demonstrated the impact of bringing together two yeast populations of *S. cerevisiae* and *L. thermotolerans* at two key points of alcoholic fermentation: at the onset of early death of non-*Saccharomyces* yeast and at the end of this phase. Owing to NMR, a single culture of *L. thermotolerans* and a co-culture were discriminated based on metabolite composition variations. On the contrary, no changes could be identified when comparing the metabolome of the single culture of *S. cerevisiae* and co-culture. In addition, they highlighted that part of the metabolite composition disappeared at the end of fermentation, suggesting that metabolic changes of co-culture occur after the death of the non-*Saccharomyces* yeasts [121]. It also appears that at different sampling times, the diversity and concentration of metabolites is very different compared to previous works on single culture [124,125]. These works highlighted that sampling time is an essential point for understanding interaction phenomena. Furthermore, studies [124,126] began to explore the differences between the endometabolome and the exometabolome associated with microorganisms involved in fermentation processes to explain the mechanisms involved in interactions. However, it remains difficult to study the endometabolome because of the complexity of sampling [127]. Therefore, recent works have focused on modelling the composition of the endometabolome based on exometabolome measurements [126].

It should also be noted that the identification of compounds detected during the metabolic profiling of the non-volatile fraction of wine remains difficult at present. The complexity of wine has been widely described and still presents many shadowy areas. The databases giving the molecular compounds present in wine remain poorly supplied and do not allow identifying all the biomarkers [102,128,129].

Targeted analysis, often associated with hypothesis verification, involved detecting and quantifying known metabolites in the wine [130–132]. Targeted metabolomics is particularly used for studying the impact of microorganisms [118,133] and interactions [134,135] during winemaking. In the context of leavening different starters to carry out fermentations in the best possible way and with the objective of managing the final quality of Syrah wines, Minnaar et al. [134] were interested in understanding the microbial interactions involved. It would appear that microbial interactions affect the production of polyphenolic compounds including anthocyanins, flavonols, and phenolic acids. Different combinations of starters were studied, involving respectively a strain of *S. cerevisiae* with *M. pulcherrima* or *Hanseniaspora uvarum (H. uvarum)*, *S. cerevisiae* and one of the non-*Saccharomyces* with a LAB strain, and *S. cerevisiae* with a lactic acid bacteria (LAB) with a single starter of *S. cerevisiae*. They identified for the mixed starter of *M. pulcherrima/S. cerevisiae* a decrease in the amount of gallic acid and of caffeic acid for *H. uvarum/S. cerevisiae*. Similarly, Nardi et al. [135] studied the impact of a co-culture of *S. cerevisiae* with a strain of *T. delbrueckii* in combination or not with a strain of *O. oeni*. They were able to demonstrate that among the extracted compounds, involved in the discrimination of the different conditions, there was an increase in certain metabolites such as amino acids like alanine and threonine, and at the same time a decrease in deleterious compounds such as acetic acid. These works described the consequences of the presence of several yeast populations in co-cultures but did not describe the mechanisms involved in the underlying interactions.

3.2. *Volatilome*

The yeast metabolome does not consist entirely of compounds from the non-volatile fraction. In fact, a wide variety of volatile organic compounds (VOCs) are released during the winemaking process and enrich the total wine composition. These compounds produced by microorganisms are grouped together under the term "volatilome" [136]. The composition of the volatilome is related to the species [47,137] and the strains of microorganisms [79,138] used to conduct alcoholic fermentation. In addition, the interactions occurring in consortia or co-cultures also influence the VOC composition of wines [139]. Many VOCs participate in the aromatic profile of wines, as developed later in the discussion [140–142]. Unfortunately, as with non-volatile omics studies, most volatile studies focus on

the impact of yeast interaction on the final VOC composition of co-cultured wines, but few explain the mechanisms of interactions and allow us only to hypothesize about the nature of yeast interactions.

VOCs can be produced by yeasts metabolizing sugars and amino acids and belong to different families of compounds including esters, higher alcohols, medium fatty acids, or aldehydes. Among these fermentation aromas, esters are widely represented, comprising acetate esters and ethyl esters. In the case of co-cultures between *S. cerevisiae* and non-*Saccharomyces* yeasts, the concentration of the major esters is mainly increased. For mixed crops in which *M. pulcherrima.* [47,143] and *H. uvarum* [144] are involved with *S. cerevisiae*, the concentration of esters increases, for example that of phenylethyl acetate. For the most part, mixed crops, including *S. bacillaris,* present higher concentrations of total esters [79,138], including ethyl octanoate or isoamyl acetate [14] compared to pure *S. cerevisiae* fermentation conditions. Conversely, when focusing on Muscat wort, Gobert et al. [55] showed a decrease in the production of isoamyl acetate in sequential fermentations of *S. bacillaris* and *S. cerevisiae.* Similarly, some esters such as isoamyl acetate and ethyl octanoate are detected in higher concentration in wines from co-fermented wort with *S. cerevisiae* and *Torulaspora delbrueckii* (*T. delbrueckii)* [145] or *L. thermotolerans* [45] and in lower concentrations in other studies [39,146] (Table 2). This different impact of co-culture on esters concentration can be explained by the use of various yeast strains and matrix [147]. In the same way, co-culture may increase the content of higher alcohols found in wines. This has been thoroughly described by Sadoudi et al. [47] for the mixed fermentations conducted with *Candida zemplinina* (*C. zemplinina)* and *S. cerevisiae*, Escribano-Viana et al. [37] for *T. delbrueckii* and *S. cerevisiae* and Englezos et al. [79,138] for *S. bacillaris* and *S. cerevisiae*. However, others studies focusing on mixed-cultures with *S. bacillaris* and *S. cerevisiae* have shown a lower concentration of certain alcohols such as 2-phenylethanol and methyl butanol [55,148]. Fatty acids have been found in lower concentrations in most of the co-cultures studied using non-*Saccharomyces* [79,135] except for the couple with *S. bacillaris* [14] and *C. zemplinina* [36] (Table 2). Varietal aromas can also be released by yeasts through the action of cleavage enzymes on odorless precursors present in the must such as terpenes, sulfur compounds, and volatile phenols [139,149]. The combination of yeast populations with a diversity of metabolism as enzymatic activity during fermentation can impact on their diversity and concentration [150]. Finally, one of the impacts studied most on VOC families in co-cultures are terpenes. The terpenes found in wines are mostly linalool, geraniol, and citronellol. In most cases they are found in higher concentrations in wines from mixed culture alcoholic fermentation [36,147].

Table 2. Impact on volatile organic compounds of different mixed starters.

Mixed Culture	VOCs Families	VOCs Impacted by Interactions	Impact	Inoculation Protocol	Mixed Culture Compared to	Matrix	SA	Ref.
S. cerevisiae/*S. cerevisiae*	esters	acetate esters	-	sim/blend	S	Sauvignon blanc	x	[123]
		ethyl dodecanoate	+					
	thiols	3-mercaptohexan-1-ol	+					
C. zemplinina/*S. cerevisiae*	thiols	3-mercaptohexan-1-ol	+	sim	S	Sauvignon		[151]
			-	seq	S and NS	Sauvignon		[47]
	higher alcohols		+	seq	S and NS	Sauvignon		[47]
	terpenes, lactones, norisoprenoids		-	seq	S and NS	Sauvignon		[47]
	lactones		+	seq	S	Shiraz		[36]
	ethyl esters, MCFA		+	seq	S	Shiraz		[36]
L. thermotolerans/*S. cerevisiae*	higher alcohols		+	sim	S and NS	Pedro Ximenez		[45]
				seq	S and NS	Pinot Grigio	x	[14]
		1-propanol, methionol		seq	S	Tempranillo		[37]
		2-methyl butanol, 3-methyl butanol, iso-butanol		sim/seq	S	Emir	x	[39]
			-	seq	S and NS	Muscat		[46]
	esters	2-phenyl ethanol acetate, ethyl acetate, isoamyl acetate, isoamyl decanoate	+	sim	S and NS	Pedro Ximenez		[45]
		ethyl octanoate		seq	S and NS	Pinot Grigio	x	[14]
		2-phenylethyl acetate, isobutyl acetate, hexyl acetate		sim/seq		moschofilero	x	[147]
				seq	S	Shiraz		[36]
				sim/seq	S	Ecolly, Cabernet Sauvignon	x	[144]
		isoamyl acetate	-	sim/seq	S	Emir	x	[39]
				seq	S	Synthetic must with precursors		[152]
				seq	S and NS	Muscat		[46]
	fatty acids		+	seq	S and NS	Muscat		[46]
			-	seq	S and NS	Pinot Grigio	x	[14]
		ethanoic acid		sim	S	synthetic must		[153]
	terpenes	geraniol, citronellol	+	sim	S and NS	Pedro Ximenez		[45]
				seq	S and NS	Pinot Grigio	x	[14]
		geraniol, damascenone		sim/seq		moschofilero	x	[147]
		geraniol, linalool, alpha terpinene		seq	S	Shiraz		[36]
		linalool, geraniol		seq	S	Synthetic must with precursors		[152]
	aldehydes/lactones		-	sim	S and NS	Pedro Ximenez		[45]
	volatile phenols/norisoprenoïds		+	sim	S and NS	Pedro Ximenez		[45]

Table 2. *Cont.*

Mixed Culture	VOCs Families	VOCs Impacted by Interactions	Impact	Inoculation Protocol	Mixed Culture Compared to	Matrix	SA	Ref.
H. uvarum/S. cerevisiae	higher alcohols		-	seq	S and NS	Rondo, Bolero, Regent	x	[122]
				seq	S	Malbec		[154]
	esters		+	sim/seq	S	Ecolly, Cabernet Sauvignon	x	[144]
		ethyl acetate, 2-phenylethyl acetate		seq	S and NS	Rondo, Bolero, Regent	x	[122]
	fatty acids		-	seq	S	Malbec		[154]
T. delbrueckii/S. cerevisiae	higher alcohols	2-phenyl ethanol, methionol	+	seq	S	Chardonnay, Soave, Vino Santo	x	[146]
		2-phenyl ethanol, 1-butanol, methionol		seq	S	Tempranillo		[37]
		2-phenyl ethanol		seq	S	Sauvignon		[47]
				seq	S and NS	Pinot Grigio	x	[14]
		phenlethyl alcohol		sim/seq	Sc	Cabernet sauvignon		[155]
	esters	acetate esters (2-phénylethyl acetate, isoamyl acetate, hexyl acetate, isobutyl acetate) and ethylesters (ethyl lactate, ethyl octanoate, ethyl hexanoate))	+	seq	S	Sauvignon, Syrah	x	[145]
				sim/seq	S	Cabernet sauvignon		[155]
		acetate esters		sim	S	Barbera	x	[135]
		acetate esters (isoamyl acetate) and ethylesters (ethyl octanoate)	-	seq	S	Chardonnay, Soave, Vino Santo	x	[146]
		acetate esters		seq	S	Sauvignon		[47]
	fatty acids	hexanoic acid and octanoic acid	-	seq	S	Chardonnay, Soave, Vino Santo	x	[146]
				seq	S and NS	Pinot Grigio	x	[14]
				sim/seq	S	Cabernet sauvignon		[155]
				sim	S	Barbera	x	[135]
	terpenes	geraniol, linalool, alpha terpinene	+	seq	S	Shiraz		[36]
		linalool, geraniol		seq	S	Synthetic must with precursors		[152]
		geraniol, nerolidol, farnesol		seq	S	Sauvignon		[47]
				seq	S and NS	Pinot Grigio	x	[14]
				sim/seq	S	Cabernet sauvignon		[155]
	volatile phenols	4-vinylphenol, 4-vinylguaiacol	-	seq	S	Chardonnay, Soave, Vino Santo	x	[146]
				sim/seq	S	Cabernet sauvignon		[155]

Table 2. *Cont.*

Mixed Culture	VOCs Families	VOCs Impacted by Interactions	Impact	Inoculation Protocol	Mixed Culture Compared to	Matrix	SA	Ref.
S. bacillaris/*S. cerevisiae*	higher alcohols		+	seq	S	Chardonnay, Riesling, Muscat, Sauvignon blanc		[79]
				seq	S	Barbera		[138]
				seq	S and NS	Pinot Grigio	x	[14]
		2-phenylethanol	-	sim	S and NS	Montepulciano	x	[148]
				sim/seq	S	Kotsifali/Mandilari		[49]
		isobutyl alcohol 1-hexanol increase, methyl butanol decrease	+/-	seq	S	Muscat		[55]
	esters		+	sim/seq	S	Kotsifali/Mandilari	x	[49]
				sim	S and NS	Montepulciano	x	[148]
				seq	S	Chardonnay, Riesling, Muscat, Sauvignon blanc		[79]
				seq	S	Barbera		[138]
		ethyl octanoate, isoamyl acetate		seq	S and NS	Pinot Grigio	x	[14]
		isobutyl acetate phenylethyl acetate, isoamyl acetate	+/-	seq	S	Muscat		[55]
	fatty acids		+	seq	S	Chardonnay, Riesling, Muscat, Sauvignon blanc		[79]
	volatile phenols, carbonyl compounds		+	seq	S and NS	Pinot Grigio	x	[14]
			-	seq	S and NS	Pinot Grigio	x	[14]
M. pulcherrima/*S. cerevisiae*	higher alcohols	1-propanol, methionol	+	seq	S	Tempranillo		[37]
				sim	S	Merlot	x	[156]
				seq	S and NS	Pinot Grigio	x	[14]
		phenylethyl alcohol, isobutyl alcohol		seq	S	Muscat		[55]
			-	sim	NS	synthetic must		[153]
	esters		+	sim	S	Merlot	x	[156]
				seq	S	Shiraz		[36]
				seq	S and NS	Pinot Grigio	x	[14]
				seq	S	Muscat		[55]
				seq	S	Sauvignon		[47]
	fatty acids		+	seq	S	Sauvignon		[47]
	terpenes		+	seq	S and NS	Pinot Grigio	x	[14]
		geraniol, linalool, alpha terpinene		seq	S	Shiraz		[36]
		linalool		seq	S	Sauvignon		[47]
	volatile phenols, carbonyl compounds		-	seq	S and NS	Pinot Grigio	x	[14]
	sulfur compounds	dimethylsulfide, ethanethiol, sulfure hydroxyde	+	sim	S	Merlot	x	[157]

Table 2. *Cont.*

Mixed Culture	VOCs Families	VOCs Impacted by Interactions	Impact	Inoculation Protocol	Mixed Culture Compared to	Matrix	SA	Ref.
Mix of four non-*Sacch* and three *S. cerevisiae*	total acids		-	seq	S and NS	synthetic must	x	[157]
	others compounds		=					
S. cerevisiae/ non-*Sacch and T.d*		methionol, ethyl lactate	-	seq	S	Tempranillo		[37]
Mix of five non-*Sacch*/*S. cerevisiae*				seq		synthetic must Sauvignon blanc	x	[64]

Ref.: References **S. *cerevisiae***: *Saccharomyces cerevisiae* **C. *zemplinina***: *Candida zemplinina* **L. *thermotolerans***: *Lachancea thermotolerans* **H. *uvarum***: *Hanseniaspora uvarum* **T. *delbrueckii***: *Torulaspora delbrueckii* **S. *bacillaris***: *Starmerella bacillaris* **M. *pulcherrima***: *Metschnikowia pulcherrrima.* **Impact on VOCs concentration**: -: decrease +: increase +/-: two cases are encountered =: no change **sim**: simultaneous/**seq**: sequential **S**: *S. cerevisiae*/**NS**: non-*Saccharomyces*. **SA**: sensory analysis. **VOCs**: volatile organic compounds.

Although most studies describe the impact of interactions on VOC composition in fermentation in co-culture, some papers have tried to explain the interaction mechanisms associated with these compositional changes. Sadoudi et al. [47] highlighted interaction mechanisms for three couples of yeast by comparing VOC concentration in simple cultures and mixed cultures. The concentration of some terpenols such as β-damascenone doubled in the co-culture of *M. pulcherrima*/*S. cerevisiae* showed a positive interaction between both strains. The synergetic effects of *T. delbrueckii*/*S. cerevisiae* have also been revealed in co-culture, exhibiting an increase in the content of terpenols, C6 compounds and 2-phenylethanol, suggesting a cumulative effect of both yeast metabolisms related to biomass. Negative interactions for *C. zemplinina*/*S. cerevisiae* were highlighted by showing a decrease in the production of farnesol in co-culture, in comparison to a pure culture of *C. zemplinina*, which could be used as a modulator of gene expression. As mentioned above, later, in 2017, the same team showed that the presence of *M. pulcherrima* induced a change in the gene expressions involved in the metabolism of acetic acid of *S. cerevisiae* in co-cultures [87]. Later, Petitgonnet et al. [46] showed the importance of cell–cell contact in VOC composition in the case of *S. cerevisiae* and *L. thermotolerans* co-culture. The study compared VOCs from wines in pure culture, conventional contact co-culture, and co-culture physically separated by dialysis rod. It was pointed out that ester and fatty acid concentrations were higher in co-culture without cell contact. Similarly, other studies [12,55] have focused on nutrient sources as well as on competition for nutrients between species in the case of co-fermentation together with their impact on VOC composition. In sequential fermentations nutrient sources, such as nitrogen, are reduced at the time of *S. cerevisiae* inoculation. Gobert et al. [55] highlighted that, in the case of sequential fermentation, amino acids such as leucine are consumed by *S. bacillaris* before *S. cerevisiae* inoculation. However, leucine is the precursor of VOCs including isoamyl acetate and 2-methylbutanol mainly synthesized by *S. cerevisiae*. The depletion of leucine before *S. cerevisiae* inoculation would therefore lead to under-expression of these VOCs in wines. On the other hand, this paper showed a possible synergetic effect between *S. bacillaris* and *S. cerevisiae* for the synthesis of isobutyl alcohol [55].

Although most of the work has focused on the interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts, few papers have explored other types of mixed fermentations. King and Capece studied the impact of co-cultures of different strains of *S. cerevisiae* on the volatilome [123,158]. In 2008, King found a positive variation in the thiol 3-mercaptohexan-1-ol (3MH) composition of Sauvignon Blanc wines in some of the co-cultures of *S. cerevisiae* strains studied [123]. Later, Escribano et al. [137] studied the co-culture of three yeasts in sequential fermentations including two non-*Saccharomyces*, *T. delbrueckii* and *L. thermotolerans* yeasts with *S. cerevisiae* yeast. Similarly, consortia including several non-*Saccharomyces* species have been considered [64,157]. Interestingly Padilla et al. [157] showed that a consortia of four non-*Saccharomyces* strains in mixed culture with three *S. cerevisiae* strains led to a decrease in total acids and did not affect the synthesis of other compounds in comparison to a pure fermentation with commercial *S. cerevisiae*.

Thus the yeast species [47] and the yeast strain [79,138] are the determining factors for the final wine VOC composition [159]. Nevertheless, the production of most of these VOCs also depends on different biotic and abiotic factors. It has been widely described that the composition of the matrix, and more particularly nitrogen sources [59], and the presence of VOC precursors have an impact on the production of VOCs [152]. In addition, the great variability in yeast inoculation protocols such as simultaneous and sequential fermentation [147], the time of adding *S. cerevisiae* in the case of sequential inoculation [39], population ratios [45,153], and environmental conditions [160] appears to play an essential role in yeast growing characteristics and subsequent VOC composition. Furthermore, the formation of these compounds occurs throughout the fermentation process. That is why some studies have aimed at identifying differences in VOC content based on the fermentation time [145]. They showed that the concentration of total esters increased by 40% from the beginning to the end of alcoholic fermentation. Another example was described by Escribano et al. [137] with the variation of the concentration of ethyl lactate for the ester formation due to an increase of higher alcohols

in the medium. All of these factors induce additional variability between the different studies and observations [122,144].

Taken as a whole, the results of the various studies of volatile compounds show divergences. The approaches to data analysis are highly diverse, thus adding to the factors described previously that influence VOC composition. Most studies have attempted to describe differences between co-cultures and single fermentations of *S. cerevisiae* but some studies have compared the impact of these co-cultures with non-*Saccharomyces* single cultures, which may lead to differences in interpretation. Studies of VOCs in co-cultures have mainly focused on providing a descriptive approach and only a few of them have tried to explain the mechanisms leading to differences in VOC composition.

3.3. Sensory Impact

For many years, the impact of micro-organisms on the sensory component of wine has been addressed by many researchers and professionals in the wine sector. These studies have aimed at characterizing the individual impact of a *S. cerevisiae* or a non-*Saccharomyces* yeast strain on the aromatic [149,161–163], visual [164] and taste [165] profiles of wines. This approach is also increasingly applied to pairs or consortia of microorganisms involved in alcoholic fermentation [64,157]. In most cases the discrimination of wines fermented by more than one species of microorganism from those fermented by a single species is described. For example, Gobbi et al. [33] attributed more spicy and acidic notes to the co-culture in commercial white grape must compared to the single culture of *S. cerevisiae*. Barbera wine was studied after alcoholic fermentation assessed by *T. delbrueckii* and *S. cerevisiae* and showed a lower intensity in attributes such as floral and red fruit aromas, as well as a change in color, becoming more intense compared to *S. cerevisiae* in pure culture [135]. The same year, Varela et al. (2017) [156] showed that wines inoculated with *M. pulcherrima* and *S. cerevisiae* were closer to uninoculated wines and associated with high scores for positive attributes such as red fruit aroma and overall fruit aroma, which are found in a minority in wines from *S. cerevisiae* in pure culture.

More specifically, in various studies it appears that the sequential fermentation path has a greater impact on the sensory profiles of wines than simultaneous inoculation [33,145,148]). Benito et al. [40] showed in a comparison between sequential fermentation and simultaneous inoculation, *S. cerevisiae* and *L. thermotolerans*, that general acidity and overall impression were increased and described as characteristic of sequential fermentations. In 2015, the same research team also attempted to determine the impact of mixed starter wines with a non-*Saccharomyces* strain on the quality of Riesling wines [38]. As observed previously, the general impression was described as better in the case of sequential fermentations with respect to a single culture control of *S. cerevisiae*. The *M. pulcherrima*/*S. cerevisiae* pair was discriminated by the terms citrus/grapefruit and pear while *L. thermotolerans*/*S. cerevisiae* pair was associated with peach/apricot [38]. This last example shows that different aromatic notes are detected from the same matrix couples involving the same strain of *S. cerevisiae*, but with different non-*Saccharomyces* strains. Binati et al. [14] were also able to discriminate wines of Pinot Grigio from different sequential fermentations involving *M. pulcherrima* and *S. bacillaris* by different VOCs such as higher alcohols and esters. King et al. [123] highlighted a difference in sensory profiles between two co-inoculations of *S. cerevisiae* involving different strains characterized by box hedge and floral aromas and with different blends of two simple cultures of the same strain themselves described by the terms white vinegar and bruised apple. There is therefore an impact of co-cultivation and therefore interactions between populations on the sensory profile in addition to the strain effect. On the contrary, when studying two strains of *T. delbrueckii* in mixed culture with *S. cerevisiae*, Azzolini et al. [146] found no differences in the sensory profile with either pair. However, complexity and persistence were found to be increased in mixed cultures compared to the single culture of *S. cerevisiae*. Likewise, Liu et al. [122] mentioned that the impact of cultivar type was greater than the strain effect. Indeed, as pointed out earlier, the matrix plays an important role in the sensory profile. Hu et al. [144] confirmed the greater role of the matrix specifically the grape varieties with the observed decrease in the vegetal component in a Cabernet Sauvignon wine as opposed to an Ecolly wine fermented with the same mixed starter.

These latest works were carried out by studying VOC composition and the sensory aspect of wines in parallel; however, few of them focused on linking these two aspects. Hu et al. [144] established that the compounds that mainly contributed to tropical fruit and floral aspects of sequential fermentation that involved *H. uvarum*/*S. cerevisiae*, were C13 norisoprenoids, terpenes, and acetate esters, while the temperate fruit notes were generated mostly by ethyl esters. Similarly, Nisiotou et al. [49] identified an association between a higher concentration of ethyl ester and the fruity aroma of wine, as previously discussed by Lytra et al. [142] for simultaneous and sequential cultivation using *L. thermotolerans*. They likewise noted a correlation between acetate esters and the floral aroma descriptor. In the work by Renault et al. [145] on the sequential fermentation of *T. delbrueckii* and *S. cerevisiae*, the over-expression of four esters (ethyl propanoate, ethyl isobutanoate, ethyl dihydrocinnamate, and isobutyl acetate), described as minor, was highlighted. Moreover, the wine was characterized and differentiated from other modalities by fruity aromas and greater complexity. Then, they added at equal concentration the sequential condition and the single culture of *S. cerevisiae* to validate the impact of these esters on the wine sensory profile. This made it possible to highlight the sensory impact of these esters and attribute them the role of aromatic biomarker. However, it should be noted that only one of its esters was present at a concentration above its detection limit in wine. Therefore, it is suggested that the other esters also lead to aromatic modulation through different interaction phenomena. Among the four esters, ethyl propanoate and isobutyl acetate were described by several studies as enhancers of fruity notes [140,142,166].

The sensory aspect of the various studies mentioned above shows changes in the sensory profile, but they do not address or make it possible to understand or explain the mechanisms of interaction between the populations involved in alcoholic fermentation. Most authors have focused more on showing a change in the sensory profile of the wines in the case of co-culture. Indeed, sensory contribution remains complicated to integrate into a process of understanding the mechanisms of interaction, since no VOC or family of VOCs can explain the aromatic profile of a wine. It is still unclear what contribution they make to the aromatic notes. Despite trends and correlations, there is not always a mirror effect between chemical composition and sensory profiles due to the existence, among other things, of interactions between these volatile aromatic compounds and non-odorous volatile compounds to form aromas [141,167,168]. These interactions between VOCs were confirmed in a very recent publication by Mc Kay et al. [169]. Associated with these interactions, various factors can induce a mismatch between the volatile composition and the sensory profile of wines, such as the detection threshold [159,163,169,170], or the masking of certain flavors associated with volatile compounds by others, as suggested by Benito et al. [38] cited above. Higher alcohols have already been described as being able to mask these fruity notes [171]. Finally, in view of the diversity of volatile aromatic compounds, many of them participate in the same aromatic note [172]. Sensory evaluation also provides different information depending on the approach selected (description, comparison, preference, or determination of product quality), [160] and the panel of selected juries (expert or naive) [173]. Sensory analysis therefore remains an essential tool to qualify the impact of co-cultures on the final product, but it does not provide information on the interaction mechanisms that may occur.

4. Conclusion and Perspectives

This review presented the state-of-the-art of yeast–yeast interactions in wine and highlighted the difficulties of studying the mechanisms involved in these phenomena. The impact of co-culture on the final matrix is now well-known but little is understood about how this happens. Indeed, it appears that all the works presented distinct methodologies, mainly in terms of biological material with the use of different yeast species and strains, leading to a plethora of results specific to each pair. Therefore, understanding these mechanisms requires further studies at this level by combining the observation of a target mechanism with the use of different strains belonging to the same species in order to draw solid conclusions. Different matrices and the application of abiotic factors also remain a major source of diversity in the results. All these variations make it difficult to generate complementary

and comparable results capable of leading to conclusions that unravel the mechanisms involved in these interactions.

During our research it became apparent that quorum sensing in yeast remains unexplained and unproven, making this gap in knowledge an important path of investigation, as discussed by Winters et al. 2019 [174]. Quorum sensing interactions have been proposed many times as a hypothesis by the authors [26,175], but neither have confirmed it. Although the direct impact of certain specific QS molecules on non-*Saccharomyces* growth has already been studied [59] when in high concentrations, experiments under conditions closer to those of winemaking have yet to be conducted. It also appears that the study of different types of interaction mechanisms such as cell–cell contact, for example, presents many contradictory results because of the use of different systems aimed at separating the different populations involved. The use of a double compartment bioreactor with a membrane making it possible to homogenize the surrounding medium in both compartments, while not denaturing the separation of microorganisms, seems to be a good strategy for understanding mechanisms. In addition, strategies at the molecular level can be considered to elucidate these mechanisms. For example, the creation of mutants of target genes that are presumed to be involved in mechanisms due to interactions such as cell–cell contact, and parietal genes, could be one avenue of investigation. Competition for nutrients is still difficult to assess when discriminating between the consumption of a nutrient by one or another of the populations involved. Monitoring this catabolic activity could be carried out, for example, by tagging amino acids for nitrogen competition. It was observed that, overall, the majority of the studies were essentially descriptive and failed to capture the interactions and their mechanisms. Technological deadlocks remain that must be overcome. The metabolomic approach is a real tool of choice and evokes metabolic pathways associated with changes in the composition of the metabolome, however, this requires further development of databases related to yeast metabolism in wine. With respect to representing the end result of all the metabolic and regulatory interactions that lead to metabolic changes, monitoring metabolic flows, currently called "fluxomics," is one of the avenues to be considered with, for example, isotopic labelling of metabolites. In addition, a question arises as to the representativeness of targeted approaches that allow the quantification of target compounds in relation to the totality of the metabolites produced. A non-targeted approach, as suggested by Suklje et al. [176], may further explain the metabolic changes that occur. Transcriptomics, an indispensable approach for understanding gene expression under established environmental conditions, is still limited from the non-*Saccharomyces* perspective, as the genomes are poorly sequenced. Data mining is also of great importance since it can lead to the establishment of models of microbial behavior in response to different individual or combined parameters. An integrated approach combining different omics techniques is a strategy of choice that was recently described by Lawson et al. [177] with the objective of better understanding the mechanisms that direct interactions within a consortium of microorganisms. However, it should be taken into account that these approaches provide additional information and do not always lead to a general combined conclusion.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/4/600/s1, Table 1S: Diversity of methodologies and results in yeast interaction experiments.

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Yeast–Yeast Interactions: Mechanisms, Methodologies and Impact on Composition

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Table S1: Diversity of methodologies and results in yeast interaction experiments

Species		*Saccharomyces cerevisiae / Metschnikowia pulcherrima*								
Matrix		Synthetic Medium						Grape Juice / Must (unspecified color)		
		SGJ	SGJ				SGJ	Grape must		
Sugar Initial Concentration (g/L)		200 (Glc 100 Fru 100)	200 (Glc 100 Fru 100)				200 (Glc 100 Fru 100)	231		
YAN / PAN (mg/L)		YAN 410					200	PAN 154 / NH_3 22		
Temperature (°C)		25	25				20	25		
Oxygenation		++	-	+	++	+++	+	+		
Inoculation delay between 2 strains (h)		0	0				0	0		
Ratio Sc/NS		1:1	1:10				1:10	1:1	1:100	1:10000
Sc population	Max	1	3	3	3	3	4	3	2	3
	Dominance		+					2	2	2
	Decrease	-	-	+	+	+	+	-	-	-
NS population	Max	1	1	1	1	1	1	7	1	3
	Decrease	2 or 1	1	3	3	3	-	7	3	3
	Detectable End	No 2 or 4						No 22	No 7	No 7
Fermentation completion							+	+	+	+
Ethanol		=	=	--	--	--	-	--	--	--
Glycerol			++	--	--	--	+	++	++	++
Organic acids	TA							--	--	--
	VA							--	--	--
	LA									
	AA									
Impacting Factors		SPE / NS	OX / SPE				SPE	RAT / SPE		
Interaction Mechanisms		TOX	COMP				COMP / TOX			
Reference		[65]	[29]				[95]	[32]		

Species		*Saccharomyces cerevisiae / Metschnikowia pulcherrima*											
Matrix		Red Grape Juice / Must					White Grape Juice / Must						
		Shiraz	Merlot	Shiraz	Tempranillo	Pinot Grigio	Sauvignon blanc	Riesling	Sauvignon blanc	Chardonnay	Muscat commercial grape juice	Sauvignon blanc	Riesling
Sugar Initial Concentration (g/L)		210	240	280 (Glc 140 Fru 140)	245	236	219 (Glc 110 Fru 109)	237	217	240 (Glc 120 Fru 120)	200 (supp)	221 (Glc 112 Fru 109)	225
YAN / PAN (mg/L)		200	190	YAN 170 (supp)	YAN 181	YAN 236	YAN 588	PAN 147	YAN 170 (supp)	260		YAN 378	YAN 250 (supp)
Temperature (°C)		22	22	25		22	20	20	15	22	20	20	20
Oxygenation		+	++	++	+	+	+	+	+	+	+	+	+
Inoculation delay between 2 strains (h)		24 days	0	96	72	48	48	24	168	24 days	72	48	48
Ratio Sc/NS		1:1	1:10	1:1	1:1	1:1	1:10	10:1	1:1	1:1	1:5	1:10	1:1
Sc population	Max							4			5	3	4
	Dominance		+	+									
	Decrease		-				-	-			-	-	-
NS population	Max												0
	Decrease	+	2	+	+		+	+	+	15	5	4	2
	Detectable End			No 7	No 4-5		No 8	No 15	No 12	No 25	Low 13	No 8	4
Fermentation completion		+		17		9	8	+	19	40	10	10	+
Ethanol		- -	-	=	- -	-	-	- -	=	-		-	=
Glycerol		-	++		++	++		++		+		++	++
Organic acids	TA						- -		+				- -
	VA	+		=	- -				++	++			
	LA												
	AA	- -	=			=	- -	=	++	- -		- -	=
Impacting Factors		GRA		SPE	SPE	SPE / NS	SPE	SPE	SPE	GRA	SPE / TEMP / DEL / MED	DEL	DEL
Interaction Mechanisms											COMP	COMP	
Reference		[155]	[153]	[36]	[37]	[14]	[47]	[38]	[41]	[155]	[55]	[87]	[44]

Species		*Saccharomyces cerevisiae / Starmerella bacillaris*									
Matrix		Synthetic Medium	Grape Juice / Must (unspecified color)			White Grape Juice / Must					
		SGJ	Grape must			Macabeo	Erbaluce / dried grape must		Sauvignon blanc	Sauvignon blanc	Muscat (commercial grape juice)	Chardonnay / Muscat / Riesling / Sauvignon blanc
Sugar Initial Concentration (g/L)		200 (Glc 100 Fru 100)	231			180	403 (Glc 210 Fru 193)		219 (Glc 110 Fru 109)	217	200 (supp)	245
YAN / PAN (mg/L)		YAN 410	PAN 154 / NH_3 22			YAN 115			YAN 588	YAN 170 (supp)		YAN 180 adj
Temperature (°C)		25	25			20	25		20	15	20 / 28	20
Oxygenation		++	+			+	+		+	+	+	+
Inoculation delay between 2 strains (h)		0	0			0	0	48	24	144	72	48
Ratio Sc/NS		1:1	1:1	1:100	1:10000	1:9	1:1		1:10	1:1	1:5	1:1
Sc population	Max		2	2	10				3		=	
	Dominance	-	-	-	-	-	-	-	-		-	=
	Decrease		1	3	10	- +						-
NS population	Max		1	2	2				1			
	Decrease	4	7	10	15	+	6	6/12	-	+	-	7
	Detectable End	No 5 / Yes	No 15	No 22	No 22	Low 10				No 14		No 14
Fermentation completion			+	+	+	10			7	24		14
Ethanol			- -	- -	- -		- -	- -	-	=	=	-
Glycerol			++	++	++	++	++	++				++
Organic acids	TA		- -	- -	- -				+			++
	VA		- -	- -	- -							
	LA											
	AA					++	= / - -	= / - -		++		
Impacting Factors		SPE / NS	RAT / SPE			SPE	DEL / SC / NS		SPE	SPE	SPE / TEMP / DEL / MED	GRA / TEMP
Interaction Mechanisms		TOX				COMP					COMP	
Reference		[65]	[32]			[48]	[23]		[47]	[41]	[55]	[79]

Species		*Saccharomyces cerevisiae / Starmerella bacillaris*																
Matrix		**Red Grape Juice / Must**																
		Merlot			Barbera		Shiraz	Barbera		Cabernet sauvignon / Merlot / Pinot noir / Shiraz		Kotsifali - Manilari 3:1				Barbera	Nebbiolo	Pinot Grigio
Sugar Initial Concentration (g/L)		240			244		280 (Glc 140 Fru 140)	246		250		220		214		234 (Glu 118 Fru 116)	234 (Glu 115 Fru 120)	236
YAN / PAN (mg/L)							YAN 170 (supp)	YAN 180 adj		YAN 180 adj		YAN 240		YAN 71 + suppl		YAN 180 adj	YAN 180 adj	YAN 236
Temperature (°C)		24			25		25	25		25		25		25		25	25	22
Oxygenation		+			+		++	+	++	+		+		+		+	+	+
Inoculation delay between 2 strains (h)		0	24	48	0	48	96	48		24	48	0	30	0	30	48	48	48
Ratio Sc/NS		1:5			1:1		1:1	1:1		1:1		1:1				1:1	1:1	1:1
Sc population	**Max**																	
	Dominance				-	-				= / +	-	+	-			-	-	
	Decrease						+	-	-	-	-		-	-	-	10	-	+
NS population	**Max**				4													
	Decrease					+	+	4	4	4	4/7	3	8	2	2	7	7	+
	Detectable End				No 21	Yes 21	No 7	No 14 / 21	No 14 / Yes 21			Low 5		Low 6	Low 7	No 10 / 14	No 9 / Yes 14	Low 5
Fermentation completion		+	+	+	-	-	17	+ / -	+ / -	7	7/10	6	13			14	14	14
Ethanol		=	- -	- -	- -	- -	=	- -	- -	- -		-	- -	- -	- -	- - / =	= / - -	-
Glycerol			++	++	=	++		++	++	++		=	++	++	++	++	++	++
Organic acids	**TA**		++	++						++		=	++			++		
	VA						++						++		=			
	LA																	
	AA							++	+++				++	=	=	- -		
Impacting Factors		NS / DEL			DEL / NS / SC		SPE	OX / NS / SC		DEL / GRA		DEL				SC		SPE / NS
Interaction Mechanisms								COMP				TOX / COMP					Not TOX / Not COMP / CCC	
Reference		[75]			[12]		[36]	[67]		[60]		[49]				[69]	[68]	[14]

Species		*Saccharomyces cerevisiae / Torulaspora delbrueckii*														
Matrix		Synthetic Medium														
		SGJ	WYPD		SGJ	SGJ									SGJ	SGJ
Sugar Initial Concentration (g/L)		210 (Glc)	200 (Glc)		210 (Glc)	220 (Glc 110 Fru 110)				200 (Glc 100 Fru 100)					200 (Glc 100 Fru 100)	200 (Glc 100 Fru 100)
YAN / PAN (mg/L)						YAN 324		YAN 176							YAN 410	200
Temperature (°C)		25	25		25	20				25					25	20
Oxygenation		+	++	+	+	+				-	+	++	+++		++	+
Inoculation delay between 2 strains (h)		0	0		0	0	0	48	48	0					0	0
Ratio Sc/NS		1:1	1:1		1:1	1:2	1:20	1:2	1:2	1:10					1:1	1:10
Sc population	Max	2	1	1	2	2	2	4	5	3	3	3	3		1/3	2
	Dominance	+	+	+	+											
	Decrease	-	-	-	-	-	-	-	-	-	+	+	+		-	-
NS population	Max	2	1	1	1	2	3	3	2	1	1	1	1		1	2
	Decrease	3	-	-	3	3	4	4	2	1	3	3	3		2/3	-
	Detectable End	Low 7			Low 6	Low 5									No 6 / Yes	
Fermentation completion		10			10	7	5	9								
Ethanol						=	=	-	=	=	- -	- -	- -		=	- -
Glycerol										++	- -	- -	- -			=
Organic acids	TA															
	VA															
	LA															
	AA					=	=	- -								
Impacting Factors						RAT / DEL				OX / SPE					SPE / NS	SPE
Interaction Mechanisms		Not TOX / Not COMP / CCC	Not TOX / Not COMP / Not QS / CCC		COMP	Not CCC / Not COMP / TOX				COMP					TOX	COMP / TOX
Reference		[25]	[26]		[27]	[51]				[29]					[65]	[95]

Species		*Saccharomyces cerevisiae / Torulaspora delbrueckii*							
Matrix		Grape Juice / Must (unspecified color)			White Grape Juice / Must			Red Grape Juice / Must	
		Grape must			Sauvignon blanc	Sauvignon blanc	Riesling	Shiraz	Tempranillo
Sugar Initial Concentration (g/L)		231			219 (Glc 110 Fru 109)	217	225	280 (Glc 140 Fru 140)	245
YAN / PAN (mg/L)		PAN 154 / NH_3 22			YAN 588	YAN 170 (supp)	YAN 250 (supp)	YAN 170 (supp)	YAN 181
Temperature (°C)		25			20	15	20	25	
Oxygenation		+			+	+	+	++	+
Inoculation delay between 2 strains (h)		0			24	120	48	96	72
Ratio Sc/NS		1:1	1:100	1:10000	1:10	1:1	1:1	1:1	1:1
Sc population	Max	3	10	3	3		4		
	Dominance	2		15				+	
	Decrease	-	-	-	-		15		
NS population	Max	3	3	3	1		0		
	Decrease	3	10	15	-	+	4	+	+
	Detectable End	No 15	No 22	No 22		14	No 6	No 7	Yes 6
Fermentation completion		+	+	+	7	19	+	17	
Ethanol		- -	- -	- -	-	=	=	=	- -
Glycerol		- -	- -	- -			++		++
Organic acids	TA	+	+	+	+	=	- -		
	VA	- -	- -	- -	- -	=		=	- -
	LA								
	AA					-	=		
Impacting Factors		RAT / SPE			SPE	SPE	DEL	SPE	SPE
Interaction Mechanisms									
Reference		[32]			[47]	[41]	[44]	[36]	[37]

Species		*Saccharomyces cerevisiae / Hanseniaspora guilliermondii*									
Matrix		Synthetic Medium								White Grape Juice / Must	
		SGJ						SGJ	SGJ	Malvasia fina - Arinto	Malvasia fina - Arinto
Sugar Initial Concentration (g/L)		200 (Glc 110 Fru 110)					100	220 (Glc 110 Fru 110)	220 (Glc 110 Fru 110)	234	234
YAN / PAN (mg/L)										YAN 175 / 387 (supp)	YAN 387
Temperature (°C)		18						20	25	20	20
Oxygenation		+						+	+	+	+
Inoculation delay between 2 strains (h)		0						0	0	0	0
Ratio Sc/NS		1:1	3:1	1:2	1:10	1:100	1:10	1:1	1:1	1:1	1:1
Sc population	Max	2	3	7	7	7			2	1	1
	Dominance										-
	Decrease	-	-	-	-	-		-	-	-	-
NS population	Max	1			3	3			2	1	1
	Decrease	1	+	1	4	15	7	+	2	2	6
	Detectable End	No 7	No 3	No 8	No 11	No 23	Yes 34	Low 4	No 4	No 12	Low 10
Fermentation completion		20	20	25	25	25		+	9	+	
Ethanol										-	
Glycerol											
Organic acids	TA									=	
	VA									=	
	LA									=	
	AA									=	
Impacting Factors		RAT								MED	
Interaction Mechanisms		TOX / Not COMP						TOX		Not COMP	
Reference		[52]						[85]	[99]	[50]	[112]

Species		*Saccharomyces cerevisiae* / *Hanseniaspora uvarum*			
Matrix		Synthetic Medium			White Grape Juice / Must
		SGJ	SGJ	SGJ	Macabeo
Sugar Initial Concentration (g/L)		200 (Glc 110 Fru 110)	200 (Glc 100 Fru 100)	200 (Glc 100 Fru 100)	180
YAN / PAN (mg/L)			YAN 410	YAN 200	YAN 114.6
Temperature (°C)		18	25	20	20
Oxygenation		+	++	+	+
Inoculation delay between 2 strains (h)		0	0	0	0
Ratio Sc/NS		4:1	1:1	1:10	1:9
Sc population	Max	2		1	
	Dominance				
	Decrease	-		8	
NS population	Max		1	1	1
	Decrease	1	2	1	5
	Detectable End	No 3	No 4	Low 10	No 10
Fermentation completion		18			+
Ethanol			=	+	=
Glycerol				-	++
Organic acids	TA				
	VA				
	LA				
	AA				++
Impacting Factors		RAT	SPE / NS	SPE	SPE
Interaction Mechanisms		TOX / Not COMP	TOX	COMP / TOX	COMP
Reference		[52]	[65]	[95]	[48]

YAN = yeast assimilable nitrogen / **PAN** = primary amino nitrogen. **Sc** = *S. cerevisiae* / **NS** = Non-*Saccharomyces*. **TA** = total acidity / **VA** = volatile acidity / **LA** = lactic acid / **AA** = acetic acid. **SGJ** = Synthetic Grape Juice / **Glc** = Glucose / **Fru** = Fructose / **WYPD** = Yeast Peptone Dextrose medium modified for wine fermentation / **Supp** = with supplementation. **Oxygen**: **-** = anaerobia, +/- semi-anaerobia, low oxygenation, + = semi-anaerobia, ++ aerobia, +++ aerobia, with higher oxygenation. **Population columns**: **Max** = maximal population reached by day x / **Dominance**: + = dominance of *S. cerevisiae*, = similar populations, "x" = dominance obtained after x days / **Decrease** = decrease since day x / **Low** = low population since day x / **No** = population not detectable since day x / **Yes** = population still detectable at day x. **Fermentation completion**: x = reached at day x, +/- = reached/not reached during experimentation. **Ethanol, glycerol and organic acids** are compared to Sc pure culture: +++ very high increase ++ high increase, + increase, +/- slight decrease, = no change, - decrease, - - high decrease. **Impacting factors**: inoculation delay (**DEL**), inoculation ratio between *S. cerevisiae* and non-*Saccharomyces* yeast (**RAT**), yeast species (**SPE**), yeast strain *S. cerevisiae* (**SC**) or non-*Saccharomyces* (**NS**), medium composition (**MED**), grape nature (**GRA**), temperature (**TEMP**), oxygenation (**OX**), type of reactor (lab, pilot, industrial) (**REAC**). **Interaction mechanisms**: involvement of quorum sensing mechanisms (**QS**), toxic compounds (including ethanol, antimicrobial peptides) (**TOX**), competition for nutrient (including oxygen) (**COMP**), cell-cell contact mechanisms (**CCC**) / **No** = mechanism involvement has been ruled out by the study.

L'exploration de ces trois axes (méthodologies existantes et en émergence pour l'étude des interactions, résultats obtenus par métabolomique et transcriptomique, et résultats récents d'analyses sensorielles) a permis de faire émerger deux conclusions importantes pour la suite des travaux présentés ici. La première est la diversité importante des méthodologies utilisées pour l'étude des interactions, tant au niveau des procédés d'analyses que des conditions de culture, ce qui limite nécessairement la possibilité de comparaison des résultats et donc l'établissement de conclusions plus globales. La seconde est l'intérêt scientifique des nouvelles méthodologies d'études appliquées à l'étude des interactions. Métabolomique et transcriptomique montrent en effet un potentiel important lorsqu'utilisées en tant qu'outils méthodologiques, et ce potentiel semble en outre décuplé lorsqu'il est possible de relier les résultats de ces méthodes à ceux d'autres méthodes complémentaires comme l'analyse sensorielle. Ces conclusions permettent donc de définir comme perspective intéressante la réalisation d'études aux conditions plus standardisées, pour faciliter à la fois leur reproductibilité et leur déclinaison sur d'autres matériels biologiques, dans un but futur de comparaison entre études. Elles montrent également l'intérêt scientifique potentiel que comportent les études adoptant une approche intégrée, c'est-à-dire faisant usage de plusieurs approches méthodologiques complémentaires qui, une fois leurs résultats regroupés, permettent de faire émerger des conclusions nouvelles.

1.4. Perspectives ouvertes par l'état de l'art et problématique de la thèse

L'analyse des travaux menés sur les fermentations mixtes et les interactions entre levures conduisent, comme discuté ci-dessus, à des résultats parfois contradictoires entre les auteurs, et à l'impossibilité actuelle de tirer des conclusions fermes sur les leviers de modulation utilisables pour contrôler les interactions levuriennes, et donc les dynamiques de population ainsi que les fermentations qui en dépendent directement. Ces travaux ayant été conduits dans des conditions de fermentation différentes, l'hypothèse que celles-ci influent sur les interactions entre souches apparaît comme crédible.

Afin de mieux comprendre et maîtriser les fermentations mixtes, il semble donc important d'étudier l'impact des conditions de fermentation sur les interactions entre levures : cet impact s'effectue-t-il directement sur les mécanismes d'interactions ou via une modification des dynamiques de population spécifiques à chaque levure ?

Parmi les conditions de fermentation, différents facteurs peuvent influencer le développement des levures et leurs interactions : la composition du milieu (concentration initiale en sucres, en azote, présence de facteurs de croissance), les paramètres environnementaux (température de fermentation, conditions d'oxygénation du milieu, d'agitation, type et taille de réacteur) et les conditions d'ensemencement (ensemencement séquentiel ou simultané des deux levures, délai d'ensemencement dans le cas d'un ensemencement séquentiel, concentration en chacune des levures). Afin d'étudier ce système complexe, il sera donc nécessaire de choisir certains facteurs parmi ceux connus pour leur impact important sur les interactions, pour les étudier plus en détail.

Afin d'étudier ces interactions, le couple *Saccharomyces cerevisiae* / *Lachancea thermotolerans* (*Lt*) a été choisi en raison des intérêts technologiques de *Lt* dans le domaine œnologique. En effet, cette levure, bien qu'ayant un rendement en éthanol au cours de la fermentation plus faible que *Sc*, présente néanmoins une bonne tolérance à cet alcool par rapport à d'autres levures non-*Saccharomyces* (García *et al.*, 2021; Hranilovic, Gambetta, *et al.*, 2018) et est capable dans certaines conditions de persister au cours de la fermentation mixte. *Lt*, de plus, a des intérêts pour la qualité organoleptique du vin fini,

puisqu'elle permet par sa production de composés d'arômes de moduler le profil aromatique du vin obtenu. *Lt* est en effet capable de produire des terpènes, des composés thiols, des esters d'acides gras et, à partir des alcools supérieurs, des esters d'acétate contribuant à la notion de fraîcheur des vins. En outre, *Lt* produit peu d'acide acétique et est même capable de le consommer par respiration, ce qui permet de réduire l'acidité volatile des vins, généralement considérée comme un critère indésirable (Morata, Bañuelos, *et al.*, 2019; Porter, 2019; Vilela, 2018).
En outre, l'une des caractéristiques majeures de *Lt* est sa capacité à produire de l'acide lactique, avec comme conséquence une augmentation de l'acidité du vin contribuant non seulement à l'équilibre des différents composants (sucres, acides, alcool) et donc à sa qualité sensorielle, mais aussi à la bonne conservation du vin. Cette production d'acide lactique ayant lieu pendant sa phase exponentielle de croissance, elle permet à *Lt* d'être compétitive vis-à-vis des autres microorganismes en début de fermentation (Kapsopoulou *et al.*, 2007; Morata, Bañuelos, *et al.*, 2019; Morata *et al.*, 2018). Le rôle de *L. thermotolerans* dans le cadre d'une bioprotection a d'ailleurs été décrit (S. Benito, 2018; Gianvito *et al.*, 2022; Nally *et al.*, 2018). Cette souche a par ailleurs été brevetée pour ses intérêts technologiques dans le cadre de la production de vin ou de bière (impact sur le profil aromatique, l'acidité) (Farber 2022, Guoliang *et al.* 2019, Sheppard *et al.* 2016, Sommer et Nielsen 2004, Yanlin *et al.* 2021). Enfin, d'un point de vue pratique, cette levure existe déjà en tant que starter commercial. Seule ou en association avec *Sc* au sein de starters proposés par Christian Hansen, AEB ou Lallemand, cette levure est d'ores-et-déjà utilisée par la profession, et les starters commerciaux l'incluant sont également utilisés par des travaux de recherche (S. Benito, 2018; Hranilovic, Li, *et al.*, 2018; Roudil *et al.*, 2020; Whitener *et al.*, 2017).

Par ailleurs, *S. cerevisiae* a été décrite par différents auteurs comme interagissant avec *L. thermotolerans* via différents mécanismes, notamment par contact cellulaire (Luyt *et al.*, 2021; Nissen & Arneborg, 2003; Petitgonnet *et al.*, 2019) ou via la production de peptides antimicrobiens (Albergaria *et al.*, 2010; Kemsawasd *et al.*, 2015). Si *S. cerevisiae* peut gêner la croissance de *L. thermotolerans*, cette dernière peut également, dans certaines conditions, affecter celle de *S. cerevisiae* (Luyt *et al.*, 2021; Romani *et al.*, 2020). Ce couple de levures constitue donc un choix intéressant pour l'étude des interactions et de leur modulation par les conditions de fermentation.
Au vu de l'état de l'art, ces travaux de thèse s'attacheront donc à répondre à deux questions principales. Comment les différents paramètres de fermentation impactent les interactions levuriennes pendant une culture mixte, et par extension le vin obtenu ? Comment mieux les étudier, et donc les comprendre pour ensuite les maîtriser ?
Cette double question demande, comme discuté ci-dessus dans l'état de l'art, un questionnement méthodologique approfondi, en visant à mieux standardiser le milieu utilisé, mais aussi à raisonner de manière multiparamétrique pour pouvoir effectuer des comparaisons d'impact entre les facteurs étudiés. Dans un second temps et pour compléter cette démarche d'amélioration méthodologique, la possibilité d'améliorer la précision et la fiabilité des systèmes de suivi des populations microbiennes sera étudiée, via la mise en place d'une automatisation des mesures de cytométrie en flux.

2. Modélisation multiparamétrique de l'impact des paramètres de fermentation sur les cultures mixtes, les interactions entre levures et le vin fini

Les paramètres de fermentation (biotiques et non biotiques) impactent la fermentation elle-même, mais également les interactions entre levures, comme le montrent les résultats contradictoires observés en comparant les résultats de différents auteurs (Bordet *et al.*, 2020) ou les travaux d'auteurs s'attachant à montrer l'impact de certains facteurs sur les interactions (Gobbi *et al.*, 2013). Il apparaît donc pertinent, comme discuté plus haut, d'adopter une approche simultanée et multiparamétrique afin d'étudier à la fois les impacts différenciés de ces paramètres, et d'éventuels effets synergiques ou antagonistes entre eux. Cette approche prend la forme d'un plan expérimental, qui permettra, outre la modélisation multiparamétrique essentielle pour l'étude, de réduire le nombre d'expérimentations nécessaires à la réalisation de celle-ci. Pour qu'un tel plan soit pertinent, il convient de correctement définir au préalable les paramètres à faire varier.

De nombreux paramètres peuvent en effet avoir un impact sur les interactions entre levures au cours de la fermentation alcoolique : composition du milieu (azote), conditions environnementales (température, oxygène), conditions d'ensemencement des levures (ratio d'ensemencement, délai entre l'ensemencement des deux levures). Toutefois, si l'étude en laboratoire est possible, certaines modulations paramétriques ne sont pas appliquées en chai (modification drastique de la température, oxygénation en continu). C'est pourquoi les paramètres, conditions et limites du plan d'expérimentation mis en place au cours de cette thèse, ont été définis en prenant en compte la possibilité d'appliquer *in fine* les résultats dans l'environnement réel de la cave de vinification. Les œnologues peuvent aisément modifier la quantité de levures ensemencées, ajouter des sources d'azote dans le jus de raisin. Ils ont également, couramment, la possibilité d'oxygéner ponctuellement le moût (agitation, bullage) ou de contrôler la température des cuves de fermentation afin d'éviter un échauffement conduisant à la mort des levures.

Un bref état de l'art (2.1.) sur les paramètres choisis sera donc effectué afin de comprendre leurs impacts déjà connus sur le couple *S. cerevisiae* / *L. thermotolerans*, et définir la plage de variation pertinente pour cette étude. Les résultats d'un premier plan d'expérimentation seront ensuite présentés (2.2). Enfin, pour affiner les résultats publiés et d'aller plus loin dans leur analyse, des expériences complémentaires seront abordées (2.3.).

2.1. Impact des paramètres de fermentation sur les fermentations mixtes *Saccharomyces cerevisiae* / *Lachancea thermotolerans* : état de l'art

L'impact connu des paramètres de fermentation sur les fermentations mixtes *Sc* / *Lt* est abordé ici sous deux angles successifs : d'abord sur les dynamiques de population, puis sur les résultats de la fermentation (modification de la composition du milieu).

Les paramètres de fermentation peuvent en effet avoir une influence sur le développement de chacune des souches en culture pure, avec des impacts différents en termes de nature et d'intensité selon la souche. Ces impacts ont nécessairement des conséquences sur la dynamique des populations en culture mixte, mais seront modulés par les différents types d'interactions qu'ont les deux souches entre

elles.
De même, les levures ayant des métabolismes différents, la transformation du moût lors d'une culture pure conduit déjà à des métabolites différents selon l'espèce et la souche utilisées. En outre, la transformation du moût par ces levures fait intervenir de nombreuses voies métaboliques. Les sucres sont transformés, via la glycolyse, en pyruvate. Ce pyruvate peut être, en présence d'oxygène (respiration) converti en acétyl-CoA (qui entrera dans le cycle de Krebs et la synthèse d'acides gras) ou, en conditions anaérobies (fermentation), transformé en acétaldéhyde puis en éthanol. Cet acétaldéhyde peut aussi être à l'origine de la formation d'acide acétique. Les acides aminés sont eux à l'origine de la formation de composés d'arômes : alcools supérieurs via la voie d'Ehrlich, puis esters en combinaison avec l'acétyl-CoA et les dérivés d'acides gras (Dzialo *et al.*, 2017). Toutes ces voies métaboliques sont gérées par des enzymes et sont donc dépendantes des conditions de fermentation (composition initiale, oxygénation, température). En culture mixte, la composition du moût est par conséquent la résultante de ces métabolismes, résultante modulée en fonction des dynamiques de population obtenues pendant la fermentation. Néanmoins, des interactions entre souches conduisent à des activités métaboliques spécifiques et impactent également la transformation du moût, empêchant ainsi de pouvoir considérer la composition finale obtenue comme une simple résultante des métabolismes différenciés et des dynamiques de population à l'œuvre (Ciani & Comitini, 2015; Gobbi *et al.*, 2013).
Pour les dynamiques de population comme pour la modification du milieu sont développés les impacts connus des quatre paramètres de fermentation choisis pour ces travaux de recherche : concentration en azote du moût initial, conditions d'ensemencement, température de fermentation et oxygénation du moût pendant la fermentation.

2.1.1. Impact des paramètres de fermentation sur les dynamiques de population

2.1.1.1. Concentration en azote du moût

La concentration en azote du milieu constitue un point clé à maîtriser pour conduire une fermentation. Il s'agit un élément essentiel qui permet la production de biomasse et augmente la vitesse d'utilisation des sucres (Comitini *et al.*, 2021). Une déficience en azote (moins de 140 ou 150 $mg \cdot L^{-1}$ dans le moût selon les auteurs) peut conduire à des cinétiques de croissance inadéquates des levures (du fait d'un blocage du système de transport des hexoses) et à des fermentations incomplètes (Bely *et al.*, 1990; Pretorius, 2000). À l'inverse, un excès d'azote minéral peut être à l'origine d'accumulation de produits indésirables (carbamate d'éthyle) (Pretorius, 2000).

Des différences dans le métabolisme de l'azote sont observées entre *S. cerevisiae* et les levures non-*Saccharomyces*. Ces différences dépendent de l'espèce mais aussi vraisemblablement de la souche. *L. thermotolerans* peut ainsi montrer une préférence pour l'azote issu des acides aminés, ou bien pour l'ammonium selon les études considérées (de Koker, 2015; Fairbairn *et al.*, 2021; Roca-Mesa *et al.*, 2020). La vitesse d'absorption des acides aminés par *Lt* est plus élevée que celle de *Sc* dans certains travaux (Prior *et al.*, 2019) ou moins élevée dans d'autres (de Koker, 2015), faisant apparaître un effet souche ou une modulation par les conditions environnementales (agitation notamment). L'impact des sources d'azote sur la croissance peut donc varier selon la nature de ces sources (acides aminés spécifiques) et selon la levure. En outre, la présence d'ammonium semble favoriser la consommation des acides aminés (Roca-Mesa *et al.*, 2020). Tous ces phénomènes se traduisent donc par une

croissance relative de chaque souche plus ou moins importante en fonction de la disponibilité des différents composés azotés et de la compétition que cela induit avec *Sc* : ainsi la décroissance de *Lt* en culture mixte séquentielle avec *Sc* est plus lente avec addition de sa source d'azote préférée (Fairbairn *et al.*, 2021).

2.1.1.2. Conditions d'ensemencement

Afin de favoriser le développement de *L. thermotolerans* en culture mixte avec *S. cerevisiae*, l'impact d'un ratio d'ensemencement en faveur de *Lt* a été étudié car il pourrait aider à l'implantation de celle-ci, au moins en début de fermentation. Une meilleure persistance de *Lt* au cours de la fermentation (15 jours au lieu de 10) et en parallèle une croissance plus lente et une population maximale plus faible de *Sc* est observée lorsque le ratio *Sc* / *Lt* est fortement diminué (de 1 à 0.01 voire 0.0001) (Comitini *et al.*, 2011). Dans d'autres conditions, la population maximale de *Lt* est augmentée lorsque le ratio diminue de 0.2 à 0.02 (Morales *et al.*, 2019).

Le mode d'ensemencement a également un impact fort sur la dynamique des populations. Si avec un ensemencement simultané, un déclin rapide de la population de *Lt* est observé en début de fermentation, un ensemencement séquentiel (ensemencement du milieu avec *Lt* puis inoculation de *Sc* après un certain délai : 24, 48, 72, 96 h) permet de limiter l'impact négatif de *Sc* sur la population de *Lt*, voire d'obtenir l'effet inverse. Avec un ratio d'ensemencement de 1, un ensemencement séquentiel conduit à une population de *Lt* supérieure et retarde son déclin (Á. Benito *et al.*, 2016; Kapsopoulou *et al.*, 2007), voire permet de maintenir la population tout au long de la fermentation (Balikci *et al.*, 2016). Ces résultats différents mettent en évidence l'influence de la souche et de la concentration d'ensemencement. Avec un ratio d'ensemencement de 0.1, la population de *Lt* peut devenir dominante pendant toute la fermentation, avec une population de *Sc* inférieure de 1 à 2 log, lorsque *Sc* est ensemencée 24 ou 48 h après *Lt* (Gobbi *et al.*, 2013). Cette même étude montre également que cet effet "mode d'ensemencement" est moins important dans le cas d'essais au stade industriel : l'ensemencement séquentiel permet d'augmenter la persistance de *Lt* mais pas de parvenir à une dominance de cette levure. L'augmentation du pouvoir de compétition de *Lt* par rapport à *Sc* dans le cas d'un ensemencement séquentiel est lié au développement plus important de *Lt* seul en début de la fermentation et donc à l'obtention d'une densité cellulaire importante lors de l'inoculation de *Sc* : la compétition avec *Sc* peut alors mettre en œuvre une compétition pour les nutriments, ou un autre mécanisme comme le contact cellulaire qui, comme discuté plus haut (cf. 1.2.2) serait favorisé par la densité cellulaire. Cet ensemencement séquentiel est utilisé par de nombreux auteurs pour favoriser l'implantation et prolonger le maintien de *Lt* en culture mixte avec *Sc*. (S. Benito *et al.*, 2015; Binati *et al.*, 2020; Dutraive *et al.*, 2019; Escribano *et al.*, 2018; Hranilovic *et al.*, 2021; Morata, Bañuelos, *et al.*, 2019).

2.1.1.3. Température

La température de conduite de la fermentation est également un facteur important à maîtriser puisque des fermentations mixtes *Sc* / *Lt* (ratio de 0.1) à 20°C et à 30°C conduisent à des dynamiques de populations différentes (Gobbi *et al.*, 2013) : dominance de *Sc* à 30°C avec diminution de la population de *Lt* après 4 jours mais persistance de *Lt* pendant toute la durée de la fermentation et population inférieure de *Sc* à 20°C. Une température de 20°C permet de supprimer l'impact négatif de *Sc* sur la croissance de *Lt* et conduit au contraire à une inhibition de la croissance de *Sc* par *Lt*, mettant ainsi en

évidence un impact de la température sur les interactions entre *Sc* et *Lt*. Une hypothèse a été émise par l'équipe de Fleet : les basses températures diminueraient la sensibilité à l'éthanol des non-*Saccharomyces*, leur permettant de persister plus longtemps (Fleet, 2003).

2.1.1.4. Oxygénation

Même si la fermentation alcoolique se déroule en grande partie en anaérobie, une oxygénation du moût est souvent effectuée en début de fermentation car elle est connue comme stimulant la croissance, et permet également d'augmenter la synthèse des acides gras insaturés contribuant ainsi à la fluidité membranaire (Valero *et al.*, 2001). Or le maintien d'une bonne intégrité membranaire joue un rôle dans la résistance à l'éthanol des levures (Branco *et al.*, 2012). L'oxygénation du milieu pourrait donc modifier la tolérance à l'éthanol des non-*Saccharomyces* et donc les dynamiques de population en culture mixte (Gianvito *et al.*, 2022).
Une oxygénation du milieu a tendance à favoriser le développement ou la persistance de *Lt* en culture mixte avec *Sc* (Nissen *et al.*, 2004), voire permet d'obtenir une population plus importante que *Sc* (Shekhawat *et al.*, 2017, 2018). Différents mécanismes sont proposés pour expliquer cet effet. Bien qu'étant anaérobie facultative et sujet à l'effet Crabtree comme *Sc*, *Lt* croît plus rapidement que *Sc* en présence d'oxygène, du fait d'une plus grande facilité de *Lt* à dévier son métabolisme vers la respiration (Shekhawat *et al.*, 2017). Des besoins plus importants d'oxygène pour la croissance et une augmentation, en milieu oxygéné, de la capacité relative (par rapport à *Sc*) de *Lt* à assimiler le glucose, permettent d'améliorer la persistance de *Lt* (Nissen *et al.*, 2004).

2.1.2. Impact des paramètres de fermentation sur la composition du moût et du vin

2.1.2.1. Concentration en azote du milieu

Des différences dans le métabolisme de l'azote sont observées entre *S. cerevisiae* et les levures non-*Saccharomyces* et dépendent de l'espèce mais aussi vraisemblablement de la souche. Selon les auteurs, *L. thermotolerans* montre une préférence pour l'azote issu des acides aminés (de Koker, 2015) ou pour l'ammonium (Fairbairn *et al.*, 2021). Ces préférences entraînent des phénomènes de compétition directe avec *Sc* pour les composés azotés lors des cultures mixtes, qui peuvent être à l'origine de changements de voies métaboliques permettant l'utilisation des composés disponibles. Ces modifications de métabolisme liées aux interactions entre souches mais aussi aux sources d'azote disponibles en début de fermentation auront comme conséquence des modifications de la composition du milieu, ainsi que du profil aromatique du vin puisque les acides aminés sont à l'origine de la synthèse de multiples composés d'arômes (Fairbairn *et al.*, 2021; Rollero *et al.*, 2021; Roullier-Gall *et al.*, 2022).
En outre, des phénomènes plus complexes de régulation des voies métaboliques de formation des composés d'arôme ont récemment été mis en évidence chez *Sc* : impact de l'ajout d'azote sur le métabolisme du carbone, sur la synthèse d'acides aminés, sur la production d'arômes, importance du moment d'ajout de l'azote, régulation des voies enzymatiques (menant notamment aux esters d'acétate ou d'éthyle) par des mécanismes différents (surexpression de gènes ou non)... (Godillot *et al.*, 2022).
À teneur en azote assimilable équivalente, la nature des composés azotés a un impact sur les composés volatils produits : une addition d'acides aminés à chaîne ramifiée permet par exemple d'augmenter les alcools supérieurs dont ils sont précurseurs (phényl-éthanol, isobutanol, alcool isoamylique) (Fairbairn *et al.*, 2021).

2.1.2.2. Conditions d'ensemencement

Le ratio d'ensemencement *Sc* / *Lt*, par son impact sur les dynamiques de population d'une culture mixte, peut significativement influencer la composition du moût après fermentation. Ainsi, un ratio favorisant *Lt* peut augmenter son impact sur la résultante de la culture mixte par rapport à une culture pure de *Sc* : réduction du pH, augmentation de l'acidité totale, des alcools supérieurs, de certains esters, diminution d'autres esters, des acides gras… Ces modifications du milieu en fonction du ratio d'ensemencement s'effectuent en parallèle d'une augmentation de la population de *Lt* et de sa persistance (Comitini *et al.*, 2011; Morales *et al.*, 2019).

L'ensemencement séquentiel (inoculation de *Sc* après *Lt*) a logiquement un impact sur la modification du milieu lié à la fermentation, du fait d'une croissance plus importante de *Lt* en début de fermentation : consommation des sucres moins rapide et moins complète, diminution plus importante de la production d'éthanol, augmentation des métabolites plus spécifiquement produits par *Lt* que par *Sc* (acide lactique, glycérol, 1-propanol, acétate d'éthyle…)... (Gobbi *et al.*, 2013). Ces résultats sont observés dans d'autres travaux (Balikci *et al.*, 2016; Á. Benito *et al.*, 2016; Kapsopoulou *et al.*, 2007), à des degrés divers (impact sur l'éthanol, le glycérol, les composés d'arômes… parfois différents), montrant donc le lien entre dynamique de population et modification du milieu mais aussi des interactions avec d'autres facteurs (souche, conditions opératoires). Par contre, pour certains composés, les fermentations séquentielles peuvent conduire à des modifications du milieu différentes de celles observées lors d'une coculture : augmentation de la production de polysaccharides, de 1-propanol, diminution de la production d'acétate d'isoamyle (Balikci *et al.*, 2016; Á. Benito *et al.*, 2016; Gobbi *et al.*, 2013). Ces résultats mettent donc en évidence un éventuel impact du mode d'ensemencement sur le métabolisme d'une ou des deux levures : ensemencement séquentiel et modification du ratio ne sont pas équivalents en termes d'impact sur le milieu.

2.1.2.3. Température

La température de fermentation, outre son impact sur le développement respectif des deux levures en présence, a également un impact sur la production de métabolites et notamment sur la production d'éthanol. Une diminution de la production d'éthanol est observée dans une coculture *Sc* / *Lt* à 20°C mais ne l'est pas à 30°C (Gobbi *et al.*, 2013). Le même type de résultats est obtenu également avec d'autres non-*Saccharomyces* par Edwards et Aplin (2022) qui observent qu'une température plus basse pendant le développement des levures non-*Saccharomyces* avant l'inoculation de *Sc* (fermentation séquentielle) semble impacter la production d'éthanol, non seulement en facilitant le développement des non-*Saccharomyces,* mais aussi en agissant sur le métabolisme oxydatif des levures (transformation des sucres en d'autres composés que l'éthanol, par d'autres voies métaboliques, encore à identifier).
L'effet de la coculture (comparaison de la production de certains métabolites en culture mixte par rapport à une culture pure de *Sc*) peut être modulé par la température (Gobbi *et al.*, 2013) : observation d'un effet à 20°C et non à 30°C (augmentation de la production de glycérol, réduction de l'acétaldéhyde, augmentation des alcools supérieurs dont le 2 phényl-éthanol, de certains esters), effet accentué à 20°C par rapport à 30°C (augmentation de la production d'acide lactique) ou inversion de l'effet (augmentation de l'acidité volatile à 20°C et diminution à 30°C). Par contre, la production de polysaccharides ne semble pas influencée par la température mais reste liée à la présence des deux levures.
Les mécanismes pouvant expliquer l'impact de la température sur les composés d'arômes n'ont pas été

étudiés lors de cultures mixtes *Sc / Lt*, mais des informations peuvent être trouvées dans les études en cultures pures. Chez *Sc*, une modification de la composition en lipides des levures pour moduler la fluidité membranaire en fonction de la température peut être impliquée : une température de fermentation basse entraîne une production plus importante d'acides gras à moyenne chaîne, qui seront ensuite transformés en esters pour diminuer leur toxicité (Beltran *et al.*, 2008; Massera *et al.*, 2021). Les profils aromatiques différents selon la température de fermentation peuvent, au moins en partie, être liés à une expression différente de certains gènes impliqués dans des voies métaboliques conduisant à la production de composés d'arômes (Molina *et al.*, 2007).
Pour les cultures pures de *Lt*,, la température a un impact sur la production de composés volatils (augmentation des alcools supérieurs, diminution des composés carbonylés et des esters lorsque la température diminue de façon générale) mais avec des variations importantes selon les souches (Vaquero *et al.*, 2020).

2.1.2.4. Oxygénation

En fonction de la présence ou non d'oxygène, les levures anaérobies facultatives peuvent consommer les sucres selon deux voies métaboliques : la respiration et la fermentation, cette dernière conduisant à la production d'éthanol. Lorsque la concentration en sucres est importante, la fermentation alcoolique est la voie préférée même en présence d'oxygène (effet Crabtree). Cet effet Crabtree, moins important chez *Lt*, inhibe donc moins chez cette levure le métabolisme respiratoire, ce qui conduit à une production d'éthanol moins importante (Vicente *et al.*, 2021).
De la même manière que les autres paramètres de fermentation, l'oxygénation du milieu impacte la production de métabolites via son impact direct sur les dynamiques de population, chaque espèce de levure ayant des facultés propres à synthétiser certains composés. Néanmoins, l'oxygénation induit également un changement de métabolisme (de fermentaire à respiratoire) et donc de modification de la production de certains composés. L'augmentation de la dégradation des sucres via la respiration, et davantage chez *Lt* que chez *Sc* entraîne une augmentation de la biomasse et de la production d'acide acétique au détriment de la production d'éthanol (Shekhawat *et al.*, 2017). En ce qui concerne les composés volatils, une augmentation des alcools supérieurs et des acides et une diminution des acides gras à moyenne chaîne et des esters est observée par ces mêmes auteurs. Ces modifications peuvent être reliées à une persistance plus importante de *Lt* mais aussi par l'induction ou la répression de certains gènes lors d'une oxygénation (facilitation de l'assimilation de précurseurs, enzymes impliquées dans la synthèse) (Shekhawat *et al.*, 2018).
A l'inverse, l'anaérobiose entraîne chez *Lt* une réponse transcriptomique similaire à celle induite chez *Sc* mais se distingue par une surexpression de gènes codant pour une lactate déshydrogénase qui lui permet de dévier le flux métabolique vers une production d'acide lactique (Shekhawat *et al.*, 2020).

2.1.3. Conclusions

En impactant les dynamiques de population de *S. cerevisiae* et de *L. thermotolerans*, les différents paramètres de fermentation biotiques et abiotiques, ont également un impact sur la production des différents métabolites issus de la fermentation (éthanol, acides, molécules volatiles…), chaque espèce, voire chaque souche de levure ayant des facultés propres à synthétiser certains composés. Néanmoins, il apparaît que ces paramètres ont également une influence sur le métabolisme propre de chaque levure, complexifiant ainsi le système et rendant très difficile la prévision des caractéristiques du vin fini.

L'état de l'art permet donc de mettre en évidence la complexité du système étudié, la fermentation alcoolique du moût de raisin avec ensemencement de deux levures différentes : interactions entre levures, impact de facteurs environnementaux sur les levures, interactions entre facteurs environnementaux et impact des facteurs environnementaux sur les interactions entre levures. Cela confirme donc la nécessité d'aborder cette problématique via une approche multiparamétrique et simultanée de toutes les facettes de la fermentation (dynamiques des populations, modification du milieu, qualité du moût fermenté) pour pouvoir différencier aux mieux les impacts respectifs des quatre paramètres étudiés.

2.2. Approche multiparamétrique et simultanée de l'impact des paramètres de fermentation sur les interactions entre *Saccharomyces cerevisiae* et *Lachancea thermotolerans*

2.2.1. Contexte

Comme discuté précédemment, un focus est effectué au cours de ces travaux sur les quatre paramètres suivants : les conditions d'ensemencement des deux levures *S. cerevisiae* et *L. thermotolerans*, la concentration en sources d'azote dans le moût, l'oxygénation du moût et la température au cours de la fermentation alcoolique.

Concernant les conditions d'ensemencement, comme le but est ici de maximiser les interactions entre *Sc* et *Lt* afin de les étudier, un ensemencement simultané est choisi pour maximiser le temps de contact entre les deux levures et s'assurer que les deux levures entrent en contact dans le même état physiologique pour toutes les conditions testées. Seul le ratio d'ensemencement *Sc* / *Lt* est donc modulé.

Ces quatre paramètres sont étudiés dans le cadre d'un plan expérimental Taguchi à deux niveaux. Comme illustré dans la figure 2, des cultures mixtes *Sc* / *Lt* sont conduites en laboratoire, avec un ensemencement simultané de ces levures (à la même concentration ou avec une concentration de *Lt* 10 fois supérieure à *Sc*) dans un moût synthétique contenant 150 ou 300 $mg \cdot L^{-1}$ d'azote total. Les fermentations sont effectuées à 18 ou 28°C et une oxygénation par bullage est effectuée sur la moitié des échantillons.

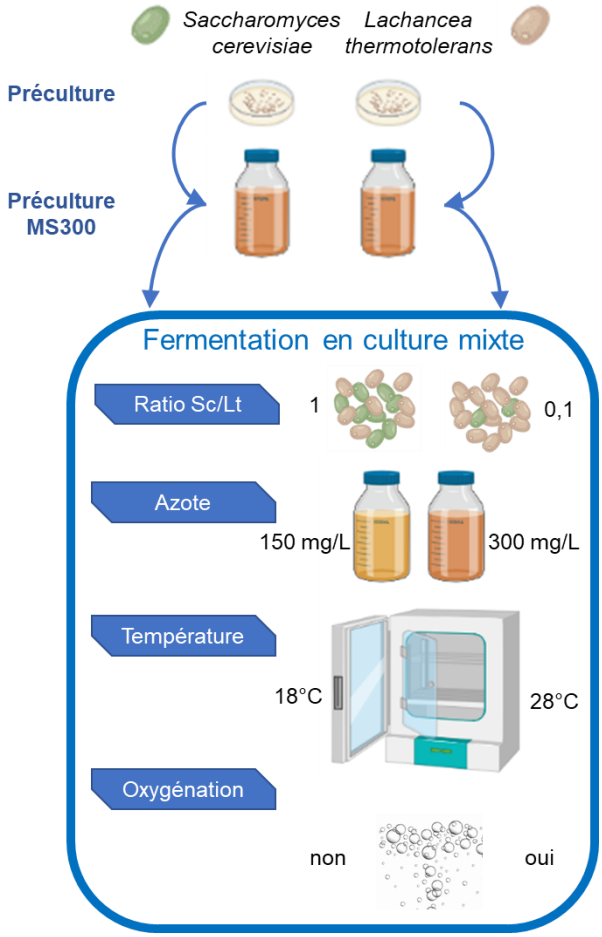

Figure 2. Paramètres étudiés et niveaux choisis pour le plan expérimental.

L'impact des différents paramètres sur ces fermentations est étudié à travers plusieurs axes d'analyse : dynamique des populations levuriennes, conduite de la fermentation (temps de fermentation, consommation des sucres, production d'éthanol, acidification), et composition en composés volatils des vins synthétiques obtenus.

La mise en place de ce plan expérimental permet de conserver une solidité statistique tout en réduisant le nombre de modalités à tester. L'utilisation d'un tel plan permet également d'étudier l'impact simultané des quatre paramètres et de comparer ces impacts via le calcul d'effets standardisés. Ces travaux ont fait l'objet d'un article scientifique présenté ci-après.

fermentation

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Article

Multiparametric Approach to Interactions between *Saccharomyces cerevisiae* and *Lachancea thermotolerans* during Fermentation

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Abstract: The aim of a significant part of current wine technology research is to better understand and monitor mixed culture fermentations and optimize the microbiological processes and characteristics of the final wine. In this context, the yeast couple formed by *Lachancea thermotolerans* and *Saccharomyces cerevisiae* is of particular interest. The diverse results observed in the literature have shown that wine characteristics are dependent on both interactions between yeasts and environmental and fermentation parameters. Here, we took a multiparametric approach to study the impact of fermentation parameters on three different but related aspects of wine fermentation: population dynamics, fermentation, and volatile compound production. An experimental design was used to assess the effects of four independent factors (temperature, oxygenation, nitrogen content, inoculum ratio) on variables representing these three aspects. Temperature and, to a lesser extent, oxygenation and the inoculum ratio, were shown to constitute key factors in optimizing the presence of *Lachancea thermotolerans* during fermentation. The inoculum ratio also appeared to greatly impact lactic acid production, while the quantity of nitrogen seemed to be involved more in the management of aroma compound production. These results showed that a global approach to mixed fermentations is not only pertinent, but also constitutes an important tool for controlling them.

Keywords: mixed culture; yeast–yeast interactions; multiparametric approach; fermentation; aroma compounds

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1. Introduction

For several years, the management of must fermentation to obtain wines with high organoleptic quality has involved the use of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts. The latter are indeed interesting for use in wine production for several reasons [1]: they increase aromatic diversity [2,3], decrease ethanol concentration [4], induce acidification [5,6], and contribute to biopreservation [7], etc. Nevertheless, cofermentations of *S. cerevisiae* and non-*Saccharomyces* yeasts are not yet completely controlled (longer fermentations, variability of product quality, etc.) due to complex and as yet not fully understood interactions between yeasts. Various interactions (competition, amensalism, mutualism, and commensalism) can be involved through different mechanisms (release of beneficial or toxic compounds, exchange of metabolites, metabolism changes linked to production of quorum sensing molecules or to cell-to-cell contacts, etc.) [8]. These interactions have an impact on the population dynamics (dominance of one species, persistence throughout fermentation), fermentation results (modulation of ethanol content, pH, etc.) and aroma production. The aroma profile in mixed *S. cerevisiae*/non-*Saccharomyces* cultures is indeed not just a simple juxtaposition of aroma compounds produced by each species in pure culture conditions, as it is also impacted by interactions between these species, either directly via population dynamics or indirectly via metabolism variations [9,10].

Among all non-*Saccharomyces* yeasts, *Lachancea thermotolerans* is of particular interest. Its unique ability to generate lactic acid as a coproduct of its sugar metabolism makes it very valuable for reducing the pH of wines, especially those from warmer viticulture areas [11]. In addition to this specificity, *L. thermotolerans* also provides many other technical benefits for vinification, such as a lower sugar-to-ethanol yield [12], high glycerol production [13], and low acetic acid production [2,11]. In addition, *L. thermotolerans* can modulate volatile compound production in wine, since it is the only species amongst non-*Saccharomyces* yeasts to potentially possess all the enzymes involved in aroma production. The following enzymes have been highlighted: lipases and esterases to produce esters from free fatty acids, aminopeptidases to release the amino acid precursors of aromas, β-glucosidase to release terpenes from precursors, and carbohydrolases to degrade the cell walls of berries and release aroma precursors [14,15]. Esters, terpenes, and higher alcohols confer fruity and floral notes to wine, thus leading to greater aroma diversity in the final product [2,11,14].

Moreover, interactions between *L. thermotolerans* and *S. cerevisiae* have already been described in the past, and both seemed to significantly impact fermentation results and vary depending on culture conditions [16–18]. This study therefore focused on *L. thermotolerans* as the non-*Saccharomyces* species of interest due to both its technical capabilities and its interactions with *Saccharomyces cerevisiae*.

Studies by different authors on cofermentations between *S. cerevisiae* (*Sc*) and *L. thermotolerans* (*Lt*) showed variable impacts on products (ethanol production, acidification, the production of metabolites as aroma compounds, etc.) [19]. These inconsistent results can be explained by strain dependence and by the impact of environmental conditions. Factors such as medium composition, temperature, oxygenation, inoculation conditions, and so forth are indeed known to have an impact on yeast metabolism, on interactions between yeasts, and consequently on product quality. For example, Gobbi et al. (2013) showed that the population dynamics observed and the aroma compounds produced within a mixed *Sc*/*Lt* culture varied with medium (must), batch volume, and temperature [16]. Other studies highlighted that oxygen played a significant role in the population dynamics [17,20], and that both the amount and nature of nitrogen sources influenced global gene expression and thus impacted yeast growth, fermentation performance, and general metabolism [21,22]. An inoculum ratio favoring one yeast or the other has also been shown to significantly impact the population dynamics over time, and then the volatile compound profiles as well [23].

However, these factors are often studied separately, and their impacts have been shown to change when other conditions vary [19]. It is thus necessary to standardize study conditions to obtain a better understanding of the mechanisms involved and to optimize wine fermentation with both non-*Saccharomyces* yeasts and *S. cerevisiae*. Some authors have already adapted their protocols for this purpose, with some of them using a synthetic medium instead of natural must [24–26], and others applying experimental designs to study how yeast populations or fermentative media are impacted by different culture parameters [27–29]. The aim of this study was to combine these standardized conditions with a multiparametric approach to environmental conditions and their consequences on multiple levels: from population and fermentation dynamics to metabolite production (ethanol, lactic acid, volatile compounds). The general objective was, overall, to better understand how mixed culture fermentations involving *S. cerevisiae* and *L. thermotolerans* can be monitored and influenced, and how the resulting final wine will be shaped as a consequence.

2. Materials and Methods

2.1. Microorganisms, Preservation, and Preculture

Two yeast species were used in this study: *Saccharomyces cerevisiae* 59A HO::eGFP (*Sc*), a haploid derivative of the commercial wine strain EC1118 (Lallemand Inc., Montréal, QC, Canada) and *Lachancea thermotolerans* BBMCZ7FA20 (*Lt*) (previously isolated and identified

by Sadoudi et al. 2012 [10]). The eGFP mutation in *S. cerevisiae* confers a green fluorescence to this strain and allows the differentiation between both yeasts in flow cytometry.

Yeasts were preserved in YPD/glycerol (50/50 *w*/*w*) at −80 °C in the lab collection.

Each strain was grown first at 28 °C on YPD agar (glucose 20 g·L^{-1}, peptone 10 g·L^{-1}, yeast extract 5 g·L^{-1}, agar 18 g·L^{-1}). Then, a preculture was obtained in MS300 liquid medium at 28 °C and agitated at 150 rpm. This synthetic must was first developed by Bely et al. (1990) [24], and is usually used in oenological research [30–33]. It contains carbon sources (glucose, fructose, organic acids), nitrogen sources (mineral and organic, 300 mg·L^{-1}), minerals, vitamins, and growth factors. Media components were purchased from Sigma^{TM} (Merck KGaA, Darmstadt, Germany). The yeasts were collected at the end of the exponential phase to inoculate the culture media.

2.2. Experimental Design

A 2-level Taguchi experimental design was used to assess the effects of 4 independent factors (temperature, oxygenation, the nitrogen content of the culture medium, and the *Sc*/*Lt* inoculation ratio) at two different levels on different variables (yeast population, medium composition, volatiles). Given this experimental design, only 8 out of 16 possible conditions had to be tested to assess the relations between the entry parameters and the observed variables, resulting in a L8(4^2) Taguchi plan. The levels for the 4 independent factors studied are summarized in Table 1. Table 2 shows the eight conditions of the experimental design and corresponding parameters.

Table 1. Parameters tested in the experimental design.

	X1	X2	X3	X4
	***Sc*/*Lt* Ratio**	**Nitrogen (mg·L^{-1})**	**Oxygenation**	**Temperature (°C)**
Level 1	0.1	150	No	18
Level 2	1	300	Yes	28

Table 2. Conditions tested in the experimental design and corresponding parameters.

Condition	*Sc*/*Lt* Ratio	Nitrogen (mg·L^{-1})	Oxygenation	Temperature (°C)
A	0.1	150	No	28
B	1	150	No	18
C	1	300	No	18
D	0.1	300	Yes	18
E	1	150	Yes	28
F	0.1	300	No	28
G	0.1	150	Yes	18
H	1	300	Yes	28

Synthetic must was prepared with 2 levels of nitrogen content: MS300 (300 mg·L^{-1} of total nitrogen) and MS150 (150 mg·L^{-1} of total nitrogen). Sterile glass flasks (Schott AG, Mainz, Germany) (GL45 screw thread, effective volume 1 L) were filled with 1 L of synthetic must. Media were inoculated with *L. thermotolerans* at 10^6 cells·mL^{-1} and with *S. cerevisiae* at 10^5 and 10^6 cells·mL^{-1} to obtain a *Sc*/*Lt* ratio of 0.1 and 1, respectively.

Cultures were carried out in these nonhermetically closed Schott flasks and in static conditions. For the oxygenated samples, air was added at the end of exponential growth by sparging (until oxygen saturation of the medium) to mimic the process in industrial conditions.

Cultures were conducted at two temperatures: 18 °C and 28 °C, which can be used in winemaking for white and red wine fermentations, respectively.

2.3. Yeast Population

The yeast population was monitored during fermentation using flow cytometry, with the eGFP mutation of *Sc* allowing for discrimination between *Sc* and *Lt*. To study yeast viability, propidium iodide dye (PI) (Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used due to its ability to bind to DNA when the cell membrane was compromised, triggering its fluorescence (maximum excitation/emission wavelengths 538/617 nm). The fermentation medium (1 mL) was centrifuged (10,000× *g*, 5 min, 4 °C), the pellet was resuspended in 1 mL phosphate-buffered saline (PBS) buffer (Fisher Scientific™, Thermo Fisher Scientific Inc.), and serial dilutions were prepared. PI (1 µL at 0.1 mg·mL^{-1} in water) was added to a 100 µL aliquot. Samples were incubated for 10 min in the dark and analyzed.

A BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used with the following conditions: a sample of 20 µL, flow 34 µL·min^{-1}, 488 nm wavelength argon laser to excite cells (autofluorescence) and dye, FL3-H long-pass filter (675 nm) for PI fluorescence, and FSC threshold of 80,000. Data were analyzed using the BD Accuri C6 software.

At the same time, the yeast population was monitored by enumeration on plates with Wallerstein Laboratory (WL) Nutrient Agar (Oxoid™, Thermo Fisher Scientific Inc.). This nutritive medium allows the identification of various yeasts via colony morphology and their aptitude to degrade bromocresol green: *Lt* appears as small blue colonies, while *Sc* colonies are larger and present a creamy-white color [34]. Serial dilutions (in PBS) of cell suspensions (100 µL) were spread on WL Nutrient Agar and incubated at 30 °C for 48 h before counting.

2.4. Chemical Analysis

Aliquots of culture media were centrifuged at 10,000× *g* for 5 min, and supernatants were stored at −20 °C until chemical analysis.

Ethanol concentration, residual sugars, and pH were determined using FTIR spectroscopy (OenoFoss™ type 4101, FOSS, Hilleroed, Denmark).

Lactic acid concentration was analyzed with an L-Lactic Acid Enzymatic Assay Kit (BioSenTec, Portet-sur-Garonne, France).

Volatile compounds were quantified using headspace–solid-phase microextraction/gas chromatography–mass spectrometry (HS–SPME/GC–MS). The extraction of volatiles was carried out using a Combi-pal autosampler (CTC Analytics, Zwingen, Switzerland). A volume of 2 mL of sample was placed in a 10 mL vial that was then fitted with a polytetrafluoroethylene (PTFE)/silicone septum and maintained under agitation at 40 °C for 10 min. After sample conditioning, an SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm, 1 cm long (DVB/CAR/PDMS, Supelco (Bellefonte, PA, USA)) was exposed for 30 min to the sample headspace and immediately desorbed in the gas chromatograph injector. Volatile compounds were analyzed by gas chromatography coupled to quadrupolar mass selective spectrometry using an Agilent 5973 Network Detector (Agilent Technologies, Palo Alto, CA, USA). Analytes were separated on a Supelcowax-10 (Supelco) 60 m × 0.25 mm I.D. with a 0.25 µm film thickness. The column temperature was held at 4 °C for 10 min, increased to 150 °C at 3 °C·min^{-1}., then to 250 °C at 15 °C·min^{-1}, and held for 5 min. The injector temperature was 260 °C, and the time of desorption of the fiber into the injection port was fixed at 5 min. The carrier gas was helium at a flow rate of 1.5 mL·min^{-1}. The temperature of the ion source was 230 °C, and that of the transfer line was 280 °C. Electron impact mass spectra were recorded at 70 eV ionization energy, 5.1 scan·s^{-1}. The GC–MS analysis was performed in the complete scanning mode (SCAN) in a mass range of 35–300 u.

The compounds were identified by comparison of their mass spectra and retention times with those of standard compounds or with those available in the Wiley 6 mass spectrum library and in the literature, respectively. The response factors of volatile compounds were calculated using a calibration curve obtained by analyzing a hydroalcoholic solution

(ethanol 10%, *v*/*v*) with different concentrations of reference compounds (ethanol 96%, 1-propanol, 3-methylbutyl acetate, 3-methylbutanol, ethyl octanoate, ethyl decanoate, 1-hexanol, 2-phenylethyl alcohol, and octanoic acid purchased from Sigma^{TM} (Merck KGaA, Darmstadt, Germany).

2.5. Result Processing and Analysis

To analyze the impact of fermentation parameters on the population dynamics, several quantitative data were calculated for each strain: maximal population (N_{max}), maximal growth rate (μ_{max}), and area under growth curve (AUC). AUC provides information about the global presence of a yeast throughout fermentation. To compare both yeasts and mitigate AUC-inherent variations depending on fermentation time, the following ratios were also calculated: $\mu_{max\,Lt}/\mu_{max\,Sc}$ and AUC_{Lt}/AUC_{Sc}.

The impact of the chosen factors on fermentation was studied as a function of the fermentation time (end of fermentation when sugar was totally consumed) and the production of ethanol and lactic acid.

Data analysis of the experimental design was performed with Ellistat software v.7.0.0 (Ellistat, Chavanod, France). To compare the effect of the fermentation parameters studied, a standardized effect was calculated, as was the corresponding *p*-value (Student's *t*-test), providing information about the significance of a given factor's effect (very significant if the *p*-value < 0.01, significant if the *p*-value < 0.05). The contribution to total variance was also expressed in percent for each factor to help visualize and compare how each of them impacted each observed or calculated variable relative to the others. Interactions between factors were not considered for any of the variables studied, as residuals contributed less to the total observed variance than other non-significant factors, and thus did not account for a sufficiently significant effect to warrant further analysis.

3. Results and Discussion

To obtain a better understanding of the impact of fermentation parameters on the interactions between *S. cerevisiae* and *L. thermotolerans*, and consequently on the transformation of must into wine, it was vital to study fermentation through a global approach. To reach this goal, the population dynamics were first studied due to the direct influence of the environment on yeast development. Then, fermentation kinetics and volatile metabolites production were assessed in that order, as they not only depend on environmental conditions, but also on the population dynamics and yeast–yeast interactions that took place during the fermentation process. The resulting combination of these three datasets made it possible to better understand the overall impacts of each factor studied.

3.1. Impact of Fermentation Parameters on Population Dynamics

The first objective was to study how fermentation parameters such as nitrogen content, inoculum ratio, oxygenation, and temperature could impact the development of both yeasts: how their maximal growth rate and maximal population were modified and, mainly, which strain would be predominant throughout fermentation. Here, the aim was not to study the impact of chosen parameters independently as in previous studies, but simultaneously through the experimental design to allow for a comparison between effects.

The impacts of fermentation parameters on variables describing the population dynamics are given in Table 3. For each parameter/variable couple, the standard effect, *p*-value, and contribution to variance are shown. The standardized effect provides information on the nature and level of the impact of the parameter on the variable: an increase in the parameter chosen leads to an increase in the variable studied when the standardized effect is positive, and to a decrease when the standardized effect is negative. The *p*-value provides information on the significance of this variation, and the contribution to variance indicates the importance of the parameter chosen in explaining the total variation of the variable studied.

Table 3. Impact of fermentation parameters on population dynamics (μ_{max}, N_{max}, AUC).

		Sc/*Lt* Ratio	Nitrogen	Oxygenation	Temperature	Residuals
$\mu_{max\ Sc}$	Stand. effect	−1.164	−1.168	−0.590	4.094	
	p-value	0.329	0.327	0.596	0.026	
	Var. contrib.	5.93%	5.97%	1.53%	73.40%	13.10%
$\mu_{max\ Lt}$	Stand. effect	−3.010	0.767	1.968	0.657	
	p-value	0.057	0.499	0.144	0.558	
	Var. contrib.	53.40%	3.47%	22.80%	2.54%	17.70%
$\mu_{max\ Lt}/\mu_{max\ Sc}$	Stand. effect	−0.590	1.510	1.259	−3.594	
	p-value	0.597	0.228	0.297	0.037	
	Var. contrib.	1.73%	11.30%	7.88%	64.20%	14.90%
$N_{max\ Sc}$	Stand. effect	1.240	0.517	−4.443	4.546	
	p-value	0.303	0.641	0.021	0.020	
	Var. contrib.	3.40%	0.59%	43.70%	45.70%	6.64%
$N_{max\ Lt}$	Stand. effect	−2.104	3.131	−0.462	−7.750	
	p-value	0.126	0.052	0.676	0.004	
	Var. contrib.	5.71%	12.60%	0.28%	77.50%	3.87%
AUC_{Lt}/AUC_{Sc}	Stand. effect	−6.999	0.888	6.822	−8.267	
	p-value	0.006	0.440	0.006	0.004	
	Var. contrib.	29.20%	0.47%	27.80%	40.80%	1.79%

Stand. effect = standardized effect; Var. contrib. = contribution to variance; N_{max} = maximal population; μ_{max} = maximal growth rate; AUC = area under growth curve. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey background), significant (*p*-value < 0.05, grey background), or very significant (*p*-value < 0.01, dark grey background).

Regarding *Saccharomyces cerevisiae*, the growth rate μ_{max} was positively and significantly impacted by temperature (stand. effect 4.094) and not by the other factors (contribution to variance of 73.4% for temperature). In contrast, the growth rate of *Lachancea thermotolerans* seemed to be impacted only by the inoculum ratio *Sc*/*Lt*, and at the limit of significance (stand. effect −3.010, *p*-value 0.057): $\mu_{max\ Lt}$ tended to be higher when the inoculum ratio favored *Lt*. In this study, oxygenation had no impact on the growth rate of either yeast, whereas previous works showed a positive effect on the μ_{max} of *Lt* [20]. No significant effect of nitrogen content was observed here, despite nitrogen sources having been previously shown to influence the growth of yeasts differently according to the nature and quantity of nitrogen compounds [21,35]. However, nitrogen content significantly impacted the growth rate only up to around 100 mg/L, depending on the species and strains of *Sc* [36]. As this "limiting concentration" was below both our chosen nitrogen levels (in line with our goal of avoiding nitrogen-scarce conditions), this explained the lack of a significant effect of this particular parameter on growth rate.

Thus, a decrease in temperature favored the faster development of *Lt* versus *Sc*: the $\mu_{max\ Lt}/\mu_{max\ Sc}$ ratio was indeed higher at 18 °C than at 28 °C (stand. effect −3.594), which supported previous results [16]. Although both *Lt* and *Sc* were able to grow at 15–20 °C and even better at 25–30 °C on YPD agar [37], temperature affected the population dynamics in mixed cultures [38,39]. *S. cerevisiae*'s ability to grow at higher temperatures than most yeasts gives it an advantage: the temperature increase during fermentation can lead to its dominance versus non-*Saccharomyces* species during wine fermentation [40–43]. On the contrary, a decrease in temperature can enhance the growth and survival of non-*Saccharomyces* yeasts by attenuating their susceptibility to ethanol [29,44]. In addition, culture at low temperature could induce an increase in the intracellular level of ROS (reactive oxygen species) and an oxidative stress response (through mechanisms that are

still not well understood) in *S. cerevisiae*: this can lead to a decrease in its maximal growth rate and reduce its competitiveness against other yeasts [36,45]. These different factors can provide an explanation for the significant impact of temperature on growth rates, both absolute ($\mu_{max\,Sc}$ and $\mu_{max\,Lt}$) and relative ($\mu_{max\,Lt}/\mu_{max\,Sc}$).

Otherwise, in the conditions tested, the maximal population of both yeasts was affected differently by the factors studied. For *Sc*, the N_{max} was significatively influenced by both temperature (positive effect, stand. effect 4.546, contrib. var. 45.7%) and oxygenation (negative effect, stand. effect −4.443, contrib. var. 43.7%). On the contrary, the maximal population of *Lt* was significantly impacted by temperature (which explained 77.5% of the variance, with a standard effect of −7.750) and with low significance by nitrogen (*p*-value 0.052, stand. effect 3.131).

Thus, an increase in temperature from 18 °C to 28 °C led to a higher *Sc* and a lower *Lt* population. Gobbi et al. (2013) [16] also described a lower maximal population of *Sc* when the temperature was decreased, but they did not observe a higher *Lt* population (they noticed the same population with a higher persistence during fermentation). In addition to temperature, in other studies [17,20] oxygenation favored the development of *Lt* and led to a higher maximal population, which was not observed as significant here. Garcia et al. (2021) [46] also showed that the survival of *Lt* in ethanol-stress conditions was enhanced in the presence of oxygen. The fact that such an impact was not observed here could be explained by differences in the oxygenation levels studied. Indeed, in this study, simple sparging of compressed air at the end of exponential growth was used to stay closer to cellar capabilities, although the works cited made use of either constant aerobic conditions [20,46] or multiple oxygen pulses over the fermentation process [17].

Concerning nitrogen, a higher concentration in the medium seemed to positively affect the $N_{max\,Lt}$ (stand. effect 3.131) but with low significance (*p*-value 0.052). As previously discussed for μ_{max}, the chosen nonscarce nitrogen conditions may explain this lack of significant impact on this population variable. The incubation ratio had no significant effect on the maximal population obtained for both yeasts, thereby supporting the work of Comitini et al. (2011) [47], who observed the same maximum population (but with longer persistence) of *Lt* when favored by the inoculum ratio.

As the effects observed on the μ_{max} and N_{max} were different, the AUC was calculated to assess the impact of fermentation parameters on the population dynamics more precisely (see additional data in Table A1). Yeast distribution over time (AUC_{Lt}/AUC_{Sc}) was significantly and mainly managed by temperature (var. contribution 40.80%, stand. effect −8.267), but also by yeast ratio (var. contribution 29.20%, stand. effect −6.999) and oxygenation (var. contribution 27.80%, stand. effect 6.822), as illustrated in Figure 1.

A lower fermentation temperature, an inoculation ratio favoring *Lt* (lower *Sc*/*Lt* ratio), and air sparging at the end of exponential growth phase all led to a significant increase in the overall presence of *Lt* compared to *Sc* throughout fermentation (higher AUC_{Lt}/AUC_{Sc}). The inoculation ratio was quite straightforward, favoring *Lt* during the early days of culture, either directly by increasing its relative population at inoculation, or more indirectly by limiting the *Sc* population early on and thus reducing its potential negative interactions toward *Lt*. Oxygenation probably acted as described in the literature, as hypothesized by Shekhawat et al., by allowing the *Lt* population to better fulfill its oxygen needs, consequently allowing for better persistence over time and leading to higher cell counts through the stimulation of the respiratory metabolism over the fermentative one [17]. Moreover, Varela et al. (2021) [48] observed the population dynamics during the spontaneous fermentation of natural grape musts and also showed that aeration led to an increased proportion of non-*Saccharomyces* yeasts in the natural consortium, possibly related to the modification of membrane lipid composition, conferring a higher ethanol tolerance to the yeasts in question. The temperature effect on the AUC ratio probably resulted from previously discussed effects on the μ_{max}, with quicker *Sc* growth inducing a quicker takeover of the culture media.

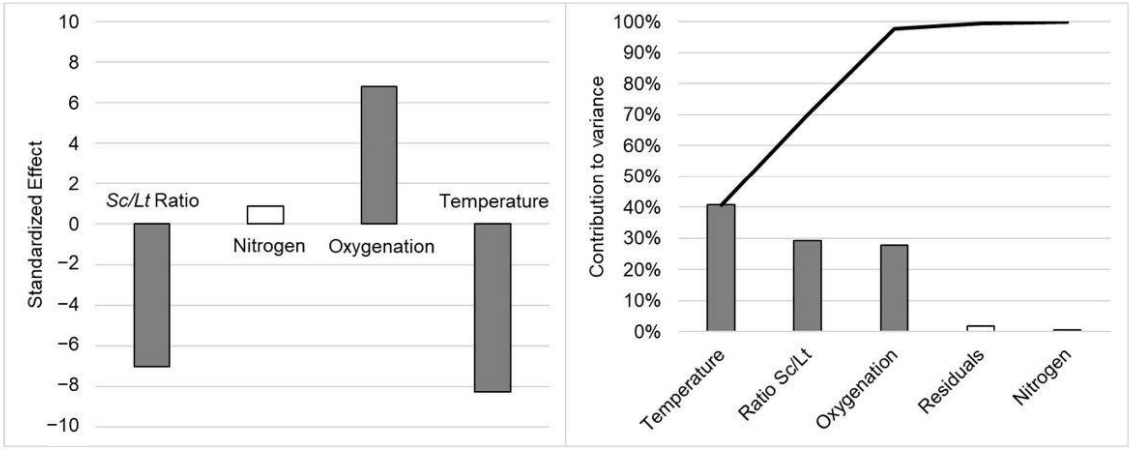

Figure 1. Impact of fermentation parameters on the AUC_{Lt}/AUC_{Sc} ratio: standardized effect (**left**) and Pareto chart of contributions to variance (**right**). Data are noted as statistically not significant (p-value > 0.1, white) or very significant (p-value < 0.01, dark grey). The black curve on the Pareto chart is the cumulated contribution to variance.

Considering these very important impacts of temperature, ratio, and oxygenation on the population dynamics (especially the AUC ratio), only two conditions (D and G: 18 °C, ratio *Sc*/*Lt* 0.1, oxygenation) led to the predominance of *Lt* on *Sc* and to the persistence of *Lt* with a high population throughout fermentation (see additional data in Figure A1). The results also showed that the amount of nitrogen in the culture medium had no significant effect on the population dynamics when modulated between the two levels chosen (150 mg·L^{-1} and 300 mg·L^{-1}), except for a small positive but nonsignificant (p-value = 0.052) effect on the N_{max} for *Lt*. As discussed for the single variables, the nature of nitrogen sources (ammonium, amino acids, or both) can impact maximal growth and maximal population of *Lt* in pure culture, but with strain-dependent variability [21,35,49]. In addition, the consumption kinetics of amino acids can vary according to the amino acid and differ from those observed in *Sc*, leading to possible competition in *Sc*/*Lt* coculture. Nevertheless, Kemsawad et al. (2015) noticed that *Lt* could grow and maintain itself even if no nitrogen was present in the synthetic medium, making use of autolysis phenomena to obtain nitrogen compounds [21]. In addition, the amount of nitrogen, even at the lowest value, is usually sufficient to allow for yeast growth and complete fermentation, as a minimum of 140 mg/L is usually recommended [22]. For all these reasons, the result observed here could be explained by the fact that the experimental conditions chosen did not aim to emulate complete nitrogen scarcity, or by a strain-specific tolerance to these lower levels of nitrogen, or both.

By studying the different fermentation factors, we expected to see an effect of all these factors on the population dynamics, as observed by other authors. Nevertheless, when all these factors were combined, their relative importance could be seen. It seemed in the conditions studied that overall, temperature was the most important factor in optimizing *Lt* presence over time during fermentation (AUC_{Lt}/AUC_{Sc} ratio). In addition to temperature, oxygenation and the inoculum ratio also appeared to be significant, but were still secondary factors in obtaining the persistence or predominance of *Lt* over *Sc* throughout the fermentation process.

To our knowledge, this is the first time information has been obtained regarding the combined effect of different fermentation parameters on the population dynamics. Such information constitutes a tool to control proper *Lt* implantation, and also to help in determining which yeast (*Sc* or *Lt*) will dominate the other during a mixed culture with given or adjustable parameters. This ensures both that *Lt* will have enough overall

persistence to potentially influence final wine quality and that *Sc* will still persist to ensure fermentation does not go on for too long or become stuck.

3.2. Impact of Fermentation Parameters on Fermentation Time and Physicochemical Variables

As mentioned in the general introduction of this study, both *Sc* and *Lt* have different fermentative capabilities when used in pure culture, with *Sc* leading to a shorter fermentation and a higher amount of ethanol, and *Lt* culture leading to less alcohol and a decrease in pH via lactic acid production, but with a higher fermentation time. The goal of an optimized mixed fermentation is to obtain a compromise between the properties of both yeasts. Thus, after having studied the influence of the different parameters on the population dynamics, it was necessary to study their general impact on fermentation to see how both these different population equilibria and the more direct impact of the yeast metabolism parameters chosen could modify the characteristics of the medium.

The effects of incubation ratio, nitrogen content, oxygenation, and temperature on fermentation are given in Table 4.

Table 4. Impact of parameters on fermentation.

		Sc/*Lt* Ratio	Nitrogen	Oxygenation	Temperature	Residuals
Fermentation time	Stand. effect	−3.057	−2.384	3.086	−7.122	
	p-Value	0.055	0.097	0.054	0.006	
	Var. contrib.	11.90%	7.26%	12.20%	64.80%	3.83%
Ethanol concentration (% *v*/*v*)	Stand. effect	2.899	0.402	−2.857	1.418	
	p-Value	0.063	0.715	0.065	0.251	
	Var. contrib.	38.70%	0.74%	37.50%	9.25%	13.80%
pH	Stand. effect	2.575	1.159	−0.644	−0.257	
	p-Value	0.082	0.330	0.566	0.813	
	Var. contrib.	57.90%	11.70%	3.62%	0.58%	26.20%
Lactic acid concentration ($g \cdot L^{-1}$)	Stand. effect	−7.322	−0.593	1.809	−2.034	
	p-Value	0.005	0.595	0.168	0.135	
	Var. contrib.	83.30%	0.55%	5.08%	6.42%	4.66%

Stand. effect = standardized effect; Var. contrib. = contribution to variance. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey background), significant (*p*-value < 0.05, grey background), or very significant (*p*-value < 0.01, dark grey background).

Fermentation took between 7 and 16 days to obtain the total consumption of sugars, depending on the conditions tested (see additional data in Figure A1 and Table A2). Thus, the choice of environmental conditions could considerably affect the resulting fermentation kinetics.

The four factors studied had different impacts on fermentation time. Oxygenation seemed to lead to longer fermentation times (stand. effect 3.086), which was to be expected, given that it was shown above to favor *Lt* more than *Sc*. On the contrary, an increase in the *Sc*/*Lt* ratio or in the amount of nitrogen resulted in a decrease in fermentation time (stand. effect −3.057 and −2.384, respectively, but with a slight significance (*p*-value 0.055 and 0.097, respectively)) due to their positive impact on the *Sc* population. An increase in temperature led to a shorter fermentation (stand. effect −7.122, *p*-value 0.006). This very significant effect (which explained 64.8% of the variance) was certainly linked to the positive effect of temperature on the *Sc* population (see Section 3.1), and supported the work of Gobbi et al. (2013) [16].

Ethanol production reached values of about 11–12% ethanol (*v*/*v*) at the end of the fermentation for all the conditions tested (see additional data in Table A2). This production was not impacted by temperature or the amount of nitrogen, but was impacted, albeit

with a low significance (p-value of 0.063 and 0.065, respectively), by the *Sc*/*Lt* ratio and oxygenation. An increase in the *Sc*/*Lt* ratio led to an increase in *Sc* at the expense of *Lt* (see Section 3.1.) and to an increase in ethanol production (stand. effect 2.899). On the contrary, oxygenation, which led to a decrease in the *Sc* compared to *Lt* population (see Section 3.1), led at the same time to a decrease in ethanol production (stand. effect −2.857). As the Crabtree effect, which was weaker in *Lt* than in *Sc*, favors respiration and leads to lower ethanol production by *Lt* [11], this lower ethanol production could be explained by the modulation of this metabolic pathway. Moreover, the ethanol yield of *Lt* has also been described as being significantly lower than for *Sc* [12]. Although the respective impacts of these two hypotheses were impossible to properly determine within the scope of this study, the overall negative impact of oxygenation on ethanol production was still observable, and could be of interest to winemakers willing to decrease the ethanol content of their wines.

The final pH was not significantly impacted by temperature, oxygenation, or nitrogen content. The *Sc*/*Lt* ratio had a positive effect on pH (stand. effect 2.575, var. contrib. 57.90%). The pH values observed when the inoculum ratio was 0.1 (predominance of *Lachancea thermotolerans*) were lower than those obtained with a ratio of 1. This pH reduction in mixed cultures *Sc*/*Lt* compared to *Sc* pure culture is often observed whatever the conditions (modalities of inoculation, temperature, medium) [16].

This decrease in pH could be linked at least partially to the production of lactic acid. *Lachancea thermotolerans*, as discussed above, is specifically known amongst non-*Saccharomyces* for its ability to transform pyruvate into this acid through lactate dehydrogenases [11,50]. As *S. cerevisiae* is not able to produce lactic acid [51] or, depending on the strain, can produce it in trace amounts only [50], the final amount of this acid can be directly related to the population dynamics, and specifically to the *Lt* population. Conditions either favoring *Lt* development compared to *Sc* or inhibiting antagonistic interactions from *Sc* that limit *Lt* growth or persistence will therefore indirectly favor lactic acid production during the fermentation process.

In the different conditions tested, the production of lactic acid varied between 0.82 and 3.52 $g{\cdot}L^{-1}$. These values are classical ones as the production of this acid is highly dependent on the strain (<0.1 to >15 $g{\cdot}L^{-1}$) and on conditions [11,50,52,53]. Lactic acid concentration was considerably impacted by the *Sc*/*Lt* ratio (var. contrib. 83.30%, stand. effect −7.322). A decrease in this ratio indeed favored the predominance of *Lt* in the earlier stages of the fermentation, and this species' lactic acid production has previously been shown to occur predominantly at the beginning of fermentation, during exponential growth [2,6,16,54]. This hypothesis was further supported by the pH dynamics shown in Figure 2, in which a rapid pH decrease during the first 72 h can be observed, with this falloff being on average significantly greater for conditions with an *Sc*/*Lt* ratio of 0.1.

Using an *Sc*/*Lt* ratio of 0.1 in mixed culture in natural must, Gobbi et al. (2013) [16] showed that the effects of the interaction between *Lt* and *Sc* on fermentation (decreases in ethanol and pH, increase in lactic acid compared to a pure culture of *Sc*) were more significant at 20 °C than at 30 °C, but this could have been related to the better persistence of *Lt* in these conditions (as also observed earlier in this study—see Section 3.1).

Morata et al. (2018) also observed that a higher lactic acid production was correlated with a more numerous *Lt* population and a higher nitrogen content (up to 500 mg/L) [54]. This impact of nitrogen was not significant here within our chosen concentration levels.

Lactic acid production did not appear to be impacted by oxygenation, although Shekhawat et al. (2020) showed an induction of lactate dehydrogenase gene expression in pure cultures of *Lt* and the redirection of metabolism toward lactic acid in response to anaerobic conditions [55]. However, the direct impact of oxygenation conditions on effective lactic acid production has not yet been shown in the current literature, and conclusions are therefore hard to draw based only on transcriptomic results.

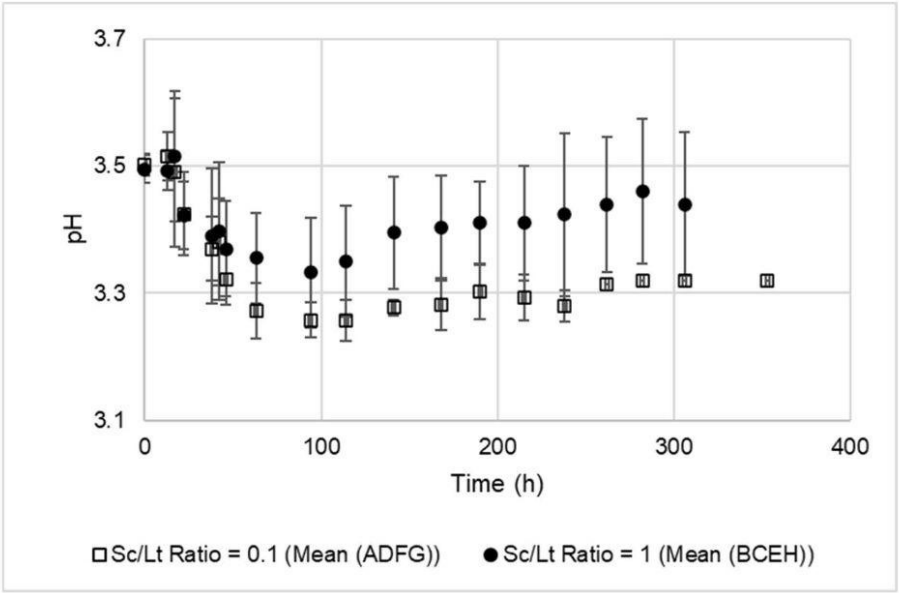

Figure 2. Evolution of pH during fermentation in mixed cultures with a *Sc/Lt* ratio of 0.1 or 1.

This study highlighted the key role of temperature to control fermentation time over other factors that did not have as much impact or significance. This role can be linked to the population dynamics seen in Section 3.1, and confirmed that overfavoring *Lt* in a mixed culture can lead to sluggish or stuck fermentations, an eventuality that must be avoided. Decreasing the ethanol concentration in these *Sc/Lt* cultures was difficult in these conditions, since the parameters chosen had no or very little impact on ethanol production. In contrast, the inoculum ratio turned out to be the most important factor in modulating lactic acid production, without it being necessary to modulate other fermentation parameters. It could be an interesting tool to control wine acidification without drastic changes in usual winemaking practices.

3.3. Impact of Fermentation Parameters on Volatile Compound Production

As the production of aroma compounds is variable according to previous studies [2], it was also interesting to observe this production in the mixed *Sc/Lt* culture and study the influence of fermentation parameters, as the volatile compounds produced modified the final sensorial characteristics of the wine. As seen above, the parameters chosen had a significant impact on the population dynamics, a modulation that also had repercussions on fermentation variables such as fermentation time and lactic acid production. We therefore assumed it would also impact the production of aroma compounds in a similar way, both directly (metabolism modifications) and indirectly (population dynamics).

The volatile compounds present in the final synthetic wines obtained with the different conditions tested were subjected to a principal component analysis. The first two principal components, F1 and F2, explained 36.4 and 25.4% of the variance, respectively, as shown in Figure 3.

Axis F1 marks a separation between four groups: condition E, which is close to γ-butyrolactone and esters with short chains; condition H, which is closer to carboxylic acids; conditions G and D, which are close to benzaldehyde (conditions corresponding to the predominance of *Lt* throughout the fermentation); and the other conditions.

Axis F2 separates condition C, which is close to esters derived from C6 to C10 carboxylic acids; condition H, which is closer to alcohols and acids; and the other conditions

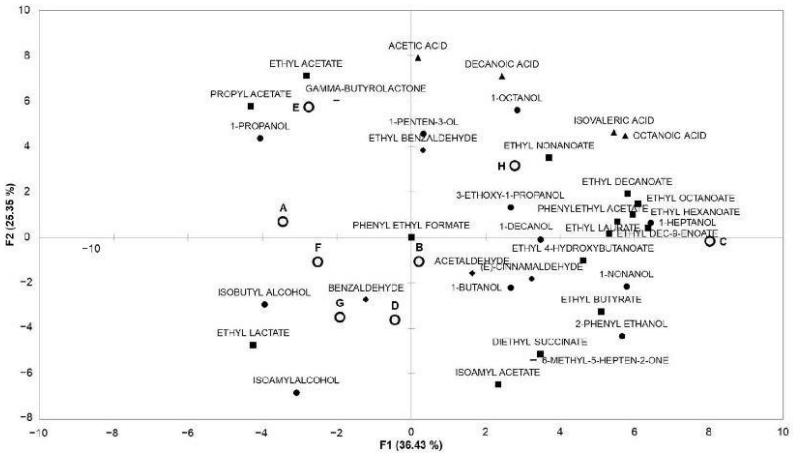

Figure 3. Principal component analysis (PCA) based on volatile compounds present in the final medium after fermentation in conditions A to H. The compounds are represented as followed: • alcohols; ♦ aldehydes; ▲ carboxylic acids; – ketones; ■ esters.

Some conditions (A, B, D, F, G) led to media with quite similar volatilomes and others (C, E, H) with different volatile compound profiles. These results showed that the fermentation parameters studied seemed to broadly impact the production of aroma compounds, and could therefore be useful in modifying the volatile composition of the final wine.

A statistical analysis was performed first on the results concerning each class (aroma compounds grouped into alcohols, aldehydes, ketones, carboxylic acids, and esters), then on the results concerning each volatile molecule. The results are presented in Tables 5–8.

Table 5. Impact of fermentation parameters on the production of volatile compounds.

		Sc/Lt Ratio	Nitrogen	Oxygenation	Temperature	Residuals
Alcohols	Stand. effect	−9.037	4.742	−2.108	−10.175	
	p-Value	0.003	0.018	0.126	0.002	
	Var. contrib.	38.00%	10.50%	2.07%	48.10%	1.39%
Aldehydes	Stand. effect	−2.026	1.113	1.581	−0.256	
	p-Value	0.136	0.347	0.212	0.814	
	Var. contrib.	37.60%	11.40%	22.90%	0.60%	27.50%
Ketones	Stand. effect	0.021	0.016	0.383	−3.825	
	p-Value	0.984	0.988	0.727	0.031	
	Var. contrib.	0.00%	0.00%	0.83%	82.30%	16.90%
Carboxylic acids	Stand. effect	6.199	1.750	0.652	2.685	
	p-Value	0.008	0.178	0.561	0.075	
	Var. contrib.	73.70%	5.87%	0.82%	13.80%	5.76%
Esters	Stand. effect	2.614	−0.848	1.837	4.514	
	p-Value	0.079	0.459	0.164	0.020	
	Var. contrib.	19.90%	2.10%	9.84%	59.40%	8.75%

Stand. effect = standardized effect; Var. contrib. = contribution to variance. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey background), significant (*p*-value < 0.05, grey background), or very significant (*p*-value < 0.01, dark grey background).

Table 6. Impact of fermentation parameters on the production of different volatile alcohols.

		*Scl*Lt Ratio	Nitrogen	Oxygenation	Temperature	Residuals
1-Propanol	Stand. Effect	0.146	−3.271	−1.220	2.392	
	p-Value	0.893	0.047	0.310	0.097	
	Var. Contrib.	0.10%	51.10%	7.12%	27.30%	14.30%
Isobutanol	Stand. Effect	−1.647	−0.324	0.402	0.558	
	p-Value	0.198	0.767	0.715	0.616	
	Var. Contrib.	43.00%	1.67%	2.57%	4.95%	47.70%
1-Penten-3-ol	Stand. Effect	1.521	−1.711	1.141	0.000	
	p-Value	0.226	0.186	0.337	1.000	
	Var. Contrib.	24.20%	30.70%	13.60%	0.00%	31.40%
1-Butanol	Stand. Effect	−0.272	0.410	−0.807	−0.484	
	p-Value	0.803	0.709	0.479	0.661	
	Var. Contrib.	2.00%	4.08%	15.80%	5.68%	72.70%
Isoamylalcohol	Stand. Effect	−9.407	−0.802	−0.591	−3.636	
	p-Value	0.003	0.481	0.596	0.036	
	Var. Contrib.	83.70%	0.61%	0.33%	12.50%	2.84%
3-Ethoxy-1-propanol	Stand. Effect	0.497	−0.508	0.255	−0.457	
	p-Value	0.654	0.647	0.815	0.679	
	Var. Contrib.	6.53%	6.83%	1.73%	5.54%	79.40%
1-Heptanol	Stand. Effect	1.632	2.472	−0.513	−0.793	
	p-Value	0.201	0.090	0.643	0.486	
	var. contrib.	21.00%	48.20%	2.08%	4.96%	23.70%
1-Octanol	Stand. Effect	1.542	2.141	0.600	2.227	
	p-Value	0.221	0.122	0.591	0.112	
	Var. Contrib.	15.60%	30.00%	2.35%	32.50%	19.60%
1-Nonanol	Stand. Effect	1.722	1.457	−0.662	−1.987	
	p-Value	0.184	0.241	0.555	0.141	
	Var. Contrib.	23.80%	17.00%	3.52%	31.60%	24.10%
1-Decanol	Stand. Effect	0.547	1.443	−0.448	0.348	
	p-Value	0.622	0.245	0.685	0.751	
	Var. Contrib.	5.25%	36.50%	3.52%	2.13%	52.60%
2-Phenylethanol	Stand. Effect	2.350	9.281	−2.367	−9.895	
	p-Value	0.100	0.003	0.099	0.002	
	Var. Contrib.	2.79%	43.50%	2.83%	49.40%	1.51%

Stand. Effect = standardized effect; Var. Contrib. = contribution to variance. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey background), significant (*p*-value < 0.05, grey background), or very significant (*p*-value < 0.01, dark grey background).

Table 7. Impact of fermentation parameters on the production of different volatile compounds (aldehydes, ketones, acids).

		Sc/*Lt* Ratio	Nitrogen	Oxygenation	Temperature	Residuals
Acetaldehyde	Stand. effect	0.031	−0.215	−0.092	−0.400	
	p-Value	0.977	0.843	0.932	0.716	
	Var. contrib.	0.03%	1.44%	0.27%	4.97%	93.30%
Benzaldehyde	Stand. effect	−2.424	1.286	2.128	−0.148	
	p-Value	0.094	0.289	0.123	0.892	
	Var. contrib.	39.00%	11.00%	30.00%	0.14%	19.90%
Ethyl benzaldehyde	*Stand. effect*	*1.000*	*−1.000*	*−1.000*	*1.000*	
	p-Value	*0.391*	*0.391*	*0.391*	*0.391*	
	Var. contrib.	*14.30%*	*14.30%*	*14.30%*	*14.30%*	*42.90%*
(E)-cinnamaldehyde	Stand. effect	−0.414	1.242	−1.656	−0.207	
	p-Value	0.707	0.302	0.196	0.849	
	Var. contrib.	2.29%	20.60%	36.60%	0.57%	40.00%
6-Methyl-5-hepten-2-one	Stand. effect	0.016	0.016	0.379	−3.838	
	p-Value	0.988	0.988	0.730	0.031	
	Var. contrib.	0.00%	0.00%	0.80%	82.40%	16.80%
γButyrolactone	*Stand. effect*	*1.000*	*−1.000*	*1.000*	*1.000*	
	p-Value	*0.391*	*0.391*	*0.391*	*0.391*	
	Var. contrib.	*14.30%*	*14.30%*	*14.30%*	*14.30%*	*42.90%*
Acetic acid	Stand. effect	20.805	−0.813	11.375	23.103	
	p-Value	0.000	0.476	0.001	0.000	
	Var. contrib.	39.40%	0.06%	11.80%	48.50%	0.27%
Isovaleric acid	Stand. effect	6.826	2.246	−1.155	0.381	
	p-Value	0.006	0.110	0.332	0.729	
	Var. contrib.	83.00%	8.99%	2.38%	0.26%	5.35%
Octanoic acid	Stand. effect	1.722	1.457	−0.662	−1.987	
	p-Value	0.184	0.241	0.555	0.141	
	Var. contrib.	73.10%	15.20%	0.61%	0.33%	10.80%
Decanoic acid	Stand. effect	3.250	1.207	1.061	3.100	
	p-Value	0.048	0.314	0.367	0.053	
	Var. contrib.	41.00%	5.65%	4.37%	37.30%	11.60%

Stand. effect = standardized effect; Var. contrib. = contribution to variance. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey background), significant (*p*-value < 0.05, grey background), or very significant (*p*-value < 0.01, dark grey background). Compounds in italics were only present in trace amounts, with values too low to perform a relevant statistical test.

Table 8. Impact of fermentation parameters on the production of different esters.

		Sc/Lt Ratio	Nitrogen	Oxygenation	Temperature	Residuals
Ethyl acetate	Stand. effect	5.914	−6.856	6.856	21.058	
	p-Value	0.010	0.006	0.006	0.000	
	Var. contrib.	6.08%	8.17%	8.17%	77.10%	0.52%
Propyl acetate	*Stand. effect*	*0.707*	*−2.828*	*0.000*	*2.828*	
	p-Value	*0.530*	*0.066*	*1.000*	*0.066*	
	Var. contrib.	*2.56%*	*41.00%*	*0.00%*	*41.00%*	*15.40%*
Isoamyl acetate	Stand. effect	−1.874	0.687	0.687	−5.746	
	p-Value	0.158	0.541	0.541	0.010	
	Var. contrib.	8.67%	1.17%	1.17%	81.60%	7.41%
Ethyl lactate	Stand. effect	−4.883	−0.740	0.888	0.000	
	p-Value	0.016	0.513	0.440	1.000	
	Var. contrib.	84.60%	2.00%	2.80%	0.00%	10.60%
Ethyl butyrate	*Stand. effect*	*3.000*	*1.000*	*−3.000*	*−7.000*	
	p-Value	*0.058*	*0.391*	*0.058*	*0.006*	
	Var. contrib.	*12.70%*	*1.41%*	*12.70%*	*69.00%*	*4.23%*
Ethyl hexanoate	Stand. effect	8.486	2.092	−4.185	−4.766	
	p-Value	0.003	0.127	0.025	0.018	
	Var. contrib.	60.20%	3.66%	14.60%	19.00%	2.51%
Ethyl octanoate	Stand. effect	1.136	1.544	−0.089	−0.396	
	p-Value	0.339	0.220	0.934	0.719	
	Var. contrib.	18.90%	34.90%	0.12%	2.00%	43.90%
Ethyl nonanoate	*Stand. effect*	*1.732*	*1.732*	*1.732*	*0.000*	
	p-Value	*0.182*	*0.182*	*0.182*	*1.000*	
	Var. contrib.	*25.00%*	*25.00%*	*25.00%*	*0.00%*	*25.00%*
Ethyl decanoate	Stand. effect	1.121	1.537	0.374	−0.208	
	p-Value	0.344	0.222	0.733	0.849	
	Var. contrib.	18.50%	34.70%	2.05%	0.63%	44.10%
Diethyl succinate	*Stand. effect*	*0.000*	*1.732*	*0.000*	*−1.732*	
	p-Value	*1.000*	*0.182*	*1.000*	*0.182*	
	Var. contrib.	*0.00%*	*33.00%*	*0.00%*	*33.00%*	*33.30%*
Ethyl dec-9-enoate	*Stand. effect*	*0.500*	*1.000*	*−0.500*	*−0.500*	
	p-Value	*0.651*	*0.391*	*0.651*	*0.651*	
	Var. contrib.	*5.26%*	*21.10%*	*5.26%*	*5.26%*	*63.20%*
Phenylethyl acetate	*Stand. effect*	*0.728*	*1.213*	*0.243*	*−0.243*	
	p-Value	*0.519*	*0.312*	*0.824*	*0.824*	
	Var. contrib.	*10.30%*	*28.70%*	*1.15%*	*1.15%*	*58.60%*
Ethyl laurate	*Stand. effect*	*5.000*	*3.000*	*−1.000*	*−3.000*	
	p-Value	*0.015*	*0.058*	*0.391*	*0.058*	
	Var. contrib.	*53.20%*	*19.00%*	*2.13%*	*19.00%*	*6.38%*

Stand. effect = standardized effect; Var. contrib. = contribution to variance. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey background), significant (*p*-value < 0.05, grey background), or very significant (*p*-value < 0.01, dark grey background). Compounds in italics were only present in trace amounts, with values too low to perform a relevant statistical test.

The *Sc/Lt* ratio had a significant positive impact on the production of carboxylic acids and esters (stand. effect 6.199 and 2.614, respectively) and a negative impact on alcohol

production (stand. effect −9.037). The *Sc* population seemed to be mainly linked to the formation of acids and esters. This was in line with the work of Morales et al. (2019) [23], who showed an increase in ethyl esters, acetals, and acids, as well as a decrease in alcohols, when the proportion of *Saccharomyces cerevisiae* increased in the inoculum. As the *Sc*/*Lt* ratio significantly impacted the proportion of each yeast in the media over time (as seen in Section 3.1), and as these two species did not produce the same aroma compounds [16,20,23], the aroma profile of a given mixed culture was indeed expected to be very different from either pure culture. However, interactions between yeasts can also change their metabolism and their aroma production, resulting in an apparent synergy (higher amount in mixed culture compared to pure cultures), as observed previously [16,23].

The amount of nitrogen had a significant effect (stand. effect 4.742) only on alcohol production. Rollero et al. (2021) highlighted the link between nitrogen sources and aroma production in cocultures of *Sc* with several non-*Saccharomyces* yeasts [56]. Higher alcohols can be produced from both sugars and amino acids: differences in the consumption kinetics of nitrogen sources and in metabolism flux according to the yeast can then lead to various productions of aroma compounds. Some authors also found that modifying nitrogen levels can lead to the production of different volatile compounds in pure *Sc* cultures [22], thus correlating with this conclusion.

Oxygenation had no significant impact on the production of the different families of volatile compounds. This was in contradiction with the results of Shekhawat et al. (2017), who noticed an increase in the production of alcohols and volatile acids and a decrease in medium-chain fatty acids and corresponding esters [20]. However, the differences in oxygenation protocols already discussed in Section 3.1 could explain this lack of a significant effect in the conditions of our study.

Temperature had a negative impact on the production of alcohols and ketones (stand. effect −10.175 and −3.825, respectively), a positive effect on ester and acid production (stand. effect 4.514 and 2.685, respectively), and no significant effect on aldehyde production. As temperature was previously shown to significantly impact population dynamics in this study, such shifts in aroma production were to be expected, as the media was fermented by very different yeast populations depending on the temperature chosen.

An increase in aroma production, notably esters, was observed previously at low temperature with some strains of *S. cerevisiae*, and this increase seemed to be related to a change in membrane lipid composition in response to the stress linked to the temperature decrease [57,58].

More specifically, regarding the volatile molecules produced during fermentation, several alcohols are produced: primary alcohols with 3 to 10 carbons, isobutyl alcohol, isoamyl alcohol, 2-phenylethanol, etc. Table 6 shows the impacts of fermentation parameters on the production of these alcohols. These results are also illustrated in Figure 4, which presents the standardized effects of each fermentation parameter on this production.

A significant impact of environmental factors was observed only on three molecules: 1-propanol, isoamyl alcohol, and 2-phenylethanol. For the other alcohols, the contribution of residuals to total variance was high, but no significant impact of interactions between factors could be observed, even when examined in more detail. This furthered the hypothesis that this lack of observed effects was due to a real absence of effects of the conditions studied on the production of these molecules.

The production of 1-propanol was significantly influenced by nitrogen content (negatively, stand. effect −3.271) and to a lesser extent (*p*-value 0.097) by temperature (positively, stand. effect 2.392). The negative influence of nitrogen content showed the complexity of forecasting the production of an aroma compound in a coculture. Propanol is indeed produced from amino acids (while other alcohols can come from both amino acids and sugars) [56]: an increase in nitrogen content should have favored its production. Gobbi et al. (2013) [16] and Balikci et al. (2016) [59] observed a higher production of propanol by *Lt* compared to *Sc* in pure culture, in contrast to the observations of Morales et al. (2019) [23] in a sugar-rich must. Vicente et al. (2021) noticed that *L. thermotolerans* usually produced

low amounts of higher alcohols, but with a high strain variability [11]. Temperature had a positive effect (stand. effect 2.392) on the production of 1-propanol, contrary to the results of Gobbi et al. (2013) [16]. All these conflicting observations highlighted the variability of propanol production, depending on strains, media, and culture conditions chosen, especially in mixed cultures [16,23,53,59].

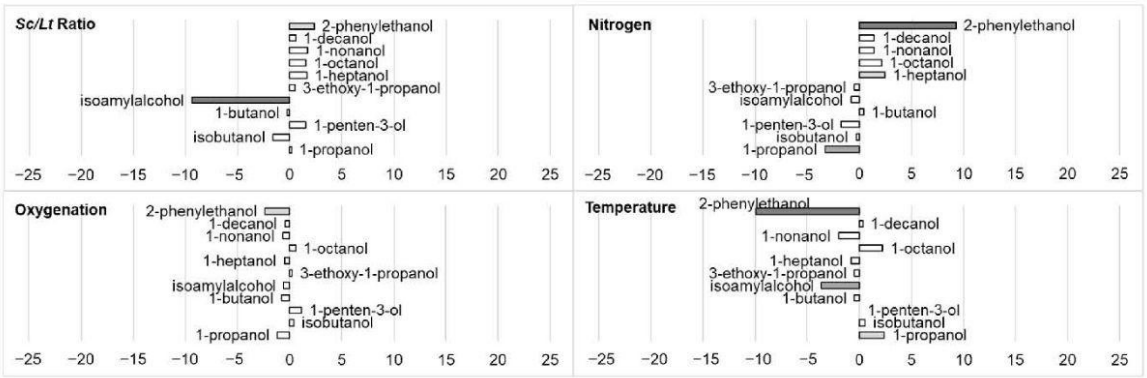

Figure 4. Impact (standardized effects) of fermentation parameters (*Sc*/*Lt* ratio, nitrogen, oxygenation, temperature) on the production of different volatile alcohols. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey), significant (*p*-value < 0.05, grey), or very significant (*p*-value < 0.01, dark grey).

The production of 2-phenylethanol was impacted mainly by nitrogen content (positively, stand. effect 9.281) and temperature (negatively, stand. effect −9.895). Gonzalez et al. (2018) observed that nutrient conditions (nitrogen sources, glucose amount) affected the production of phenylethanol (a molecule that can play a quorum-sensing role) with other non-*Saccharomyces* yeasts [60]. Fairbairn et al. (2021) noticed an increase in the production of 2-phenyl ethanol by *Lt* when the medium was supplemented with phenylalanine (its precursor) compared to a medium with an addition of ammonium only [49]. In a mixed culture *Sc*/*Lt* under anaerobic conditions compared to a pure culture of *Lt*, Shekhawat et al. (2019) [61] observed an upregulation of genes involved in the metabolism of amino acids, and especially those involved in the conversion of phenylalanine into phenylethanol. This suggested that this upregulation, which probably was linked to interactions, could be lower in the case of aerobic conditions. This could then explain the negative impact that oxygenation seemed to have on phenylethanol production in this study (stand. effect −2.367, with a lower significance: *p*-value 0.099).

Temperature had a negative effect on 2-phenylethanol production (stand. effect −9.895). Gobbi et al. (2013) observed no effect on this production [16]. However, they observed a higher concentration of 2-phenylethanol in the presence of *Lt* compared to a pure culture of *Sc* under all of the conditions tested (inoculation modalities, temperature of fermentation, different grape juices). They explained that this production was due to synergy between *Sc* and *Lt*, as both of these yeasts are low producers of this compound. On the other hand, Morales et al. (2019) observed an increase in phenylethanol production in natural must when the *Sc*/*Lt* ratio decreased in the inoculum from 0.2 to 0.02 [23]: the results here showed a slight inverse trend (stand. effect 2.350, *p*-value 0.1), although it did not seem to be the most impactive factor, accounting for only 2.79% of the observed variance. Both these impacts of temperature and *Sc*/*Lt* ratio on 2-phenylethanol production may, at first glance, be perceived as the sole result of the impact of temperature and *Sc*/*Lt* ratio on yeast populations shown in Section 3.1. However, metabolism modifications via yeast–yeast interactions tend to complexify the phenomenon, as discussed earlier in this study.

The production of isoamyl alcohol decreased when the inoculum ratio favored *Sc* (stand. effect −9.407, var. contrib. 83.70%) or when temperature was increased (stand. effect −3.636, var. contrib. 12.50%). A negative impact of the *Sc*/*Lt* ratio on isoamyl alcohol production was also observed by Morales et al. (2019) [23]. Temperature had a negative effect on isoamyl alcohol, but Gobbi et al. (2013) did not observe any impact of temperature on the production of this molecule [16]. Fairbairn et al. (2021) noticed an increase in the production of isoamyl alcohol by *Lt* when the medium was supplemented with the amino acid precursors of this alcohol (leucine, isoleucine) compared to a medium with an addition of ammonium only [49]: such an impact of nitrogen was not observed here. Shekhawat et al. (2017) [20] observed, in mixed *Sc*/*Lt* cultures, that oxygenation resulted in an increase in higher alcohols, particularly isoamyl alcohol, 2-phenylethanol, and isobutanol: the absence of an effect of oxygenation observed here can be linked to different conditions of temperature and medium composition (yeast extract as a nitrogen source versus individual amino acids), to the differences in oxygenation levels mentioned previously, or to a strain-dependence factor.

Table 7 shows the impacts of fermentation parameters on the production of various volatile compounds (aldehydes, ketones, and acids). These results are also illustrated in Figure 5, which presents the standardized effects of each fermentation parameter on this production.

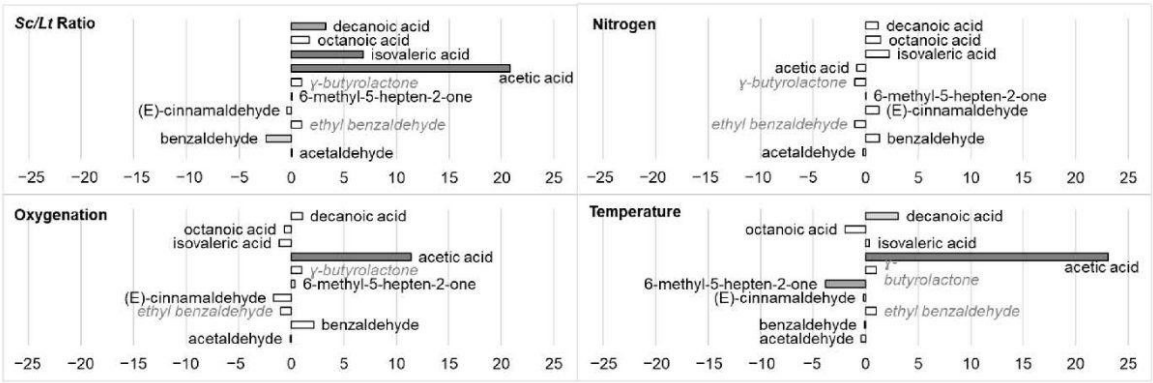

Figure 5. Impact (standardized effects) of fermentation parameters (*Sc*/*Lt* ratio, nitrogen, oxygenation, temperature) on the production of different volatile compounds (aldehydes, ketones, acids). Data are identified as statistically almost significant ($0.05 < p\text{-value} < 0.1$, light grey), significant (p-value < 0.05, grey), or very significant (p-value < 0.01, dark grey). Compounds in italics and in grey were only present in trace amounts, with values too low to perform a relevant statistical test.

During fermentation in the different conditions tested, aldehydes (acetaldehyde, benzaldehyde) and some ketones were produced. Concerning the global production of aldehydes, none of the four factors tested had a significant effect on their production. As far acetaldehyde is concerned, the *Sc*/*Lt* ratio had a positive effect on its production according to Morales et al. (2019) [23], but this was not observed here. The results concerning the production of acetaldehyde in mixed *Sc*/*Lt* cultures indeed seemed to be variable and strain-dependent [11]. A slight effect of the *Sc*/*Lt* ratio on benzaldehyde production (stand. effect −2.424, p-value 0.094) was nevertheless observed.

Regarding ketones, the production of 6-methyl-5-hepten-2-one was negatively impacted by an increase in temperature (stand. effect −3.838), but not by the other factors. Morales et al. 2019 [23] showed that this compound was produced more by *Sc* than *Lt* in pure culture, and that in mixed culture with different *Sc*/*Lt* ratios, it led to lower quantities than in *Lt* culture, leading to the assumption that it may be regulated by an interaction between the two species.

The fermentation of the different synthetic musts in tested conditions led to the production of various organic acids: isovaleric acid, octanoic acid, decanoic acid, and acetic acid. Octanoic acid was not affected by the factors studied. Decanoic acid production was impacted by the *Sc*/*Lt* ratio as isovaleric acid (stand. effects 3.250 and 6.826, *p*-value 0.048 and 0.006, respectively) and also by temperature (stand. effect 3.100, *p*-value 0.053). Regarding acetic acid, its final content was managed by temperature and oxygenation (stand. effect 23.103 and 11.375, respectively). Its production also increased when the *Sc*/*Lt* ratio increased (stand. effect 20.805), supporting previous results [23,47]. *Lt* is indeed known to produce acetic acid at low concentrations, leading to a lower volatile acidity of wine [2,14]; however, high strain variability was observed in previous studies [11]. The positive impact of oxygenation on acetic acid production was unexpected, as this parameter favors the overall presence of *Lt* over time. *Lt* does not produce as much acetic acid as *Sc* [20,23]. However, it has also been shown in previous works that oxygenation significantly modulated acetic acid metabolism and increased its production for both species in both pure and mixed cultures. Moreover, this modulation was linked to a decrease in ethanol production [20], a tendency noted in Section 3.2 (albeit with low significance), which further corroborated this hypothesis.

Table 8 shows the impacts of fermentation parameters on the production of esters. These results are also illustrated in Figure 6, which presents the standardized effects of each fermentation parameter on this production.

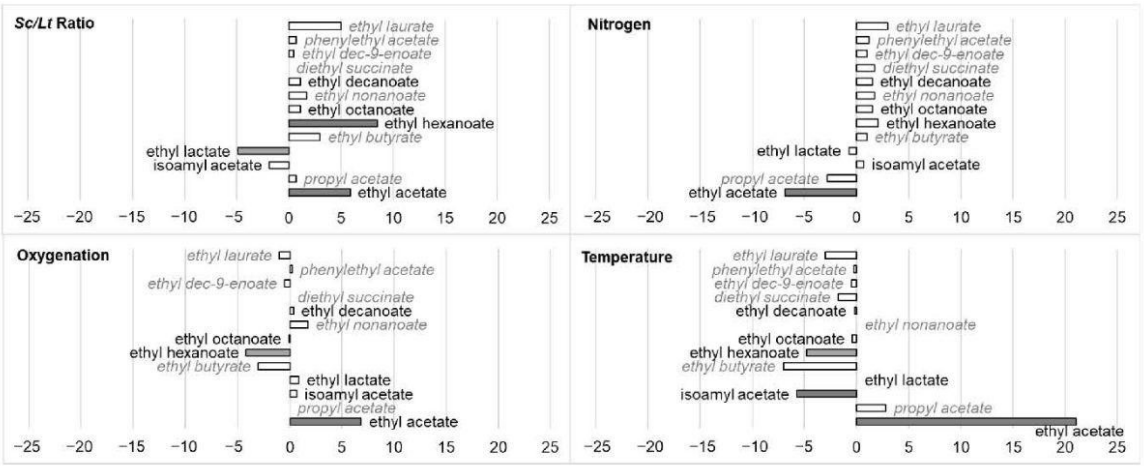

Figure 6. Impact (standardized effects) of fermentation parameters (*Sc*/*Lt* ratio, nitrogen, oxygenation, temperature) on the production of different esters. Data are identified as statistically almost significant (0.05 < *p*-value < 0.1, light grey), significant (*p*-value < 0.05, grey), or very significant (*p*-value < 0.01, dark grey). Compounds in italics and in grey were only present in trace amounts, with values too low to perform a relevant statistical test.

After fermentation, the media contained different esters derived from different acids and fatty acids: ethyl acetate, isoamyl acetate, ethyl lactate, ethyl hexanoate, octanoate, etc. Although some factors influenced the production of fatty acids such as decanoic acid, no significant effect was observed on esters from these acids. The environmental factors studied significantly impacted only four molecules: ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl lactate. Ethyl acetate production increased with an increase in the *Sc*/*Lt* ratio, oxygenation, and particularly temperature (stand. effects 5.914, 6.856 and 21.058, respectively), but with a decrease in nitrogen content (stand. effect −6.856). These results were in contradiction with previous works [16,23] in which an increase in ethyl acetate was observed in conditions favoring the development of *Lt* (low temperature, low *Sc*/*Lt* ratio). However, other studies have also shown that the population dynamics were

not the only factor in play here, and that synergy effects could occur as mixed *Sc*/*Lt* cultures can end up with higher ethyl acetate content than either of the pure ones [20,59]. Isoamyl acetate production was negatively impacted by temperature (stand. effect −5.746), while ethyl hexanoate was influenced mostly and positively by the *Sc*/*Lt* ratio (stand. effect 8.486), and negatively by oxygenation and temperature (stand. effects −4.185 and −4.766, respectively). Ethyl lactate production was negatively influenced by the *Sc*/*Lt* ratio (stand. effect −4.883). This result should be linked to results concerning lactic acid, since it is a precursor of ethyl lactate. Hranilovic et al. (2021) [53] showed that sequential inoculation, which allows *Lt* to develop better at the beginning of fermentation, led to an increase in ethyl lactate, but these authors also noted that the production of this aroma compound was strain-dependent.

Overall, the production of aroma compounds was impacted by fermentation parameters, but in very different ways depending on both the modulated factor and the volatile molecule studied. Some impacts could be explained by the population dynamics, but interactions between *Lt* and *Sc* often occurred. These interactions led to metabolism changes and consequently to volatile production, which was different from what would be expected. As far as the production of higher alcohols is concerned, nitrogen content seemed to be a key factor to control. As was shown in Section 3.1 nitrogen content did not significantly impact the population dynamics; this suggested an interaction between *Sc* and *Lt*, and more specifically competition for amino acids known to be aroma precursors [22,35]. These results then highlighted both the possibility of impacting the final wine volatilome by modulating specific fermentation parameters depending on the goal sought: carboxylic acid production could be modulated by modifying only the inoculum ratio (in a way similar to that of lactic acid, as noted in Section 3.2), whereas for ketones and esters, temperature seemed to be the most important (and only significant) lever of action. The presence of higher alcohols could be impacted by both of these parameters and, as discussed above, by supplementation with nitrogen, which could offset competition between *Sc* and *Lt* for specific precursors.

4. Conclusions

Through a multiparametric approach making use of experimental design, this work provided a better understanding of the impact of fermentation parameters on wine fermentation that simultaneously involved *Saccharomyces cerevisiae* and *Lachancea thermotolerans*. In contrast to most studies, factors here were studied simultaneously and not separately. This global approach allowed us to establish discrepancies between the relative impacts of each parameter studied for the first time. Indeed, previous works mostly focused on one parameter at a time, and not always in controlled and repeatable conditions (natural must, for example). Although these methods had their obvious advantages (better characterization of the single effect of a given factor, closer to real conditions, etc.), they were often unable to conclude globally by comparing different impacts of factors studied; moreover, the natural media induced variability and hindered reproducibility. The choices made in this study were meant to fill this gap by relying on experimental design as well as the use of a synthetic medium and controlled conditions to help establish how the chosen parameters (inoculum ratio, nitrogen, oxygenation, temperature) could influence fermentation on many interconnected levels (population dynamics, fermentation dynamics and results, final volatile compound profiles).

This study suggested that fermentation parameters could constitute tools to control various variables of interest in winemaking, such as the proper implantation of a yeast strain in must, the persistence or dominance of one yeast over another, fermentation time, acidification, and even the characteristics of the final wine volatilome. The population dynamics, as shown, were mostly and strongly impacted by temperature, and to a limited extent by oxygenation. The fermentation dynamics were influenced by temperature and the *Sc*/*Lt* ratio, while lactic acid and pH were dependent on the *Sc*/*Lt* ratio only. The production of volatile compounds (especially alcohols) seemed to be partly modulated by

nitrogen content, and this area would therefore be an interesting perspective to explore in further studies. Analyzing precisely how the nitrogen content of the media fluctuates over time during the fermentation process [62] would provide valuable insight by helping to establish links between the modulation of specific compounds and the availability of specific amino acids. Thanks to the global approach taken, this study also highlighted that the production of certain volatile compounds was not only related to the population dynamics, but also was most likely impacted by interactions between species. Further works could eventually go as far as using a sensorial analysis to attempt to link the chosen fermentation parameters and resulting population dynamics to the final wine obtained and its subsequent perception by oenologists and consumers.

These results showed that the effects of fermentation parameters were very different depending on which variable was studied, which could explain the variability of the results observed in previous different studies.

To manage a mixed culture between *L. thermotolerans* and *S. cerevisiae*, it is then necessary to follow a global approach (population dynamics, fermentation kinetics and products, physicochemical parameters, aroma compounds, etc.) to find the best compromise between manageable conditions, leading to an optimized wine. For example, the two conditions allowing for the persistence of *Lt* until the end were also those that took the most time to complete, and the risk of them becoming sluggish or even stuck was non-negligible. On the other hand, the results showed that the lactic acid content of the final wine could very well be modulated by modifying only the inoculation ratios, without any regard for other factors. Indeed, ensuring adequate *Lt* persistence over time did not seem vital to achieving such a goal. Thus, this study showed that monitoring mixed-culture fermentations seems to be achievable, and can even impact specific variables of interest through the modification of certain precise fermentation conditions.

Overall, although this was not the main goal of this study, it still paved the way toward establishing a potential model for simulating population dynamics and obtaining final synthetic wines in similarly controlled conditions. Building such a model and ensuring its reliability would demand both a broadening of the experimental design given above by including other external conditions to be studied and refining it by including more levels per factor, and thus characterizing the linearity or non-linearity of responses to the external conditions chosen. Of course, either one of these perspectives would also be interesting to pursue separately, and could be the focus of future studies.

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Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article and its Appendix A. The raw data are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

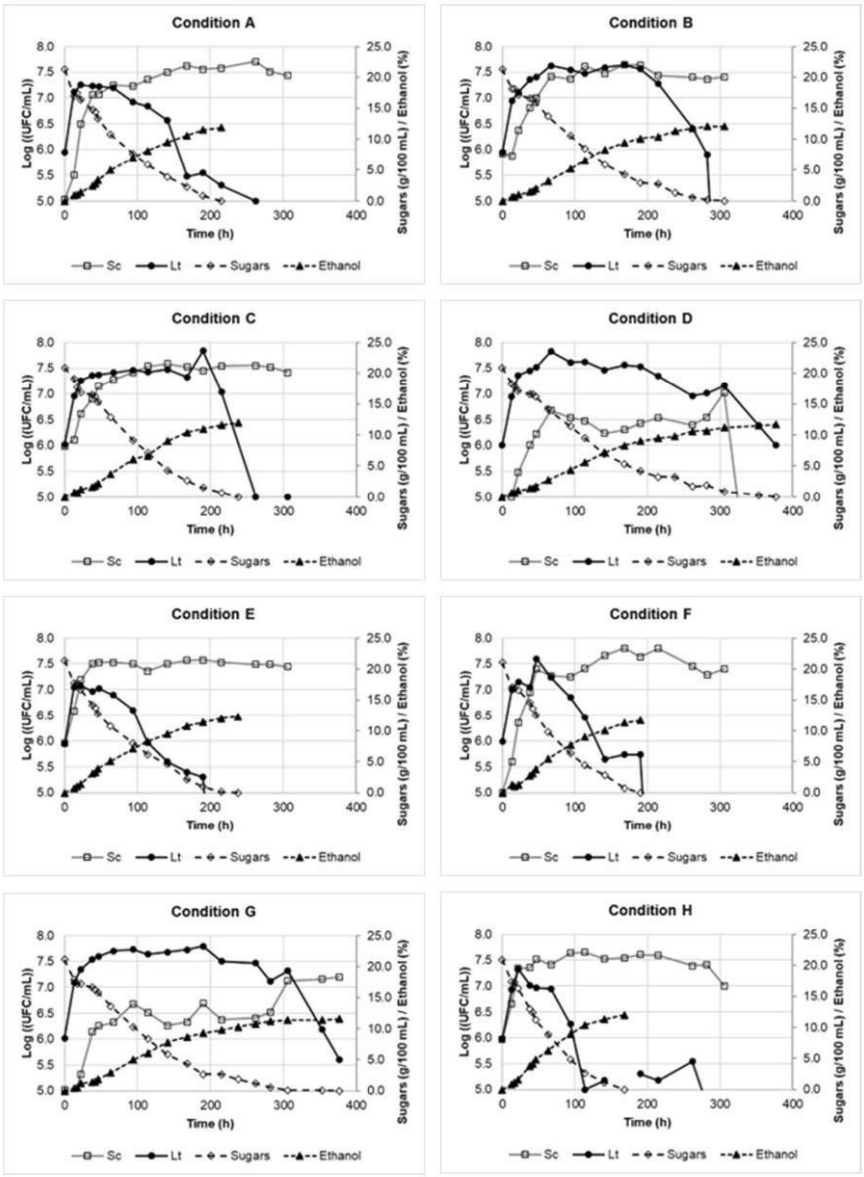

Figure A1. Evolution of both yeast populations, sugar concentrations, and ethanol concentrations during coculture of *S. cerevisiae* and *L. thermotolerans* in the different conditions of the experimental design (**A**–**H**).

Table A1. Quantitative data related to population dynamics in the different conditions of the experimental design (A to H): maximal population (N_{max}), maximal growth rate (μ_{max}), area under growth curve (AUC), $\mu_{max\,Sc}/\mu_{max\,Lt}$ ratio, AUC_{Lt}/AUC_{Sc} ratio.

	Sc/*Lt* Ratio	Nitrogen ($mg \cdot L^{-1}$)	Oxygenation	Temperature (°C)	$\mu_{max\,Sc}$ (h^{-1})	$\mu_{max\,Lt}$ (h^{-1})	$\mu_{max\,Lt}/\mu_{max\,Sc}$	$N_{max\,Sc}$ ($UFC \cdot mL^{-1}$)	$N_{max\,Lt}$ ($UFC \cdot mL^{-1}$)	AUC_{Sc}	AUC_{Lt}	AUC_{Lt}/AUC_{Sc}
A	0.1	150	No	28	0.278	0.201	0.72	5.00×10^7	1.80×10^7	9.14×10^9	1.67×10^9	0.18
B	1	150	No	18	0.063	0.073	1.16	4.40×10^7	4.40×10^7	8.52×10^9	7.16×10^9	0.84
C	1	300	No	18	0.052	0.162	3.12	3.80×10^7	6.90×10^7	8.88×10^9	6.21×10^9	0.70
D	0.1	300	Yes	18	0.082	0.243	2.96	1.10×10^7	6.70×10^7	1.12×10^9	9.16×10^9	8.18
E	1	150	Yes	28	0.286	0.198	0.69	3.80×10^7	1.20×10^7	9.80×10^9	8.70×10^8	0.09
F	0.1	300	No	28	0.268	0.216	0.81	6.40×10^7	4.00×10^7	1.03×10^{10}	1.70×10^9	0.17
G	0.1	150	Yes	18	0.080	0.240	3.00	1.60×10^7	6.20×10^7	1.92×10^9	1.22×10^{10}	6.35
H	1	300	Yes	28	0.116	0.168	1.45	4.50×10^7	2.10×10^7	9.65×10^9	9.00×10^8	0.09

Table A2. Quantitative data related to fermentation in the different conditions of the experimental design (A to H): fermentation time (time of total consumption of initial sugars), ethanol concentration, pH, and lactic acid concentration at the end of the fermentation.

	Sc/*Lt* Ratio	Nitrogen ($mg \cdot L^{-1}$)	Oxygenation	Temperature (°C)	Fermentation Time (h)	Ethanol (%)	pH	Lactic Acid ($g \cdot L^{-1}$)
A	0.1	150	No	28	215	11.93	3.34	2.02
B	1	150	No	18	306	11.98	3.44	0.93
C	1	300	No	18	238	12.08	3.45	0.85
D	0.1	300	Yes	18	377	11.53	3.40	2.69
E	1	150	Yes	28	238	11.92	3.40	0.82
F	0.1	300	No	28	190	11.78	3.33	2.33
G	0.1	150	Yes	18	377	11.17	3.24	3.52
H	1	300	Yes	28	168	11.80	3.42	0.85

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2.2.3. Principales conclusions de l'étude

Ces travaux ont permis de mettre en évidence de multiples impacts des paramètres de fermentation (ratio *Sc* / *Lt*, azote, température, oxygénation), qui diffèrent en fonction des variables étudiées (populations, fermentation, composés volatils).

Les dynamiques de populations levuriennes sont impactées fortement par la température, et, dans une moindre mesure, le ratio d'ensemencement et l'oxygénation du milieu. En revanche, les conditions qui favorisent la persistance de *Lt* allongent également le temps nécessaire pour terminer la fermentation.

En ce qui concerne l'impact sur le vin synthétique produit, les paramètres étudiés ont permis de dégager les conclusions suivantes. Les effets sur la production d'éthanol ne sont pas significatifs mais le ratio *Sc* / *Lt* et l'oxygénation semblent jouer un rôle dans cette production. Le ratio d'ensemencement *Sc* / *Lt* influence fortement la production d'acide lactique dans le milieu et a tendance à impacter le pH de ce dernier, alors que les autres paramètres sont sans effet observable sur ces deux critères.

De la même manière, la production de composés volatils est influencée par les paramètres de fermentation, mais avec des effets différents selon le paramètre et les molécules étudiés. Certains impacts peuvent être expliqués par les dynamiques de populations observées mais mettent également en évidence l'existence d'interactions entre *Sc* et *Lt*, car conduisant à des volatilomes différents lorsqu'on modifie le taux d'azote, paramètre n'impactant pas significativement les dynamiques de population.

Ces résultats illustrent la complexité de la maîtrise de telles fermentations mixtes. Des compromis doivent être trouvés pour obtenir, via un développement des souches approprié, les caractéristiques souhaitées pour le moût fermenté (éthanol, acidification, composés volatils), tout en évitant des temps de fermentation trop importants, voire des fermentations languissantes.

Cependant, cette étude ne suffit évidemment pas à elle seule à comprendre les dynamiques multi-factorielles à l'œuvre pendant une fermentation alcoolique en culture mixte. Une perspective intéressante serait, en premier lieu, de compléter le plan expérimental avec les huit modalités non réalisées ici. Cela permettrait de solidifier statistiquement les conclusions et d'étudier les interactions éventuelles entre paramètres de fermentation (effet "synergie"). Il serait également intéressant, au-delà de la solidification des résultats, d'élargir l'étude, soit via les paramètres de fermentations étudiés, soit via les variables mesurées expérimentalement. Par exemple, ajouter au plan d'expérimentation un niveau intermédiaire pour les paramètres étudiés, ou un nouveau paramètre tel que le taux de glucides du moût initial (déjà montré comme impactant significativement les cultures mixtes *S. cerevisiae* / *Hanseniaspora guilliermondii* (Barbosa *et al.*, 2022)) permettraient d'affiner le modèle statistique obtenu et donc les conclusions qui en découlent. L'influence des vitamines sur les cultures mixtes est un autre sujet d'étude exploré dans la littérature (Barbosa *et al.*, 2015; Medina *et al.*, 2012) et leur impact semble interagir avec celui de l'azote. De la même façon, l'impact du taux de lipides du moût a déjà été montré comme significatif à plusieurs degrés dans une autre étude multiparamétrique portant sur le couple *S. cerevisiae* / *Metschnikowia pulcherrima* (Seguinot *et al.*, 2020). Les lipides étant impliqués dans les mécanismes de réponse des levures aux stress (Shekhawat *et al.*, 2020), ils pourraient également moduler les interactions entre celles-ci. La concentration de ces deux familles de nutriments dans le moût semblent donc aussi être des paramètres de fermentation pertinents pour affiner le modèle. Par ailleurs, l'introduction de conditions en ensemencement séquentiel constitue une piste pour approfondir l'étude de l'impact des conditions d'ensemencement au-delà du ratio *Sc* / *N.S.*.

Par ailleurs, des champs expérimentaux tels que l'évaluation sensorielle ou la consommation des

différentes sources azotées n'ont pas été explorées durant cette étude, et leur implémentation sur un tel plan expérimental permettrait d'aller plus loin dans la discussion des résultats.

2.3. Approche multiparamétrique et simultanée de l'impact des paramètres de fermentation sur les interactions entre *Saccharomyces cerevisiae* et *Lachancea thermotolerans* / Résultats complémentaires

2.3.1. Contexte

A l'issue des essais précédents, des expérimentations supplémentaires ont été menées afin de compléter les connaissances à différents niveaux.
Un plan d'expérimentation complémentaire (plan 2) de celui décrit précédemment (plan 1) permettra d'étudier un plan complet et de solidifier d'un point de vue statistique les conclusions. La réalisation de ce plan à une période différente (changements de conditions de manipulations (aliquote de levures, batch de milieux de culture, environnement, manipulateur)) permettra également d'évaluer la reproductibilité des résultats. Enfin, l'étude d'un plan complet devrait conduire à la mise en évidence d'interactions entre facteurs.
En outre, une approche sensorielle sera effectuée sur les vins synthétiques issus des conditions de fermentation du plan 2, afin d'évaluer qualitativement leur composition aromatique, et de les discriminer en fonction de leur acidité.

La figure 3 schématise les différentes analyses effectuées sur les plans 1 et 2.

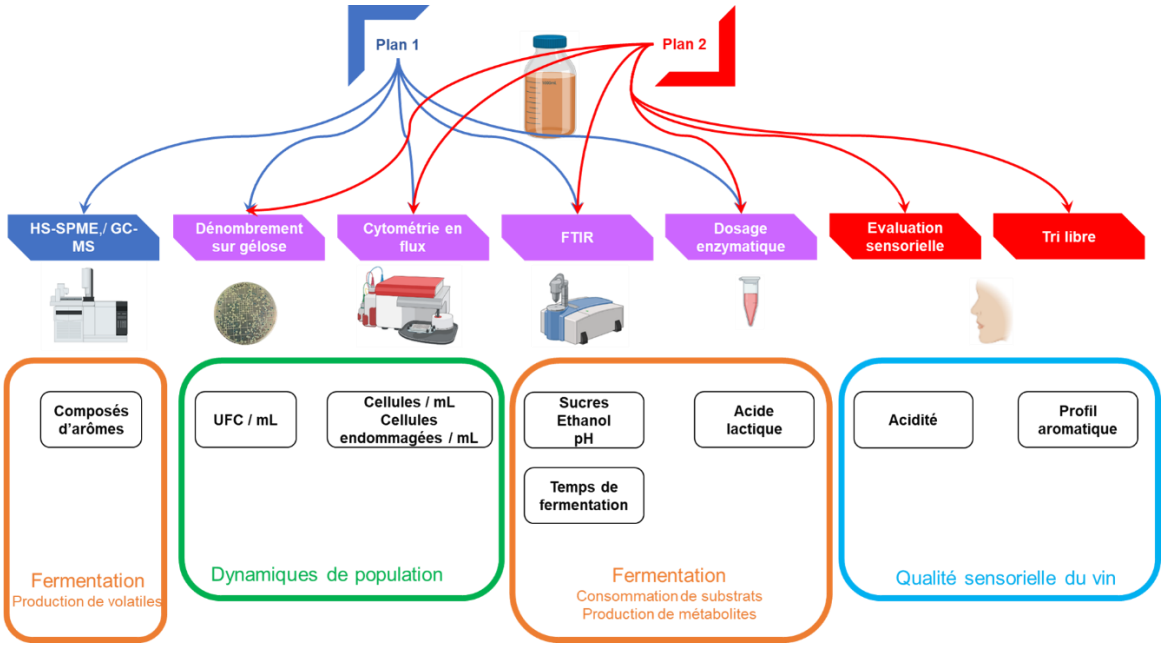

Figure 3. Schéma des différentes techniques d'analyses mises en œuvre sur les échantillons issus des plans expérimentaux 1 et 2 et résultats quantitatifs en découlant.

2.3.2. Matériel et Méthodes

2.3.2.1. Matériel

Les microorganismes utilisés sont les levures *S. cerevisiae* et *L. thermotolerans* utilisées dans les expériences relatées dans la publication Joran *et al.* 2022 (cf. 2.2.).
Les cultures ont été menées dans des conditions identiques à celles utilisées lors de ces travaux : fermentations en flacons Schott de 1L.

2.3.2.2. Méthodes

2.3.2.2.1. Cultures. Plan expérimental

Les expériences ont été menées dans des conditions similaires à celles présentées dans l'article Joran *et al.* 2022 (cf. 2.2) : préculture des levures sur milieu YPD en boîte de Pétri puis en moût synthétique MS300, à 28°C sous agitation, jusqu'en fin de phase exponentielle et transfert de la biomasse dans les conditions du plan d'expérimentation (Tableau 1).

Tableau 1. Conditions testées dans le plan d'expérimentation et paramètres correspondants

Condition	Ratio *Sc* / *Lt*	Azote ($mg \cdot L^{-1}$)	Oxygénation	Température (°C)
Plan 1				
A	0.1	150	Non	28
B	1	150	Non	18
C	1	300	Non	18
D	0.1	300	Oui	18
E	1	150	Oui	28
F	0.1	300	Non	28
G	0.1	150	Oui	18
H	1	300	Oui	28
Plan 2				
I	0.1	150	Non	18
J	0.1	150	Oui	28
K	0.1	300	Non	18
L	0.1	300	Oui	28
M	1	150	Non	28
N	1	150	Oui	18
O	1	300	Non	28
P	1	300	Oui	18

L'ensemencement des milieux de culture mixte s'effectue à raison de 10^6 $cellules \cdot mL^{-1}$ de *L. thermotolerans* et de 10^5 ou 10^6 $cellules \cdot mL^{-1}$ de *S. cerevisiae* pour obtenir un ratio de 0.1 ou 1. Le moût synthétique contient 150 (MS150) ou 300 $mg \cdot L^{-1}$ (MS300) d'azote assimilable (organique et minéral). Les cultures sont menées à 18°C ou 28°C. Toutes les fermentations sont conduites sans agitation. L'oxygénation est effectuée en fin de phase exponentielle par bullage d'air pendant le temps

nécessaire à la saturation en oxygène d'1 L de milieu stérile inerté à l'azote (ici, 12 min). La concentration en oxygène dissous a été mesurée en continu par un analyseur d'oxygène HI 9146 (Hanna Instruments, Woonsocket, RI, États-Unis), et la saturation définie après stabilisation de la mesure pendant 30 s.
Des fermentations en culture pure sont également effectuées pour comparaison, dans le cadre de l'étude, de la quantité d'acide lactique produite dans les différentes conditions. Dans ce cas, un milieu MS300 est ensemencé avec 10^6 cellules·mL^{-1} de levure (*Sc* ou *Lt*) et la culture est conduite à 18°C sans oxygénation.

2.3.2.2.2. Suivi des populations

La population levurienne est suivie comme précisé dans la publication Joran *et al.* 2022 (cf. 2.2) par énumération sur milieu WL (qui permet de distinguer les deux espèces de levures via la morphologie des colonies) et par cytométrie en flux. Dans ce cas, la mutation eGFP de *Sc* permet de la distinguer de *Lt* et un marquage par l'iodure de propidium permet d'évaluer les cellules endommagées. Le protocole de prélèvement, dilution et marquage est identique à celui décrit ci-dessus dans Joran *et al.* (2022).

2.3.2.2.3. Suivi de la composition du milieu

Le suivi de la composition du milieu est effectué comme précisé dans la publication Joran *et al.* 2022 (cf. 2.2) : spectroscopie infrarouge à transformée de Fourier pour déterminer la concentration en éthanol, sucres résiduels et le pH et kit enzymatique pour évaluer la concentration en acide lactique.

2.3.2.2.4. Evaluation sensorielle

Des échantillons de moût fermentés, issus des différentes conditions du plan d'expérimentation 2 ainsi que ceux issus de cultures pures soit 10 échantillons, ont été présentés, dans un ordre aléatoire à un jury expert (étudiants en DNO) de 14 personnes. Il leur a été demandé de classer les échantillons selon l'acidité perçue en bouche. Un test de Friedman a été effectué afin d'évaluer si les différences entre échantillons sont significatives.

Un autre test (tri libre) visant à décrire le profil aromatique des différents échantillons de moûts fermentés issus du plan d'expérimentation 2 a également été effectué. Les 10 échantillons ont été présentés, dans un ordre aléatoire, au même jury. Il leur est demandé de regrouper les échantillons qui leur semblent similaires puis de décrire leur odeur en générant les descripteurs qui leur conviennent. Les descripteurs sont ensuite analysés afin de les regrouper par synonymes et de décrire le profil par un nombre limité de notes aromatiques. Le nombre d'occurrences de chaque descripteur est ensuite déterminé et les descripteurs utilisés par moins de 20 % des juges pour un échantillon sont éliminés. Une analyse factorielle des correspondances (AFC) est ensuite effectuée afin de relier les notes aromatiques (10 descripteurs au final) et les échantillons.

2.3.2.2.5. Analyse des résultats

Les résultats du plan d'expérimentation complet sont analysés, avec le logiciel Ellistat, comme indiqué dans l'article Joran *et al.* 2022 (cf. 2.2). Afin d'établir le modèle en régression linéaire multiple, le modèle est tout d'abord effectué avec les 4 facteurs uniquement, puis les interactions entre facteurs significatifs

sont incluses dans le modèle si elles aussi sont significatives, les autres étant exclues pour éviter de disperser ladite modélisation avec des interactions inexistantes.

Comme illustré dans la figure 4, les résultats bruts issus des différentes analyses des échantillons issus du plan expérimental sont agrégés afin de déterminer et calculer des indicateurs quantitatifs. En ce qui concerne les dynamiques de population, les vitesses de croissance maximales et les populations maximales sont déterminées pour les deux levures ainsi que l'aire sous la courbe de croissance (Area Under Curve (AUC)) qui renseigne sur la présence des levures au cours de la fermentation. Les ratios $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$ et AUC_{Lt} / AUC_{Sc} sont des indicateurs quantitatifs calculés afin de donner des informations sur la croissance relative et la persistance relative de *L. thermotolerans* par rapport à *S. cerevisiae*.

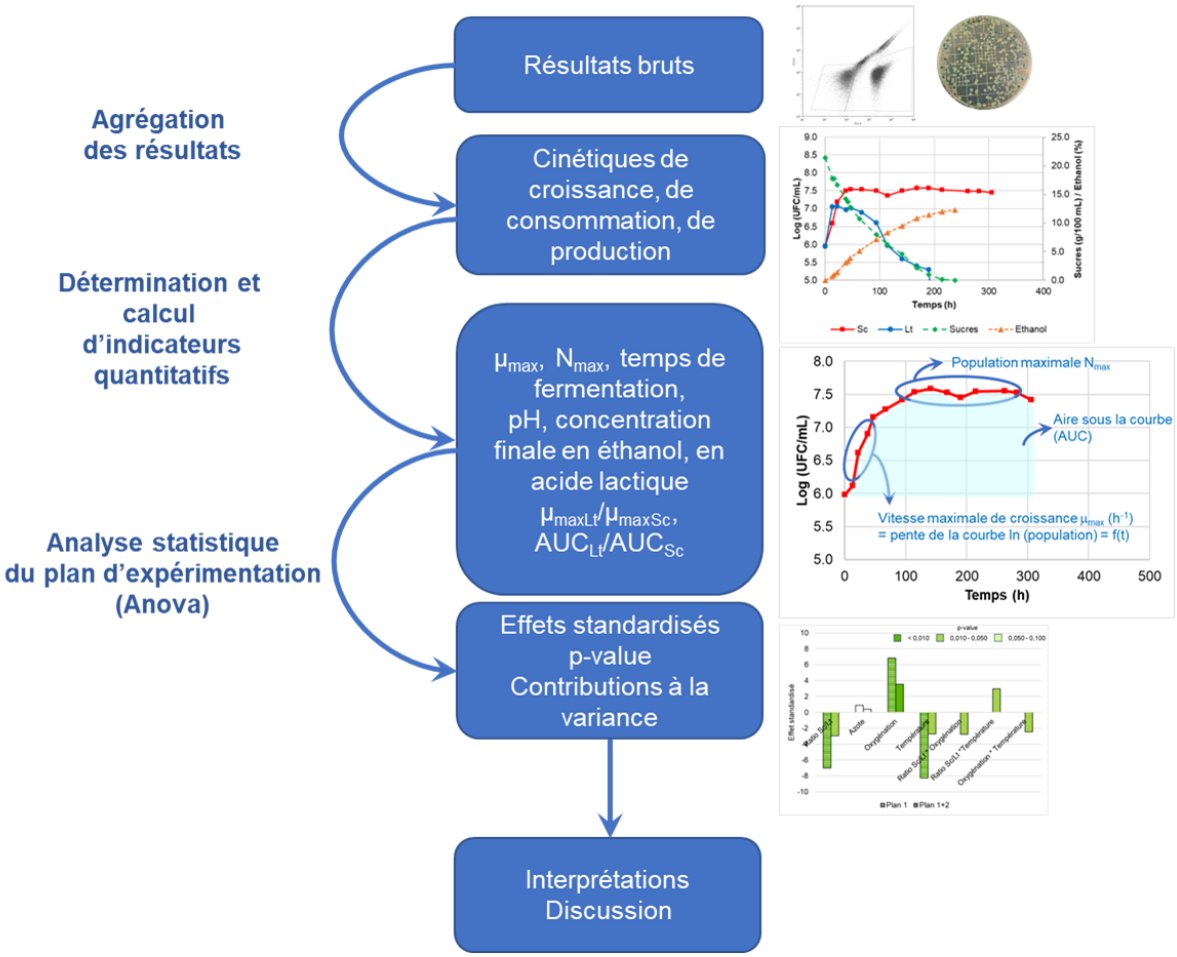

Figure 4. Démarche de traitement des résultats bruts.

2.3.3. Résultats

L'objectif est d'étudier simultanément l'impact des quatre facteurs étudiés (ratio *Sc* / *Lt*, concentration en azote du milieu, oxygénation, température) sur les dynamiques de population, la composition du milieu et sur les caractéristiques sensorielles du "vin synthétique" produit.
Les différentes conditions expérimentales testées (cultures mixtes selon les plans d'expérimentation 1 et 2 et cultures pures) conduisent, comme illustré dans la figure 5, à des conduites de fermentation différentes en termes de dynamiques de population, de consommation des sucres et de production d'éthanol. La culture pure de *S. cerevisiae* permet d'atteindre la fin de fermentation assez rapidement alors que la culture de *L. thermotolerans* conduit à un moût contenant encore des sucres après 500 h de fermentation. En ce qui concerne les cultures mixtes, quelles que soient les conditions, les fermentations sont complètes (consommation de tous les sucres) et *S. cerevisiae* domine en fin de fermentation, celle-ci durant de 200 à 500 h environ. *L. thermotolerans* se développe en début de fermentation puis sa population chute plus ou moins rapidement (persistance faible à forte au cours de la fermentation). Seules quelques conditions (D, G, I, K) permettent de conserver *L. thermotolerans* bien présente dans le milieu pendant la majorité de la fermentation.

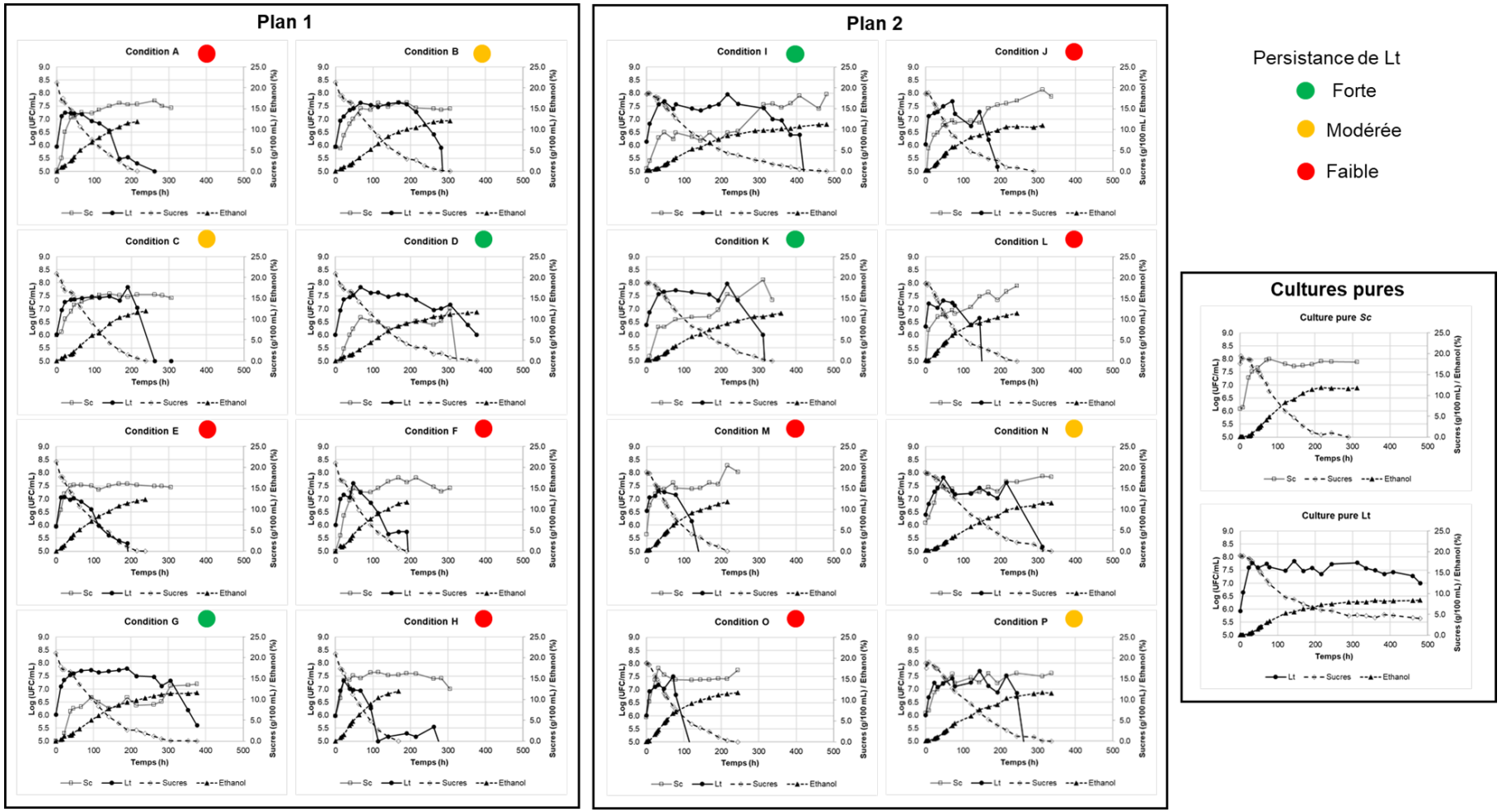

Figure 5. Cinétiques de fermentation (populations de *Saccharomyces cerevisiae* et de *Lachancea thermotolerans*, concentration en sucres et en éthanol) obtenues en culture pure et en culture mixte, dans les différentes conditions des plans d'expérimentations. La persistance de *Lt* est estimée comme faible, moyenne ou forte, si *Lt* persiste à une concentration supérieure à 10^6 cellules·mL^{-1} pendant respectivement moins de 66 %, entre 66% et 90 % inclus, et plus de 90 % du temps de fermentation.

Les résultats obtenus pour les différents critères observés (valeurs quantitatives) sont donnés dans les tableaux 2 et 3, pour respectivement les données relatives aux dynamiques de population et aux paramètres de fermentation.

Tableau 2. Données quantitatives relatives aux dynamiques de populations dans les différentes conditions du plan expérimental complet : vitesse de croissance maximale, ratio des vitesses de croissance, population maximale, ratio des aires sous la courbe

Condition	Ratio *Sc*/*Lt*	Azote ($mg \cdot L^{-1}$)	Oxygénation	Température (°C)	$\mu_{max\ Sc}$ (h^{-1})	$\mu_{max\ Lt}$ (h^{-1})	$\mu_{max\ Lt}$ / $\mu_{max\ Sc}$	$N_{max\ Sc}$ ($UFC \cdot mL^{-1}$)	$N_{max\ Lt}$ ($UFC \cdot mL^{-1}$)	AUC_{Lt} / AUC_{Sc}
A	0.1	150	Non	28	0.278	0.201	0.72	5.00×10^7	1.80×10^7	0.18
B	1	150	Non	18	0.063	0.073	1.16	4.40×10^7	4.40×10^7	0.84
C	1	300	Non	18	0.052	0.162	3.11	3.80×10^7	6.90×10^7	0.70
D	0.1	300	Oui	18	0.082	0.243	2.98	1.10×10^7	6.70×10^7	8.18
E	1	150	Oui	28	0.286	0.198	0.69	3.80×10^7	1.20×10^7	0.09
F	0.1	300	Non	28	0.268	0.216	0.81	6.40×10^7	4.00×10^7	0.17
G	0.1	150	Oui	18	0.080	0.240	2.98	1.60×10^7	6.20×10^7	6.35
H	1	300	Oui	28	0.116	0.168	1.45	4.50×10^7	2.10×10^7	0.09
I	0.1	150	Non	18	0.048	0.127	2.64	9.00×10^7	9.00×10^7	1.24
J	0.1	150	Oui	28	0.142	0.176	1.24	1.36×10^8	4.90×10^7	0.23
K	0.1	300	Non	18	0.100	0.143	1.43	1.29×10^8	5.10×10^7	1.20
L	0.1	300	Oui	28	0.155	0.191	1.23	7.80×10^7	2.10×10^7	0.31
M	1	150	Non	28	0.119	0.147	1.24	1.90×10^8	2.00×10^7	0.13
N	1	150	Oui	18	0.074	0.134	1.83	7.30×10^7	6.50×10^7	0.48
O	1	300	Non	28	0.131	0.241	1.84	6.50×10^7	3.15×10^7	0.15
P	1	300	Oui	18	0.048	0.197	4.10	4.15×10^7	5.00×10^7	0.54

Tableau 3. Données quantitatives relatives à la fermentation dans les différentes conditions du plan expérimental complet : temps de fermentation (temps correspondant à la consommation totale des sucres initiaux), concentration en éthanol, pH, concentration en acide lactique en fin de fermentation

Condition	Ratio *Sc*/Lt	Azote ($mg \cdot L^{-1}$)	Oxygénation	Température (°C)	Temps de fermentation (h)	Ethanol (%)	pH	Acide lactique ($mg \cdot L^{-1}$)
A	0.1	150	Non	28	215	11.93	3.34	2.02
B	1	150	Non	18	306	11.98	3.44	0.93
C	1	300	Non	18	238	12.08	3.45	0.85
D	0.1	300	Oui	18	377	11.53	3.40	2.69
E	1	150	Oui	28	238	11.92	3.40	0.82
F	0.1	300	Non	28	190	11.78	3.33	2.33
G	0.1	150	Oui	18	377	11.17	3.24	3.52
H	1	300	Oui	28	168	11.80	3.42	0.85
I	0.1	150	Non	18	480	11.24	3.12	5.76
J	0.1	150	Oui	28	289	10.96	3.24	5.28
K	0.1	300	Non	18	336	11.42	3.11	6.02
L	0.1	300	Oui	28	244	11.50	3.17	4.50
M	1	150	Non	28	216	11.80	3.30	2.21
N	1	150	Oui	18	336	11.53	3.40	2.51
O	1	300	Non	28	244	11.80	3.43	0.88
P	1	300	Oui	18	336	11.48	3.42	0.35

2.3.3.1. Dynamiques des populations

L'impact des facteurs étudiés sur les dynamiques des populations est évalué, après traitement statistique, à travers un effet standardisé. Ces valeurs sont reprises dans le tableau 4 avec, pour chaque facteur, la p-value correspondante et la contribution à la variance.
L'analyse du plan complet (1+2) conduit à l'observation d'effets significatifs des paramètres de fermentation choisis sur les variables étudiées, et permet également de mettre en évidence des interactions significatives entre ces paramètres, malgré des résidus plus élevés (26 points de plus en moyenne) que lors de l'analyse du plan 1 seul. Cette augmentation de la contribution à la variance des résidus peut être reliée aux aléas de deux expérimentations faites à des périodes différentes : levures issues de cryotubes et de précultures différentes, batchs de milieux différents, manipulateur différent, conditions environnementales différentes lors des analyses… Même si ces aléas pris individuellement sont négligeables et difficilement évitables, cela contribue à la difficulté d'avoir une reproductibilité exacte lors de travaux sur matériel biologique.

L'analyse des résultats concernant les taux de croissance maximale des levures est effectuée sans le facteur oxygénation. L'apport d'air ayant lieu en fin de phase exponentielle, il n'a nécessairement aucun impact sur la période de croissance maximale, et ne doit donc pas être pris en compte dans le modèle.
En ce qui concerne *S. cerevisiae*, l'impact positif de la température (effet standardisé 4.475, p-value 0.011) sur le taux de croissance maximal observé lors du plan 1 est confirmé, avec une forte significativité, par le plan complet (effet standardisé 4.239, p-value 0.001). Les deux autres facteurs (ratio d'ensemencement et concentration en azote) n'influencent pas significativement la croissance de *Sc*, et contribuent très faiblement à la variance. Par contre, les facteurs oxygénation et température qui impactent la population maximale dans le cas du plan 1 (effets standardisés -4.443 et 4.546, p-values 0.021 et 0.020 respectivement), ne sont plus significatifs dans le plan complet et masqués par un taux de résidus très important.
Dans le cas de *L. thermotolerans*, l'effet faiblement significatif (effet standardisé -2.296, p-value 0.083), dans le plan 1, du ratio *Sc* / *Lt* sur le taux de croissance maximal, n'apparaît plus significatif dans le plan complet. Aucun facteur n'a d'impact significatif sur ce taux de croissance. La population maximale de *Lt* est, quant à elle, impactée négativement et de façon très significative par la température (effet standardisé -5.197, p-value 0.001) comme, dans l'étude du plan 1 mais l'effet de la concentration d'azote n'est plus observé dans le plan complet.

Le rapport des taux de croissance maximal $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$, qui donne une indication sur la vitesse de croissance relative de *Lt* par rapport à *Sc*, est, lui, impacté négativement par la température, de façon encore plus significative que lors de l'analyse du plan 1 (effet standardisé -4.738, p-value 0.001). Une température élevée a donc tendance à favoriser la croissance de *Sc* comparativement à celle de *Lt*, ce qui est en accord avec la littérature (voir Joran *et al.* 2022). Le plan complet fait apparaître en outre un impact négatif du ratio *Sc* / *Lt* (effet standardisé -2.556, p-value 0.027) et une interaction positive entre ce ratio et la concentration en azote du milieu (effet standardisé 2.893, p-value 0.015), comme illustré dans la figure 6.
L'augmentation de la proportion de *Sc* dans la flore d'ensemencement a donc tendance à diminuer la vitesse de croissance relative de *Lt* par rapport à *Sc*, alors qu'aucun effet significatif n'est visible sur les vitesses de croissance prises individuellement. Des phénomènes d'interactions entre les deux souches peuvent expliquer ces résultats : accélération de la croissance de *Sc* en présence de *Lt* ou, plus

Tableau 4. Impact des paramètres de fermentation sur les dynamiques des populations

p-value
< 0,010
0,010 - 0,050
0,050 - 0,100

		Plan 1					Plan 1+2									
		Ratio *Sc/Lt*	Azote	Oxygénation	Température	Résidus	Ratio *Sc/Lt*	Azote	Oxygénation	Température	Ratio *Sc/Lt* * Azote	Ratio *Sc/Lt* * Oxygénation	Ratio *Sc/Lt* * Température	Azote * Oxygénation	Azote * Température	Oxygénation * Température	Résidus
$\mu_{max\ Sc}$	Effet stand.	−1.272	−1.276		4.475		-1.181	-0.622		4.239							
	p-value	0.272	0.271		0.011		0.260	0.546		0.001							
	Contrib. Var.	5.93%	5.97%		73.40%	14.70%	4.40%	1.22%		56.60%							37.80%
$\mu_{max\ Lt}$	Effet stand.	-2.296	0.585		0.501		-1.248	1.521		1.265							
	p-value	0.083	0.590		0.643		0.236	0.154		0.230							
	Contrib. Var.	53.40%	3.47%		2.54%	40.50%	8.92%	13.20%		9.15%							68.70%
$\mu_{max\ Lt}$ / $\mu_{max\ Sc}$	Effet stand.	−0.556	1.440		−3.388		-2.556	-1.026		-4.738	2.893						
	p-value	0.608	0.223		0.028		0.027	0.327		0.001	0.015						
	Contrib. Var.	1.73%	11.60%		64.30%	22.40%	13.20%	2.13%		45.40%	16.90%						22.30%
$N_{max\ Sc}$	Effet stand.	1.240	0.517	−4.443	4.546		-0.205	-0.860	-1.203	1.162							
	p-value	0.303	0.641	0.021	0.020		0.841	0.408	0.254	0.270							
	Contrib. Var.	3.40%	0.59%	43.70%	45.70%	6.64%	0.29%	5.08%	9.93%	9.26%							75.44%
$N_{max\ Lt}$	Effet stand.	−2.104	3.131	−0.462	−7.750		-1.556	-0.173	-0.300	-5.197							
	p-value	0.126	0.052	0.676	0.004		0.148	0.866	0.770	0.001							
	Contrib. Var.	5.71%	12.60%	0.28%	77.50%	3.87%	5.97%	0.07%	0.22%	66.60%							27.13%
AUC_{Lt} / AUC_{Sc}	Effet stand.	−6.999	0.888	6.822	−8.267		-2.940	0.381	3.522	-2.719		-2.752	2.986			-2.451	
	p-value	0.006	0.440	0.006	0.004		0.019	0.713	0.008	0.026		0.025	0.017			0.040	
	Contrib. Var.	29.20%	0.47%	27.80%	40.80%	1.79%	14.60%	0.25%	21.00%	12.50%		12.80%	15.10%			10.20%	13.55%

Effet stand. = effet standardisé ; Contrib. Var. = contribution à la variance ; N_{max} = population maximale ; μ_{max} = vitesse de croissance maximale ; AUC = aire sous la courbe.
Paramètre 1 * Paramètre 2 = interaction entre le paramètre 1 et le paramètre 2.
Le code couleur des cases est établi en fonction de la p-value.

probablement une décélération de celle de *Lt* (puisqu'une tendance négative mais non significative est observée sur son μ_{max} seul).

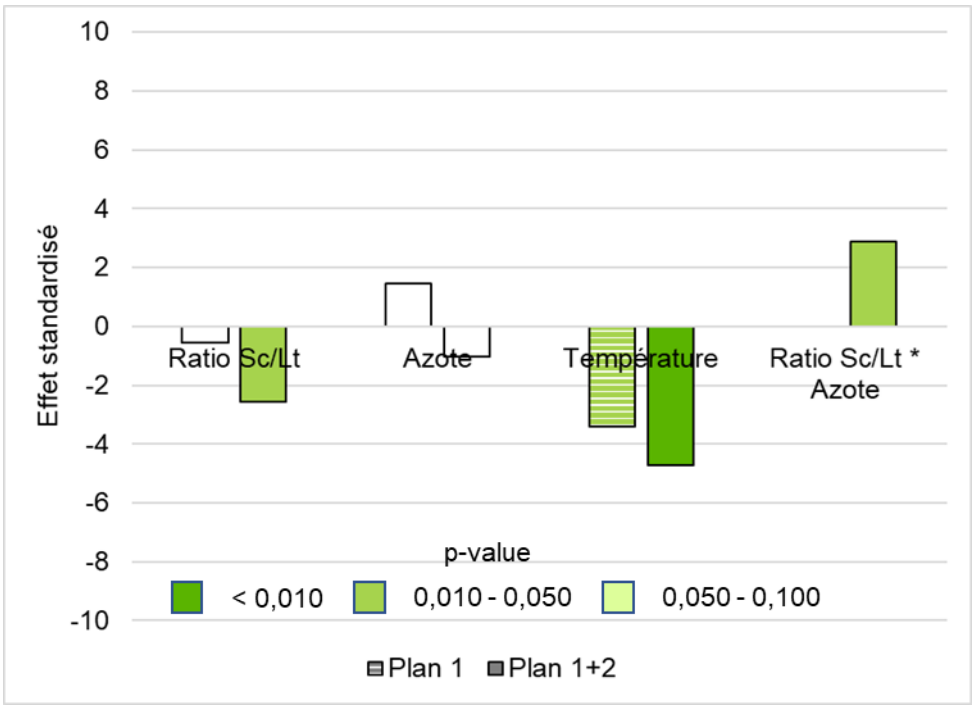

Figure 6. Impact des paramètres de fermentation sur le ratio $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$ (effets standardisés).

Des phénomènes de compétition vis à vis des éléments nutritifs peuvent également être impliqués puisqu'une interaction entre le ratio *Sc* / *Lt* et la concentration en azote est mise en évidence. C'est d'ailleurs la seule interaction significative entre facteurs, comme illustré dans la figure 7.

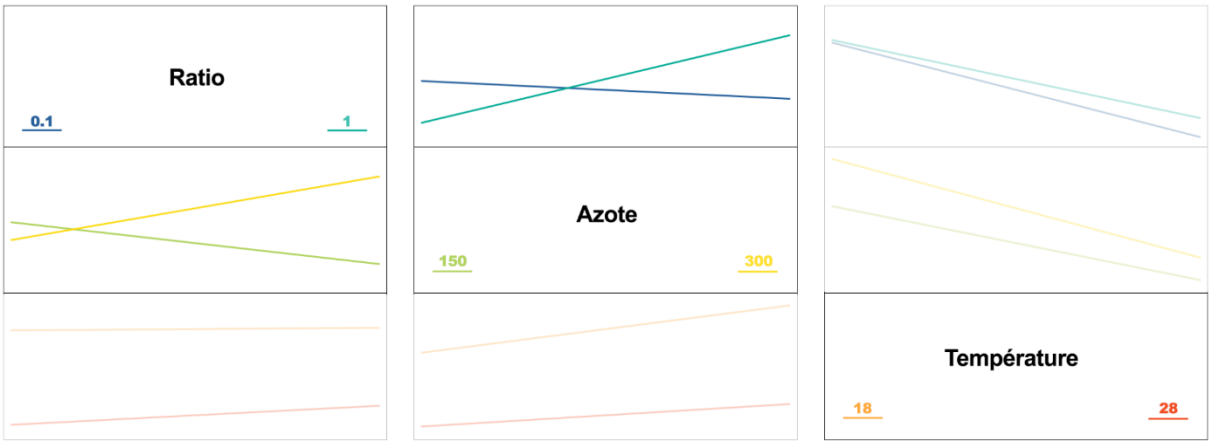

Figure 7. Graphe des interactions entre facteurs impactant le ratio $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$.

Chaque graphe, à l'intersection entre deux paramètres, représente l'interaction entre ceux-ci. L'axe des ordonnées représente l'effet standardisé sur la variable choisie (ici le ratio $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$). L'axe en abscisse représente le paramètre à la verticale du graphe. Le code couleur des courbes correspond au code couleur des valeurs du paramètre situé à l'horizontale du graphe. Les graphes correspondant à des interactions non-significatives sont représentés en transparence.

Les conditions augmentant le ratio $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$ favorisent la croissance de *Lt* par rapport à *Sc* et donc son implantation. C'est le cas lorsque l'ensemencement est effectué avec un ratio *Sc* / *Lt* de 0.1. Une augmentation de l'azote a alors peu d'impact sur le ratio $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$. Par contre, quand le ratio *Sc* / *Lt* est égal à 1, une augmentation de l'azote permet d'augmenter le taux de croissance maximal de *Lt* de façon plus importante que celui de *Sc*, alors que l'azote ne semble avoir aucun impact significatif sur les vitesses de croissance prises individuellement. Quand l'ensemencement est effectué avec la même concentration de chacune des deux levures, une concentration en azote plus élevée dans le milieu tend à augmenter le ratio $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$. Ces résultats laissent penser que des phénomènes de compétition pour les sources azotées existent entre les deux souches lorsqu'elles sont introduites dans le milieu simultanément et à concentration équivalente. Une explication peut être donnée par les travaux d'autres chercheurs (Peng *et al.*, 2019) qui ont, après une étude protéomique, formulé l'hypothèse que *Sc* répondait au stress induit par une coculture avec *Lt*, par une stratégie basée sur la compétition en nutriments qui lui permet de rester la levure dominante. *Sc* réagit en effet à la présence de *Lt* en augmentant la synthèse des protéines de stress (Heat Shock Proteins) et de protéines permettant soit d'augmenter la disponibilité de nutriments (utilisation de sources de carbone et d'azote comme la glycine), soit de favoriser l'endocytose et donc l'entrée des nutriments dans la cellule. Cette synthèse diminue ensuite lorsque la population de *Lt* commence à décliner.

Afin d'étudier les dynamiques de population, un autre critère semble pertinent à suivre : le rapport des aires sous la courbe de croissance de *Lt* et de *Sc* (AUC_{Lt} / AUC_{Sc}), qui rend compte de la persistance globale de *Lt* au cours de la fermentation. Pour ce critère, le plan complet permet de confirmer les effets observés dans le plan 1 : effet négatif du ratio *Sc* / *Lt* et de la température (effets standardisés -2.940 et -2.719, p-value 0.019 et 0.026 respectivement) et effet positif de l'oxygénation (effet standardisé 3.522, p-value 0.008). La significativité est diminuée car le plan complet met en évidence des interactions entre ces 3 facteurs (positive entre ratio et température et négatives pour les couples ratio/oxygénation et oxygénation/température), comme illustré dans la figure 8.

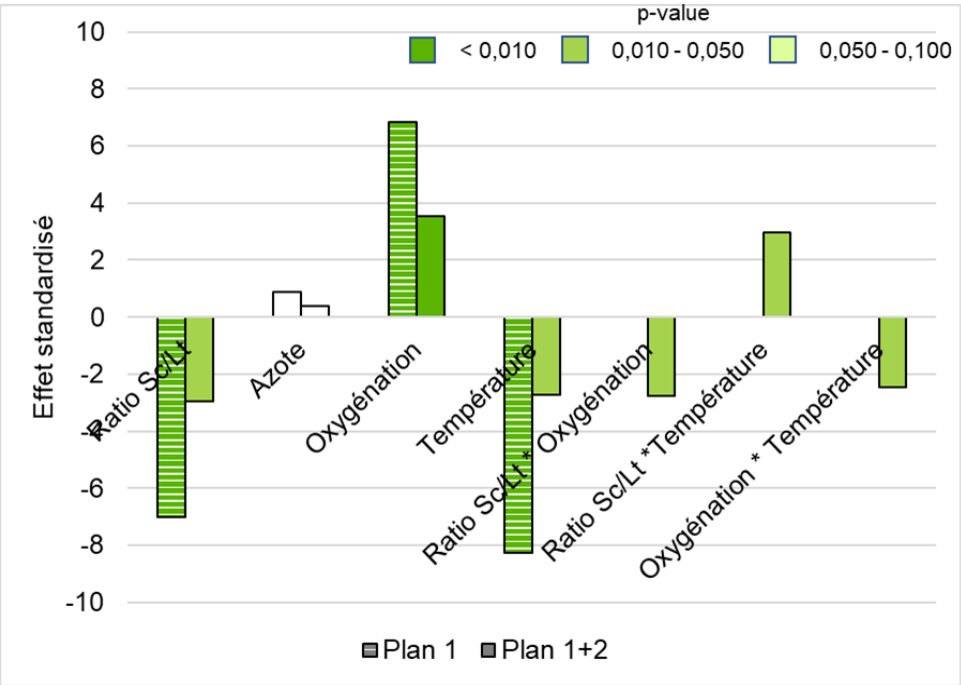

Figure 8. Impact des paramètres de fermentation sur le ratio AUC_{Lt} / AUC_{Sc} (effets standardisés).

La composition en azote du milieu n'a aucun impact significatif sur ce critère ni lorsqu'il est considéré seul, ni dans le cadre d'interactions avec les autres facteurs ce qui confirme l'absence d'influence de l'azote sur la persistance de *L. thermotolerans* dans le milieu de fermentation, dans les conditions testées.

Les interactions entre facteurs sont précisées dans la figure 9.

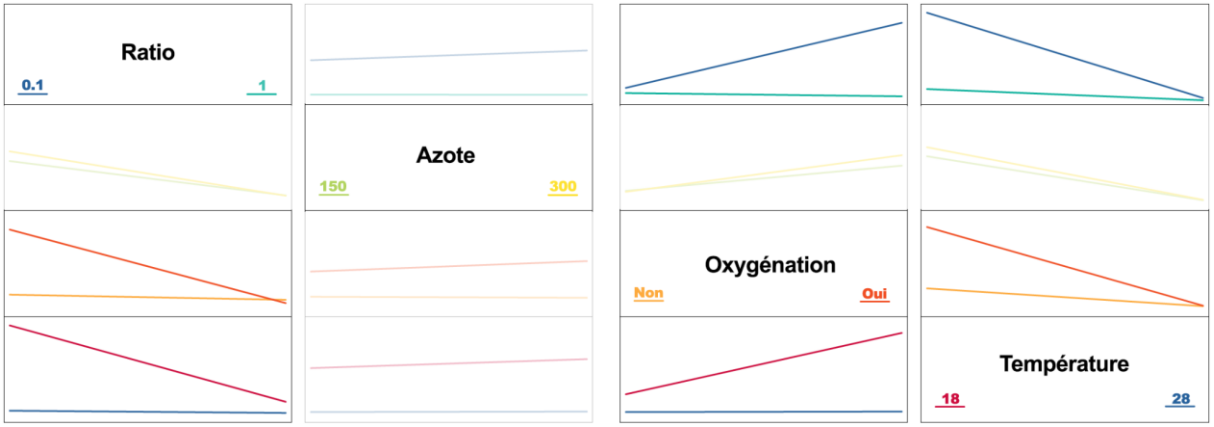

Figure 9. Graphe des interactions entre facteurs impactant le ratio AUC_{Lt} / AUC_{Sc}.
Chaque graphe, à l'intersection entre deux paramètres, représente l'interaction entre ceux-ci. L'axe des ordonnées représente l'effet standardisé sur la variable choisie (ici le ratio AUC_{Lt} / AUC_{Sc}). L'axe en abscisse représente le paramètre à la verticale du graphe. Le code couleur des courbes correspond au code couleur des valeurs du paramètre situé à l'horizontale du graphe. Les graphes correspondant à des interactions non-significatives sont représentés en transparence.

Lorsque le ratio d'ensemencement est en faveur de *Lt* (ratio *Sc* / *Lt* égal à 0.1), la persistance de *Lt* est augmentée dans le cas d'une oxygénation du milieu et d'une température plus faible. En revanche, lorsque l'ensemencement privilégie *Sc* (ratio *Sc* / *Lt* égal à 1), l'oxygénation et la température n'ont pas d'impact. En l'absence d'oxygénation, le ratio d'ensemencement n'a pas d'impact et la température a un léger impact négatif sur le ratio AUC_{Lt} / AUC_{Sc}. Lorsque le milieu de fermentation est oxygéné, par contre, un ratio *Sc* / *Lt* de 0.1 et une température de 18°C permettent d'augmenter la persistance de *Lt* par rapport aux valeurs obtenues pour un ratio de 1 et une température de 28°C.
Le ratio d'ensemencement et l'oxygénation n'ont pas d'impact sur la persistance de *Lt* lorsque la température de fermentation est de 28°C, mais ont une influence à 18°C : le ratio AUC_{Lt} / AUC_{Sc} est augmenté lorsque le ratio d'ensemencement est diminué et que le milieu est oxygéné.
Si l'un des facteurs favorise *Sc* (ratio *Sc* / *Lt* égal à 1 ou température de 28°C ou absence d'oxygénation), les autres facteurs n'ont plus d'impact sur la persistance de *Lt*, ce qui montre l'efficacité des mécanismes mis en œuvre par *Sc* pour favoriser sa survie en présence de *Lt*. *Sc* est par exemple, capable d'induire la mort de *Lt* par contact cellulaire en accumulant certains peptides à sa surface (Branco, Kemsawasd, *et al.*, 2017) : cet impact n'intervenant qu'après accumulation et donc lorsque *Sc* est en phase stationnaire, toute condition permettant de ralentir la croissance de *Sc* en début de fermentation devrait permettre d'augmenter la persistance de *Lt*.
Un ratio d'ensemencement favorisant *Lt*, une température plus basse et une oxygénation du milieu sont donc des conditions permettant d'obtenir une persistance plus importante de *Lt* au cours de la fermentation. Toutefois les interactions entre ces facteurs rendent plus complexe la gestion de la fermentation mixte pour obtenir une persistance de *Lt* jusqu'en fin de fermentation. *Lt* est présent en fin de fermentation pour uniquement 4 conditions (D, G, I, K : cf. Figure 5).

2.3.3.2. Fermentation

L'impact des facteurs étudiés sur la fermentation alcoolique (temps de fermentation et composition du milieu) est évalué, après analyse statistique, via un effet standardisé avec la p-value correspondante et via la contribution à la variance, comme indiqué dans le tableau 5.
L'analyse du plan complet (1+2) permet de confirmer la plupart des effets observés lors de l'analyse du plan 1, avec une augmentation de la contribution à la variance des résidus, liée à la reproductibilité des expérimentations comme évoqué ci-dessus (2.2.3.1.). Toutefois, ce plan complet ne permet pas de mettre en évidence d'interactions entre les facteurs étudiés.

La concentration en azote n'impacte aucun des critères étudiés (temps de fermentation, concentration en éthanol, pH, concentration en acide lactique) après analyse du plan complet.

L'oxygénation impacte uniquement la concentration en éthanol, avec un effet négatif et une significativité plus élevée que lors de l'analyse du plan 1 (effet standardisé -2.274, p-value 0.044). Cette diminution de la concentration en éthanol peut être reliée à une présence relative de *Lt* par rapport à *Sc* plus importante que sans oxygénation (étant donné l'impact positif de l'oxygénation sur le ratio AUC_{Lt}/AUC_{Sc} discuté plus haut), cette souche *Lt* ayant un rendement en éthanol plus faible que *Sc*. Cela peut être également lié à une modification du métabolisme levurien, la présence d'oxygène favorisant la respiration plutôt que la fermentation, comme discuté ci-dessus dans Joran *et al.* 2022.

La température influence de façon importante le temps de fermentation (effet standardisé -5.265, p-value 0.001). La conduite de la fermentation à 28°C a pour effet de conduire à des fermentations plus courtes : ces résultats sont à mettre en relation avec l'impact de la température sur les dynamiques de population décrites précédemment (cf. 2.2.3.1.) : en favorisant le développement de *Sc* par rapport à *Lt*, une température plus élevée permet une fermentation alcoolique plus rapide, comme discuté également dans Joran *et al.* 2022.

Tableau 5. Impact des facteurs étudiés (ratio *Sc* / *Lt*, concentration en azote, oxygénation, température) sur la fermentation alcoolique en culture mixte

p-value
< 0,010
0,010 - 0,050
0,050 - 0,100

		Plan 1					Plan 1+2									
		Ratio *Sc*/*Lt*	Azote	Oxygénation	Température	Résidus	Ratio *Sc*/*Lt*	Azote	Oxygénation	Température	Ratio *Sc*/*Lt* * Azote	Ratio *Sc*/*Lt* * Oxygénation	Ratio *Sc*/*Lt* * Température	Azote * Oxygénation	Azote * Température	Oxygénation * Température	Résidus
Temps de fermentation	Effet stand.	−3.057	−2.384	3.086	−7.122		-2.284	-1.737	0.751	-5.265							
	p-value	0.055	0.097	0.054	0.006		0.043	0.110	0.469	0.001							
	Contrib. Var.	11.90%	7.26%	12.20%	64.80%	3.83%	11.00%	6.35%	1.19%	58.30%							23.16%
Concentration en éthanol	Effet stand.	2.899	0.402	−2.857	1.418		3.039	0.914	-2.274	1.126							
	p-value	0.063	0.715	0.065	0.251		0.011	0.380	0.044	0.284							
	Contrib. Var.	38.70%	0.74%	37.50%	9.25%	13.80%	33.60%	3.04%	18.80%	4.61%							39.95%
pH	Effet stand.	2.575	1.159	−0.644	−0.257		3.635	0.694	0.472	0.139							
	p-value	0.082	0.330	0.566	0.813		0.004	0.502	0.646	0.892							
	Contrib. Var.	57.90%	11.70%	3.62%	0.58%	26.20%	53.00%	1.93%	0.89%	0.08%							44.10%
Concentration en acide lactique	Effet stand.	−7.322	−0.593	1.809	−2.034		-4.257	-0.859	-0.089	-0.703							
	p-value	0.005	0.595	0.168	0.135		0.001	0.409	0.931	0.497							
	Contrib. Var.	83.30%	0.55%	5.08%	6.42%	4.66%	59.70%	2.43%	0.03%	1.63%							36.21%

Effet stand. = effet standardisé. Contrib. Var. = contribution à la variance
Paramètre 1 * Paramètre 2 = interaction entre le paramètre 1 et le paramètre 2
Le code couleur des cases est établi en fonction de la p-value.

Le ratio d'ensemencement *Sc* / *Lt* est un paramètre clé pour le déroulement de la fermentation alcoolique puisqu'il a une influence significative sur tous les critères étudiés, comme illustré dans la figure 10.

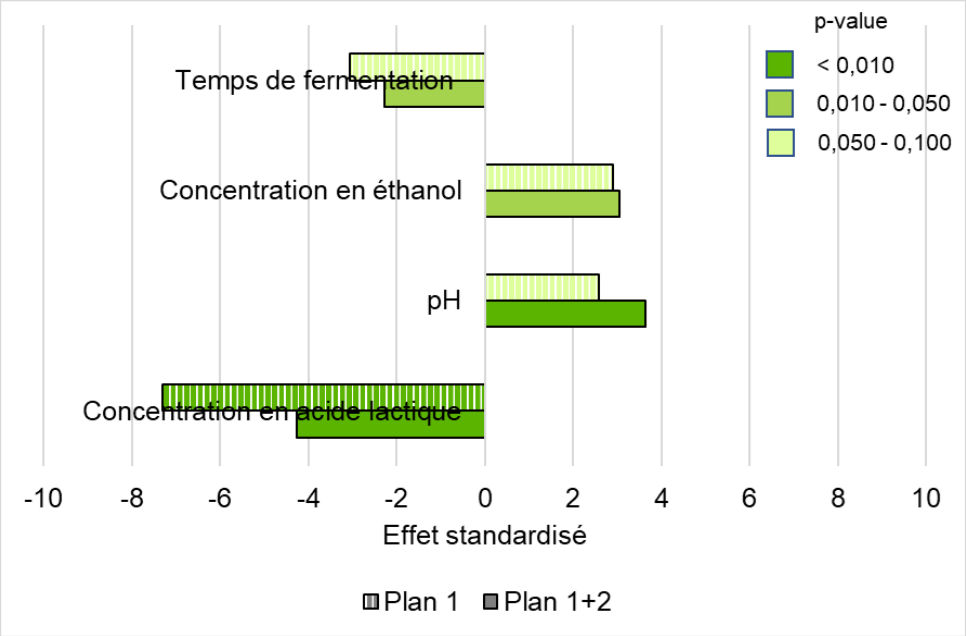

Figure 10. Impact du ratio d'ensemencement *Sc* / *Lt* sur la fermentation alcoolique (temps de fermentation, concentration en éthanol, pH, acide lactique) (effets standardisés).

Le ratio d'ensemencement a un impact négatif sur le temps de fermentation et un impact positif sur la concentration en éthanol, confirmant ainsi les tendances peu significatives observées dans le plan n°1. Un ratio *Sc / Lt* élevé a, en effet, tendance à favoriser le développement de *Sc* par rapport à *Lt* (cf. 2.2.3.1.) et *Sc* est connue pour avoir des capacités supérieures à *Lt* en termes de fermentation (effet Crabtree supérieur (Vicente *et al.*, 2021) et de production d'éthanol (rendement en éthanol plus important (Hranilovic, Gambetta, *et al.*, 2018).

Le ratio *Sc /Lt* influence également, de façon très significative, le pH (effet standardisé 3.635, p-value 0.004) et la concentration en acide lactique (effet standardisé -4.257, p-value 0.001). L'ensemencement du milieu avec une population levurienne favorisant *Lt* permet une production d'acide lactique plus importante et une acidification supérieure du milieu (diminution du pH). En effet, ces conditions permettent l'implantation rapide de *Lt* dans le moût et donc la production d'acide lactique pendant sa phase exponentielle de croissance, confirmant ainsi les résultats discutés dans Joran *et al.* (2022).

Cette production d'acide lactique et l'acidification du milieu sont des objectifs à atteindre pour la profession puisqu'elles permettent à la fois d'obtenir des vins avec des caractéristiques sensorielles intéressantes et de limiter les risques de contamination microbiologique du moût (Gianvito *et al.*, 2022).

2.3.3.3. Caractéristiques sensorielles

Afin de compléter les données concernant l'impact des paramètres étudiés sur les populations levuriennes et le déroulement de la fermentation, une analyse des caractéristiques sensorielles a été effectuée sur les différents échantillons de vins synthétiques issus du plan n°2.
Les résultats présentés précédemment ont permis de montrer un impact important de certains paramètres sur la production d'acide lactique au cours de la fermentation. Il paraît donc important dans un premier temps d'évaluer si cette production influence l'acidité du vin ressentie sensoriellement. Ensuite, l'odeur des vins synthétiques sera évaluée sensoriellement afin de mettre en évidence l'influence éventuelle de certains paramètres de fermentation sur le profil aromatique.

Les différents vins synthétiques sont soumis à une évaluation de l'acidité par un jury expert. Celui-ci a classé les échantillons issus des différentes conditions expérimentales du plan n°2 selon leur acidité croissante. La figure 11 donne la note d'acidité obtenue (somme des rangs obtenus pour chaque échantillon) ainsi que la concentration en acide lactique des vins, issues de l'analyse de ce composé via un kit enzymatique.

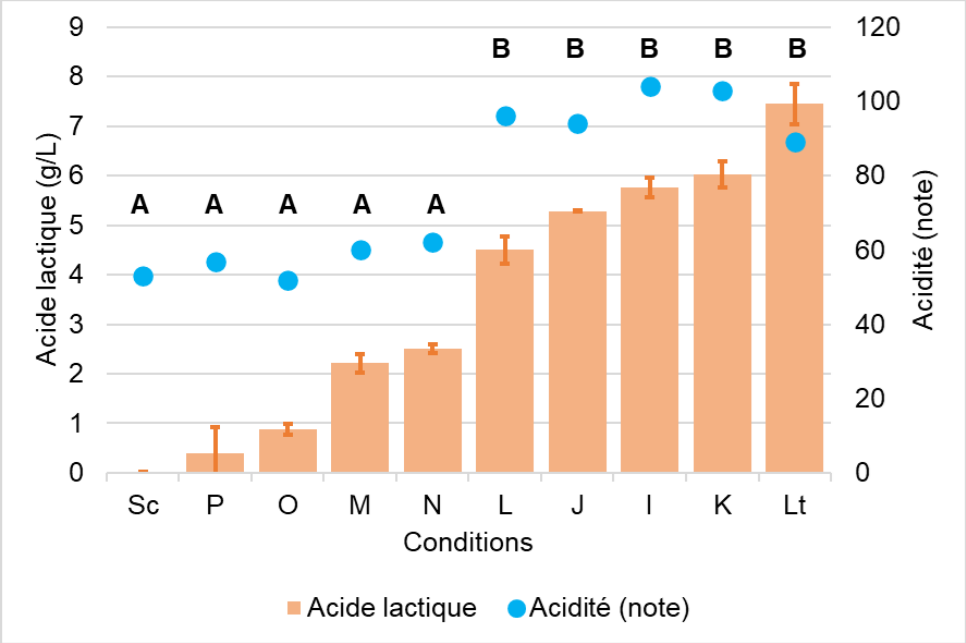

Figure 11. Acidité des vins synthétiques évaluée sensoriellement et concentration en acide lactique

Un test de Friedman effectué sur les notes d'acidité met en évidence deux groupes d'échantillons significativement différents : le groupe A avec des notes entre 50 et 60 et le groupe B avec des notes entre 80 et 110. L'existence de ces deux groupes statistiques souligne l'impact du ratio d'ensemencement sur l'acidité du vin obtenu. En effet, le groupe A, avec une acidité plus faible, regroupe le vin issu de la culture pure de *Sc* et les échantillons M, N, O, P issus de cultures mixtes ensemencées avec un ratio *Sc* / *Lt* de 1. Au contraire, les échantillons I, J, K, L, ensemencés avec un ratio de 0.1 ont une acidité plus importante et proche de celle de *Lt*.
Les concentrations en acide lactique déterminées pour les différents échantillons sont entre 0 et 7.5 $g \cdot L^{-1}$. La valeur quasiment nulle observée pour la culture de *Sc* est en accord avec les résultats de

la littérature puisque cette levure est connue pour, selon la souche, ne pas être capable de produire cet acide ou de le produire en très faible quantité (Sauer *et al.*, 2010; Sgouros *et al.*, 2020). *Lt*, au contraire, est capable de produire de grandes quantités d'acide lactique (Gobbi *et al.*, 2013; Sgouros *et al.*, 2020) ce qui est confirmé par la concentration observée ici (environ 7.5 $g \cdot L^{-1}$). Les vins synthétiques issus de cultures mixtes présentent des concentrations avec des valeurs intermédiaires, qui permettent là également de distinguer 2 groupes : les échantillons M, N, O, P ensemencés avec un ratio *Sc* / *Lt* de 1, qui présentent des valeurs faibles en acide lactique (0.3 - 2.5 $g \cdot L^{-1}$) et les échantillons I, J, K, L ensemencés avec un ratio de 0.1 qui présentent des concentrations plus élevées (4.5 - 6.0 $g \cdot L^{-1}$). La culture pure de *Lt* se distingue par une acidité perçue plus faible que celle attendue compte-tenu de la forte concentration réelle en acide lactique : la concentration en sucres résiduels (4.0 $g \cdot L^{-1}$) lors du prélèvement (due à une fermentation incomplète) explique probablement la moindre acidité ressentie sensoriellement (Noordeloos & Nagel, 1972).
De manière générale, les valeurs d'acidité perçues sensoriellement sont donc en accord avec les concentrations en acide lactique, et confirment les résultats obtenus dans le plan d'expériences. Ainsi, parmi les quatre facteurs étudiés, le ratio d'ensemencement impacte de manière significative à la fois l'acidité réelle et l'acidité perçue, appuyant ainsi la conclusion susmentionnée : si l'objectif initial de la culture mixte est l'acidification du moût, la persistance de *Lt* tout au long de la fermentation n'est pas un prérequis essentiel, contrairement à sa présence en début de fermentation (très impactée par le ratio d'ensemencement).

Les échantillons issus du plan n°2 sont ensuite soumis à une évaluation sensorielle par le même jury expert, afin de définir leur profil olfactif. Pour cela, la méthode du tri libre est utilisée : les juges regroupent les échantillons ayant une odeur similaire et caractérisent celle-ci avec des descripteurs. Après différents traitements des données brutes, une Analyse Factorielle des Correspondances (AFC) entre descripteurs de l'arôme et échantillons est effectuée et illustrée dans la figure 12.

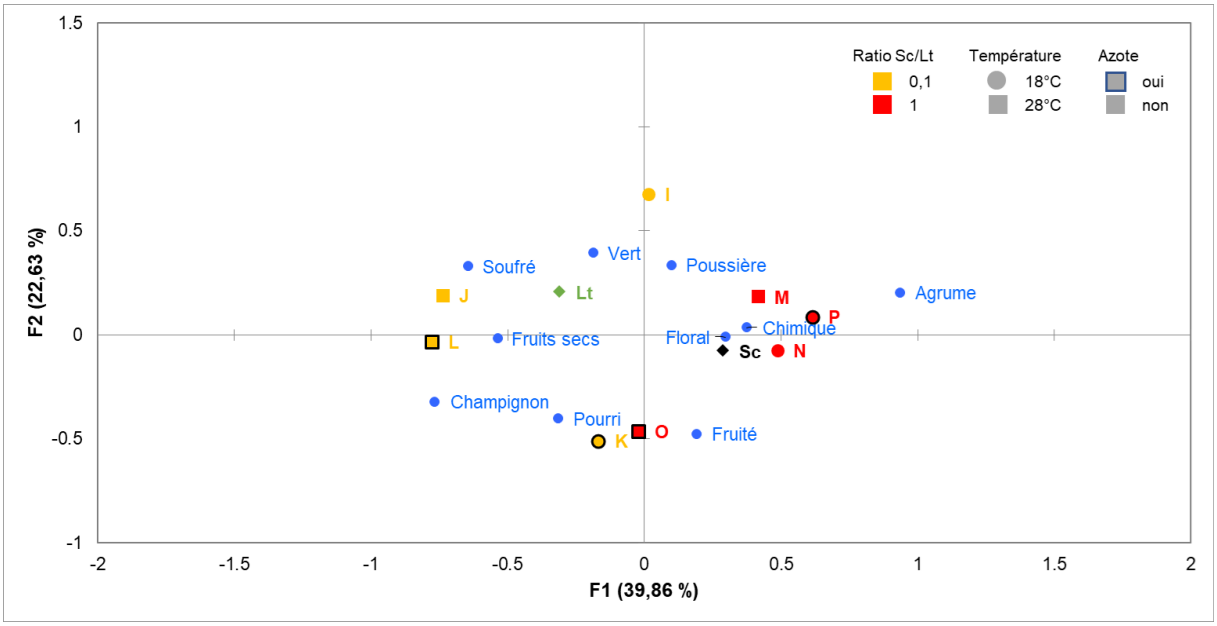

Figure 12. Analyse Factorielle des Correspondances (AFC) entre descripteurs de l'arôme et échantillons de vin synthétique issus du plan d'expérimentation n°2

Les deux axes F1 et F2 de l'AFC expliquent 62.49 % de la variance. L'axe F1 permet de séparer des notes recherchées (fruité, floral…) de notes aromatiques moins désirables (moisi, soufré…).
Les différentes conditions du plan expérimental en culture mixte *Sc* / *Lt* conduisent à des échantillons qui ont des profils aromatiques différents entre eux et différents des cultures pures de chacune des 2 levures. La culture pure de *Sc* est caractérisée par des notes fruitées alors que *Lt* a un arôme plus proche des notes vertes.

L'axe F1 a tendance à séparer les conditions avec un ratio d'ensemencement différent : les échantillons avec un ratio de 0.1 et de 1 sont représentés respectivement en jaune et en rouge.
Les conditions I et K favorisent la persistance de *Lt* au cours du temps (rapports AUC_{Lt}/AUC_{Sc} les plus élevés du plan n°2 et assez proches : 1.24 et 1.20 respectivement) et pourtant conduisent à des produits avec des profils aromatiques bien distincts, et différents de celui de *Lt*. Les conditions J et L pour lesquelles la persistance de *Lt* est faible ont un profil aromatique proche mais assez éloigné de celui de *Sc*, qui pourtant domine au cours de la fermentation. Les conditions N et P conduisent à une persistance modérée de *Lt* et pourtant à un profil aromatique proche de *Sc*. Ces exemples mettent en évidence les interactions entre les deux levures en ce qui concerne les composés volatils produits : la persistance ou la dominance de l'un ou l'autre souche ne permet pas de prédire le profil aromatique du produit puisque des changements de métabolisme sont induits par la présence simultanée des deux levures dans le milieu.
Cette AFC ne permet pas de mettre clairement en évidence un impact de la température.
Les échantillons, sans oxygénation, I et K d'une part et M et O d'autre part diffèrent dans chacun des couples par la concentration initiale du milieu en azote et sont séparés par l'axe F2. Par contre, cet axe ne sépare pas les échantillons des couples avec oxygénation (J et L, N et P). L'azote semble donc avoir un impact sur le profil sensoriel, mais une interaction avec l'oxygénation est observée.

Ces résultats sont en accord avec les conclusions issues du plan d'expérimentation n°1 concernant la production des différentes molécules volatiles : interactions entre souches, impact du ratio d'ensemencement. Les effets contrastés de la température et de l'oxygénation sur les différents composés volatils observés dans le plan n°1 conduisent à des profils sensoriels différents et difficilement prévisibles. L'impact de l'azote sur les composés d'arômes avait été mis en évidence également mais seulement sur la production d'alcools supérieurs, en accord avec la littérature (Fairbairn *et al.*, 2021; Gobert *et al.*, 2019; Rollero *et al.*, 2021).

2.4. Conclusions, perspectives

Les résultats décrits dans Joran *et al.* 2022 ainsi que dans les travaux complémentaires réalisés ensuite montrent ainsi de multiples effets de la part des quatre paramètres étudiés (ratio d'ensemencement, concentration en azote, oxygénation, température) sur plusieurs métriques essentielles rendant compte à la fois du déroulé correct d'une fermentation en culture mixte, et des caractéristiques du vin fini. La modélisation multiparamétrique et l'étude statistique de ces impacts et de leurs interactions a permis non seulement d'évaluer leur significativité, mais aussi de comparer leurs impacts respectifs sur les métriques étudiées. Cela permet de dégager des conclusions à la fois sur les dynamiques de population et sur les paramètres constituant des leviers d'action potentiellement exploitables pour moduler les caractéristiques du vin fini.

En ce qui concerne les dynamiques de population, le plan expérimental complet fait apparaître un effet positif de la température sur la vitesse de croissance maximale de *Sc* et par conséquent un impact négatif sur la vitesse de croissance relative de *Lt* par rapport à *Sc* (rapport $\mu_{max\ Lt}$ / $\mu_{max\ Sc}$). L'implantation de *Lt* dans le moût est facilitée par un ratio d'ensemencement en faveur de *Lt* (ratio *Sc* / *Lt* de 0.1) mais, dans le cas où les deux levures sont ensemencées à la même concentration, des phénomènes de compétition pour les sources azotées semblent avoir lieu, ce qui défavorise la croissance relative de *Lt* par rapport à *Sc* si la concentration d'azote dans le moût est faible.
Le rapport des aires sous les courbes de croissance des deux levures (AUC_{Lt} / AUC_{Sc}) est un indicateur de la persistance de *Lt* au cours de la fermentation. La concentration initiale en azote, bien que pouvant faciliter l'implantation de *Lt*, n'a aucun effet sur sa persistance, vraisemblablement du fait d'une consommation de ces sources azotées pendant la phase exponentielle de croissance. La persistance de *Lt* peut cependant être modulée par les trois autres paramètres de fermentation, mais avec des interactions entre ces facteurs. En revanche, lorsque l'un des paramètres est en faveur de *Sc*, les deux autres paramètres n'agissent plus sur la persistance de *Lt,* montrant ainsi l'efficacité des mécanismes propres à *Sc* lui donnant un avantage sur *Lt* en culture mixte.

Même si la modulation de la présence globale de *L. thermotolerans* au cours de la fermentation (ratio des AUC) semble complexe, car non seulement dépendante de tous les paramètres étudiés sauf l'azote, mais aussi d'interactions synergiques ou antagonistes entre ces paramètres, les résultats obtenus en analysant le milieu permettent quant à eux d'identifier des leviers d'action plus clairs. Selon la métrique étudiée, tous les paramètres étudiés n'ont en effet pas la même importance, allant d'une absence totale d'impact à un effet si significatif que la maîtrise du paramètre apparaît comme cruciale pour l'obtention de l'effet souhaité sur le vin fini.
Ainsi, pour moduler le volatilome, la température et le ratio d'ensemencement apparaissent comme les deux principaux leviers à actionner (avec l'azote pour les alcools supérieurs), mais l'impact simultané de ces deux leviers (notamment la température) sur le temps de fermentation oblige à adopter une approche globale et à trouver, via la modélisation multiparamétrique, un compromis acceptable pour éviter une fermentation trop longue voire languissante.
Pour une réduction d'éthanol, la température n'a plus d'effet significatif, et la modulation du ratio ainsi que l'oxygénation du moût en fin de phase exponentielle impactent nettement plus le résultat final obtenu.
En ce qui concerne l'utilisation spécifique de *L. thermotolerans* pour produire de l'acide lactique, la modulation semble ici beaucoup plus simple à mettre en œuvre car elle ne dépend que du ratio d'ensemencement initial, un paramètre aisément modifiable par tout conducteur de fermentation sans matériel ou suivi particulier.
Un autre objectif pourrait impliquer la volonté de maintenir *L. thermotolerans* présente tout au long de la fermentation : son action antimicrobienne (via la production d'acide lactique, de composés toxiques…) afin d'éviter le développement de microorganismes indésirables. L'optimisation de son rôle de bioprotection pourrait permettre de supprimer ou diminuer l'utilisation de SO_2 dans le vin (Rubio-Bretón *et al.*, 2018). L'ensemencement en faible quantité de *L. thermotolerans* (ratio *Sc* / *Lt* de 100), permettrait de bénéficier de son action de bioprotection tout en n'affectant pas le développement de *S. cerevisiae* et la qualité du vin obtenu (Nally *et al.*, 2018).

En somme, la complexité de la maîtrise technique d'une coculture dépend drastiquement de l'objectif technique défini pour celle-ci, comme illustré par la figure 13.

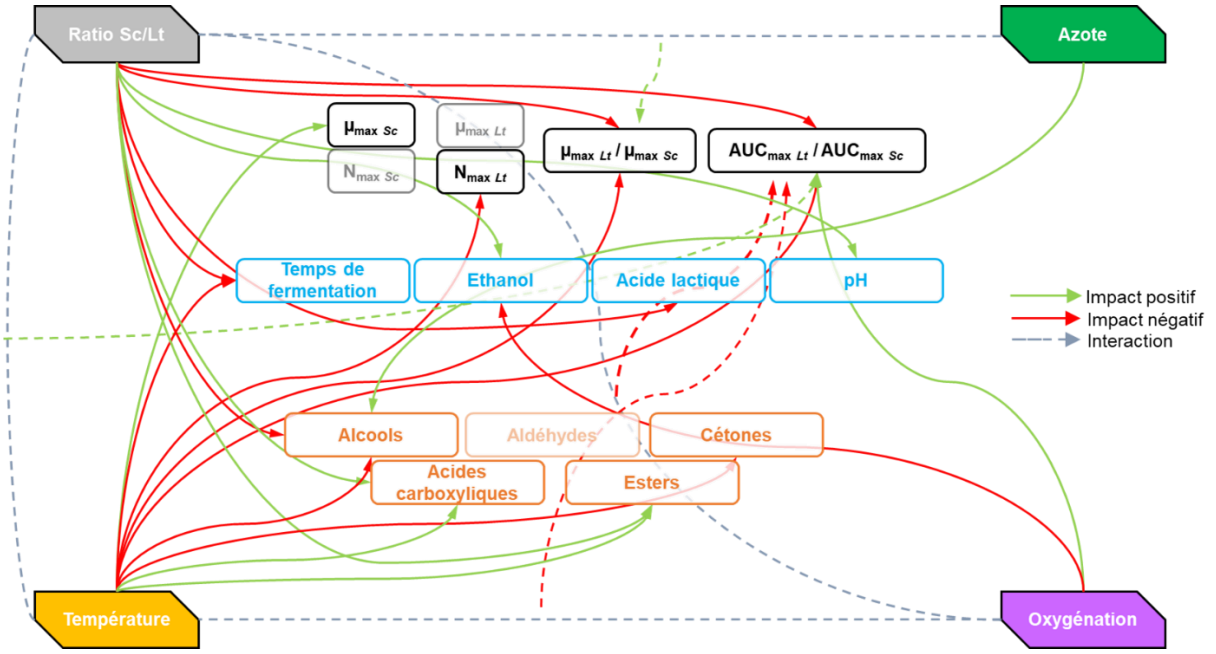

Figure 13. Schéma récapitulatif des impacts des différents paramètres sur les dynamiques de population, le déroulé de la fermentation et la production de molécules volatiles.

Par ailleurs, différents points déjà en partie abordés dans les conclusions de la première étude (2.3) restent à approfondir. Il est toujours possible, évidemment, de solidifier encore plus les résultats obtenus par une reproduction totale du plan complet sans le scinder en deux parties expérimentales. Il serait en outre intéressant de valider les résultats obtenus lors d'une étude sur moût réel et/ou à l'échelle pilote, afin de s'assurer que les conclusions tirées à partir de fermentations standardisées sur moût synthétique résistent à des conditions d'expérimentation plus proches des conditions réelles.

Il est également possible de développer ce plan et d'affiner le modèle obtenu en conservant les quatre paramètres étudiés, mais en modifiant les niveaux étudiés ou en ajoutant des niveaux intermédiaires. L'ajout d'un niveau intermédiaire apparaît comme particulièrement intéressant, en permettant d'observer des réponses potentiellement non linéaires de certaines variables étudiées lors de la modulation d'un paramètre.

En outre, l'impact d'autres paramètres pourrait être étudié afin de compléter les connaissances et d'intégrer le plus de paramètres possibles dans le modèle : concentration d'autres composés dans le moût (sucres, lipides, vitamines…), nature des sources azotées, moment et intensité de l'oxygénation…

Le choix s'était porté ici sur des fermentations mixtes avec un ensemencement simultané des deux levures mais la même démarche pourrait être appliquée à des conduites de fermentation avec ensemencement séquentiel, déjà explorées dans plusieurs autres études (Oliveira & Ferreira, 2019; Vaquero *et al.*, 2021; Zhang *et al.*, 2022).

Un autre volet d'étude intéressant, déjà partiellement abordé dans cette étude, est l'évaluation sensorielle des vins obtenus. En effet, même si une évaluation sensorielle a été conduite et a permis de dégager des conclusions intéressantes, notamment sur l'acidité perçue en bouche, elle reste très exploratoire, et ne permet pas de dresser de conclusions fermes sur le profil olfactif des vins synthétiques obtenus. Cela est dû à la fois à la taille réduite du jury convoqué pour l'étude, et à l'utilisation d'un moût synthétique ayant pu dérouter les membres de celui-ci (Fairbairn *et al.*, 2022). Afin de développer ce volet, vital pour conclure proprement sur l'impact sensoriel réel perçu sur le vin

fermenté en culture mixte, et interpréter correctement les résultats obtenus lors de l'analyse du volatilome par GCMS, il serait donc intéressant de pallier ces deux limites. Ainsi, utiliser un nombre de juges plus important et évaluer la qualité organoleptique de vins issus d'un plan d'expérimentation en moût réel constituent deux pistes à sérieusement considérer pour de futures études.

Pour finir, l'étude des dynamiques de population présentée dans ce volet des travaux de thèse gagneraient, de manière générale, à comporter plus de points d'échantillonnage ainsi qu'une amplitude de suivi plus importante (voire jour et nuit). En effet, la précision d'évaluation des métriques choisies pour évaluer ce pan de l'étude dépendant directement de la fréquence de prélèvement, augmenter cette fréquence le plus possible diminuerait donc leur variabilité. Identifier et appliquer à une future étude de nouvelles méthodologies d'évaluation des populations levuriennes permettrait donc également de dresser des conclusions encore plus solides statistiquement.

3. Suivi automatisé de populations microbiennes

3.1. Contexte

Le suivi et la maîtrise des populations microbiennes est un point clé pour conduire des fermentations, que ce soit au niveau du laboratoire pour des travaux de recherche ou au niveau de la cave de vinification (industrielle ou non). En effet, les dynamiques de population impactent fortement le déroulement de la fermentation, la consommation des substrats présents dans le moût de raisin et la production de métabolites à partir de ces substrats. Les travaux présentés précédemment ont notamment montré l'impact de différents paramètres de fermentation sur les dynamiques de population et sur les caractéristiques des vins obtenus (éthanol, acidité, profil aromatique…). Afin de mieux comprendre les phénomènes d'interactions entre souches, mais aussi optimiser la fermentation (par exemple, étudier l'impact du moment où doit se faire l'oxygénation lors de cocultures, notamment lorsque les populations ne sont pas dans les mêmes phases physiologiques), il est nécessaire de faciliter et améliorer le suivi des populations. La cytométrie en flux est une méthode rapide mais qui nécessite de la main d'œuvre pour effectuer les dilutions et les marquages et qui donc ne peut pas assurer facilement un suivi en continu, 24 heures sur 24, de la fermentation. Il paraissait donc opportun de s'intéresser à la mise au point d'une technologie automatisée permettant de suivre en continu les différentes populations levuriennes dans le moût.
De tels systèmes automatisés existent et ont été utilisés pour le contrôle de la qualité de l'eau par exemple, ou pour suivre certaines cultures de microorganismes, mais aucun travail n'a été publié concernant des applications dans le domaine du vin. De plus, appliquer ces systèmes au vin pose des défis techniques : des durées d'expérimentation longues (plusieurs jours à plusieurs semaines, voire plusieurs mois) combinées ou non avec des milieux riches en sucres et en particules peuvent être à l'origine de phénomènes d'encrassement et/ou induire des contaminations du système de mesure. En outre, la production de gaz carbonique inhérente au processus fermentaire peut potentiellement interférer avec le bon déroulement du protocole d'échantillonnage.

Des travaux ont donc été menés afin de valider la faisabilité de suivi des microorganismes dans le domaine du vin par une technologie de cytométrie en flux automatisée. Cette faisabilité a été étudiée en complexifiant progressivement les conditions expérimentales. Les protocoles du système automatisé (prélèvement, dilution, marquage des microorganismes, paramètres de cytométrie) ont été tout d'abord validés sur des suspensions aqueuses de deux microorganismes clés en œnologie (la levure *Saccharomyces cerevisiae* et la bactérie *Oenococcus oeni*). Les expérimentations ont ensuite permis de montrer la possibilité de suivre les populations dans des conditions fermentaires identiques à celles utilisées dans la partie 2 de ces travaux, puis des suivis plus espacés et plus longs ont été réalisés sur du vin rouge dans le but de simuler la surveillance du vieillissement d'un vin fini.
Ces travaux ont fait l'objet de la rédaction d'un article soumis à la revue OENO-One et présenté en 3.2.

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OENO One - Automated microbial monitoring throughout the winemaking process

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Abstract

Management of microbial flora is a key point in the winemaking process. It impacts process time, fermentation quality (e.g., production of alcohol, development of aroma compounds, absence of undesirable microorganisms), and overall quality of the final wine product. For this management of the flora, real-time microbial monitoring is crucial. Currently used, classical methods (e.g., cultivation on plates) or even more recent technologies (e.g., flow cytometry) do not allow for optimal flora sampling frequency and measurements around the clock without heavy labor cost. The objective of this work was to evaluate the feasibility of automated microbial monitoring based on an online flow cytometry system in different applications in the field of winemaking. Initially, the protocol of automated sampling, double-staining, and analysis was validated on yeast and bacterial populations after rehydration of starter cultures. The system was then tested on a variety of increasingly complex biological systems, simulating wine-related applications. First, a yeast starter culture preparation for "prise de mousse" was tested. Then, a mixed-culture alcoholic fermentation was monitored. Finally, a microbial-focused observation of wine aging was emulated. By overcoming specific constraints linked to must medium (e.g., sugars, alcohol contents, production of gas, fermentation duration), this work shows the potential of this technology for (1) automated yeast or bacterial monitoring in a wide range of wine environments, (2) simultaneous monitoring of both total and intact populations of multiple microorganisms, (3) long observation periods, and (4) high sampling frequencies for high-resolution data. This study also opens the door for the application of this kind of technology beyond such research applications to cellar use. It can be particularly useful for facilitating and improving

quality control on potential contaminants or stuck fermentations, as well as better piloting starter preparation, alcoholic fermentation, or malolactic fermentation.

automation, flow cytometry, monitoring, wine, yeast, bacteria, real time

Introduction

Producing wine of controlled and constant quality heavily relies on properly managing the development of microorganisms as well as their subsequent impact on the must (Belda *et al.*, 2017). Monitoring microbial populations (yeasts and bacteria) all along the process (alcoholic fermentation and malolactic fermentation) is hence key to ensure that the process goes as expected and to guarantee the quality of the final wine product. Microbial monitoring is used at many stages of the process, to control proper implementation of fermentation starters, to avoid the development of unwanted or alteration flora, and to follow and manage the evolution of mixed cultures over time (Bordet *et al.*, 2020; Ciani *et al.*, 2010; Ciani and Comitini, 2015).

Fermentation is usually monitored either directly through enumeration of the microbial population or indirectly through physico-chemical parameters (e.g., sugars, CO_2). These last parameters can be followed online, but continuous monitoring of wine microorganisms in a more direct way has not been implemented yet in cellars. Traditional microbial techniques are already used for this purpose, but they have multiple shortcomings that are limiting monitoring: they are labor-intensive (e.g., microscopy), results are obtained with a significant delay (e.g., culture on Petri dish) or do not allow for flora identification (e.g., OD measurements). Recently, flow cytometry has allowed researchers to reduce most of these shortcomings (Longin *et al.*, 2017). Flow cytometry is a rapid, cultivation-independent method, capable of single-cell resolution, hence also allowing the differentiation of subpopulations of yeasts and bacteria. Although a fast measurement technology, flow cytometry still requires manual labor, especially for sampling, diluting, and staining, which limits measurement frequency and makes monitoring around-the-clock challenging.

Automation of flow cytometry can overcome these limitations and enable autonomous sample collection, staining, and analysis. Samples are collected from the studied medium at regular intervals, then mixed with a staining solution, incubated at appropriate temperature, and finally pumped into a flow cytometer for analysis. Such systems have been used to monitor water quality (Besmer *et al.*, 2014) but also bioreactor processes (Heins *et al.*, 2022). Other studies include automated monitoring involving fermentations and/or use of *Saccharomyces cerevisiae* (Abu-Absi *et al.*, 2003; Freitas *et*

al., 2013; Kacmar *et al.*, 2004). For winemaking however, no studies of the application of an automated system exist yet.

The aim of this work was to study the feasibility of online microbial monitoring in various applications relevant in winemaking: (1) rehydration of active dried microorganisms (yeasts, bacteria), (2) adaptation of active dried yeasts before inoculation in wine, (3) monitoring of alcoholic fermentation, and (4) tracking microbial populations during wine aging.

Materials and methods

1. Monitoring of microbial populations by automated flow cytometry

For all experiments, microbial monitoring was implemented with the following workflow: (1) sample collection at regular intervals, (2) dilution (if applicable), (3) staining and incubation, (4) measurement by flow cytometry, and (5) data processing and analysis.

An onCyt OC-300 automation unit (onCyt Microbiology AG, Dübendorf, Switzerland) was used (schematic in Figure S1) as the interface between sample containers and the flow cytometer. The system collected the sample automatically from the medium following a programmable interval. Here, the interval was 25 min except for the monitoring of the wine aging where samples were taken every 12 hours. Before each sampling, all fluidic parts of the automation system were cleaned with a sodium hypochlorite solution (1 % active chlorine) and then a sodium thiosulfate solution (50 mM) followed by rinsing with ultrapure water to avoid contamination from the previous sample. Before each measurement, the complete dead volume of the sample inside the sample tubing was replaced (575 µl). After being retrieved, samples were used without dilution or automatically diluted 10-fold, 100-fold, or 1'000-fold in serial dilution steps in TRIS buffer (10 mM, pH 8) depending on expected concentrations in a given experiment (see Materials and methods Section 2.). The sample was then stained twice within 2 minutes to apply multiple staining assays (see below). Assuming no substantial change of the sample within this short period, the resulting concentrations from both assays were interpreted as belonging to the same time point.

Diluted samples were then stained with an established assay using SYBR Green I (SG) stain and a mix of SG and Propidium Iodide (PI) stains respectively (Barbesti *et al.*, 2000; Gatza *et al.*, 2013; Nescerecka *et al.*, 2016). SG specifically binds to nucleic acids and in this state emits strong green

fluorescence signals upon excitation by a blue laser (488 nm). As SG can enter all cells regardless of their biological state, all particles with high green fluorescence signals in the absence of PI stain are considered as the "total" population. PI also specifically binds to nucleic acids but emits stronger red fluorescence signals upon excitation by a blue laser (488 nm). PI can only enter cells with compromised membranes where it will occupy nucleic acid binding sites instead of SG resulting in lower green fluorescence but higher red fluorescence. Hence, all particles that retain high green fluorescence signals caused by SG despite the presence of PI stain are considered as the "intact" population. In contrast, the intact population subtracted from the total population quantifies the "damaged" or "dead" cells, which shifted into the signal space of low green and high red fluorescence (Longin *et al.*, 2017). In the case of mixed fermentation (see Materials and methods Section 2.3.), only PI staining was used (no SG), as yeasts can be detected through their autofluorescence or the fluorescence related to the presence of GFP protein. SG and PI (Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA, USA) were prepared at respectively 3.92 and 24 $\mu mol \cdot L^{-1}$ concentration in sterile TRIS buffer (10 mM, pH 8) to obtain 2X staining solutions to be used within the automated system and mixed 1:1 with the sample. Stained samples were incubated for 10 min at 37 °C before being pumped to the flow cytometer.

A BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used with the following settings: analyzed volume of 20 µL, flow rate of 34 $\mu L \cdot min^{-1}$, excitation by a 488 nm wavelength argon laser, detection by the FL1 long-pass filter (530 nm) for green fluorescence and the FL3 long-pass filter (670 nm) for red fluorescence. The trigger was set on FSC for yeast analysis, with a FSC-H threshold of 80,000. For bacteria, the trigger was set on FL1, with FSC-H and FL1-H thresholds of respectively 10,000 and 600.

Data were analyzed using the cyPlot v3.08 software (onCyt Microbiology AG, Dübendorf, Switzerland) for quantitative analysis, and the BD Accuri C6 software for qualitative evaluations. Fixed gates were defined for each type of experiment (bacteria with SG+PI, yeasts with SG+PI, yeasts with GFP+PI) to discriminate the microbial populations from background signals and differentiate between the aforementioned total, intact and damaged populations. Summary statistics for gated FCM files were then exported to .csv files and population concentration calculated based on dilution level used for each sample.

2. Studied biological systems

2.1. Active dried yeasts and bacteria after rehydration

Dried active yeasts *Saccharomyces cerevisiae* 18-2007 from IOC (Épernay, France), often used as a fermentation starter, were investigated. 1 g of dried yeasts was rehydrated in 100 mL of physiological water at 37 °C. The yeast suspension was placed on a Variomag (Port Orange, FL, USA) shaking table (150 rpm). Monitoring of microbial population was started after 30 min of homogenization and lasted for 5 h. The automated flow cytometry system was set to dilute the samples 100-fold before staining. Two flasks were monitored in parallel.

Lactic bacteria involved in malolactic fermentation (*Oenococcus oeni*, "Maxiflore Elite" from IOC) were used. 1 g of dried active bacteria was rehydrated in 100 mL of an activation solution (based on inactivated yeasts), at 20 °C. The bacterial suspension was placed on a Variomag shaking table (150 rpm). Monitoring of microbial population was started after 30 min of homogenization and lasted for 25 h. The automated flow cytometry system was set to dilute the samples 100-fold before staining. Three biological replicates were monitored in parallel.

2.2. Yeast starter culture for "prise de mousse"

Dried active yeasts *Saccharomyces cerevisiae* 18-2007 from IOC were investigated. 1 g of dried yeasts was first rehydrated in 100 mL of physiological water at 37 °C. The yeast suspension was placed on a Variomag shaking table (150 rpm) for 15 min.

Then, the first step of preparation of the yeast starter culture was an adaptation of the yeast to a "wine environment", in a medium containing high sugar concentration and alcohol. The yeast suspension was mixed with 80 mL of sucrose syrup (600 $g \cdot L^{-1}$) and 100 mL of wine, resulting in a final volume of 280 mL. This wine was obtained from the alcoholic fermentation of a white grape juice (standardized by sucrose up to 200 $g \cdot L^{-1}$ of total sugars) by the same yeast strain. A nitrogen source (ammonium sulfate) was added at 100 $mg \cdot L^{-1}$. Media components were purchased from Sigma™ (Merck KGaA, Darmstadt, Germany). The mix was kept homogenized at 20 °C in a sterile 500 mL Erlenmeyer flask and monitored until the density dropped to 1.030 (measurement with an DMA 35 density meter (Anton Paar GmbH, Graz, Austria)). The automated flow cytometry system was set to dilute the samples 100-fold before staining. Three biological replicates were monitored in parallel.

Subsequently, the second step of preparation of the yeast starter culture was made in a medium allowing adaptation and growth. A 56 mL-aliquot of the previous culture (adaptation medium) was used to inoculate a wine medium composed of 624 mL of wine, 160 mL of water, and 160 mL of sucrose syrup, resulting in a final volume of 1 L. A nitrogen source (ammonium sulfate) was added

at 150 mg·L^{-1}. This medium was kept homogenized at 20 °C in a sterile 1 L Erlenmeyer flask and monitored until the density dropped and stabilized. The automated flow cytometry system was set to dilute the samples 10-fold initially, then set to dilute the sample 100-fold after 49 h, when the events rate was reaching values too high for reliable measurement by the cytometer (> 1000 events·µL^{-1}). Three biological replicates were monitored in parallel.

2.3. Mixed culture of two yeasts during alcoholic fermentation

Two yeast species, well-known for their importance in must fermentation, were investigated in this study: *Saccharomyces cerevisiae* 59A HO::eGFP (*Sc*), a haploid derivative of the commercial wine strain EC1118 (Lallemand Inc., Montréal, QC, Canada) and *Lachancea thermotolerans* BBMCZ7FA20 (*Lt*) (previously isolated and identified by Sadoudi *et al.* (Sadoudi *et al.*, 2012)). The eGFP mutation in *S. cerevisiae* confers a green fluorescence to this strain and allows the differentiation between the two yeast species by flow cytometry. Yeasts were preserved in YPD/glycerol (50/50 w/w) at -80 °C in the lab collection.

Each strain was first grown at 28 °C on YPD agar (glucose 20 g·L^{-1}, peptone 10 g·L^{-1}, yeast extract 5 g·L^{-1}, agar 18 g·L^{-1}). Then a preculture was prepared in MS300 liquid medium at 28 °C and agitated at 150 rpm. This synthetic must was first developed by Bely *et al.* (Bely *et al.*, 1990) and is commonly used in oenological research (Alonso-del-Real *et al.*, 2019; Taillandier *et al.*, 2014; Vendramini *et al.*, 2017; Zupan *et al.*, 2013). It contains carbon sources (glucose, fructose, organic acids), nitrogen sources (mineral and organic, 300 mg·L^{-1}), minerals, vitamins, and other growth factors. Media components were purchased from Sigma^{TM}. The yeasts were collected at the end of the exponential phase to inoculate the culture media.

Sterile glass flasks (Schott AG, Mainz, Germany) (GL45 screw thread, effective volume 1 L) were filled with 1 L of synthetic must. Media were inoculated with *L. thermotolerans* and with *S. cerevisiae* at 10^6 cells·mL^{-1} each. Cultures were carried out in these non-hermetically closed Schott flasks, kept homogenized and at a temperature of 20 °C. The automated flow cytometry system was set to first dilute the samples 10-fold, and then to automatically increase the dilution when the measured event concentrations reached 1000 events·µL^{-1}, to ensure reliable measurement by the cytometer. Three biological replicates were monitored in parallel.

2.4. Wine during aging

Monitoring of microorganisms was performed for 13 days on the natural flora of 200 mL of red wine from a bottle produced by University of Burgundy, kept homogenized at 20 °C. Only one 500 mL

flask was monitored and kept open to encourage natural inoculation and simulate contamination. The samples were not diluted before staining.

Results

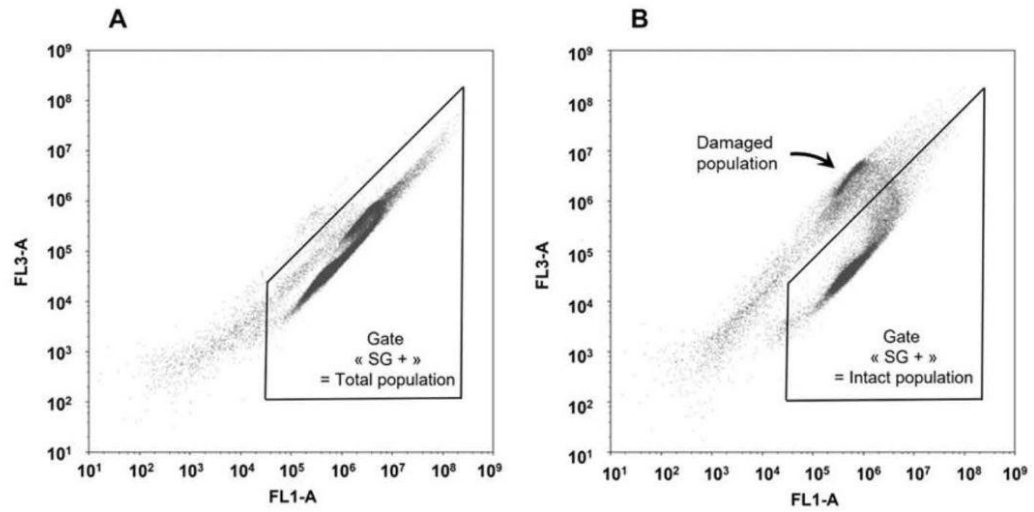
The aim of this work was to study the potential of automated flow cytometry for microbial monitoring in wine production. The first step was to validate the chosen automation and detection system for both bacteria and yeast enumeration, differentiation, and viability characterisation (section 1). In a second step, the system was used for monitoring near-real environments representing various applications in wine production: preparation of a yeast inoculum for sparkling wine (section 2), monitoring of a mixed culture alcoholic fermentation (section 3), and tracking microbial populations during wine aging (section 4).

1. Tuning and validation of methods for automated monitoring of wine microorganisms

The first step was to validate the feasibility of automatically monitoring microorganisms important for wine production: the yeast *Saccharomyces cerevisiae*, the main microorganism involved in alcoholic fermentation, and the bacterium *Oenococcus oeni* involved in malolactic fermentation.

Preliminary experiments (data not shown) allowed the optimization of the following parameters for the automated analysis by flow cytometry: (1) sampling volume, (2) sampling frequency, (3) sample preparation (dilution, staining, mixing, incubation). It is also necessary to validate the method of data analysis. To this end, a simple and short analysis of post-rehydration suspensions of microorganisms in physiological water was conducted.

First of all, the feasibility of detecting and enumerating the total and intact population of *Saccharomyces cerevisiae* by the automated system was investigated.



211

212 **Figure 1. Exemplary density plots (t = 3h45) obtained with automated flow cytometry during**
213 **monitoring of rehydrated yeast (*Saccharomyces cerevisiae*) after staining with only SG (A) and**
214 **both SG and PI (B).**

215 Each dot represents the fluorescence intensity of a given particle in the green fluorescent range (x-
216 axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The gate "SG
217 +" delineates the microbial population (yeast cells in this experiment) labeled by SG. This
218 corresponds to the total population for SG staining only and to the intact population for SG-PI
219 staining.

220 The density plots presented in Figure 1 show the signals corresponding to green and red fluorescence
221 (axis FL1-A and FL3-A respectively) for each event. Gate "SG +" was chosen to select the yeast
222 population labeled by SG. When the sample is stained with SG only (Figure 1A), the gate "SG +"
223 corresponds to the total population. When a double staining is made (SG and PI, Figure 1B), the gate
224 "SG +" corresponds to the intact population. Damaged cells increase in red fluorescence and decrease
225 in green fluorescence due to binding by PI, leading to a shift out of the gate. Some of these cells are
226 visible upwards and to the left outside of the gate in Figure 1B.

227 The density plots in Figure 1 show clear separation between stained cells and background for SG-
228 staining (Figure 1A) and between intact cells and damaged cells for SG-PI-staining (Figure 1B). Only
229 marginal numbers of events were located at the border of the gates for quantification of cells. Such
230 clear separation is needed to ensure the reliability and representativeness of the calculated cell
231 concentrations. This was observed for all measurements over the course of the experiment and
232 allowed for the monitoring of total and intact yeast population depicted in Figure 2.

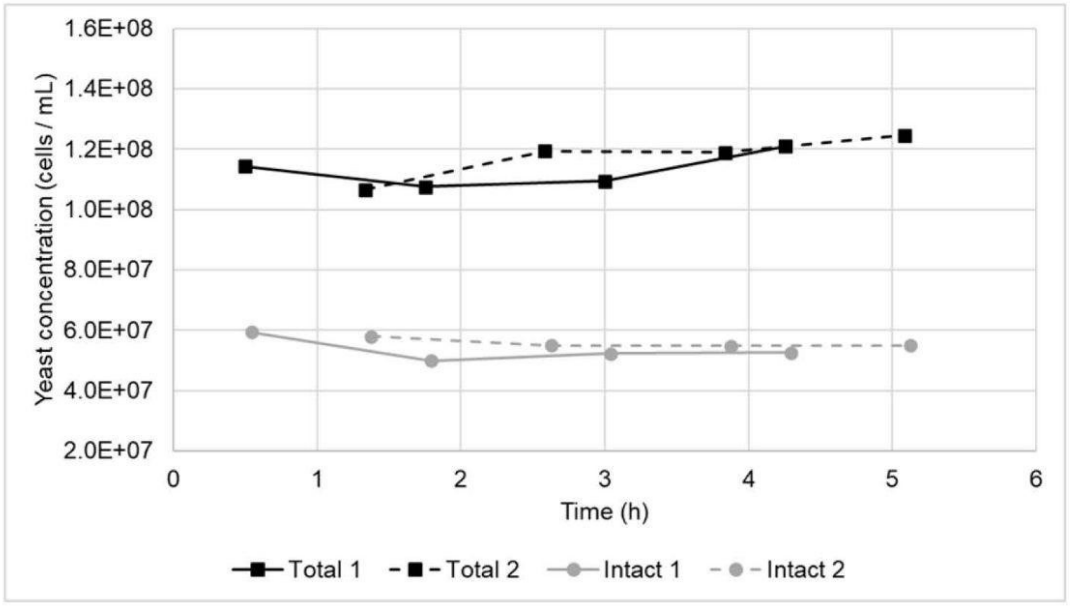
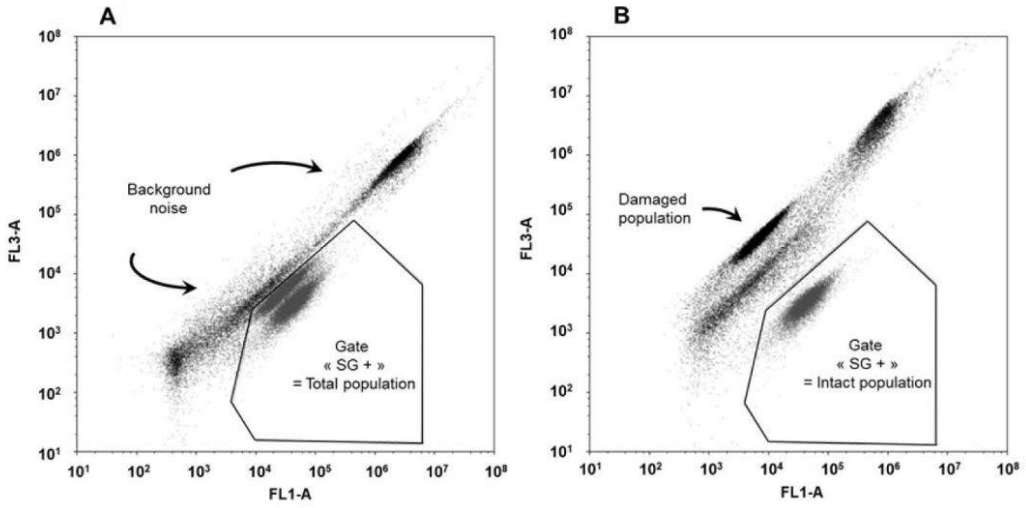


Figure 2. Total and intact yeast concentration of a fermentation starter (*Saccharomyces cerevisiae*), monitored by automated flow cytometry for 5 h

Black squared and grey round dots represent respectively total and intact yeast concentration. Continuous and dashed lines correspond to two biological replicates. For each replicate, a measurement was taken every 75 min. t = 0 h equals to the start of rehydration.

The yeast concentrations in Figure 2 show that the automated flow cytometry system produces stable and repeatable results for both total and intact cell concentration (1.1 x 10^8 and 5.5 x 10^7 cell·mL^{-1}), over the course of 5 h. The variation coefficient between all collected samples is 5.3 % and 8.0 % for intact and total population respectively. Even assuming the biological population is constant over the course of the experiment and thus inputting this variation solely to the machine, these values are acceptable and validate the system reliability. The fraction of the intact population in the total population is about 55 % after rehydration, which is comparable to values obtained previously in similar conditions with non-automated flow cytometry (Attfield *et al.*, 2000).

The same protocol was applied to a bacterial population, as in winemaking, a bacterial inoculation is often made to control the starting and the reproducibility of malolactic fermentation. The population of *Oenococcus oeni* was monitored with the automated flow cytometry system.



250

251 **Figure 3. Exemplary density plots (t = 3h00) obtained with automated flow cytometry during**
252 **monitoring of rehydrated bacteria (*Oenococcus oeni*) after staining with only SG (A) and both**
253 **SG and PI (B).**

254 Each dot represents the fluorescence intensity of a given particle in the green fluorescent range (x-
255 axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The gate "SG
256 +" delineates the microbial population (bacterial cells in this experiment) labeled by SG. This
257 corresponds to the total population for SG staining only and to the intact population for SG-PI
258 staining.

259 As presented in the cytogram in Figure 3, a large amount of background noise (event clouds outside
260 the "SG +" gate in Figure 3A) was observed in this case. Based on blank measurements of the
261 rehydration medium, it was possible to show that these background signals were due to the activation
262 solution (containing inactivated yeasts) whose presence in the rehydration medium is necessary for
263 proper rehydration of *Oenococcus oeni*. The gate "SG +" accounts for the total bacteria population
264 when they are stained with only SG (Figure 3A) and for the intact population when a double staining
265 SG - PI is used (Figure 3B). As for the monitoring of yeasts (see Figure 1), some of the damaged cells
266 that take up PI and thus increase in red fluorescence and decrease in green fluorescence can be seen
267 upwards and to the left outside of the gate.

268 A clear separation was visible between stained cells and background for SG-staining (Figure 3A) and
269 between intact cells, damaged cells, and background for SG-PI-staining (Figure 3B). Only marginal
270 numbers of events were located at the border of the gates for quantification of cells. This clear
271 separation is particularly needed in samples containing higher concentrations of background particles.

This was observed for all measurements over the course of the experiment and allowed for the monitoring of total and intact bacterial population depicted in Figure 4.

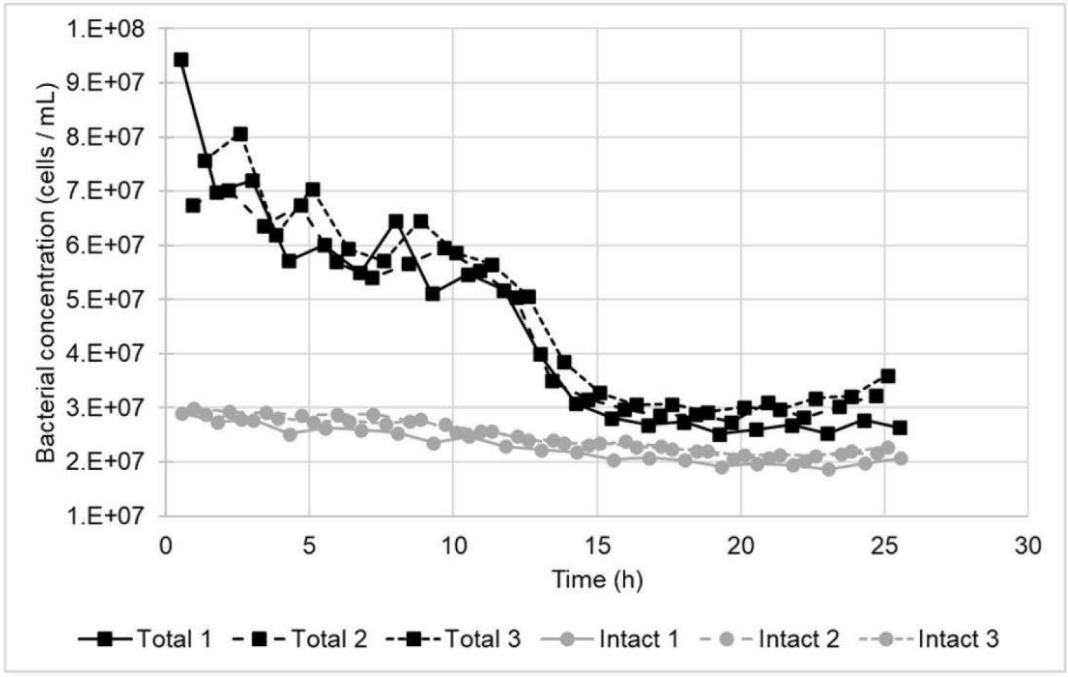


Figure 4. Total and intact bacterial concentration of a fermentation starter (*Oenococcus oeni*), monitored by automated flow cytometry for 25 h.

Black squared and grey round dots represent respectively total and intact yeast concentration. Continuous and dashed lines correspond to three different biological replicates. For each replicate, a measurement was taken every 85 min. t = 0 h equals to the start of rehydration.

As can be seen from Figure 4, the automated system monitored the total bacterial population during the experiment time (25 hours). There was little dispersion between the results of the three biological replicates: the average variation coefficient between replicates was 4.8 % and 7.3 % for the intact and total populations respectively. The fraction of the intact population in the total population is about 40 %, a common viability value for *Oenococcus oeni* after rehydration (Zhao and Zhang, 2005). The total bacterial concentration, initially close to 8×10^7 cells·mL^{-1}, decreased during the experiment and stabilized after 15 hours at about 3×10^7 cells·mL^{-1}. The intact bacterial concentration was more stable, between 2×10^7 and 3×10^7 cells·mL^{-1}.

2. Microbial monitoring during starter culture preparation for "prise de mousse"

After having shown that the automated flow cytometry system can monitor a yeast population in a simple medium (physiological water), the next step was to apply this system in a real application: the preparation of a yeast inoculum for the stage "prise de mousse" of sparkling wine. This preparation is a two-step protocol with first an adaptation phase of the yeasts inoculated in a medium containing sugar and alcohol and then a transfer of these yeasts to a second adaptation medium that is sugar- and alcohol-rich, where cells will start to grow.

During the adaptation step, basic yeast monitoring with the automated flow cytometry was achieved over several days with minimal growth, in order to validate the reliability of the system over longer periods of time. It also constitutes a first test of sampling a more complex medium than physiological water (high sugar content, wine, production of CO_2).

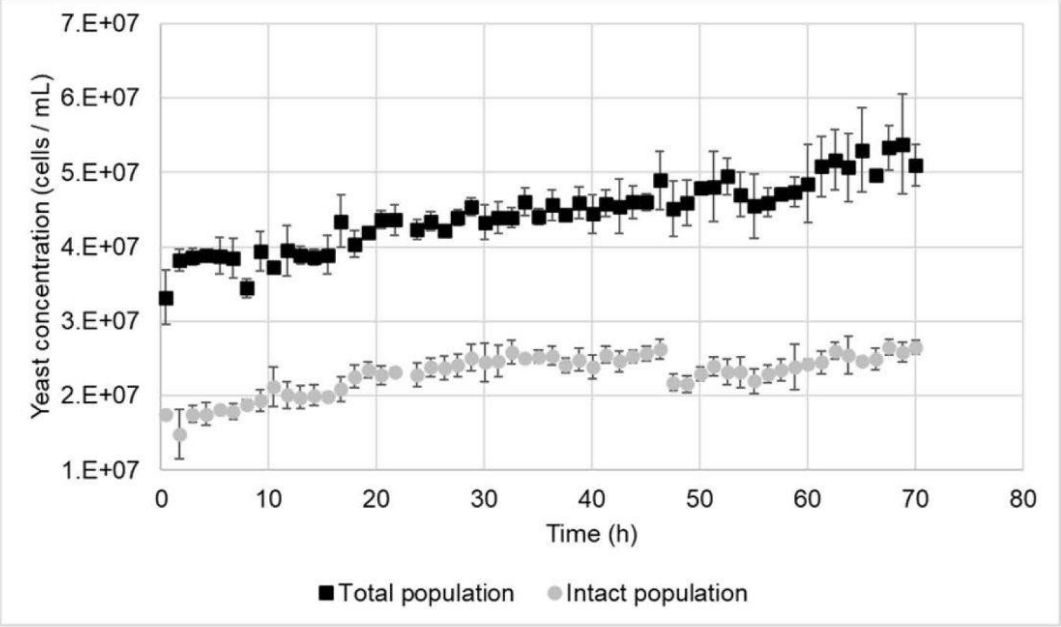


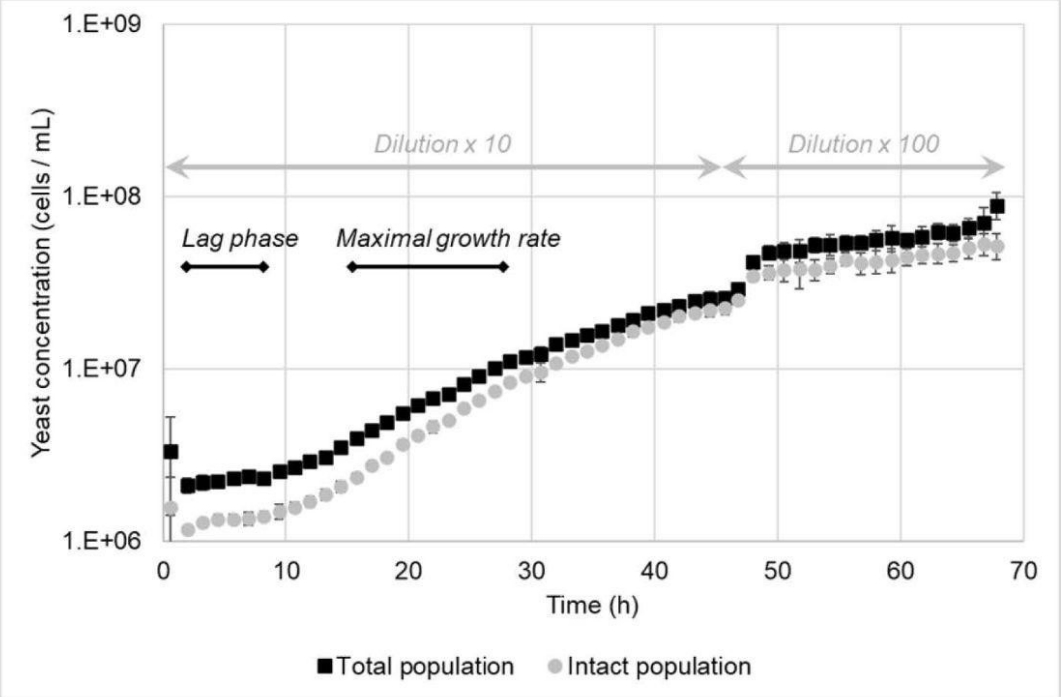
Figure 5. Total and intact yeast concentration (*Saccharomyces cerevisiae*) during adaptation phase, monitored by automated flow cytometry.

Black squared and grey round dots represent respectively total and intact yeast concentration. Each dot is the mean of 3 biological replicates. For each replicate, a measurement was taken every 85 min.

At the beginning of the adaptation step, the initial yeast concentration measured by the automated system was about 4×10^7 cells·mL^{-1} (Figure 5). This was in line with the concentration of the dehydrated yeast and the dilutions used for preparation. During the adaptation phase, the total and intact population increased slightly. There was little variability between the three biological replicates

309 (average variation coefficient between replicates of 5.1 % and 6.1 % for the total and intact
310 populations respectively), thus confirming the three sampling lines results did not drift apart during
311 the experimental process due to potential technical issues such as fouling by yeast accumulation,
312 tubing deformation, etc. This experiment lasted about 3 days without outputting outlier values,
313 indicating that the clean-in-place system and protocol are efficient.

314 These results showed that the studied system is able to automatically monitor total and intact yeast
315 populations in a complex medium containing sugar and alcohol, over several days without drift. The
316 system could thus be used for the following experiments, which involved monitoring during even
317 longer durations, and, as yeast growth (albeit quite slow) occurred, aimed to establish if a mid-
318 experiment change in dilution level could significantly impact results.



319

320 **Figure 6. Total and intact yeast concentration (*Saccharomyces cerevisiae*) during the growth**
321 **step in sugar- and ethanol-rich medium, monitored by automated flow cytometry.**

322 Black squared and grey round dots represent respectively total and intact yeast concentration. Each
323 dot is the mean of 3 biological replicates. For each replicate, a measurement was taken every 85 min.

324 At the beginning of this growth step, the total yeast population measured by the automated flow
325 cytometry system was 2.2 x 10^6 cells·mL^{-1} as expected taking into account the dilutions used for
326 preparation. Very low variability was observed between the three biological repetitions (average
327 variation coefficient between replicates of 7.7 % and 8.0 % for the total and intact populations

respectively), but the very first data point shows instability (its variation coefficients being above 50 %) and can be considered an outlier. The system was able to monitor the growth of total and intact population for the whole growth step (3 days), until a total population of about 9 x 10^7 cells·mL^{-1} was reached. The high-frequency monitoring allowed for precise and accurate determination of the different phases of growth. After a lag phase of about 8 hours at about 1.3 x 10^6 cells·mL^{-1}, the intact population increased with a maximal growth rate μ_{max} of 0.10 h^{-1} (see additional data, figure S3), and seemed to be reaching the stationary phase at the end of the experiment.

After 47 h, the automated dilution of the sample was switched from 10-fold to 100-fold without stopping the monitoring. This caused a jump in concentration calculated from the actual measurements and the nominal dilution factor. This suggests that the two dilution regimes do not work equally well.

3. Monitoring of two different yeast populations during a mixed culture alcoholic fermentation

This experiment had two objectives. First, it aimed to determine if the system allowed for complete and proper monitoring of one of the most studied process steps in wine research: alcoholic fermentation.

Second, as mixed culture fermentations constitute a highly studied subject in today's literature, it was also interesting to see if the system allowed for proper differentiation of two different yeast species over time, as increasing acquisition frequency is both very valuable and quite challenging for studies on this subject.

Thus, the automated flow cytometry system was used for monitoring a mixed culture alcoholic fermentation involving two yeasts: a mutant GFP+ of *Saccharomyces cerevisiae* and *Lachancea thermotolerans*.

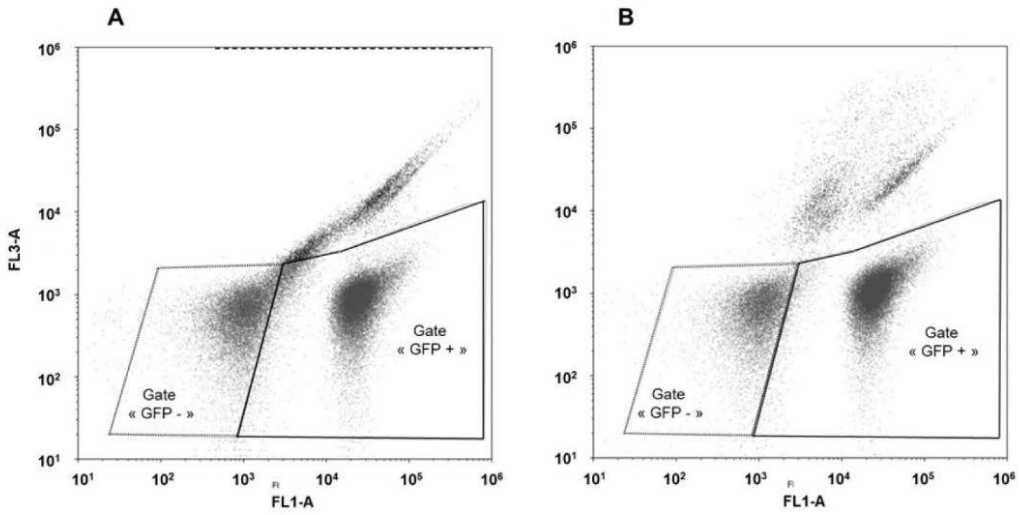


Figure 7. Exemplary density plots (t = 22h00) obtained with automated flow cytometry during monitoring of a mixed culture alcoholic fermentation involving *Saccharomyces cerevisiae* and *Lachancea thermotolerans* without staining SG (A) and after staining with PI (B).

Each dot represents the fluorescence intensity of a given particle in the green fluorescent range (x-axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The gates "GFP +" and "GFP -" delineate the yeast population which emits respectively more green fluorescence because of the presence of the GFP protein (*S. cerevisiae*) or no specific fluorescence (*L. thermotolerans*). Some of the damaged cells that take up PI and thus increase in red fluorescence and decrease in green fluorescence can be seen upwards and to the left outside of the gate.

As presented in the density plots (Figure 7), the PI staining allowed to sort out "damaged" yeasts via the FL3 channel. Amongst intact cells, the gates "GFP -" and "GFP +" were used to estimate respectively *L. thermotolerans* and *S. cerevisiae* population.

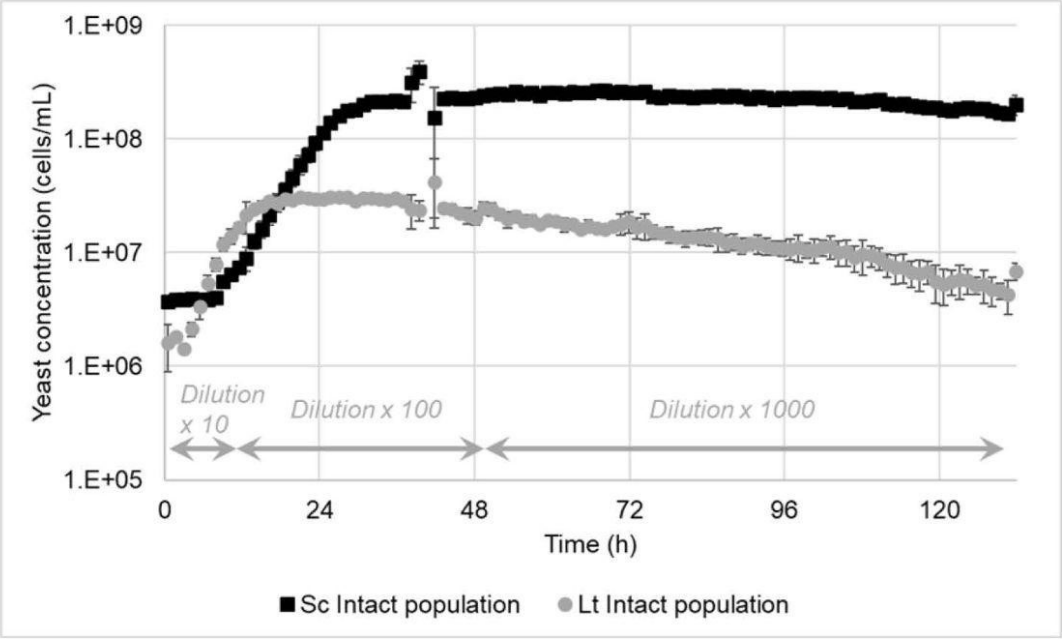


Figure 8. Intact population of *Saccharomyces cerevisiae* and of *Lachancea thermotolerans* monitored by automated flow cytometry during mixed fermentation.

Black squared and grey round dots represent respectively total and intact yeast concentration. Each dot is the mean of 3 biological replicates. For each replicate, a measurement was taken every 85 min.

These results showed that it is possible to monitor the population of two different yeasts in the same time all along the duration of the fermentation, by the automated flow cytometry. The system gave repeatable results for more than 5 days (average variation coefficient between replicates of 8.3 and 16.2 % for *Sc* and *Lt* respectively). This automated monitoring with such a frequency of sampling here also allowed for precise determination of the different phases of growth for both yeasts. For *Saccharomyces cerevisiae*, after a lag phase of about 8 hours at 4×10^6 cells·mL^{-1}, the intact population increased with a maximum growth rate (see additional data figure S4) μ_{max} of 0.22 h^{-1}, before reaching the stationary phase at about 32 h and stabilizing at 2×10^8 cells·mL^{-1}. The growth of *Lachancea thermotolerans* was faster at the beginning (lag phase of only 3 hours, μ_{max} of 0.35 h^{-1}) but reached a maximum of 3×10^7 cells·mL^{-1} more quickly (after 16 h) (most probably because of interactions due to *S. cerevisiae* development, as seen in previous works (Comitini *et al.*, 2011; Fairbairn *et al.*, 2021; Gobbi *et al.*, 2013; Kemsawasd *et al.*, 2015) and the population tended to decrease until the end of the experiment.

4. Microbial monitoring during wine aging

After having studied high-frequency and low duration monitorings, the goal of the final experiment was to explore system stability over long periods of time, especially with less frequent acquisitions. It also allowed for studying yet another matrix, red wine, and validated compatibility of this technology with this matrix.

Dot plots presented in Figure 9A show the signals obtained during the monitoring of microbial population in a sample of red wine. The gate "SG +" allowed to discriminate the microbial population from the expected background noise of the complex medium, red wine (autofluorescence of numerous compounds, cellular debris…).

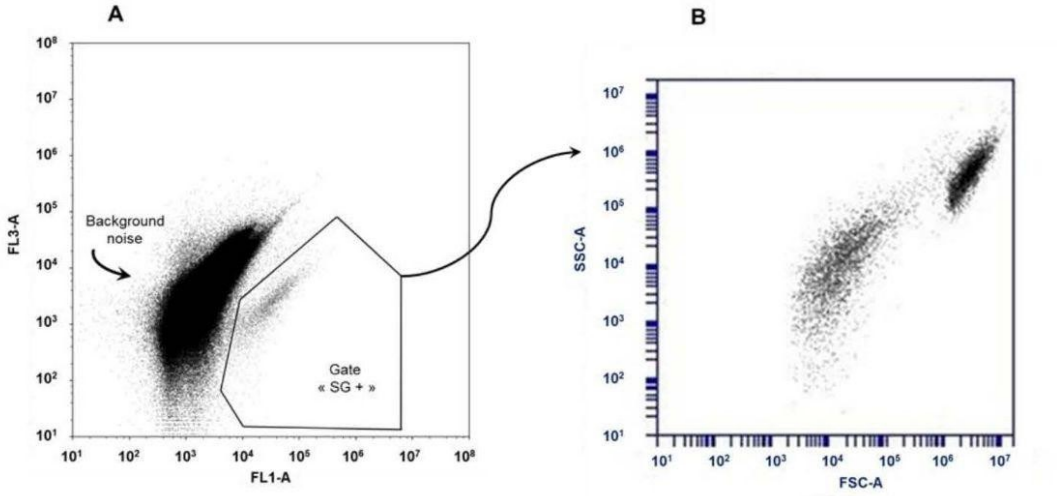


Figure 9. Exemplary density plots (t = 256 h) obtained with automated flow cytometry during monitoring of microbial population of red wine during aging after staining with SG (A) and size distribution of events from the gate "SG +".

In Figure 9A, each dot represents the fluorescence intensity of a given particle in the green fluorescent range (x-axis, FL1-A, logarithmic) and the red fluorescence range (y-axis, FL3-A, logarithmic). The gate "SG +" delineates the total microbial population labeled by SG. In Figure 9B, forward scatter is plotted on the x-axis and side scatter on the y-axis, both with logarithmic scales.

Microbial population presenting a green fluorescence in presence of SG consisted of two populations, small particles and bigger particles, which could be associated respectively with bacterial population and yeast population, given the FSC and SSC values of the corresponding events (Figure 9B).

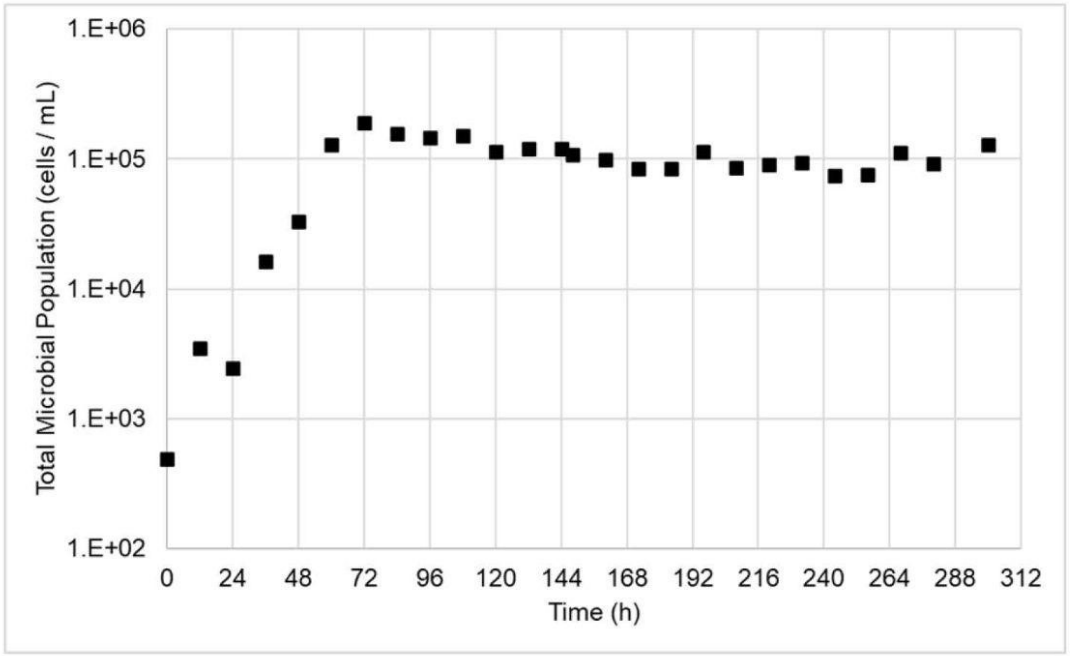


Figure 10. Total microbial population (yeast and bacteria) ("SG +" gate of Figure 9) during wine aging, monitored by automated flow cytometry.

A measurement was taken every 12 hours.

Total microbial population (Figure 10) increased during the first 72 hours and then remained stable during the following 10 days. Some instabilities in the estimated population were observed at the beginning of the experiment, which could be explained by the very low level of population, both absolute and relative to the background noise.

Discussion

The overarching goal of this study was to evaluate the potential and feasibility of automated flow cytometry applications towards wine-related environments, through a wide range of increasingly complex case studies, each aiming to explore different technical challenges. Overall, even if flow cytometry is nowadays widely used in wine-related scientific experiments (Bordet *et al.*, 2020; Guzzon and Larcher, 2015; Longin *et al.*, 2017) and even began to work its way into more and more cellars labs, automated flow cytometry has not yet seen any use in these conditions (Heins *et al.*, 2022). It indeed poses a few challenges, like high-sugar medium sampling, dissolved CO_2 management or high growth, but this research area is in need of facilitation for higher-frequency

microbial sampling and analysis, and around-the-clock measurements. Flow cytometry automation could constitute part of the answer to both problems.

First experiments (Results Section 1.) conducted in simple conditions (presence of one type of microorganism, physiological water matrix) allowed for validation of protocols for sampling, staining, and flow cytometry analysis. The data show that it is possible with the automated flow cytometry technology to follow a yeast population for several hours after rehydration, with a good repeatability. The double staining method can be used to quantify total and intact yeast concentrations. The experiment applied on bacteria leads to similar conclusions about method validation and also highlights a decrease of total population although the intact bacterial population remains constant. These results can be explained by a very long monitoring with respect to the usual rehydration duration. Rapid consumption of the growth factor and subsequent nutrient scarcity leads to a decrease of total bacterial concentration, and a resulting possible autolysis phenomenon (already observed in *O. oeni* (Crouigneau *et al.*, 2000) can explain the meanwhile relatively stable intact population. This hypothesis is further confirmed by comparing the density plot of Figure 3 with one of the density plots generated at the end of the experiment (see supplementary data, Figure S2), and observing the disappearance of the "damaged bacteria" event cloud. So, validation of the chosen automated flow cytometry method through analysis of both yeasts and bacteria in simple media, as it had been done in other works (Besmer *et al.*, 2014), proved the studied system was capable of accurately evaluating microbial populations in "ideal" conditions. Such results were essential, as it paved the way for further works presented in this paper to be put in place.

The second part of the study (Results Section 2.) explored the possibility of monitoring microbial populations over multiple days, on a more complex medium (water-sugar-wine mix). The results here were of significant importance for multiple reasons.

First, they validated the possibility for the system to monitor microbial cultures, both total and intact populations overnight during 72 hours, without drift, showing the efficiency of the clean-in-place protocol.

Second, the results showed that the studied system is able to automatically monitor yeast populations in a complex medium (containing high concentrations of sugars, ethanol, with presence of gas bubbles…). This represents a quite significant novelty as it is the first time automated flow cytometry has been applied with success to wine must under fermentation, which is a medium with high sugar

content, and consequently a higher viscosity than the minimal media used in previous experiments involving microbial growth (Baert *et al.*, 2015; Freitas *et al.*, 2013; Kacmar *et al.*, 2004).

In addition, these experiments validated the possibility to monitor exponential growth by automatic adaptation of the dilution protocol to avoid saturation of the cytometer. However, the automated switch of dilution (from 10-fold to 100-fold) without stopping the monitoring, caused a jump in calculated concentration. This could be explained by an underestimation of the population before this higher sample dilution, by overcrowding of the cytometer's sensors or a poor separation of microorganisms (leading to yeast duets and triplets being measured as a single event). Possibly, the higher dilution leads to a higher yeast population because the ratio of cells to background in the SG gate is higher, hence with a multiplication of 100 instead of 10 leading to a stronger effect in the back-calculated concentration. Moreover, in terms of applications, these results highlight the possibility of using such a system for short-term high-frequency monitoring of yeast population during a levain preparation, especially for "Prise de Mousse". This could help the execution of future experiments, as such frequent microbial monitoring is very labor intensive and time consuming (even with flow cytometry), thus not always easy to put in place. For this reason, existing studies often favor lower analysis frequency (Benucci *et al.*, 2016; Kemp *et al.*, 2020), and similar future ones could benefit from easier access to higher sampling frequencies.

The third part of the study (Results Section 3.) built upon the aforementioned growth-monitoring capabilities, and allowed for longer monitoring, with higher growth and population levels than the previous one on levain preparation. The instability of results observed at about 40 h is quickly self-resolved, and could come from multiple external causes to the analysis system, from a manipulation error when refilling the reagents, to a bubble making its way into the sampling system, or maybe a punctual agitation problem. The automated system is then able to analyze yeast population with variations of 2 log in the population. This was possible thanks to an on-line modification of sample dilution settings during the experiment that did not lead to significant perturbations.

In addition, this experiment evaluated the capacity for alcoholic fermentation monitoring, which had not been explored yet through the prism of automated flow cytometry (especially involving a mixed culture). Such a monitoring constitutes indeed a technical challenge: rapid microbial growth during the first few days of fermentation, then high stability, CO_2 production, transition from a high-sugar to a low-sugar medium, high ethanol media, long duration (especially at 20 °C). This experiment highlights the possibility for this system to monitor total and intact yeast populations over the course

484 of the alcoholic fermentation process, two key variables for properly managing this crucial step of
485 winemaking.

486 Furthermore, proper differentiation of two different yeast species over time was obtained. This
487 possibility to follow several microbial populations at the same time all along their growth and
488 subsequent stationary phase is also of great importance to monitor mixed cultures, which are more
489 and more studied nowadays in wine fields to improve organoleptic characteristics of wine. Increasing
490 acquisition frequency is both very valuable and quite challenging for studies on this subject.

491 The lack of any significant technical issue during this experiment and the overall precision of the
492 results shows that the system could very well be used for experiments involving alcoholic
493 fermentations in the future. Indeed they could, like levain monitorings mentioned above, also benefit
494 from higher frequency microbial samplings, especially during the exponential growth phase where
495 the μ_{max} is often evaluated. Flow cytometry use is already quite developed for such experiments
496 (Bordet *et al.*, 2020; Pérez-Torrado *et al.*, 2017), thus only minimal adjustments to existing methods
497 would be required.

498 However, a limit to this perspective would be the permanent agitation required for such a monitoring,
499 getting in the way of the usual static conditions and sedimentation during the later phases of
500 fermentation, and then modifying the very biological system meant to be observed (Varela *et al.*,
501 2021). In this regard, automated flow cytometry is indeed no different from standard high-frequency
502 manual sampling, which suffers from the same dilemma. Possible ways to indirectly deal with this
503 would be either to accept the possible differences and check for them through a static control, to lower
504 the acquisition frequency and link the agitation to the sampling system to simulate lower frequency
505 manual samplings, or to keep the static conditions and only sample the supernatant (Veloso *et al.*,
506 2020).

507

508 The fourth and final part of the study (Results Section 4.) focused on wine aging and tested radically
509 different experimental conditions from the other ones: low population dynamics, longer time gaps
510 between samples, and very long monitoring duration (as real applications could last up to several
511 months). Automated flow cytometry system allowed for proper monitoring of the global microbial
512 population in a red wine for a long time (13 days): no outlier results were observed indicating that
513 sampling, analysis and cleaning protocol seem to be efficient even during several weeks and at
514 considerably lower sampling frequency. These results indicate that such a system, with proper setup
515 and method development, could be used to follow and study wine as a microbial system all along the

aging proces. It would be especially interesting for monitoring abnormal microbial development, or even specific alteration floras if identifiable by flow cytometry, such as *Brettanomyces bruxellensis* with RNA-FISH probes, for example (Branco *et al.*, 2020). The potential differentiation between bacteria and yeast thanks to their size differences could also help detect a premature start of the malolactic fermentation, or, on the other hand, ensure it takes place properly when needed.

However, the experiment as it stands suffers from two small shortcomings. The first one is the very high background noise, which was expected given the analyzed sample was unfiltered and barely diluted red wine, containing debris and particles of the same size as microorganisms (Salma *et al.*, 2013). SYBR Green I staining helped alleviate this problem and identify microorganisms among the other particles, but saturation of the cytometer detectors and aggregation between microorganisms and particles is still to consider. Second, the experiment has only been run for 13 days, albeit a cellar wine monitoring would in theory usually need to run for multiple weeks, even months. In this interval, some grime build-up could occur over time even if it was not observed within the timeframe of this study, and the system could then need some supplementary maintenance outside of the clean-in-place protocol used here.

Conclusions

These works show the technical potential of the use of automated flow cytometry in the wine field. This system allows for monitoring of bacteria and yeasts involved in wine production, in a complex sugar- and ethanol-rich medium, for a long time (up to 2 weeks). A variety of applications, common in this field, were tested. Results show the applicability of the studied automated system for both high-frequency and around-the-clock samplings. Even if some adjustments and method refinings remain to be done, these results hint at a high diversity of potential applications: monitoring microbial starter pre-adaptation, alcoholic and malolactic fermentations, wine aging… Such uses in both lab and cellar conditions could also be coupled with other existing on-line analysis techniques, such as FTIR for multiple physico-chemical characteristics of the wine (Veale *et al.*, 2007; Veloso *et al.*, 2020), or thermal conductivity for carbon dioxide monitoring (Descoins *et al.*, 2006), thus fully automatizing most of the tedious parts of the monitoring process. Data analysis automation such as automatic gating could also be an interesting addition to this technique, as mentioned by Heins *et al.* in their recent review (Heins *et al.*, 2022). Overall, this study constitutes a first step towards

automation of flow cytometry analysis in both wine research and industry, which could in a near future constitute the next tool of interest for both microbiology studies and on-line quality control.

Acknowledgements

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Supplementary Data

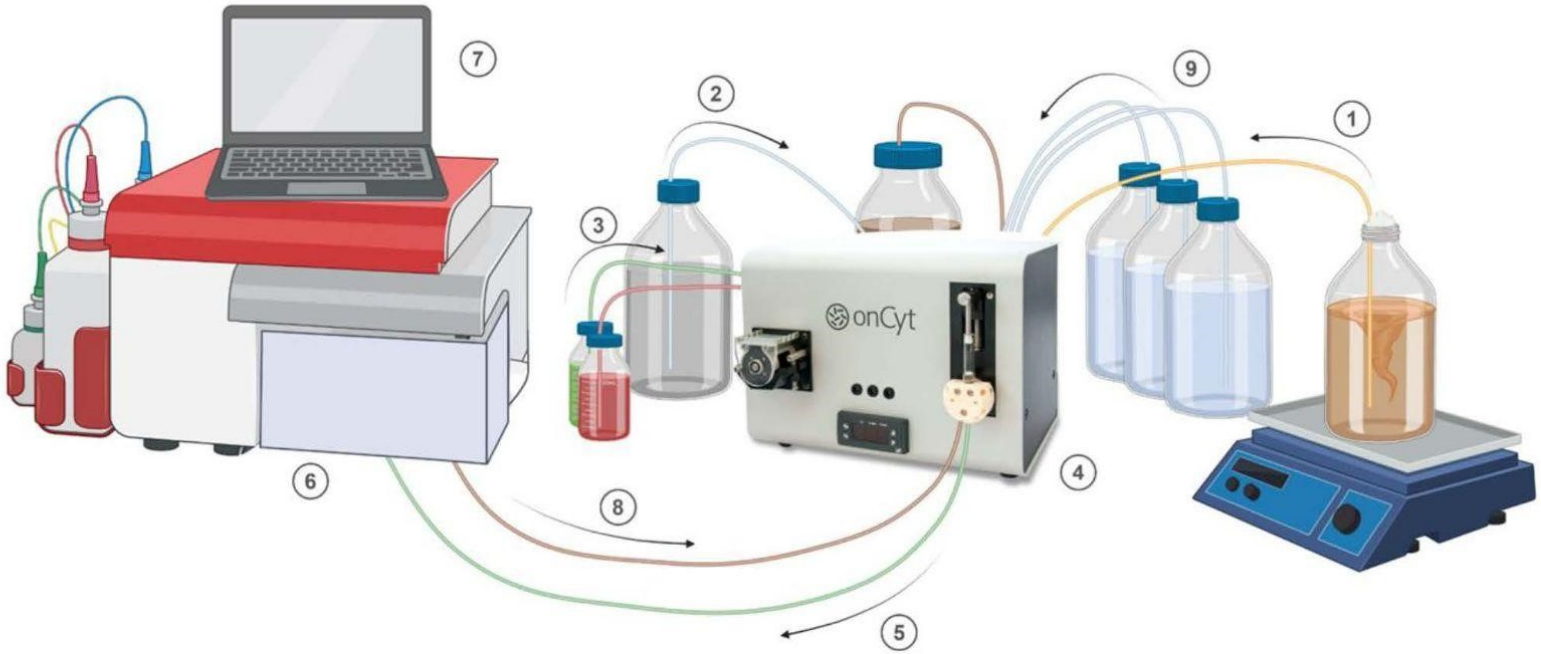

Figure S1. Detailed process of the automated flow cytometry system.

Sampling (1), dilution (2), staining (3), incubation (4), transfer to the acquisition chamber (5), acquisition by the cytometer (6), results processing (7), waste disposal (8), cleaning cycle (9). This diagram was created with BioRender.com.

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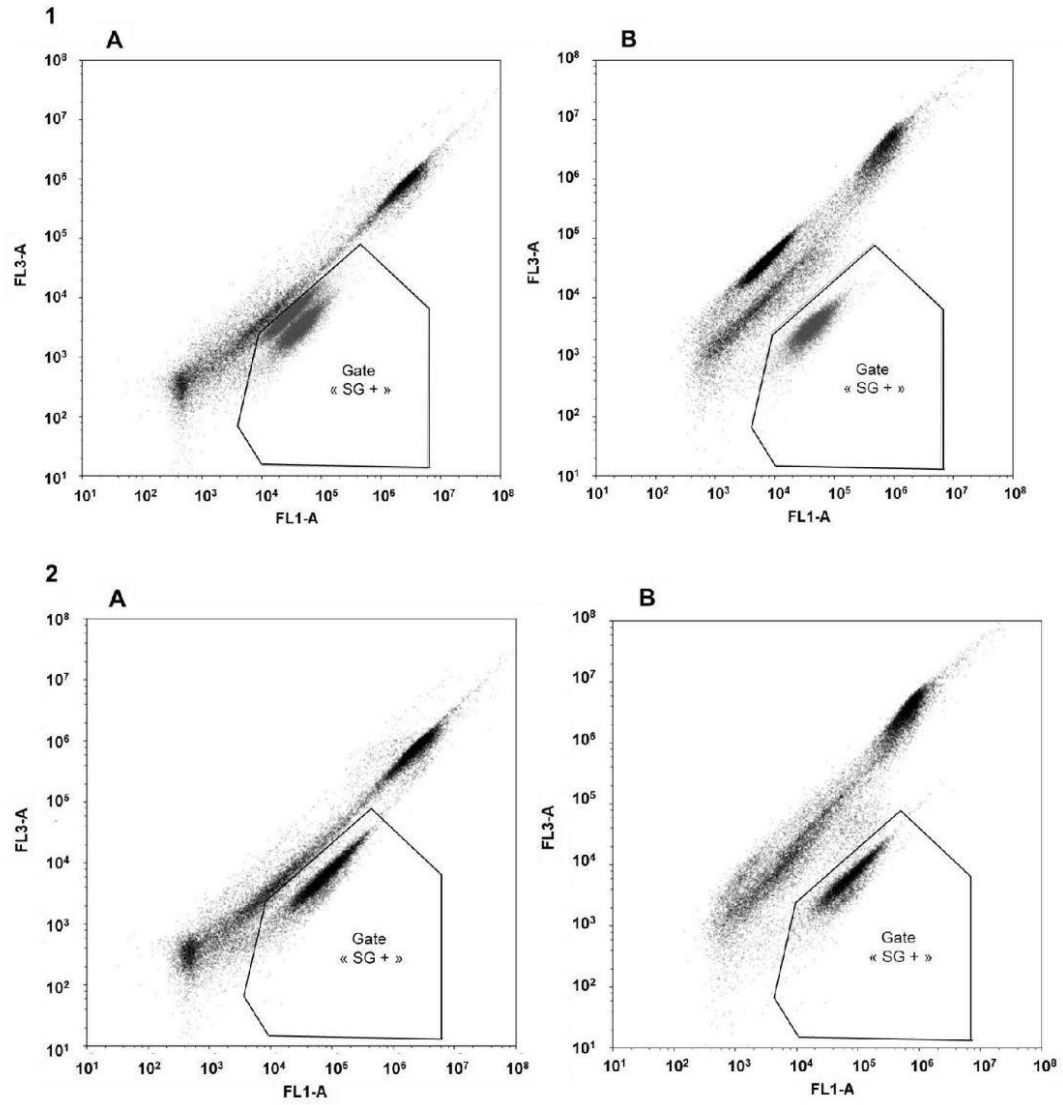

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692

693 **Figure S2. Density plots obtained with automated flow cytometry during monitoring of**
694 **rehydrated bacteria, at the beginning (1) and at the end (2) of the experiment, and after staining**
695 **with only SG (A) or both SG and PI (B).**

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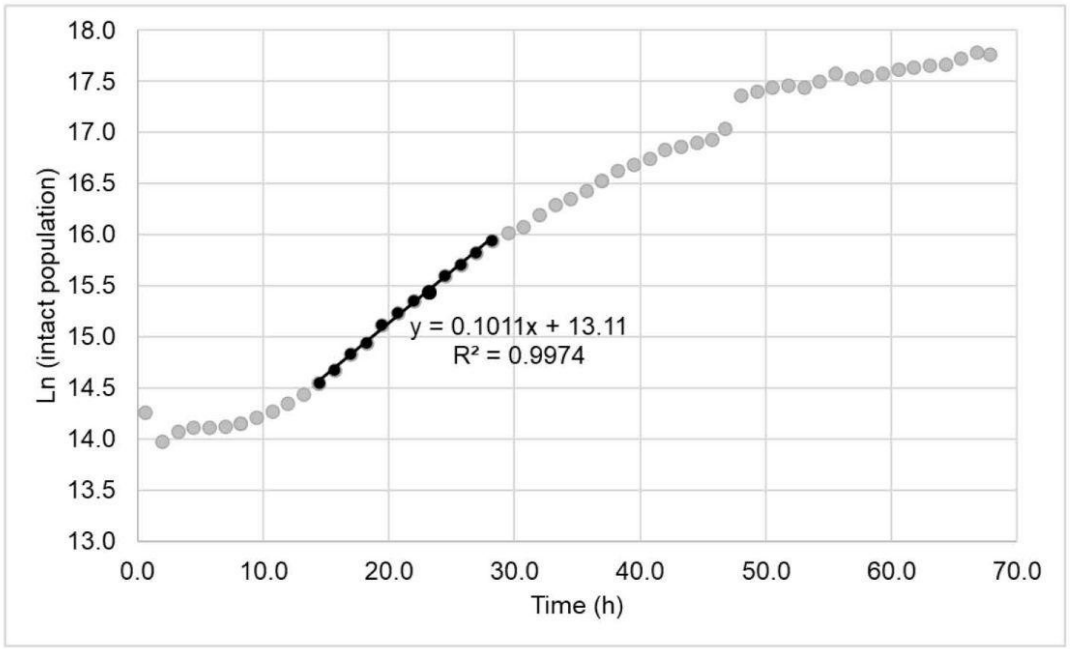
697



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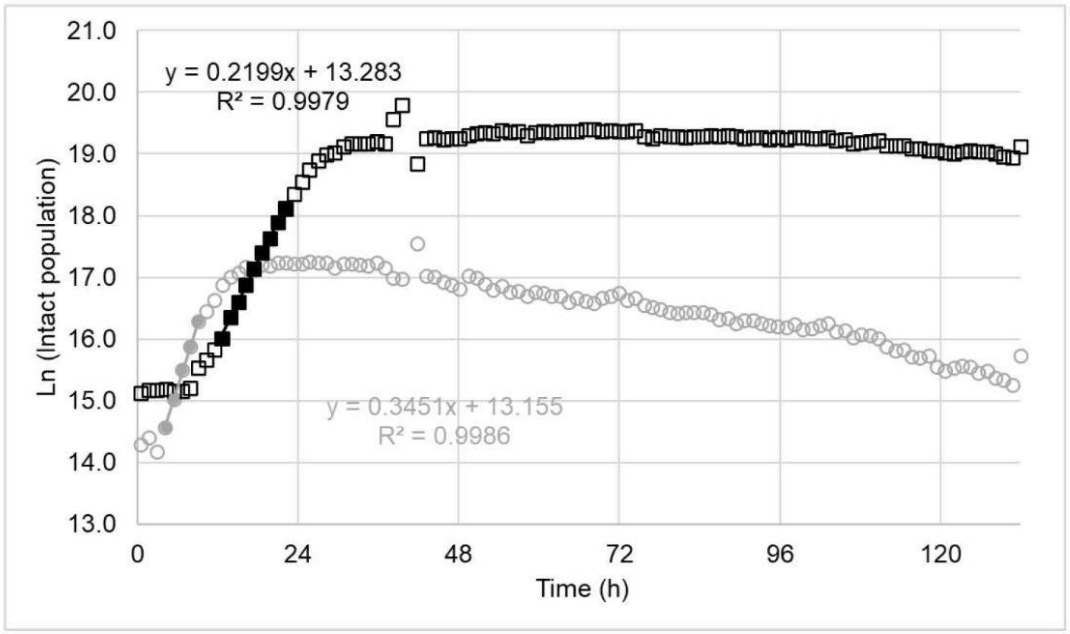
699

700 **Figure S3. Determination of maximal growth rate of intact yeast population (*Saccharomyces*
701 *cerevisiae*), during the multiplication phase in sugar- and ethanol-rich medium, monitored by**
702 **automated flow cytometry.**

703 Each dot is the mean of 3 biological replicates.

704

705



706

707 **Figure S4. Determination of maximal growth rate of intact population of *Saccharomyces***
708 ***cerevisiae* and of *Lachancea thermotolerans* during mixed fermentation, monitored by**
709 **automated flow cytometry.**

710 Each dot is the mean of 3 biological replicates.

711

712

3.3. Conclusions, perspectives

Les travaux ont permis de montrer la faisabilité d'un suivi automatisé par cytométrie en flux des populations microbiennes dans diverses conditions liées à l'élaboration du vin.
Cette technique montre un intérêt certain pour des activités de recherche, afin de mieux comprendre les interactions entre levures tout au long d'une fermentation mixte et potentiellement approfondir les travaux présentés dans la partie 2 de ce manuscrit de thèse (voir 2.4).
En outre, un tel système pourrait être applicable en cave, dans diverses situations œnologiques : contrôle des populations microbiennes lors de la préparation des pieds de cuve, de l'ensemencement des cuves, au cours des fermentations (alcoolique, malolactique en cuve, et fermentation en bouteille pour les vins effervescents) voire au cours du vieillissement du vin. Les attentes sont variées : s'assurer de la présence d'une concentration souhaitée de microorganismes, du développement après ensemencement, de l'absence de contaminations, ou bien suivre différentes populations simultanément afin de pouvoir à l'avenir les contrôler en agissant, en continu, sur les conditions de fermentation.
Cette facilitation des analyses microbiologiques présente également un intérêt évident pour les laboratoires œnologiques, qui pourraient améliorer ainsi leurs protocoles d'analyses pour les applications citées ci-dessus. L'intérêt est, pour ces laboratoires, encore plus marqué que pour les laboratoires de recherche, car les analyses réalisées y sont généralement plus standardisées et réalisées avec une fréquence et une régularité supérieure à celles des applications en recherche, aux protocoles plus variables. Ainsi, l'automatisation y est potentiellement à la fois plus facilement réalisable et davantage optimisable.
Des améliorations du système sont envisageables et des verrous techniques restent cependant à lever, notamment au niveau de la fiabilité des dilutions et de l'étude de systèmes produisant du CO_2 mais en conditions statiques, non testés dans cette étude. Cependant, le potentiel d'une telle automatisation reste clairement visible à travers ces travaux, que ce soit pour la recherche, le contrôle qualité, ou encore d'éventuelles applications industrielles.

4. Conclusion générale et perspectives

Ces travaux de thèse ont permis d'approfondir les connaissances existantes sur le déroulé des fermentations alcooliques en culture mixte entre *Saccharomyces cerevisiae* et *Lachancea thermotolerans.* L'étude multiparamétrique a permis de mettre en évidence pour la première fois les impacts complexes des facteurs étudiés sur les dynamiques de population à l'œuvre dans une telle coculture, leurs éventuelles synergies ou antagonismes, ainsi qu'un probable phénomène de compétition pour les sources azotées dans certaines des conditions étudiées. Sur un plan plus technique, des leviers d'actions potentiels ont également été identifiés pour contrôler l'impact de cette coculture sur certaines caractéristiques d'intérêt du vin fini (volatilome, éthanol, acidité). Certaines zones restent à explorer, notamment la consommation différenciée des sources azotées, pouvant offrir des précisions sur la compétition pour ces sources lors de la phase exponentielle, ainsi que la confirmation de l'impact de la modification du volatilome sur les caractéristiques sensorielles du vin. En effet, même si une évaluation sensorielle a bien été réalisée lors de cette thèse et a permis d'entrevoir quelques hypothèses et tendances, elle ne permet pas de dresser de conclusions fermes et précises et mériterait donc d'être approfondie.

Ces travaux ont également permis d'explorer des pistes méthodologiques et techniques potentielles pour le suivi de ces fermentations, d'abord dans un but d'étude scientifique, mais pouvant potentiellement être appliquées à un but de contrôle technique en cave ou de contrôle qualité en laboratoire œnologique. Tout d'abord l'approche par standardisation des conditions et modélisation des conséquences des paramètres étudiés montre, comme vu ci-dessus, un intérêt scientifique évident. Cette étude constitue un premier pas dans cette direction, et le développement d'approches similaires pourraient à l'avenir constituer des pistes de recherche intéressantes. Il serait en effet envisageable, comme discuté dans les conclusions de la partie 2 de ces travaux, de développer l'étude et le modèle obtenu en élargissant le champ d'étude, soit au niveau des paramètres inclus dans le modèle, soit au niveau des métriques choisies. Affiner le modèle de cette manière permettrait non seulement de dégager de nouvelles conclusions scientifiques et d'élargir notre compréhension des mécanismes à l'œuvre dans les fermentations en culture mixte, mais également d'améliorer sa précision et donc sa capacité potentielle de prédiction théorique pour des applications en cave. En effet, la possibilité d'utiliser un modèle similaire mais affiné et confirmé pour définir à l'avance, par exemple, le ratio exact d'ensemencement *Sc / Lt* à utiliser sur un moût dont on a mesuré les caractéristiques pour obtenir une quantité voulue d'acide lactique a un intérêt technique non-négligeable.
Une autre perspective serait l'application de cette méthodologie générale à d'autres systèmes biologiques. Tout d'abord, *Lachancea thermotolerans* n'est pas la seule non-*Saccharomyces* décrite dans la littérature comme présentant un intérêt scientifique ou technique (C. Varela, 2016), ni la seule dont les interactions directes ou indirectes avec *Saccharomyces* complexifient les dynamiques de population lors d'une culture mixte (Zilelidou & Nisiotou, 2021). Une approche multiparamétrique standardisée appliquée à d'autres couples levuriens pourrait permettre, comme dans les travaux présentés ici, de confirmer des leviers de modulation déjà mis en évidence indépendamment dans la littérature ou en dégager de nouveaux, puis de comparer l'intensité de leurs impacts et étudier les éventuelles synergies entre ces effets. La perspective d'utiliser cette approche pour faciliter l'étude de consortiums microbiens plus complexes (trois micro-organismes ou plus) n'est également pas à

négliger, ainsi que la possibilité de l'appliquer à d'autres matrices fermentaires faisant également de manière occasionnelle ou systématique l'objet de cultures mixtes (bière, kombucha, kéfir…).
Dans un second temps, la technique de mesure de populations microbiennes par cytométrie en flux automatisée a montré son potentiel, à travers de multiples applications liées au processus d'élaboration du vin. Si cette preuve de concept reste encore bien sûr à confirmer et des verrous technologiques restent à lever, l'intérêt de cette technique à la fois pour la cave et le laboratoire semble présent. En effet, elle libère du temps et/ou de la main d'œuvre qualifiée, en plus de permettre des suivis de population avec une fréquence de prélèvement et une amplitude temporelle de suivi supérieures à celle d'un opérateur humain. En outre, d'un point de vue scientifique, placer le processus de prélèvement-marquage-dilution entre les mains d'un robot permet d'améliorer la reproductibilité et donc la fiabilité des résultats en standardisant de manière plus poussée l'exécution de ce protocole. La fréquence et l'amplitude élevée des mesures peuvent également permettre d'obtenir des courbes de population bien plus détaillées, et donc de déterminer plus précisément certaines métriques secondaires (vitesse de croissance maximale par exemple), ce qui peut se révéler crucial lors d'une expérimentation. Par exemple, utiliser cette automatisation lors de l'étude multiparamétrique aurait pu permettre à la fois d'établir plus précisément les variables étudiées pour définir les dynamiques de population, et de limiter partiellement la variance résiduelle induite par la réalisation du plan complet en deux parties.

A plus longue échéance, l'articulation des deux volets de cette thèse pourrait également révéler son potentiel par la combinaison du modèle détaillé décrit plus haut et l'obtention de résultats de prélèvements en quasi-temps réel, nuit et jour. Il est ainsi possible d'imaginer à terme la mise au point d'une boucle de rétroaction modulant par exemple en temps réel, à partir du modèle théorique préétabli et des mesures automatisées de cytométrie, un levier d'action pilotable tel que la température de fermentation afin de maintenir la proportion de levures à un niveau constant défini à l'avance. Cette perspective, bien que probablement lointaine pour une application fiable en cave, ne relève pas de l'impossible à une échelle laboratoire dans un premier temps, et pourrait permettre d'ouvrir la porte à des études fixant deux populations à une proportion donnée pendant l'ensemble de la fermentation, afin d'étudier de manière plus fiable d'éventuelles modifications métaboliques induites par des interactions en comparaison avec des cultures pures.

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